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Research article

Identification of wild-species introgressions in the Mi-1 region of tomato breeding lines using a simple polymerase chain reaction-based method

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Abstract

A polymerase chain reaction (PCR) marker, PMIF/PMIR (tightly linked to the *Mi-1.2* gene, which provides resistance to the root knot nematode) was developed. PCR primers were designed in intron 1 of the *Mi-1.2* gene. PCR using these primers produced six different profiles for different tomato lines. These profiles allowed discrimination among lines of *Solanum lycopersicum* with no introgressions from wild species in the *Mi-1.2* gene region and lines with introgressions from *S. peruvianum*, *S. chilense* and *S. habrochaites*. Furthermore, these PCR profiles distinguished between resistant (Mi/Mi, Mi/+) and susceptible hybrids (+/+) of root knot nematode. Sequences of the 780-bp PCR-amplified fragment had 99% identity with intron 1 of the *Mi-1.2* gene, which confirmed the tight linkage of the markers to the studied locus. The information generated by these primers could be used in tomato breeding programs for detection of introgressions from wild species in the *Mi-1.2* region of chromosome 6.

Introduction

Tomato, *Solanum lycopersicum* (previously *Lycopersicum esculentum*), is one of the most important vegetable crops of the Solanaceae and is widely used as a food ingredient throughout the world and more than 100 million t are produced annually, with the USA, several European countries, Japan and China being among the most important tomato-producing countries (De Carvalho et al., 2015). However, many tomato hybrids are highly susceptible to a number of pathogens, including bacteria, viruses, fungi, nematodes and insect pests. Resistance alleles are primarily present in wild tomato species (Foolad, 2007). Thus, breeding for disease resistance is an important objective in tomato improvement. Many disease resistance genes have been identified in wild species such as *Solanum peruvianum* (Sp) (Barham and Winstead 1957; Seah et al., 2004), *Solanum chilense* (Sc) (Zamir et al., 1994; Grandillo et al., 2011), *Solanum habrochaites* (Sh) (Hanson et al., 2006), *Solanum pennellii* (Parniske et al., 1999), and *Solanum pimpinellifolium* (Chunwongse et al., 2002; Foolad 2007) and introgressed into the cultivated tomato, *S. lycopersicum* (Sl).

Root knot nematodes (*Meloidogyne* spp.) are economically important plant pathogens that cause severe damage to tomato crops especially in tropical, sub-tropical and warm climates (De Carvalho

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et al., 2015). Yield losses of 50% have been reported (Darekar and Mhase 1988) as a result of root deformation and induced-susceptibility to other pathogens (Johnson, 1998). The use of root knot nematode (RKN)-resistant tomato hybrids is a powerful tool for nematode management. Thus, much effort has been invested into identifying host resistance against RKN in tomato wild species (Fassuliotis, 1985). The single dominant locus *Mi-1* confers resistance to three of the most damaging RKN species in cultivated tomato, *M. incognita*, *M. javanica* and *M. arenaria* (Roberts and Thomason, 1986). In addition, this locus was reported to also provide resistance to certain isolates of the potato aphid *Macrosiphum euphorbiae* (Rossi et al., 1998; Vos et al., 1998) and to two biotypes of the whitefly *Bemisia tabaci* (Nombela et al., 2003).

The *Mi-1* locus was introgressed into the cultivated tomato from a nematode-resistant accession (P.I. 128657) of the complex species *S. peruvianum*, using embryo rescue to obtain a breeding line of these normally incompatible species (Smith, 1944). Genetic and physical mapping located *Mi-1* locus in the introgressed region on the short arm of chromosome 6. There are seven homologs of the *Mi-1* gene arranged in two clusters (cluster 1p and cluster 2p) in the resistant cultivar Motelle, which has the introgressions from Sp (Seah et al., 2004). The functional gene *Mi-1.2* is localized in cluster 1p (Milligan et al., 1998, Seah et al., 2007). A similar arrangement of the seven homologs (cluster 1e and cluster 2e) is present in the susceptible cultivar Moneymaker (Sl). The organization of the *Mi-1* locus in Motelle (with the Sp introgression) and Sl is a 300-kb region inverted in Sl.

Selecting desirable resistance genes in a commercial tomato breeding program requires several generations to develop appropriate inbred lines (Devran et al., 2013). Thus, molecular markers and their ability to tag resistance genes as well as to define the contents of wild-species introgressions in the tomato germplasm can help reduce the number of generations required for selecting favorable resistance alleles without using biological assays.

Marker-assisted selection for RKN resistance began with the use of the Aps-1 marker for acid phosphatase more than three decades ago (Medina-Filho and Tanksley, 1983) and then DNA-based markers were first used in 1994 (Williamson et al., 1994). Several DNA-based markers that are tightly linked to the *Mi* locus have been developed and used for marker-assisted selection for RKN resistance (Williamson et al., 1994; Goggin et al., 2004; Bendezu, 2004; El Mehrach et al., 2005; Seah et al., 2007). Although these DNA-based markers were able to discriminate between resistance and susceptible varieties, they did not provide any information on the introgression present in the Mi-1 region. In addition, the CAPS marker REX-1, which is one of the commonly used markers (Williamson et al., 1994), produced false positives with several begomovirus-resistant lines having an introgression from Sc (El Mehrach et al., 2005; Devran et al., 2013). While some other markers did not discriminate between homozygous and heterozygous genotypes (Devran and Elekçioglu 2004), the Mi23 co-dominant SCAR marker was able to discriminate between *Mi1.2* plants and plants with introgressions from Sc (Seah et al., 2007); unfortunately, it was not tested with Sh germplasm.

Thus, the aim of the current study was to develop polymerase chain reaction (PCR)-based molecular markers that allow the detection of the *Mi-1.2*-resistant gene in tomato breeding lines as well as to distinguish between the tomato genotypes that have introgressions from the wild species *S. peruvianum*, *S. chilense*, and *S. habrochaites* in the *Mi-1* locus region.

Materials and Methods

Plant materials used

Several tomato lines, varieties and hybrids with different known introgressions in the *Mi-1* region of chromosome 6 were used to evaluate the efficiency and reliability of PCR primers:

The RKN homozygous resistant cultivars were: Motelle [see accession LA2823 at Tomato Genetic Resource Center (TGRC)] and Anahu (LA0655 at TGRC) (*Mi/Mi*), which have a *S. peruvianum* introgression in the *Mi-1* locus region.

The RKN susceptible lines, cultivars or hybrids of *S. lycopersicum* (+/+) were: Moneymaker, Nainemor, M82, TY50 and the wild species *Solanum cerasiformae*.

The RKN heterozygous resistant commercial hybrids (*Mi*/+) were: Better Boy [V1, F1, N, ASC, St (N= RKN resistance)], Dominique [V,F1,F2,TMV,N] and Marina [F1,F2,N,ASC,GLS,BS1,Ve].

The RKN-susceptible germplasm types but with resistance to begomoviruses with S. *chilense* introgression were: TY52 (*Ty1* introgression from LA1969, Zamir et al., 1994); and Gc9 (*Ty3* introgression from LA2779 and selected from Fla 595-2), Gc16 (selected from Fla 658-2BK) and Gc171-C1 (*Ty3a* introgression from LA1932 and selected from Fla 8348) (Mejía et al., 2005).

The RKN susceptible wild species was *S. habrochaites* accession (LA1223 TGRC).

The RKN susceptible (see Seah et al., 2007), begomovirus resistant inbred line was Ih902 (Vidavsky and Czosnek 1998), containing the *Ty3* begomovirus-resistance allele introgression from *S. chilense* LA2779 (Martin et al., 2007).

The inbred line selected in Guatemala Gh2 has RKN and begomovirus resistance. Gh2 contains a *Ty3* introgression derived from Ih902, and also has RKN resistance derived from a begomovirus-susceptible parent provided by F. Vidavsky, The Hebrew University of Jerusalem, Israel (Mejía et al., 2005). Gh2 was homozygous for the markers for REX-1, Mi23, Ty1 (TG97 region) and Ty-3 FLUW25 (Martin et al., 2007). Martin et al. (2007) reported that a bioassay by V. Williamson, Univ. of California-Davis showed that Gh2 was resistant to RKN. The sequence of the fragment with the Mi23 primers for Gh2 had the same sequence as the fragment from Motelle (Seah et al., 2007).

Gh13 and Gh1 are inbred lines selected in Guatemala; they are RKN susceptible and begomovirus resistant (resistance derived from Ih902; Mejía et al., 2005).

Mh2082 is a begomovirus-resistant germplasm selected in Morocco from a cross between Ih902 and the RKN-resistant hybrid cultivar Daniella (Hazera Co, Israel). It is unknown if this line had the introgression for RKN resistance.

DNA Extraction

DNA was extracted from fresh leaves from plants grown in a plant growth chamber at the University of Wisconsin-Madison, USA. Samples of 50 mg of tissue were frozen in liquid nitrogen in a microfuge tube, then ground with a sterilized Kontes™ micropestle (Kontes Glass, Vineland, NJ, USA), and extracted using a the PUREGENE® DNA Purification Kit (Gentra Systems, Inc.; Minneapolis, MN, USA) following the manufacturer's instructions. DNA concentrations were adjusted to 10 ng/µl and extracts were stored at -20°C.

Development of polymerase chain reaction-based method and primer design

Primer design was done using the differences between the sequences of the intron 1 segment of the *Mi-1.2* and *Mi-1.1* genes (Milligan et al., 1998). The intron 1 segment occurs in the untranslated region of the *Mi1-2* gene between nt 14,521 and 16,084 for Motelle (U81378). A set of primers, PMIF(5'TCCATTAAGCCCAAGTCGA GATAG3') and PMIR(5'GTCCTGCTCGTTTACC ATTACTTTTCC 3'), was designed in the insertion of 738 nt found in the intron 1 segment of the *Mi-1.2* gene between 15,071 and 15,852 (Motelle, U81378). PMIF and PMIR had 100% identity with intron 1 in the promoter region of *Mi-1.2* of Motelle (U831378) and also with several regions in the Mi-1 locus such as cluster 1e of *S. lycopersicum* M82 (chromosome 6, HG975518), and cluster 2p (Mi-1.6 and Mi-1.4 genes) of Motelle (AY729690) and *Solanum sp.* VFNT (DQ, 863287). Moreover, 100% nucleotide identity was found with *S. pennellii* (HG975445) and *S. arcanum* (EF028060) sequences. PCR with this set of primers should amplify a 782-bp fragment when the *Mi-1.2* gene is present. Furthermore, the primers were expected to give several fragments from other regions of Mi-1 locus which could help to distinguish between different tomato wild-species introgressions. PCR primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Polymerase chain reaction analysis

PCR reactions were performed in a total volume of 50 µL containing 5 µl 10x buffer, 5 µL 2.5 mM deoxynucleotide triphosphates (dNTPs), 5 µL 25 mM $MgCl_2$, 1 Unit *Taq* DNA polymerase, 5 µL each of forward and reverse sense primers at 10 µM, 5-7 µL of DNA template (extract), and H_2O. All molecular biology chemicals for PCR were purchased from Promega, Corp. (Madison, WI, USA). PCR was conducted using an MJ DNA Engine PT200 Thermocycler™ (MJ Research Inc.; Waltham, MA, USA) using the following cycling profile: 3 min initial denaturation at 94°C and 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 50°C or 53°C, 1 min extension at 72°C, followed by a final extension of 10 min at 72°C. PCR-amplified fragments were electrophoresed in 1.5% Seakem LE™ agarose gel (BioWhittaker Molecular Applications; Rockland, ME, USA) in 0.5X TBE buffer, stained with ethidium bromide, and visualized with an Eastman Kodak; Rochester, NY, USA.

Sequence analysis

PCR fragments of PMIF-PMIR were first cloned into two different plasmids: plasmid pGEM®-T Easy vector (~ 3 kb) and plasmid pCR® 2.1-TOPO (3.9 kb). Recombinant plasmids were identified using enzymatic digestion. PCR fragments were then sequenced using a Big Dye Sequencing Kit™ (Biotechnology Center; Madison, WI, USA). Analysis of the sample sequences was accomplished by comparison with known DNA sequences through the National Center for Biotechnology Information BLAST program and the DNAMAN software (Lynnon Corp.; Quebec City, QC, Canada).

Results

The PCR amplification results with PMIF/PMIR primers showed three different electrophoretic profiles for RKN-resistant and RKN-susceptible germplasm without introgressions from Sc in this region. The profile for the RKN-resistant cultivar, Motelle (*Mi-1.2*/*Mi-1-2*, Fig. 1 lane 1), had five fragments: 900 bp, 780 bp, 708 bp, 627 bp and 597 bp (the RKN profile). The RKN-susceptible cultivar (Moneymaker (+/+), Fig. 1 lane 2) had a distinctly different profile with four fragments: 876 bp, 756bp, 605 bp and 434 bp (the Sl profile). For the heterozygous RKN-resistant cultivar (*Mi-1.2*/+) Better Boy (Fig. 1 lane 5) there was a PCR fragment profile, which combined the 434-bp fragment detected in the RKN-susceptible germplasm (the H-RKN profile).

Since it is known that there are begomovirus-resistance genes from Sc in the *Mi-1*-locus region of chromosome 6, breeding lines with introgression from Sc accessions were evaluated with the PMIF/PMIR primers. For Ty52, which carries a *S. chilense Ty-1* introgression from LA1969 that spans this region, five bands were detected (Fig. 1 lane 4). Three of these fragments (567 bp, 552 bp, 522 bp; the Sc-Ty1 profile) were not associated with Sl or RKN-resistant germplasm. Moreover, the breeding line Ih902 (Fig. 1 lane 3) with a begomovirus-resistance locus in chromosome 6 for *Ty-3* and known to have Sp sequences in the REX-1 marker region had a different profile, which was a combination of the Sc-*Ty1* and RKN profiles (902 profile). It did have the 780-bp fragment that is associated with the RKN resistant cultivar Motelle, but Ih902 is susceptible to RKN. It was originally reported that Ih902 had an introgression from *S. habrochaites* (Sh), but later it was determined to have an introgression from Sc Because of the distinct fragment profile for Ih902, it seems that it may have an introgression from both Sp and Sc. *S. habrochaites* accession LA1223 only gave two fragments (780 bp and 616 bp, Sh profile) with the PMIF/PMIR primers (Table 1, Fig. 3 lane 12).

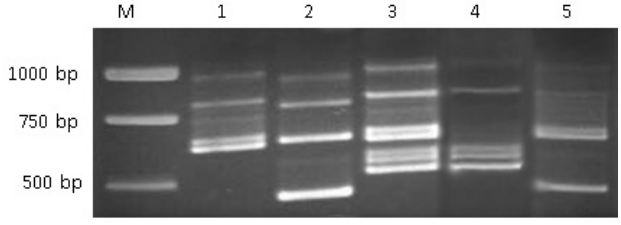

Fig. 1 Polymerase chain reaction results with primers PMIF/PMIR designed to evaluate germplasm in the *Mi-1* locus region for introgressions from wild species.

Table 1 Polymerase chain reaction results using PMIF-PMIR primers to evaluate germplasm in the *Mi-1* locus region for introgressions from wild species

Germplasm	Genotype	PMIF/PMIR profile[a]
M82	+/+	Sl
Nainemor	+/+	Sl
Ty50	+/+	Sl
S. cerasiformae	+/+	Sl
Gh2	Mi/Mi and Ty3/Ty3	RKN
Gh13	Ty3/Ty3	Sl
Gh1	Ty3/Ty3	902
Gc16	unknown	Sl
Gc9	Ty3/Ty3	Sc
Gc171-C1	Ty3a/Ty3a	Sl
Anahu	Mi/Mi	RKN
Mh2082	unknown	902
S. habrochaites	unknown	Sh

[a] Sl = *S. lycopersicum*; RKN = root knot nematode-resistant line; 902 = Ih902 ; Sc = Sc introgression in the Mi-1 locus region; Sh = two PCR fragment of 780 bp and 616 bp, from *S. habrochaites* LA1223.

From the above results six fragment profiles that corresponded to germplasm with different known sequences in the *Mi-1*-locus region of chromosome 6 were obtained. Thus, these primers discriminated between introgressions from Sp, Sc and Sh as well as RKN-resistant and RKN-susceptible germplasms.

The effectiveness of these primers was evaluated with a set of 13 RKN-resistant and RKN-susceptible germplasm with different introgressions in *Mi-1* region of chromosome 6 (Table 1, Figs. 2 and 3). Begomovirus-resistant germplasm, Gc16, Gc9, and Gc171, which are known to have an introgression from Sc in chromosome 6, were evaluated with the PMIF-PMIR (Table 1, Fig. 2). Gc16 was derived from an original cross of Sl and Sc and had the Sl profile (Fig. 2 lane 3), which would indicate that it did not have an Sc introgression in the region of the *Mi-1* locus. Gc9 (Fig. 2 lane 4) had the Sc-*Ty1* profile and it is known from sequence data to have an introgression from Sc in the *Mi-1* region (C. Martin and D.P. Maxwell, personal communication; Ji et al., 2007). Gc171-C1 (Fig. 3 lane 3) has the Ty3a locus from Sc (Ji et al., 2007) and the Sl profile was obtained, which was expected as the Sc introgression was not in the region of the *Mi-1* locus.

For line Ty50 (Fig. 2 lane 9) a Sl profile was revealed, and this line is known not to have an introgression in this region

The presence of *Mi-1.2* was also evaluated in the three inbred breeding lines, Gh1, Gh2 and Gh13, selected in Guatemala for resistance to begomoviruses. These lines were derived from a cross between the begomovirus-resistant line Ih902 and begomovirus-susceptible germplasm. The PCR-fragment profile obtained with Gh2 (Fig. 2 lane 2) was identical to the RKN-profile for the *Mi-1.2* homozygous germplasm Motelle, which indicated that Gh2 had the *Mi-1.2* gene. This result was in agreement with data reported by Seah et al., (2007), where the sequence of the fragment with the Mi23 primers for Gh2 had the same sequence as the fragment from Motelle. The line Gh1 (Fig. 3 lane 4) had the 902 profile, which was consistent with it having Ih902 in its background. The electrophoretic Sl profile obtained for Gh13 (Fig. 3 lane 6) was identical to that of the RKN-susceptible cultivar Moneymaker, and thus, it was concluded that Gh13 had Sl sequences in the *Mi-1* locus region. This finding was confirmed by the sequence data for chromosome 6 for Gh13.

The PCR fragments with primers PMIF-PMIR for the *Mi-1* region were cloned and sequenced to determine their sequence identities. Seven bands amplified from four tomato lines with different sequences in the Mi-1 region were chosen: the 627-bp and 780 bp fragments from Motelle (with Sp introgression for *Mi-1.2*), the 434-bp, 605-bp and 756-bp

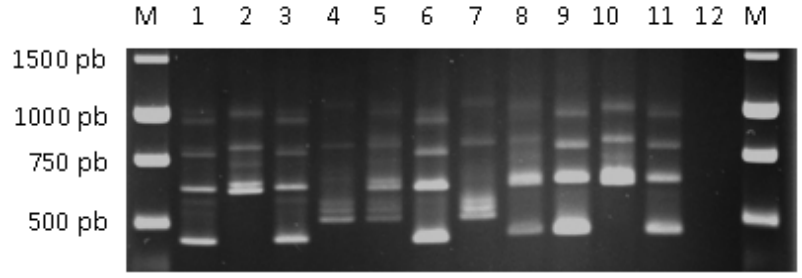

Fig. 2 Gel electrophoresis image showing the effectiveness of the PMIF/PMIR primers to evaluate germplasm in the *Mi-1* locus region for introgressions from wild species

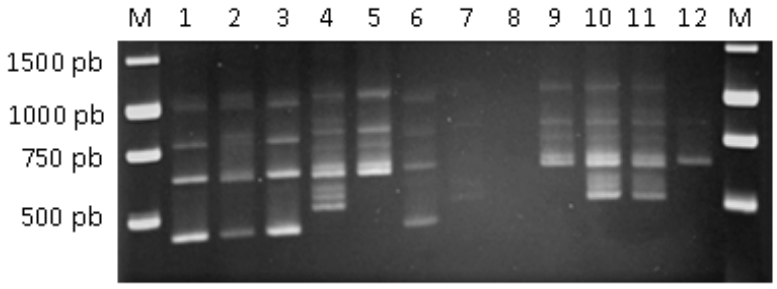

Fig. 3 Gel electrophoresis image showing the effectiveness of the PMIF/PMIR primers to evaluate germplasm in the *Mi-1* locus region for introgressions from wild species

fragments from *S. cerasiformae* (non-breeding line closely related to Sl), the 522-bp fragment from TY52 (with Sc introgression for *Ty1*) and the 616-bp fragment from Sh accession LA1223. Sequences were compared with known DNA sequences at the National Center for Biotechnology Information with the BLAST program. The sequence of the 627-bp fragment from Motelle (Fig. 4) had 99% identity for 594 nt with the *Mi-1.6* gene of *S.* sp. VFNT (DQ863288), 95% identity for 577 nt with the intron 1 of the *Mi-1.2* gene from Motelle (U81378) and 88% nucleotide identity for Heinz 1706 for 338 nt and there was 13-bp indel (SGN SL3.0). Heinz 1706 does not have the *Mi-1.2* resistance locus. The 780-bp fragment from Motelle (Fig. 5) had 99% nt identity with the *Mi 1-2* gene from Motelle (U81378) and 93% nt identity with Sl M82 with 9 small indels and 2 large indels as well as 16 SNPs (Fig. 6). For *S. cerasiformae* three bands were sequenced: 434 bp, 605 bp and 756 bp (Figs. 7, 8, 9). These fragments had 100% with cluster 1e of Sl M82 (HG975518) and 92-94% nt identity with the *Mi-1.2* gene from Motelle (U65668). Furthermore, the sequences of 522-bp band from *S. chilense* (TY52, Fig. 10) had identities of 98% with *S.pennelli* (HG975445) and 95% with *S. arcanum* (EF028059), Sl M82 (HG975518) and Motelle (U65668). The 616-bp fragment from the Sh LA1223 accession (Fig. 11) had its highest identity with *S. arcanum* (EF028060) at 97%, and 95% with Sl M82 (HG975518). These sequence results confirmed the tight linkage of the 780-bp fragment with the *Mi1.2* gene and that the primers amplified fragments from other regions of the genome inside and outside the *Mi1.2* gene for both RKN-susceptible and resistant germplasm.

```
1       TCCATTAAGC CCAAGTCGAG ATAGGAGAAA AATATTATTA GAGAGATTAT TAATTTAATG
61      ACATTTTACT TAGATTTTTT TACCAAAATA AGGGAATAAT ATCCCGTTAT TTAACTAGCT
121     TTTAAGCATT ATGGGTGGAA AGTAGAAAGA AGAAACATAG TAGAATAGAC AGTAAGTTAT
181     GCTTTAATGA GTAGATTTGT ATATGATTAC ATATTTTGTT TGACTTTTCG GTGTTTCGAT
241     TAGAAAACTT ACAAGTTCTT AATACATGTA TCATTTGTTG ATTTGTCCGT TTGGCACGTC
301     ATTTGTGGTT ACAAGTCACA TATGAAGTAT GTCCACGAAC ACACCGATGT CAAGTATAGA
361     TTTCTACTTG ATACATTTGA TGTTGTTTTC CATTCTCATT CTCTCTTTAT TTTTTTTTCT
421     TTACATTCAC ACGCACAATA ATTTTCTTAC AGGCTCCTTA TAAGCCATAT GCACATAGAC
481     GAATCTAGGA TTTGATGTTT ACAAGTTTCT ATGTCGACGT CATATTAATA TCAATAATAA
541     TTAGATTGAC AATCACATAT TTATAATATT AAGTCGATAA CTTTCTTCTT TGTATAGGT
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Fig. 4 Sequence of 627-bp polymerase chain reaction fragment amplified using PMIF/PMIR from root knot nematode-homozygous cultivar Motelle (introgression from *S. peruvianum*)

```
1       TCCATTAAGC CCAAGTCGAG ATAGGAGAAA AATATTATTA GAGAGATTAT TAATTTAATG
61      ACATTTTACT CTAGTTTTTT ATCAAAATAA GGGAATAATA TCCTGTTATT TAACTACCTT
121     TTAAGCATTA TGGGTGGAAA GTAGAAAGAA GAAACATAAC AGAACAGACA GTAAGTTATG
181     CTTTAATGAG TAGATCTGTA TAGGATTACA TATTTGTTTG ACTTTTCGGT GTTTCGATTA
241     GAAAACTTAC AAGTTTTTAA TACATGTATC ATTTGTTGAT TTGTCCGTTT GGCACGTCAT
301     CTGTGGTTAC AAGTCACATA TGAAGTATGT CCACGAGACA CACCGAATGT CAAGTATAGA
361     TTTCTACTTG ATCATACACA ACTTTATCTG AGGTTGATGC CAAATTTAAA TGACTACCTA
421     AAGCTGATAT TTTAAACATT AATCTTGTAC ACGAAAACAT TATTCCTATT ACTGTTTTCT
481     TTACCTTTAC CTTATAGACT TTTCTGGCAG AAAAAAGTTA GACAGATACA TTTGATGATG
541     TTTACCATTC TCATTCTCTC TTTATTTTAT TTTCTTTACA TTCACACGCG CAATAATTTT
601     CTTGTAGGTT CCTTATATGC CATATGCACA TAGACGAATC TAGGATTTGA TATTTACAAG
661     TTTCTATGTC GACGTCATAT TAATATCAAT AATAATTAGA TTGACAATCA CATATTTATA
721     ATATTAAGTC GATAACTTTC TTCTTTGTAT AGGTTGGAAA AGTAATGGTA AACGAGCAGG
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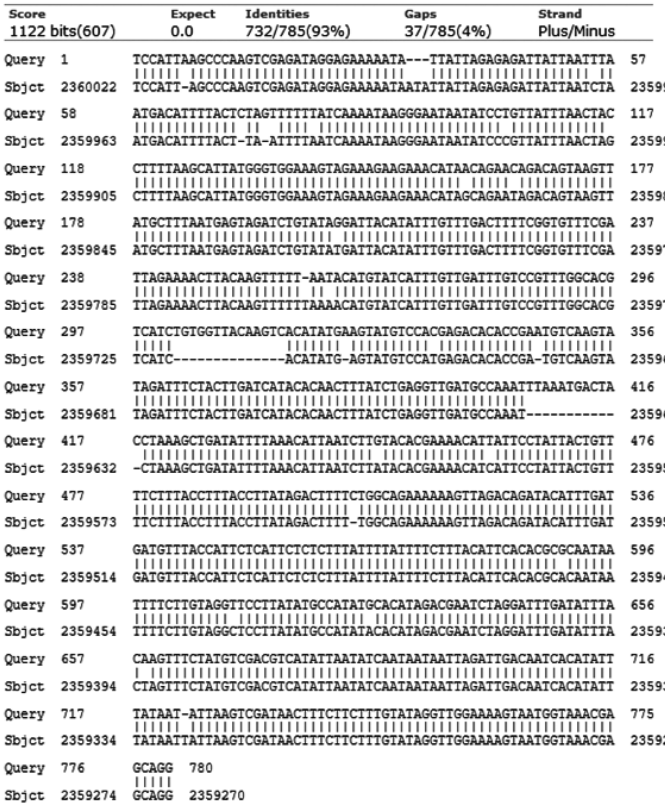
Fig. 5 Sequence of 780-bp polymerase chain reaction fragment amplified using PMIF/PMIR from root knot nematode homozygous cultivar Motelle (*S. peruvianum*)



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Query  357      TAGATTTCTACTTGATCATACACAACTTTATCTGAGGTTGATGCCAAATTTAAATGACTA  416
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Sbjct  2359681  TAGATTTCTACTTGATCATACACAACTTTATCTGAGGTTGATGCCAAAT-----------  2359633

Query  417      CCTAAAGCTGATATTTTAAACATTAATCTTGTACACGAAAACATTATTCCTATTACTGTT  476
                 |||||||||||||||||||||||||||| |||||||||||| ||||||||||||||||
Sbjct  2359632  -CTAAAGCTGATATTTTAAACATTAATCTTATACACGAAAACATCATTCCTATTACTGTT  2359574

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                ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  2359573  TTCTTTACCTTTACCTTATAGACTTTT-TGGCAGAAAAAAGTTAGACAGATACATTTGAT  2359515

Query  537      GATGTTTACCATTCTCATTCTCTCTTTATTTTATTTTCTTTACATTCACACGCGCAATAA  596
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  2359514  GATGTTTACCATTCTCATTCTCTCTTTATTTTATTTTCTTTACATTCACACGCACAATAA  2359455

Query  597      TTTTCTTGTAGGTTCCTTATATGCCATATGCACATAGACGAATCTAGGATTTGATATTTA  656
                ||||||||||| ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  2359454  TTTTCTTGTAGGCTCCTTATATGCCATATACACATAGACGAATCTAGGATTTGATATTTA  2359395

Query  657      CAAGTTTCTATGTCGACGTCATATTAATATCAATAATAATTAGATTGACAATCACATATT  716
                | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2359394  CTAGTTTCTATGTCGACGTCATATTAATATCAATAATAATTAGATTGACAATCACATATT  2359335

Query  717      TATAAT-ATTAAGTCGATAACTTTCTTCTTTGTATAGGTTGGAAAAGTAATGGTAAACGA  775
                ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2359334  TATAATTATTAAGTCGATAACTTTCTTCTTTGTATAGGTTGGAAAAGTAATGGTAAACGA  2359275

Query  776      GCAGG  780
                |||||
Sbjct  2359274  GCAGG  2359270
```

Fig. 6 Sequence-alignment of 780 bp polymerase chain reaction fragment from Motelle and M82 (Chromosome 6, HG975518) sequence, using BLAST program, with query sequence: 780bp PCR fragment from Motelle and subject sequence: Sl-M82 (Chromosome 6, HG975518) sequence

```
1       TCCATTAAGC CCAAGTCGAG ATAGGAGAAA AATATTATTA GAGAGATTAT TAATTTAATG
61      ACATTTTACT ACCTAAAGTT GATATTTTAA ACATTAATCT TGTACTCCAA AACACTATTT
121     CTATCACTGT TTTCTTTACT TTTACTTTAT AGACCTTTTT GTCAGAAAAA AGTTAGACGG
181     ATACATTTGA TGTTGTTTTC CATTCTCATT CTCTCTTTAT TCTTTTTTCT TTACATTCAC
241     ACGCACAATA ATTTTCTTGT AGGCTCCTTA TAAGCCATAT GCACATAGAC GAATCTAGGA
301     TCTGATGTTT ACAAGTTTCT ATGTCGACGT CATATTAATA TCGATAATAA TTAGATTGAC
361     AATCACATAT TTATAATTAT TAAGTCGATA ACGTTCTTCT TTGTATAGGT TGGAAAAGTA
```

Fig. 7 Sequence of 434-bp polymerase chain reaction fragment amplified from the root knot nematode-susceptible germplasm *S. cerasiformae* using PMIF/PMIR primers

```
1       ATAGGAGAAA ATTATTAGAG AGATTATTAA TCTAATGACA TTTTACTTAG ATTTTAATCA
61      AAATAAGGGA ATAATATCCT GTTATTTAAC TAGCTTTTAA GAATTATGGG TGGAAAGTAG
121     AAAGAAGAAA CATAACAGAA TAGACAGTAA GTTATGCTTT AATGAGTAGA TTTGTATATG
181     ATTACATATT TTGTTTGACT TTTCGGTGTT TCGATTAGAC TTACATGTTT TTAATACATG
241     TATCATTTGT TGATTTGTCC GTTTGGCCCG TCATCACATA TGAAGTATGT CCATGAGACA
301     CACCGATGTC AAGTATAGAT TTCTACTTGA TCATACACAA CTTTATCTGA GGTTGATGCC
361     AAATTTACCT AAACTTGATA TTTTACATTC ACACGCACAA TAATTTTCTT GTAGGCTCCT
421     TATATGCCCA CATAGACGAA TCTAGGATTT GATATTTACA AGTTTCTATG TCGACGTCAT
481     ATTAATATCA ATAATAATTA GATTCACAAT CACATATTTA TAATTATTAA GTCGATAACT
541     TTCTTCTTTG TATAGGTTGG AAAAGTAATG
```

GTAAGC

Fig. 8 Sequence of 605-bp polymerase chain reaction fragment amplified from the root knot nematode-susceptible germplasm *S. cerasiformae* using PMIF/PMIR primers

```
1       TCCATTAAGC CCAAGTCGAG ATAGGAGAAA AATAATATTA TTAGAGAGAT TATTAATCTA
61      ATGACATTTT ACTTAATTTT AATCAAAATA AGGGAATAAT ATCCCGTTAT TTAACTAGCT
121     TTTAAGCATT ATGGGTGGAA AGTAGAAAGA AGAAACATAG CAGAATAGAC AGTAAGTTAT
181     GCTTTAATGA GTAGATCTGT ATATGATTAC ATATTTGTTT GACTTTTCGG TGTTTCGATT
241     AGAAAACTTA CAAGTTTTTT AAAACATGTA TCATTTGTTG ATTTGTCCGT TTGGCACGTC
301     ATCACATATG AGTATGTCCA TGAGACACAC CGATGTCAAG TATAGATTTC TACTTGATCA
361     TACACAACTT TATCTGAGGT TGATGCCAAA TCTAAAGCTG ATATTTTAAA CATTAATCTT
421     ATACACGAAA ACATCATTCC TATTACTGTT TTCTTTACCT TTACCTTATA GACTTTTTGG
481     CAGAAAAAAG TTAGACAGAT ACATTTGATG ATGTTTACCA TTCTCATTCT CTCTTTATTT
541     TATTTTCTTT ACATTCACAC GCACAATAAT TTTCTTGTAG GCTCCTTATA TGCCATATAC
601     ACATAGACGA ATCTAGGATT TGATATTTAC TAGTTTCTAT GTCGACGTCA TATTAATATC
661     AATAATAATT AGATTGACAA TCACATATTT ATAATTATTA AGTCGATAAC TTTCTTCTTA
721     GTATAGGTTG GAAAAGTAAT GGTAAACGAG CAGGAC
```

Fig. 9 Sequence of 756-bp polymerase chain reaction fragment amplified from the root knot nematode-susceptible germplasm *S. cerasiformae* using PMIF/PMIR primers

```
1       TCCATTAAGC CCAAGTCGAG ATAGGAGAAA AATATTATTA GAGAGATTAT TAATCTAATG
61      ACATTTTACT TAGATTTTTA TCAAAATAAG GGAATAATAT CCCGTTATTT AACTAGCTTT
121     TAAGAATTAT GGATGGAAAG TAGAAAGAAG AAACATAACA GAGACAGTAA GTTATGCTTT
181     AATGAGTAGA TTTGTATATG ATTACCAAAA CACTATTCTT TACTTTTACC TTATAGACTT
241     TTTGGGCAGA AAAAAGTTTA GACTGATACA TTTGATGATG TTTTCCATTC TCATTCTCTC
301     TTTATTTATT TTTTTTACAT TCACACGCAC AACAATTTTC TTACAGGCTC CTTATAAGCC
361     ATATGCACAT ACACGAATCT AGGATTTGAT ATTTACAAGT TTCTATGTCG ACGTCATATT
421     AATATCAATA ATAATTAGAT TGACAATCAC ATATTTATAA TTATTAAGTC GATAACTTTC
481     TTCTTTGTAT AGGATGGAAA
```

Fig. 10 Sequence of 522-bp band obtained with PMIF/PMIR from the susceptible cultivar TY52 (*Ty1* introgression from *S. chilense*)

```
1       TCCATTAAGC CCAAGTCGAG ATAGGAGAAA AATATTATTA GAGAGATTAT TAATTTAATG
61      ACATTTTACT TAGATTTTTT ATCAAAATAA GGGAATAATA TCCTGTTATT TAACTAGCTT
121     TTAAGCATTA TGGGTGGAAA GTAGAAAGAA GAAACATAGC AGAATAGACA GTAAGTTATG
181     CTTTAATGAG TAGATCTGTA TATGATTACA TATTTGTTTG ACTTTTCGGT GTTTCGATTA
241     GAAAACTTAC AAGTTTTTAA TACATGTATC ATTTGTTGAT TTGTCCATTT GGCACGTCAT
301     CACATATGAA GTATGTCCAT GAGACACACC GATGTCAAGT ATAGATTTCT ACTTGATCAT
361     ACACAACTTT ATCTGAGGTT GATGCCAAAT TTACCTAAAC TTGATATTTT ACATTCACAC
421     GCACAATAAT TTTCTTGTAG GCTCCTTATA TGCCATATGC ACATAGACGA ATCTAGGATT
481     TGATATTTAC AAGTTTCTAT GTCGACGCCA TATTAATATC AATAATAATT AGATTGACAA
541     TCACATATTT ATAATTACTA AGTCGATAAC TTTCTTCTTT GTATAGGTTG GAAAAGTAAT
```

Fig. 11 Sequence of 616-bp fragment obtained with PMIF/PMIR from the susceptible LA1223 accession of *S. habrochaites*

Discussion

The development of RKN-resistant tomato hybrids is considered the most cost-effective and sustainable option for nematode management (Cook, 2000). The identification of the RKN-resistant gene in tomato plants can depend on the use of biological assays, which is expensive and time consuming. Additionally, previous studies have shown that evaluation of disease-resistant traits in biological assays is not always straightforward because assays are often affected by environmental factors (Arens et al., 2010). Furthermore, screening of many recombinant plants and selection of breeding inbred lines can be very tedious and labor intensive. Thus, marker-assisted selection (MAS) represents a powerful tool to overcome some limitations of traditional breeding methods and is successfully adopted by tomato breeding programs. MAS is most efficient when the marker is tightly linked to the studied trait (Kelly, 1995). The present study reported the development of a PCR marker (PMIF/PMIR), which is tightly linked to the *Mi-1.2* gene. Although different molecular markers that are linked to the *Mi-1* locus have been developed for selection, some of them have limitations (Williamson et al., 1994; El Mehrach et al., 2005). Indeed, in earlier studies, Williamson et al., (1994) used the C1/C2 and C2/S4 primers to screen tomato lines for RKN resistance. They were able to distinguish resistant genotypes from the susceptible ones but were not able to distinguish resistant homozygous plants from resistant heterozygous ones. The PCR PMIF/PMIR primers discriminated between these genotypes and without enzymatic digest as is the case for the REX-1 marker (Williamson et al., 1994). The REX-1 marker was reported to give false positives with begomovirus-resistant germplasm (El Mehrach et al., 2005; Seah et al., 2007). This problem was resolved with the PMIF/PMIR marker that gave distinct fragment profiles with some begomovirus-resistant germplasm, for example, TY52 and Gh13 (Zamir et al., 1994; Mejía et al., 2005). However, Ih902 remains an anomaly as it has a combination of profiles from Motelle and Ty52, but it is susceptible to RKN. One possibility could be that there is a mutation in the *Mi1.2* gene but the primers still have the ability to anneal. In the other hand, Sl profile revealed in Gh13, was confirmed by the sequence data reported by Menda et al., (2014) for chromosome 6. Other PCR markers specific for the *Mi-1.2* locus (El Mehrach et al., 2005; Seah et al., 2007) were able to discriminate resistant and susceptible germplasm without giving false positives with begomovirus-resistant breeding lines. However, the PMIF/PMIR primers had the advantage of detecting germplasm that had different introgressions from wild species in this region of chromosome 6, such as Sp, Sc-*Ty1* and 902-profiles. Thus, these markers could be used in a breeding program to select inbred lines with begomovirus-resistance genes as well as the RKN-resistance gene.

The current study developed a PCR-based marker for the *Mi-1.2* gene that could be used effectively in a tomato breeding program and would eliminate the need for bioassays and markers that require enzyme digestion of PCR products. It also effectively distinguished among several different introgressions from wild tomato species that could be present in the *Mi-1* region of chromosome 6.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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