Differential expression of *MYB33* and *AP2* genes and response of Ty resistant plants to beet curly top Iran virus infection in tomato

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**SUMMARY**

*Beet curly top Iran virus* (BCTIV) is a member of genus *Becurtovirus*, family *Geminiviridae*, which constrains host crop production in various geographical regions in Iran. This virus causes significant crop loss in sugar beet and has been also reported from other hosts including tomato. Various plant factors such as resistance genes and microRNA pathways were found to be involved in plant–geminivirus interaction. *Ty* resistant hybrids were found to confer resistance to both monopartite and bipartite begomoviruses through increasing cytosine methylation of the viral genome. In this study we investigated the response of various tomato cultivars and *Ty* resistant hybrids to BCTIV infection. In addition, the effect of the virus on the regulation of microRNA target genes in plant development was investigated. Based on the evaluated disease severity index and coefficient of infection, *Ty* resistant hybrids were grouped as moderately susceptible plants and produced leaf curling symptoms after BCTIV infection. However, no clear symptom was observed in the cultivar Super Chief. In addition, a significantly lower level of virus accumulation was observed in Super Chief plants compared to the susceptible cultivar, Grosse Lisse. Expression analysis of miRNA target genes in infected plants showed that *AGO1* was induced in both cultivars. However, *MYB33* and *AP2* were differentially regulated in the susceptible and moderately resistant cultivars. The importance of regulation of these miRNA target genes in viral symptom development is discussed.

**Keywords:** Agroinfection, Geminivirus, microRNA, resistance.

**INTRODUCTION**

*Geminiviruses* are single-stranded DNA viruses with twinned icosahedral particles. They cause major constraint on production of various crops worldwide. Based on the genome sequences and architectures, the family *Geminiviridae* was grouped into seven genera including genus *Becurtovirus* (Varsani et al., 2014).

*Beet curly top Iran virus* (BCTIV) is a member of becurtoviruses. The BCTIV genome contains five open reading frames (ORFs), two ORFs (called C1 and C2) on the complementary-sense strand and three ORFs (called V1, V2 and V3) on the virion-sense strand. However, the genome lacks C4 ORF which encodes symptom determinant proteins in curtoviruses (Bolok Yazdi et al., 2008). BCTIV is a widespread virus in various geographical regions in Iran, where it has been isolated from sugar beet (*Beta vulgaris*) and other dicotyledonous crops including tomato (*Solanum lycopersicum*), cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*). (Gharouni Kardani et al., 2013). Resistance genes and pathways including ubiquitin/26S proteasome and microRNA pathways were found to be involved in plant-geminivirus interaction, reviewed by Sahu et al. (2014). In tomato plants, the first resistance study in begomovirus was reported by Zamir et al. (1994). The resistance locus, called *Ty-1*, originated from a wild tomato, *S. chilense*. The *Ty-1* gene confers partially dominant resistance to tomato yellow leaf curl virus (TYLCV) and has been introgressed into cultivated tomato plants. Since then, five more resistance/tolerance loci including *Ty-2* to *Ty-6* have been mapped and introgressed from different wild tomatoes including *S. chilense*, *S. habrochaites* and *S. peruvianum* into cultivated tomato plants (Butterbacha et al., 2014). A low level of TYLCV accumulation has been reported in plants containing all these resistance genes. Therefore, they exhibit more of a tolerance phenotype rather than immune or resistance phenotypes.

Plant microRNAs play important roles in adaptation to both biotic and abiotic stresses (Sunkar et al., 2012), and also in plant development including leaf morphogenesis, reviewed by Jin et al. (2013) and Zhang et al. (2006). Leaf morphogenesis is regulated by several microRNAs, including miR159, which is a conserved phenomenon in plants even with very different leaf forms (Palatnik et al., 2003). Plant viruses were shown to interfere with microRNA
mediated regulation of host genes to induce developmental defects in infected plants (Chapman et al., 2004; Kasschau et al., 2003; Mallory et al., 2002).

In tomato plants, BCTIV infection produces leaf distortion, leaf curling and stunting, thereby causing major yield losses. BCTIV is a relatively newly identified virus (Bolok Yazdil et al., 2008) and therefore resistance or tolerance phenotypes to the viral infection and also the interaction with host factors have not been studied yet. In this study we investigated the response of tomato cultivars and Ty resistant hybrids to BCTIV infection; and also regulation of selected miRNA target genes in infected plants, which have role in leaf morphogenesis and plant development.

MATERIALS AND METHODS

Plant material and virus isolate. Tomato seeds were grown in pots containing vermiculite, loamy sand, and coco peat (1:1:1). The common tomato cultivars used in this study were included: Calj, Moneymaker, Super Chief and Grosse Lisse, provided by the Behta Company (Tehran, Iran) and two tomato homozygous hybrids, AVTO-1002 and AVTO-1007, provided by AVRDC (Taiwan), which contain the Ty-1,2,3 and Ty-2,3 resistance genes, respectively.

The infectious clone of BCTIV-Kaf was constructed after isolating a full length BCTIV genome (GenBank accession No. KP410285) from tomato plants in the Farce province of Iran (Eini et al., 2016). 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 pBin20-1.4BC-TIV-Kaf construct. The resulting construct was then introduced into Agrobacterium tumefaciens strain C58 by electroporation. Transformed cells were selected and used for inoculation of tomato plants.

Experimental design and virus inoculation. To test the response of tomato plants to BCTIV-Kaf infection, seven tomato cultivars and two tomato homozygous Ty resistant hybrids were grown in pots. On a completely randomized design experiment, for each cultivar 12 plants were included in three replicates. This experiment was repeated using the same experimental design and number of plants. For agroinoculation, A. tumefaciens cells containing pBin20-1.4 BCTIV, were grown. The optical density of bacterial cells at 600 nm was measured and adjusted to 0.2. Five microliters of these cells were used to agroinoculate each plant at four-leaf stage as described by Kheyr-Pour et al. (1991).

Detection and quantification of virus in tomato plants. To confirm the accumulation and spread of virus, total DNA was extracted from the newly emerged leaf tissues at 14 and 21 days after agroinoculation using a modified CTAB method (Rouhibakhsh et al., 2008). The extracted DNAs were tested for the BCTIV-Kaf infection using the polymerase chain reaction (PCR) with a specific primer pair, BCP-F/BCP-R (Table 1), to amplify the full length coat protein gene.

To compare the viral DNA accumulation in susceptible and moderately resistant tomato cultivars by quantitative real-time PCR (qPCR), total DNA was extracted from three individual infected plants from two cultivars at 14 and 21 dpi (days post inoculation). For each sample, 100 nanograms of DNA was used in a reaction containing 26.6 pmol of BC-F and BC-R primers (Table 1) and Absolute QPCR SYBR Green buffer (ABgene). The reactions were carried out in a RotorGene 2000 qPCR instrument (Corbett Research). A melting curve was recorded at the end of each run to assess reaction specificity. PCR efficiency was determined using standard curves prepared by serial dilution of DNA from infected plants. The level of viral accumulation was normalized to that of reference gene, SlEF-α.

The relative accumulation of virus for each sample was calculated using the 2−ΔΔCt method as described by Livak and Schmittgen (2001). Three biological repeats were tested for each target gene. For statistical analysis the mean of biological replicates was tested by t-test (P<0.5%) using SAS software.

Disease evaluation and data analysis. Symptom development was monitored from the second week and

| Table 1. Oligonucleotide primers used in this study. |
|---|---|
| Primers | Size (nt) | Sequences (5’ to 3’) |
| AP2-F | 21 | GCCAACTGATCTTGATCTGA |
| AP2-R | 21 | ATGGAAAGAAGAAGCTTG |
| AGO1-F | 23 | GCCAGGAAATTTGATATAGAT |
| AGO1-R | 23 | CAAGGGGAAATTCGCTAGATC |
| MYB33-F | 20 | GGAAGAACACATTGTTGAT |
| MYB33-R | 22 | GATTGATCGATAGAATTC |
| GAPDH-F | 19 | GGCCTGCAATCAAGGAGGAA |
| GAPDH-R | 21 | AAATCAAATCACGGGAAACTG |
| BC-F | 20 | ATGGGAACCTGTTTTTCAAC |
| BC-R | 19 | TTAGAAAATATATTTG |
| BCP-F | 21 | ATGGCGGTTCAAGGTCAGAAG |
| BCP-R | 20 | TCAATGAAATAAAGCCTAC |
| SlEFa1-F | 20 | TACTGGGTGTGTGTAAGC |
| SlEFa1-R | 24 | AACTTCTTTCAGATTTCATC |

| Table 2. Coefficient of infection rate based of the PDI and PDS for the tested tomato cultivars and lines in two experiments. PDI is the number of infected plants per total number of inoculated plants × 100; PDS is equal to the sum of numerical rating per (total number of observed × maximum disease grade) × 100, and CI was calculated by multiplying PDI to PDS. |
|---|---|---|
| Cultivar | CI | PDI | PDS |
| Super Chief | 15.56 | 83 | 12.58 |
| Moneymaker | 56.25 | 100 | 56.25 |
| AVTO-1002 | 37.5 | 100 | 37.5 |
| Calj | 68.5 | 100 | 68.5 |
| AVTO-1007 | 37.5 | 100 | 37.5 |
| Grosse Lisse | 68.5 | 100 | 68.5 |

1 Coefficient of Infection; 2 Percent Disease Incidence; 3 Percent Disease Severity.
Table 3. Response of tomato cultivars to BCTIV infection based on Coefficient of Infection (CI).

<table>
<thead>
<tr>
<th>Host reaction</th>
<th>CI</th>
<th>Tomato cultivars and line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance</td>
<td>0-10</td>
<td>None</td>
</tr>
<tr>
<td>Moderately resistant</td>
<td>10.1-30</td>
<td>Super Chief</td>
</tr>
<tr>
<td>Moderately susceptible</td>
<td>30.1-60</td>
<td>AVTO-1007, AVTO-1002, Moneymaker</td>
</tr>
<tr>
<td>Susceptible</td>
<td>60.1-80</td>
<td>Calj, Grosse Lisse</td>
</tr>
<tr>
<td>Highly susceptible</td>
<td>80.1-100</td>
<td>None</td>
</tr>
</tbody>
</table>

evaluated at 28 dpi. The severity of disease symptoms in the infected plants was scored according to the following scale as suggested by Friedmann et al. (1998): zero for symptomless; one for mild leaf thickening and yellowing; two for leaf thickening, yellowing and mild leaf curling; three for leaf thickening, yellowing and severe leaf curling; four for leaf thickening, yellowing, epinasty, stunting and severe leaf curling. The recorded scores were used to calculate the disease severity (DS) index, disease incidence (DI) and coefficient of infection (CI) by the following formulae as previously described (Arunachalam et al., 2002).

\[ \text{PDI} = \frac{\text{Number of infected plants}}{\text{total number of plants}} \times 100 \]

\[ \text{PDS} = \frac{\text{Sum of numerical rating}}{\text{(total number of observed} \times \text{maximum disease grade}} \times 100 \]

\[ \text{CI} = \frac{\text{PDI} \times \text{PDS}}{100} \]

Based on the obtained CI (Table 2) in two experiments, infected tomato plants were grouped into five levels of resistance (Table 3). This grouping system was suggested by Kanakala et al. (2013). In addition, an analysis of variance (ANOVA) for the calculated and normalized (Arc \( \sin x^{0.5} \)) PDS index was conducted. The means were compared by Fisher’s least significant difference (LSD) test and differences at \( P < 0.05 \) were considered significant using SAS (9.1) software.

Quantitative reverse transcription-PCR of miRNA target genes. Based on the results of disease evaluation in tomato cultivars and hybrids, cultivars Grosse Lisse and Super Chief were selected for gene expression analysis. Leaf tissues were collected from BCTIV infected and healthy plants at 14 and 21 dpi. Total RNA was extracted from leaf tissues using RNX-Plus reagents (Cinaclone, IRI). Two micrograms of the extracted RNAs were treated with DNase I and then used for oligo-dT-primed first-strand cDNA synthesis with SuperScript III reverse transcriptase (Vivantis Technologies, Malaysia). The quality of the produced cDNAs was tested by PCR using GAPDH F/R primers (Table 1).

The qPCR amplification of target genes including AG01, MYB33 and AP2 was performed using the prepared cDNA and their specific primers (Table 1). The expression level of target genes for each sample was normalized to that of reference gene, GAPDH (Table 1). The relative amount of target gene for each sample was calculated using the \( 2^{-\Delta \Delta Ct} \) method as described by Livak and Schmittgen (2001). Three biological repeats were tested for each target gene. The identity of the PCR products was confirmed by sequencing. For statistical analysis, the mean of biological replicates was tested by t-test (\( P < 0.5\% \)) using SAS software.

RESULTS

Phenotypic responses of tomato cultivars and \( Ty \) resistant hybrids to BCTIV infection. Various symptoms were observed in tomato cultivars and \( Ty \) resistant hybrids infected with BCTIV. Infected plants produced yellowing, leaf thickening, leaf curling and stunting (Fig. 1). In tomato cultivars Grosse Lisse and Moneymaker, and also tomato hybrids AVTO-1002 and AVTO-1007, the leaf curling symptoms became visible at 14 dpi, whereas in the cultivar Super Chief, the first mild leaf curling symptoms were obtained at 21 to 24 dpi.

Based on the obtained CI (Table 2) and the grouping system suggested by Kanakala et al. (2013), the tested tomato cultivars and \( Ty \) resistant hybrids were placed into three groups (Table 3). For resistant and moderately resistant plants, the calculated CI was in the range of 0-10 and 10.1-30, respectively (Kanakala et al., 2013). Based on this grouping system, tomato cultivars Grosse Lisse and Calj were grouped as susceptible and AVTO-1007, AVTO-1002 and Moneymaker grouped as moderately susceptible to BCTIV-Kaf infection. Only Super Chief was grouped as moderately resistant (Table 3). In addition, an ANOVA for the calculated and normalized PDS index was conducted. There was no significant (\( P < 5\% \)) difference between two experiments, therefore the normalized PDS data from both experiments were pooled and then analyzed using least significant difference (LSD) at the 5% level of significance. Table 2 shows the obtained disease severity index for the tested cultivars and \( Ty \) resistant hybrids was in a range of 18.75 to 68.5. The lowest disease severity was obtained for Super Chief, while Grosse Lisse and Calj showed the highest disease severity. The AVTO-1007 hybrid (containing \( Ty-1,2,3 \)) showed a lower PDS compared to AVTO-1002 (containing \( Ty-2,3 \)). The calculated DS index displayed statistically (\( P < 5\% \)) significant variation in response to BCTIV infection for the tested tomato cultivars and \( Ty \) resistant hybrids. In this indexing system, developed by Kanakala et al. (2013), disease severity scores less than 10 represent resistant phenotypes.

Based on these results, we selected Grosse Lisse, a susceptible cultivar, and Super Chief, a moderately resistant cultivar, for further analysis.

Accumulation level of BCTIV in tomato cultivars. Virus accumulation and spread was tested by PCR in the newly emerged leaf tissues at 14 and 21 dpi. PCR results
showed that BCTIV was present in all inoculated plants in the tested tomato cultivars and hybrids at 21 dpi (Table 2). However, Super Chief displayed the lowest rate of viral infection at 83%. At 14 dpi, BCTIV accumulation was clearly detected by PCR in the susceptible cultivar Grosse Lisse whereas only a very faint band was observed for Super Chief plants. This was hardly visible after using five times more DNA template in the PCR reaction (Fig. 2) and using a more sensitive method, qPCR.

Comparison of BCTIV accumulation in the moderately resistant and susceptible cultivars by qPCR confirmed that the concentration of viral DNA was clearly lower in Super Chief, than that in Grosse Lisse at 21 and 14 dpi (Fig. 3).

**MiRNA target genes expression in infected plants.** Regulation of selected miRNA target genes which are known to have a role in leaf morphogenesis and plant development was tested by qPCR. Fig. 4 shows that the level of AGO1 was induced significantly in both cultivars at 21 dpi. In both cultivars, at the early stage of infection (14 dpi), the level of AGO1 was similar to that of the control plant. However, the levels of AP2 and MYB33 were differentially regulated in the susceptible and moderately resistant plants infected with BCTIV compared to that of healthy plants. The same pattern of MYB33 and AP2 expression was found in both cultivars at 14 and 21 dpi.

**DISCUSSION**

Beet curly top Iran virus is a widespread virus and a major constraint to crop production in Iran (Gharouni Kardani et al., 2013; Heydarnejad et al., 2007; Soleimani et al., 2013). The diversity and wide occurrence of viruses such as BCTIV provide a significant challenge for breeders to develop cultivars with resistant or tolerant traits, to enable management of these viral diseases (Strausbaugh et al., 2008).

Tomato plants infected with BCTIV displayed a range of symptoms (Fig. 1), which reflected the differential response of tomato cultivars and Ty resistant hybrids to the viral infection. Based on the obtained CI and DS index (Table 2), and the response to infection with BCTIV-Kaf (Table 3), none of the tested tomato cultivars and Ty resistant hybrids were found to be highly resistant. This finding, together with the results from screening the most common tomato cultivars for resistance to BCTIV (Khoshnazar and Eini, 2016) indicate the lack of resistance
source/s to BCTIV infection in commonly cultivated tomato plants. This supports previous reports, which claim almost all geminivirus resistance in tomato has been derived from wild species (Bian et al., 2007). Providing further support to our results, pepper cultivars have also been found to be moderately to highly susceptible to beet curly top virus (BCTV) infection and no resistant phenotype has been observed in these cultivated plants (Wang et al., 1999).

In tomato, six resistance/tolerance genes (Ty-1 to Ty-6) to TYLCV infection have been described (Caro et al., 2015). Ty-1 and Ty-3 were shown to encode for RNA-dependent RNA polymerases (Verlaan et al., 2013). Ty-1 represents a novel class of R-genes which confer resistance to both monopartite and bipartite begomoviruses through increasing cytosine methylation of viral genome and transcriptional gene silencing (Butterbacha et al., 2014). Depending on the type of begomoviruses, the response of Ty resistant plants was shown to be varied (Shahid et al., 2013). Based on the calculated CI, both AVTO-1002 and AVTO-1007 hybrids were grouped as moderately susceptible plants to BCTIV infection (Table 3). This means BCTIV can infect and produce clear symptoms in these plants, possibly due to suppression of host gene silencing or escaping viral genome methylation. Supporting this assumption, TYLCV was shown to evade the host defence through a population of de novo synthesized unmethylated viral DNA (Bian et al., 2007). In addition, Ty1-based resistance against TYLCV was also shown to be compromised in mixed infection with cucumber mosaic virus (CMV), which encodes a suppressor of the gene silencing protein, 2b (Butterbach et al., 2014).

A significantly lower level of BCTIV accumulation in Super Chief, a moderately resistant cultivar (Fig. 3), was positively correlated with mild or no symptoms, compared to that of the susceptible cultivar, Grosse Lisse. Supporting this result, symptom development and severity were found to be positively correlated with viral DNA accumulation in Arabidopsis plants infected with BCTV (Lee et al., 1994). A low level of TLCV accumulation was also reported for tomato genotypes such as TY172, classified as having a TLCV and TYLCV-resistant genotype (Bian et al., 2007).

Investigating the roles of host miRNAs in plant viral immunity/sensitivity is a new research subject that may shed a light on disease initiation and symptom development (Naqvi et al., 2010). Argonaute 1 (AGO1) is an essential component of the microRNA (miRNA) pathway and can also exhibit antiviral function. The AGO proteins regulate plant development and innate immunity by targetting endogenous transcripts (Pumplin and Voinnet, 2013), which may indirectly affect susceptibility to viruses. Some AGO proteins with an antiviral role were found to load virus-derived siRNA to directly target viral RNA (Garcia-Ruiz et al., 2015; Pantaleo et al., 2007). The level of AGO1 was induced in both susceptible and moderately resistant tomato cultivars infected with BCTIV-Kaf (Fig. 4), which is consistent with the induction of this gene in plants infected with various groups of plant viruses, including TMV, cymbidium ringspot virus, turnip crinkle virus,
tobacco etch virus, CMV and tomato leaf curl New Delhi virus (Naqvi et al., 2010; Vaucheret et al., 2004). It was discovered that over-accumulation of AGO1 exhibited developmental defects in Arabidopsis plants (Vaucheret et al., 2004). Hence, the higher level of AGO1 induction in Grosse Lisse compared to that of Super Chief may partially explain the severe symptoms in this cultivar. A large excess of AGO1 protein was found to interfere with the function of RISC or sequester miRNAs or other RISC components (Vaucheret et al., 2004).

MYB transcription factors are well known in determining leaf morphology (Allen et al., 2007; Millar and Gubler, 2005) and the importance of these miRNA target genes in viral pathogenesis has been established (Du et al., 2014). Some host miRNAs including mir159 which regulates MYB factors have been predicted to bind BCTIV genome (Amirnia et al., 2016). Our gene expression analysis also showed a positive correlation between higher accumulation of MYB33 and the induction of severe disease symptoms in susceptible tomato plants. Supporting this result, a higher level of accumulation of MYB33 was directly correlated with the production of severe disease symptoms caused by CMV (Du et al., 2014).

APETALA2 (AP2) genes play important roles in various developmental processes, including signalling, stress response and regulation of disease resistance pathways (Gutterson and Reuber, 2004). Members of this stress-related transcription also play significant roles in plant resistance to both biotic and abiotic stresses (Sharoni et al., 2011). For example, in rice plants infected with rice stripe virus, expression of AP2 gene members was differentially regulated (Sharoni et al., 2011). Our results show that AP2 was also differentially regulated in susceptible and moderately resistant tomato cultivars in response to BCTIV-Kaf infection (Fig. 4). A lower level of AP2 gene accumulation was observed in susceptible plants infected with BCTIV and these plants displayed more severe disease symptoms and a higher level of viral accumulation. Gao et al. (2016) also report that turnip crinkle virus (TCV)-infected plants, which contained lower AP2 gene expression, displayed more severe symptoms and higher viral accumulation (Gao et al., 2016). Low levels of AP2 in the infected plants can be due to a higher accumulation of miR172 at the later stage of infection. Supporting our results, the accumulation of miR172 was also shown to increase with the number of days after inoculation of ToLCNDV in tomato plants (Naqvi et al., 2010) and the level of AP2 gene was clearly decreased in the susceptible tomato plants infected with ToLCNDV (Naqvi et al., 2010).

Differential regulation of AP2 and MYB33 in susceptible and moderately resistant tomato cultivars infected with BCTIV reflects the difference in the regulation of miRNA and their target genes. Recently, it was shown that depending on the type of plant tissue, microRNAs can regulate plant development differently (Yao et al., 2016). In conclusion, induction of MYB33 and AGO1 and diminution of AP2 in the leaf tissue of the susceptible cultivar may explain the abnormal leaf growth and severe symptoms in the plants infected with BCTIV. Testing more miRNA target genes in plant development and also their response to other geminiviruses would contribute to a greater understanding of viral symptom development in plants.

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