A tomato NBS-LRR gene *Mi-9* confers heat-stable resistance to root-knot nematodes[1]

Shudong Chen[1, 2, 3*], Yupan Zou[1, 3*], Xin Tong[1, 2, 3], Cao Xu[1, 3#]

[1] State Key Laboratory of Plant Genomics, National Center for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, Beijing100000, China

[2] University of Chinese Academy of Sciences, Beijing 100000, China

[3] CAS-JIC Centre of Excellence for Plant and Microbial Science (CEPAMS), Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100000, China

Abstract

Root-knot nematodes (RKNs) are the most widespread soil-borne obligate endoparasites. They can infect the roots of many crops and cause significant yield losses. In tomato, the only commercially available RKN resistant gene *Mi-1.2* fails at soil temperatures above 28°C. We cloned the heat stable RKN-resistant gene *Mi-9* from a gene cluster composed of seven nucleotide-binding site and leucine-rich repeat (NBS-LRR) type resistant genes in *Solanum arcunum* accession LA2157. Screening nematode infections in individual & combinatorial knockouts of five NBS-LRR genes showed that *Mi-9 Candidate 4 (MiC-4)* alone is sufficient to confer heat stable RKN resistance. Our study identifies a new source of heat stable resistance to RKN in tomato for challenging environmental conditions. We also showcase a roadmap for rapid characterization of resistance genes by combining comparative genomics and genome editing, with the potential to be utilized in other crops.

Keywords: tomato, nematode, heat-stable resistance, *Mi-9*, genome editing

1. Introduction

Root-knot nematode (RKN) is one of the most destructive and widespread types of parasites of many crops (Fuller *et al.* 2008). They cause massive yield losses and cost farmers billions of dollars annually. In tomatoes, the only commercially available RKN resistance (R) gene *Mi-1.2*, encoding a

[1] *These authors contributed equally to this work.

Correspondence Cao Xu, E-mail: caoxu@genetics.ac.cn

nucleotide-binding site and leucine-rich repeat (NBS-LRR) protein, fails at soil temperatures above 28°C (Fuller *et al.* 2008, Jablonska *et al.* 2007). Soil temperature is frequently higher than 28℃ in both greenhouse and field tomato production systems, and rising temperatures caused by global climate change are making the situation worse. In *Solanum (S.) arcanum* accession LA2157, a previous study has narrowed down the heat-stable resistance locus *Mi-9* to the telomeric distal end and centromeric proximal end of the short arm of chromosome 6, a similar genetic interval as *Mi-1.2*, where a gene cluster of multiple NBS-LRR type R genes is located (Jablonska et al., 2007). A recent study reported the cloning of the *Mi-9* gene and the inference of its resistance function by virus-induced gene silencing (VIGS) in LA2157 and ectopic expression in another wild species *Solanum pimpinellifolium* (Jiang *et al.* 2023). Due to the high sequence similarity of the NBS-LRR genes out of the cluster where *Mi-9* resides, VIGS may not be able to accurately pinpoint the exact functional resistance gene(s) from the cluster due to its non-specificity and unstable inheritance. Here we overcome the lack of recombination by characterizing the genomic structural changes of the gene cluster across multiple tomato species, then precisely knocking out cluster members individually and combinatorially to pinpoint the resistance gene using genome editing (Fig. 1-A).

2. Materials and methods

2.1 Plant materials and growth conditions

Solanum arcanum (LA2157) seeds were provided by Tomato Genetics Resource Center (TGRC), UC Davis. Plants were grown in the greenhouse under natural light with supplementation from LED on a 12 h light/12 h dark photoperiod. The climate chamber (CORVIRON) was used for high-temperature (34℃) treatment.

2.2 Genome assembly

Fresh leaves of LA2157 plants were collected for genomic DNA fine-extraction. A 60-kb insert SMRTbell library was generated for SMRT PacBio Sequel II sequencing and 120.57 Gb of PacBio long reads were obtained. Software CANU (version 1.8), HERA and RaGOO (v1.1) was used to update the contigs to super-contigs and orient the super-contigs with respect to the SL4.0 Heinz1706 reference genome (Alonge *et al.* 2019, Du and Liang 2019, Koren *et al.* 2017). Circos (version 0.69) was used to show the genomic features (Krzywinski *et al.* 2009). The raw sequencing data in this paper have been submitted to Genome Sequence Archive (GSA) under the accession

number CRA016303(PRJCA017398). The assemblies generated in this paper genome were deposited in the Genome Warehouse (GWH) under the accession number GWHESQA00000000 (PRJCA017398).

2.3 Comparative genomics and genome evolution analysis

The representative genomes of *Solanaceae* were downloaded from the database (http://caastomato.biocloud.net/home) (Li *et al.* 2023). The syntenic relationships between LA2157 and other genomes were calculated by MUMmer 4.0.0beta2 (Marcais *et al.* 2018). The homologs of *Mi-1.2* were predicted using BLASTN (blast 2.60+) with an E-value cutoff of 10^{-5} (McGinnis and Madden 2004). Sequences were aligned using ClustalW and the phylogenetic tree was constructed using MEGA7 by the maximum likelihood (ML) statistical method with 1000 bootstrap samples and complete deletion (Kumar *et al.* 2016).

2.4 Real-time quantitative PCR

Tomato roots with or without nematode infection and tobacco leaves 3 days after agrobacterium infection were frozen in liquid nitrogen. Quantitative PCR was performed with 10 ng of cDNA input using SYBR Premix Ex TaqII kit and the gene-specific primers were listed in Appendices. The PCR program was carried out on a CFX Connect PCR system (BIO-RAD). Quantitative data were calculated from the number of PCR cycles (Ct) and normalized against internal reference (*Actin*).

2.5 Detection and quantification of hypersensitive response in tobacco leaves

To make the constructs for *pSuper-MiCs*, the coding sequences were ligated into the *pSuper1300* vector by In-Fusion cloning (Vazyme). Point mutations on NBS domain (D mutated to V) were generated by site-directed mutagenesis as previously reported (van Ooijen *et al.* 2008). The *Agrobacterium tumefaciens* strains (GV3101) were resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES-KOH, pH 5.8; adding 150 μM acetosyringone right before use) to an OD600 of 0.3. For electrolyte leakage rate detection, tobacco leaves from independent plants expressing proteins were harvested in ddH_2O and shaken at 37℃for 2 hours. The basal electrolyte content was measured by conductometer. And then, the sample were boiled for 10 mins and shaken for 2 h at 37℃ to release all electrolytes. The final electrolyte content was measured as above. The ratio of basal to final electrolyte content was used to represent leakage rate.

2.6 Generation and genotyping of CRISPR mutants

Sequence alignment was carried out to assess the similarity among five *MiCs*. And then, PAM sites

were first screened on LRR domain by manual operation and confirmed by blasting against the whole LA2157 genome. Guide RNAs targeting five *MiCs* simultaneously and single *MiC* individually were ligated into pDIRECT-22C vector respectively as previously reported (Cermak *et al.* 2017). Transplantation of tissue-culture-regenerated seedlings were conducted as previously described (Brooks *et al.* 2014). Sanger sequencing was carried out to identify mutations in target genes of CRISPR plants.

2.7 Nematode subculture, infection and micro-examination

Meloidogyne incognita was propagated on water cabbage and tomato in greenhouse. Egg masses were collected and hatched in water for 7 days. Pre-parasitic second-stage juveniles (pre-J2s) were injected nearby the root tips. For nematode infection in soil, tomato plants with 4 fully unfolded leaves were chosen for nematode incubation. Nematode infection assay on 1/2 MS was modified from previously reported (Lilley *et al.* 2018). Pre-J2 nematodes were sterilized using 0.15 mM mercuric chloride for 10 mins and washed by sterilized water for 5 times. Sterilized tomato seeds were planted on 1/2 MS. The culture dishes were half-shaded by aluminum foil and placed vertically to mimic the underground surroundings. The plants were placed at 26 and 34℃ climate incubators for normal development and high-temperature treatment. Plant roots with nematodes at different developmental stages were stained with different dyes for microscopy.

2.8 Root staining

For trypan blue staining, the plant samples were boiled for 10 mins in a 1:1 mixture of ethanol and 0.5 mg/ml trypan blue in lactophenol, followed by destaining overnight in 2.5 g/ml chloral hydrate in ethanol. For DAB (3,3'-diaminobenzidine) staining, the plant samples were incubated in the DAB staining buffer (1 mg/ml DAB and 200 mM Na_2HPO_4, pH 6.5) for 30 mins. The roots were washed off the soil first and stained by 0.35% fuchsine acid dissolved in ddH_2O for 10 mins. The staining buffer can be warmed for better and faster staining. The stained roots were destained and stored in a 1:1 mixture of ddH_2O and glycerol in a weakly acidic pH for phtography. The pictures were carried out by Olympus microscope (SteREO Discovery, v.12). Quantification of signal intensity were performed by ImageJ (Fiji Win64).

For more details, see Appendices.

3. Results

3.1 The rapid cloning of *Mi-9* from wild tomato species

To clone the heat-stable RKN resistance gene from LA2157, we performed a *de novo* genome sequencing using the PacBio Sequel II platform and obtained an 864.66 Mb genome assembly with an N50 of 5.8 Mb (Fig 1-B, Appendix 1). A total of 33,613 genes were identified with an average length of 3889.2 bp (Appendix 1). We then used the *Mi-1.2* as a query to perform a global search (BLAST) in LA2157 genome. Seven NBS-LRR homologs, namely *Mi-9 Candidate 1-7 (MiC1-7),* were detected in a cluster, separated into two sub-clusters by a 330 kb interval (Fig 1-C). An in-depth comparative genomic analysis was performed to identify the syntenic blocks encompassing this 330 kb inversion in 13 representative *Solanaceae* species (Fig 1-C). The inversion is present in *S. arcanum* LA2157 and *S. tuberosum*, *S. lycopersicoides*, *S. pennellii*, *S. pimpinelifolium*, *S. galapagense* as well as *S. lycopersium,* but absent in *S. chilense*, *S. corneliomulleri*, *S. neorichii* and *S. chmielewskii*, suggesting that two independent intra-chromosomal inversion events might have occurred before and after the emergence of LA2157, respectively (Fig 1-C, D, Appendix 2). We then compared the spatial distribution, direction and sequence integrity of *Mi-1.2* homologs within the cluster across all species listed above. Notably, some wild tomato species like *S. chilense* show high similarity to LA2157 (Fig 1-C), implying their RKN resistance potential. In support, a *S. chilense* accession LA2884 has been reported to have RKN resistance (J.C. Veremis 1996). But tomato cultivars like Heinz1706 and ZY65, as well as wild species such as *S. pimpinelifolium* and *S. galapagense* that do not have RKN resistance (J.C. Veremis 1996), were significantly different from that of LA2157 (Fig 1-C, D, Appendix 2), suggesting that two rounds of gene rearrangements (inversions) within this cluster might account for the gain and/or loss of heat-stable RKN resistance. We reconstructed a phylogenetic tree using all complete homologs in LA2157 and Heinz1706, in which *MiC-6* loci from two species are sister genes with 100% bootstrap support, indicating that they were not impacted by the second inversion (Appendix 3). Since *MiC-1*, *-2* and *-3* all have the same orientation in LA2157 and Heinz1706, we hypothesize that structural variations of *MiC-4* and/or *MiC-5* may be the key to heat-stable RKN resistance (Fig 1-C).

Sequence analysis showed that *MiC-1* and *MiC-3*, lacking the N terminal including starting codon, had already degraded into pseudogenes in LA2157 (Fig 1-E). We then focused on the remaining five *MiCs*. To obtain accurate genomic and transcript information of *MiCs* with extremely high sequence similarity, we performed full-length transcript sequencing and found that all five *MiCs*

were expressed in roots at 26℃ (Fig 1-E, Appendix 4). Their expression can be induced by nematode infection at 26℃ and 34℃ (Fig 1-F). We took advantage of hypersensitive responses (HR) of R genes and designed a transient expression system to rapidly test the function of five *MiCs* by expressing autoactivated versions of *MiCs* in tobacco leaves. Only expression of *MiC-4* induces typical HR like that of *Mi-1.2* with apparent necrosis, ROS burst and severe electrolyte leakage ((Fig 1-G, H, I), suggesting that *MiC-4 (LA2157_010834)* is likely to be the gene conferring RKN resistance at *Mi-9* locus.

3.2 The function identification of *Mi-9*

The fact that five *MiCs* share more than 94% DNA sequence identity on average makes RNA interference, even genome editing, difficult to impossible to specifically target each *MiC* gene. To overcome this, we utilized a multiplex CRISPR/Cas9 system to simultaneously target and edit homologous genes both individually and combinatorically. Comprehensive sequence analyses revealed that the LRR domains of *MiCs* showed slightly lower sequence identities, ranging from 85% to 93%, making this region a possible target to distinguish different cluster members (Fig 2-A). We designed specific gRNAs to target the LRR domain of each *MiC* (Fig 2-A). By assembling eight gRNAs targeting all five *MiCs* simultaneously into one construct or two gRNAs targeting individual *MiCs* into five constructs, we generated both quintuple and single mutants of *MiCs* in LA2157 (Fig 2-B, Appendix 5). None of these mutants show notable morphological differences from wild-type plants under normal conditions without nematode infection (Fig 2-C). However, only quintuple and *MiC-4* single mutants show delayed growth after nematode infection at 34°C, a typical phenotype of nematode-sensitive tomato plants after infection (Fig 2-D). Root staining and observation showed that root-knots developed only in the quintuple and *MiC-4* single mutants (Fig 2-E). Importantly, the *MiC-4* single mutant shows non-distinctive phenotypes from the quintuple mutant neither in delayed growth nor root-knots after infection. Similar susceptible phenotypes of the quintuple and *MiC-4* single mutants were also identified at 1/2 MS medium (Appendix 6). We performed sequence alignments between *MiC-4 and Sarc_034200* (*Mi-9* published by Jiang et al) (Appendix 7). These two genes share the same DNA sequence and genetic coordinates (Appendix 7). Combined with the phenotypic data, we demonstrated that the *MiC-4* is the same gene as *Mi-9* (Fig 2-E).

To explore when and how *MiC-4* functions to confer resistance to nematodes, we compared the nematode infection processes between the susceptible cultivar Ailsa Craig (AC) and LA2157 in soil

at 34°C (Appendix 8). More than 60% of nematodes invaded the plants from root tips both in AC and LA2157 (Appendix 8). The infected nematodes normally developed into mature females in AC, while their growth ceased at Juvenile stage 2 in LA2157 (Appendix 8). To precisely control the infection and compare immune responses, we synchronously treated LA2157 and *CR-MiC-4* mutants with the same amounts of nematodes on sterilized solid mediums under 34°C. Consistently, the roots of LA2157 showed rapid ROS burst and necrosis after nematode infection rather than the *CR-MiC-4* mutants, indicating the immune response triggered by *MiC-4* occurs at the very early stage of infection (Fig 2-F, G, H, Appendix 9).

4. Discussion

We overcome the lack of recombination by characterizing the genomic structural changes of the gene cluster across multiple tomato species, then accurately targeting and knocking out cluster members individually and combinatorically to pinpoint the resistance gene using CRISPR/Cas9 mediated editing. Our study identified a heat-stable RKN resistance gene from wild tomato species by integrating comparative genomics with multiplex genome editing. We showed a new source of heat-stable resistance to RKN in tomatoes for challenging environmental conditions. We also showcase a roadmap for the rapid characterization of resistance genes from complicated genomic regions by combining comparative genomics and genome editing, with the potential to be utilized in other crops.

Interestingly, the expression of *Mi-9 (MiC-4)* was induced by nematode infection as *Mi-1.2* whether at normal or high temperature. Sequence alignment showed 35 SNPs caused amino acid substitutions between *Mi-1.2 and Mi-9 (MiC-4)*, which may be responsible for the differentiation on high temperature resistance. In addition, ROS homeostasis and bursting were reported to be involved in cell fate determination and response to environmental cues (Waszczak *et al.* 2018). We found *Mi-9 (MiC-4)* mediated quick ROS accumulation during cell necrosis, these responses were impaired in *CR-MiC-4* mutants. It is worthy investigating that how the ROS signal was regulated by *Mi-9 (MiC-4)* will benefit the fundamental research on the interaction between the plants and parasitic nematodes.

5. Conclusion

Collectively, we cloned the heat-stable RKN-resistance gene *Mi-9* from a gene cluster composed of NBS-LRR type resistance genes in LA2157. Screening nematode infections in individual &

combinatorial knockouts of five NBS-LRR genes showed that *Mi-9 Candidate 4 (MiC-4)* alone is sufficient to confer heat-stable RKN resistance and it blocks RKN invasion at the very early stage of infection.

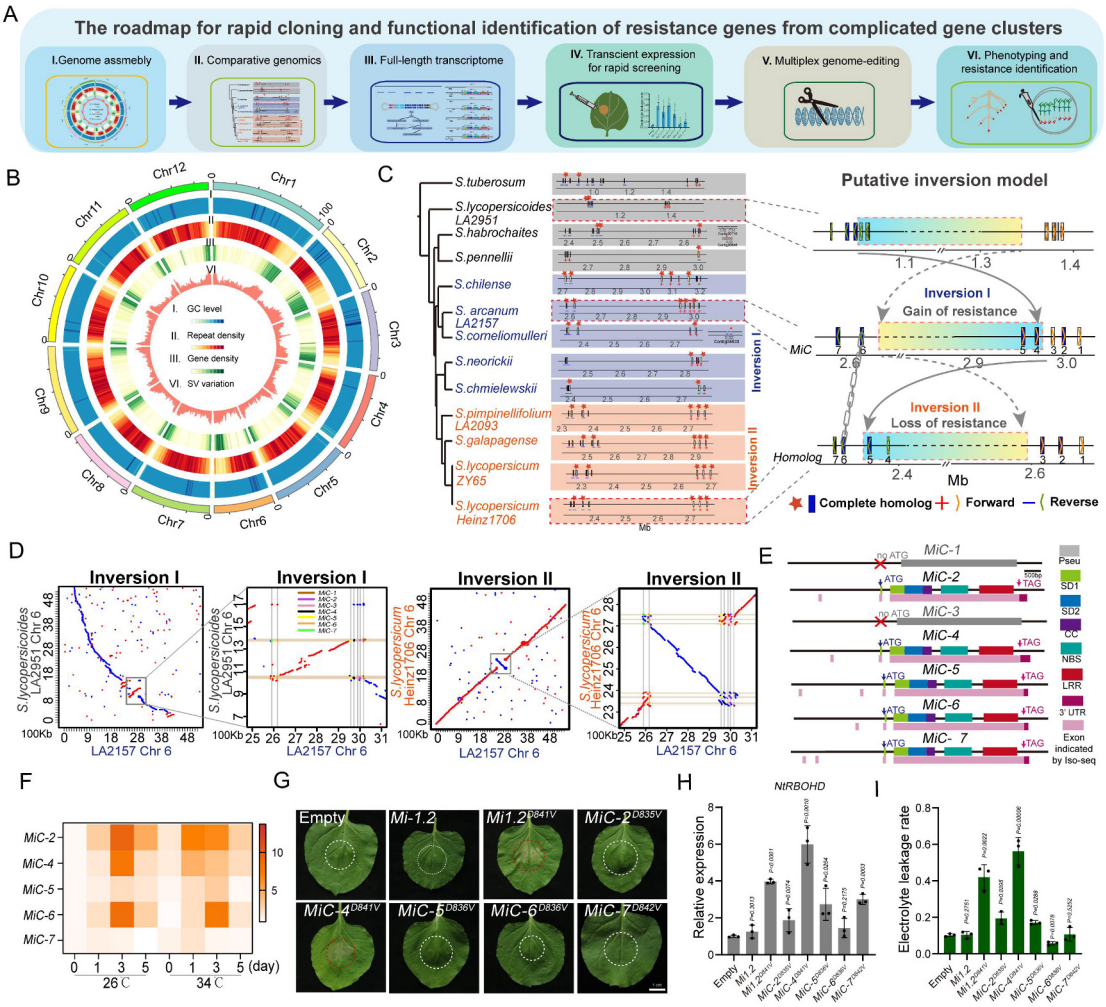

Fig 1 The rapid cloning of *Mi-9* from wild tomato species. A, The roadmap of the study. B, Features of the LA2157 genome. C, Comparative genomic analysis of *Mi-1.2* homologs in representative species of *Solanaceae*. D, Syntenic dot plots of resistance candidate region on chr6 between LA2157 and *S. lycopersicoides* (LA2951) as well as Heinz1706. E, Full-length transcripts of *MiCs* revealed by Iso-Seq. Colored boxes are used to represent respective domains. Pseu, pseudogene, SD, solanaceae domain, CC, coiled-coil, NBS, nucleotide binding domain, LRR, leucin-riced repeat. G-I, Hypersensitive response assessment indicated by visual photos (H), ROS related gene expression I) and electrolyte leakage assay (J). Autoactivated *Mi-1.2* and five *MiCs* were transiently expressed by mutating the key amino acid, aspartic acid (D) to valine (V) as previous reported (Van Ooijen et al. 2008). NtActin served as a control. Data are presented as means (±s.d.) (n = 3, two-tailed *t*-test). Scale bars, 0.5 cm.

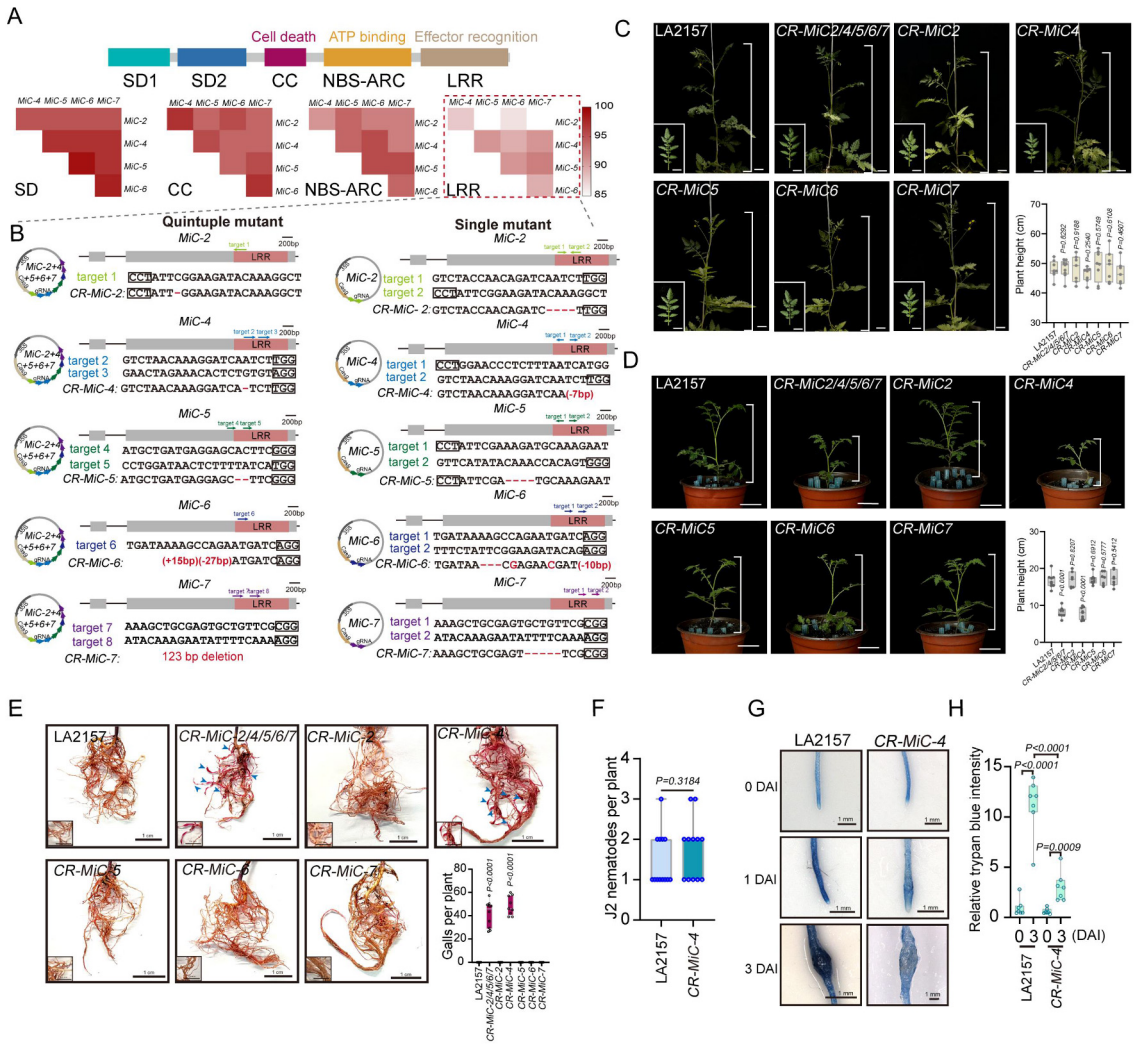

Fig 2 Genetic identification of *Mi-9*. A, Schematic and sequence similarity assessment of five *MiCs*. B, Target design and genotype of T1 knockout mutants of five *MiCs*. C-D, Plant morphology of *CR-MiCs* mutants without and with nematode infection. E, Root-knots staining and statistics of galls per plant of *CR-MiCs* mutants with nematode incubation. Plants were incubated with 500 Pre-J2 nematodes and placed at 34℃ for 3 weeks. Blue arrowheads indicate the macroscopic root-knots. F, quantification of J2 nematodes per plant. G-H, representative photos and quantification showing cell death indicated by trypan blue staining. DAI, day after infection. Data are presented as means (± s.d.) (n ≥ 6, two-tailed *t*-test).

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Declaration of competing interest

The authors declare no conflict of interest.

References

Alonge M, Soyk S, Ramakrishnan S, Wang X, Goodwin S, Sedlazeck F J, Lippman Z B, Schatz M C. 2019. RaGOO: fast and accurate reference-guided scaffolding of draft genomes. *Genome Biol,* **20**, 224. doi,10.1186/s13059-019-1829-6.

Brooks C, Nekrasov V, Lippman Z B, Van Eck J. 2014. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol,* **166**, 1292-1297. doi,10.1104/pp.114.247577.

Cermak T, Curtin S J, Gil-Humanes J, Cegan R, Kono T J Y, Konecna E, Belanto J J, Starker C G, Mathre J W, Greenstein R L, Voytas D F. 2017. A Multipurpose Toolkit to Enable Advanced Genome Engineering in Plants. *Plant Cell,* **29**, 1196-1217. doi,10.1105/tpc.16.00922.

Du H, Liang C. 2019. Assembly of chromosome-scale contigs by efficiently resolving repetitive sequences with long reads. *Nat Commun,* **10**, 5360. doi,10.1038/s41467-019-13355-3.

Fuller V L, Lilley C J, Urwin P E. 2008. Nematode resistance. *New Phytol,* **180**, 27-44. doi,10.1111/j.1469-8137.2008.02508.x.

J.C. Veremis P A R. 1996. Identification of resistance to Meloidogyne javanica. *Theor Appl Genet,* **93**.

Jablonska B, Ammiraju J S, Bhattarai K K, Mantelin S, Martinez de Ilarduya O, Roberts P A, Kaloshian I. 2007. The Mi-9 gene from Solanum arcanum conferring heat-stable resistance to root-knot nematodes is a homolog of Mi-1. *Plant Physiol,* **143**, 1044-1054. doi,10.1104/pp.106.089615.

Jiang L, Ling J, Zhao J, Yang Y, Yang Y, Li Y, Jiao Y, Mao Z, Wang Y, Xie B. 2023. Chromosome-scale genome assembly-assisted identification of Mi-9 gene in Solanum arcanum accession LA2157, conferring heat-stable resistance to Meloidogyne incognita. *Plant Biotechnol J*. doi,10.1111/pbi.14055.

Koren S, Walenz B P, Berlin K, Miller J R, Bergman N H, Phillippy A M. 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research,* **27**, 722-736. doi,10.1101/gr.215087.116. (in English)

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones S J, Marra M A. 2009. Circos: an information aesthetic for comparative genomics. *Genome Res,* **19**, 1639-1645. doi,10.1101/gr.092759.109.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution,* **33**, 1870-1874. doi,10.1093/molbev/msw054. (in English)

Li N, He Q, Wang J, Wang B, Zhao J, Huang S, Yang T, Tang Y, Yang S, Aisimutuola P, Xu R, Hu J, Jia C, Ma K, Li Z, Jiang F, Gao J, Lan H, Zhou Y, Zhang X, Huang S, Fei Z, Wang H, Li H, Yu Q. 2023. Super-pangenome analyses highlight genomic diversity and structural variation across wild and cultivated tomato species. *Nat Genet,* **55**, 852-860. doi,10.1038/s41588-023-01340-y.

Lilley C J, Maqbool A, Wu D, Yusup H B, Jones L M, Birch P R J, Banfield M J, Urwin P E, Eves-van den Akker S. 2018. Effector gene birth in plant parasitic nematodes: Neofunctionalization of a housekeeping glutathione synthetase gene. *PLoS Genet,* **14**, e1007310. doi,10.1371/journal.pgen.1007310.

Marcais G, Delcher A L, Phillippy A M, Coston R, Salzberg S L, Zimin A. 2018. MUMmer4: A fast and versatile genome alignment system. *PLoS Comput Biol,* **14**, e1005944. doi,10.1371/journal.pcbi.1005944.

McGinnis S, Madden T L. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis

tools. *Nucleic Acids Research,* **32**, W20-W25. doi,10.1093/nar/gkh435. (in English)

van Ooijen G, Mayr G, Albrecht M, Cornelissen B J, Takken F L. 2008. Transcomplementation, but not physical association of the CC-NB-ARC and LRR domains of tomato R protein Mi-1.2 is altered by mutations in the ARC2 subdomain. *Mol Plant,* **1**, 401-410. doi,10.1093/mp/ssn009.

Waszczak C, Carmody M, Kangasjarvi J. 2018. Reactive Oxygen Species in Plant Signaling. *Annu Rev Plant Biol,* **69**, 209-236. doi,10.1146/annurev-arplant-042817-040322.