Identification of resistance in tomato against root knot nematode (*Meloidogyne incognita*) and comparison of molecular markers for *Mi* gene

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Abstract

Tomato production is limited by many biotic stresses of which root knot nematode (RKN, *Meloidogyne incognita*) is a major pest. The present study aimed to identify resistance sources in controlled conditions and compare molecular markers for efficient and rapid screening of *M. incognita* resistance. Among the ten genotypes evaluated, HAT-310 and HAT-311 were found immune to *M. incognita* infestation. Further, six crosses with these two resistant sources, (HAT-311 x Swarna Lalima, HAT-296 x HAT-311, EC-596747 x HAT-311, Swarna Lalima x HAT-310, EC-596743 x HAT-310 and Swarna Lalima x HAT-311), exhibited immune responses against *M. incognita*. Four molecular markers viz., JB-1, REX-1, PMi12 and Mi23 were employed in eighteen germplasm to characterise resistance and susceptibility of the genotypes against infestation by *M. incognita*. JB-1 yielded 420 bp in all the genotypes after digestion and hence could not be used to differentiate between nematode resistance and susceptibility. Marker PMi12 yielded additional DNA fragments besides the expected bands and did not give consistent results. REX-1 and Mi23 markers successfully differentiated between nematode resistant and susceptible genotypes. Moreover, Mi23 can also separate homozygous and heterozygous resistance sources since the restriction enzyme analysis was not needed. The resistant genotypes identified from the present study may be used further in nematode resistance breeding programme of tomato. And also, the Mi23 marker can be used for rapid screening of the germplasm.

Keywords: *Meloidogyne incognita*, *Solanum lycopersicum,* SCAR marker, Mi23, Polymorphism.

Introduction

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop with worldwide production of 161.8 million ton (FAO STAT 2012). Tomato production is limited by many biotic stresses, of which the root knot nematode (RKN), *Meloidogyne incognita* is a major pest and is reported to cause yield losses ranging from 25-100% (Jablonska et al. 2007; Seid et al. 2015). Root knot nematodes are sedentary endo-parasites. Use of resistant varieties is the best viable method to control these soil borne pathogens without increasing the cost of cultivation and provides an economically and environmentally viable approach for the management of nematodes. Thus, screening for nematode resistance is required while transferring resistance gene(s) into breeding lines in resistance breeding programmes.

Many modern tomato varieties carry a single, dominant gene called *Mi* that confers effective field resistance to RKN (Barham and Winstead 1957; Laterrot and Pecaut 1965; Dropkin 1969). This gene confers resistance to three of the most damaging species of root knot nematodes (*M. hapla*, *M. incognita* and *M. enterolobii*) (Roberts and Thomason 1986) and was introgressed into cultivated tomato from *Solanum peruvianum* in the 1940s (Smith 1944). It is currently the only source of RKN resistance in cultivated tomatoes. This gene was mapped on the short arm of chromosome 6 (Kaloshian et al. 1998). Sequence analyses have showed existence of genes called *Mi-1.1, Mi-1.2* and *Mi-1.3* in Mi locus. Out of these, only *Mi-1.2* gene confers resistance to RKN (Milligan et al. 1998). Further, it was found to be tightly linked to *Ty-1* gene which confers resistance to tomato leaf curl virus (Zamir et al. 1994). Therefore, there is a problem of introgression of both *Mi-1* and *Ty-1* genes.

Marker assisted selection (MAS) is a powerful tool in plant breeding (Francia et al. 2005). MAS in tomato was initiated with the isozyme marker Aps-1 (Medina- Filho and Tanksle 1983). Since then, DNA markers, such as Rex-1, which is Cleaved Amplified Polymorphic Sequences (CAPS), has been applied into many modern tomato cultivars (Williamson et al. 1994). The Rex-1 marker has widely been used to assay the *Mi-1* gene in tomato breeding and was proven relatively reliable (Williamson et al. 1994). However, a study showed that the Rex-1 marker gave false positives for the presence of *Mi-1* in some of the begomovirus-resistant germplasm derived from Ih902 (El Mehrach et al. 2005).

Thereafter, Mi23, which is co-dominant Sequence Characterised Amplified Region (SCAR) marker for the *Mi-1*, was developed so that it can be used to distinguish the presence of *Mi-1* plants bearing *Ty-1* (Seah et al. 2007). Hence, the present study aims to characterise tomato genotypes including crosses using different PCR based molecular markers *viz.,* JB-1, REX-1, PMi12 and Mi23 and their comparison with each other for efficient identification of resistance conferred by the *Mi* gene.

Materials and Methods

Culturing of root-knot nematode

The isolate of *M. incognita* used in this study was collected from the infected tomato fields of ICAR-RCER, Research Centre, Ranchi, India. The species was identified as *M. incognita* with the help of perineal pattern of adult female nematode (Jepson 1987). The nematode, *M. incognita* was isolated and reproduced from a single egg mass from susceptible tomato variety Pusa Ruby grown on sterile media. To ensure sufficient infestation levels for further experimentation, the nematode species was mass produced on susceptible tomato variety Pusa Ruby grown in 15 cm diameter pots containing one kg sterilized sandy soil (sand, farm yard manure and sand mixture in 2:1:1 ratio) and inoculated with the infective juveniles collected from the stock and maintained at 25^0C $\pm$ 2.

Nematode extraction and counting

For collection of egg masses, the cultured root knot nematode infected tomato plants were uprooted and gently washed in water to remove the soil. The roots were air dried for two hours and the egg masses were collected for hatching. The culture was maintained regularly and counting of juveniles was done on a rectangular counting disc using a stereo microscope.

Screening of Tomato Genotypes against Root-Knot Nematode

The experiment was conducted under the net house of ICAR RCER, RC Ranchi, Jharkhand (23.35º N and 85.33º E at 629 m above mean sea level) during the main season of 2013-14 and 2014-15. Total annual rainfall was 1430 mm with 1100 mm during June to September and the average minimum and maximum temperatures were 37ºC and 40ºC respectively. A total of ten genotypes (EC-596747, Swarna Kanchan, Swarna Lalima, Swarna Anmol, HAT-302, EC-596743, HAT-294, HAT-296, HAT-310 and HAT-311) of tomato obtained from the germplasm collections of ICAR RCER, RC Ranchi and eight crosses (Swarna Lalima x HAT-310, HAT-296 x HAT-302, HAT-296 x EC-596743, EC-596743 x HAT-310, HAT-311 x Swarna Lalima, HAT-296 x HAT-311, EC-596747 x HAT-311 and Swarna Lalima x HAT-

311) involving these collections were screened for resistance against root knot nematode, *M. incognita*. Pusa Ruby was used as susceptible control.

All the tomato genotypes were sown separately with three seeds per pot of 15 cm diameter filled with soil as previously described for nematode cultures. After germination, thinning was done with one seedling per pot. There were three replications of each genotype in a completely randomised design. Twenty five days after germination the plants were inoculated into three holes near the root zone with freshly hatched J2 of *M. incognita* at the rate of 1000 J2/pot (1 J2/g of soil mixture) and covered with the same soil. The plants were watered regularly to keep the soil moist.

Plant reaction to root knot nematode

Plants were uprooted 45 days after inoculation and washed gently to remove the soil under tap water. After drying of roots, root galls and egg masses were counted in all the replications and average was subjected to gall index. Gall indices were assessed using a visual rating based on six-point rating scale (0–5) according to Taylor and Sasser (1978) [0 = no gall or no infection (Immune; I); 1 = 1–2 galls (Highly Resistant; HR); 2 = 3–10 galls (Resistant; R); 3 = 11–30 galls (Moderately Resistant; MR); 4 = 31–100 galls (Susceptible; S), and 5 = 100 and above galls (Highly Susceptible; HS)]. Nematode eggs were extracted from per gram of root sample as previously described and counted using a stereomicroscope. Resistance and susceptibility of tomato varieties was based on a reproduction index (RI), which was calculated as number of eggs per gram of tomato root divided by the number of eggs per gram of susceptible control roots multiplied by 100. The disease reaction is classified as RI = 0 (immune), RI < 1 (highly resistant), $1 \leq RI < 10$ (very resistant), $10 \leq RI < 25$ (moderately resistant), $25 \leq RI < 50$ (slightly resistant) and $RI \geq 50$ (susceptible) (Taylor, 1967).

Statistical analysis

Data on galling index, number of egg masses, number of eggs per gram of root and reproduction index were analysed. All the characters were log(x+1) transformed before the analysis. The data was subjected to analysis of variance (ANOVA) using IBM SPSS Statistics version 21.0 software. Means were compared using Fisher's least significant difference tests for paired comparisons at probability level of 5%.

Fig.1 (A) Resistance reaction to *M. incognita* (no galls) in line HAT-310 (B) Susceptible reaction to *M. incognita* (heavy galls) in Swarna Lalima

DNA Extraction and Marker Analysis

Leaves of eighteen tomato genotypes i.e., germplasm collections and their crosses were surface sterilized with 0.1% of $HgCl_2$ and used for the isolation of genomic DNA using CTAB method (Doyle and Doyle 1990) followed by RNase treatment (Healey et al. 2014). Quantification of genomic DNAs was determined by NanoDrop 8000 spectrophotometer. The isolated DNA was checked on 0.8% agarose gel and was subjected to PCR amplification.

Table 1 PCR based markers used for root knot nematode characterisation

Primer name	Marker	Restriction Enzyme	Primer sequence	Amplicon size (bp)
JB-1	CAPS	Taq 1	F: AACCATTATCCGGTTCACTC R: TTTCCATTCCTTGTTTCTCTG	900
REX-1	CAPS	Taq 1	F: TCGGAGCCTTGGTCTGAATT R: ATGCCAGAGATGATTCGTGA	720
PMi12	SCAR	--	F: CCTGCTCGTTTACCATTACTTTTCCAACC R: CTGCTCGTTTACCATTACTTTTCCAACC	620 and 720
Mi23	SCAR	--	F: TGGAAAAATGTTGAATTTCTTTTG R: GCATACTATATGGCTTGTTTACCC	380 and 430

PCR amplification was performed in a total volume of 25 µL containing 10XPCR Buffer, 0.2 mM dNTPs, 0.4 mM of each primer, 2 mM $MgCl_2$, 20 ng of template DNA and 1 Unit *Taq* DNA Polymerase. PCR amplification was carried out using a thermocycler (DNA Engine PTC-200, Bio-Rad Laboratories, USA). Reaction conditions consisted of 35 cycles with denaturation at 94^0C for 30 sec, annealing at 52^0C (Rex-1, JB-1, and PMi12) and 56^0C

(Mi23) for 30 sec, and polymerisation at 72^0C for 1 min with a final extension at 72^0C for 5 min. Primarily, 5 µL of each primer reaction was loaded onto a 1.5% agarose gel to ascertain whether PCR amplification was successful. PCR products obtained from PMi12 and Mi23 were not digested by any restriction enzymes, but 10 µL of each PCR product obtained from REX-1 and JB-1 markers were digested with *Taq*I following the manufacturer's instructions (Thermo Fisher Scientific., USA). All the PCR products were visualised by electrophoresis on 2 % agarose gel and ethidium bromide staining. The gels were run at constant 5 V/cm for 30 min in 1 X TAE buffer. PCR products of resistant genotypes of Mi23 marker was sequenced (Chromous Biotech Pvt Ltd., India), and sequencing analysis was performed using Geneious R8 (Biomatters. NZ) software. Furthermore, MEGA 6.06 (Tamura et al., 2013) was used for phylogenetic analysis of the sequenced 380 bp and 430 bp of the resistant parents and crosses along with the reference sequences.

Results

Phenotypic evaluation of tomato genotype against root knot nematode

Reaction of genotypes to *M. incognita* was significantly different based on number of galls, mean gall index, number of egg masses per plant, number of eggs per gram of root and reproduction index. Genotypes were classified into significantly different groups based on Fischers Least Square Difference test (LSD) (Table 2). Among ten genotypes screened, HAT-310 and HAT-311 showed a immune reaction to *M. incognita* as no egg masses were observed. Among eight crosses, six crosses, HAT-311 x Swarna Lalima, HAT-296 x HAT-311, EC-596747 x HAT-311, Swarna Lalima x HAT-310, EC-596743 x HAT-310 and Swarna Lalima x HAT-311 were found immune to *M. incognita* (Table 2).

Table 2: Number of galls, number of egg masses, number of eggs per gram of root, Reproduction Index (RI) and disease reaction of tomato genotypes against *M. incognita*

Genotype/Character	Number of galls per plant*	Number of egg masses per plant	Number of eggs per gram of root	Reproduction Index (RI)**	Disease Reaction (based on GI/RI)***
EC-596747	367.67 (2.57^{bcd})	25.67 (1.42^{b})	2723.67 (3.44^{b})	74.49 (1.87^{b})	**HS/S**
Swarna Kanchan	585.00 (2.77^{a})	26.33 (1.44^{b})	2401.00 (3.38^{c})	65.70 (1.82^{c})	**HS/S**
Swarna Lalima	342.67 (2.53^{cd})	13.67 (1.16^{cd})	1398.67 (3.15^{e})	38.32 (1.60^{e})	**HS/S**
Swarna Anmol	408.33 (2.60^{abcd})	21.33 (1.34^{b})	1565.67 (3.19^{d})	42.79 (1.65^{d})	**HS/S**
HAT-302	368.67	13.33	991.33	27.12	**HS/S**

	(2.56^{bcd})	(1.16^{cd})	(2.99^{fg})	(1.44^{f})	
EC-596743	154.00 (2.19^{e})	25.00 (1.41^{b})	2427.67 (3.38^{c})	66.38 (1.82^{c})	**HS/S**
HAT-294	333.33 (2.53^{cd})	26.00 (1.43^{b})	2753.67 (3.44^{b})	75.37 (1.88^{b})	**HS/S**
HAT-310	0.00 (0.00^{i})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
HAT-311	0.00 (0.00^{i})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
HAT-296	322.33 (2.51^{d})	16.33 (1.23^{c})	1565.33 (3.20^{d})	42.49 (1.64^{d})	**HS/S**
Swarna Lalima x HAT-310	2.67 (0.53^{gh})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
HAT-296 x HAT-302	508.33 (2.71^{ab})	11.33 (1.08^{d})	963.00 (2.98^{g})	26.34 (1.43^{f})	**HS/S**
HAT-296 x EC-596743	447.67 (2.66^{abcd})	12.67 (1.13^{cd})	1060.00 (3.03^{f})	29.00 (1.47^{f})	**HS/S**
EC-596743 x HAT-310	2.33 (0.49^{h})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
HAT-311 x Swarna Lalima	2.67 (0.53^{gh})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
HAT-296 x HAT-311	4.00 (0.69^{fg})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
EC-596747 x HAT-311	5.67 (0.82^{f})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
Swarna Lalima x HAT-311	3.67 (0.65^{fgh})	0.00 (0.00^{e})	0.00 (0.00^{h})	0.00 (0.00^{g})	**I/I**
Pusa Ruby (Susceptible control)	488.67 (2.69^{abc})	338.33 (2.52^{a})	3657.0 (3.56^{a})	100.00 (2.00^{a})	**HS/S**
LSD (*P*=0.05)	0.06	0.06	0.05	0.05	
F value	360.36	451.06	15263.72	3895.43	
df	38,56	38,56	38,56	38,56	

Values are means of three replicates.

Numbers of galls, number of egg masses per plant, number of eggs per gram of root and reproduction index were log transformed and log transformed values indicated in parenthesis

Values sharing common do not differ significantly at P < 0.05 according to Fisher's test.

.*Gall indices were assessed using a visual rating based on six-point rating scale (0–5) according to Taylor and Sasser (1978) [0 = no gall or no infection (Immune; I); 1 = 1–2 galls (Highly Resistant; HR); 2 = 3–10 galls (Resistant; R); 3 = 11–30 galls (Moderately Resistant; MR); 4 = 31–100 galls (Susceptible; S), and 5 = 100 and above galls (Highly Susceptible; HS)]

** RI: Reproduction index = (number of eggs per gram of root of each tomato genotype)/ (number of eggs per gram of root of susceptible Pusa Ruby) x 100. The disease reaction is classified as RI = 0 (immune), RI < 1 (highly resistant), 1 < RI < 10 (very resistant), 10 < RI < 25 (moderately resistant), 25 < RI < 50 (slightly resistant) and RI > 50 (susceptible) (Taylor, 1967)

***Disease reaction based on Galling Index (GI) and Reproduction Index (RI)

Molecular Evaluation of root knot nematode resistance in tomato genotypes

PCR based molecular markers viz., JB-1, REX-1, PMi12 and Mi23 were used to evaluate the nematode resistance in tomato genotypes. PCR with JB-1 primers yielded about 900 bp DNA fragment (Fig. 2A). PCR products after digestion with *Taq*I restriction enzyme yielded nearly 420 bp in all the genotypes (Fig. 2B).

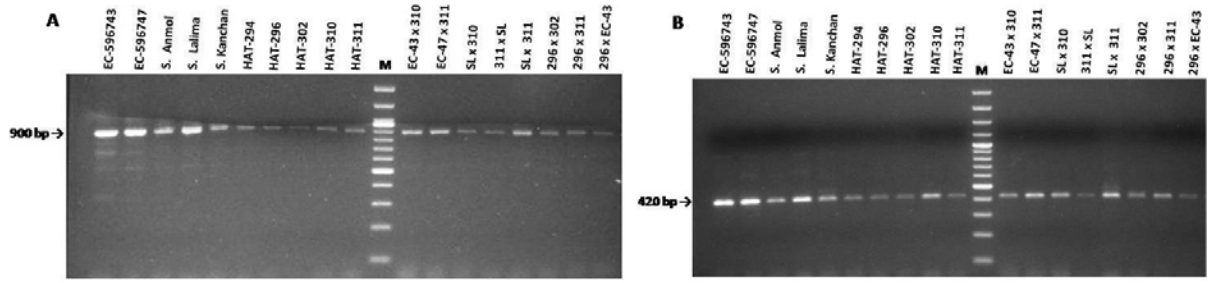

Fig. 2 (A) PCR product obtained using JB-1 marker and (B) Digestion of JB-1 PCR products with *Taq*I

PCR with the REX-1 marker produced the band of about 720 bp in all tomato hybrids and lines used in the study (Fig. 3A). Digestion of PCR products with *Taq*I yielded 720, 560 and 160 bp fragments (Fig. 3B) in resistant genotypes i.e., HAT-310 and HAT-311. The susceptible plants produced a 720 bp fragment (EC-596747, Swarna Kanchan, Swarna Lalima, Swarna Anmol, HAT-302, EC-596743, HAT-294 and HAT-296) while segregating material yielded three bands including 720, 560, and 160 bp (EC-596743 x HAT-310, HAT-311 x Swarna Lalima, HAT-296 x HAT-311, EC-596747 x HAT-311 and Swarna Lalima x HAT-311) (Fig. 3).

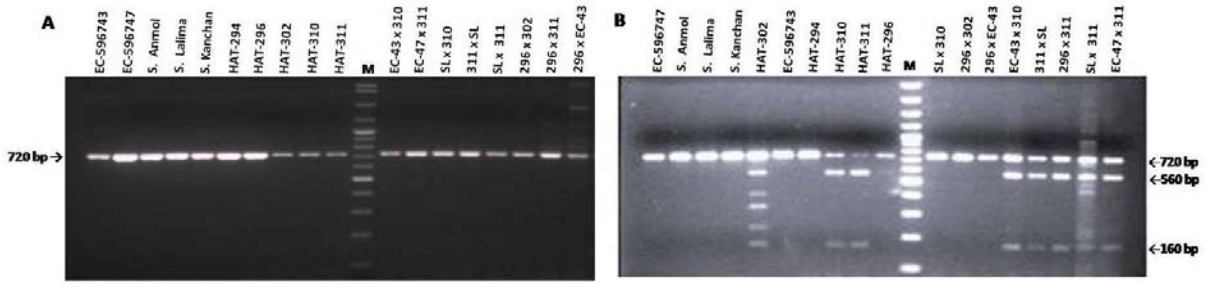

Fig. 3 (A) PCR product obtained using REX-1 marker and (B) Digestion of REX-1 PCR products with *Taq*I

PCR with PMi12 yielded 620 bp fragment with resistant genotypes (HAT-310 and HAT-311) and 720 bp fragment with susceptible genotypes. Heterozygous plants (EC-596743 x HAT-310, HAT-311 x Swarna Lalima, HAT-296x HAT-311, EC-596747 x HAT-311 and Swarna Lalima x HAT-311) produced both 620 and 720 bp fragments along with extra bands which are about 800, 900, and 1050 bp in analysed samples. Despite repeated efforts, the bands were not clear and extra bands were observed.

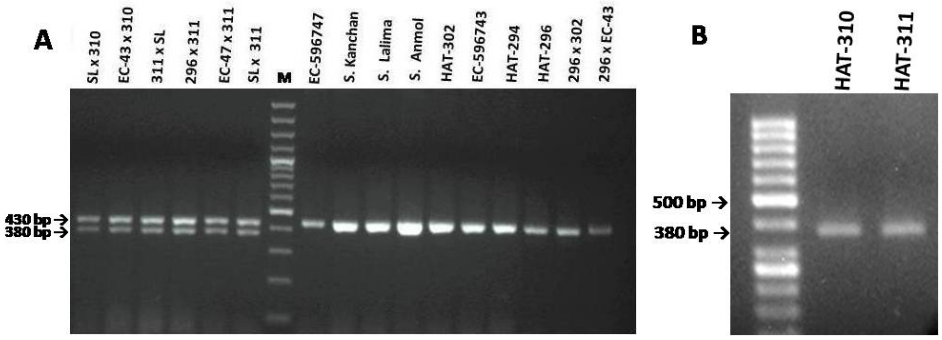

Fig. 4 PCR product obtained using Mi23 marker in segregating material and susceptible genotypes (B) PCR product obtained using Mi23 marker in resistant genotypes

Mi23 produced 380 bp fragments for the homozygous genotype (Mi/Mi) i.e HAT-310 and HAT-311 (Fig. 4B). Tomato plants which lack the Mi-1 locus yielded 430 bp fragment (EC-596747, Swarna Kanchan, Swarna Lalima, Swarna Anmol, HAT-302, EC-596743, HAT-294 and HAT-296, HAT-296 x HAT-302, HAT-296 xEC-596743). Heterozygous resistance genotypes yielded two fragments 380 bp and 430 bp (Swarna Lalima x HAT-310, EC-596743 x HAT-310, HAT-311 x Swarna Lalima, HAT-296 x HAT-311, EC-596747 x HAT-311, Swarna Lalima x HAT-311) (Fig. 4A).

PCR amplified product (380 bp) of the resistant genotypes HAT- 310 and HAT- 311 and crosses were sequenced. Sequences from present study were BLAST analyzed in NCBI for confirming the similarity with Mi23 gene. There was 100% similarity and E-value 0.0 with *S. lycopersicum* cultivar inbred Gh2 Mi23 locus marker genomic sequence with a total score of 512 and 652 for HAT-310 (Accession number: MF471636) and HAT-311(Accession number: MF471637) respectively. The gene sequences of 380/430bp of the six resistant crosses were also registered in NCBI GenBank repository (Accession numbers: MG557820 to MG557831).

Phylogenetic relationship study through neighbour joining method based phylogenetics of Mi gene in eight genotypes of the present study with reference gene showed that all the parents and crosses are clearly divided into two groups i.e. cluster A and cluster B (Fig. 5). Cluster A has the resistant fragment 380bp and cluster B had the susceptible fragment 430bp of all the genotypes respectively. In cluster A, RKN resistant parent HAT-310 and reference sequence EU033926.1:1-322 *S. lycopersicum* cultivar inbred Gh2 Mi23 locus for 380bp formed a separate clade. Also the resistant cross Swarna Lalima x HAT-310 grouped with HAT-310. The remaining five resistant crosses grouped with HAT-311

indicating the transfer of RKN resistance gene in these crosses. The crosses HAT-311xSwarna Lalima, HAT-296xHAT-311 and the genotype HAT-311 are more closely linked forming a separate sub cluster within the cluster A.

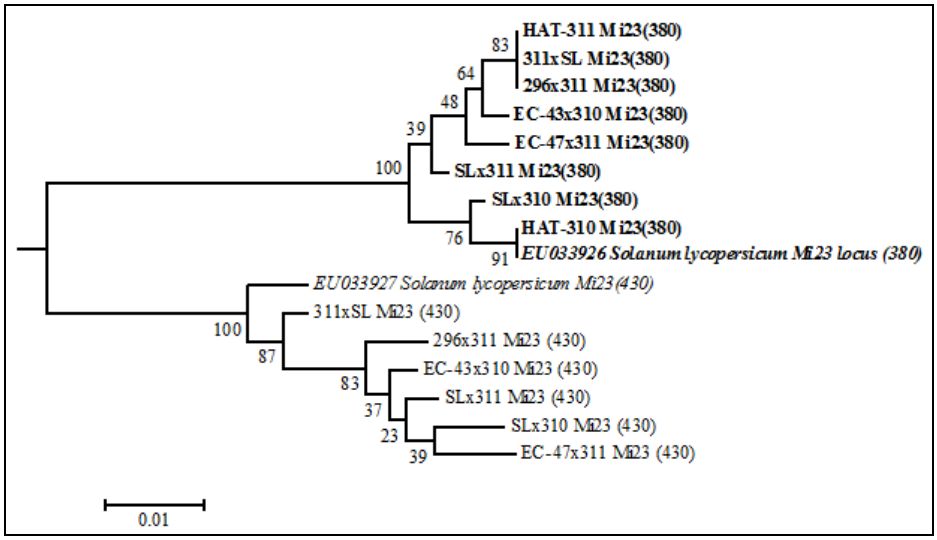

Fig. 5 Molecular Phylogenetic analysis by Neighbour joining method showing the relationships of resistant parents and crosses for 380bp and 430bp of Mi23 locus along with reference sequences in reference to *M. incognita*.

Discussion

Germplasm screening of the present study identified two new sources (HAT-310 and HAT-311) of genetic resistance to *M. incognita*. All the crosses (six) involving HAT-310 and HAT -311 were found immune to *M. incognita* indicating the presence of a single dominant resistant gene. Several groups reported *Mi* as a single, dominant gene conferring effective field resistance to *M. incognita* (Barham and Winstead, 1957; Laterrot and Pecaut, 1965; and Dropkin, 1969). Hence simple crossing with the resistant genotype can ensure the transfer of this resistant gene. The availability of genetic resistance against root knot nematode is of utmost importance for breeding resistant varieties of tomato. These new sources of resistance can be further incorporated in breeding programmes to develop nematode resistance in tomato like hybrid development, gene pyramiding etc.

To confirm the genetic resistance, molecular markers namely REX-1, JB-1, PMi12 and Mi23 for RKN resistance were used to characterise the phenotypically resistant germplasm. Also these molecular markers were compared to identify the best suitable marker for *M. incognita* resistance. PCR products of JB-1 after digestion with *Taq*I restriction enzyme yielded nearly 420 bp in all the genotypes. According to Devran et al (2013) 500 bp

band was reported in resistant homozygous or heterozygous condition of tomato yellow leaf curl virus along with the 420 bp band which was common in all the genotypes. The difference in the results is expected since the germplasm selected for the present study has not been characterised for tomato leaf curl virus resistance and hence it could not be confirmed for the presence of *Ty-1* gene. JB-1 marker was previously reported as a specific marker for the screening of *Ty-1* gene (Perez de Castro et al., 2007). This marker led to a successful separation on resistant and susceptible plants to TYLCV, however, marker JB-1 is dominant, and it cannot distinguish homozygous and heterozygous resistant genotypes. Hence, it is concluded that JB-1 could not differentiate between the nematode resistance and susceptibility.

Digestion of PCR products of REX-1 with *TaqI* yielded 720, 560 and 160 bp fragments in resistant genotypes, 700 bp fragment in susceptible genotypes while segregating material yielded three bands including 720, 560, and 160 bp. The present study was in accordance with Devran et al (2013). Williamson et al (1994) reported the use of REX-F1/REX-R2 primers to amplify the REX-1 locus closely linked to the Mi-1 locus, and *Taq*I restriction site exists in resistant plants. Another related study showed that REX-1 marker is appropriate for screening of *Mi-1* gene (Skupinova et al. 2004). Our findings are in confirmation with the previous studies. However, in one cross, Swarna Lalima x HAT-310 and in one genotype, HAT-302 the desired band pattern was not obtained. The reason may be attributed to the presence of *Ty-1* gene, since the germplasm was not characterised for tomato leaf curl virus resistance. El Mehrach et al (2005) reported that marker REX-1 could not be used in tomato hybrid lines with introgressions of *Solanum habrochaites* and *S. chilense* on chromosome 6 because the marker gave false-positive results in plants containing *Ty-1* gene. The above findings by different scientists suggest that this marker cannot be confidently used to screen plants bearing *Ty-1* gene for nematode resistance in tomato breeding program. This may be the reason for the different banding pattern of HAT-302 and the cross Swarna Lalima x HAT-310. Hence, REX-1 could be used only to differentiate between nematode resistant and susceptible genotypes.

Marker PMi12 was reported to have given the expected DNA fragment in plants bearing *Ty-1* gene for selection of RKN resistance (El Mehrach et al. 2005). However, the marker results in the production of additional bands in analysed plants. This can be due to different homologues in the tomato genome; which may cause false evaluation of PCR results (Devran et al. 2013). As anticipated from the previous studies, Marker PMi12 yielded

297 additional DNA fragments in addition to the expected bands and did not give consistent
298 results in the present study.

299 Furthermore, Mi23 produced 380 bp fragment in homozygous resistant genotypes and
300 430 bp in susceptible genotypes. It yielded both the bands in the heterozygous resistant
301 crosses. This co-dominant marker was employed for identification of *Mi-1* gene in all plants
302 (Seah et al. 2007). This marker successfully distinguished all alleles including homozygous,
303 heterozygous, and susceptible in tomato plants bearing *Ty-1*. Similar results were obtained by
304 Reddy et al (2016). Since both *Mi-1* and *Ty-1* are located on chromosome 6 and very close to
305 each other, theoretically, molecular markers linked to these genes could be used for screening
306 of nematode resistance and TYLCV resistance. However, markers linked to *Mi-1* gene have
307 not given consistent results for identification of *Ty-1* (Pe´rez de Castro et al. 2007).
308 Introgression between *Ty-1* gene and *Mi-1* may be the reason. Seah et al (2007) reported that
309 the Mi23 marker did not give a false-positive fragments with the begomovirus-resistant
310 breeding lines derived from *S. habrochaites* (Vidavsky and Czosnek 1998) and *S. chilense*
311 (Ty-1 locus) (Agrama and Scott 2006) for Mi-1 gene.

312 Phylogenetic relationship studies of the alleles with Mi 23 marker clearly indicated
313 that the resistant fragments grouped with reference gene EU033926.1:1-322 *S. lycopersicum*
314 cultivar inbred Gh2 Mi23 locus for 380bp and susceptible fragments with the reference gene
315 EU033927 *S. lycopersicum* cultivar M82-1-8 Mi23 locus marker genomic sequence for
316 430bp (Garcia el al. 2007). The results clearly indicated that the genetic resistance for *M.*
317 *incognita* has been successfully transferred to their crosses.

318 In the present study, HAT-310 and HAT-311 were identified to be new sources of
319 genetic resistance to *M. incognita*. The six resistant crosses (HAT-311 x Swarna Lalima,
320 HAT-296 x HAT-311, EC-596747 x HAT-311, Swarna Lalima x HAT-310, EC-596743 x
321 HAT-310 and Swarna Lalima x HAT-311) will be further selected for fruit quality and yield
322 to produce high yielding *M. incognita* resistant tomato hybrids.

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