

Artículo científico

Identification of *Mi-1* homologs in various *Solanum* speciesIdentificación de homólogos del gen *Mi-1* en varias especies de Solanáceas

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doi.org/10.26807/remcb.v39i1.566

Recibido 6-02-2018 ; Aceptado 21-05-2018

ABSTRACT.- In Ecuador, several solanaceous crops, including naranjilla (*Solanum quitoense*), are attacked by the nematode *Meloidogyne* sp. Resistant cultivars are not available, and there is a need to identify potential sources of resistance that can be incorporated into breeding programs. The *Mi-1* gene from *S. peruvianum* is known to confer resistance to *Meloidogyne* in tomato (*S. lycopersicum*). In this study, 42 plant accessions of wild and cultivated *Solanum* species were screened to identify the presence of this gene, its diversification, expression, and evidence of gene recombination. The *Mi-1* gene (exon 3) was identified in 34 accessions. Sixteen alleles were identified, which encode 15 different amino acid sequences, all of which encode NBS-LRR proteins and share 72-96% homology with the tomato *Mi-1* protein. The *Mi-1* locus was highly polymorphic; most polymorphisms tend to accumulate in the NBS rather than in the LRR region. Genetic recombination was detected among the tomato and potato related sequences, but was absent in the naranjilla group. Homologs identified expressed in both non-infected roots and leaves of some accessions, suggesting the constitutive expression of the gene.

KEYWORDS: homologs, *Meloidogyne*, *Mi-1* gene, resistance, *Solanum*.

RESUMEN.- En el Ecuador, varios cultivos de solanáceas –incluyendo la naranjilla (*Solanum quitoense*)– son atacados por nematodos del género *Meloidogyne* sp. Cultivares resistentes no están disponibles y existe una necesidad importante de identificar fuentes potenciales de resistencia para ser incorporadas en los programas de mejoramiento. El gen *Mi-1* de *S. peruvianum* es conocido por la resistencia a *Meloidogyne* que confiere en el tomate (*S. lycopersicum*). En este estudio, 42 accesiones de plantas silvestres y cultivadas de especies de *Solanum*, fueron analizadas para identificar la presencia de este gen, su diversificación, expresión y evidencia de recombinación. El gen *Mi-1* (exón 3) fue identificado en 34 accesiones. Dieciséis alelos fueron identificados, de los cuales 15 codificaron secuencias aminoácidas diferentes. Todas incluyeron proteínas NBS-LRR y compartieron una homología del 72%-96% con la proteína *Mi-1* del tomate.

El locus de *Mi-1* resultó altamente polimórfico. La mayoría de los polimorfismos se acumularon en la región NBS en vez de LRR. La recombinación genética fue detectada entre secuencias relacionadas con el tomate y la papa. Sin embargo, esta no se presentó en el grupo de la naranjilla. Homólogos fueron identificados tanto en hojas como en raíces de algunas accesiones de plantas no infectadas, sugiriendo una expresión constitutiva del gen.

PALABRAS CLAVES: homólogos, *Meloidogyne*, *Mi-1* gen, resistencia, *Solanum*.

INTRODUCTION

In the highland region of Ecuador, large areas of land are cultivated with local economically important *Solanum* crops such as the potato (*S. tuberosum* and *S. phureja*), tomato (*S. lycopersicum*), tree tomato (*S. betaceum*), pear melon (*S. muricatum*), and naranjilla (*S. quitoense*). In 2016, 3514 ha of tree tomato were planted which produced 28512 t (INEC, 2014). There is no current information on naranjilla production, but the crop is cultivated on approximately 9460 ha by more than 7100 families (INEC, 2000). In both crops, production has been constrained, among other factors, by the attack of the root-knot nematode (RKN) *Meloidogyne* sp. The nematode restricts the plants' absorption of nutrients and water uptake from the soil, resulting in a reduction in yield and in the useful life of crops (Trudgill, 1991). In naranjilla, for instance, *M. incognita* is one of the main diseases causing estimated yield losses of 30%, which double when the nematode interacts with *Fusarium oxysporum*, and reducing plant life by 50% (Revelo et al. 2003). In the 70s, the lack of cultivars resistant to this and other significant diseases was the principal cause of naranjilla production collapse in Ecuador (Ochoa et al., 2008). In tree tomato plantations, *M. incognita* incidence can be 100%, yield losses as high as 90% in some cultivars, and plant life can be reduced by half (Revelo et al., 2003; Revelo et al., 2004). The nematode has traditionally been controlled in both crops by highly toxic nematicides, and in the case of the naranjilla, by clearing primary forests to introduce the crop in virgin soils (Vásquez et al., 2011). Resistant cultivars, accepted by farmers, are not available in both crops, and national breeding programs are seeking new sources of resistance.

Resistance to plant-parasitic nematodes is defined as the ability of a plant to suppress its development or reproduction (Roberts, 2002), and is currently the most effective and environmentally safe method for controlling nematode infestation. For example, tomato resistant cultivars have proven commercially successful in the control of the most damaging *Meloidogyne* species (Castagnone-Sereno 2002).

Plants have evolved several defense mechanisms against a broad range of pathogens, including resistance genes or R-genes. One of these genes is the tomato *Mi-1* gene, which was originally identified in the wild tomato *S. peruvianum*, and then introduced into cultivated tomato using embryo rescue (Smith, 1944). *Mi-1* confers resistance to three RKNs (*M. arenaria*, *M. incognita* and *M. javanica*) (Dropkin, 1969a), to the potato aphid *Macrosiphum euphorbiae* (Rossi et al., 1998) and the whitefly *Bemisia tabaci* (Nombela et al., 2003). The use of resistant tomato cultivars with this gene has proven highly

effective as a nematode management strategy, and resistant cultivars yield normally on infested land (Roberts and May, 1986). Nowadays, all commercially available tomato cultivars resistant to RKNs carry the *Mi-1* gene and it is the only commercially available source of resistance to these nematodes in the crop. *Mi-1* has been exploited extensively over the last two decades for modern tomato cultivar development (Jablonska et al., 2006), but new sources of resistance against nematodes, which can be incorporated in breeding programs, are required.

The *Mi-1* gene encodes a 1257 amino acid protein and has three exons (two are translated to protein). It belongs to a major class of plant R-genes (NBS-LRR) that encode nucleotide binding sites and leucine-rich repeats (Kaloshian et al., 1998; Milligan, 1998), and a putative coiled-coil domain preceding the NBS. NBS-LRR proteins mediate pathogen recognition and initiate defense signaling that leads to host resistance (Belkhadir et al., 2004). In plant R proteins, the NBS is part of a larger, highly conserved domain of approximately 320 amino acids (Leipe et al., 2004), whereas the LRR domain is generally the most variable region among closely related R genes (Bergelson 2001).

Mi-1 is located in the short arm of chromosome 6 of the tomato (Kaloshian et al., 1998). The short arm of chromosome 6 in various *Solanum* species is an important hot spot of R-genes effective against diverse and economically significant crop pathogens (Seah et al., 2007). In the *Mi* locus, three genes, *Mi-1.1*, *Mi-1.2*, and *Mi-1.3* were identified, but only the *Mi-1.2* gene is able to confer resistance to RKNs (Milligan, 1998; Rossi et al., 1998). This gene will henceforth be referred to as *Mi-1* in the text. Several *Mi* homolog genes have been mapped in the vicinity of the *Mi-1* in *S. lycopersicum* and *S. peruvianum* (Gleason et al., 2004). With the exception of some *Mi* homologs considered pseudogenes, the identity of the DNA sequences for all *Mi-1* homologs is extremely high, ranging from 92.9% to 96.7% (Seah et al., 2007)

In tomato, the function of *Mi-1* and of other RKN R-genes is lost at high temperature (Ammiraju et al., 2003). For instance, *Mi-1* is only effective at soil temperatures below 28 °C (Holtzmann 1965; Dropkin 1969b). However, the *Mi-9* gene from *S. arcanum*, a homolog of *Mi-1* and localized in the same chromosomal interval as *Mi-1*, confers heat-stable resistance to RKNs at 25 °C and 32 °C (Ammiraju et al., 2003; Jablonska et al., 2006). There are also some *Mi* homologs discovered in chromosome 12 that inhibit reproduction of virulent nematode isolates and maintain a phenotypic resistance response when soil temperatures are above 28 °C (e.g. *Mi-3* and *Mi-5*) (Jablonska et al., 2006).

In the potato, gene *Rpi-blb2* of the wild potato *S. bulbocastanum*, which confers broad resistance to *Phytophthora infestans*, also maps to this region. This gene is the nearest homolog to the gene *Mi-1* known in the potato, sharing 82% amino acid sequence identity with the tomato gene *Mi-1* (Vossen et al., 2005). Resistance genes against *M. incognita* have not yet been reported in tuber-bearing potatoes. However, 59 *Mi-1* homologs were described and studied in the cultivated potato species *S. tuberosum* ssp. *tuberosum* and *S. phureja* (Sanchez-Puerta and Masuelli, 2011).

The principal aim of this study was to provide insight into the presence and expression of the *Mi-1* gene among different plant species of the genus *Solanum* (Solanaceae) in order to identify potential sources of resistance to *Meloidogyne* spp., particularly in naranjilla. To accomplish this, 42 plant accessions of wild and cultivated *Solanum* species were screened to determine its presence, diversification, expression, and evidence of recombination.

MATERIALS AND METHODS

Plant Material.-A total of 42 wild and cultivated *Solanum* plant accessions, belonging to 25 species and 9 sections, were used in this study (Table 1). *Solanum* seed were derived either from the Germplasm Bank of the National Department of Phytogenetic Resources of the National Autonomous Institute for Agricultural Research (Departamento Nacional de Recursos Fitogenéticos del Instituto Nacional Autónomo de Investigaciones Agropecuarias - INIAP-DENAREF Quito) or the International Potato Center - Quito (CIP-Quito). In the specific case of tomato, seeds from commercial cultivars were used. Seeds were germinated, and plants were grown in a greenhouse until they reached an approximate height of 10 cm.

Isolation of Nucleic Acids.- The genomic DNA of each accession (Table 1) was obtained from 100 mg of fresh leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) protocol, diluted to 5 ng.μL⁻¹ and stored at -20 °C until use. RNA was extracted from non-infected leaves (13 plant accessions) and roots (only two accessions) (Table 1) with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) protocols, respectively. Quality and quantity of DNA and RNA was checked using the NanoVue Spectrophotometer (General Electric Company, Little Chalfont, UK) and by agarose gel (1% for DNA and 2% for RNA) electrophoresis.

PCR Analyses and Sequencing.- To search for *Mi-1* homolog sequences, primers corresponding to a conserved region (1Kb) of exon 3 of the *Mi-1* gene (Williamson

and Kumar, 2006) were designed. Primer sequences used were 1F 5'-AACTCGAGAAAAGGAAGTGG-3' and 1R 5'-CAAGATTGATCCTTTGTTAGACAC-3'. Reactions were performed in a final volume of 20 μl containing 150 μM dNTPs, 1.5 mM MgCl₂, 1 μM of each primer, 1 U *Taq* DNA polymerase and 1X DNA polymerase buffer. For F1 and R1, cycling conditions were: 94 °C for 5 min; 35x (92 °C, 0.45 min; 61 °C, 1 min; 72 °C, 1 min); and 72 °C, 10 min. Amplicons were analyzed by electrophoresis in 1.5% agarose gels containing SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA USA) in TBE 1X buffer, and visualized under UV light. The selected amplicons were recovered from gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The fragments obtained were then sequenced on both strands by MacroGen S.A. (Seoul, Korea).

Diversity Analysis.- Nucleotide sequences were analyzed with the software BioEdit (Hall, 1999) and translated into amino acid sequences. All sequences were aligned using MUSCLE (Edgar, 2004). BlastN and BlastP search strategies were conducted to identify DNA and/or protein sequences homolog to *Mi-1*. Putative homologs were checked for significance, and only sequences with at least 70% coverage of the query sequence and with an E-value of 1.e-05 as the cutoff were considered as true homologs. The same strategy was used to identify protein domains. The number of polymorphic amino acid sites and gaps were identified using ClustalX 2.0 (Larkin et al., 2007).

Phylogenetic Analysis.- Maximum likelihood analyses were performed with RAxML (Stamatakis et al., 2008) under the General Time Reversible model with parameters for invariant sites and gamma-distributed rate heterogeneity (Tamura et al., 2011). One hundred bootstrap replicates were performed. The trees were visualized using FigTree 1.4.3 (Rambaut, 2009). For these analyses, 11 *Mi-1* gene homologs obtained from this study, plus 23 *Mi-1* homologs belonging to *S. tuberosum*, *S. lycopersicum*, *S. peruvianum* and *S. phureja* (Sanchez-Puerta and Masuelli 2011) were used. *Mi-1.2* from *S. lycopersicum*, *Rpi-blb2* from *S. bulbocastanum* (Vossen et al., 2005), *CaMi* (Chen et al., 2007) and *Me* (Mao et al., 2008) from *Capsicum annuum* were used for comparison.

Test of Gene Recombination.- Recombination events of the *Mi-1* loci within the different clades were analyzed using the Split Decomposition Method (Huson, 1998) integrated in the Splits Tree4 program (Huson and Bryant, 2006). A significant threshold of 80% was used as a cutoff for finding evidence of genetic recombination.

Gene Expression analysis.- RNA was extracted from 100mg of leaf (13 *Solanum* accessions) and leaf and root

Table 1. Plant accessions belonging to different *Solanum* sections used for *Mi-1* locus screening and gene expression analysis.

	Code	Species	Section	Gene expression analysis
1	S45	<i>S. mamosum</i>	<i>Acanthophora</i>	
2	A1	<i>S. brevifolium</i>	<i>Anarrichomenum</i>	
3	A4	<i>S. brevifolium</i>	<i>Anarrichomenum</i>	
4	A2	<i>S. sodiroi</i>	<i>Anarrichomenum</i>	
5	S12	<i>Solanum</i> spp./tomatillo	<i>Anarrichomenum</i>	
6	S17	<i>S. caripense</i>	<i>Basarthurm</i>	x
7	J2	<i>S. juglandifolium</i>	<i>Juglandifolia</i>	
8	J3	<i>S. juglandifolium</i>	<i>Juglandifolia</i>	
9	J4	<i>S. juglandifolium</i>	<i>Juglandifolia</i>	
10	S28	<i>S. juglandifolium</i>	<i>Juglandifolia</i>	x
11	S29	<i>S. ochrantum</i>	<i>Juglandifolia</i>	
12	La15	<i>S. gradiflorum</i>	<i>Lasiocarpa</i>	
13	La17	<i>S. hirtum</i>	<i>Lasiocarpa</i>	
14	La16	<i>S. hyporhodium</i>	<i>Lasiocarpa</i>	
15	S35	<i>S. hyporhodium</i>	<i>Lasiocarpa</i>	x
16	La21	<i>S. pectinatum</i>	<i>Lasiocarpa</i>	
17	La22	<i>S. pectinatum</i>	<i>Lasiocarpa</i>	
18	S37	<i>S. pseudolulo</i>	<i>Lasiocarpa</i>	x
19	S32	<i>S. quitoense</i>	<i>Lasiocarpa</i>	
20	S33	<i>S. quitoense</i>	<i>Lasiocarpa</i>	x*
21	S42	<i>S. quitoense</i>	<i>Lasiocarpa</i>	x
22	S34	<i>S. sessiliflorum</i>	<i>Lasiocarpa</i>	x
23	S38	<i>S. vestisimum</i>	<i>Lasiocarpa</i>	x
24	S36	<i>Solanum</i> sp.	<i>Lasiocarpa</i>	
25	S19	<i>Solanum</i> spp. #61	<i>Lasiocarpa</i>	
26	Ly23	<i>S. habrochaites</i> -like	<i>Lycopersicon</i>	
27	Ly24	<i>S. habrochaites</i>	<i>Lycopersicon</i>	
28	S57	<i>S. lycopersicum</i> (cv. Advantage)	<i>Lycopersicon</i>	
29	S59	<i>S. lycopersicum</i> (cv. Flora Dade)	<i>Lycopersicon</i>	x*
30	Ly10	<i>S. peruvianum</i>	<i>Lycopersicon</i>	
31	S8	<i>S. melongena</i>	<i>Melongena</i>	x
32	S1	<i>S. andreanum</i>	<i>Petota</i>	
33	P7	<i>S. andreanum</i>	<i>Petota</i>	
34	S18	<i>S. andreanum</i>	<i>Petota</i>	x
35	S16	<i>S. colombianum</i>	<i>Petota</i>	x
36	P8	<i>S. minutifolium</i>	<i>Petota</i>	
37	S3	<i>S. paucijugum</i>	<i>Petota</i>	
38	S15	<i>S. paucijugum</i>	<i>Petota</i>	x
39	S14	<i>S. solisii</i>	<i>Petota</i>	
40	T1	<i>S. hispidum</i> -like	<i>Torva</i>	
41	T2	<i>S. hispidum</i> -like	<i>Torva</i>	
42	T3	<i>S. hispidum</i> -like	<i>Torva</i>	

* RNA extracted from leaf and root tissue

(2 accessions) tissue (Table 1) as stated above. RNA was quantified and pre-treated with 1 μ L DNase I (Invitrogen, Carlsbad, CA, USA) and 1 μ L 10X buffer. Additionally, DEPC water was added to a final volume of 10 μ L. The reaction was incubated for 15 min at room temperature and DNase I inactivation was performed using 1 μ L of EDTA at 65 °C. Superscript III reverse transcriptase Kit (Invitrogen, Carlsbad, CA USA) was used to perform cDNA synthesis in a final volume of 12 μ L. Initially, 1 μ L of oligo (dT)18 primer (0.5 μ L. μ L⁻¹) and 1 μ L dNTPs (10 mM) were added and incubated for 5 minutes at 65 °C. It was immediately placed on ice for 1 minute and 4 μ L of first strand 5X buffer, 1 μ L of DTT (0.1 M), and 1 μ L of Superscript III reverse transcriptase (200 U. μ L⁻¹) were added to a final volume of 20 μ L. Reaction tubes were vortexed, incubated at 50 °C for 15 min and at 70 °C for 15 min. cDNA was stored at -20 °C until use. Quality and quantity of cDNA was verified by agarose gel (2%). cDNA was subsequently used to amplify GAPDH and *Mi-1* genes. GAPDH gene was used as a constitutively expressed endogenous control. PCR conditions were the same as cited above and amplicons were visualized in 2% agarose gels.

RESULTS AND DISCUSSION

Mi-1 Gene Homologs Are Present in *Solanum* Species.- Identification of the potential presence of *Mi-1*

locus in the *Solanum* accessions was performed through an amplification of a conserved 1 Kb region of exon 3 of the *Mi-1* tomato gene. This fragment spans amino acid 672-964 and includes the NBS (672 to 802) and the LRR (803 to 964) regions. The 1 Kb amplicon was detected in 34 out of the 42 accessions examined (Table 2). It was present in all the *Anarrichomenum* and wild potato accessions (Petota) analysed, as well as in most accessions of *S. quitoense* and its close relatives (*Lasiocarpa section*). Four accessions showed an additional 0.9 Kb fragment, and eight accessions did not show any amplification band (Table 2). The 0.9 Kb fragments detected may correspond to uncharacterized paralogs or pseudogenes (Meyers, 1998; Noel, 1999), which seems to be common among resistance genes. Pseudogenes may be potential sources of variation (Meyers, 1998; Michelmore and Meyers, 1998) and a rapid way to achieve new specificities (Ota and Nei, 1994) because they tend to evolve more quickly than the functional genes.

To verify if the amplicons obtained corresponded to *Mi-1* homologs, the 1 Kb and 0.9 Kb fragments of 12 plant accessions were sequenced (Table 3); some of these accessions were relevant for the national breeding programs. Sequencing resulted in mixed sequences in few accessions, indicating that the primers amplified multiple alleles or *Mi-1* paralogs.

Table 2.- Description of the *Mi-1* exonic amplification in the 42 accessions of *Solanum* analyzed in this study.

Section	Plant accession	Presence of a 1 Kb fragment	Presence of a 0.9 Kb fragment	None
<i>Anarrichomenum</i>	A1, A2, A4	x		
	S12	x	x	
<i>Basarthurm</i>	S17	x		
<i>Juglandifolia</i>	J2, J3, S28, S29	x		
	J4			x
<i>Acanthophora</i>	S45			x
<i>Melongena</i>	S8	x		
<i>Petota</i>	S1, S16, S18, P7, P8	x		
	S3, S15, S14	x	x	
<i>Torva</i>	T3	x		
	T1, T2			x
<i>Lycopersicon</i>	S57, S59, Ly10	x		
	Ly23, Ly24			x
<i>Lasiocarpa</i>	S19, S32, S33, S34, S35, S36, S37, S38, S42, La17, La21, La22	x		
	La15, La16			x

Multiple alignment of the nucleotide sequences with the Mi-1 exon using BlastN revealed the existence of 16 different alleles (Table 3). Chromatogram analyses indicated that most accessions were homozygous, whereas two accessions (S17 of *S. caripense* and S18 of *S. andreaenum*) were heterozygous (Table 3).

The 16 allele sequences were translated into amino acids. MUSCLE alignment showed that they encoded 15 different amino acid sequences; sequences S57 and S59 were the same (Table 3). Sequences from A1, S12-1, and S18 presented premature stop codons or frameshift mutations and therefore were classified as pseudogenes. They were excluded from further analyses.

A BlastP search against non-redundant protein sequences showed that all resultant amino acid sequences encoded NBS-LRR proteins and shared 72%–96% homology with the tomato Mi-1 protein that confers resistance to *Meloidogyne*. The sequences analyzed corresponded to the final part of the NBS-conserved region and the initial part of the LRR region of these proteins.

Multiple amino acid sequence alignments between the Mi-1 protein and the 15 proteins identified in this study revealed a total of 117 polymorphic amino acid sites (Fi-

gure 1a). Most polymorphisms tended to accumulate in the NBS region (57/131) rather than in the LRR region (60/163), although difference was not clear-cut. Additionally, 71 gaps were found in the NBS region and 80 in the LRR region. These results show that, compared to the NBS portion, the LRR portion tended to be less divergent among the *Mi-1* homologs from the different *Solanum* accessions analyzed.

To investigate the phylogenetic relationships among the *Mi-1* loci, a phylogenetic tree was constructed using the nucleotide sequences from 11 *Solanum* accessions (Table 3), 23 *Mi-1* homologs from *S. tuberosum*, *S. lycopersicum*, *S. peruvianum* and *S. phureja* (Sanchez-Puerta and Masuelli, 2011) and the *Mi-1.2*, *Rpi-blb2*, *CaMi* and *Me* genes. The resulting tree topology is shown in Figure 1b. *Mi-1* sequences obtained from different *Solanum* sections are distributed all across the tree and were closely related to the *Mi-1* homologs. The sequences were grouped into three main clusters. The first main cluster, the tomato cluster (TC), included the *CaMi* gene from *C. annuum* (which is similar to the gene *Mi-1* from tomato), the *Mi-1.2* gene from *S. lycopersicum*, one accession from *S. peruvianum*, two accessions from *S. lycopersicum*, and various tomato *Mi-1* related sequences; one sequence from *S. paucijugum* and one

Table 3. Polymorphisms identified in the *Mi-1* locus in 12 *Solanum* accessions.

Section	Species	Code	Nucleotide sequence		Amino acid sequence	
			Allele 1	Allele 2	Allele 1	Allele 2
	<i>S. brevifolium</i>	A1	<i>brf_nal</i>		<i>brf_pal</i> ^a	
<i>Anarrichomenum</i>	<i>S. spp/tomatillo</i>	S12-1	<i>toll_nal</i>		<i>toll_pal</i> ^a	
	<i>S. spp/tomatillo</i>	S12-2	<i>tol2_nal</i>		<i>tol2_pal</i>	
<i>Basarthurm</i>	<i>S. caripense</i>	S17	<i>car_nal</i>	<i>car_na2</i>	<i>car_pal</i>	<i>car_pa2</i>
	<i>S. sessiliflorum</i>	S34	<i>ses_nal</i>		<i>ses_pal</i>	
<i>Lasiocarpa</i>	<i>S. hyporhodium</i>	S35	<i>hyp_nal</i>		<i>hyp_pal</i>	
	<i>S. quitoense</i>	S42	<i>qui_nal</i>		<i>qui_pal</i>	
<i>Melongena</i>	<i>S. melongena</i>	S8	<i>mel_nal</i>		<i>mel_pal</i>	
	<i>S. lycopersicum</i> (Advantage)	S57	<i>adv_nal</i>		<i>adv/fda_pa</i> <i>1</i> ^b	
<i>Lycopersicon</i>	<i>S. lycopersicum</i> (Flora Dade)	S59	<i>fda_nal</i>		<i>adv/fda_pa</i> <i>1</i> ^b	
	<i>S. peruvianum</i>	Ly10	<i>per_nal</i>		<i>per_pal</i>	
<i>Petota</i>	<i>S. paucijugum</i>	S15-1	<i>paj1_nal</i>		<i>paj1_pal</i>	
	<i>S. paucijugum</i>	S15-2	<i>paj2_nal</i>		<i>paj2_pal</i>	
	<i>S. andreaenum</i>	S18	<i>and_nal</i>	<i>and_na2</i>	<i>and_pal</i> ^a	<i>and_pa2</i> ^a

^a Sequences classified as pseudogenes.

^b Amino acid sequences were the same.

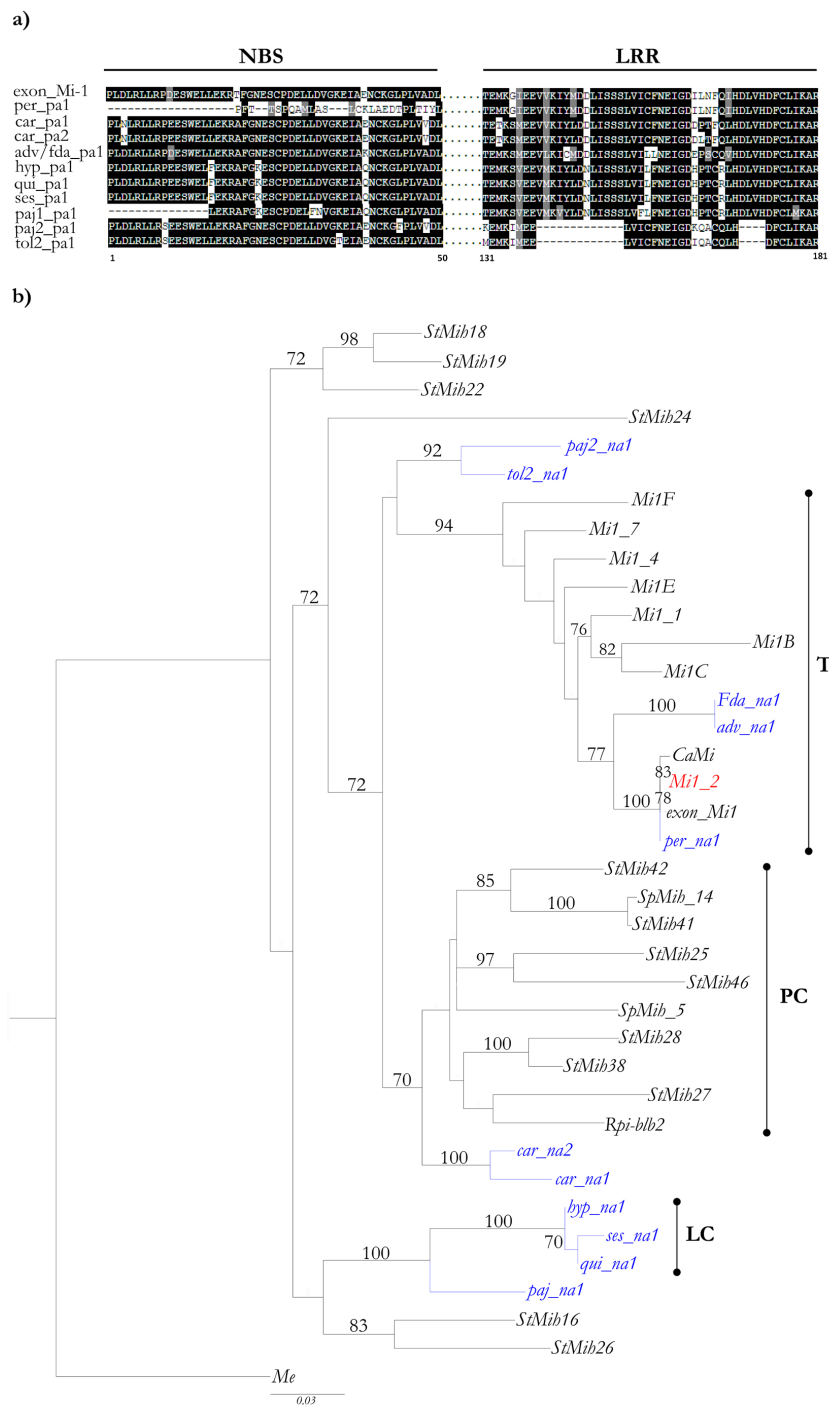


Fig. 1. Multiple sequence alignment of the NBS and LRR regions of the predicted *Mi-1* homologs, and nucleotide phylogenetic tree. **(a)** Multiple alignments of the predicted *Mi-1* homologs amino acid sequences revealed a highly polymorphic family. **(b)** Phylogenetic analysis of nucleotide sequences from 11 *Mi-1* homologs found in this study (in blue), 23 *Mi-1* homolog sequences from *Solanum tuberosum* (*StMi1h* genes), *S. lycopersicum* (*Mi* genes), *S. phureja* (*SpMi1h*) (Sanchez-Puerta & Williams, 2011) and *S. peruvianum*, and four sequences of the genes *Mi-1.2* from *S. lycopersicum*, *Rpi-blb2* from *S. bulbocastanum* (van der Vossen et al., 2005), *CaMi* (Chen et al., 2007), and *Me* (Mao et al., 2008) from *Capsicum annuum*, which were used for comparison. The tree was rooted with gen *Me*. Analysis was performed with RAXML (Stamatakis et al., 2008). Numbers on branches correspond to bootstrap support values >59% from 100 bootstrap replicates. TC: tomato cluster; PC: potato cluster; LC: *Lasiocarpa* cluster.

from “tomatillo” were closely related. The second main cluster, the potato cluster (PC), included the *Rpi-blb2* gene, *S. tuberosum* and *S. phureja* related sequences, but surprisingly, *S. caripense* members were very close related. The third cluster, the Lasiocarpa cluster (LC), was poorly resolved and grouped three sequences coming from plant accessions of section Lasiocarpa; *S. paucijugum* was strongly related to this cluster. Each cluster had bootstrapping values over 70%.

Additionally, phylogenetic trees were constructed using only the sequences of NBS and LRR domains to determine if these would maintain the same functional distribution. As expected, both trees showed the same topology (data not shown). Phylogenetic reconstruction of exon 3 is consistent with the idea that *Mi-1* is a complex loci with several members distributed among *Solanum* species.

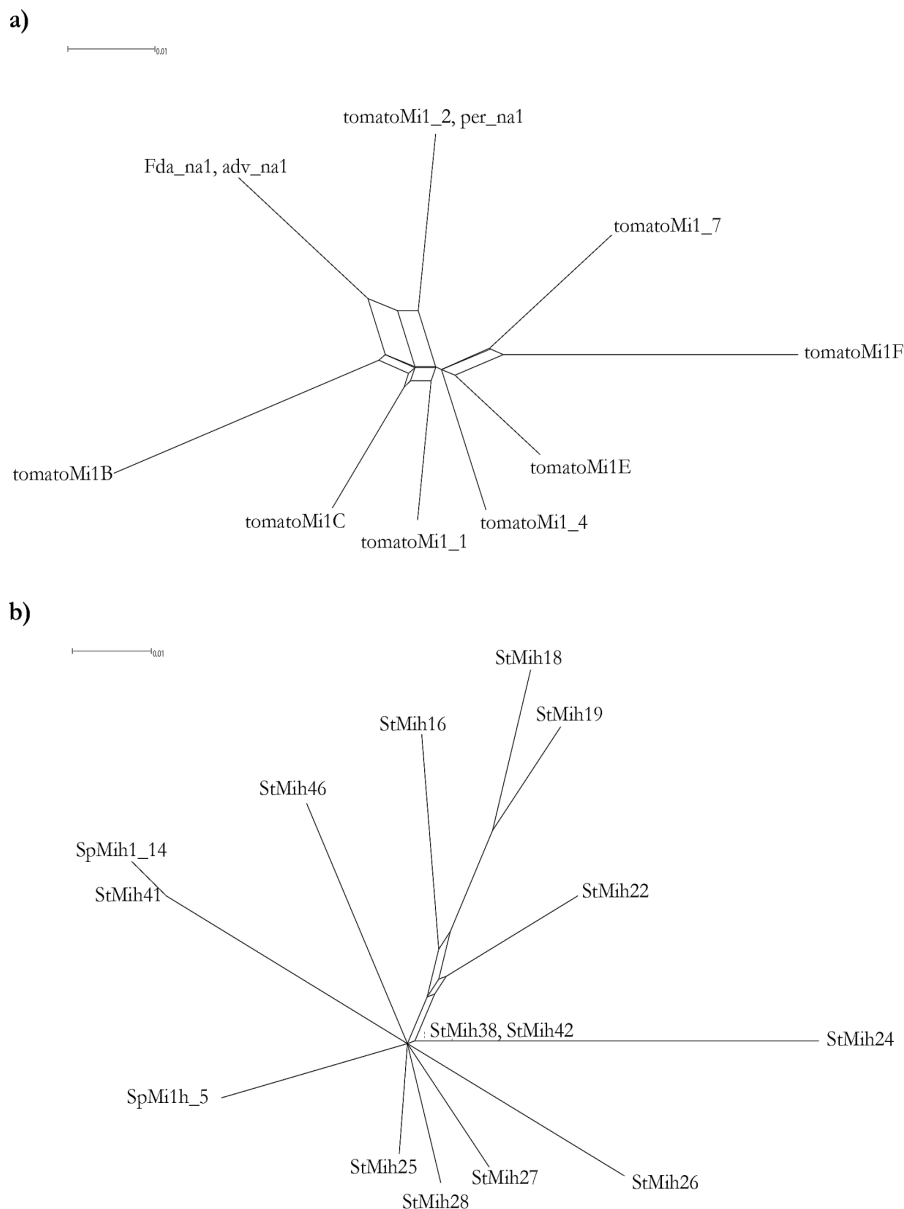


Fig. 2. Recombination analysis with the Split Decomposition Method (Huson, 1998). Trees show an interconnected network between some accessions due to possible recombination events. A threshold of 80% as cutoff was used to find evidence for genetic recombination. **(a)** Tomato clade. Eleven accessions show evidence of recombination with 73.8% fit. **(b)** Potato clade. Fifteen accessions show evidence of recombination with 40.5% fit. Scale bar shows the percent difference among the sequence.

The high number of alleles identified at the *Mi-1* locus can be explained by the fact that the accessions belonged to different sections of the genus *Solanum*. The identification of *Mi-1* homologs in sections as distant as *Petota* or *Lasiocarpa* (Bohs, 2005), suggests that this gene is highly conserved in the genus *Solanum* and that it may have originated from an ancestral common gene which might have evolved in different ways to improve pathogen recognition. The presence of polymorphic and conserved regions among the sequences analyzed also suggests that all *Mi-1*-like sequences evolved from a common ancestor (Michelmore and Meyers, 1998).

The tree *Mi-1* homologs detected in *Lasiocarpa* are potential sources of resistance to *Meloidogyne* in the naranjilla crop. The three *Mi-1* homologs identified in *Petota* in the wild potatoes *S. paucijugum* and *S. andreaeanum* are also potential sources of resistance to the nematode. Previously, *Mi-1* homologs have been identified only in *S. bulbocastanum* (Vossen et al., 2005), and the cultivated potatoes *S. phureja* and *S. tuberosum* ssp. *tuberosum* (Sanchez-Puerta and Masuelli, 2011). Further cloning and functional analysis of these genes will assess its role in plant defense.

Recombination Contribution to Sequence Diversity in the *Mi-1* loci. Investigation of *Mi-1* loci recombination rate within the different clades was performed using the Split Decomposition Method (Huson 1998). This method evaluates the fit percentage of each of the selected clades to a phylogenetic tree topology. The higher the fit percentage, the lower the evidence of recombination. Using a significant threshold of 80% as cutoff, evidence for genetic recombination was found only within the tomato (73.8%) and potato (40.5%) clusters, whereas no evidence was observed in the *Lasiocarpa* cluster (100%). According to the network diagrams, a group of 11, 15, and zero sequences showed evidence of recombination in the tomato, potato, and *Lasiocarpa* clusters, respectively (Figure 2; *Lasiocarpa* cluster not shown). This means that most of the *Mi-1* homologs identified in this study generated by interallelic recombination and not by mutation, and that recombination appears to be the predominant mechanism in the generation of allelic variation. Recombination was absent in the *Lasiocarpa* sequences analyzed, implying that the *Mi-1* homologs identified in this section can be considered an excellent target for cloning; and hence, potential sources of resistance to *Meloidogyne* in the naranjilla crop.

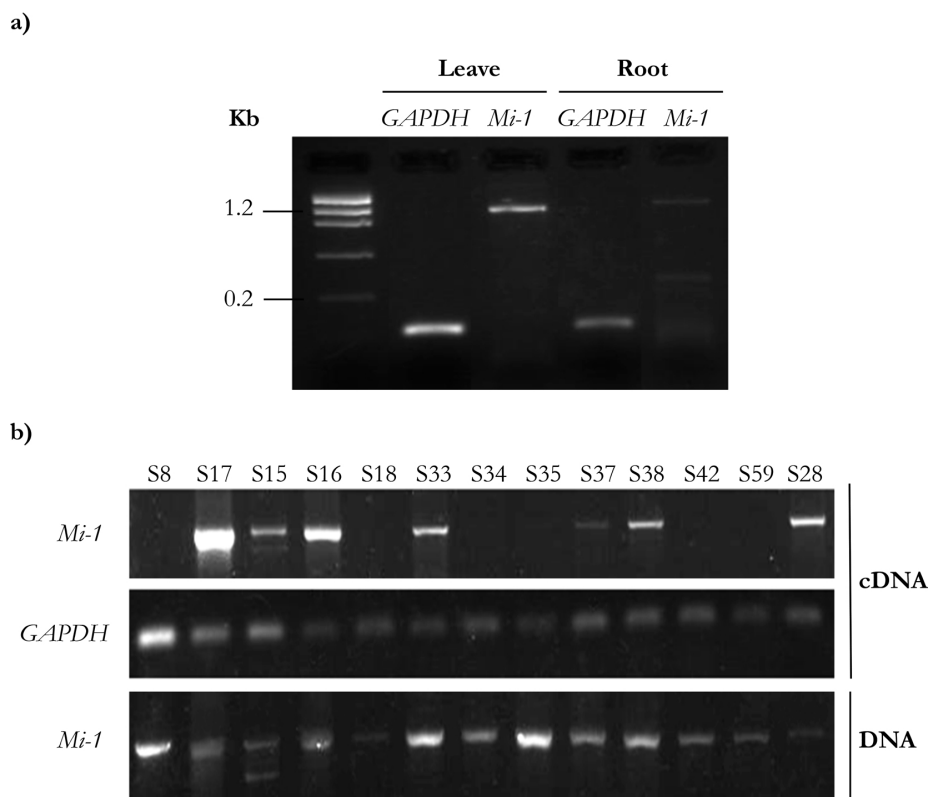


Fig. 3. Expression of *Mi-1* and *GAPDH* genes in non-infected leaves and roots of some *Solanum* accessions. **(a)** Expression of the *Mi-1* gene in non-infected leaves and roots of the tomato plant *S. lycopersicum* (S59, Flora Dade); *GAPDH* gene was used as a constitutively expressed endogenous control. Low DNA mass ladder (Invitrogen, Carlsbad, CA USA) was used to determine the molecular weight of fragments. **(b)** Transcript accumulation of *Mi-1* homologs in 13 different *Solanum* accessions (non-infected leaves).

***Mi-1* Gene Homologs Are Constitutively Expressed in Some *Solanum* Accessions.**- Gene expression of the *Mi-1* gene was evaluated in some plant accessions. For this, RNA was extracted from non-infected leaves and/or roots and cDNA was synthesized. The *GAPDH* gene was used as endogenous control for constitutive expression. In a first assay, gene expression was analyzed in non-infected leaves and roots of tomato (S59) and naranjilla (S33) (Table 1). Amplification showed expression of both genes in both roots and leaves, suggesting the constitutive expression of the *Mi-1* gene (Figure 3a; results of naranjilla not shown).

In a second assay, non-infected leaves of 13 accessions of 12 species were evaluated. The *Mi-1* gene was expressed in only seven samples (Figure 3b) even though the *Mi-1* gene was present in all samples tested at genomic level.

Gene expression in non-infected leaves of some species suggests that pathogen infection is not required to induce *Mi-1* expression and that the gene is constitutively expressed. Expression of *Mi-1* homologs at the plant level makes them good candidates for cloning.

CONCLUSIONS

The *Mi-1* gene was detected in 34 wild and cultivated plant accessions of *Solanum*, distributed in seven sections of the genus.

Sequencing revealed 16 different alleles encoding 15 different amino acid sequences. All amino acid sequences identified encode NBS-LRR type proteins that share 72%–96% homology with the tomato *Mi-1* amino acid sequence.

The *Mi-1* homologs identified are highly polymorphic. Most polymorphism tends to accumulate in the NBS rather than in the LRR region.

Evidence of genetic recombination was detected in the tomato and potato clusters, but not in the *Lasiocarpa* cluster. The three *Mi-1* homologs identified in *Lasiocarpa* are good targets for gene cloning, and hence potential sources of resistance to *Meloidogyne* in naranjilla.

Gene expression of some *Mi-1* homologs in non-infected tissues suggests its constitutive expression in the plant. These genes may also be considered as potential sources of resistance against the nematode.

ACKNOWLEDGEMENTS

The authors would like to thank the National Department of Phytogenetic Resources of the National Autonomous

Institute for Agricultural Research (INIAP-DENAREF Quito) and the International Potato Center - Quito (CIP-Quito), for providing the seeds from their Germplasm Bank. This project was funded by Universidad de las Fuerzas Armadas - ESPE, Sangolquí, Ecuador.

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