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ORIGINAL ARTICLE

Comparative transcriptomics of susceptible and resistant *Cucumis metuliferus* upon *Meloidogyne incognita* infection

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Abstract

Main conclusion **Comparative transcriptomics has identified several candidate genes contributing to the resistance of *Cucumis metuliferus* against *Meloidogyne incognita*.**

Abstract The Southern root-knot nematode (*Meloidogyne incognita*) is a significant threat to Cucurbitaceae crops. The African horned melon (*Cucumis metuliferus*), a wild relative, exhibits high resistance to this nematode. To explore the resistance mechanism, phenotypic analyses were conducted on a susceptible inbred line (CM27) and a resistant inbred line (CM3). CM3 exhibited enhanced root biomass and significantly higher resistance compared to CM27, with poor nematode development observed in CM3 roots. Transcriptomic profiling at multiple post-infection time points revealed 2243 and 3700 differentially expressed genes (DEGs) in CM3 and CM27, respectively. Among these, the top ten DEGs upregulated exclusively in CM3 were functionally analyzed using virus-induced gene silencing (VIGS). Silencing of *EVM0019904* or *EVM0017058* in CM3 led to susceptibility to *M. incognita*. These findings provide novel insights into the resistance mechanisms of *M. incognita* in *C. metuliferus* and offer potential resources for breeding nematode-resistant Cucurbitaceae crops.

Keywords *Cucumis metuliferus* · *Meloidogyne incognita* · Comparative transcriptomics · VIGS

Abbreviations

RKN	Root-knot nematode
CM	*Cucumis metuliferus*
DEGs	Differentially expressed genes
VIGS	Virus-induced gene silencing
J2	Second-stage juveniles
SJ2	Swollen second-stage juveniles
J3	Third stage juveniles
J4	Fourth stage juveniles
PDS	Phytoene dehydrogenase

Introduction

Root-knot nematodes (*Meloidogyne* spp.) infect the roots of almost all cultivated plants by penetrating and migrating between root cells (Abad et al. 2008; Jones et al. 2013). These nematodes induce the formation of giant cells, which supply nutrients essential for their development (Abad et al. 2008; Jones and Payne 1978). Infection impairs nutrient and water assimilation, leading to suboptimal plant growth and significant yield losses (Abad et al. 2009). Within host root systems, the infective second-stage juvenile (J2) progresses through molting stages (J3 and J4) to adulthood (Moens et al. 2009). The swollen J2 (SJ2) represents a transitional stage as J2 develops into J3 (Collett et al. 2023).

Cucumis sativus (cucumber) and Cucumis melo (melon), key members of the Cucurbitaceae family, are economically important crops. Losses caused by *Meloidogyne* spp. in these crops range from 10 to 98% (Exposito et al. 2020; Ploeg and Phillips 2001; Kumar et al. 2020; Sasser 1979). Among the nematodes, *M. incognita* predominantly infects Cucurbitaceae crops, including cucumber and melon (Kayani et al. 2013; Liu et al. 2014). Effective management remains

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challenging (Shrestha et al. 2020). Although nematicides are highly effective, their use is increasingly restricted due to environmental and human health concerns (Shrestha et al. 2020; Nyczepir and Thomas 2009; Zasada et al. 2010; Desaeger et al. 2020; Hamill and Dickson. 2005). Biological control offers a potential alternative but is often inconsistent and slower-acting compared to chemical methods (Askary 2015). Crop rotation is another management strategy but is hindered by the difficulty of selecting rotations that minimize nematode damage and align with farming practices (Bridge 1996). Grafting, while promising, is constrained by seasonal and rootstock selection considerations (Ayala-Doñas et al. 2020; Exposito et al. 2020). Additionally, no commercial rootstock resistant to *M. incognita* exists for cucumber or melon (Expósito Creo 2021), and grafting may affect fruit quality (Expósito et al. 2018; Guan et al. 2014). The cultivation of *M. incognita*-resistant varieties presents the most sustainable, economical, and environmentally friendly solution (Phani et al. 2021). Many efforts to identify cultivated cucumber or melon resources with resistance to *M. incognita* have been undertaken (Mukhtar et al. 2013; Walters and Wehner 2002; Wehner et al. 1991; Fassuliotis and Rau 1963; Nugent and Dukes 1997; Thomason and McKinney 1959; Walters et al. 1993; Shen et al. 2007). Unfortunately, no *M. incognita*-resistant cucumber or melon materials were found and hence the breeding of varieties with resistance to *M. incognita* is currently difficult to achieve (Mukhtar et al. 2013).

Cucumis metuliferus, a wild Cucurbitaceae relative, exhibits high resistance to *M. incognita* (Fassuliotis 1970; Wehner et al. 1991; Walters et al. 2006). This resistance is associated with suppression of larval development beyond the second stage, delayed adult development, and increased stimulation of male development (Walters et al. 2006; Ling et al. 2017; Li et al. 2021; Ye et al. 2017; Fassuliotis 1970). While penetration rates of *M. incognita* into *C. metuliferus* roots are similar to those in cucumber and melon (Fassuliotis 1970; Ye et al. 2017). A recent report showed *M. incognita* nematodes exerted a strong preference for cucumber roots rather than for *C. metuliferus* roots in vitro. Moreover, root volatiles from *C. metuliferus* have been shown to repel or kill *M. incognita*, and their application to cucumber roots enhances resistance (Xie et al. 2022). Comparative transcriptomic studies suggest that resistance in *C. metuliferus* involves genes related to salicylic acid (SA) and jasmonic acid (JA) signaling, Ca^{2+} signaling, cytoskeleton biosynthesis, phenylpropanoid biosynthesis, plant hormone signal transduction, and plant-pathogen interaction (Li et al. 2021; Ling et al. 2017; Ye et al. 2017).

In this study, RNA-seq and VIGS were utilized to investigate *M. incognita* resistance in two inbred *C. metuliferus* lines: CM3 (resistant) and CM27 (susceptible). Phenotypic analyses evaluated nematode development at various stages post-infection. Transcriptome profiling identified resistance-related DEGs in CM3, and ten top DEGs were functionally analyzed via VIGS. The findings can be utilized to dissect a novel resistance mechanism in *C. metuliferus* and provide a strong basis for the breeding of *M. incognita*-resistant Cucurbitaceae crops.

Materials and methods

Plant materials and nematode preparation

Two inbred lines of *C. metuliferus*, CM3 (resistant) and CM27 (susceptible), were used in this study. These lines were kindly provided by Prof. Xingfang Gu from the Department of Cucurbits Genetics and Breeding, Institute of Vegetable and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. Seedlings were grown in round plastic pots (6 cm diameter × 10 cm height) and maintained under controlled conditions in a phytotron with day/night temperatures of 28 °C/22 °C and 85% relative humidity. All experiments were conducted using seedlings at the two-leaf stage.

Water spinach (Ipomoea aquatica Forssk.), highly susceptible to *M. incognita*, was cultivated to generate nematodes. Egg masses extracted from the roots were incubated in distilled water at 28 °C to hatch. Second-stage juveniles (J2) were collected and stored in a 200 mL conical flask for subsequent use.

Nematode inoculation assays

To ensure sufficient nematode presence in the roots, each CM3 and CM27 seedling was inoculated with 1000 J2 nematodes. The inoculation procedure followed a previously described protocol (Li et al. 2021; Xie et al. 2022). Roots were harvested and carefully cleaned at 3-, 7-, 14-, and 28 days post-inoculation (dpi). Nematode development was assessed by staining the roots using the sodium-hypochlorite-acid-fuchsin method (Bybd Jr et al. 1983). The number and body width (μm) of nematodes were evaluated under a microscope (OLYMPUS IX53, Japan). At 7-, 14- and 28 dpi, nematodes in the roots were categorized into motile second-stage juveniles (J2), swollen second-stage juveniles (SJ2), third-stage juveniles (J3), fourth-stage juveniles (J4), and adults (A), following established criteria (Collett et al. 2023; Khan et al. 2006). Between 347 and 592 nematodes were classified at each time point. At approximately 55 dpi, the number of galls and egg masses per plant was recorded for 31–34 plants. Additionally, root length (cm) and fresh root weight (g) were measured.

Transcriptome sequencing and de novo assembly

Total RNA (1 µg per sample) extracted from roots was used for library preparation with a NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's protocol. Raw sequence data were processed into clean reads, which were mapped to the *C. metuliferus* reference genome using Hisat2 tools software (Ling et al. 2021).

Identification and functional analysis of DEGs

Gene expression levels were quantified using fragments per kilobase of transcript per million mapped fragments (FPKM) values, as described previously (Pertea et al., 2016). Differentially expressed genes (DEGs) were identified using thresholds of $P \leq 0.01$ and |log2 fold change| ≥ 2. Functional annotation of DEGs was conducted using the Pfam database.

Virus-induced gene silencing (VIGS)

The VIGS vector pV190, provided by Dr. Qinsheng Gu (Zhengzhou Fruit Research Institute, Zhengzhou, China), has been successfully employed in several Cucurbitaceae species (Bi et al. 2022; Liu et al. 2020; Xu et al. 2021; Zhang et al. 2021; Zhu et al. 2021). In this study, pV190 was used to investigate candidate gene functions in *C. metuliferus* for the first time. A 186-bp fragment of *CmPDS* (Phytoene dehydrogenase) was cloned into pV190 using homologous recombination, generating the construct pV190::CmPDS186. This construct was introduced into *Agrobacterium tumefaciens* strain GV3101, and the *A. tumefaciens* suspension was used to infiltrate cotyledons of 14-day-old *C. metuliferus* seedlings, following established methods (Liu et al. 2020).

Genes uniquely upregulated in CM3 at one or more time points post-inoculation were targeted for functional validation via VIGS. Constructs containing approximately 300-bp coding sequences of these genes were introduced into seedlings. Gene silencing efficiency was confirmed by qPCR, and plants with significantly reduced target gene expression were inoculated with nematodes. Plants treated with the empty pV190 vector served as controls. Each experiment was performed in triplicate, using 12–21 plants per replicate.

Primers

All primers used in this study were designed via the online software tool Primer3Plus (https://www.primer3plus.com) and are summarized in Supplementary Table S1.

Statistical analysis

Data analyses were performed using Microsoft Excel 2019, GraphPad Prism 8.0 and R statistical software 4.1.2.

Results

Disease evaluation between CM3 and CM27

To evaluate the differences in root response to *M. incognita* infection between CM3 and CM27, root fresh weight, root length, and gall numbers were measured. A previous study showed that few egg masses were produced by *M. incognita* on *C. metuliferus* seven weeks after inoculation (Faske 2013). Consequently, roots were harvested at 55 dpi for egg production assessment in this study. The fresh weight and length of CM3 roots were significantly greater than those of CM27 (Supplementary Figures S1A and S1B). On average, 96 galls were observed on CM3 roots, compared to 274 galls on CM27 roots (Supplementary Figure S1C). Notably, the gall count in CM3 was significantly lower than in CM27 ($P < 0.01$).

To account for root size variability, gall numbers were normalized per centimeter and per gram of root tissue (Fig. 1A and B). The normalized gall counts were significantly lower in CM3 than in CM27 ($P < 0.01$). Egg masses were observed on over 50% of CM27 roots but on less than 10% of CM3 roots (Fig. 1C). These findings collectively indicate that CM3 exhibits greater resistance to *M. incognita* compared to CM27.

Penetration and development of M. incognita in CM3 and CM27

To further reveal the differences in infection dynamics between CM3 and CM27, the number and development of nematodes in roots were analyzed. The nematode numbers increased from 3 to 14 dpi in both lines but plateaued thereafter (Fig. 2A). Significant differences in nematode numbers were observed at 14 dpi and 28 dpi between the two lines.

Nematode body width was used as an indicator of developmental progression. The nematodes in CM3 roots exhibited smaller body widths compared to those in CM27 roots (Fig. 2B). Developmental stage analysis further highlighted delayed progression in CM3. At 7 dpi, approximately 75% of nematodes in CM3 remained at the second stage, while in CM27, about 50% had developed into SJ2s, and over 5% had progressed to J3s. At 14 dpi, approximately 60% of nematodes in CM27 were at the J3 stage, whereas fewer than 20% had reached J3 in CM3. By 28 dpi, over 40% of nematodes in CM27 were at the J4 stage, compared to only about 10%

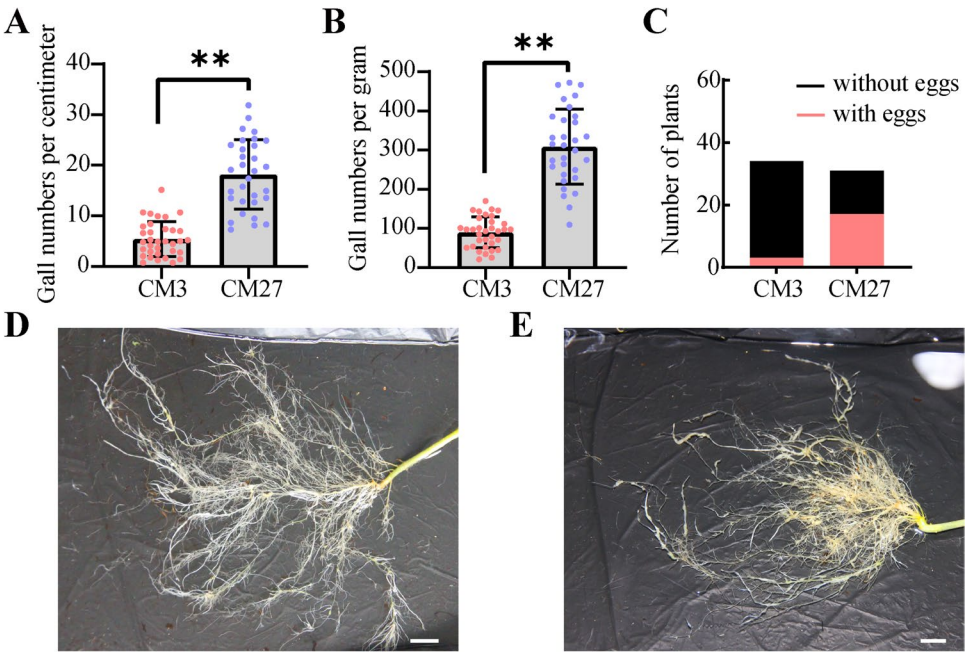

Fig. 1 Symptoms of *Meloidogyne incognita* infection in resistant CM3 and susceptible CM27. **A**-**B** Number of galls per centimeter (**A**–**C**) and per gram of CM3 and CM27 tissue at 55 days post-infection (dpi). Data are presented as means ± SD. $*P<0.05$; $**P<0.01$ (Student's *t*-test). **C** Number of plants with or without egg masses of CM3 and CM27 at 55 dpi. **D**-**E** Root images of CM3 and CM27 at 55 dpi. Scale bar = 2 cm

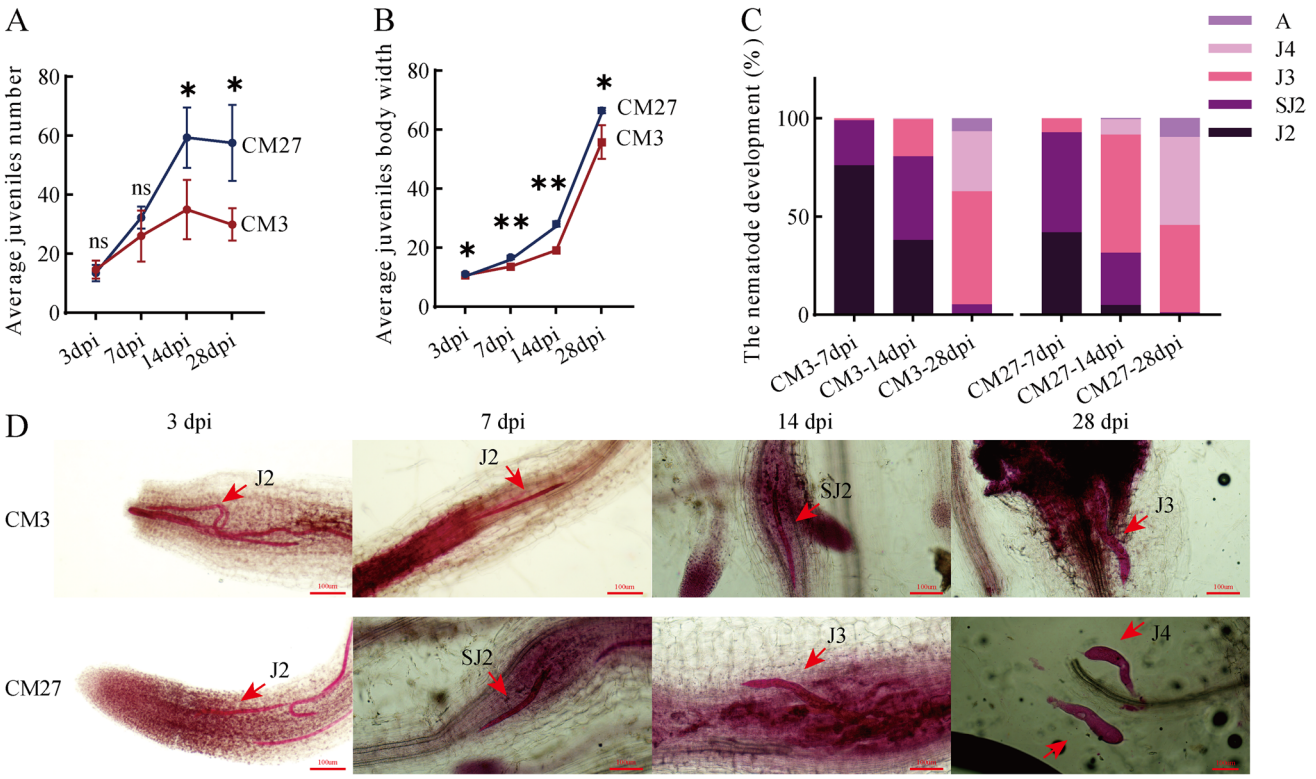

Fig. 2 Penetration and development of *M. incognita* in CM3 and CM27 roots. **A**-**B** Nematode count and body width of nematodes at different developmental stages at days post inoculation (dpi). Data are presented as means ± SD. $*P<0.05$; $**P<0.01$; ns, not significant (Student's t-test). **C** Proportions of different life stages: second-stage juveniles (J2), swollen second-stage juveniles (SJ2), third-stage juveniles (J3), fourth-stage juveniles (J4), and adults (**A**). **D** Acid fuchsin-stained penetrated nematodes at different life stages. Scale bar = 100 µm

in CM3. These results indicate that nematode development

was substantially impaired in CM3 roots compared to CM27 (Fig. 2C and D).

Transcriptome sequencing of CM3 and CM27 infected by M. incognita

To investigate the molecular mechanisms underlying *M. incognita* resistance in CM3, transcriptome profiles of root tissues from CM3 and CM27 were compared dynamically. RNA was extracted from roots at 0-, 3-, 7-, 14- and 28 dpi, generating a total of 30 sequencing libraries. Illumina RNA-Seq yielded 193.64 Gb of clean reads, with each sample producing at least 5.82 Gb of clean data (Supplementary Table S2). The clean reads were deposited in the NCBI Sequence Reads Archive (SRA) database (Accession number: PRJNA1021046) and aligned to the *C. metuliferus* reference genome. Alignment efficiencies ranged from 82.45% to 94.77% (Supplementary Table S3).

DEGs were identified by comparing inoculated samples with their respective non-inoculated controls in CM3 and

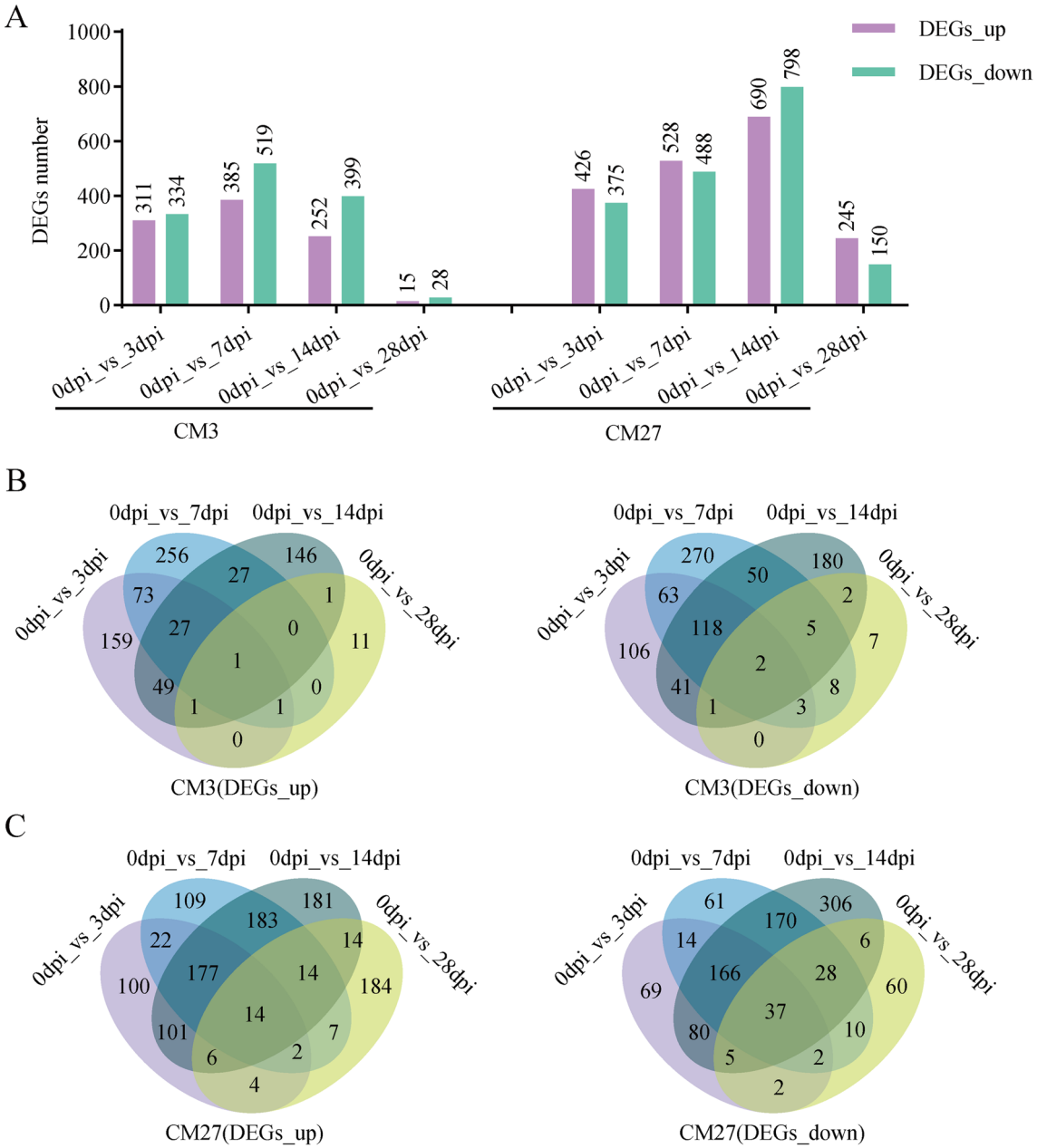

Fig. 3 Differentially expressed genes (DEGs) after *Meloidogyne incognita* infection in CM3 and CM27. **A** Numbers of upregulated and downregulated DEGs across different time points ($P < 0.01$). **B**-**C** Venn diagrams showing specific and common DEGs at each time point, generated using EVenn (http://www.ehbio.com/test/venn)

CM27 at each time point (Fig. 3A; Supplementary Table S4). In CM3, 645 DEGs were detected at 3 dpi, increasing to 904 at 7 dpi, followed by a decline to 651 at 14 dpi and 43 at 28 dpi. By contrast, CM27 exhibited a higher number of DEGs, ranging from 395 to 1488 across the time points. The number of DEGs in CM27 increased gradually until 14 dpi and then declined significantly at 28 dpi. Venn diagrams of the upregulated and downregulated DEGs at 3-, 7-, 14-, and 28 dpi revealed distinct expression patterns between the two cultivars. In CM3, 963 genes were upregulated and 1,280 were downregulated, with only one upregulated gene and two downregulated genes shared across all time points (Fig. 3B). In CM27, 1889 genes were upregulated and 1811 were downregulated, with 14 upregulated and 37 downregulated genes shared at all time points (Fig. 3C). These results indicate that *M. incognita* infection induced more extensive transcriptomic changes in the susceptible CM27 than in the resistant CM3. Moreover, the number of DEGs peaked at 7 dpi in CM3 and at 14 dpi in CM27, suggesting differing temporal responses to infection. The DEGs that were consistently upregulated or downregulated across all time points are summarized in Supplementary Table S4.

Identification of the top ten upregulated genes in CM3

It was hypothesized that the upregulated DEGs unique to the resistant CM3, and absent in the susceptible CM27, could be critical for conferring resistance. Based on expression levels, the top ten candidate genes were identified, all of which were exclusively upregulated in CM3, exhibiting up to a fivefold differential expression compared to non-inoculated control (Table 1). Among these, four genes (*EVM0022963*, *EVM0019904*, *EVM0027941* and *EVM0005552*) had unknown functions, while three genes (*EVM0017058*, *EVM0026123* and *EVM0010549*) belonged to the Hsp20/alpha-crystallin family (HSP20). Of the remaining three genes, *EVM0004550* contains a O-methyltransferase domain, *EVM0016021* contains a WRKY DNA-binding domain, and *EVM0023153* belongs to Glycine-rich protein family. Notably, HSP20 genes constituted the largest proportion among the top ten candidates, suggesting their vital role in regulating resistance to nematode infection. Further analysis identified 15 upregulated HSP20 genes in CM3 following *M. incognita* infection, compared to only nine in CM27 (Supplementary Table S4). These findings underscore the importance of HSP20 family genes in the nematode resistance mechanism of CM3.

Functional verification analysis

To validate the use of the VIGS vector pV190 for functional gene analysis in *C. metuliferus*, the *CmPDS* gene was selected as a model. Although pV190 has been successfully employed in various cucurbits (Bi et al. 2022; Xu et al. 2021; Zhang et al. 2021; Zhu et al. 2021), it was not certain that the silencing system would work in *C. metuliferus*. In *C. metuliferus*, *EVM0010091* encoding the PDS protein was used to construct the *CmPDS* silencing vector. Referring to a previous study, the *CmPDS* silencing vector, pV190::CmPDS186, was completed according to Fig. 4A and B (Liu et al. 2020). To evaluate the silencing efficiency, the expression level of *CmPDS* was examined in roots and leaves at 14 dpi. Results revealed a significant downregulation of *CmPDS* expression in both tissues (Fig. 4C). Photobleaching was observed in 21 out of 24 seedlings. Furthermore, the silencing effect of the *CmPDS* gene persisted for over 40 days (Fig. 4D). These findings demonstrate that the VIGS system is a reliable and effective tool for functional gene analysis in *C. metuliferus*.

To further investigate the function of the top ten upregulated genes in CM3, their expression levels were silenced in CM3 using VIGS. Silencing efficiencies were evaluated at 14 dpi by qPCR, with reductions in expression ranging from 34.5 to 99.5% relative to pV190 controls (Fig. 5A,

Table 1 Details of the top ten upregulated genes in CM3

Gene ID	Pfam_annotation	log_2 fold change in CM3		
		0dpi_vs_3dpi	0dpi_vs_7dpi	0dpi_vs_14dpi
EVM0022963	Unknown function	Inf*	–	–
EVM0019904	Unknown function	Inf	–	–
EVM0027941	Unknown function	Inf	–	–
EVM0017058	Hsp20/alpha crystallin family	6.5523	–	–
EVM0004550	O-methyltransferase domain	6.0004	–	–
EVM0026123	Hsp20/alpha crystallin family	5.9231	–	–
EVM0010549	Hsp20/alpha crystallin family	5.3823	–	–
EVM0016021	WRKY DNA -binding domain	–	6.3012	–
EVM0005552	Unknown function	–	5.0672	–
EVM0023153	Glycine rich protein family	–	5.7419	5.4147

*Indication of infinity (Inf) in log_2 fold change

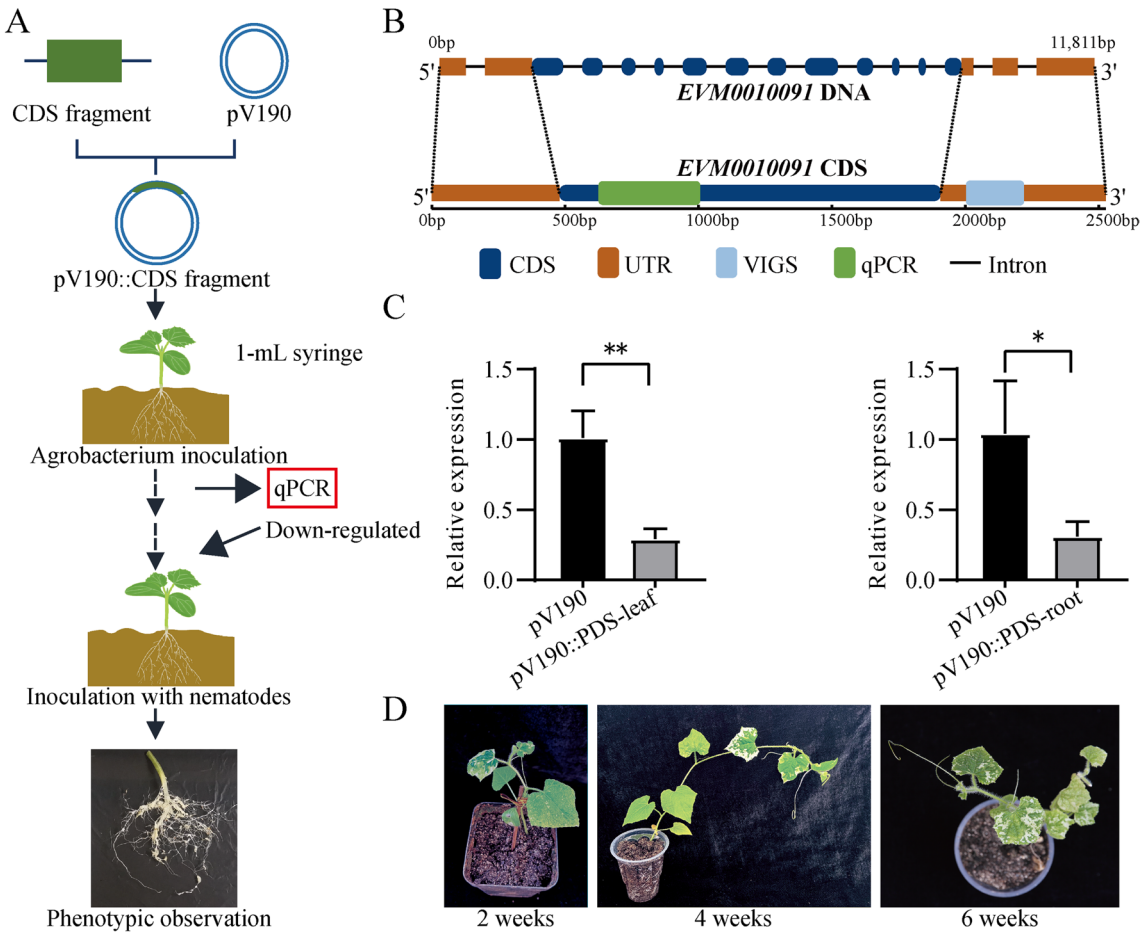

Fig. 4 An overview of the CGMMV-based VIGS protocol in *Cucumis metuliferus*. **A** Main phase of VIGS in *C. metuliferus*. **B** Positions of qPCR and silencing fragments in *CmPDS* (*EVM0010091*). **C** qPCR analysis of *CmPDS* expression in leaves and roots of silenced plants and controls at 2 weeks post inoculation. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). Relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method. Gene expressions were normalized to the beta-actin gene of *C. metuliferus*, and subsequently normalized to pV190 plants. **D** Photobleaching was observed and photographed in *C. metuliferus* 2 weeks, 4 weeks and 6 weeks after inoculation, respectively

C, E). At 40 dpi, the gall numbers and root fresh weight per root were measured, and the gall numbers per gram were calculated. The mean of gall numbers per gram on non-infected plants, pV190 plants and *CmPDS*-silenced plants were similar. As shown in Fig. 5B, D and F, the gall numbers per gram on *EVM0019904*- and *EVM0017058*-silenced plants in the three replicates were significantly increased compared with pV190 plants ($P < 0.05$). Similarly, silencing of *EVM0010549*, *EVM0005552*, or *EVM0016021* led to increased gall numbers in two of the three replicates. However, no significant differences in gall numbers were observed upon silencing of the remaining genes compared to the controls. These results suggest that specific genes, such as *EVM0019904* and *EVM0017058*, may play critical roles in nematode resistance in CM3.

Discussion

Unlike most Cucurbitaceae crops, such as cucumber and melon, *C. metuliferus* exhibits high-level resistance to *M. incognita* (Fassuliotis 1970; Nugent and Dukes 1997; Walters et al. 1993; Xie et al. 2022). Previous studies have shown that the resistance of *C. metuliferus* is mainly reflected in the arrested development of nematodes (Fassuliotis 1970; Walters et al. 2006; Ye et al. 2017). However, these investigations utilized interspecies comparisons between *C. metuliferus* and susceptible species such as cucumber or melon, potentially introducing confounding factors. This study adopts an intraspecific approach, comparing resistant (*C. metuliferus* CM3) and susceptible (*C.*

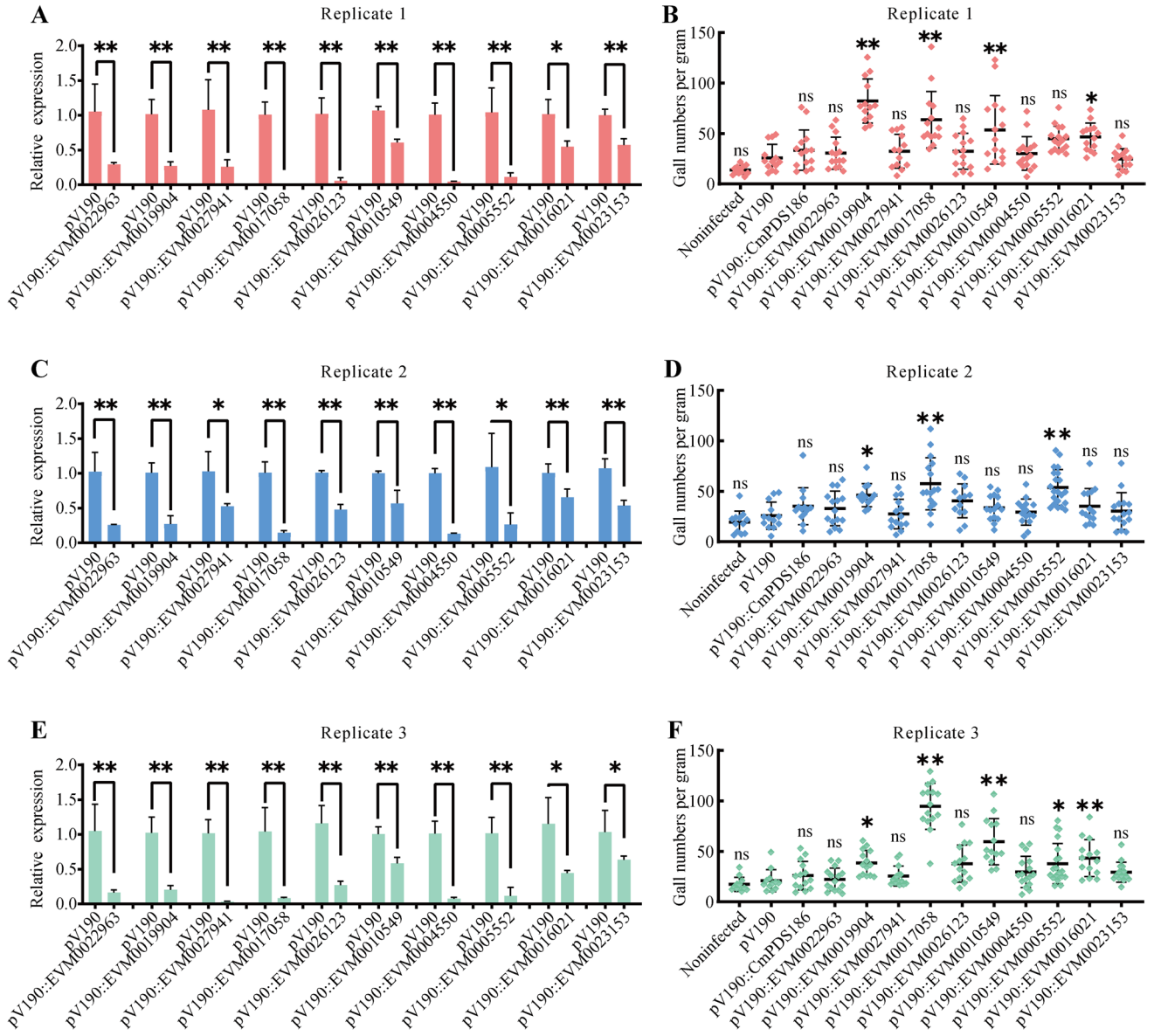

Fig. 5 Silencing of the top ten upregulated genes in CM3. **A**, **C** and **E** Relative expression levels of the top ten upregulated genes compared with pV190 plants (control). Relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method. Gene expressions were normalized to the beta-actin gene of *Cucumis metuliferus*, and subsequently normalized to pV190 plants. Data are presented as means ± SD ($n = 3/4$). *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). **D**, **E** and **F** Gall numbers per gram of silenced plants at 40 dpi. Data are presented as means ± SD. *, $P < 0.05$; **, $P < 0.01$; ns, not significant (one-way ANOVA followed by Dunnett's multiple comparisons test). # denotes the control

metuliferus CM27) inbred lines. Such a comparison minimizes errors arising from interspecific differences. Additionally, unlike previous studies that focused on pathways without identifying specific functional genes, this research employs VIGS to directly confirm the roles of candidate genes. The findings provide critical insights into the resistance mechanisms of *C. metuliferus* and identify promising resistance genes.

Here, CM3 was identified to be highly resistant to *M. incognita* compared with CM27, according to the comparison of galls and egg masses on roots. In addition, the number of nematodes in roots during the first 7 dpi did not differ significantly between the two lines, indicating that early-stage resistance was not due to reduced nematode invasion. Indeed, similar results have been identified in the comparison of *C. metuliferus* accession PI482443 and cucumber Jinlv No.3 from 2 to 4 dpi (Ye et al. 2017). After 14 dpi, a substantial difference in nematode penetration was observed between CM3 and CM27. The difference at the later stages might due to the reason that larvae in

CM3 roots prefer to develop into males and emigrate from roots to the soil. Under certain stresses, including host stress or nutrient deprivation, the stimulation toward maleness is triggered (Davide and Triantaphyllou 1967; Snyder et al. 2006). The adult males will migrate from host roots to soil for the population's survival (Snyder et al. 2006; Eisenback 1985). Similarly, this masculinization has been found in *C. metuliferus* (Ye et al. 2017; Li et al. 2021; Fassuliotis 1970). These conclusions are consistent with the above hypothesis. Nematode development in CM3 and CM27 roots was further assessed by analyzing body width and life stage at multiple post-infection time points. The nematodes in the resistant *C. metuliferus* CM3 were poorly developed compared to those in CM27, indicating that the resistance might be associated with hindrance of nematodes development. There was a significant difference in body width at 3 dpi, showing the hindrance of nematode development might be induced once infection occurs. In conclusion, the resistance in CM3 is associated with the suppression of *M. incognita* development beyond the second stage, delaying the development of J2s to J3s/J4s, compared to that in CM27.

Several attempts have been made to compare the mechanisms involved in response to *M. incognita* between *C. metuliferus* and cucumber/melon via comparative transcriptomics (Ye et al. 2017; Li et al. 2021; Ling et al. 2017). The genes associated with salicylic acid (SA) signaling, jasmonic acid (JA) signaling, Ca^{2+} signaling, cytoskeleton biosynthesis, phenylpropanoid biosynthesis, plant hormone signal transduction and plant-pathogen interaction may be involved in the resistance in *C. metuliferus*. Nevertheless, the function of these candidate genes has not been further validated. In contrast, this study uniquely utilizes two *C. metuliferus* accessions with significantly different phenotypes to conduct comparative transcriptome studies for the first time. By comparing the DEGs in the two materials, the top ten of only upregulated genes in the resistant accession CM3 were selected for further functional validation.

Among the exclusive top ten upregulated DEGs identified in CM3, three genes were annotated as HSP20 proteins which belong to the superfamily of Heat shock proteins (HSPs). The *HSP20s* can be induced by plant parasitic nematode infection (Barcala et al. 2008; Hishinuma-Silva et al. 2020; Lopes-Caitar et al. 2013). According to RNA-sequencing analysis, more *HSP20* genes were induced in the reniform nematode resistant cotton genotype (LONREN-1) compared with the susceptible cotton genotype (DP90 & SG747) (Li et al. 2015). Similarly, more *HSP20s* were induced by *M. incognita* in CM3 in this study. The promoter of sunflower *Hahsp17.7G4* is activated in giant cells of tobacco plants infected by *M. incognita* from 1 to 20 dpi, indicating its activity spans from early to middle infection stages. This suggests that the promoter activity may be more related to giant cell differentiation than to an immediate response to infection (Escobar et al. 2003). However, the three *HSP20s* were induced only at 3 dpi in this study. Moreover, we did not verify the expression of these three genes in giant cells, so, it is uncertain whether the three *HSP20s* achieve nematode resistance by affecting giant cells differentiation in *C. metuliferus*. Soybean gene *GmHsp22.4* is involved in nematode infestation responses, possibly acting as the first line of cellular defense by capturing unfolded proteins and reducing protein aggregation sizes (Hishinuma-Silva et al. 2020), but our results do not allow for similar speculation. Here, the expression of three *HSP20* genes (*EVM0017058, EVM0026123* and *EVM0010549*) was induced by *M. incognita*, and silencing of *EVM0017058* and *EVM0010549* enhanced susceptibility in three and two replicates, respectively. This suggests that *HSP20* genes, especially *EVM0017058* and *EVM0010549* may be key transcriptional regulators in responding to *M. incognita* infection in CM3. However, the mechanism of action of these genes remains unclear. In the future, the expression of *HSP20s* in giant cells could be investigated to confirm whether these genes are involved in giant cells differentiation. Furthermore, the potential role of the identified candidate *HSP20* genes in resistance could be further confirmed by analyzing transgenic overexpressed or knockout mutant plants.

In addition to the three *HSP20* genes, the functionally unannotated genes *EVM0019904* and *EVM0005552* were induced and upregulated at 3 dpi and 7 dpi, respectively. The silenced plants of the two genes exhibited an increase in number of galls in three and two replicates, respectively. Moreover, A *WRKY* DNA-binding gene (*EVM0016021*) was also one of the top ten upregulated genes in CM3. The *WRKY* transcription factors are a vital gene family that actively participates in various metabolic pathways in plants. Different *WRKYs* play different roles in response to nematode infection in plant. Transgenic soybean lines overexpressing three *WRKY* transgenes displayed increased resistance to *Heterodera glycines* (Yang et al. 2017). On the contrary, the nematode-responsive gene *WRKY45* enhances susceptibility to *M. javanica* in tomato (Chinnapandi et al. 2017). Here, the expression of *EVM0016021* was induced at 7 dpi and the gall numbers per gram on *EVM0016021*-silenced plants in two replicates were significantly increased compared with pV190 plants. Taken together, the two *HSP20* genes, the WRKY DNA-binding gene and the two genes with unknown function were likely to play an important role in CM3 defense against *M. incognita*. Overall, those results identified a number of candidate genes that could explain the resistance in *C. metuliferus* to *M. incognita*. In the future, these candidate genes could be potential resistant sources to the pest for cucumber or melon improvement through biological techniques such as transgenic technology or interspecific hybridization.

Conclusion

In this study, we conducted a phenotypic comparison between a *C. metuliferus* inbred line (CM27) and a resistant inbred line (CM3). The results revealed that CM3 demonstrates a higher resistance to *M. incognita* in comparison to CM27. Furthermore, nematodes in the roots of CM3 developed poorly. The results of comparative transcriptomic and function verification showed some potential resistance genes. These findings can enhance the understanding of resistance in *C. metuliferus* CM3. In addition, these candidate genes could be used to obtain resistance to *M. incognita* in cucumber or melon via biotechnological techniques. In summary, in contrast to previous studies that mainly focused on related metabolic pathways, the primary contribution of this study is to identify and provide potential resistance genes for *M. incognita*-resistant breeding in Cucurbitaceae crops.

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Author contributions X.X. and B.X. designed, performed experiments and analyzed the data. S.Z. and Q.Y. assisted in investigating the data. B.X., J.Z. Y.L. and Z.M. supervised this work and provided input and expertise. R.L., J.L. and X.X. analyzed the transcript data. Y.B., B.X. and R.G.F.V. were responsible for the development and guidance of the project. X.X. wrote the manuscript. Y.B., R.G.F.V. and B.X. revised the manuscript. All authors discussed the results and edited the manuscript.

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Data availability The RNA-seq data used in this study were deposited in the NCBI public database with the project accession number PRJNA1021046. All data described in the study have been provided as supplementary material.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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