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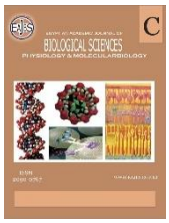

Isolation and Transformation Mi gene Using Chitosan Nanoparticle Into Cucumber to Resist Root-Knot Nematode

Amira H. Ayad[1], Ashraf B. Abdel Razik[1], Eman T. Hussein[2], Entesar H. Taha[3], Samir A. Ibrahim [1], and Sawsan Y. Elateek [1*]

[1]Department of Genetics, Faculty of Agriculture, Ain Shams University, Egypt.
[2]Department of Botany and Microbiology, Faculty of Science, Helwan University, Egypt.
[3]Department of Plant Protection, Faculty of Agriculture, Ain Shams University, Egypt.
***E-mail:** amirahesham799@gmail.com

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ABSTRACT

Many plants around the world suffer significantly from plant parasitic nematodes, so transgenic crops were suggested as an application of biotechnology to make plants more resistant to nematodes in a safe way and without causing any changes in the ecosystem of plants. There are a few strategies for controlling nematodes in cucumber, and one of these methods uses natural resistance genes (R-genes) to resist nematodes. Our study was conducted with an isolated mi gene from nematode-resistant tomato plants, which produce proteins capable of preventing nematode feeding and detected with the PCR method then the resistant gene was inserted into a pEGAD vector and carried out in chitosan nanoparticles to be transformed into plants (cucumber cultivars) using Injections. The transgenic plants were tested by using spore suspension on a leaf. As a result of this study, a UV/visible spectroscope was used to characterise chitosan nanoparticles, and GFP gene florescent light found in transgenic plants compared to control. This will be a new approach to controlling nematodes, not only with genetic engineering but also with chitosan nanoparticles to get new cucumber cultivars to resist nematodes.

INTRODUCTION

(PPNs) Plant-parasitic nematodes are endo-parasitic pathogens causing serious damage and a decrease in crop yields ranging from 8 to 20% (Sasser and Freckman, 1987). These parasites get their nutrition from the cytoplasm of living plant cells. The pecuniary damage is brought on by sedentary *Heteroderidae* endoparasites. This family was divided into the Root-Knot Nematodes and the Cyst nematodes, which included *Heterodera* and *Globodera* (Parkinson *et al .,* 2004). Root -Knot nematodes were called that since they cause Root galls or Root-Knots, These galls grow on a variety of hosts, infect a wide range of plant species, and seriously harm a number of crops all over the world (Trudgill, 1991). In the study of plant nematology, the importance of genetic engineering in producing transgenic plants with nematode resistance genes has thus been constructed. (Atkinson *et al*., 1998). Control strategies can be separated into three categories: The target of the nematode, an interface between nematode-plant, and finally the reactions of plants (McCarter *et al* ., 2005) Various nematode resistance (Nem-R) genes have been identified in plants, and they all give resistance against endoparasites that are stationary.

Different plant species have effectively for the cloning of R genes. Various plant species have successfully by the cloning R genes (DeWit, 1997). Most genes that code for proteins contain a structural motif called Leucine-Rich Repeat that repeats 20–30 amino acids (LRR). R genes contain LRR separated into two categories: extracellular LRR that has a membrane anchor with an amino-terminal and products of resistance gene (R gene) that are cytoplasmic (Bent, 1996). Due to this, wild species of tomatoes (*Lycopersicon peruvianum L.*) were where the RKN resistance to tomatoes was initially identified. (Perry and Maurice, 2013). The dominant single gene called Mi is found in tomato varieties, localized in a small region of tomato's chromosome 6. Mi gene was identified using recombinant DNA sequence analysis. Mi-gene has been detected in some tomato cultivars (Type D, Hybrid Ajyad 7, Hybrid Ajyad 19, and Type A99) genotypes against root-knot nematodes. By using resistant varieties, such as the tomato variety, The plants create their first line of defense against RKN penetration by interactions with external receptor proteins, receptor-like kinases, receptor-like proteins, and nematode effectors. Next, the plant initiates direct (gene-for-gene) interactions as the second line of defense. (Rashed *et al*., 2017). Gene delivery using the nanoparticles is considered non-viral delivery and it can be used via vehicles such as chitosan. Chitosan has been developed for managing crop diseases as an alternative to chemical pesticides. Nanoparticles can enter plant cells and leaves, and transport DNA and chemicals successfully (Riva *et al*., 2011). Our aim in this study is to direct the interaction between tomato receptor proteins against nematode effectors, using chitosan nanoparticles.

MATERIALS AND METHODS

Plant Materials:

Tomato cultivars (*Solanum lycopersicum*, Type D, Hybrid Ajyad 7, Hybrid Ajyad 19, Type A99) which were used in this study and were purchased (obtained) from (Horticultural Research Institute, Research Department of Vegetable Breeding and medicinal aromatic Plants (Agriculture Research center). The seeds were grown in a mixture of clay soil, sandy soil, and peat moss. They were cultivated to isolate the Mi gene and then transport it into three cultivars of cucumber cultivars (Hybrid F1, 20 rough and Sam 19) which were obtained from (SAKATA company, Horticultural Research Institute and Monarch Seed Company). Seeds are grown in a mixture of clay soil, sandy soil, and peat moss as well.

Preparation of Experiment Field and Identification of Nematode:

The tested species of Root-knot nematode was *Meloidogyne*. Adult females were collected in order to identify the species of nematode-based on morphological characteristics that corresponded with the female perineal pattern. (Taylor and Sasser, 1978).

DNA Extraction from Root-knot Nematode (RKN):

The work was carried out at the Nematology lab. DNA extraction was carried out utilizing QIAquick® PCR Purification Kit (50) Cat. no.28104 from Qiagen DNA of female root-knot nematodes was isolated from cucumber plants concurring with the method of (Robin *et al*., 2005).

Mi gene Isolation and Cloning into pEGAD Vector:

Genomic DNA was extracted from tomato leaves and used to amplify the Mi gene according to the procedure of Edward (Lu *et al*., 2011). Each 25µl PCR reaction required the addition of 50ng of template DNA. 12.5 µl of 2x master mix (Biolene), 0.25 µl of Taq polymerase, 1 µl of each 50 nmole forward and reverse primers, and up to 25 µl of sterile water are included in each reaction mixture. Specifically for the Mi 1.2 gene, the primers were designed by snap gene® (2.3.3) utilizing the commonly used gene sequence that can be accessed in the NCBI database under the GenBank accession

number (NM_001247134). The primers were designed using the following sequences: (Mi-F) 5′-GGGTTCTCTAGCTAAACTTC-3′ and (Mi-R) 3′-GAGGAATCTCATCACAGGAT-5′ These primers purchased from Bioline by using the (Kit Cat#BIO-41026). The Mi gene PCR process was run for 35 cycles, which included 30 seconds at 94℃,30 seconds at 64℃, and 50 seconds at 72℃. On a 1.4% (w/v) agarose gel, the PCR result was examined in comparison to the hyper ladder 1Kb by using (Cat. #BIO-33053). The Gene JETTM PCR Purification Kit (Cat. # Thermo K0701) was then used to purify the insert. The ligation protocol was as follows: The Mi gene insert was ligated into pEGAD vector were digested with the same restriction enzymes (EcoRI and HindIII), according to the manual instruction with antibiotic selectable gene Ampicillin, in a concentration of 50 µg/ml; with constitutive promoter which is active in vivo in all conditions (Tawfik *et al*., 2022).

Verified Transformation with Bacterial Method:

Colony PCR was utilized to distinguish recombinant from non-recombinant colonies. The recombinant bacteria were cultured with 100µg/ml ampicillin on LB agar plates. After growing, a single colony from bacterial transformation was utilized as a template for the confirmatory PCR reaction. After that, the product was electrophoresed using the ladder (1 kb) on a 1.4% gel.

Chitosan Nanoparticles:

Characterization of the Chitosan Nanoparticles:

The degree of Deacetylation (DD) of chitosan nanoparticles was determined according to Czechowska-Biskup *et al*., 2012 The titration technique was used by dissolving 0.2 g of powdered chitosan in 25 ml of 0.1M HCl with continuous stirring for 30 min. The solution was titrated with a 0.1 mol dm^3 sodium hydroxide solution. Once the chitosan was completely dissolved, the DD of the chitosan was calculated using the formula:

$$DA[\%] = 2.03 \cdot \frac{V_2 - V_1}{m + 0{,}0042 \cdot (V_2 - V_1)}$$

The sample weight is represented by m, the volumes of 0.1 mol·dm-3 sodium hydroxide solution that corresponds to the deflection points are represented by V1 and V2, the molecular weight of the chitin monomer units is the coefficient of 2.03, and the difference between the molecular weights of the chitin and chitosan monomer units is the coefficient of 0.0042.

Construction of (Chitosan/Plasmid DNA) (CS/pDNA):

Mansouri *et al*., (2006) stated that chitosan was dissolved in 25 mM acetic acid at a working concentration of 0.08 percent, and the pH was adjusted to 5.5 in order to produce CS/DNA. In a water bath, the CS and recombinant pEGAD were incubated for 15 minutes at 55°C. The process of creating the CS/pDNA combination involved adding 0.08 percent CS to a corresponding volume of pDNA solution (50 mg/ml) dissolved in 50 mM sodium sulphate, vortex mixing for one minute, and then using 1% agarose gel electrophoresis to measure the binding between pDNA and CS (X.W. Li *et al* ., 2003).

Transformation of the Chitosan/pDNA into A Cucumber:

Abdel-Razik *et al*., (2017) described the transfection of chitosan nanoparticles containing the recombinant plasmid into cucumber plant as follows: 2 weeks germinated seedlings of cucumber (Hybrid F1, 20 rough and Sam 19) were injected by 300 µl of CS/pDNA using a syringe at the top of the plant stem. The injected plants were left to grow for 4-5 weeks to see the effect of genes in plants. The (GFP) labeled gene showed green fluorescence under a fluorescence microscope, indicating that the cs/pDNA that have been transferred with the green fluorescent protein (GFP) tagged vector.

Molecular Screening of Successfully Transgenic Cucumber:

PCR Analysis:

Tawfik *et al*., (2022) reported that transformants for the Mi gene were screened using a polymerase chain reaction (PCR). the genomic DNAs of transgenic cucumbers aged

four to five weeks were extracted. (Rogers and Bendich, 1985).The following primers were employed in the PCR, which had genomic DNA as its target : (Mi-F) 5′-GGGTTCTCTAGCTAAACTTC-3′ and (Mi-R) 3′-GAGGAATCTCATCACAGGAT-5′. The Oligo 7 program was used to create these primers. 40 cycles have consisted of PCR amplification reactions: denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and finally post-extension at 72 °C for 10 min. All PCR procedures made use of Taq DNA polymerase. The resultant PCR products were separated using agarose gel electrophoresis at a 1.2% (w/v) concentration.

RT-PCR Detection of Transgenic Cucumber Plant:

The RT-PCR Reaction was carried out in three steps as follows: RNA extraction kit (QIAamp ccfDNA/RNA Kit 50 cat. No. 55184), cDNA synthesis and real-time reaction. After four weeks of cultivation, RNA was extracted from the transgenic cucumber using the method described by Jordan *et al*., (2015). The cDNA was produced from total RNA using the SuperScript® III One-Step RT-PCR System and Platinum® Taq DNA Polymerase (Thermo Scientific). Next, Real-Time PCR procedures were carried out to quantify a chosen subset of gene transcripts using PikoReal Real-Time (Thermo Scientific) and SYBER-green as the fluorescent dye, quadrate by the manufacturer's instructions. Using a 1% agarose gel, the amplified PCR products were separated. After each run, a melting curve was collected to exclude the primers that produced non-specific PCR results.

Pathogenicity Test For Root-Knot Nematode In Cucumber Plant:

This test was applied to determine of infection rate and disease index of Root-Knot Nematode on transgenic and non-transgenic Cucumber plants by (the Blender method) (Taylor and Sasser, 1978). Different Nematode pathogens were applied to the transgenes of cucumber cultivars. The incidence was calculated through the following equation:

Infection % = Number of infected plants ÷ Total number of tested plants × 100

RESULTS AND DISCUSSION

This study manipulated the transformation of Mi gene from Tomato (*Solanum lycopersicum*, Type D, Hybrid Ajyad7, Hybrid Ajyad 19, Type A99) to Cucumber cultivars to resist Root-Knot Nematode. This gene was isolated as a Resistance gene from some tomato cultivars (*Solanum lycopersicum*, Type D, Hybrid Ajyad7, Hybrid Ajyad 19, Type A99) and transformed into different cucumber Cultivars (Hybrid Victor F1, Cucumber Rough 20, Sam19) and this transformation occurred using chitosan nanoparticles.

Plant Material and Growth Conditions Infested with RKN:

Tomato seeds (*Solanum lycopersicum*, Type D, Hybrid Ajyad 7, Hybrid Ajyad 19, Type A99) and cucumber seeds (Hybrid F1, 20 rough and Sam 19) were grown in a mixture of clay soil, sandy soil, and peat moss in plant pot as shown in Table (1). Similar to (Weng *et al*., 2021) research, in which he and others grew cucumber from 18°C to 28 °C temperature cycle and from 8hr to 16 hr in darkness. Then seedlings were placed in greenhouse pots.

Table 1. Breeding of plants.

	Tomato					Cucumber		
	Solanum Lycopersicum	Type D	Hybrid Ajyad 7	Hybrid Ajyad 19	Type A99	Cucumber Sam 19	Cucumber Hybrid Victor F1	Cucumber strain 20 rough
Reproductive period	One-month	One-month	One-month	One-month	One-month	One-month	One-month	One-month
Mi Gene	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Detected Mi gene	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Resistance	(+)	(-)	(-)	(-)	(+)	(-)	(+)	(+)

Mi gene Detection and Cloning:

Total genomic DNA from tomato cultivars was extracted and PCR with Mi gene-specific primers was run on 1.2% agarose gel with 1Kb ladder (NEB). The Mi gene was detected at 1000 bp. However, our results were consistent with those of Tawfik and Fathy (2022), who reported that using UidA-specific primers and polymerase chain reaction analysis, the UidA gene was found in *Escherichia coli* (K12 strain), and its molecular weight was 1812 bp. Colony PCR was used to verify transformation into bacterial cells using the selected transformed bacterial colonies that were cultivated on LB medium supplemented with 100 µg/L ampicillin. A toxic mini gene and pEGAD, a small bacterial vector, are utilized for high-efficiency cloning and the subsequent in vitro transcription of PCR results. Colony PCR was performed using specific modified bacterial colonies that were cultivated on LB medium supplemented with 100 µg/L ampicillin, in a manner similar to that described by (Fathy *et al*., 2022) who used pMiniT which is a compact bacterial vector that employs a toxic minigene for high-efficiency cloning and subsequent in vitro transcription of PCR products. It contains SP6 constitutive promoter (for bacteriophage SP6 RNA polymerase).

Transformation of CS/pDNA into Cucumber Plant:

In this study, CS/pDNA was successfully generated under specific instructions and used as a reliable gene delivery mechanism. Chitosan nanoparticles were used to modify plant genes. This was in agreement with (Abdel Razek *et al*., 2017), who transformed *Paulownia sp.* and *Echinacea purpurea* plants using chitosan nanoparticles as a carrier of pBDG expressing the GFP gene. Using a transmission electron microscope, the following parameters of the medium molecular weight (501 KDa) and pH 4.4 particles were measured: average DD of 51.23 ± 5.765%; average particle size of 43.98 ± 1.24 nm (Fathy, 2022). It was found that a 1:1 ratio of chitosan polymers to plasmid (pDNA) was optimal for complex formation. Positive-charged chitosan polymers were shown to have a high propensity to form complexes with DNA (-ve charge). The molecular weight of CS determines the capacity of pDNA complexes Chitosan polymers with a positive charge were shown to have a high propensity to form complexes with DNA (-ve charge). According to agarose gel electrophoresis of complexes, the ideal complex formation ratio between CS and plasmid (pDNA) was 1:1. The molecular weight of CS is typically used to measure its capacity to encapsulate pDNA complexes.

According to (Czechowska-Biskup *et al*., 2012), A simple titration process was employed to quantify DD evaluations of amine or acetyl amine groups on the glycoside unit of chitosan nanoparticles. The positive charge of this group and how it interacts with the negative charge of DNA dictate the affinity between the nanoparticles and pDNA.These results were consistent with the research of (Kiang, 2004), who recommended keeping a constant DNA concentration while changing the concentration of chitosan. According to (Hallaj-Nezhadia *et al*., 2011), DNA binding is dependent on the chitosan's molecular weight and degree of deacetylation, indicating that the shape and size of the particles play a major role in transferring genes into cells.

Confirmation of Transformation of CS/pDNA into Transgenic Cucumber:

Molecular Analysis of Transgenic Cucumber: The presence of the Mi gene was detected in transformed cucumber plants using the conventional PCR technique. Although it was the same method Mansouri *et al*., (2006) reported. Transformation succeeded in the three cucumber cultivars lines (Hybrid F1, 20 rough, and Sam F1) and showed 2800 bp (Fig. 1). These results are similar to Cingel *et al*., (2010), who used PCR to verify that the OC-II and OC-I genes had been transformed into potato plants. In order to determine whether the transformed potato plants contained the plasmid pSKI074, Cingel *et al*.,(2010) used PCR.

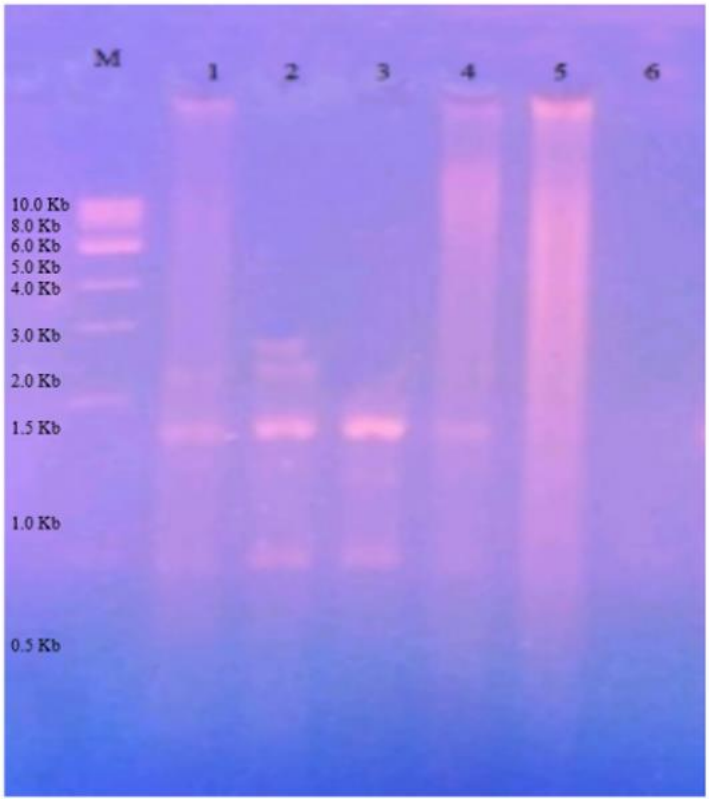

Fig 1. PCR for Mi gene detection in cucumber cultivars 1, 2, 3 were; transgenic; and 4, 5, 6 were; non-transgenic; cucumber. 1: transgenic infected Hybrid F1, 2: transgenic infected 20 Rough, 3: transgenic infected Sam F1, 4: non-transgenic infected Hybrid F1, 5: non-transgenic infected 20 rough, 6: non-transgenic infected Sam F1 (M: Berus 1kb ladder from willow fort company).

CS/pDNA Into Transgenic Cucumber Line:

After 4 weeks, the regenerated cucumber plants are fully grown and multiply, and Figure 2 shows that the blue color is expressed due to the expression of the GFP gene. The (GFP) labeled gene showed green fluorescence under a fluorescence microscope, indicating that the cs/pDNA that have been transferred with the green fluorescent protein (GFP) tagged vector. The percentage of shoots or leaves that were GFP-positive was used to assess the efficiency of transformation (TE%), and the percentage of explants used to represent control plant transformation was computed as (T0%) (Selvaraj *et al*., 2010). In certain studies, GFP was also used as a reporter gene. The number of confirmed or validated T0 or advanced-generation transgenic plants is used to estimate TE. Given that a single seed may produce numerous transplants, the number of seeds and explants utilized in a study should also be specified for a simple comparison. Nanasato *et al*., (2013) discovered that the transformation efficiency of the Japanese cucumber line Shinhokusei No. 1 ranged from 7.5-16%, with an average of 11.9 ± 3.5%. The TE was 21% or 8.5% for Agrobacterium strains LBA4404 or EHA105 in PS76.

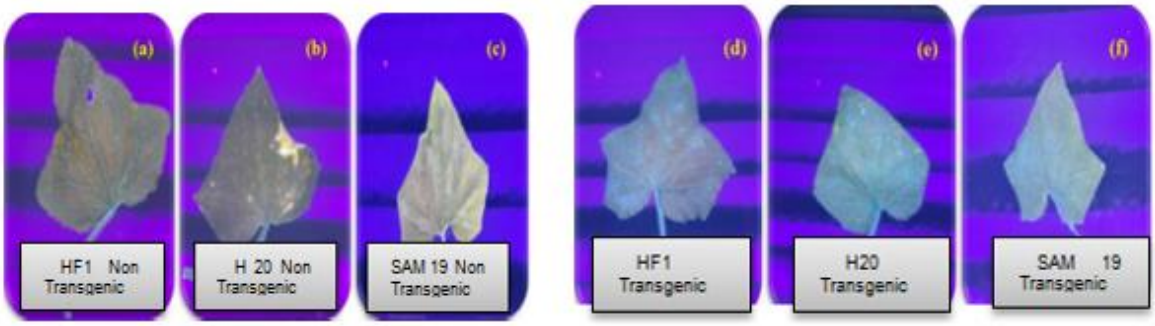

Fig 2. Expression of GFP reporter gene in transgenic cucumber cultivars lines compared to non-transgenic lines.
(a) non-transgenic infected Hybrid F1, (b) non-transgenic infected Hybrid 20 rough, (c) non-transgenic infected Sam 19, (d) transgenic infected Hybrid F1,(e) transgenic infected Hybrid 20 rough,(f) transgenic infected Sam 19.

RT-PCR:

The one-step pre-amplification reaction for qPCR was optimized in this study, and the universal RT-qPCR reaction technique was modified. The following were the most suitable RT-qPCR amplification Thermo cycling conditions: Reverse transcription was run for 5 minutes at 52°C. Following that, the PCR step, which consisted of 10s denaturation cycles at 95°C, 15 cycles of 5 s at 95°C and 30 s at 60°C that did not collect fluorescence signals, and 40 cycles of 5 s at 95°C and 30 s at 60°C that did to identify the transcript expression, RNAs were reverse-transcribed into cDNA and selected regions of the Mi gene were amplified. A product was revealed by the RT-PCR results (green "sample 2," red "sample 3," and black "sample 4") (Fig. 3). These results are consistent with studies by Wang *et al.,* (2019),
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These findings are in line with research conducted by Wang *et al*., (2019), who assessed the level of LL gene expression in LLOE transgenic plants using quantitative real-time PCR (qPCR). Thermo Fisher Scientific, located in Waltham, Massachusetts, USA, provided both the reverse transcription kit and the micro kit for plant RNA purification. They served to separate total RNA by using the cucumber Cs Actin gene as an internal reference, the qPCR was performed on a Quant Studio™ 3 Real-time PCR instrument according to Yang *et al*., (2018) method using the $2^{-\Delta\Delta CT}$ approach, the relative expression level was determined. Pairwise t-tests were used to establish significance, and transformation efficiency (TE%) was calculated as the proportion of plantlets that tested positive for PCR across all separate transformation cycles

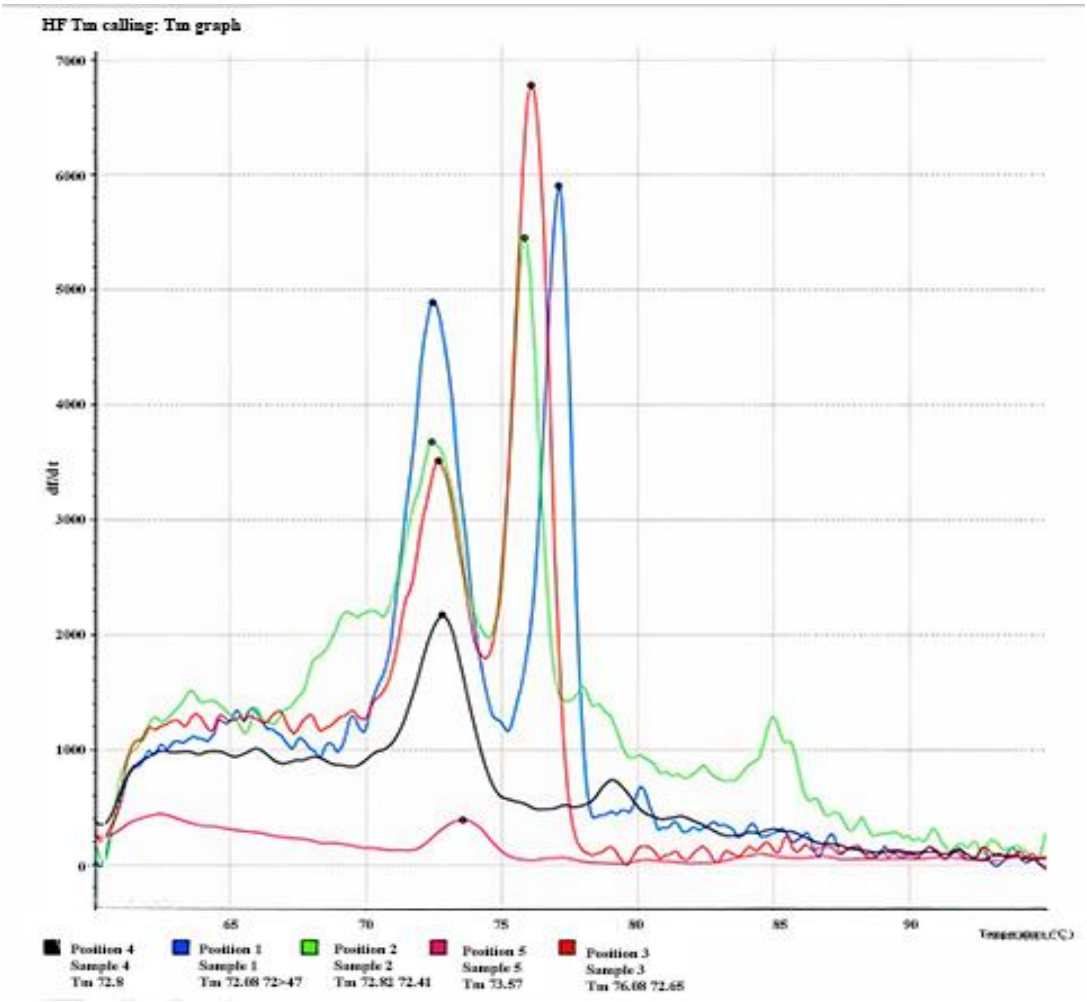

Fig 3. RT-PCR melting curve

The figure illustrated the SYBR Green technique applied to detect the quantitative expression of Mi gene within the transgenic cucumber lines. The distinct fusion curves for the PCR products of Mi gene from Transgenic cucumber lines (showing distinct colors). Amplification reactions can be seen for a specific product (green "sample 2 HF1 at 72.41℃", red "sample 3 H_2O at 72.65 ℃ ", black "sample 4 Sam 19 at 72.8℃ ").

Pathogenicity Test:

Morphological Measurement of Cucumber Cultivars:

The rate of infection of transgenic and non-transgenic cucumber plants with root-knot nematode was calculated after transferring mi gene to it with chitosan molecules to find out the effect of nematodes on resistant and non-resistant cucumber varieties to nematode damage according to the following equation in Table 2 :

Infection % = Number of infected plants ÷ Total number of tested plants × 100

According to Taylor and Sasser (1978), a plant is considered slightly resistant (SR) if nematode reproduction on it is between 25 and 50% of that on the susceptible host; moderately resistant (MR) if it is between 10 and 25%; very resistant (VR) if it is between 1 and 10%; highly resistant (JIR) if it is less than 1%; and immune if it is 0%. Our results were in line with these findings. In this technique, nematode reproduction is represented by the total number of eggs and juveniles extracted from the soil and roots of afflicted plants. Depending on the susceptible cultivar selected for comparison, the rating of resistance assigned to a specific cultivar can differ significantly as indicated in Table (3), as this scale was created somewhat arbitrarily

both transgenic and non-transgenic cucumber cultivar lines' measured morphological characteristics were examined. Transgenic lines were found to be higher in the parameters that were examined, and the nematode's effects were morphologically shown through fresh weight, shoot length, leaf number, and symptoms of cucumber plants after nematode infection as illustrated in Table (4).

Table 2. The Effect of Nematode in transgenic cucumber.

Cultivar	Field of study	Percentage of Infection	Pathological Evidence	(MR) or (SR)
C. strain 20 Rough	Agricultural Land	66%	2	Moderately Resistant (MR)
C. strain 20 rough duplicate	Agricultural Land	60%	2	slightly resistant plant (SR)
Hybrid F1	Agricultural Land	60%	2	Moderately Resistant (MR)
Hybrid F1 duplicate	Agricultural Land	53%	2	slightly resistant plant (SR)
Sam 19	Agricultural Land	50%	2	slightly resistant plant (SR)
Sam19 duplicate	Agricultural Land	37%	2	slightly resistant plant (SR)

Table 3. Comparison of morphological Cucumber cultivars.

Pathogenic nematode	Hybrid F1	20 rough	Sam 19
Transgenic cucumber			
Non-transgenic cucumber			

Table 4. Measurement of morphological parameters of Cucumber cultivars

Cultivar	Line	Fresh weight (g)	Shoot length (cm)	Leaf number
Hybrid F1	Transgenic	33.406±5.0056	39.233±6.057	10±0.04
	Non-transgenic	23.906±6.0056	22.733± 5.11	8±0.005
C. strain 20 rough	Transgenic	44.84±6.0173	54.13±0.115	16±0.07
	Non-transgenic	42.456±8.643	48.9±8.034	6±0.014
Sam 19	Transgenic	51.543±7.014	51.466±9.05	6±0.025
	Non-transgenic	43.54±7.0173	46.233±9.115	10±0.04

Conclusion

In the current study, a new transformation method was used to transform mi gene (resistant nematode gene) using chitosan particles. The aim was to transfer the nematode-resistant genes from resistant nematodes plants such as cultivars (*Solanum lycopersicum*, Type D, Hybrid Ajyad7, Hybrid Ajyad 19 and Type A99) in tomato plants acquired from *Solanum lycopersicum*, Type 99 into sensitive to nematode infection such as cultivars (Hybrid F1, 20 rough and Sam 19) in cucumber plants. Our results prove that some of the plants had been transgenes while others failed to have the gene transferred to them. In the future experiment, we will proceed to explore more cultivars of nematode-sensitive plants to try to transfer the resistant genes to them using the same technique, chitosan nanoparticles.

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