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Genetic Transformation and siRNA-Mediated Gene Silencing for Aphid Resistance in Tomato

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Abstract: We explored the ability of RNA interference (RNAi) to silence the *Acetylcholinesterase* 1 (*Ace 1*) gene in aphid *Myzus persicae* and developed transgenic tomato plants resistant to aphid infestation. Three plasmid constructs, T-449: a single *Ace 1* fragment (forward orientation), T-452: two *Ace 1* fragments (reverse and forward orientations), and T455: a single inverted *Ace 1* fragment, were developed and transformed into two tomato cultivars, Jamila and Tomaland. PCR, northern blotting, and small interfering RNAs (siRNA) analysis were performed to validate the success of *Agrobacterium*-mediated transformation. The efficiency of transformation was highest for the T-452 construct. In vivo effects of the transformed constructs were confirmed in feeding experiments, and there was significant downregulation of the *Ace 1* gene. In addition, an aphid challenge assay was conducted to investigate the siRNA-mediated silencing of the target gene (*Ace 1*) in the inhibition of fecundity in *M. persicae*. We found that the plants that were transformed with the T-452 vector had 37.5% and 26.4% lower fecundity at 27 °C in the Jamila and Tomaland, respectively. Our results strongly indicated that the plant-mediated silencing of aphid-RNA might be a robust and effective approach for developing pest and disease resistant in plants.

Keywords: *Agrobacterium tumefaciens*; genetic transformation; horticulture crop; northern blotting; RNAi

1. Introduction

Tomato (*Solanum lycopersicum* L.; Solanaceae) is considered to be one of the most important vegetable crops in the world, as it ranks first in terms of production and cultivation area. According to the FAOSTAT database (http://www.fao.org/faostat/en/), the total harvested area of tomatoes reached 4.8 million ha, with total production exceeding 182 million tons in 2017. As a rich source of vitamins (especially A and C), minerals, amino acids, and dietary fibers, the tomato is also considered as one of the most important nutritive crops and is of great economic value [1].

Tomato cultivation suffers from serious losses due to infestation by insects and pests and the diseases that they transmit. *Myzus persicae* (aphids), sap-sucking bugs of the order Hemiptera, has been considered a significant crop pest in terms of direct feeding damage and transmission of plant infections [2]. It can bolster on over 40 distinctive plant families [3] and is capable of effectively transmitting over 100 sorts of plant viruses [4]. In the aphid, the *Ace 1* gene is one of two genes coding for acetylcholinesterases (*AChEs*). *AChEs* (EC 3.1.1.7) are the main receptors of some

pesticides, including carbamate and organophosphate insecticides. Their main function is to catalyze the hydrolysis of a few choline esters, counting acetylcholine, which basically work as neurotransmitters in insects. Hence, inhibition of *AChEs* leads to the obstruction of neurotransmission and death of the insects [5]. Management of insect–pest problems in the tomato is an important concern. One approach is genetic enhancement through the use of genetic engineering that offers a novel method to urge disease-resistant plants that not only display high resistance to multiple pathogen attacks but that are ecologically secure and sound. The *Agrobacterium*-mediated genetic transformation method has several advantages over other approaches to deal with transgenic plant production; it is fairly straightforward, easy to use, has a limited cost of equipment, and generally results in a single or low duplicate of the transgene being inserted. Because of its nutritive and economic significance, the tomato was one of the first plants to be genetically modified, which was achieved using *Agrobacterium* [6]. Thereafter, studies used different protocols and explants to produce genetically modified tomato plants in order to enhance the quality or develop disease resistance [7–9].

Small non-coding RNA molecules (siRNA and miRNA) are evolutionarily-conserved homology-dependent unique phenomena of gene silencing, demonstrating its vital role in genetic expression termed as RNA silencing or RNA interference "RNAi" [10]. It involves dsRNA-initiated sequence-specific degradation of cognate RNA via 21 nucleotide (nt) length effector molecules with the aid of cellular protein machinery. The silencing phenomenon results from either transcription inhibition (Transcriptional Gene Silencing; TGS) or from RNA degradation (Post-transcriptional Gene Silencing; PTGS) and has been correlated with the accumulation of siRNAs [11,12]. Transgenic plants expressing small RNA molecules designed to silence insect targeted genes gain more attention and are considered as the next generation of pest management strategies, considering their beneficial effects as compared to other chemical and biological strategies [13]. Host-induced gene silencing (HIGS) utilizing the RNA hushing mechanisms and, particularly, silencing the targets of attacking pathogens, have been effectively used in the management of plant viral diseases [14]. HIGS is a process in which the host plant is genetically transformed to produce small RNA molecules to target transcripts in insects and/or parasites. siRNA-mediated gene silencing for aphid resistance has been successfully reported in several crops, including tobacco [15–19], *Arabidopsis* [20,21], wheat [22], and barley [23] to reduce fecundity and parthenogenetic population of aphids. Tomatoes showed a significant fungal resistance after expressing small RNAs targeting the dicer-like transcripts of *Botrytis cinerea* [24]. Furthermore, tomato lines expressing short tandem targets mimicking RNAs from the miR482/2118 microRNAs family showed enhanced resistance to bacterial and oomycetes infections [25]. Viral outbreaks on crops of commercial interest are becoming increasingly common as a result of climate change and of increased global trade, both of which are capable of increasing the dispersal rate of plant viruses and their insect vectors, geographically as well as temporally during the growing season [26]. Commercial agriculture in Saudi Arabia has become a highly mechanized and technological enterprise that depends on the use of selected high quality and adapted plant varieties and supply of inputs that compensate for the agriculturally unfavorable environmental conditions for agriculture that occur throughout most of the kingdom. Viral disease outbreaks can potentially cause serious losses in yields and/or quality in densely planted monocultures.

This study aimed to develop an efficient and robust genetic transformation system in two elite tomato cultivars grown in Saudi Arabia, and assess the degree of silencing of a specific gene (*Myzus persicae Ace 1* gene) in aphids, the effect of silencing on the fitness of the insect colonies, and their ability to disseminate viral disease.

2. Materials and Methods

2.1. Bacterial Strain and Construct

Agrobacterium tumefaciens strain LB4404 (ElectroMax, Invitrogen Life Technologies, Carlsbad, CA, USA) was used in the transformation experiments. Three plasmid constructs were developed and

transfected into the bacterial strain. The Construct T-448 is a pBluescript II SK (+) vector, which is a phagemid excised from lambda ZAPII (Accession: X52324.1) [27] containing an insert between XhoI and XbaI sites of its polylinker corresponding to a cDNA fragment of the *Acetylcholinesterase gene* (*Ace 1* fragment) obtained by RT-PCR from the aphid *M. persicae* (Figure 1). The fragment consists of nucleotides 1095 to 1677 of the published sequence with the accession number AY 147,797 [5]. Constructs T-449, T-452, and T-455 were based on the binary vector pFGC5941 (accession AY310901: phosphinothricin acetyl transferase (*BAR*) and aminoglycoside phosphotransferase (*aadA*) genes [28].

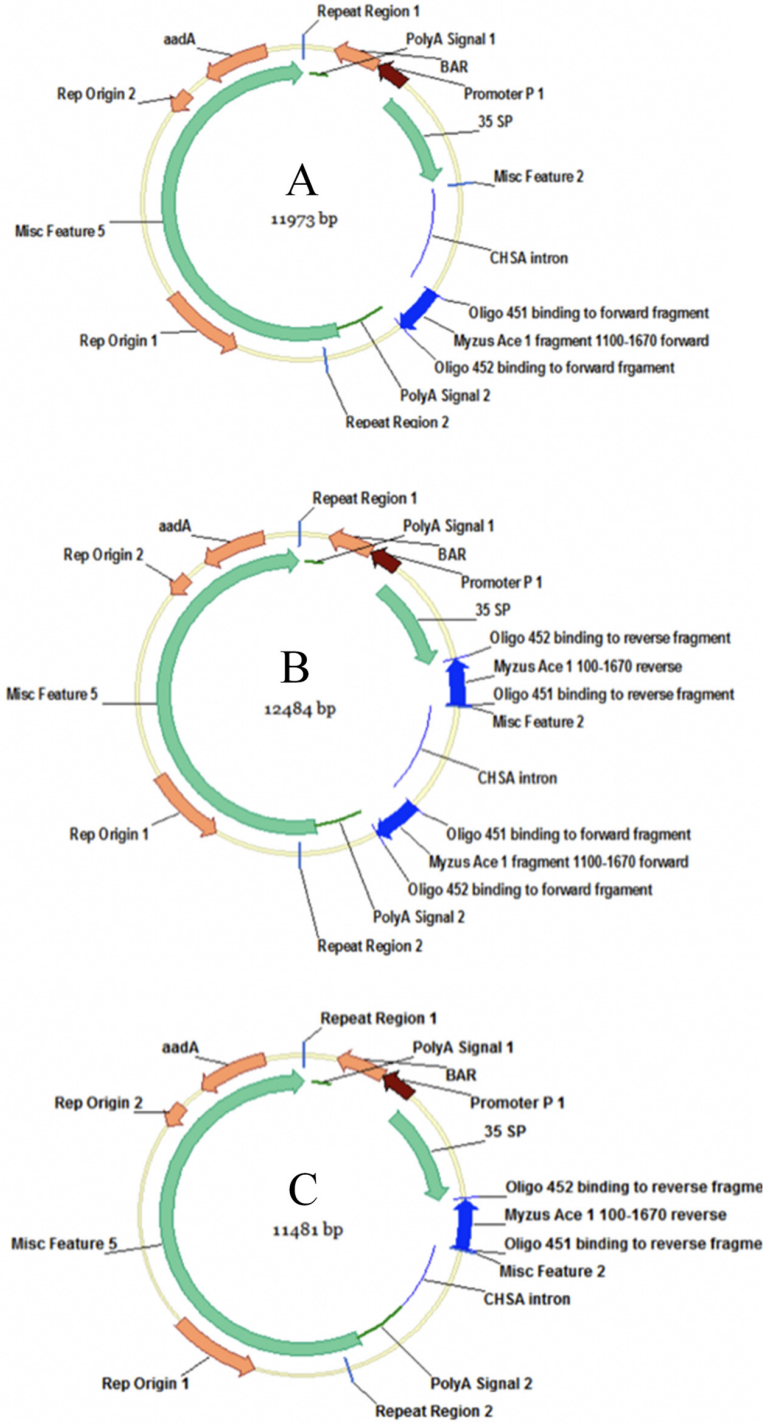

Figure 1. Schematic representation of constructs: (**A**) T-449: pGCS5941 binary with single *Myzus persicae Ace I* fragment 1100–1670; (**B**) T-452: pGCS5941 binary with double *Myzus persicae Ace 1* fragment 1100–1670; (**C**) T-455: pGCS5941 binary with single inverted *Myzus persicae Ace 1* fragment 1100–1670.

Construct T-449 contains a single *Ace 1* fragment in the forward orientation (Figure 1A). Construct T-452 contains two *Ace 1* fragments in the reverse and forward orientations, respectively, flanking the plasmid intron sequence (Figure 1B). Construct T-455 contains a single inverted *Ace 1* fragment only (Figure 1C).

2.2. Electroporation (Transformation) and Selection of Positive Colonies

Agrobacterium tumefaciens LB4404 was transformed with each binary vector by electroporation. Electroporation was performed in a 0.1 cm MicroPulser^{TM} cuvette (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA, USA) using a MicroPulser Electroporator (Bio-Rad) under the following conditions: 2.0 kV, 200 Ω, 25 μF. After electroporation, the cells were transferred to a 15 mL tube with 1.0 mL of LB medium incubated at 220 rpm (30 °C) for 3 h with shaking. The transformed cells were then spread on pre-warmed LB-agar plates supplemented with Kanamycin (50 μg/mL) and Streptomycin (200 μg/mL) and incubated for 48–56 h. Colonies that had been transformed and were carrying a binary vector were grown on selective LB-agar plates and stored for co-cultivation and transformation experiments.

2.3. Plant Materials and Explants Preparation

Seeds of tomato cultivars Jamila and Tomaland were cleaned in running tap water for 20 min, followed by soaking in 5% (v/v) detergent solution for 10 min. After thorough rinsing with sterile ultrapure pure H_2O, the seeds were surface disinfected with 2.5% (v/v) sodium hypochlorite (Clorox Co., Jeddah, Saudi Arabia) for 10 min and then rinsed 4–5 times with sterile ultrapure H_2O to remove the disinfectant. The seeds were then germinated on sterile cotton moistened with sterile half-strength Murashige and Skoog (MS) medium [29].

In our preliminary in vitro culture experiments, considerable efforts were devoted to optimizing the most suitable conditions for plant regeneration, and cotyledonary leaf explants were found to be more responsive explants [1]. Therefore, cotyledonary leaves (CL) from aseptic seedlings that were 8–10 d old were used as explants. The leaf discs were incubated on in vitro regeneration medium for 2 days at a 24 °C temperature in a growth chamber (Conviron Adaptis, Winnipeg, MB, Canada) before co-cultivation with *Agrobacterium.* Proper care was taken while placing leaf discs on the medium so that the dorsal surface was always in contact with the media.

2.4. Co-Cultivation of CL Explants with Agrobacterium

Agrobacterium-containing constructs were grown in LB with Kanamycin (50 μg/mL) and Streptomycin (200 μg/mL) at 28 °C and 200 rpm overnight. The culture (50 mL) was then pelleted at 4 °C temperature at 6000 rpm for 10 min. Leaf discs were transferred into approximately 20 mL autoclaved MS liquid (MS salt without agar) and 200 μL of the culture incubated for 20 min with occasional slow shaking to induce proper infection. The leaf discs were then transferred to another plate and dried using sterile blotting paper. After blot drying, the leaf discs were transferred onto the selective medium MS + BA (5.0 μM) + IBA (2.5 μM) + Kinetin (10.0 μM) + Augmentin (200 μg/mL) + Kanamycin (50 μg/mL) and incubated in dark for proper transfer.

2.5. Selection and Regeneration of Plantlets

After 48 h of co-cultivation, the leaf discs were transferred onto selection medium. They were then aseptically relocated onto shoot-regeneration medium for multiplication and plant regeneration. The culture were maintained in a growth chamber under 50 μmol m^{-2} s^{-1} light provided by cool white LEDs for a photoperiod of 16 h at 22 ± 2 °C, and the regenerated buds were regularly sub-cultured onto the freshly prepared media.

2.6. Rooting and Establishment of Transgenic Plants

For rooting, the regenerated shoots were individually transferred to $\frac{1}{2}$MS medium containing 0.5 µM indole-3-butyric acid (IBA). The rooted transgenic shoots were transferred to pots containing sterile potting soil (Planta Guard) and kept in a growth chamber at 22 ± 2 °C with diffuse light of 50 µmol m^{-2} s^{-1} for a photoperiod 16 h for acclimation. The in vitro generated transgenic plants (T0) were used for PCR characterization and northern blotting, and the homozygous seeds (T1) were further selected and used for aphid challenge assay and qRT-PCR analysis of the target gene.

2.7. Characterization of Transgenic Plants by PCR Analysis

DNA was isolated from 300 mg of fresh leaf tissues from the transformed plants (T0) following the method of Doyle and Doyle [30], and the isolated DNA was quantified using a NanoDrop™ 200c spectrophotometer (Thermo Scientific™, Waltham, MA, USA). PCR reactions were carried out in a thermocycler (T-100, Bio-Rad Laboratories Inc., Hercules, CA, USA) in a 50 µL of reaction mixture containing 25 ng of DNA, Tris-SO_4 (60 mM) (pH 8.9), $(NH_4)_2SO_4$ (18 mM), $MgSO_4$ (2 mM), dNTP mix (250 nmol), primer (1 µM of each), and 5 U of Taq DNA polymerase (Fermentas, Waltham, MA, USA). Primer pairs used for the confirmation of *BAR* genes as follows; GGTCTGCACCATCGTCAACC-F and CTGCCAGAAACCCACGTCAT-R. The reaction mixture was amplified at 95 °C (5 min), followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension of 5 min at 72 °C. The amplified products were electrophorized at 60 V for 1 h in 1× TBE buffer using 1% agarose gels and stained with ethidium bromide (0.5 µg/mL) and photographed using a Gel Documentation System (G-Box, Syngene, Cambridge, UK). The housekeeping gene *GAPDH* was run as control to ensure the equal loading of samples.

2.8. Characterization of Transgenic Plants by Northern Blotting

A total of 100 mg of leaf tissues collected from T0 plants were crushed in liquid nitrogen, and total RNAs were extracted by adding 400 µL of 0.1 M Tris-HCl (pH 8.0), 10 mM EDTA, 0.1 M LiCl, 2-mercaptoethanol (1%), and SDS (1%) at room temperature. For northern blotting, 400 µL of extract was mixed with an equal volume of phenol/chloroform and vortexed for 10 min. Samples were centrifuged at 12,000 rpm for 10 min, and supernatants were carefully collected and re-extracted with an equal volume of phenol/chloroform followed by centrifugation at 12,000 rpm for 5 min. Supernatants were then aspirated without taking debris from the interface. Ethanol precipitation of supernatants were performed by adding 7.5 µL of sodium acetate (3 M) and 3 volumes of absolute ethanol. The solution was kept at −80 °C for 30 min, followed by centrifugation and washing with ethanol (70% v/v). The pellets were air dried and finally resuspended in 100 µL of autoclaved MilliQ water, and frozen until use. Samples (30 µL) were separated on polyacrylamide gels (15%) containing urea (7 M). Before transfer, equal loading of samples was confirmed by staining the gel with ethidium bromide. The gel was transferred to Amersham Hybond-N+ membranes (GE Healthcare, Buckinghamshire, UK) using 0.5× TBE buffer at 300 mA for 40 min. The wet membranes were cross-linked by UV exposure. The probe was obtained using T3 RNA polymerase after linearization of the pSK-Achl (T448) fragment with the restriction enzyme *BamHI*. Blots were prehybridized and hybridized with a DNA probe (digoxigenin-11-UTP) prepared following the manufacturer's instructions (DIG Northern Starter Kit, Roche Diagnostics GmbH, Mannheim, Germany).

2.9. qRT-PCR Analysis of Ace 1 Expression in Aphids Fed on Transgenic Plants

Transcriptional variation of in *M. persicae Acetylcholinesterase 1* gene (*MpAce 1*) level was analyzed using qRT-PCR. Aphids were fed on transgenic plants (T1) to analyze their *MpAce 1* expression. After 14 days, 5 adult aphids were selected from the tomato plants for the aphid challenge assay. Total RNA was isolated from selected aphids using Triazole reagent followed by treatment with a TURBO DNA free kit (Ambion Inc., Austin, TX, USA). One step RT-qPCR was performed in a 15 µL

of reaction mixture containing, SYBER Green (7.5 µL), primer (5 µM) of each primer of target gene (CCGTTGGGACAATACAAACC-F, GGATTCCACATTGTAGCAC-R), DTT (0.15 µL of 100 mM), RT Mix (0.75 µL), RNAase-free water (1.8 µL), RNA (3 µL) (approximately 10 ng/µL). Expressional analysis was performed using a Rotor-Gene Q thermal cycler (Qiagen, Venlo, Limburg, The Netherlands) with the following parameters; 50 °C for 10 min, 95 °C for 3 min, 40 cycles of 95 °C for 10 s, and 60 °C for 20 s, and a final ramp from 60 °C to 95 °C rising 1 °C every 5 s. Reactions were performed in triplicate for all samples with duplicate replicates in each run. Relative expression of *MpAce 1* was analyzed using the $2^{-\Delta\Delta CT}$ method of the Rotor-Gene Q Series Software (Qiagen). Statistical analyses were performed using SPSS statistics software, Version 24 (SPSS Inc. Chicago, IL, USA). The housekeeping *GAPDH* gene was used as an internal control.

2.10. Aphid Challenge Assay

To screen the insect resistance against transgenic tomato plants and determine the effect of varying temperatures on aphid fitness, 5 plants (T1) from each construct (T-455, T-452, and T-449) were grown in individual pots at 2 different temperatures (22 °C and 27 °C). With the help of writing brush, seedlings at the 5-leaf stage were inoculated with 5 adult aphids. After 7 d, all adults were removed, and 5 two-day-old nymphs were left on leaves of each line to grow for 2 weeks. After 14 d, the total number of the aphids grown on seedling leaves was counted. Their numbers were recorded as the fecundity of 5 nymphs. Empty vector transgenic plants and untransformed tomato plants were inoculated in parallel as negative controls.

2.11. Data Analysis

For tomato transformation, 50 explants were used for each construct, and the experiments were performed in duplicate. The data for plant regeneration was recorded after 8 weeks and statistically evaluated using SPSS, and the values were compared using a multiple range test (DMRT) at 5% significance. Percent transformation efficiencies (TE) were calculated on the basis of co-cultivated explants producing transgenic shoots on selective media using the following equation:

$$TE(\%) = \frac{Number\ of\ transgenic\ plants\ regenrated}{\text{Total number of explants used for transformation}} \times 100\%.$$

For the molecular data, differences in expression were determined by using Dunnett's multiple comparison test (Sigma Plot 11.0; Systat Software Inc., San Jose, CA, USA) with a significance threshold of $p < 0.05$ unless otherwise stated. * $p < 0.05$, ** $p < 0.01$ verses normal plant (NM).

3. Results and Discussion

3.1. Generation of Transgenic Plants and PCR Confirmation

Schematic representation of the tomato transformation is depicted in Figure 2. A total of 100 CL explants of cultivars Jamila and Tomaland derived from 8–10 d old aseptic seedlings were co-cultivated with *Agrobacterium* carrying the constructs T-449, T-452, and T-455. Twenty CL explants were cultured for regeneration as a control. The co-cultivated explants were cultured on selective media MS + BA (5.0 µM) + IBA (2.5 µM) + Kinetin (10.0 µM) + Augmentin (200 µg/mL) + Kanamycin (50 µg/mL) (Figure 3A). After 2 weeks, the co-cultivated explants were selected and transferred onto the medium supplemented with BA (5.0 µM) + IBA (2.5 µM) + Kinetin (10.0 µM) for shoot regeneration. Shoot buds were observed two weeks after transfer onto a shoot induction medium (Figure 3B). The frequency of co-cultivated explants producing shoots differed widely between the genotypes and constructs. As shown in Table 1, varying transformation efficiencies were obtained in both the cultivars. Such variations can be caused by tomato genotypes and the use of different constructs, which can be understood by 63% transformation efficiency with T-452 in Jamila, while it was approximately 58% in cv. Tomaland (Table 1). In addition, the lowest efficiency of 40% was found in cultivar Tomaland by

using the binary vector T-449. The results obtained were in agreement with earlier findings of genetic manipulation in tomato, suggesting differences of transformation efficiency with various plasmid constructs [31,32]. About 90% of the transgenic plants survived to ex vitro condition and did not show any immediate detectable variation with respect to morphology or growth characteristics (Figure 3D). Further, confirmation of transgenic plants was carried out using PCR analysis. The PCR bands in corresponding gels unequivocally demonstrated the successful transformation of different constructs in tomato plants (Figure 3E).

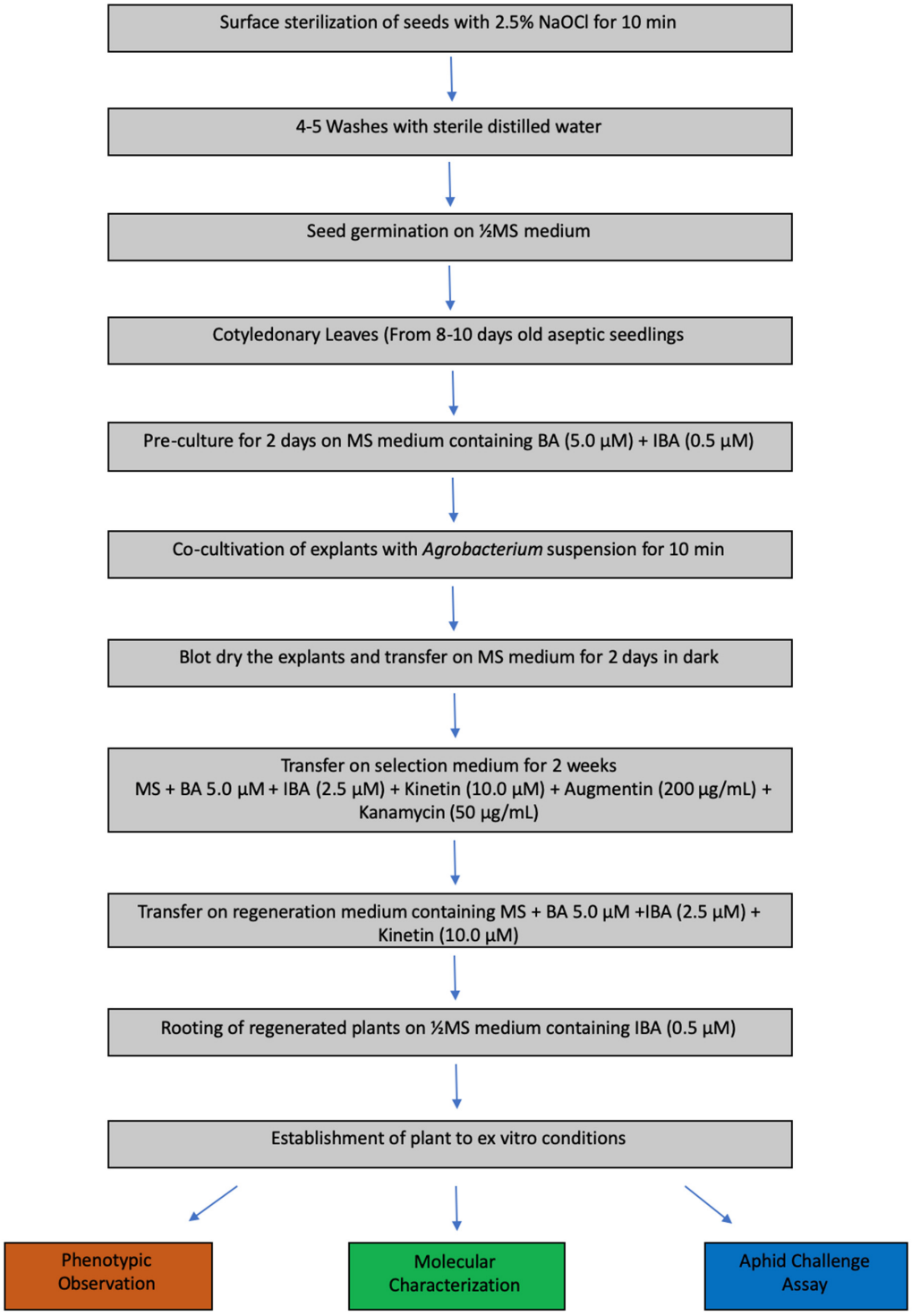

Figure 2. Schematic representation of genetic transformation in tomato.

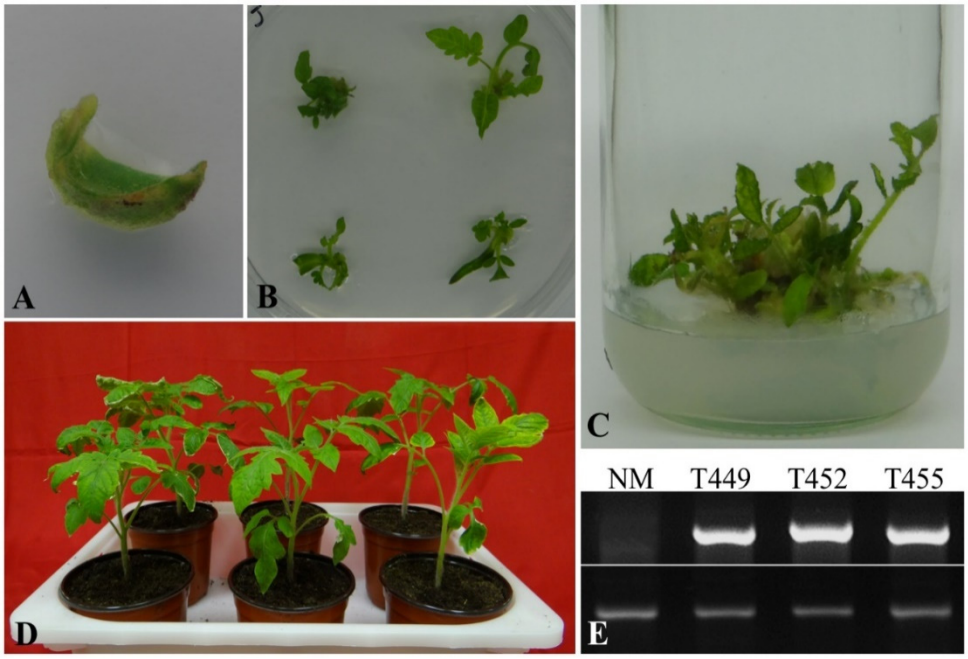

Figure 3. Genetic transformation in tomato (**A**) cotyledonary leaves (CL) explants cultured on selective media after co-cultivation; (**B**) induction of multiple shoots on regeneration medium; (**C**) proliferation of shoots on regeneration medium; (**D**) regenerated transgenic plants established to ex vitro conditions; (**E**) PCR confirmation of transgenic plants generation with the T-449, T-452, and T-455 constructs in a 1% agarose gel. The housekeeping gene *GAPDH* was run as control to ensure loading of samples. NM refers to control.

Table 1. Transformation efficiency of different constructs in tomato cultivars Jamila and Tomaland.

Plasmid Constructs	Number of Explants Co-Cultivated	Mean Number of Explants Produce Shoots		Mean Transformation Efficiency (%)	
		Jamila	Tomaland	Jamila	Tomaland
T-449	100	45^{b}	40^{b}	45^{b}	40^{b}
T-452	100	63^{a}	58^{a}	63^{a}	58^{a}
T-455	100	52^{c}	49^{c}	52^{c}	49^{c}

[1] Data represent the average of two independent experiments. Values denoted the same letters are not significantly different according to Duncan's multiple range test (DMRT) at the 5% level.

3.2. Northern Blotting of Transgenic Plants

The presence of siRNA produced in independent lines of constructs T-455, T-452, and T-449 in transgenic tomato cultivars Jamila and Tomaland was confirmed by northern blot analysis. The chemiluminescence analysis of northern blots revealed the abundance of siRNAs in T-452 and T-455, while the abundance was less for T-449 (Figure 4). The northern data conforms with the previous study detecting siRNAs targeting the *MpAChE2* gene of *Myzus persicae* in tobacco [17]. Also, our data is in agreement with RNA dot blot assay for siRNA detection of the *AChE* gene in whitefly [33]. Additionally, the data is also supported by the expression of siRNA in *A. thaliana* transgenic lines targeting the *MpC002* and *Rack-1* [16].

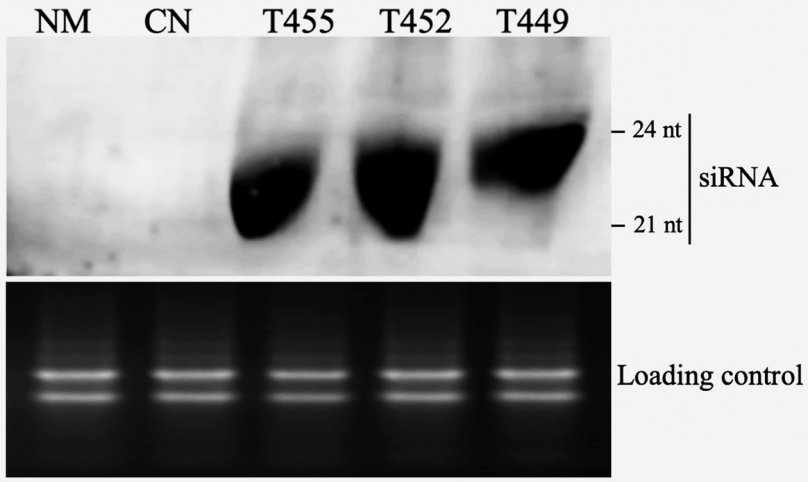

Figure 4. Northern blot detection of *Ace 1* siRNAs in transgenic plants with *Ace 1* specific probe. NM = normal plant; CN = plant with empty vector. Total RNA stained with ethidium bromide serve as the loading control.

3.3. Transcriptional Analysis of Aphids Feeding on Transgenic Tomato Plants

qRT-PCR data revealed that the expression levels of the target gene (*Ace 1*) decreased in aphids fed on either cultivars of transgenic plants, as compared to those fed on normal plants and on plants with an empty vector (Figure 5). The maximum downregulation of *Ace 1* in aphids fed on construct T-452 (double insert) was found to be significant, followed by construct T-455 (inverted insert) in both cultivars. Comparatively, aphids that fed on plants with the T-449 construct did not exhibit significant downregulation of *Ace 1*. The higher expression level and stem stability of the siRNA and the specificity to the target may be contributing factors to these results [17]. These results are similar to those observed in microinjection and artificial feeding of siRNAs to insects. For example, chemically synthesized siRNAs resulted in *Ace 1* gene downregulation with high mortality, in addition to substantial growth inhibition of survivor larvae in *Plutella xylostella* [34] and *Helicoverpa armigera* [35]. Moreover, lower gene expression of the target gene in tomato plants was corroborated with findings of Malik, Raza, Amin, Scheffler, Scheffler, Brown, and Mansoor [33] in which whiteflies that fed on tobacco plants exhibited a significant knockdown of *Ache* and *EcR* genes.

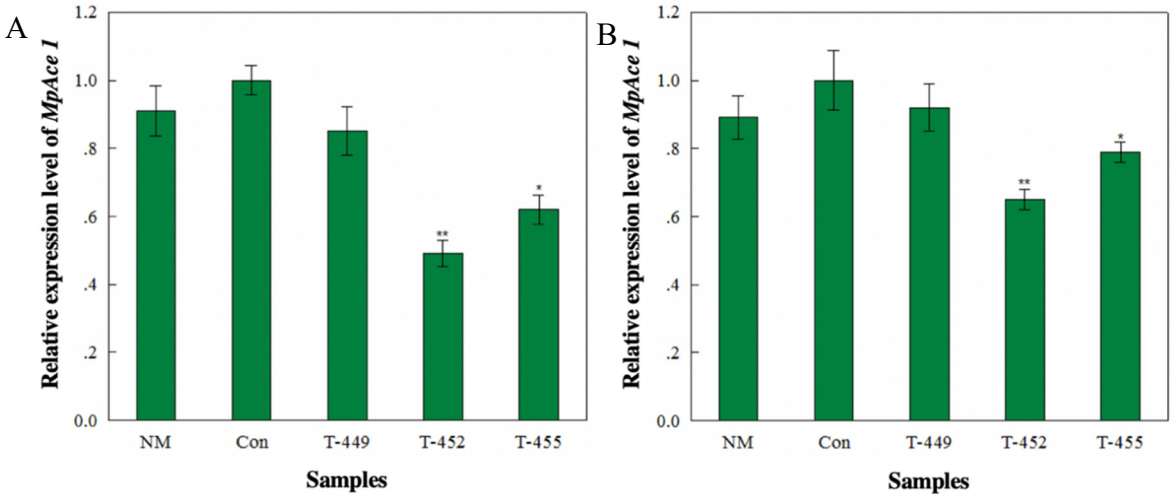

Figure 5. Knockdown of *Myzus persicae Acetylcholinesterase 1 (Mp Ace 1)* expression in aphids that fed on tomato cultivars Jamila (**A**) and Tomaland (**B**). NM refers to untreated plants and Con to plants transfected with an empty vector. Each histogram represents mean ± S.E of three replicates where * denotes significantly different expression to that of NM at $p < 0.05$ and ** at $p < 0.01$.

3.4. Aphid Resistance Against Transgenic Tomatoes

An aphid challenge assay was conducted to investigate the plant-mediated silencing of the target gene (*Ace 1*) on the fecundity of *M. persicae*. Three independent lines of both of the cultivars were transformed with T-449, T-452, and T-455 constructs and were tested at optimal (22 °C) and high (27 °C) temperatures. Five individual aphids were caged on transgenic plants (Figure 6), and their reproductive capacity was monitored and compared to the normal and control (with empty vector) plants. Fecundity data showed that the cultivars Jamila and Tomaland transformed with the T-452 (double hair loop) constructs exhibited a significant reduction in the number of aphids nymphs produced per adult by 34.2% and 26.5% at 22 °C, respectively (Figure 7). Interestingly, at high temperature (27 °C) the cultivars Jamila and Tomaland transformed with the T-452 construct showed a 37.5% and 26.4% decline in nymph production, respectively. Comparatively, cultivars Jamila and Tomaland transformed with vector T-455 reduced the nymph production by only 23.6% and 20% at 22 °C respectively. Higher temperature resulted in a decline of 23.5% in nymph production in T-455 transgenic plants (Figure 7). However, plants transformed with the T-449 (direct insert) construct did not affect the number of nymphs produced at either temperature.

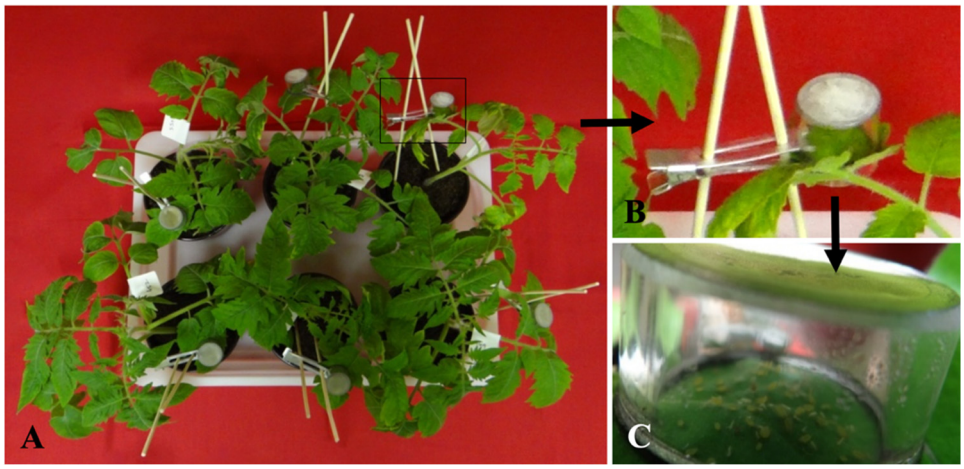

Figure 6. (**A**) In vivo bioassay (Aphid fitness test) of T1 transgenic plants with aphids. (**B**,**C**) Enlarged views of the inset showing aphid colonies.

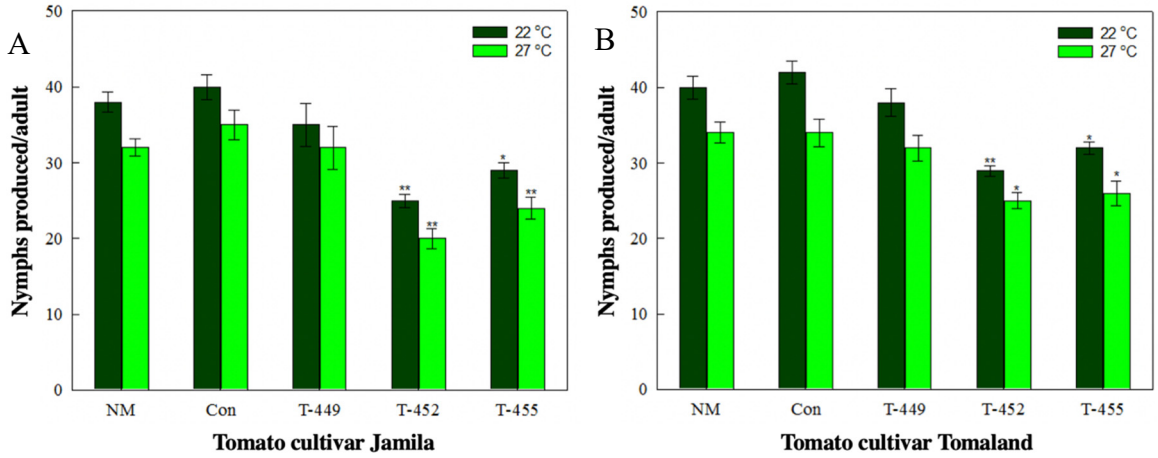

Figure 7. Effect of various constructs (T-449, T-452, and T-455) on the fecundity of aphid's nymphs at different temperatures feeding on tomato cultivars Jamila (**A**) and Tomaland (**B**) NM refers to untreated plants and Con to plants transfected with an empty vector. Each histogram represents mean ± SE of three replicates, where * denotes significantly different expression to that of the NM treatment at $p < 0.05$, and ** at $p < 0.01$.

These temperatures (22 and 27 °C) were selected due to aphids being unlikely to survive or produce nymphs at temperatures above 30 °C [36]. Insects are ectotherms, and temperature variations play a critical role in its survival, distribution, and population dynamics [37,38]. Hence, understanding the effect of temperature variation on an individual's performance and the plastic responses of populations to different temperatures is critical [39]. We selected two temperatures for study because the extreme climatic conditions (heat waves and daily fluctuations) of Saudi Arabia can impact aphids in various ways, including reduced fecundity reduction and population growth, slow development, community structure, and interruption to trophic cascades [40–43]. Aphids are more sensitive to acute changes in temperature, making them a suitable test organism for our study [40]. Aphids have been mainly controlled by the application of several classes of pesticides, including neonicotinoids, organophosphates, pyrethroids, and carbamates. However, their application has lost effectiveness, toxicological effects on non-target organisms have been revealed, and severe threats to the environment have been exposed. Our molecular data (qPCR and northern blotting) on transgenic tomato showed successful expression of siRNA targeting of the *Ace 1* gene in aphids. Feeding on transgenic plants exhibited a significant reduction in nymphs' production compared to aphids that fed on non-transgenic control plants. Such declines can easily be understood with consideration of the downregulation of the target genes (*MpAce 1*) in aphids that fed on plants producing siRNA. Reduction fecundity in aphids feeding on transgenic plants is in line with previous reports exhibiting the fall of the whitefly population when fed on transgenic tobacco plants [17,33]. The response of insects to extreme environmental conditions relies on a combination of different factors, such as changes in membrane structure and osmolyte compounds and molecular processes (gene expression, heat shock proteins, and enzymes), to survive and recover from unfavorable conditions [44]. However, the above molecular changes warrant an in-depth study to establish a positive role of siRNA in reducing aphid fecundity at high temperatures.

4. Conclusions

In conclusion, a robust *Agrobacterium*-mediated genetic transformation system was developed for aphid resistance in two different cultivars of tomato. Three different constructs were investigated for their transformation efficiency in both the cultivars. A significant reduction in aphid fecundity was achieved by plant-mediated aphid-RNA silencing using the T-452 construct containing two *Ace 1* fragments in the reverse and forward orientations. The use of this construct could dramatically decrease aphid population growth and lead to a substantial reduction in agricultural losses. These results obtained strongly suggest that the plant-mediated aphid-RNA silencing might be an effective way to develop disease resistance in various agricultural crops.

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