

Protocol for *Mi*-gene Detection (nematode resistance) in Tomatoes

Description

Root knot nematodes comprise a group of endoparasitic roundworms that cause major economic damage to crops around the world. These microscopic organisms penetrate the roots of thousands of plant species and migrate to the vascular cylinder, where they initiate a series of changes in the root, resulting in the formation of galls (or root knots) as well as the development of specialized feeding cells, called “giant cells,” in their hosts. These alterations grossly affect nutrient partitioning and water uptake in the host. Many modern tomato varieties carry a single, dominant gene called *Mi*. This gene confers resistance to three of the most damaging species of root knot nematodes (*Meloidogyne* spp). The *Mi*-gene was introduced into cultivated tomato, *Lycopersicon esculentum*, from its wild relative *L. peruvianum* in the early 1940s. With the assistance of linked markers, beginning with the isozyme marker (IEF) *Aps-1* and, more recently, with the DNA marker, *Rex-1*, *Mi* has been incorporated into many modern tomato cultivars. In both resistant and non-resistant cultivars, nematodes are attracted to and penetrate roots after which they then migrate to a feeding site within the roots. While in non-resistant cultivars the nematodes develop a feeding site normally, in resistant cultivars there is localized tissue necrosis or a hypersensitive response (HR). As a consequence of this HR, the nematodes are unable to develop a feeding site this and subsequently either die or leave the roots.

The *Mi*-gene is very similar to *Prf*, a tomato gene required for resistance to *Pseudomonas syringae*. Both genes share several structural motifs including a nucleotide binding site and a leucine-rich repeat region, that are characteristic of a family of plant proteins, including several that are required for resistance against viruses, bacteria, fungi, and now, nematodes. It has also been reported that presence of the *mi*-gene also confers resistance to aphids (and thus reduced incidence of aphid-borne viruses such as PVY and CMV).

While the *Mi*-gene confers resistance (but not complete) to nematodes in a heterozygous state, gene expression in the homozygous state offers moderately better resistance. It should be remembered that the expression of this gene is temperature sensitive; at soil temperatures at or above 28°C, gene expression (and therefore nematode resistance) is reduced.

The below protocol detects the *Mi*-locus. Presence of the gene in its heterozygous or homozygous state can be detected by the addition of a restriction enzyme digest. Time taken for the detection is approximately one day. It is more efficient, time-wise, to do up to 93 samples at a time this being the number of wells in the microtitre plate used for the extraction as well as the number of wells in the PCR machine. Doing ten samples takes nearly as long as 93 samples!! If you have confirmed by using this method that both parents are homozygous for *Mi*, there is no need to confirm

the status of their progeny. Similar if one of that parents is homozygous and the other negative for Mi (and that has been confirmed using this method), then there is no need to confirm the status of their progeny.

Protocol

1. Tomato leaf DNA extraction

Extract tomato leaf (any stage) DNA using a modified NaOH lysis procedure.

1.1 Label each plant.

1.2 Cut a small “disc” of tomato leaf tissue using a paper hole punch.

1.3 Place the leaf disc into a 96 well plate as a pre-designated.

1.4 Add 50 µl of 0.5 M NaOH into each well.

1.5 Grind leaf tissue to a liquid consistency using a pestle. Optionally, a pestle in a small bench-top hand drill can be used.

1.6 After each sample is ground, wash the pestle in a 0.1% chlorine (final) solution and rinse three times with R/O.

1.7 Precipitate the leaf extracts by centrifuging in a microtitre plate benchtop centrifuge at 200 rpm, 4°C for 10 minutes.

1.8 Transfer 10 µl of plant extract to a 96 well plate-containing 90 µl of 0.1 M Tris-HCl, pH 8.0 (1:10 dilution). Mix by pipetting.

1.9 Centrifuge at 200 rpm., 4°C for 10 minutes.

1.10 Store at –20°C or 4°C overnight.

NB. In all cases, a positive control (**known** positive control for *Mi-gene*) and negative control (**known** negative control for the *Mi-gene*) **MUST** be included. In both cases these should be extracted at the same time as the samples. Controls can be leaf discs stored at -20°C.

Controls: Negative – Peto 94 Heterozygous – Extra (EWS) Homozygous -

2. PCR

Primers*

Primer REX-F1: 5' TCG GAG CCT TGG TCT GAA TT 3'

Primer REX-R3: 5' ATG CCA GAG ATG ATT CGT GA 3'

Preparation of PCR master mix: as follows:

Reagents	25 μ l Reaction	Master mix (50 reactions) of 25 μ l each
10 mM dNTPs	0.5	25
10X Buffer	2.5	125
50 mM MgCl ₂	1.0	50
REX-F1 primer (30 pmole/ μ l)	1.0	50
REX-R3 primer (30 pmole/ μ l)	1.0	50
dH ₂ O	16.5	825
Taq DNA Polymerase** (2 u/ μ l)	0.5	25
Total	23	1150
DNA template	2	
Total volume	25	

- Pipette 23 μ l of PCR master mix to PCR tubes.
- Add 2 μ l of DNA template.

* Williamson et al., 1994. A PCR-based marker tightly linked to the nematode resistance gene, *Mi*, in tomato. *Theor. Appl. Genet.* 87:757-763.

** Check the stock *Taq* DNA Polymerase concentration first

PCR reaction:

PCR reaction performed in a PCR machine using the following profile:

1 cycle: 94°C for 3 minutes

30 cycles: 94°C for 1 minute
65°C for 2 minutes

72°C for 2 minutes

1 cycle: 72°C for 10 minutes

Amplified DNA should be approximately 750 bp.

3. Restriction enzyme digestion of PCR amplified products

Digest PCR products with *TaqI* restriction enzyme.

TaqI restriction enzyme site:



Preparation of restriction digestion master mix: as follows:

Reagents***	Single Reaction (μl)	Master mix (50 Reactions) of 12 μl each
10X Buffer	1.2	60
dH ₂ O	0.8	40
<i>TaqI</i> (10U/μl)	0.2	10
PCR amplified DNA	10	-
Total	12.0	110

- Pipette 10 μl of PCR product to eppendorf tubes containing 2 μl of “master mix”
- Gently vortex and spot spin
- Incubate at 65°C for 2 hours using PCR machine.
- Add 2 μl of 6X gel loading buffer
- Run gel. Alternatively digests can be stored at 4°C.

*** Check manufacturer’s specifications of *TaqI*

4. Agarose Gel Electrophoresis

Electrophoresis DNA in 1% agarose gels at 150 volt for approximately 40 minutes (this will vary depending on the electrophoresis unit used).

- Prepare 1% agarose gel in 1X TAE buffer and dissolve by heating in a microwave.
- Cool the molten agarose to 50 – 60°C, pour into the mould, insert the comb and allow the gel to set.
- Submerge the gel in 1X TAE buffer and remove the comb.
- Load the DNA approximately 14 μl into the wells. Include the appropriate molecular weight marker as well as the negative and positive controls.
- Run the gel at 150 volt for approximately 40 minutes until the bromophenol blue dye front is within 1 cm of the end of the gel.

5. Visualisation of DNA using Ethidium Bromide

- Submerge the gel and stain in ethidium bromide solution for 20 minutes.
- Rinse the gel in dH₂O.
- Visualize the restricted amplified DNA fragments under ultraviolet-illumination
- Verify results against DNA molecular weight and positive, negative control.

6. Analysis of DNA fragments pattern

- Verify results against DNA molecular weight markers (MW) and the positive and negative controls. This indicates the absence or presence of the loci (Figure 1).

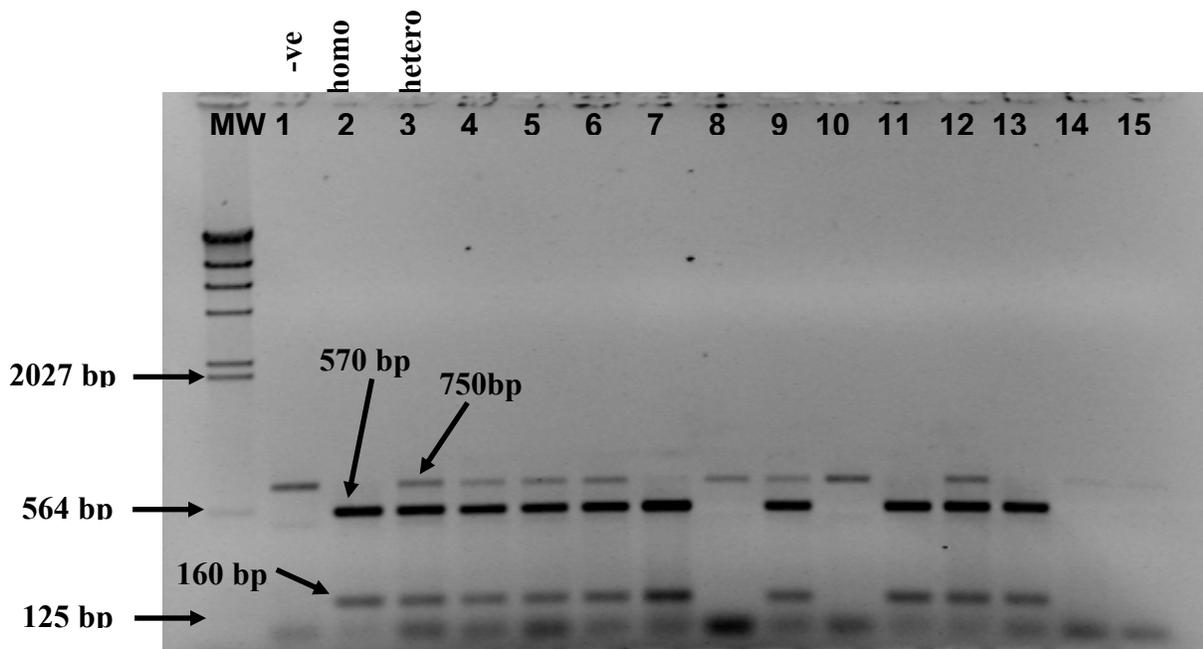


Figure 1. Agarose gel electrophoresis of PCR amplified DNA associated *Mi-gene* detection in tomato. Controls include, negative (no *Mi*) lane 1, homozygous (lane 2) and heterozygous, lane 3. Three DNA different DNA patterns are possible:

- **Negative result:** A single band of approximately 750 bp indicates an absence of the *Mi* gene. Lanes 1, 8, 10, 14 and 15.
- **Homozygous:** Two bands of approximately 570 and 160 bp indicates the homozygous state. Lanes 2, 7, 11 and 13.
- **Heterozygous:** Three bands of approximately 750, 570 and 160 bp indicates the heterozygous state. Lanes 3 – 6, 9 and 12.

7. Summary

- Tomato lines that have **DO NOT contain the *Mi-gene*** are “**nematode susceptible**”.
 - Tomato lines that **contain the *Mi-gene*** either in its homozygous (two bands at 570 bp and 160 bp) or heterozygous (three bands at 750bp, 570 bp and 160 bp) states are “**nematode resistant**”.
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Trouble Shooting

1. If it is believed that both parents are homozygous for the *Mi-gene* but the progeny is either heterozygous or are segregating for homozygous and heterozygous, then the original assumption of both parents being homozygous is incorrect.
2. On occasions you will observe a faint band or smear associated with the upper 750 bp band. Firstly check the positive control and other samples. If the band is of a lower concentration than the other heterozygous and negative samples, the sample in question is homozygous. Also investigate whether the restriction digest was done at the correct temperature and for correct period of time? If in real doubt, repeat the sample being sure to add additional *TaqI* restriction enzyme. If there is a problem with all the samples, check the use-by-date and the efficiency of the restriction enzyme.