Tfg-Mi, a root-knot nematode resistance gene from fenugreek (*Trigonella foenum-graecum*) confers nematode resistance in tomato

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ABSTRACT

Several root-knot nematode (Meloidogyne spp.) resistance genes have been discovered in different plants. For the first time, the Mi gene from fenugreek (Trigonella foenum-graecum) was detected in this study. A candidate root-knot nematode resistance gene (designated as Tfg-Mi) was isolated from the resistant fenugreek line Giza 3 by degenerate PCR amplification combined with the RACE technique. Also, another two candidate root-knot nematode resistance genes (designated as To-Mi11 and To-Mi12) were isolated from the resistant tomato (Lycopersicon esculentum) line Nemagard. Expression profiling analysis revealed that both genes were highly expressed in roots, leaves and flowers and expressed at a lower level in stems, but are not detectable in fruits. To verify the function of Tfg-Mi, a sense vector containing the genomic DNA spanning the full coding region of Tfg-Mi was constructed and transferred into root-knot nematode susceptible tomato plants. Sixteen transgenic plants carrying one to five copies of T-DNA inserts were generated from two nematode susceptible tomato cultivars. RT-PCR analysis revealed that the expression levels of Tfg-Mi gene varied in different transgenic plants. PCR assays showed that the resistance to root-knot nematodes was significantly improved in some transgenic lines compared to untransformed susceptible plants, and that the resistance was heritable.

Keywords: *Tfg-Mi, To-Mi11 and To-Mi12, gene sequencing, cloning, root-knot nematode, fenugreek, transgenic tomato.*

INTRODUCTION

Plant parasitic nematodes are important endoparasitic pests of many crop species and cause crop losses about 100 billion US dollars each year all over the world (Cai *et al.*, 1997). Among them, root-knot nematodes (*Meloidogyne* spp.) are the most contributors and spread world wide. These parasites are prevalent in the open fields and in controlled environment production systems (Ammiraju *et al.*, 2003). Disease symptoms are characterized by the presence of galls or root knots on infected plants.

These root knots alter the uptake of water and nutrients and interfere with the translocation of minerals and photosynthates in the host (Milligan *et al.*, 1998), resulting in

plant poor yield, stunted growth, wilting, and susceptibility to other pathogens.

Current control of root-knot nematodes is deficient and needs an integration of the combined of several pest management strategies. The cultural control is widely practiced, but rotation is of limited value for nematodes with a host range as wide as that of *Meloidigyne* spp. (Trudgill, 1997). Another control is dependent on environment hazardous chemical pesticides. But this has become difficult due to the withdrawal of effective nematicides and soil fumigants from the market (Oka and Cohen, 2001).

Potentially, the use of resistance cultivars is the most efficient and environmentally safe control measure to retard invasion by the root-knot nematodes (Holliday, 1989). However, only *Mi* gene confers resistance to root-knot nematodes, which and it was introgressed to *Lycopersicon esculentum* from a wild relative *L. peruvicanum* in the early 1940s by embryo rescue (Smith, 1944). It is difficult to be introduced into other susceptible cultivars by cross breeding between *L. esculentum* and *L. perurianum* DNA (Messeguer *et al.*, 1991). Milligan *et al.* (1998) had cloned the *Mi* gene from the resistant tomato. But in Egypt, there is no report about this gene, besides, no resistance cultivar can be used.

Resistance to root-knot nematode was first identified 60 years ago in an accession of *Lycopersicum peruvianum Mill*, a wild relative of cultivated tomato (*L. esculentum Mill*) and originated in the western coastal region of South America (Watts, 1947). The single dominant *Mi* gene of tomato confers resistance to three major root-knot nematodes (Gilbert and McGuire, 1956) and has been isolated by positional cloning approach (Milligan, *et al.* 1998). It shares several structural motifs with other *R* genes, including NBS and LRR domains, which are characteristic of a family of plant proteins that are required for resistance against viruses, bacteria, fungi and nematodes. The *Mi* locus contains three open-reading frames. Two of them, *Mi-1.1* and *Mi-1.2*, appear to be intact genes; while the third is a pseudogene. Complementation studies revealed that *Mi-1.2*, not Mi-*1.1*, was sufficient in conferring resistance to *M. javanica*. When nematodes became attracted to and penetrate the roots, the *Mi* gene triggers a localized tissue necrosis or hypersensitive response. Nematodes failed in such cases to establish feeding sites and then either died or leave the roots. In *Capsicum*, several root-knot nematode resistance genes have been discovered (Fery and Dukes, 1984; Thies *et al.*, 1997). As early as Hare (1956) identified a dominant gene (named *N*), which confers resistance to *M. incognita* in the *C. frutescens* L. "Santanka XS" line. More recently, Di Vito *et al.* (1992) discovered high levels of resistance to root-knot nematode in some lines of *C. chacoense* Hunz., *C. chinense* Jacq. and *C. frutescens*. Hendy *et al.* (1983) found that two *C. annuum* lines, were resistant to a wide variety of root-knot nematode populations.

Scope of the study

In this study, we screened various accessions of fenugreek (*Trigonella foenum-graecum*) for nematode resistance and then cloned a root-knot nematode resistance gene (designated as *Tfg-Mi*) and also from tomato as a control. The function of this candidate gene was further verified by transforming the genomic DNA containing the full coding region of *Tfg-Mi* in sense orientation into susceptible tomato cultivars.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of fenugreek and tomato were grown at 25±2^{O}C for 7 days in dark on MS medium. For isolation of the gene and its

expression analysis in tomato and fenugreek, the root, stem, leave, flower and fruit of the root-knot nematode resistant tomato line Nemagard were harvested from plants growing in the greenhouses of the National Research Centre,Giza,Egypt. Two root-knot nematode susceptible tomato cultivars Edkawy and Super strain B were used for generating sense *Mi* transgenic plants.

Isolation of plant genomic DNA

Genomic DNA was isolated on a mini-prep scale as mentioned by Murray and Thompson (1980) with some modifications. Small pieces of leaf tissues 0.5 gm) were frozen in liquid nitrogen in Eppendorf tubes and homogenized in (500 μl extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl, pH 8.0, 100 mM βME). The extract was incubated at 60^{O}C for 20 min and then 500 μl of phenol: chloroform: isoamyl alcohol were added (24:24:1) and mixed by vortexing for 30 sec followed by centrifugation at 10,000x g for 5 min at room temperature. The aqueous phase was transferred to another tube and this was once again extracted with 500 μl of chloroform: isoamyl alcohol (24:1) in Eppendorf tube. To the aqueous phase, 0.6 volume of isopropanol was added to precipitate the genomic DNA and spooled the fibrous genomic DNA. Genomic DNA was then washed thrice with 70% ethanol, dried vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37^{O}C for 30 min, followed by extraction with phenol: chloroform: isoamyl alcohol (24:24:1) and the aqueous phase was transferred to a fresh tube. There after, the genomic DNA was precipitated by adding 0.3 M sodium acetate, pH 5.2 and 2.5 vol of ethanol and collected by centrifugation at 10,000 x g for 20 min at 4^{O}C. The pellet was washed with 70 % ethanol, vacuum dried and dissolved in TE.

Isolation of *Tfg-Mi* and *Mi* genes

Genomic DNA was extracted from young leaves of fenugreek and tomato plants using the method described by Fulton *et al.*, (1995). A pair of specific primers was designed according to the public sequence of *Mi* gene (AF039682) with an additional 12 bp sequence from the *attB* core region at the 5' end. Amplification reaction (25 µl) containing PCR buffer (2.0 mM MgSO4, 0.2 pM of AM-FW1 and AM-RV1 primers, 0.2 mM dNTPs, 1.0 U *Taq* polymerase, 0.25 U *pfu* polymerase, and 100 ng template DNA). PCR was performed as follows: 94 °C for 5 min; then 94°C for 30 s, 49 °C 1min, 72 °C for 2 min, 35 cycles; followed by 72°C for 10 min and held at 4°C using a PTC-100 Thermal. Combination with the specific primers, (5 µl) this PCR product was used as a template, to perform the second-step PCR as follows: 94°C for 5 min; then 94°C for 30s, 45°C for 1min, 72°C for 7min, 5 cycles; 94°C for 30s, 55°C for 1min, 72°C for 7min, 15 cycles; then 72°C for 10 min and held at 4°C.

The target PCR product was cleaned up by gel extraction kit (Promega) and cloned into the pGEM-T vector kit (Promega), then transformed into *E. coli* DH5α. Positive clones were confirmed by restriction analysis and sequencing.

Bacterial strain

DH5α competent cells were made by the protocol of Hanahan (1985).

Agrobacterium tumefaciens strain LBA4404 with pBI121 (Jefferson, 1987) was used in this study. *A. tumefaciens* was grown for 2 d in YM medium (0.4 g/L yeast extract, 10 g/L mannitol, 0.1 g/ L NaCl, 0.2 g MgSo4, 0.5 g/L) containing the appropriate antibiotics at 28 °C on a rotary shaker (220 rpm) until an OD600 = 1.0 was obtained.

Plant Transformation

Explants were isolated and cultured onto the preculture media for a one-day preculture period. At the end of the one-day preculture, the explants were dipped in an *Agrobacterium* culture, blotted and recultured on the same media for a 3-day co-cultivation period. The explants were then transferred to fresh media of different plant growth regulator combinations containing 100 mg kanamycin with 300 mg Carbenicillin to control *Agrobacterium* growth.

Establishment of the protocol of callus and shoot formation and regeneration conditions for tomato *(Lycopersicon esculentum)*

Seeds of two tomato cultivars (Super strain B and Edkawy Egyptian cultivar) were surface-sterilized in 2% sodium hypochlorite for 10 min and rinsed three times with sterile distilled water.

After germination, the midribs of the leaves were removed and the remaining parts of the leaves were sectioned into 1-2 cm^2 random pieces. These leaf pieces were referred to as explants. Such explants were used for production of callus culture according to the methods described by EL-Kazzaz *et al.* (2001). For callus and shoot formation, leaf explants were cultured (upside-down) on a Murashige and Skoog- (1962) (MS) medium, supplemented with 1.0 mg/l benzyl amino purine (BAP), 0.1 mg/l Naphthaleneacetic acid (NAA), 3% sucrose and 0.7% (w/v) agar (MSA medium). pH was adjusted to 5.7 with NaOH and the medium was sterilized by autoclaving at 121°C for 15 min at 1 kg/cm2 pressure. The cultured plates were incubated for 16 hr light cycle and were subcultured every 3 weeks. After callus and shoot development, the shoots were excised from the callus and transferred to MSA medium without growth regulators (MSC-) or with IAA (MSC+) for root formation and further development of the shoots.

Cloning of Mi 11 and Mi12-genes

Taq polymerase, dNTPs (deoxynucleoside triphosphate) and convergent primers achieved amplification of the DNA fragment. The reaction conditions for PCR involved denaturation at $94^{O}C$ for 30 sec, annealing at $52^{O}C$ for 30 seconds and extension at $72^{O}C$ for either 2 min for cloning DNA in plasmid or 2.5 min (for both *-mi gene* amplification) and 2 min (for *-Mi* genes + *GUS* fusion). After 30 cycles of amplification, an aliquot of this reaction mix was loaded onto a 0.8 % agarose gel and checked.

Establishment of the protocol of *Agrobacterium – mediated* transformation for tomato (*Lycopersicon Esculentum)*

The transformation procedure was followed as described previously by Hsieh *et al.*, (2002).

Plasmids pBI121

Plasmid pBI121 was obtained from ICGEB, New Delhi, India. which contains the gene of interest *gus-Tfg-Mi* controlled by *CaMV 35S* and the *nos* terminator. It also harbors the selectable marker gene *nptII* under the control of the *nos* promoter and *nos* terminator (Fig. 1).

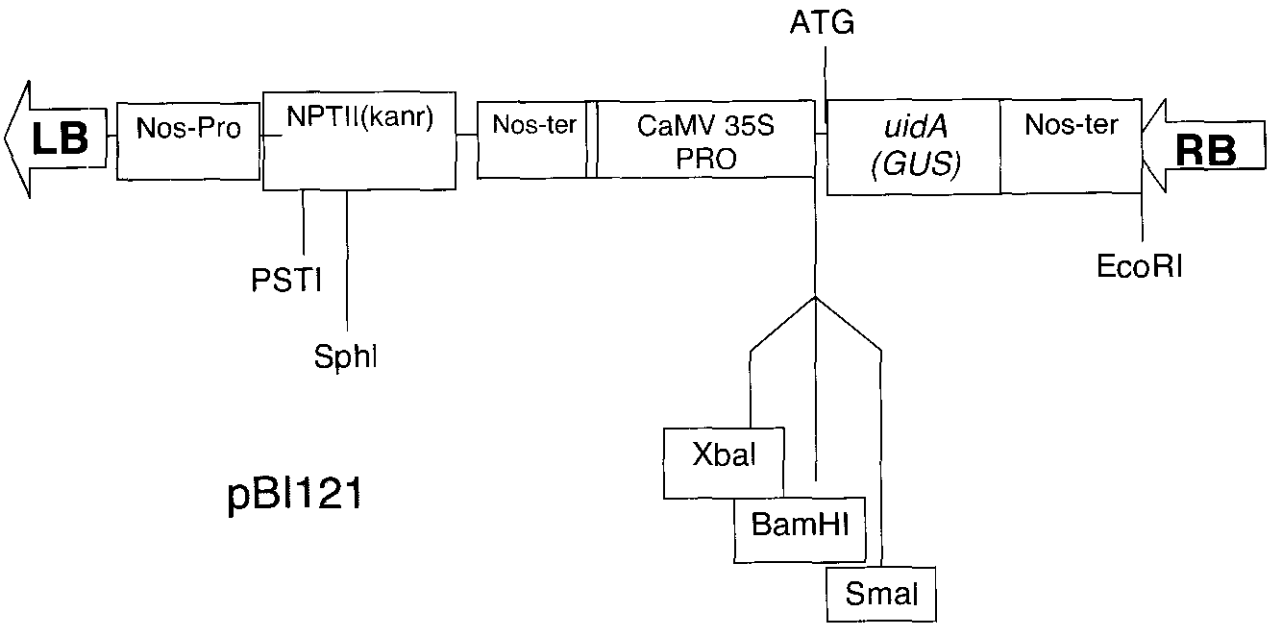

Fig. (1): The pBI121 plasmid map. Positions of restriction sites are indicated.

Isolation of plasmid DNA

This method was adopted from Sambrook *et al.* (1989).

Preparation of *Agrobacterium*

Single colonies were produced by streaking out the bacteria from a 20% glycerol stock kept at -80°C. Semi-solid YM medium was supplemented with kanamycin at 50 μg/ml for pBI121 and for pBI121- *Mi12 and Tom-Mi12* genes kanamycin and streptomycin at 100 μg/ml and 300 μg/ml. The Petri dishes were incubated 48 hr at 28°C and 3 ml liquid YM cultures were inoculated with appearing colonies. After 24 hr of incubation at 28°C and 210 rpm, cultures were diluted 20-fold in liquid YM medium supplemented with the appropriate antibiotics.

Transformation of tomato explants with the *Tfg-Mi12* and *Tom-Mi12* genes

Genomic DNA fragments containing the full-length *Tfg-Mi12* and *Tom- Mi12* genes were introduced into the nematode susceptible tomato cultivar Edkawy with the pBI121 T-DNA construct by *Agrobacterium* transformation.The two transgenic tomato lines harboring this construct are termed pBI121-*Mi11, Tfg-Mi, and Mi12* genes, and pBI121-*Mi12* and *Tom- Mi12* was constructed by subcloning a 1050 bp fragment from the genomic DNA clone bearing the *Mi12* gene and 1450 bp fragment from the genomic DNA *Mi12* gene into the T-DNA vector pBI121. pBI121 was chosen from four transgenic tomato lines for these studies because it is the largest of two clones that have show to confer nematode resistance in transgenic tobacco and tomato plants.

Amplification of an *Mi*-Specific PCR fragment in transgenic tomato explants

Mi11 and Mi12 genes were isolated by reverse transcriptase-PCR from 4-week-old tomato leaves as described previously (Whitham *et al.*, 1996). The presence or absence of the pBI121 T-DNA construct bearing the *Mi11 and Mi12* genes was

determined by isolation of DNA from different progeny and analysis by PCR using the *Mi1 and Mi2* specific primers : *Mi1F* (5'-GGA AAA GAC GGA GAT GAA GG-3') and *Mi1R* (5'-TCA CAA CAG AGG ACC CAC AG-3') for *Mi1* gene and *Mi2F* (5'-ATC ATT CTT TGG GGA TGC TG -3') and *Mi2R* (5'-AGC AAT CGA AGG TCA AGA GG -3') for *Mi2* gene to amplify a 1050 and 1450-bp product, respectively. The parameters of the PCRs were 94°C for 1 min, 52°C for 30 sec, and 72°C for 2 min for 35 cycles with 250 ng of tomato genomic DNA and 0.5 pM of each primer.

5'and 3' RACE PCR to isolate cDNA of *Mi 12 gene*

Based on the sequence data of the isolated *Mi12* gene, two primers were synthesized for the 3¯ RACE (mi12r1 5'-AGC AAT CGA AGG TCA AGA GG -3'; 5'- ATC ATT CTT TGG GGA TGC TG -3') and two for the 5'RACE (5'- GGA AAA GAC GGA GAT GAA GG -3 '; 5'- TCA CAA CAG AGG ACC CAC AG -3'). Total RNA from fenugreek and tomato was used in the RACE analysis. The cDNA synthesis and subsequent PCRs were performed according to the kit protocol (GibcoBRL, Rockville, MD). Several clones from the 5¯ and 3¯ RACE PCR were analyzed and an overlapping consensus sequence of 850 bp was assembled. Based on this sequence, two primers (5'- ATC ATT CTT TGG GGA TGC TG -3'; 5'- TCA CAA CAG AGG ACC CAC AG -3') were designed to amplify a cDNA from fenugreek and tomato. The 2.186 -kb fragment was isolated from a lowmelting agarose gel and purified with the Wizard PCR preps (Promega), cloned into pGEM-T- vector (Promega) and sequenced as described above.

Confirmation of transgenic plants by PCR amplification for the coding sequence from fenugreek and tomato *Mi12,* Tfg-Mi, *and Mi12* genes

Based upon the sequence of the N-terminus and C-terminus, two specific oligonucleotides were synthesized; one for the N-terminus region of - *Mi11,* Tfg-Mi, *and Mi12* genes and the other for the C-terminus region. Using these primers the complete coding sequence for the tomato - *Mi11 and Mi12* genes were PCR amplified (0.5 pM of each primer, 200 µM dNTps, 2.5U Taq DNA polymerase in a 50 µl reaction; 94°C for 1 min; 55°C for 1 min and 72°C for 4 min; 25 cycles) using tomato total gDNA as a template. Each of the 1.05 and 1.450 kb amplified product was gel purified and cloned into pGEM -T-easy vector (Promega, USA) as per the manufacturer's instructions.

Homology and structural comparison of fenugreek *Tfg-Mi* to other tomato *Mi11* and *Mi12* genes

Most of the sequence (protein and DNA) analyses were performed using CLCVector program and Genbank database. Homology searches were done using FASTA. Multiple sequence alignment was done using CLUSTALW.

RESULTS AND DISCUSSION

Root-knot nematodes of the genus Meloidogyne are economically important pathogens for a wide range of crops. Infective second-stage juveniles of these obligate endoparasites penetrate the roots of the host and migrate intercellularly to the vascular cylinder (Williamson and Hussey, 1996). The primary symptom of root-knot nematode infection is the formation of typical root galls on the roots of susceptible host plants. Their invasion of the root system of the host plants

results in a shallow, knotted root system and susceptibility to other pathogens. Nutrient and water uptake are substantially reduced because of the damaged root system, resulting in weak and poor yielding plants (Ammiraju *et al.*, 2003).

Screening the accessions of fenugreek and tomato against root-knot nematodes

Eight tomato lines and the fenugreek cultivar, Giza 3, were tested for their resistance to root-knot nematodes. After the *M. incognita* infection, six tomato lines and the fenugreek line Giza 3 showed resistance while the other two tomato lines (Super strain B and Edkawy Egyptian cultivar) were all susceptible. And this was confirmed by the PCR for the presence of the *Mi11 and Mi12* genes,where the *Mi11* was found in all the plant lines especially in tomato plant . it was found that the absence of the *Mi12* leads to susceptible plant (Fig 3A and 3B) the presence or absence of the *Mi11 and Mi12* genes was determined by isolation of DNA from different progeny and analysis by PCR using the *Mi11* and *Mi12* specific primers: *Mi11F* and *Mi11R* for *Mi11* gene and *Mi12F* and *Mi12R* for *Mi12* gene to amplify a 850 and 1450-bp product, respectively.

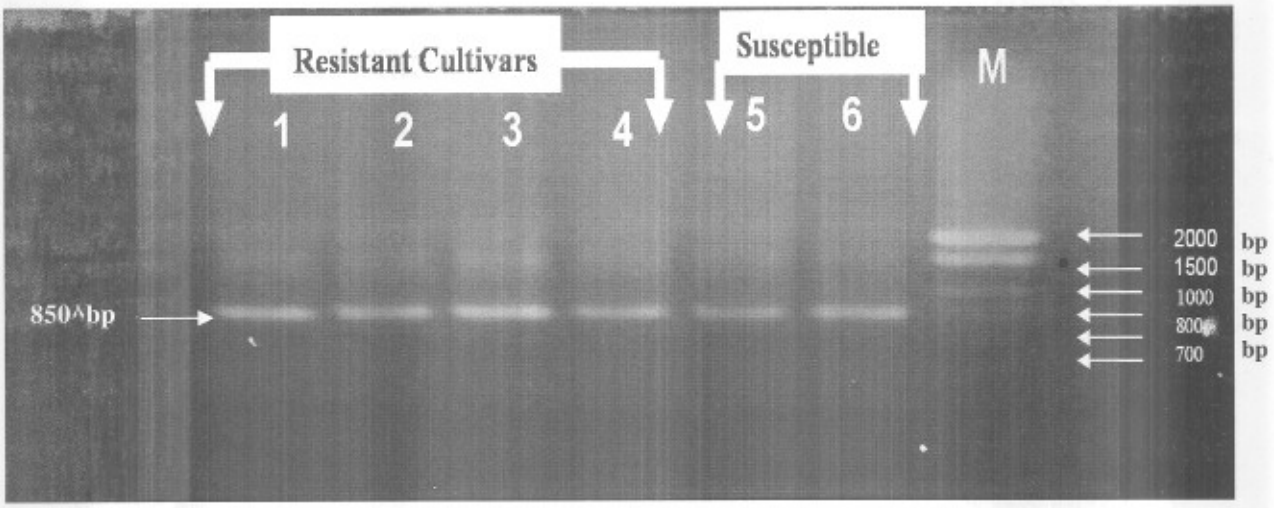

Fig.(2A): PCR amplification for screening of the presence of Mi11 gene with different tomato species . Lanes from 1 to 6 using Mi11-F and Mi11-R primers and M refer to DNA standard. The samples from 1 to 6 refer to: 1-Malika, 2-Fayrouz, 3-Nemagard, 4-Jampact, 5-Super Marmand and 6-Super Strain B.

Isolation of fenugreek *Tfg-Mi* and tomato *Mi11* and *Mi12* genes and sequencing analysis

The resistant tomato and fenugreek lines were used to isolate the fenugreek *Tfg-Mi* and tomato *Mi11 and Mi12* genes. Using the specific primers to amplified the Mi11, *Tfg-Mi and Mi12* by *Mi11F* and *Mi11R* for *Mi11* gene and *Mi12F* and *Mi12R* for *Mi12* and *Tfg-Mi* genes were used to amplify a 850 and 1450-bp product, respectively. The parameters of the PCRs were 94°C for 1 min, 52°C for 30 sec., and 72°C for 2 min for 35 cycles with 250 ng of tomato genomic DNA and 0.5 pM of each primer (Fig. 3 A and B) .

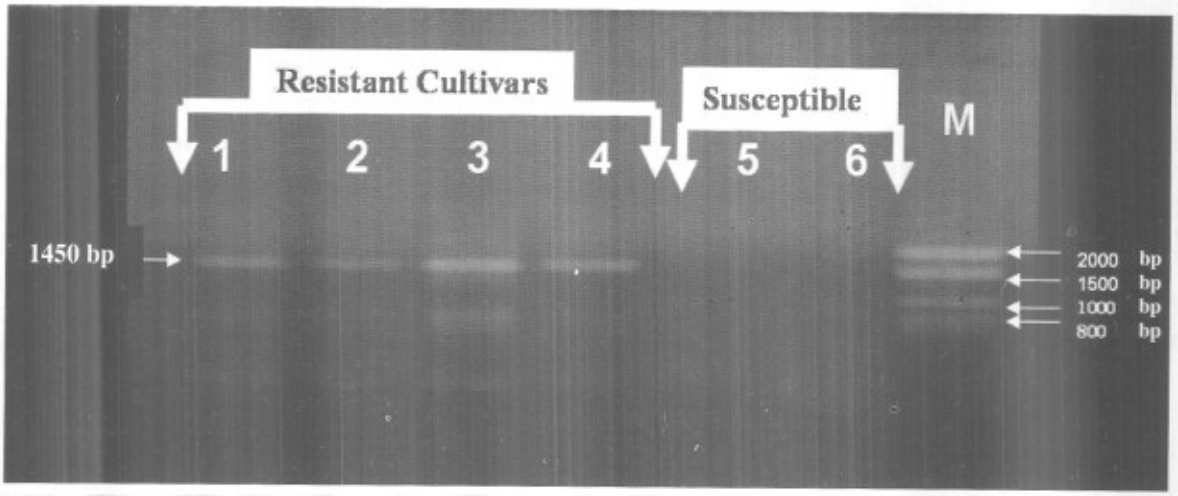

Fig.(2B): PCR amplification for screenings of the presence of MI 12 gene with different tomato species . Lanes from 1 to 6 using MI12-F and MI12-R primers and M refer to DNA standard. The sample from 1 to 6 refer to: 1-Malika , 2-Fayrouz ,3-Nemagard ,4-Jampact ,5-Super Marmand and 6-Super Strain B .

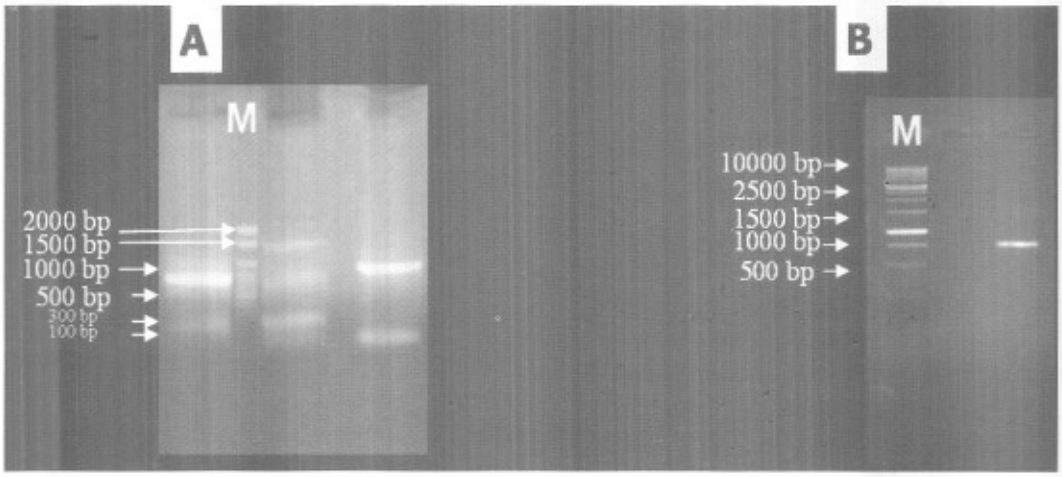

Fig. (3): (A) - Photograph of a gel showing PCR products from tomato (1), and Fenugreek (2) (B) - Photograph of a gel showing the purification of the PCR products from tomato (3) before going to pGEM-T cloning and sequencing.

The fragment was cloned into the pGEM-T easy vector with site-specific recombination. Recombinant plasmid pGEM-T was confirmed by digestion of *XbaI* and *BamHI* (Fig. 4A and B) and by sequencing.

PCR Screening using different primers of pGEM-T-Mi12 construct (Fig. 5).(T7 and Sp6) primers present in the pGEM-T-easy-vector.

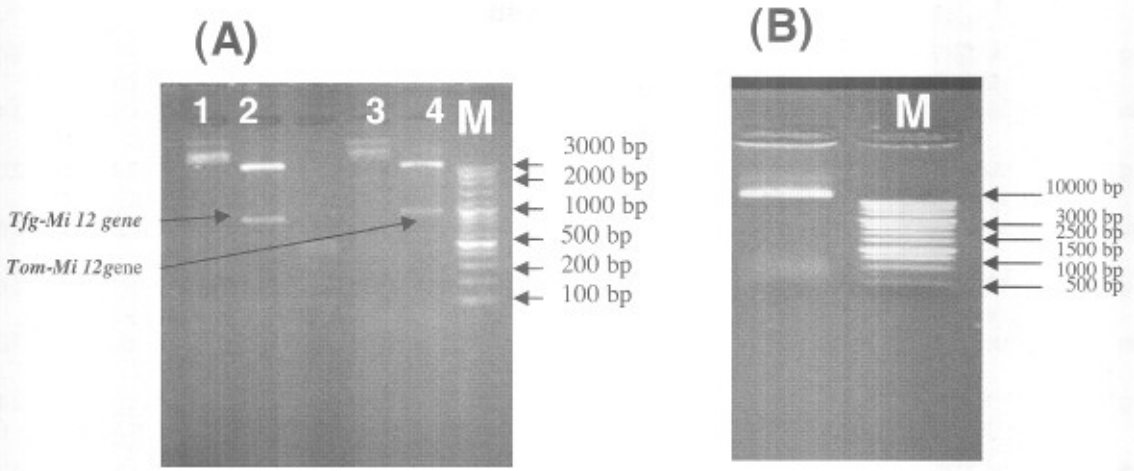

Fig. (4): A gel showing restriction digests of (A) pGEMT plasmid containing the Tfg-Mi12 gene and TO-Mi12 gene 1,3 undigested clones 2,4 digested clones (B) pBI121 plasmid by XbaI and BamHI Enzyme.

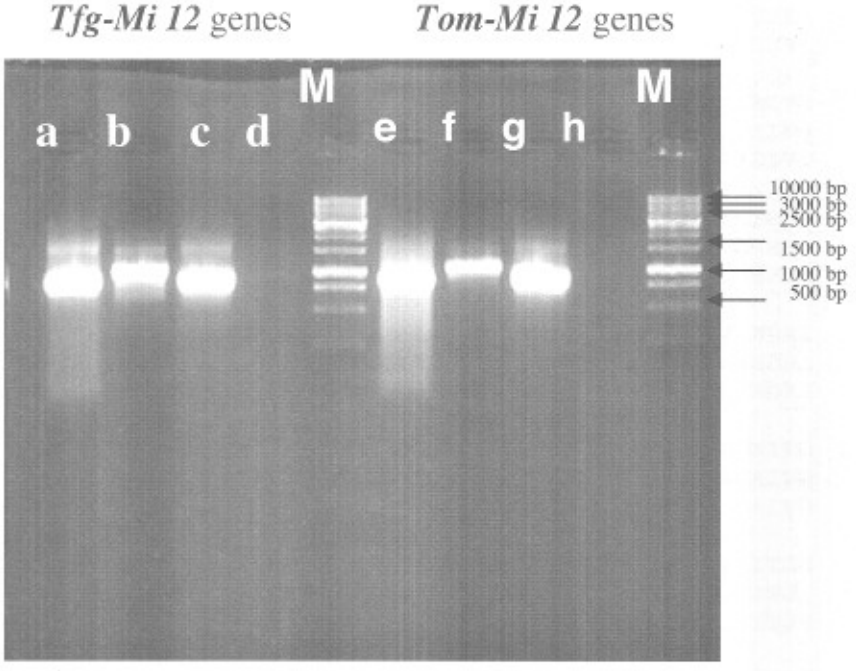

Fig. (5): PCR amplification of Tfg-Mi12 and To-Mi12 genes cloned into pGEMT--Mi12 construct using different primers after DH5á bacteria transformation.
1- For Tfg-Mi12 F gene. a) Tfg-Mi12 F and Mi12 R b) T7 and Sp6 primers c) T7 and Tfg-Mi12 F d) T7 and Tfg-Mi12 R.
2 - For To-Mi12 gene. e) To-Mi12 F and To-Mi12 R f) T7 and Sp6 primers g) T7 and To-Mi12 F h) T7 and To-Mi12 R.

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Tfg 1      MADRVGHFLWDDQTDEDSRLSELDEDEQNDRDSRLFKLAHLLLKIVPVELEVIHICYTNL  60
           MA+RVGHFLW+DQTDEDSRLSELDEDE NDRDSRLF+L HLLLKIVP ELEV+HICYTNL
Tom 184    MAERVGHFLWEDQTDEDSRLSELDEDEHNDRDSRLFQLTHLLLKIVPTELEVMHICYTNL  243

Tfg  61    KASTSAEVGLFIKQLLETSPDILREYLIPLQEHMVTVITPSTSGARNIHVMMEFLLLILS 120
           KASTSAEVG FIK+LLETSPDILREY+I LQEHM+TVI PST GARNIHVMMEFLLLILS
Tom  244   KASTSAEVGRFIKKLLETSPDILREYIIQLQEHMLTVIPPSTLGARNIHVMMEFLLLILS  303

Tfg 121    DMPKDFIHHDKLFDLLDRVGVLTREVSTLVRDLEEEPRNKEGNNQTNCATLDLLENIELL  180
           DMPKDFIHHDKLFDLL  VG LTREVSTLVRDLEE+ RNKEGNNQTNCATLDLLENIELL
Tom 304    DMPKDFIHHDKLFDLLAHVGTLTREVSTLVRDLEEKLRNKEGNNQTNCATLDLLENIELL  363

Tfg  181    KKDLKHVYLKALDSSQCCFPMSDGPLFMHLLHIHLNDLLDSNAYSIALIKEEIELVKQDL 240
           KKDLKHVYLKA +SSQCCFPMSDGPLFMHLLH+HLNDLLDSNAYSI+LIKEEIELV Q+L
Tom  364   KKDLKHVYLKAPNSSQCCFPMSDGPLFMHLLHMHLNDLLDSNAYSISLIKEEIELVSQEL 423

Tfg  241   KFIRSFFVDA-EQGLYKDLWARVLDVAYEAKDVIDSIIVRDNGLLHLIFSLPITIKKIKL 299
           +FIRSFF DA EQGLYKD+WARVLDVAYEAKDVIDSIIVRDNGLLHLIFSLPITIKKIKL
Tom  424   EFIRSFFGDAAEQGLYKDIWARVLDVAYEAKDVIDSIIVRDNGLLHLIFSLPITIKKIKL 483

Tfg  300   IKEEISALDENIPKDRGLIVVNSPKKPVERKSLTTDKITVGFEEETNLILRKLTSGSADL 359
           IKEEISALDENIPKDRGLIVVNSPKKPVERKSLTTDKI VGFEEETNLILRKLTSG ADL
Tom  484   IKEEISALDENIPKDRGLIVVNSPKKPVERKSLTTDKIIVGFEEETNLILRKLTSGPADL 543

Tfg  360   DVISITGMPGSGKTTLAYKVYNDKSVSSRFDLRAWCTVDQGCDEKKLLNTIFSQVSDSDS 419
           DVISITGMPGSGKTTLAYKVYNDKSVS  FDLRAWCTVDQG D+KKLL+TIFSQVS SDS
Tom  544   DVISITGMPGSGKTTLAYKVYNDKSVSRHFDLRAWCTVDQGYDDKKLLDTIFSQVSGSDS 603

Tfg  420   KLSENIDVADKLRKQLFGKRYLIVLDDVWDTTTWDELTRPFPESKKGSRIILTTREKEVA 479
            LSENIDVADKLRKQLFGKRYLIVLDDVWDTTT DELTRPFPE+KKGSRIILTTREKEVA
Tom  604   NLSENIDVADKLRKQLFGKRYLIVLDDVWDTTTLDELTRPFPEAKKGSRIILTTREKEVA 663

Tfg  480   LHGKLNTDPLDLRLLRPDESWELLEKRAFGNESCPDELLDVGKEIAENCKGLPLVADLIA 539
           LHGKLNTDPLDLRLLRPDESWELL+KR FGNESCPDELLDVGKEIAENCKGLPLVADLIA
Tom  664   LHGKLNTDPLDLRLLRPDESWELLDKRTFGNESCPDELLDVGKEIAENCKGLPLVADLIA 723

Tfg  540   GVIAGREKKRSVWLEVQSSLSSFILNSEVEVMKVIELSYDHLPHHLKPCLLYFASFPKDT 599
           GVIAGREKKRSVWLEVQSSLSSFILNSEVEVMKVIELSYDHLPHHLKPCLL+FAS+PKDT
Tom  724   GVIAGREKKRSVWLEVQSSLSSFILNSEVEVMKVIELSYDHLPHHLKPCLLHFASWPKDT 783

Tfg  600   SLTIYELNVYFGAEGFVGKTEMNSMEEVVKIYMDDLIYSSLVICFNEIGYALNFQIHDLV 659
            LTIY   VY GAEGFV KTEM  +EEVVKIYMDDLI SSLVICFNEIG  LNFQIHDLV
Tom  784   PLTIYLFTVYLGAEGFVEKTEMKGIEEVVKIYMDDLISSSLVICFNEIGDILNFQIHDLV 843

Tfg  660   HDFCLIKARKENLFDQIRSSAPSDLLPRQITIDCD-EEEHFGLNFVMFDSNKKRHSGKHL 718
           HDFCLIKARKENLFD+IRSSAPSDLLPRQITID D EEEHFGLNFVMFDSNKKRHSGKHL
Tom  844   HDFCLIKARKENLFDRIRSSAPSDLLPRQITIDYDEEEEHFGLNFVMFDSNKKRHSGKHL 903

Tfg  719   YSLRIIGDQLDDSVSDAFHLRHLRLLRVLDLHTSFIMVKDSLLNEICMLNHLRYLSIDTQ 778
           YSLRI GDQLDDSVSDAFHLRHLRL+RVLDL  S IMV DSLLNEICMLNHLRYL I TQ
Tom  904   YSLRINGDQLDDSVSDAFHLRHLRLIRVLDLEPSLIMVNDSLLNEICMLNHLRYLRIRTQ 963

Tfg 779    VKYLPLSFSNLWNLESLFVSTNRSILVLLPRILDLVKLRVLSVDACSFFDMDADESILIA  838
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```
          VKYLP SFSNLWNLESLFVS   SILVLLPRILDLVKLRVLSV ACSFFDMDADESILIA
Tom  964  VKYLPFSFSNLWNLESLFVSNKGSILVLLPRILDLVKLRVLSVGACSFFDMDADESILIA 1023

Tfg  839   EDTKLENLRILTELLISYSKDTKNIFKRFPNLQLLSFELKESWDYSTEQHWFSELDFLTE 898
           +DTKLENLRIL ELLISYSKDT NIFKRFPNLQ+L FELKESWDYSTEQHWF +LD LTE
Tom  1024  KDTKLENLRILGELLISYSKDTMNIFKRFPNLQVLQFELKESWDYSTEQHWFPKLDCLTE1083
```

Fig. (6): Comparison of the predicted amino acid sequences of Tfg-Mi with Mi-1.2, a root-knot nematode resistance gene from tomato (Accession number: 543551 Mi-1.2). The deduced amino acid sequence of the Tfg-Mi gene product is shown and the amino acids that differ from the Mi-1.2 gene product are indicated. The positions of a potential leucine zipper motif are underlined and bold.

Results showed that the fragment was 850bp long for *Mi11* and 1450bp long for *Mi12* and *Tfg-Mi* genes. GenScan and CLC analysis both predicted that the DNA fragment had two the same active and leucine rich repeat sids, contained a full-length open reading frame (ORF) of 3774bp encoding 1257 amino acids. BLAST result showed that the predicted ORF had 99% identity to the published tomato root-knot nematode resistance gene *Mi* (AF039682), which is a member of the Leucine Zipper, Nucleotide Binding, Leucine-Rich Repeat family.

A 850 and 1450 bp fragments were obtained through PCR amplification,. The full-length cDNA sequence of *Tfg-Mi* was obtained using RACE amplification. The full-length cDNA of *Tfg-Mi* was 2186 bp long, which included the putative transcription start site, a potential open reading frame of 2774 bp.The potential open reading frame encoded for 1257 amino acid residues and contained a potential leucine zipper and a heptad repeat motif. The deduced amino acids sequence showed extensive homology with each other (Fig. 6). The conserved active leucine rich repeats, which forms covalent binding during DNA cleavage is located at the 791st residue (is denoted by overhead arrow in Fig. 6). The genomic DNA sequence of *Tfg-Mi* shared 98% identity and the deduced amino acids shared 99% identity (Fig. 6), with *Mi-1.2* (accession number: AF039682), the root-knot nematode resistance gene from tomato.

Using the specific primers designed based on *Tfg-Mi* sequence information; a genomic fragment with approximately 2.3 kb in length was amplified from the fenugreek line Giza 3. The genomic sequence of fenugreek will be deposited into the GenBank database soon. Our results show the isolation and characterization of *Mi-1.1* and *Mi-1.2* from tomato (*Lycopersicon peruvianum*) and *Tfg-Mi* from Fenugreek (*Trigonella foenum-graecum*) as shown in (Fig. 7).

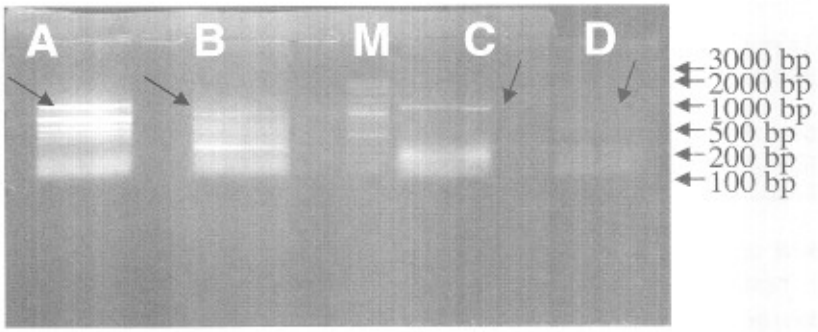

Fig. (7): PCR amplification for screening of the presence of Tfg-Mi 12 and To-Mi 12 genes with fenugreek and tomato.
Ianes A and B using Mi12-F and Mi12-R primers for Mi12 gene from tomato and fenugreek and Lanes C and D using Mi12-F and Mi12-R primers for Mi12 gene and lane M DNA 1 KB Marker.

Production of transgenic plants

In our case, the efficient introduction of constructed plasmids into *A. tumefaciens* is of great practical importance. Transformation was done with the calcium chloride treatment with freeze-thaw (Horsch *et al.*, 1985). Transformation frequencies of up to 10^7 transformants/µg DNA using electroporation have been reported. Although the transfer of naked DNA into plants by particle bombardment can be used as a rapid test for protein expression prior to stable transformation, it is not suitable for the expression of large amounts of foreign proteins in plants. This technique has received far more attention for the regeneration of transgenic plants, in particular cereal crops. However, the obtained results refer to the successful transformation in tomato plants (Fig. 89).

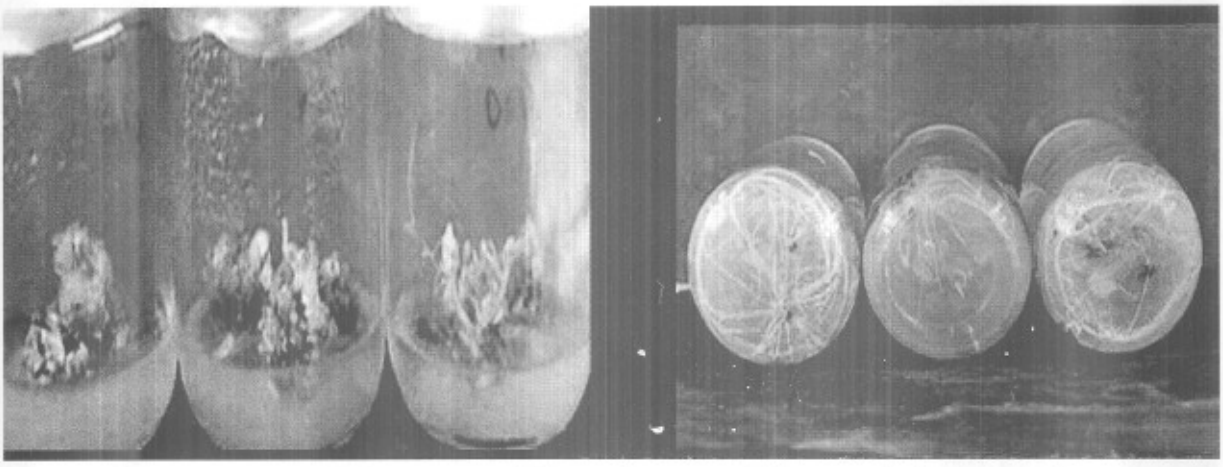

Fig. (8): Establishment of the protocol for culture conditions and Agrobacterium – mediatd transformation for tomato. Tomato explants on MS regeneration medium containing BA 1.0 / NAA 0.1 mg / L (left) and on MS modified regeneration medium for rooting (right).

Establishment of the protocol for culture conditions and Agrobacterium–mediated transformation in tomato

Cotyledon and hypocotyl explants from 10-day-old seedlings and tomato leaves from 4-week-old plants were cultured on a preculture medium. Cotyledon and leaf pieces were placed on the media with the abaxial surface of the leaf in contact with the medium. The cotyledons and leaves were cut, and two explants were obtained from each. At least, three independent experiments were carried out to test each plant growth regulator combination, and at least 100 explants were tested each time.

Culture media and conditions for transformation of tomato before cloning

Plant growth regulators were examined to obtain adventitious shoot initiation from the calli and explants following *Agrobacterium* cocultivation.

The plant growth regulator combinations examined were in mgL–1: BA 1.0/NAA 0.1; BA 1.0/NAA 0.2; BA 1.0/IAA 0.2; BA 2.5/IAA 0.2.

For evaluation of the effect of a second combination of plant growth regulators on transformation efficiency, the plant growth regulator combinations examined were in mg L–1: BA 1.0/NAA 0.1 it is the best medium. Using nonchemical methods to control root-knot nematodes becomes an important measure in view of constraints on use of nematicides and soil fumigants because of the toxic to the environment (Sorribas *et al.*, 2005) Utilizing natural resistance gene would be an efficient, durable, simple and nonpolluting method of control. But until now, only *Mi* gene is the known source of root-knot nematode resistance in cultivated tomato (Goggin *et al.*, 2004). All currently available fresh-market and processing tomato cultivars resistant to root-knot nematodes were derived from an interspecific hybrid, while none was from transgenic approach (Ernst *et al.*,2002) Ferrier-Cana *et al.*,2003).

Transgenic tomato plant confirmation

Taq-polymerase, dNTPs (deoxynucleotide triphosphate) and convergent primers achieved amplification of the DNA fragment. The reaction conditions for PCR involved denaturation at 94^{O}C for 30 seconds, annealing at 52^{O}C for 30 seconds and extension at 72^{O}C for either 30 seconds for cloned DNA in plasmid or 2.5 min. After 30 cycles of amplification, an aliquot of this reaction mix., was loaded onto a 0.8 % agarose gel and checked.

Cloning of the Mi–GUS cassette was further confirmed by PCR for the plasmid DNA (*Mi–GUS*) from both the ends using the GUS5 and GUS3 primers present in the pBI121 vector. After the confirmation, one of the positive colonies (pBI121-Mi–GUS) was selected for further step of cloning (Fig. 9).

It looks promising that resistance of the transgenic plants was partially enhanced at the high soil temperature environment, indicating that the instability of native promoter is one of the factors affecting heat instability of *Mi* gene that confers resistance to root-knot nematodes. In addition, Goggin *et al.* (2004) reported that the *Mi*-mediated nematode resistance is instable in transformed tomato lines and decreased from generation to generation. In this study, however we did not detect any reduction of nematode resistance in transgenic progenies.

The main reason of this difference is probably due to the change of the promoters used in the experiments (Chen *et al.*, 2006).

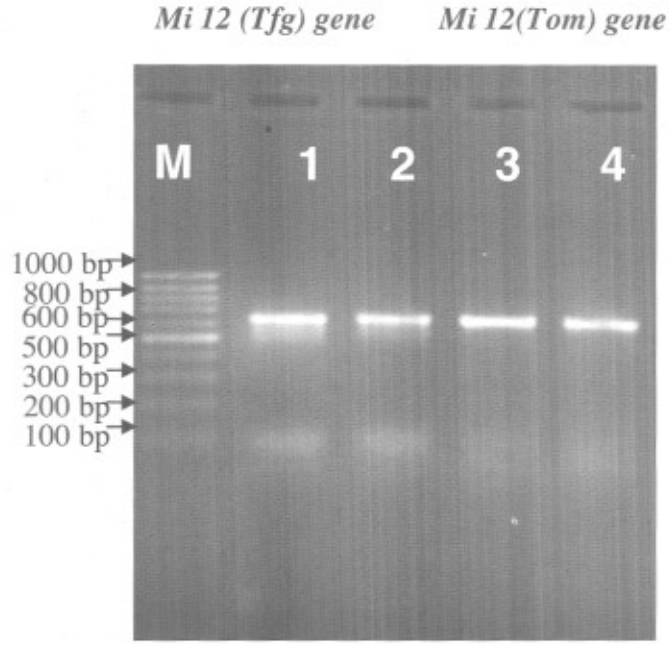

Fig.(9): PCR confirmation of Mi12(Tfg) and Mi12(Tom) genes cloned into pBI121-Tfg-Mi12 (lanes 1 and 2) and pBI121-tom-Mi12 (lanes 3 and 4) construct using Mi12(Tfg) and Mi12(Tom) primers after LB4404 Agrobacterium tumefaciens transformation

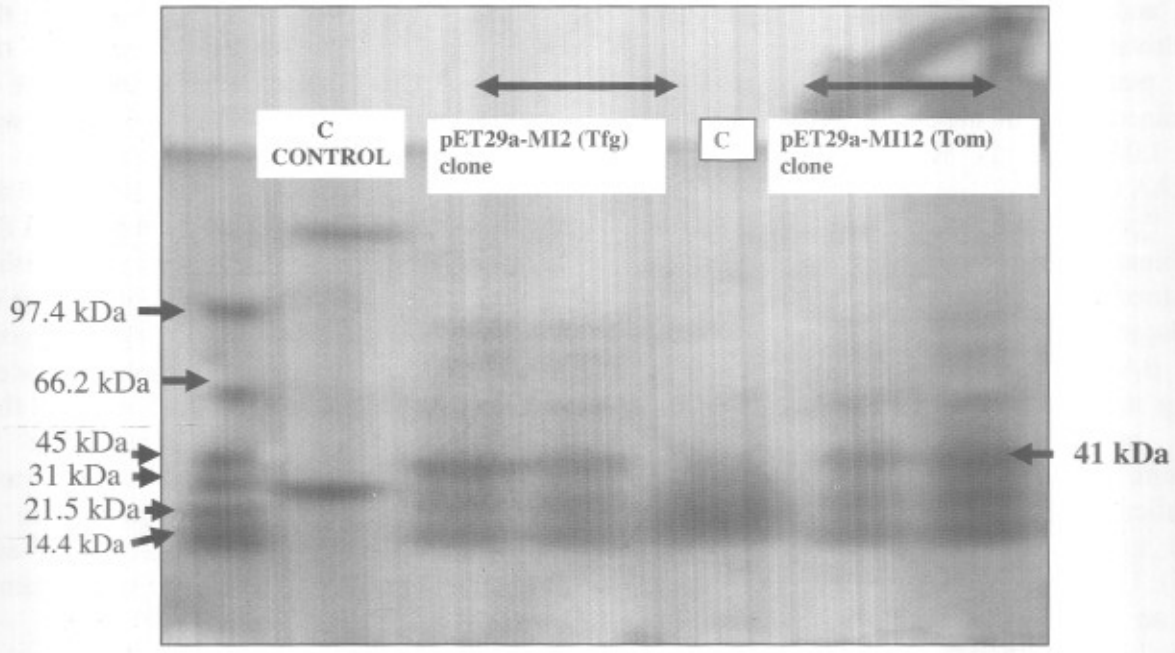

Fig. (10):Expression of Mi 12 (Tfg) and Mi12 (Tom)genes in pET29a . Mi 12(Tfg) and Mi12(Tom) genes were cloned under T7 promoter in the NcoI / BamHI sites of pET29a.

Confirmation of transgenic plants

The nematode susceptible tomato cultivars of Super strain B and Edkawye (Egyptian cultivars) were transformed using *Agrobacterium tumefaciens* carrying the sense construct pBI121. Following plant transformation and kanamycin selection, 35 independent transformants were obtained.

PCR analysis

To confirm the presence of T-DNA in the genomes of the transformants plants were screened for *NPT*II gene by PCR. Thirty-one among 35 independent transformants showed positive results. It has been found that when the gene or T-DNA inserted copies were more than three, most of the transgenic plants turned to susceptible to root-knot nematode and the transcript levels of *Mi* gene were very low and even could not be detected. This indicated that the nematode resistance of transgenic plants was correlated with the copy numbers of T-DNA and the transcript abundance of *Mi* mRNA. The resistance unattained in transgenic plants with multiple copies of, the gene was possibly due to the transcriptional gene silencing. Some researches reported an inverse correlation between copy number and the level of gene expression, which suggests that increasing the number of copies of the T-DNA could lead to gene silencing (Herve and Mathilde, 2001). The mechanism of transcriptional gene silencing is probably the interaction of closely linked copies (DNA-DNA pairing) leading to the formation of secondary DNA structures that attract methylation and heterochromatic components. Methylation is probably involved in the maintenance of the silent state (Herve, 1997). In order to avoid or reduce the transcriptional gene silencing, this needs large numbers of independent transformants for each construct and selected the single copy one, for subsequent analysis.

DNA digestion

The full-length gDNA of *Mi* gene was digested from the pGEMT vector with *Xba*I and *BamH*I, and cloned into the binary vector pBI121 by fusion of the *MI* with the GUS gene. The resulted plasmid construct was designated pBI121Mi (Fig.11).

All the constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. *Agrobacterium*-mediated tomato transformation was performed as described by Fillatti *et al.* (1996). Super strain B and Edkawy were used for transformation with the vector pBI121 (sense gene). Regenerated transgenic plants were screened for *GUS* gene by PCR. Moreover, to our knowledge there is no available root-knot nematodes resistant cultivar in our country.

Our present results suggest that transgenic method is a feasible approach to improve the root-knot nematode resistance in tomato. In addition, this method offered a new way to breed root-knot nematodes resistant varieties for other crops that can be seriously damaged by root-knot nematodes and for which no genetic resources of resistance have been identified, like eggplant, cotton, cucumber and potato, etc. We have attempted to transfer this gene into susceptible lettuce and now have obtained resistant transgenic plants (data not shown).

However, it has been reported that when the soil temperature is above 28°C, the resistance of *Mi* gene in tomato plants might partially lost (Dropkin, 1969). But the mechanism is still unknown. A possible explanation for this was either protein encoded by this gene was unstable or the native promoter could not properly work, or some other factors involved in its pathway not properly work. In this study, we replaced the native promoter of the gene with cauliflower mosaic virus (CaMV) 35S promoter, a stronger and constitutive promo-

ter, and tried to figure out whether the instability is due to the gene expression or the activity of the promoter.

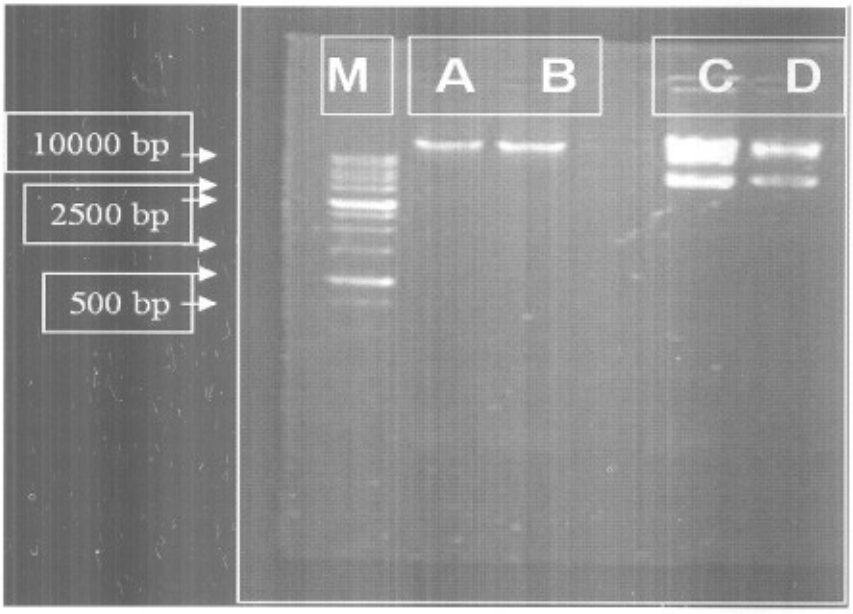

Fig. (11): A gel showing restriction digests of pBI121 plasmid digests by XbaI and BamHI Enzymes.
Lane A and B contains pBI121 plasmid digests by XbaI and BamHI Enzymes.
Lane C and D contains undigested pBI121 plasmid.
Lane M contains DNA Marker.

In susceptible tomato lines the root-knot nematode resistance gene, *Mi*, is associated with an inverted chromosomal segment (Seah *et al. 2004*). However, in fenugreek, we were not able to determine this kind of inversion. By using the susceptible fenugreek DNA as template, we failed to obtain any bands with the primers *Mi12F* and *Mi12R* by PCR amplification (data not shown). A possible explanation for this observation is that either the fragment of *Tfg-Mi* gene is not present in the genome of susceptible fenugreek lines or this gene developed mutations, thus losing its function of resistance to root-knot nematodes.

ACKNOWLEDGMENT

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الملخص العربي

جين *Tfg – Mi* لمقاومة نيماتودا تعقد الجذور من الحلبة للحصول على نباتات طماطم تقاوم الإصابة

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من المعروف أنه تم عزل العديد من جينات مقاومة النيماتودا من النباتات وبصفة خاصة من الطماطم.في هذا البحث تم عزل ولأول مرة جين من نفس العائلة ولكن من نبات الحلبة وقد تم تسميه هذا الجين باسم *Tfg – Mi* كاختصار إلى أنه ينتمي إلى جينات مقاومة جنس النيماتودا *Meloidogyne spp.* وتم عزله من الحلبة *Trigonella foenum – graecum* وقد تم عزل هذا الجين من صنف جيزة ٣ للحلبة باستخدام تقنيات الـ PCR المعتمد على وجود بعض من البريمر المعروف التتابع الجيني له مع بعض أنواع البريمر التي تم مقارنتها مع الموجود في بنك الجينات وكذلك من نفس جينات الطماطم المعزولة سابقا. كذلك تم عزل نفس الجين من نباتات الطماطم حتى يكون كنترول في معظم عمليات النقل الوراثي ومقارنه الجينات. عند تحليل التعبير الجيني لهذا الجين في الأجزاء النباتية المختلفة لوحظ أن له تعبيرا جينيا عالي وخاصة في منطقة الجذور مقارنة بتعبير جيني محدود في باقي الأجزاء النباتية مثل الأوراق والأزهار ولم يلاحظ في الثمار. لتأكيد فاعلية عمل جين *Tfg – Mi* تم استخدام هذا الجين في محاولة إنتاج نباتات طماطم مقاومة للنيماتودا وخاصة للنباتات الحساسة من الطماطم والتي لا يوجد بها تعبير جيني وخاصة لجين *Mi 12*. وقد تم إدخال هذا الجين في بلازميدات للآجروبكتريم حتى يمكن استخدامها في عمليات النقل الوراثي ٠في النهاية امكن الحصول على نباتات الطماطم محوله وراثيا والكشف عنها عن طريق استخدام الطرق المختلفة من الـ RT – PCR و الـ PCR العادية للتأكد من وجود الجين وكذلك للتأكد من التعبير الجيني للـ *Tfg – Mi* في هذه النباتات المحورة وراثيا والتأكد أنها أصبحت صفة مورثة في النبات.