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Artificial microRNA-Based RNA Interference and Specific Gene Silencing for Developing Insect Resistance in *Solanum lycopersicum*

Mohammad Faisal *, **Eslam M. Abdel-Salam** and **Abdulrahman A. Alatar**

Department of Botany & Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia; eabdelsalam@ksu.edu.sa (E.M.A.-S.); aalatar@ksu.edu.sa (A.A.A.)
* Correspondence: faisalm15@yahoo.com; Tel.: +966-(011)-4675877

Abstract: RNA Interference (RNAi), which works against invading nucleic acids or modulates the expression of endogenous genes, is a natural eukaryotic regulating system, and it works by noncoding smaller RNA molecules. Plant-mediated gene silencing through RNAi can be used to develop plants with insect tolerance at transcriptional or post-transcriptional levels. In this study, we selected *Myzus persicae's acetylcholinesterase* 1 gene (*Ace 1*) as a silencing target to develop transgenic *Solanum lycopersicum* L. plants' resistance to aphids. An RNAi plasmid vector containing an artificial microRNA (amiRNA) sequence was engineered and successfully transformed into Jamila and Tomaland, two elite tomato cultivars. A northern blot analysis and PCR were carried out to check the efficacy of *Agrobacterium*-mediated transformation in T_0 transgenic plants. The quantitative PCR data showed a substantial downregulation of the *Ace 1* gene in aphids fed in clip cages on T_1 transgenic plants. Furthermore, there was a substantial drop in aphid colonies that were fed on T_1 transgenic plants of both the cultivars. These findings strongly suggest that transgenic plants that express amiRNA could be an important tool for engineering plants resistant to aphids and possibly for the prevention of viral disease in other plant-infested pests.

Keywords: *Agrobacterium*; artificial microRNA; gene silencing; northern blotting; aphid resistance

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1. Introduction

Small RNA (sRNA) molecules play a crucial role in gene regulation through a process known as the RNA interference (RNAi) in order to partially or completely silence targeted genes. The RNAi is an evolution-conserved homolog-dependent gene-silencing phenomenon that has been observed in all eukaryotes and operates by the noncoding small RNA molecules (sRNA). In plants, a sRNA class was found in *Arabidopsis*, which included small endogenous RNA molecules of normally 20–24 nt, known as microRNAs (miRNAs) [1]. Since then, several studies have investigated the biogenesis and potential role of miRNAs and found that modifications of the hairpin stem loop nucleotides seem to have some effect on the biosynthesis of these molecules [2]. The artificial development of miRNAs was thus feasible, and the resulting molecules were called artificial miRNAs (amiRNAs). For amiRNAs, an endogenous miRNA precursor can be used when a particular miRNA sequence complements the desired silencing gene without altering the secondary structure of the miRNA molecule [3]. Artificial miRNAs were developed first in animals [4] and then in plants [5]. More experiments have subsequently shown the high efficiency of amiRNAs in the silencing of targeted genes or gene families. For example, the use of DICER-like protein 1 to produce amiRNAs that target the viral mRNA of the turtle mosaic and turnip yellow mosaic viruses was induced in *Arabidopsis thaliana* by changing miR159 precurrents [6]. In another study about *A. thaliana*, amiRNA molecules were used to mute the miRNA endogenous families (MiR159 and MiR164) [7]. Transgenic tomatoes that express two amiRNAs targeting the 2a and 2b genes in the Cucumber mosaic virus

(CMV) demonstrated a substantial resistance both alone and when combined to a CMV infection, such as tomato yellow leaf curl virus and tobacco mosaic virus [8]. In apples, amiRNAs developed based on the endogenous Md-miR156h miRNA backbone were used to silence various genes and to explore their possible functions [2]. Virus outbreaks in crops of commercial interest are growing more and more frequent due to global warming and increased economic growth, which may raise the dispersal rate of microbial pathogens and their vectors geographically and spatially during the vegetative growth. In recent years, RNAi-mediated gene silencing via the expression of amiRNA has been considered a realistic approach to engineering plants resistant to pests. Several systematic reviews were published to show thorough efforts to establish and use amiRNAs for gene silencing and crop improvement, e.g., [1,9].

The cultivation of tomato (*Solanum lycopersicum* L.) crop is affected by a range of biotic factors, including bacteria, fungi, nematodes and viruses that cause devastating diseases leading to major economic losses. Tomatoes appear to be an ideal haven for many viruses, and so far more than 10 major viruses have been found to infect tomatoes naturally, and some of these viruses have a large number of distinct strains [10]. Most of these viruses are transmitted by pests. Aphids are sap-sucking insects of the order *Hemiptera* and are important crop pests in terms of direct feeding damage and also the transmission of plant viruses [11]. One such example is aphid *M. persicae*, which can feed on over 40 different plant families [12] and is capable of efficiently transmitting over 100 types of plant viruses [13]. Hence, *M. persicae* is one of the most important aphid pests in agricultural crops. *M. persicae* is the transmission vector of many plant viruses. In particular, aphids transmit many single-stranded plant RNA viruses that cause important losses in commercial crops, such as potyviruses and cucumoviruses. The management of insect–pest problems in tomatoes is an important concern, and one approach would be genetic enhancement through the modern tools of genetic engineering. *Acetylcholinesterase* is an enzyme in the insect's central nervous system that catalyzes the breakdown of neurotransmitter acetylcholine into its acetyl-CoA and acetate components, thereby clearing the residual neurotransmitter molecules from the synaptic cleft that regulates normal behavior. Dysfunction in acetylcholine will result in muscle atrophy, due to the continuous activation of muscles and glands, and will cause the paralysis and even the death of the insect [14,15]. *Acetylcholinesterase* is encoded by two acetylcholinesterase (*Ace 1* and *Ace 2*) genes in most insects and is differentially expressed in different insect species [16]. According to several studies, *Ace 1* would possibly encode a key catalytic enzyme, and its expression is far higher than that of *Ace 2* in many insect species [17–19]. *Agrobacterium tumefaciens*-mediated genetic transformation is an effective and widely used approach to introduce foreign DNA into dicotyledons plants. *Agrobacterium* delivery of T-DNAs remains one of the favored pathways for introducing and transmitting exogenous genes within plant cells. One of the reasons for this is that the wide spectrum of plants that are prone to transformation by this bacterium has been successfully applied for producing genetically modified tomato plants with an improved nutritional value and disease resistance [20–23]. Being a model plant, the tomato has been transformed with different genes, such as antiripening [24,25], insect [26] and herbicide tolerance [27], virus resistance [28–30] and salt tolerance [31–33]. The goal of the present study was to investigate RNAi's persistence in the *Agrobacterium*-mediated silencing *acetylcholinesterase* 1 (*Ace 1*) gene of *Myzus persicae* using an artificial microRNA (amiRNA) vector for the production of aphid-resistant tomatoes, followed by the evaluation of the degree of silencing of the target gene and its effect on the aphid population.

2. Materials and Methods

2.1. Vector Designed and Baterial Strain

The plant amiRNA vector was based on the binary vector *pFGC5941 (accession AY310901:* phosphinothricin acetyl transferase (*BAR*) and aminoglycoside phosphotransferase [(*aadA*) genes, complete cds]) [34]. The vector contains an artificial microRNA to a 21 nucleotide of the *Ace 1* sequence (Figures 1 and 2). An artificial *Ace 1* pre-miRNA sequence was obtained

by PCR amplification, using as a template plasmid pAmiR159 pUC118 [35] and *Myzus persicae* amiRNA 1508 primers: forward (5′-AAGATAGATCTTGATCTGACGATGGAAGTACAG GCCATAATCAACATTACATGAGTTGAGCAGGGTA-3′) and reverse (5′-AAGAGTCGACA TGTAATGTTGTAT ATGGCCTGTAGAAGAGTAAAGCCATTA-3′). The primers contain miR159a backbone sequences with Sal I and Bgl II restriction sites in the reverse and forward primers, respectively harboring the 21-nt of the amiRNA targeting *MpAce 1* gene. The PCR fragment was digested with Sal I and Bgl II and cloned under the transcription control of the CMV 35S promoter into the binary vector *pFGC5941* linearized with XhoI and BamHI (that have compatible cohesive ends with Sal I and Bgl II, respectively), thus creating the amiRNA vector. The *agrobacterium* strain LB4404 was transfected with the amiRNA vector by electroporation. The electroporated cells were grown for 48–56 h on LB-agar plates containing kanamycin (K50; 50 µg/mL) and streptomycin (S200; 200 µg/mL), and the positive colonies carrying the amiRNA vector were selected and stored at an ultralow temperature (–80 °C) for further experiments.

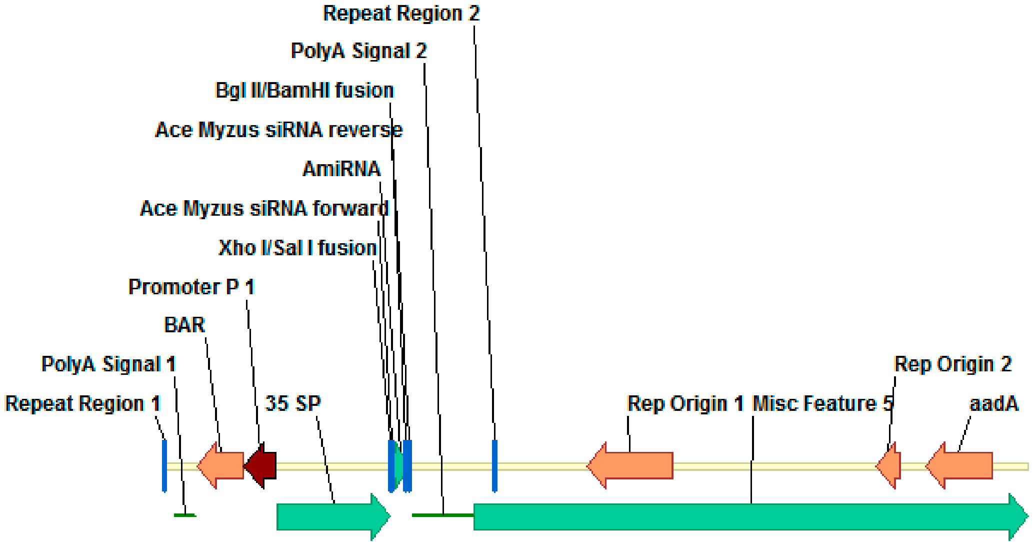

Figure 1. Schematic representation of the binary vector *pFGC5941* with amiRNA.

tcgacatg**cggcggttaaatcggctatag**gaagagtaaaagccattaaagggcaagttaaagctcctgagatatgcaatcca
tataaccaaatcctttttactttacaaggattagggcttatgggatccatagcttagcagcttttctttaccctgctcaactcatg**cta**
tagccgatttaaccgccgcttccatcgtcagatcaagatc

Figure 2. Nucleotide sequence of the artificial microRNA to an *Ace 1* gene sequence (highlighted in bold) from *M. persicae*, inserted in the binary vector.

2.2. Plant Materials and Genetic Transformation

The tomato seed cultivars viz., Jamila and Tomaland (Syngenta Seeds BV, BK Enkhuizen, The Netherlands), obtained from an authorized seed store in Riyadh, Saudi Arabia, were disinfected with sodium hypochlorite (NaOCl) solution (5%; *w*/*v*) by gradual shaking in a rocker for 10 min, followed by removal of the disinfectant through washing with ultrapure steam-sterilized water. The sterile tomato seeds were put in magenta boxes on half-strength Murashige and Skoog (MS; Duchefa BV, Haarlem, The Netherlands) medium-moistened [36] cotton and incubated in the dark for two days and later transferred to the growth chamber (Conviron Adaptis, Winnipeg, MB, Canada) at 24 °C under a 16/8 day-night cycle with a light intensity of 50 µmol m^{-2} s^{-1}.

The in vitro plant was produced according to our previously described method, with slight modifications [37]. Cotyledonary leaves (CL) excised from 10-days-old aseptically

germinated seedlings were used as explants for cocultivation and shoot regeneration. Prior to cocultivation, the CL explants were cultured on MS medium and incubated in a growth chamber at 24 °C for 48 h. The precultured explants were co-cultivated with *Agrobacterium* (LB4404) containing the amiRNA vector for 20 min with gradual shaking in a rocker for infection, followed by being transferred on MS media and kept at 24 °C under dark conditions. After 48 h, the agro-cultivated CL explants were aseptically transferred on antibiotic selective MS media containing 6-benzyladenine (BA, 5.0 μM) + 6-furfurylaminopurine (Kinetin, 10.0 μM; Duchefa BV, Haarlem, The Netherlands) + indole-3-butyric acid (IBA, 2.5 μM; Duchefa BV, Haarlem, The Netherlands) + K50 + Augmentin (200 μg/mL) and were incubated in a growth chamber-Conviron Adaptis under cool white ELD light with a 16/8 day-night cycle. After 14 days, the CL explants were transferred to a fresh media devoid of antibiotics for further shoot regeneration and multiplication for six weeks. The regenerated shoots were rooted in 0.5 μM of indole-3-butyric acid supplied half-MS medium and were successfully developed ex vitro.

2.3. Molecular Analysis of Transgenic Plants

PCR and northern blotting were used to analyze the putative transgenic plants in order to validate the stable transformation. For the PCR analysis, DNA was isolated from 100 mg leaf tissues of T_0 transgenic and control plants using a Qiagen DNeasy Plant Mini Kit (Qiagen, MD, USA). PCR reactions were performed in a thermocycler (T-100, Bio-Rad, Hercules, CA, USA), as described earlier [37]. For confirmation of the BAR gene, the primers are; forward 5′GGTCTGCACCATCGTCAACC3′ and reverse 5′CTGCCAGAAACCCACGTCAT3′. The PCR products were electrophorized in agarose gel (1 %; *w*/*v*) at 60 V for 1 h and were analyzed using a G-Box Gel Documentation System (Syngene, UK).

To detect the amiRNA in transgenic tomato plants, northern blot hybridization was performed using a DIG Northern Starter Kit (Roche, Mannheim, Germany). Total RNA was extracted from young leaf tissues of T_0 transgenic and control plants and was prepared for blot analysis, as previously described [37]. A specific DNA probe was obtained using T3 RNA polymerase after the linearization of the *pSK-Ach I* fragment with *BamHI* (PCR DIG Probe Synthesis Kit, Roche, Roche, Mannheim, Germany). The blots were hybridized with the DNA probe (digoxigenin-11-UTP) prepared according to the instruction manual provided by the manufacturer. The hybridized membranes were washed at room temperature with SSC buffer containing 0.2 % SDS, followed by being washed at 42 °C with SSC buffer containing 0.1% SDS for 10 min and being detected immunologically on X-ray film using chemiluminescence substrate.

2.4. qRT-PCR Analyses of Target Ace 1 Gene in Aphids

To investigate the expression of the target *Ace 1* gene by quantitative real-time (qRT-PCR) analysis, the total RNA was isolated from five aphids fed on T_1 transgenic and control plants using TRIzol^{TM} reagent (Life Technologies, Carlsbad, CA, USA), followed by a treatment with a TURBO DNA-free^{TM} kit to remove the DNA (Life Technologies, Carlsbad, CA, USA). qRT-PCR analyses were laid out in 96-well plates containing 15 μL of reaction mixture: 7.5 μL of SYBER Green, 0.15 μL of 100 mM of DTT, 1.8 μL of RNAase-free water, 3 μL RNA (approximately 10 ng/μL) and 5 μM of primers (each): forward—CCGTTGGGACAATACAAACC, reverse—GGATTCCACATTGTAGCAC, in a Rotor-Gene Q thermal cycler (Qiagen, The Netherlands) with the following conditions; 10 min at 50 °C, 3 min at 95 °C, 40 cycles for 10 s at 95 °C and 20 s at 60 °C, and the final ramp with a raise of 1 °C every 5 s from 60 °C to 95 °C. For each sample with identical replicates, triplicate reactions were made in each process. Using Rotor-Gene Q Series Software (Qiagen), the relative expression of the *Ace 1* gene was analyzed by the $2^{-\Delta CT}$ process.

2.5. Aphid Performance on T_1 Transgenic Plants

To assess the aphid performance, five T_1 transgenic plants carrying the amiRNA vector were separately grown in plastic pots at 24 °C under a 16/8 day-night cycle with a light

intensity of 50 μmol m^{-2} s^{-1}. Five adult aphids were placed in clip cages (diameter 20 mm, height 15 mm and length 70 mm) on T_1 transgenic, empty vector nontransgenic and control plants with the help of a soft painting brush, and their reproductive ability was monitored. All the adults were removed after seven days, and only five two-day-old nymphs remained. The population of aphids was counted in each clip cage after 14 days, and their numbers were recorded as the fecundity of five nymphs.

2.6. Data and Statistics

Transformation experiment was conducted on 50 explants in duplicates. Statistical analysis was carried out using Sigma Plot 14.0 (Systat Software Inc., San Jose, CA, USA), and significant differences were compared by Dunnett's multiple comparison test at a significance level of $p < 0.05$ and $p < 0.01$.

3. Results and Discussion

3.1. Transformation and Regeneration of Transgenic Plants

For an efficient process of genetic transformation in crop, a successful selection strategy is very important, which can be accomplished by choosing suitable explants for in vitro regeneration. The choice of explants depends on the quality of the plants, and various plant species' explants behave differently in vitro [38–40]. In this study, after cocultivation, the explants endured a 48-h rest time in the dark on MS medium without selection agent in order to enhance the regeneration efficiency of the *agrobacterium*-infected CL explants. Direct transfers to the selective medium are recorded as resulting in tissue necrosis of the explants after coculture [41].

The *agrobacterium*-infected explants began to form shoot buds after two weeks of transfer on the selective regeneration medium (Figure 3A). The induced bud started develop shoots within 4–6 weeks (Figure 3B). The two studied cultivars revealed different regeneration responses in terms of the number of shoots regenerated from the *agrobacterium*-infected explants. There are several earlier studies on *agrobacterium*-mediated genetic transformation in various tomato cultivars, though the regeneration of plants and transformation rates were also found to vary significantly with respect to genotypes and explants [42–45]. In this study, the largest number of shoots (6.47 $\pm$ 0.51) was produced from CL explants of the Jamila cultivar infected with *agrobacterium* containing amiRNA. The cultivar of explants is deemed essential and cannot be resolved or replaced by optimizing other external influences, e.g., by treating extremely virulent strains or by optimizing in vitro culture conditions [46–48]. Similarly, cotyledons derived from axenic seedlings have been successfully used for genetic transformation in tomatoes [37,43].

All the shoots generated on the selective medium produced roots when transferred onto $\frac{1}{2}$MS medium containing 0.5 μM indole-3-butyric acid (IBA) (Figure 3C). Plantlets with developed shoots and roots carrying amiRNA vector were ready for transfer to the greenhouse within 10–12 weeks after the *agrobacterium* infection. So far, over 50 transgenic plants were developed that grew normally after transplanting to ex vitro conditions, with a survival rate of 90 percent (Figure 3D).

3.2. PCR Characterization of T_0 Transgenic Plants

To validate the presence of the transgene, a PCR analysis was performed with the genomic DNA of putative transgenic and nontransgenic plants. Amplified products suggesting the presence of the transgene without escape plants were found in all the tested transgenic plants. The PCR analysis showed that both Jamila and Tomaland cultivars had been successfully transformed with the designed amiRNA construct, as supported by the gel banding analysis. Jamila showed a better response (65% positive) than Tomaland, which showed a weak response (50% positive) to the PCR analysis. Meanwhile, no bands were observed in the case of the control plants and the plants transformed with an empty vector. Although there were no bands observed for the control explants, the obtained

finding was consistent with previous observations of tomato transformation that indicated transformation efficiency discrepancies among different cultivars [34].

Figure 3. *Agrobacterium*-mediated genetic transformation from CL explants of the *Solanum lycopersicum* cultivar Jamila. (**A**) Shoot bud induction on regeneration medium; (**B**) Multiplication of shoots on regeneration medium; (**C**) In vitro rooted shoot; and (**D**) T_0 transgenic plants transferred to plastic pots.

3.3. Northern Blotting of T_0 Transgenic Plants

The PCR positive individual T_0 transgenic lines carrying amiRNA were confirmed by northern blot hybridization. The northern blots' chemiluminescence analysis showed unique hybridization patterns, indicating the abundance of amiRNA in the transgenic tomato plants of both cultivars. No signal was observed in either the control plants or the plants transformed with an empty vector (Figure 4). Similarly, Vu et al. [49] reported the expression of artificial microRNAs in tomato plants for the silencing of the precoat and coat proteins of a begomovirus. The findings obtained are also consistent with some previous research showing the expression of siRNA by northern blotting for the *MpAChE1* gene in tomato plants [37] and the *MpAChE2* gene in tobacco plants [50].

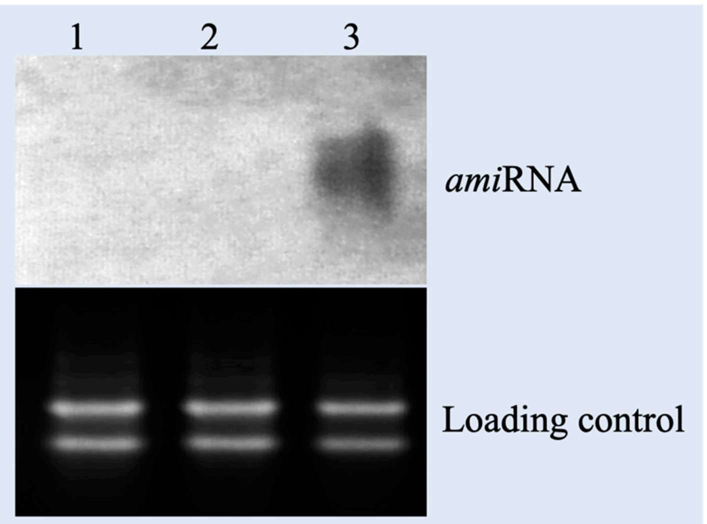

Figure 4. Northern blot analysis of amiRNA in tomato transgenic plants. Lane 1 = Control plant; Lane 2 = Plant with empty vector, Lane 3 = Transgenic plant.

3.4. qRT-PCR Analysis of Aphids Fed on Plants Expressing amiRNA

In order to check the expressional changes in the *Ace 1* mRNA level, the aphids fed on transgenic plants were analyzed using a reverse transcription quantitative real-time PCR (qRT-PCR). In T_1 transgenic tomato plants developing amiRNA against *Ace 1* gene, aphids were allowed to feed. The qRT-PCR analysis showed a substantial degradation of the target gene (*Ace 1*) expression levels in aphids that were fed on transgenic plants when compared with the aphids fed on normal plants or pants transformed with an empty vector (Figure 5). The differences in the degradation of the *Ace 1* gene may be challenging, but the higher expression levels, amiRNA stem stability and target precision may play a significant role [50]. This finding is in line with the expression profiles of the acetylcholinesterase 2 coding gene (*MpAChE2*), where *MpAChE2* transcript levels decreased in aphids feeding on tobacco plants expressing amiRNA when compared with those feeding on control plants. Similarly, in *Schistocerca americana* and *Rhodnius prolixus*, the downregulation of target genes was also revealed after the microinjection or artificial feeding of siRNA, respectively [51,52].

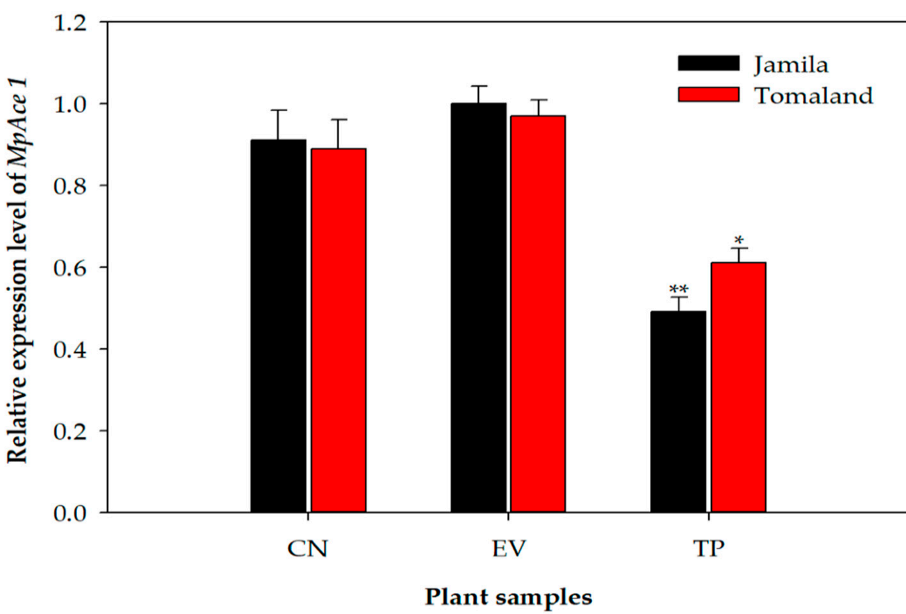

Figure 5. Relative expression level of the *Ace 1* gene in aphids fed on tomato cultivars. CN; control plant, EV; plant with empty vector; TP; transgenic plant. Bar represents mean ± S.E of three replicates. Asterisks (* and **) represent the significant differences at $p < 0.05$ and $p < 0.01$ with respect to that of CN.

3.5. Aphid Resistance Against T_1 Transgenic Tomato

To ascertain whether the plant-mediated silencing of the *Ace 1* gene inhibited the fecundity of aphids, the performance of aphids feeding on transgenic plants was evaluated. T_1 transgenic plants were inoculated with five individual aphids in clip cages, and their reproductive potential was documented and compared with the normal plants and plants transformed with an empty vector (Figures 6 and 7). The reproduction rate is an easily measured absolute fitness factor, and antibiotics are classified as an aphid resistance category that adversely affects the reproduction of aphids [53,54]. In this study, it was observed that the populations of aphids that fed on amiRNA-expressing tomato plants significantly decreased. After 14 days of feeding on control plants of the cultivar Jamila, aphids had a mean total of 37 offspring, while aphids feeding on transgenic plants had a mean total of only 21 offspring (Figure 7). In contrast, aphid feeding on the cultivar Tomaland resulted in an average of 26 offspring. The findings of this study are comparable to those of Amer et al. [55] and Akbar et al. [56], which recorded the differences in aphid populations on different varieties of *Brassica*. In addition, the modulation of target genes in transgenic plants was corroborated by a fitness assay, in which the aphid population decreased dramatically through a feeding exposure to plants expressing amiRNA when compared to control plants. The results of the qRT-PCR revealed that the downregulation of the *Ace 1* gene in aphids fed on tomato plants carrying the amiRNA vector was linked to early mortality. The decline in the aphid population feeding on tomato transgenic plants is comparable to previous studies that indicate that the population of aphids and white flies declines when fed on tobacco plants [50,57].

Figure 6. Aphid performance assay on T_1 transgenic tomato plants. Inset is the larger image of the aphids' colony in a clip cage.

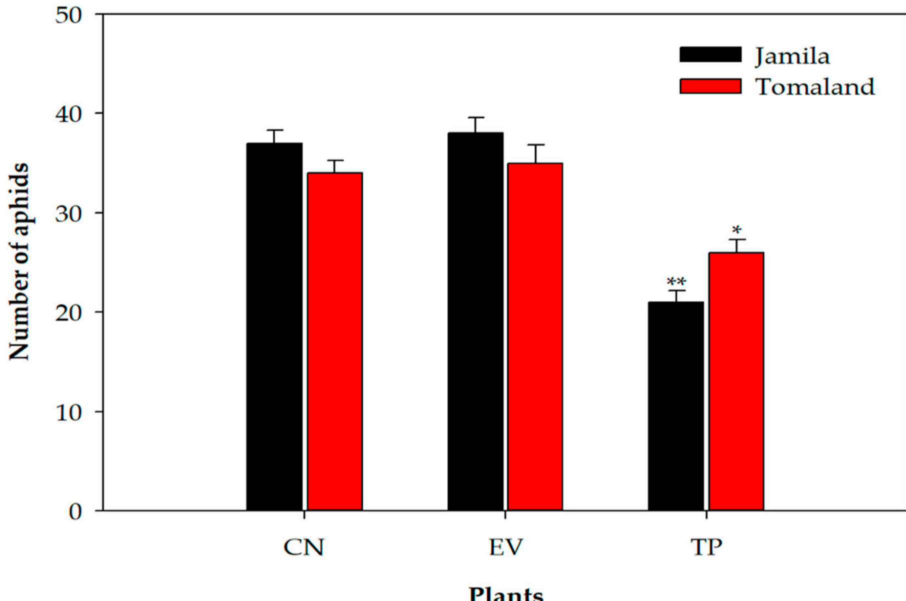

Figure 7. Effect of gene silencing using amiRNA on aphid populations after 14 days of feeding on transgenic tomato plants. CN; control plant, EV; plant with empty vector; TP; transgenic plant. Bar represents mean ± S.E of three replicates. Asterisks (* and **) represent the significant differences at $p < 0.05$ and $p < 0.01$ with respect to that of CN.

4. Conclusions

In conclusion, we developed an easy-to-use *Agrobacterium*–mediated transformation system for tomatoes using cotyledonary leaf explants. In two different cultivars, the binary vector containing the artificial micro-RNA (amiRNA) to a 21 nucleotide of the *Ace 1* gene sequence was successfully transformed, and the transgenic plants revealed the amiRNA

integration and expression. The transgenic plants with amiRNA have a beneficial impact on the silencing of the target gene and have resulted in a significant reduction in the aphid population. To the best of our knowledge, this is the first report on artificial microRNA-based gene silence and stable genetic transformation in tomatoes. Plant-mediated silencing of the target gene through the use of amiRNA has the ability to minimize insect infestation and virus dissemination as a biopesticide, breaking down the neurotransmission and the transcriptional regulation of developmental genes in aphids.

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