DETECTION OF THE TOMATO *MI 1.2* GENE BY PCR USING NON-ORGANIC DNA PURIFICATION

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ABSTRACT

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A PCR based method using DNA extracted with non-organic reagents was developed to detect the presence of the two alleles (*Mi* and *mi*) of the tomato *Mi 1.2* resistance gene in plant material. Plant tissue from young leaves of tomato plants was blotted onto a FTA® Card (Whatman, Inc.) and processed according to the manufacturer's instructions. Individual samples from the FTA® Card containing the DNA template were used in PCR reactions. The forward primer (IMOF1) anneals to the 3' end of the conserved region and the beginning of the intron, and the reverse (IMOR1) anneals to the C terminal of the leucine-rich repeat (LRR), amplifying a 998 bp DNA fragment corresponding to the marker for the resistance allele (*Mi*) and a 900 bp fragment corresponding to the marker for the susceptible allele (*mi*). Tomato cultivars Motelle and Motaci (isogenic for the *Mi 1.2* locus), *L. peruvianium f. glandulosum* cv. Canta, *L. esculentum var. cerasiforme* VFNT Cherry, Mossol, Motabo, G-178 and VFN-8 showed the marker for the homozygous resistant genotype (*Mi/Mi*). Tomato cultivars Alicante, Harzfeuer F1, Marglobe, Marmande and Rutgers showed the marker for the homozygous susceptible genotype (*mi/mi*) and cultivar Big Boy showed both markers, corresponding to the heterozygous resistant genotype (*Mi/mi*). The chi-square value using these markers was $\chi^2 = 1.2$. Two advanced tomato lines P1 (*Mi/Mi*) and P2 (*mi/mi*) and 13 individual plants of their hybrid F_1 progeny were also analyzed, with the *Mi* and *mi* markers present in the resistant (P1) and susceptible parent (P2), respectively, and both markers present in all the heterozygous F_1 individuals. This PCR and FTA® Card combination results in a fast, accurate, cost effective and reliable method for use in marker-assisted selection in breeding programmes and plant model studies of the host root-knot nematode interaction.

Key words: germplasm, *Lycopersicon*, *Meloidogyne*, nematode, resistance, root-knot, susceptibility.

RESUMEN

Bendezu, I. F. 2004. Detección del gen *Mi 1.2* en tomate usando PCR y la extracción inorgánica de ADN. Nematropica 34:23-30.

Tejido joven de plantas de tomate fue prensado sobre el papel de FTA® Card siguiendo las recomendaciones de uso del producto. Muestras de papel de FTA® Card con ADN fueron usadas en reacciones de PCR. Los oligonucleotidos IMOF1 y IMOR1 amplificaron una banda de 998 pares de bases correspondiente al marcador de resistencia (*Mi*) y una banda de 900 pb como marcador de susceptibilidad (*mi*). Cultivares de tomate Motelle y Motaci (isogénicos para el *Mi 1.2* locus), *L. peruvianium f. glandulosum* cv. Canta, *L. esculentum var. cerasiforme* VFNT Cherry, Mossol, Motabo, G-178 y VFN-8 muestran el marcador de resistencia en el estado homozigote (*Mi/Mi*). Cultivares Alicante, Harzfeuer F1, Marglobe, Marmande y Rutgers muestran el marcador de susceptibilidad (*mi/mi*) en estado homozigote y el cultivar Big Boy muestra ambos marcadores correspondiendo al genotipo heterozigote (*Mi/mi*). El valor de chi-cuadrado usando estos marcadores es $\chi^2 = 1.2$. Las lineas avanzadas de mejoramiento de tomate P1 (*Mi/Mi*) y P2 (*mi/mi*) y trece de sus descendientes hibridos F_1 fueron analizados. Los parentales resistente y susceptible mostraron los marcadores correspondientes y la progenie mostró ambos marcadores, confirmando su naturaleza heterozigota. Esta combinación de

PCR y el método inorganico de extracción de ADN usando la FTA® Card resulta en un protocolo rapido, exacto, de bajo costo y confiable que puede ser usado en la selección masal de individuos resistentes en programas de mejoramiento e ingenieria genetica, para la producción de tomates transgénicos, o en el estudio de la interacción de hospedero y nematodo usando plantas modelo.

Palabras clave: agallador, germoplasma, *Lycopersicon*, *Meloidogyne*, nemátodo, resistencia, susceptibilidad.

INTRODUCTION

The genus *Meloidogyne* is the most important nematode pest in horticultural crops worldwide (Sasser and Freckman, 1987). Genetic resistance in tomato against these pests is efficient in reducing their populations densities and thereby reducing the need for pesticide application (Medina-Filho and Tanksley, 1983; Roberts *et al.,* 1986). The most important source of resistance is conferred by the *Mi* family of genes from the wild tomato *Lycopersicon peruvianum*, providing effective resistance to *M. incognita, M. javanica* and *M. arenaria* (Hadisoeganda and Sasser, 1982) and secondary opportunistic organisms such as the soil-borne bacterial pathogen *Ralstonia solanacearum* (Deberdt *et al.,* 2003). *Mi* also provides resistance to the aphid *Macrosiphum euphorbiae* (Rossi *et al.*, 1998) and biotypes Q (Nombela *et al.*, 2003) and B (Jiang *et al.*, 2001) of *Bemisia tabaci.*

Most commercial tomato cultivars now available come from a cross by which the *Mi* gene was introgressed from *L. peruvianum* into *L. esculentum* using embryo culture (Medina-Filho and Stevens, 1980). The *Mi* locus is located at least 40 Mbp from the linked *Aps-1* gene (Zhong *et al.,* 1999), which codes for the enzyme acid phosphatase and has been used as a marker for root-knot nematode in the past (Rick and Fobes, 1974). With this marker it has been found that false negatives can be obtained because not all the hybrids carrying the *Mi* also carry the *Aps-1* gene. This is due to the variability in the length of the DNA fragments from *L. peruvianum* that have been introgressed in those hybrids (Messeguer *et al.,* 1991). Later, a PCR protocol for identifying plants with the *Mi* gene was developed using the marker *REX-1* (Williamson *et al.*, 1994), but this method requires additional digestion of the PCR product with the enzyme *TaqI.* A recent report, using cDNA and reverse transcriptase-polymerase chain reaction, seems to distinguish between resistant and susceptible genotypes (Martinez and Kaloshian, 2001) but is laborious, time-consuming and certainly not cost-effective or applicable for mass screening.

PCR amplification using sequence characterized regions is considered to be a convenient system of using markers for screening in commercial breeding programmes (Heer *et al.*, 1998; Huang *et al.*, 2000; Matthews *et al.*, 1998; Paran and Michelmore, 1993). Plant genetic improvement can benefit from the use of PCR markers because it is efficient, can be carried out using young plant material (raised in Petri dishes instead of pots), and is non-destructive, faster and cheaper to run than standard bioassays (Hussey and Janssen, 2002). PCR technology in conjunction with the use of FTA® Cards has been shown to be useful and very efficient for the rapid diagnosis of plant disease resistance genes (Lin *et al.,* 2000). FTA® Cards are square pieces of cellulose-based matrix impregnated with non-organic chemicals that lyse cells and also protects and stabilizes DNA for long term storage.

The objective of this study was to develop a fast and cost-effective PCR protocol for detection of the *Mi 1.2* gene in

tomato using young tomato leaves and a DNA extraction method that does not require use of organic reagents.

MATERIALS AND METHODS

Germplasm

A total of 17 commercial cultivars, advanced germplasm and isogenic lines were used for this study and were mostly provided by the Tomato Germplasm Resource Centre (TGRC) of the University of California-Davis (Table 1). Tomato cv. Motelle was used as an isogenic resistant (*Mi/Mi*) control, cultivar Big Boy was considered heterozygous resistant (*Mi/mi)* and cvs. Marglobe and Rutgers were considered homozygous susceptible (*mi/mi*) controls respectively.

DNA Extraction using FTA® Card

Circular sections (4 mm in diameter) were taken with sterile cork borers from young leaves of 10-day-old tomato plants grown from seeds in Petri dishes containing moist pieces of sterile filter paper and crushed onto the FTA® Card (Whatman, Inc.) using a metal rod wrapped in parafilm. The FTA® Cards containing the samples were left to dry at room temperature for two hours before taking 2.0 mm diameter disks using a Harris UNI-CORE (Whatman, Inc.) and proceeding with the DNA purification following the manufacturer's recommendations. After the last wash, the samples were left to dry at room temperature for 1 hour and then individually used in the PCR analysis or stored at -20°C inside a sealed multi-barrier pouch containing a desiccant packet (Whatman, Inc.).

Table 1. Tomato cultivars and advanced germplasm used in the study.

Specie	Phenotype	Cultivar/line	Source
L. esculentum	S	Alicante	commercial
L. esculentum	R	Big Boy	commercial
L. esculentum	R	G178	TGRC
L. esculentum	S	Harzfeuer F1	commercial
L. esculentum	S	Marglobe	TGRC
L. esculentum	S	Marmande	TGRC
L. esculentum	R	Mossol	TGRC
L. esculentum	R	Motabo	TGRC
L. esculentum	R	Motaci	TGRC
L. esculentum	R	Motelle	TGRC/V. Williamson
L. esculentum	S	Rutgers	TGRC
L. esculentum	R	VFN-8	TGRC
L. peruvianium f. glandulosum	R	Canta	TGRC
L. esculentum var. cerasiforme	R	VFNT Cherry	TGRC
L. esculentum	R	P1	Mayford Seeds
L. esculentum	S	P2	Mayford Seeds
L. esculentum	R	P1/P2 progeny	Mayford Seeds

Standard DNA extraction of the controls was performed following the protocol published by Burow *et al.* (2001) with the modification that the DNA was resuspended in sterile distilled water.

Primer Design and PCR Amplification of Genomic DNA

A region in the tomato *Mi 1.2* gene (GENBANK Accession number AF039682) was used to design four pairs of specific oligonucleotide primers (Table 2) with zero or very low 3' self complementarity and melting temperatures between 50°C and 60°C using the shareware software Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR reactions contained sterile distilled water 34.75 µl, PCR buffer B (Promega Corp.) 10× 5.0 µl, Mg_2Cl_2 5.0 µl (25 mM), dNTPs (Pharmacia, Inc.) 2.0 µl (100 mM each), the two primer combinations 3.0 µl (1.5 µl each = 150 ng), Taq DNA polymerase (Promega Corp.) 0.25 µl (1.25 Units). One single sample (disk) was used per PCR reaction and when using controls, 2.0 µl of DNA template (~4 ng) was added to the reaction. PCR cycles were 94°C for 5 minutes; 40 cycles of: 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes; 72°C for 5 minutes. These products were electrophoresed immediately or stored at 4°C. Ten microlitres from each PCR reaction was loaded onto a 2.0% agarose 1× TBE gel (0.5 µg/ml ethidium bromide) and run for 3 hours at 80 volts, after which gels were exposed to UV light and photographs taken using a digital camera.

RESULTS

DNA Extraction using FTA® Card

A few samples (<5%) required additional washes (1 or 2) before being left to dry and later used in the PCR reaction. This was because the presence of yellow or green color in the last wash indicated the presence of polyphenols and carbohydrates, and these proved to inhibit the PCR reaction (data not shown).

PCR Amplification of Target Region

Pairs of primers were tested in different combinations (Table 2) before a single pair was selected based on positive discrimination of the resistant and susceptible alleles. Using DNA extracted by the conventional method (Burow *et al.,* 2001), primers IMOF1 and IMOR1 amplified 998

Table 2. Twenty-mer oligonucleotide primers designed using Primer 3.0 and the DNA sequence of the *Mi 1.2* gene.

Name	Single nucleotide sequence (5'-3')	GC%	Melting temperature
IMOF1	agccatgcttgcttcacttt	45	56.2
IMOR1	agaggacccacagtggtttg	55	57.7
IMOF2	tagcactttaggggctcgaa	50	56.1
IMOR2	tccgcaatttatcagcaaca	40	53.4
IMOF3	gtatgccgggttcaggtaaa	50	55.5
IMOR3	agtgctcctcctcctcatca	55	57.7
IMOF4	tatgccgggttcaggtaaaa	45	54.6
IMOR4	agtgctcctcctcctcatca	55	57.7

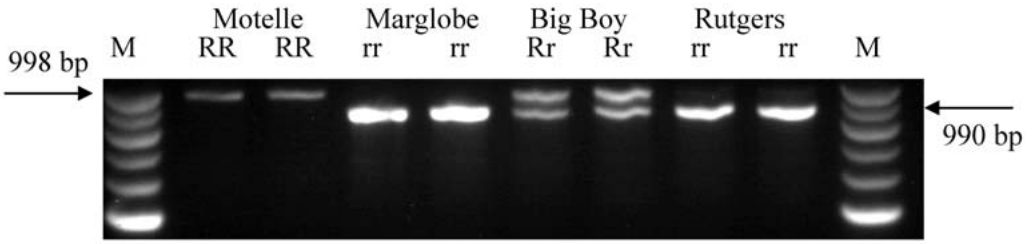

Fig. 1. PCR amplification of a region of the Mi 1.2 gene using the standard DNA extraction method and primers IMOF1 and IMOR1. From the left (samples in duplicates): resistant cv. Motelle, susceptible cv. Marglobe, heterozygous resistant cv. Big Boy and susceptible cv. Rutgers. M = marker 100 bp ladder.

bp and 900 bp DNA fragments for the resistant and the susceptible alleles, respectively (Fig. 1). The forward primer (IMOF1) anneals to the 3' end of the conserved region and the beginning of the intron, and the reverse (IMOR1) anneals to the C terminal of the leucine-rich repeat (LRR), which has been reported to be the most divergent between susceptible and resistant genotypes (Milligan *et al.,* 1998).

When the FTA® Card samples were used, these primers resulted in the consistent amplification of the 998 bp fragment in all the resistant allele bearing genotypes and the isogenic resistant control tomato cv. Motelle, and the 900 bp fragment in all the non-resistant allele bearing genotypes and the isogenic susceptible control cv. Marglobe (Fig. 2). Cultivar Rutgers appeared to have in some PCR reactions what seems to be the marker for the resistant allele. In order to confirm the hypothesis that nonspecific amplification was a possible source of the resistant allele marker in Rutgers, further experiments with annealing temperatures of 55°C and 60°C were carried out but in all cases visualization of the resistant marker was not readily apparent and its presence in the susceptible cv. Rutgers could not be confirmed. In any case since the melting temperatures of the two primers used are 56.2°C (forward) and 57.7°C (reverse), any PCR product obtained with annealing temperatures above those are considered non-specific.

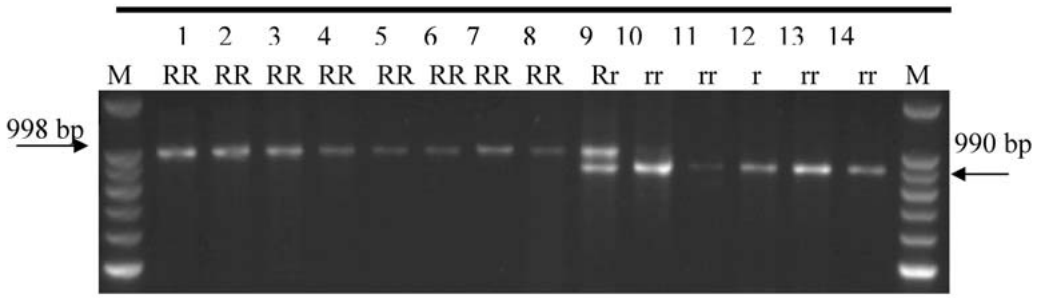

Fig. 2. PCR amplicons obtained with DNA isolated using FTA® Cards and primers IMOF1 and IMOR1. From left to right: cvs. Canta (1), Mossol (2), Motabo (3), Motaci (4), Motelle (5), G-178 (6), VFN-8 (7), VFNT Cherry (8), Big Boy (9), Rutgers (10), Alicante (11), Harzfeuer F1 (12), Marglobe (13) and Marmande (14). M = marker 100 bp ladder. Chi square value $\chi^2 = 1.2$ (alpha = 0.05). RR = homozygous resistant, Rr= heterozygous resistant, rr = homozygous susceptible.

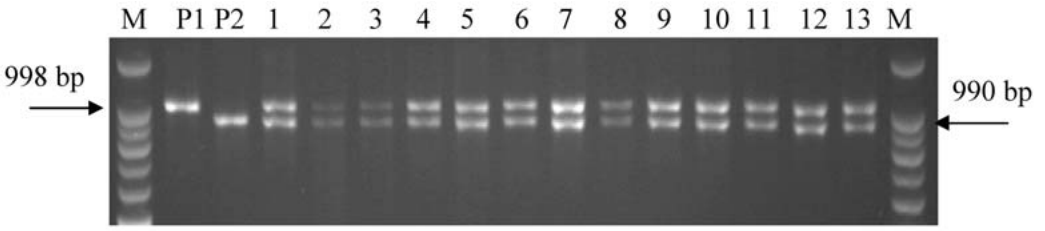

Fig. 3. PCR amplicons obtained with DNA isolated using FTA® Cards from a homozygous resistant (P1) and a homozygous susceptible (P2) tomato line and 13 heterozygous F_1 progeny. M = marker 100 bp ladder.

The two advanced resistant and susceptible tomato parents and their hybrid F_1 progeny were also analyzed using the FTA® Card methodology. The homozygous resistant P1 (*Mi/Mi*) and the homozygous susceptible P2 (*mi/mi*) parents show the markers for the corresponding alleles (Fig. 3), and their progeny show the presence of both markers (*Mi/mi*). The absence of any segregation of the frequencies of the male and female alleles does not depart from what is expected in tomato crosses (Gadish and Zamir, 1986; Ganal and Tanksley, 1996).

The smaller size of the susceptible allele could be explained by the fact that non-resistant genotypes have always shown deletions and a shorter intron between the conserved region and the LRR (Milligan *et al.,* 1998), the latter being involved in regulation of the transmission of the resistance response as well as in recognition of the nematode (Hwang and Williamson, 2003).

The whole FTA® Card extraction and PCR procedure takes a day from obtaining the samples to visualization of the PCR products after electrophoresis. This molecular procedure greatly reduces the time and expense for the routine screening for nematode resistant tomato germplasm. Standard bioassays used for screening of tomato germplasm segregating for resistance to root-knot nematodes require at least 1-3 months (Bost and Triantaphyllou, 1982; Hussey and Barker, 1973) and involve a considerable amount of labour. Therefore, this procedure represents a precise and efficient tool that tomato breeders and researchers will find inexpensive and easy to use, not only in routine screening for marker-assisted selection in breeding programmes, but also in the analysis of transgenic tomato plants (Milligan *et al.*, 1998) and other genetically modified model plants (e.g., *Arabidopsis thaliana*, *Lotus japonicus*), used in the study of the interaction between host and virulent/avirulent populations of root-knot nematodes. Potential for robotic manipulation can not be ignored since it has already been suggested for very similar procedures (Lange *et al.,* 1998).

For the near future it is envisioned that the use of new technologies based on fluorescent reporter systems such as Molecular Beacons, Scorpions, TaqMan or 'DNA chips' (Lee *et al.*, 1993; Livak *et al.*, 1995; Young and Mudge, 2002; Winzeler *et al.*, 1998), automated for high-throughput genotyping, should optimize even more the process of tomato germplasm screening by reducing the cost and time of the analytical procedure.

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