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mgr-mir-228-regulated transthyretin-like protein in *Meloidogyne graminicola* suppresses ROS generation and enhances parasitism

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Abstract

The transthyretin-like protein family is highly conserved across plant parasitic nematodes, yet its role in *Meloidogyne graminicola*, a significant plant parasitic nematode in rice, remains underexplored. In this study, we identified and characterized the *MgTTL1* gene, which encodes a transthyretin-like protein with a distinctive N-terminal domain predicted to facilitate plasma membrane localization. Expression analysis revealed that *MgTTL1* is specifically localized in the esophageal gland cells and is significantly upregulated during the parasitic stages of the nematode. Using deep sequencing data, we identified *mgr-mir-228* as a miRNA that interacts with *MgTTL1* mRNA, as validated by dual-luciferase reporter assays. Notably, *mgr-mir-228* is highly expressed during the J2 stage but downregulated in the J3/J4 stages, likely to suppress *MgTTL1* expression before host root invasion, suggesting its potential as a resistance trait in managing root-knot nematodes in rice. Functional assays, including in vitro RNA interference and *mgr-mir-228* mimics treatment, demonstrated that reducing *MgTTL1* levels impacts nematode reproduction. Overexpression of MgTTL1 in planta resulted in a suppression of reactive oxygen species (ROS) production in response to pathogen-associated molecular patterns, thereby significantly increasing susceptibility to nematode and bacterial pathogens. These findings suggest that *MgTTL1*, specifically regulated by the newly identified miRNA *mgr-mir-228*, functions as a negative regulator of ROS-mediated plant immunity and plays a pivotal role in modulating plant susceptibility to nematode infections.

Keywords *Meloidogyne graminicola*, Transthyretin-like protein, microRNA, Plant immunity, Reactive oxygen species

Background

Transthyretin-like (TTL) proteins, with their unique conserved domain, emerged from the foundational work analyzing protein domains within *Caenorhabditis elegans* (Sonnhammer and Durbin 1997; McCarter et al. 2003). This initial discovery set the stage for a broader investigation that uncovered a variety of TTL homologs across a spectrum of nematodes, including those parasitizing animals and plants, through comprehensive bioinformatics analyses (Gao et al. 2003; Yatsuda et al. 2003; Bellafiore et al. 2008; Hewitson et al. 2008; Jones et al. 2009; Mitreva et al. 2011). These proteins, while structurally mirroring the transthyretin,

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exhibit divergent functions, suggesting a fascinating complexity (Li et al. 2013).

Delving into the functionalities of TTL proteins reveals their significant roles across different nematodes: In *C. elegans*, they act as crucial connectors in the immune response, mediating the recognition and removal of apoptotic cells by linking surface phosphatidylserine (Wang et al. 2010). In *Haemonchus contortus*, TTL proteins are pivotal in the regulation of larval development post-embryogenesis and safeguarding the gonad from oxidative damage (Shi et al. 2021). Furthermore, in *Meloidogyne javanica*, a TTL protein operates as an effector molecule, dampening the plant's innate defenses to facilitate nematode invasion (Lin et al. 2016). Despite these insights, the specific mechanisms through which TTL proteins enhance nematode parasitism remain an intriguing puzzle, hinting at unexplored avenues in understanding and potentially combating nematode infections.

Root-knot nematodes (RKNs) represent a formidable group within the plant-parasitic nematodes (PPNs), known for their extensive damage and significant economic losses in agriculture (Castagnone-Sereno et al. 2013; Tapia-Vazquez et al. 2022; Khan et al. 2023). Among these, *M. graminicola* stands out as a major threat to rice production, classified as a quarantine pest for its devastating impact on rice yields across major rice-growing regions (Bridge and Page 1982; Pokharel et al. 2010; Kyndt et al. 2014). The battle between plants and RKNs involves complex immune responses, akin to those against bacterial and fungal invaders, specifically pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Goverse and Smant 2014; Przybylska and Obrepalska-Steplowska 2020; Khan et al. 2023).

The battle between host plants and RKNs has prompted researchers to delve into the arsenal of effector proteins employed by *M. graminicola* to breach plant defenses. Noteworthy discoveries include MgGPP and MgMO237, which have been shown to facilitate the nematode's parasitic endeavors by inhibiting host cell death and suppressing plant immunity, respectively (Chen et al. 2017, 2018). Another protein, MgPDI2, has been found to trigger cell death in *Nicotiana benthamiana*, demonstrating a unique strategy to facilitate parasitism (Tian et al. 2020). Additionally, MgMO289, which is more active during later juvenile stages, targets essential host defense mechanisms by interfering with a copper metallochaperone (Song et al. 2021). Despite these advances, the detailed regulatory mechanisms governing the timing and localization of effector protein expression by *M. graminicola* remain largely unexplored, presenting a tantalizing area for future research endeavors.

In this study, we successfully isolated and analyzed a gene responsible for a transthyretin-like protein, which we have termed *MgTTL1*, from the nematode *M. graminicola*. Our research presents compelling evidence that MgTTL1 plays a pivotal role as an effector in the parasitic strategy of *M. graminicola*. Furthermore, we uncovered that the expression of the *MgTTL1* gene is modulated by the microRNA *mgr-mir-228*, a regulatory mechanism that significantly contributes to the nematode's ability to infect rice plants. The findings of our investigation offer fresh insights into the mechanisms of nematode parasitism and open new avenues for developing strategies to combat *M. graminicola* infections.

Results

Cloning and in silico analysis of the *M. graminicola* TTL1 gene

The *MgTTL1* DNA, measuring 2345 base pairs (GenBank accession No. PP550866), was successfully cloned from *M. graminicola* genomic DNA. This cDNA comprises a 480-base pair open reading frame (ORF), a 70-base pair 5' untranslated region (UTR), and a 1795-base pair 3' UTR that includes a polyadenylation signal (AAT TAAAAAAA). The derived MgTTL1 polypeptide, predicted to be 159 amino acids in length, has an estimated molecular weight of 18.1 kDa and a theoretical isoelectric point (pI) of 6.2. The MgTTL1 protein is anticipated to contain a transthyretin-like protein 52 (TTR-52) domain between amino acid positions 48 and 125, along with a 26-amino acid secretion signal peptide at the N-terminus, suggesting its potential for secretion into host cells by the nematode. Phylogenetic analysis grouped various TTL proteins into five clusters, with MgTTL1 clustering closely with MjTTL1 and RsTTL1, indicating a close evolutionary relationship (Additional file 1: Figure S1). Sequence alignment analysis revealed that MgTTL1 shares 74.5% and 69.7% sequence identity with MjTTL1 and RsTTL1, respectively.

Expression and upregulation of *MgTTL1* in the esophageal gland during parasitic stages of *M. graminicola*

The tissue localization of *MgTTL1* transcripts in *M. graminicola* was examined through in situ hybridization. A strong accumulation of transcripts was detected in the esophageal gland cells of pre-parasitic second-stage juveniles (J2s, Fig. 1a right). Probing with sense single-stranded DNA (ssDNA) served as a negative control and produced no signal (Fig. 1a left).

The expression levels of *MgTTL1* were measured across five different developmental stages of *M. graminicola* using quantitative RT-PCR (qRT-PCR) analysis with gene-specific primers. The stage-specific analyses revealed that the transcript levels were

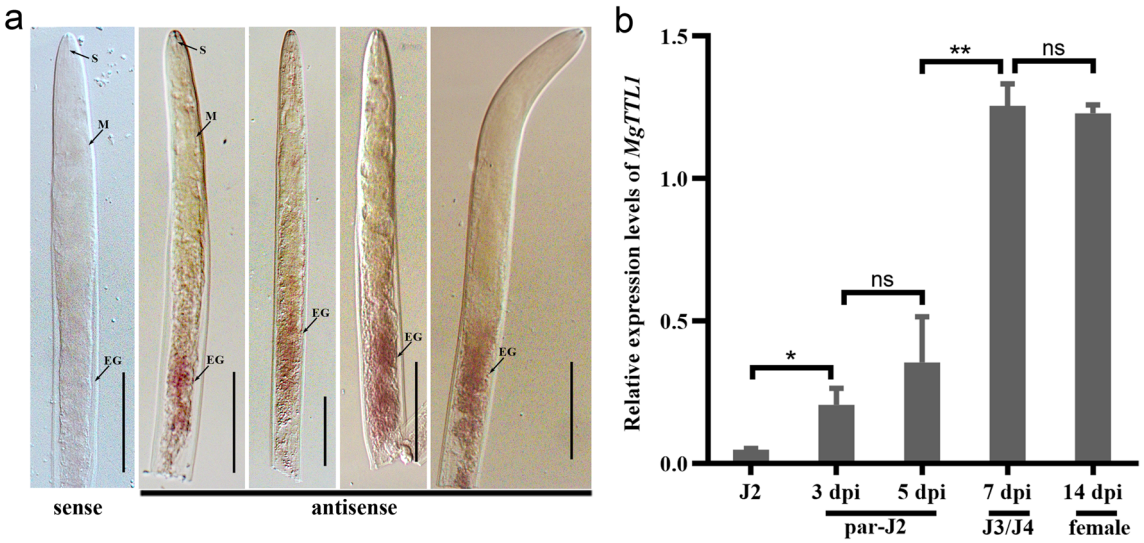

Fig. 1 In situ hybridization and analysis of the developmental expression pattern of *MgTTL1*. **a** Localization of *MgTTL1* expression in the esophageal gland of pre-parasitic second-stage juveniles of *M. graminicola* using in situ hybridization. Fixed nematodes were hybridized with sense (left) and antisense (right) DNA probes targeting *MgTTL1*. Scale bars represent 50 µm. EG, esophageal gland cells; M, metacorpus; S, stylet. **b** The relative expression levels of *MgTTL1* were detected using qRT-PCR in five different developmental stages of *M. graminicola* (J2, par-J2, par-J3/4, and female). Data shown are the means of three repeats plus standard deviation (SD) (n = 3). Samples were compared with the Tukey-test (*$P < 0.05$; **$P < 0.01$). par-J2, par-J3 and par-J4: parasitic second-, third- and fourth-stage juveniles

significantly lower in pre-parasitic second-stage juveniles (J2s) compared to other stages (Fig. 1b). The transcript levels of *MgTTL1* were four times higher at 3 days post-infection compared to J2s (Fig. 1b). This expression pattern of *MgTTL1* suggests that it may have a role throughout the infection process.

Prediction and functional localization of the unique N-terminal domain of MgTTL1 in the plasma membrane

The structure of the MgTTL1 protein was predicted using AlphaFold3 (https://golgi.sandbox.google.com/), which revealed a distinct N-terminal alpha-helix (Additional file 1: Figure S2a). TMHMM 2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) further indicated that this N-terminal region contains a transmembrane domain, with residues 7–24 forming the transmembrane helix (Additional file 1: Figure S2b). SignalP 6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/) also predicted a high probability of this N-terminal region functioning as a signal peptide, similar to those found in conventional nematode-secreted proteins (Additional file 1: Figure S2c). When GFP-tagged MgTTL1 was transiently expressed in *N. benthamiana* alongside the membrane marker PM-RK, it localized to the plasma membrane, displaying punctate structures, in contrast to the cytoplasmic localization observed with the GFP control (Fig. 2).

Impact of *MgTTL1* on the parasitic dynamics of *M. graminicola*

We conducted in vitro RNA interference (RNAi) experiments on *MgTTL1* to investigate its role in the parasitic process of *M. graminicola*. The qRT-PCR analysis showed that *MgTTL1* expression was reduced by approximately 50% following treatment with ds*MgTTL1* compared to the control (Fig. 3a). Following this, rice roots were exposed to J2s that had been soaked in double-stranded RNA (dsRNA) targeting *MgTTL1* or GFP, and the nematode multiplication factor (MF) at 15 days post-infection (dpi) was assessed. There was a significant decrease in the MF in rice infected with nematodes treated with *MgTTL1* dsRNA (MF = 4.46) as opposed to those treated with GFP dsRNA control (MF = 9.01) (Fig. 3b). These results indicate that *MgTTL1* plays a crucial role in the parasitic infection of *M. graminicola*.

Interaction between *mgr-mir-228* and *MgTTL1* mRNA in *M. graminicola*

Previously, we demonstrated that miRNAs play a role in regulating *M. graminicola* infection in rice by targeting specific critical effector genes of the nematode. For instance, *mgr-mir-9* was pinpointed as a miRNA targeting the effector gene *MgPDI*, which leads to a reduction in *MgPDI* expression and decreased reproductive capability in nematodes treated with *mgr-mir-9* mimics. We also discovered that *mgr-mir-228* targets several

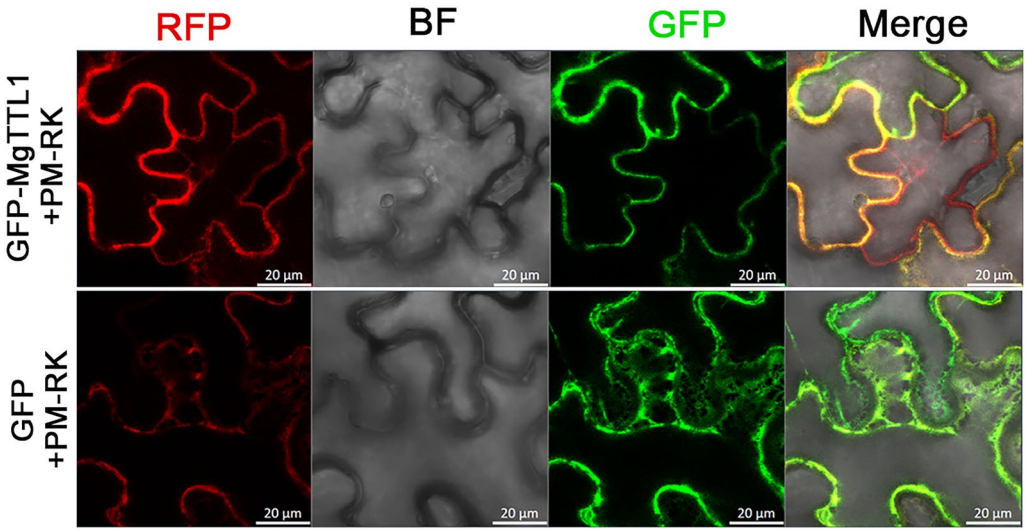

Fig. 2 Subcellular localization of MgTTL1 in planta**.** The subcellular localization of GFP-tagged MgTTL1, co-infiltrated with the membrane marker PM-RK in *N. benthamiana*, revealed its presence at the plasma membrane, in contrast to the predominantly cytoplasmic localization observed with GFP alone. Scale bars represent 20 μm

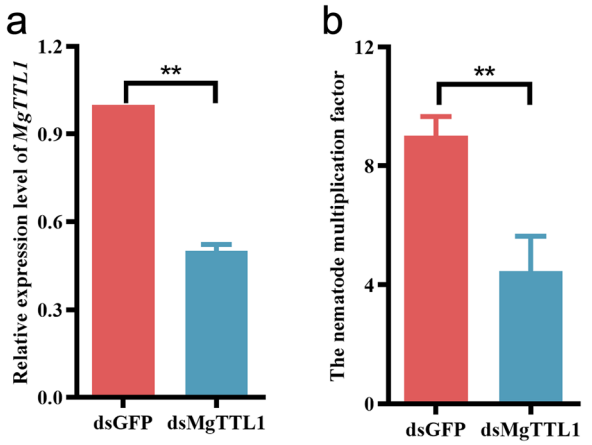

Fig. 3 Impact of MgTTL1 on the parasitic dynamics of *M. graminicola*. **a** The relative expression level of *MgTTL1* post RNAi. **b** Average MF values at 15 dpi post infection with differentially treated *M. graminicola*. Asterisks indicate significant differences based on Tukey's test (**$P < 0.01$)

intriguing genes. Building on this, we utilized three miRNA target prediction algorithms—RNAhybrid, TargetScan, and miRanda—to identify potential miRNA regulators within libraries reported in a previous study (Tian et al. 2023). The analysis using Targetscan and miRanda indicated that *mgr-mir-228* could target the *MgTTL1* mRNA (Fig. 4a). Further examination revealed that the *mgr-mir-228* Uni-miRNA (AAUGGC ACUAGAUGAAUUCACGG) was the most prevalent (Table 1), based on miRNA sequence reads from three *M. graminicola* J2 libraries (Tian et al. 2023).

Additionally, a dual-luciferase reporter assay was conducted in vitro to confirm the interaction between *mgr-mir-228* and *MgTTL1* mRNA. The assay results demonstrated that co-transfection with the luciferase reporter gene vector containing the wild-type *MgTTL1* 3′ UTR and *mgr-mir-228* resulted in reduced *MgTTL1* expression levels compared to the negative controls (Fig. 4b). Expression levels of *mgr-mir-228* were also measured at the J2 stage and par-J3/J4 stage (5dpi), revealing a significant decrease at the par-J3/J4 stage (5 dpi) (Fig. 4c). These findings underscore the regulatory role of *mgr-mir-228* in targeting *MgTTL1* during *M. graminicola* early infection stage.

Influence of *mgr-mir-228* on *MgTTL1* expression and nematode reproductivity in *M. graminicola*

To assess the in vivo effects of *mgr-mir-228* on its target gene *MgTTL1*, newly hatched J2 stage nematodes were treated with either *mgr-mir-228* mimics or negative control mimics for 24 h. Subsequently, qRT-PCR was used to measure the accumulation of *mgr-mir-228* in the nematodes. The results showed a marked increase in *mgr-mir-228* levels in the treated J2s compared to the controls (Fig. 5a). As expected, *mgr-mir-228* mimics treatment led to a decrease in *MgTTL1* expression (Fig. 5b). Additionally, the reproductive capacity of the nematodes was significantly reduced following treatment with *mgr-mir-228* mimics (MF = 7.23) compared to those treated with the negative control (MF = 10.82) (Fig. 5c). These findings

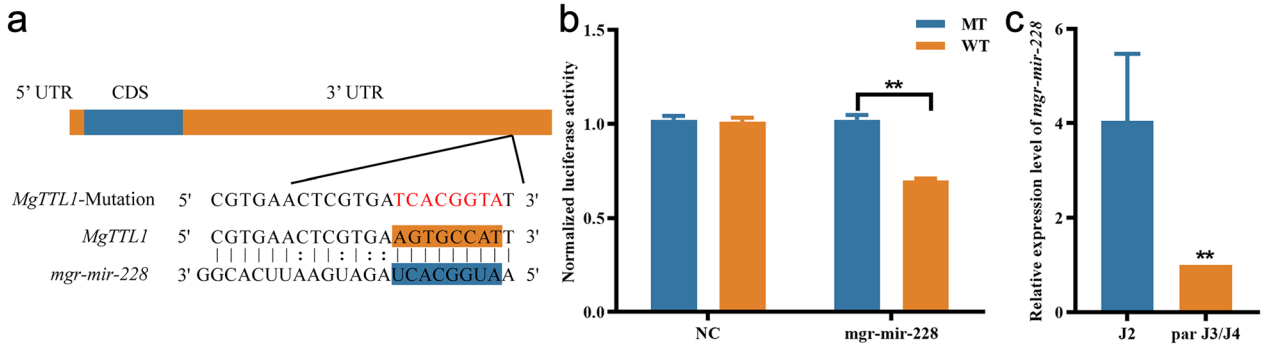

Fig. 4 The role of *mgr-mir-228* in regulating *MgTTL1* expression and nematode infection processes. **a** Pairing schemes of *mgr-mir-228* and *MgTTL1* mRNA interactions are illustrated. The matching position of *MgTTL1* mRNA starts at 3′ UTR. **b** *MgTTL1* is the direct target of *mgr-mir-228*. *pMIR-REPOR-MgTTL1* luciferase constructs containing a wild type (WT) or mutant type (MT) target sequence were transfected into HEK293 cells. Results showed the mean values of four independent experiments ± SD (n = 4). **c** The expression level of *mgr-mir-228* at the J2 stage and pra-J3/J4 stage (5 dpi). Data shown are means ± SD (n = 3). All data were analyzed by ANOVA (one-way) with a Tukey's test (***P* < 0.01)

Table 1 The relative abundance of different Uni-miRNA of *mgr-mir-228* family

Sequence (5′–3′)	The relative abundance (%)		
	J2a	J2b	J2c
AAUGGCACUAGAUGAAUUCACGG	53.85	52.53	49.44
AAUGGCACUAGAUGAAUUCACG	34.68	38.02	39.33
AAUGGCACUAGAUGAAUUCACGGU	6.47	4.84	6.37
AAUGGCACUAGAUGAAUUCAC	4.15	4.15	4.12
AAUGGCACUAGAUGAAUUCACGGUU	0.61	–	–
AAUGGCACUAGAUGAAUUCA	0.24	–	0.75
AAUGGCACUAGAUGAAUU	–	0.46	–

collectively indicate a significant role for *mgr-mir-228* in vivo.

Regulation of reactive oxygen species responses by TTL1 in planta

To further elucidate the role of TTL1 in modulating susceptibility through reactive oxygen species (ROS) responses, we conducted comprehensive ROS assays. We specifically investigated how TTL1 modulates ROS production in response to the pathogen-associated molecular pattern flg22. Using a luminol-based chemiluminescence assay, we measured ROS production in leaf discs of *Agrobacterium*-infiltrated *N. benthamiana*. The

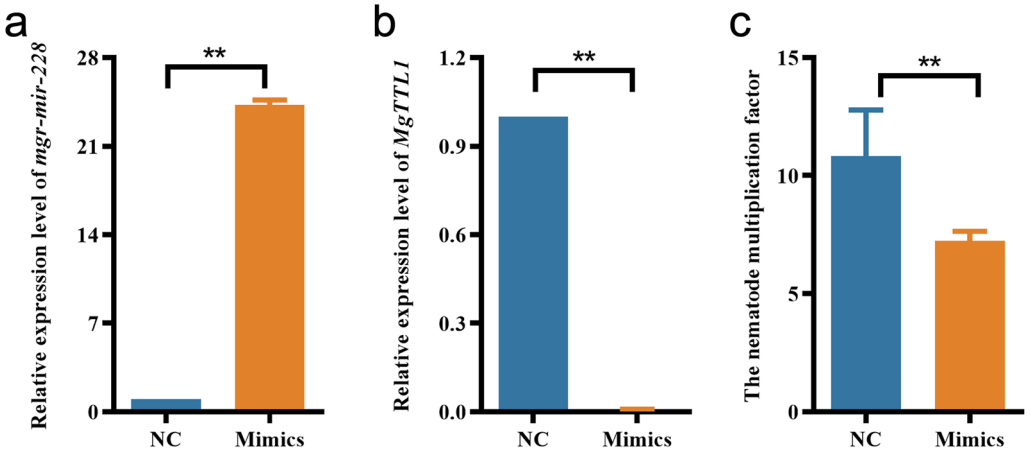

Fig. 5 The impact of *mgr-mir-228* on nematode infection processes. **a** The relative accumulation levels of *mgr-mir-228* at 24 h post treatment. **b** Expression analysis of *MgTTL1* at 24 h post treatment. **c** The multiplication factor values at 15 dpi. Mimics: *mgr-mir-228* mimics, NC: negative control mimic. Data shown are means ± SD. Statistical analysis was performed using ANOVA (one-way) with a Tukey's test. Significant differences are indicated by asterisks (***P* < 0.01)

results were quantified as accumulated relative luminescence units (RLU). Our findings revealed that, upon flg22 stimulation, the control group exhibited a rapid spike in ROS levels at 5 min post-treatment, with a sustained increase up to 10 min. In contrast, the group treated to express TTL1 showed only a marginal increase in ROS levels, despite the presence of flg22 (Fig. 6a). This suggests that TTL1 might play a regulatory role in attenuating the ROS burst typically triggered during the plant immune response.

To determine whether TTL1 inhibits PTI ROS and may serve as a fundamental suppressor of plant basal immunity, we overexpressed MgTTL1 in various plant-pathogen interaction systems. Since MgTTL1 shares 67% sequence identity with TTL1 from *Heterodera glycines* (GenBank accession No. AAF76925), which is a putative secretory protein from the esophageal gland cells (Wang et al. 2001), we speculate that TTL1 may play a similar role in the ROS response of plant hosts to parasitic nematode infection. Then we transiently overexpressed MgTTL1 in soybean roots and assessed the development of soybean cyst nematode (*Heterodera glycines*, SCN) past the J2 stage. The results indicated that, compared to the empty vector control, roots overexpressing MgTTL1 exhibited increased susceptibility to SCN infection (Fig. 6b). Second, we generated stable transgenic *Arabidopsis thaliana* lines overexpressing MgTTL1. Two positively transformed lines were selected for further analysis. We conducted direct luminescence measurements of *Arabidopsis* leaf tissues inoculated with bioluminescent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*-lux) and performed bacterial titer assays. Both MgTTL1 overexpression lines showed enhanced bacterial growth in direct luminescence

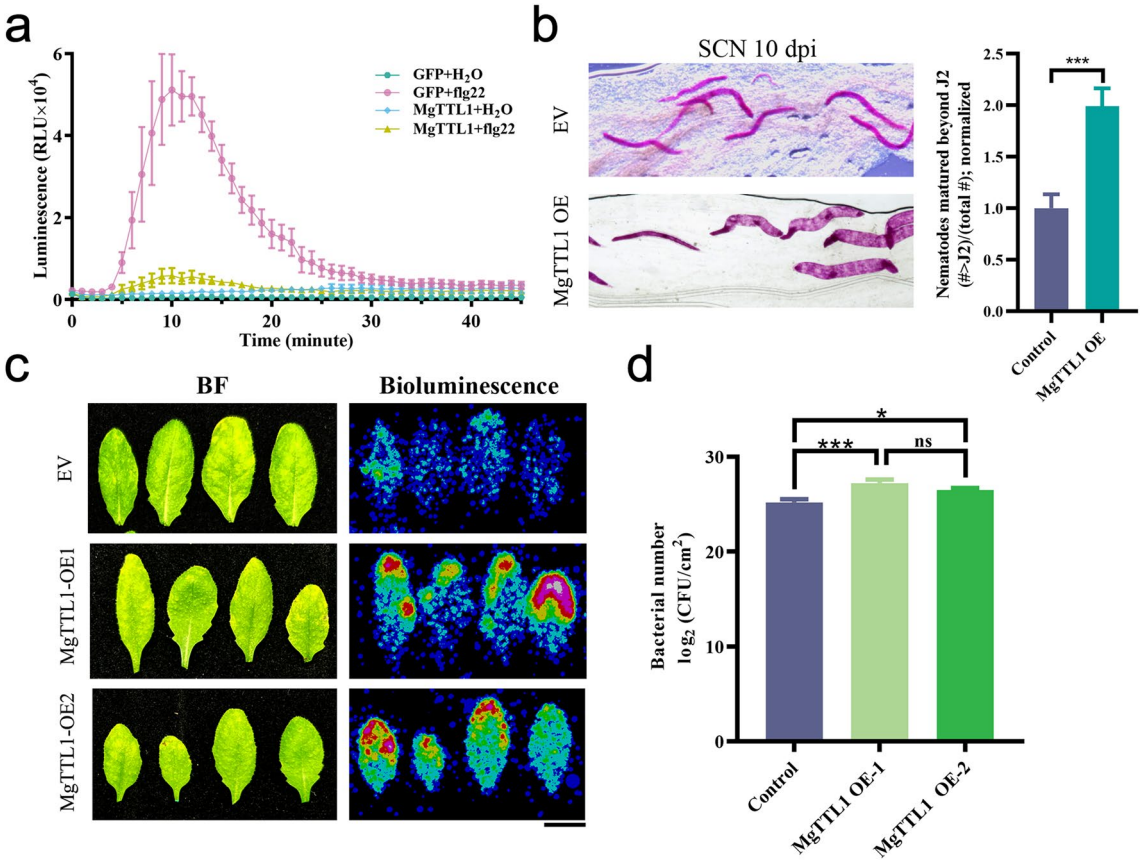

Fig. 6 MgTTL1 as a negative regulator of ROS-mediated plant immunity. **a** Detection of ROS generation following flg22 stimulation. Leaf discs were cut from *N. benthamiana* plants three days after infiltration with *A. tumefaciens* overexpressing MgTTL1 or GFP (control). **b** Assessment of soybean cyst nematode (SCN) development past the J2 stage at 10 dpi in soybean roots transiently overexpressing MgTTL1 or empty vector (control). Nematode matured beyond J2 (# > J2)/(total #): the proportion of J3 and J4 in the total nematodes. Scale bars represent 100 µm. **c** Representative images and luminescence readings of Arabidopsis leaves of transgenic lines overexpressing MgTTL1 (MgTTL1-OE1 and MgTTL1-OE2) compared to the empty vector control (EV). Bar = 1 cm. **d** Bacterial counts for Arabidopsis transgenic lines overexpressing MgTTL1 or empty vector controls. Statistical analysis was performed using ANOVA (one-way) with a Tukey's test. Significant differences are indicated by asterisks (*$P < 0.05$; ***$P < 0.001$)

measurements (Fig. 6c) and bacterial titer assays (Fig. 6d).

To further investigate the role of MgTTL1, we tested its overexpression (OE) in soybean transgenic roots and challenged with *M. graminicola*. Soybean roots were inoculated with approximately 200 M. *graminicola* J2s, and infection was analyzed 14 days post-inoculation (dpi). In the mock control roots, fewer than 10 J2s successfully infected the roots, demonstrating very high host resistance to *M. graminicola* in soybean, with most nematodes remaining at the J2 stage. In contrast, MgTTL1-OE roots showed significantly higher numbers of J2s infecting the roots, although most nematodes stillremained at the J2 stage (Additional file 1: Figure S3). This indicates that MgTTL1 overexpression compromises soybean resistance at early stages by increasing nematode penetration but does not affect the overall resistance to nematode development, as most nematodes fail to progress beyond the J2 stage.

Collectively, these findings demonstrate that TTL1 acts as a negative regulator of ROS responses, potentially increasing susceptibility to pathogen attacks by modulating the plant's innate immunity against nematode, bacterial, and fungal pathogens.

Discussion

M. graminicola, a sedentary endoparasite obligatory to rice, inflicts significant damage in rice-producing regions (Pokharel et al. 2010; Kyndt et al. 2014). This nematode completes its life cycle within the rice roots, necessitating a continuous suppression of host immunity. Plants have developed two principal resistance mechanisms, PTI and ETI, to defend themselves against pathogen attacks (Goverse and Smant 2014; Przybylska and Obrepalska-Steplowska 2020; Khan et al. 2023). Nevertheless, PPNs have evolved a specialized strategy to undermine host immunity by directly injecting effectors into host cells through their stylets (Goverse and Smant 2014). An increasing number of effector genes from *M. graminicola* have been identified. In this context, we have identified an effector gene, *MgTTL1*, which plays a role in suppressing host PTI immunity. In situ hybridization revealed that MgTTL1 is expressed in the esophageal gland cells of *M. graminicola* pre-parasitic J2s, suggesting its role as a nematode secretory effector protein. The predicted and functional localization of MgTTL1 collectively suggest that its distinctive N-terminal domain is essential for membrane association, likely facilitating its role in cellular signaling or interactions at the plasma membrane.

Building on our previous research, we have also demonstrated that miRNAs contribute to the regulation of *M. graminicola* infection in rice by specifically targeting crucial effector genes of the nematode. Notably, *mgr-mir-9* targets the effector gene *MgPDI*, leading to a decrease in *MgPDI* expression and reduced reproductive capability in nematodes treated with *mgr-mir-9* mimics. Furthermore, in this study we first reported that *mgr-mir-228* targets *MgTTL1*, a gene that encodes a critical structural domain characteristic of transthyretin-like family proteins specifically expressed in the esophageal gland cells of *M. graminicola* at the J2 stage (Fig. 1a). As the nematode develops, the expression level of *mgr-mir-228* decreases (Fig. 4b), while its target *MgTTL1* is upregulated during the J3/J4 and later female stages (Fig. 1b). Both the application of dsRNA to induce RNAi of *MgTTL1* and the application of *mgr-mir-228* mimics significantly reduced the MF of *M. graminicola* (Figs. 3b and 5c). These results suggest that MgTTL1 plays a crucial role in modulating the susceptibility of *M. graminicola* to host defenses. These findings highlight the potential of targeting *MgTTL1* through miRNA-based or RNAi approaches as a promising strategy for controlling *M. graminicola* infections in rice.

Transthyretin-like proteins were first identified in *C. elegans* (Sonnhammer and Durbin 1997). While TTL homologs have been identified in numerous nematode species, (Gao et al. 2003; Furlanetto et al. 2005; Jacob et al. 2007; Bellafiore et al. 2008; Jones et al. 2009; Lin et al. 2016). This study represents the first report of a *TTL* gene in *M. graminicola.* Previously, five distinct TTL proteins were recognized within *M. javanica* (Lin et al. 2016). Phylogenetic analysis in this study classifies the TTL from our research as TTL1, with MgTTL1 demonstrating high similarity to the MjTTL1 and RsTTL1 proteins. According to BLAST searches, MgTTL1 homologs appear to be exclusive to nematodes. However, the differential expression of *TTL1* genes in different nematode species highlights the potential diversity in their functional roles. In *Radopholus similis*, *RsTTL1* is expressed in tissues surrounding the vulva, suggesting a possible involvement in reproductive processes or local cellular functions (Jacob et al. 2007). Although MgTTL1 shares 61.9% sequence identity with RsTTL1, the *MgTTL1* in *M. graminicola* is localized to the esophageal gland cells, pointing towards a role in parasitism or gland-specific activities. The difference in localization between RsTTL1 and MgTTL1 is understandable, given the low sequence identity (18.5%) between their signal peptides—MgTTL1 (MINIFNFNYLLFSLIFIFNYLILINSN) and RsTTL1 (MAPMFLPTVSVFLLLLVVVQQSLLVLA). Additionally, the promoter sequences of *RsTTL1* and *MgTTL1* differ significantly (Additional file 1: Figure S4). The 2 kb regions encompassing the promoter and 5'UTR of *TTL1* genes from *R. similis* (PRJNA541590, GCA_013357305.1) and *M. graminicola* (PRJNA411966, GCA_002778205.2) exhibit only 49.5% sequence similarity, reflecting

significant divergence despite their shared classification as plant-parasitic nematodes (Additional file 1: Figure S4). Signal peptides are critical for directing subcellular localization and are subject to evolutionary adaptations that enable proteins to perform distinct physiological roles. However, tissue localization depends on broader factors, such as gene expression patterns, regulatory elements, and interactions with tissue-specific receptors. These factors may explain why certain members of large protein families exhibit distinct localization patterns.

Additionally, the HgTTL homolog MgTTL1 from another plant-parasitic nematode shares 67% sequence identity with TTL1 from *Heterodera glycines* (GenBank accession No. AAF76925), a putative secretory protein also located in the esophageal gland cells (Wang et al. 2001). This suggests that certain TTL proteins may function as effectors localized in the esophageal gland cells. qRT-PCR analysis reveals that *MgTTL1* transcription levels increase as the parasitic phase progresses, reaching a peak at the J3/J4 stages. This pattern aligns with the expression behaviors of other known *M. graminicola* effectors like MgMO237 and MgMO289 (Chen et al. 2018; Song et al. 2021), suggesting that MgTTL1 might contribute consistently throughout nematode infection. Additionally, our experiments demonstrated that rice infected with nematodes treated with MgTTL1 dsRNA exhibited a marked reduction in multiplication factor compared to GFP dsRNA-treated controls, underscoring the essential role of MgTTL1 in *M. graminicola* parasitism. These variations in tissue localization underscore the evolutionary adaptations of *TTL* genes to the distinct physiological needs of each species, offering valuable insights into their functional divergence.

TTL proteins from *M. javanica* (MjTTL5) have been previously shown to suppress plant ROS bursts, similar to how the effector MgMO237 enhance nematode parasitism by inhibiting these bursts (Lin et al. 2016; Chen et al. 2018). Notably, MjTTL5 has been found to interact with AtFTRc, significantly boosting the plant's ROS-scavenging activity (Lin et al. 2016). In this study, we report for the first time that MgTTL1 also contributes to *M. graminicola* parasitism by suppressing plant ROS burst activity. Our results indicate that the RLU of the control group, triggered by flg22, increased sharply, whereas the RLU of the group treated with MgTTL1 showed only a slight increase despite flg22 also being used as the elicitor.

The regulatory mechanisms of nematode effector gene expression, particularly through miRNA regulation pathways, remain largely unexplored. miRNAs are known to function as endogenous gene regulators and are pivotal in various biological processes including stress response, reproduction, development, and disease pathogenesis (Carrington and Ambros 2003; Ambros 2004; Bartel 2004; Chen 2005; Zhang et al. 2007; Zhang and Wang 2015; Gebert and MacRae 2019). Recent research on *M. incognita* has suggested that miRNAs could regulate specific target gene levels during nematode infection (Wang et al. 2015; Subramanian et al. 2016; Zhang et al. 2016). It has also been reported that certain miRNAs, including *mgr-mir-9*, may regulate the expression of secreted effectors like MgPDI to enhance *M. graminicola* infection (Tian et al. 2023). In this study, we hypothesize that *MgTTL1* expression might be tightly regulated by specific miRNAs. *mgr-mir-228*, in particular, was predicted to be a potential regulator of *MgTTL1* mRNA by miRNA target prediction algorithms (TargetScan and miRanda) used on miRNA libraries from a previous study (Tian et al. 2023). The interaction between *mgr-mir-228* and *MgTTL1* was confirmed using an in vitro dual-luciferase reporter assay. Notably, the expression patterns of *MgTTL1* and *mgr-mir-228* were inversely correlated (Tian et al. 2023), strongly suggesting that *mgr-mir-228* acts as a transcriptional regulator of *MgTTL1*. Furthermore, when *M. graminicola* J2s were treated with exogenous *mgr-mir-228*, a significant reduction in *MgTTL1* expression was observed. Specifically, this treatment resulted in a substantial decrease in the reproductive capacity of the nematodes, with a MF of 7.23, compared to the control treatment with an MF of 10.82. These findings collectively indicate that *mgr-mir-228* plays a critical role in the infection process of *M. graminicola* by modulating its effector target, MgTTL1. It is particularly intriguing that nematodes could suppress their own effector genes in the J2 stage and, post-infection, downregulate miRNAs to increase the expression of effectors, thereby facilitating the infection process. Further research is needed to understand how miRNAs are downregulated or the deeper mechanisms through which miRNA regulation affects infection-related effectors.

Conclusions

In conclusion, this study has identified and characterized the *MgTTL1* gene, which encodes a transthyretin-like protein that localizes to the plasma membrane and is upregulated during *M. graminicola* parasitism in rice. Furthermore, we discovered that the nematode miRNA, *mgr-mir-228*, regulates *MgTTL1* expression, with its expression being stage-specific. Our findings reveal that *mgr-mir-228* miRNA controls *MgTTL1*, which suppresses ROS-mediated plant immunity. This provides insights into nematode-triggered PTI suppression and highlights the potential of nematode miRNAs as a resource for developing resistance in crop protection.

Methods

M. graminicola and plant materials

The *M. graminicola* strain ZJJH was cultivated on rice plants (*Oryza sativa* cultivar 'Nipponbare') in a glasshouse, using cultivation methods (Huang et al. 2016). Rice cultivation followed the protocol specified by Tian et al. (2023). Seeds were placed on damp filter paper and kept at a temperature of 28°C for 8 days to stimulate germination. After germination, the seedlings were relocated to potting soil and grown in a controlled environment chamber, which was maintained at temperatures of 28°C during the day and 26°C at night, with 75% humidity, and subjected to a photoperiod consisting of 16 h of light followed by 8 h of darkness.

Gene amplification and characterization

Total RNA from *M. graminicola* was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), adhering strictly to the instructions provided by the manufacturer. The sequence of *MgTTL1* was elucidated using the Rapid Amplification of cDNA Ends (RACE) technique, employing the SMART RACE cDNA Amplification kit (Clontech, Madison, WI, USA) as per the manufacturer's recommendations. The identification of *MgTTL1* was accomplished through the analysis of transcriptome data of *M. graminicola* produced in our lab. The primer sequences utilized in this research are detailed in Additional file 2: Table S1. DNA fragments amplified via PCR were inserted into the pGEM-T Easy Vector (Promega, Madison, WI, USA) for cloning and subsequent sequencing.

Analysis of the *MgTTL1* sequence and its inferred amino acid composition was conducted using DNASTAR software (Version 5.02; DNASTAR Inc., Madison, USA). To assess the sequence similarity of the predicted protein, searches were carried out using BLASTx, BLASTn, and tBLASTn against the databases at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Sequence alignment and phylogenetic tree construction were performed using ClustalW and MEGA 6.0 software. The amino acid sequences of transthyretin-like (TTL) homologs included in the phylogenetic analysis are enumerated in Additional file 2: Table S2. Prediction of conserved domains was facilitated by the NCBI's Conserved Domains database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The potential signal peptide of MgTTL1 was predicted with the SIGNALP 6.0 server (http://www.cbs.dtu.dk/services/SignalP/).

Arabidopsis transformation

CDS sequence of *TTL1* was amplified by KOD One (Toyobo No.KMM-101) PCR, and each ORF was assembled with the double CaMV 35S promoter, TMV omega enhancer (pICH51288), nopaline synthase (NOS) terminator, and a spectinomycin selection gene into the binary vector pAGM4673 (MoClo Tool Kit) using the Golden Gate cloning method (Weber et al. 2011). The inserts were sequenced to confirm correct incorporation. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, and transgenic *Arabidopsis* plants were generated using the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998).

Generation of transgenic soybean roots

Using established methods, transgenic roots were generated with the *A. rhizogenes* strain ARqua1 (Han et al. 2023). Cotyledons from Williams 82 soybeans were transformed with either an RFP empty vector or vectors designed for overexpression, resulting in root development according to the procedures outlined by Han et al. (2023).

SCN assay inoculum

SCN eggs from the Hg 0 populations were sourced from Congli Wang at the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences. These eggs were placed in a hatching buffer containing 3 mM $ZnCl_2$ and incubated for five days at ambient temperature. Infective J2 stage SCNs were then collected and disinfected on the surface using a sterilization buffer composed of 0.1 g/L $HgCl_2$ and 0.01% sodium azide for three minutes. Following two rinses with water, the J2 stage SCNs were suspended in 0.05% sterile agarose water, preparing them for root inoculation.

The nematode demographic assay was detailed in a previous study (Cook et al. 2012). Nematode infection and development within the root systems were monitored by clearing and staining with acid fuchsin approximately 14 days post-inoculation. Results were expressed as the percentage of nematodes that progressed beyond the J2 stage, calculated as ([J3 + adult males + adult females] / [J2 + J3 + adult males + adult females]). Each data point was normalized to the mean from Williams 82 roots transformed with the empty vector in the same experiment. All reported data are derived from at least two independent biological replicates, with more than 12 independently transformed roots represented for each bar in the bar graph.

Pseudomonas assay

P. syringae pv. *tomato* (*Pto*) strain DC3000, expressing the LuxCDABE operon, was used as previously described (Anderson et al. 2011). Three days before infection, a glycerol stock of *Pto*-Lux was streaked on King's B medium (KBM) agar plates with kanamycin (50 μg/mL)

and rifampicin (60 μg/mL) and incubated at 30°C for 2 days, then at room temperature for 1 day. Before infection, *Pto* was scraped from the agar plate, resuspended in sterile water to an OD_{600} of 0.02. *Arabidopsis* seedlings are used for experiments after about 20 days of growth. Six plants were inoculated per group. Disease symptoms were observed after 3 days and the bioluminescence was measured using a single-photon imaging system. For *Pto* growth measurements, seedlings were homogenized in 500 μl 10 mM $MgCl_2$, serially diluted, and spotted on antibiotic-containing KBM agar plates. Colonies were counted after 2 days of incubation at 30°C.

Developmental expression analysis

RNA specimens were collected from various developmental phases of *M. graminicola* (par-J2: 3–5 dpi; par-J3/J4: 7 dpi; female: 14 dpi), utilizing the TRIzol extraction procedure (Invitrogen, Carlsbad, CA, USA). The conversion of RNA to cDNA was facilitated by the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). To specifically target and amplify *MgTTL1* transcripts, qRT-PCR was employed using the primer pair MgTTL1-Q-F/MgTTL1-Q-R, as listed in Additional file 2: Table S1. For normalization purposes, the *actin* gene of *M. graminicola* was amplified using the primer pair Mg-ACT-Q-F/Mg-ACT-Q-R, serving as the reference gene (Haegeman et al. 2013; Petitot et al. 2016). These qRT-PCR assays were performed on a CFX Connect real-time PCR system (BIO-RAD, Hercules, CA, USA) with the SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Tokyo, Japan), under the following thermal cycling conditions: an initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. The relative expression levels of *MgTTL1* transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

RNAi and infection assay

The process of silencing *MgTTL1* via RNAi was carried out in line with the methods described (Tian et al. 2020). To achieve this, dsRNA specifically targeting *MgTTL1* and a control (GFP) were synthesized and subsequently purified utilizing the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). Approximately 20,000 freshly hatched second-stage juveniles (J2s) of *M. graminicola* were submerged in the dsRNA concoction for 36 h at ambient temperature in a dark environment, with a gentle rotation. Post-treatment, half of these J2s were subjected to qRT-PCR analyses to assess the RNA interference efficacy, while the other half were designated for subsequent plant infection studies.

For the infection trials, a Pluronic F-127 (PF-127) gel (Sigma-Aldrich, St. Louis, MO, USA) was employed, adhering to methodologies previously established (Wang et al. 2009; Dutta et al. 2011; Tian et al. 2017). To examine nematode development, two-week-old rice seedlings were each inoculated with 80 treated J2s, and rice roots were subsequently stained with acid fuchsin at 15 dpi (Tian et al. 2019). The resulting galls were carefully dissected to calculate the nematode multiplication factor (MF), using the formula: MF = (total number of egg masses × average number of eggs per egg mass) divided by the initial inoculation count. Representative staining images used for MF calculation are shown in Additional file 1: Figure S5. For soybean root inoculation, approximately 200 J2s of soybean cyst nematode were inoculated into transgenic soybean root by pipetting a suspension near the root zone. Fourteen days post-inoculation, roots were washed and stained with acid fuchsin, then observed under a stereomicroscope to assess the development of soybean cyst nematode past the J2 stage.

Agrobacterium-mediated transient expression

The technique of *Agrobacterium*-mediated transient expression was employed to investigate the suppressive role of MgTTL1 on the ROS burst and to determine its subcellular localization within the leaves of *N. benthamiana*, in accordance with the methods established by (Tian et al. 2020). In summary, the coding sequence of *MgTTL1* was integrated into the pGD-eGFP vector, and this resulting MgTTL1::pGD-eGFP fusion construct was introduced into the *A. tumefaciens* strain EHA 105. Leaves of six-week-old *N. benthamiana* plants were infiltrated with this *A. tumefaciens* suspension to enable the transient expression of the MgTTL1::pGD-eGFP. Following infiltration, the plants were placed in a growth chamber set to a 16-h light/8-h dark photoperiod at 25°C for optimal growth conditions. The PM-RK plasmids were prepared as described (Nelson et al. 2007).

Detection of ROS using a luminol-based chemiluminescence assay

To assess ROS generation, a luminol-based chemiluminescence assay was conducted, incorporating modifications from an established method (Liang et al. 2013). Leaf discs (0.25 cm^2) were cut from *N. benthamiana* plants three days after infiltration with *A. tumefaciens* and were then incubated overnight in water within a 96-well plate. Following this, a mixture consisting of 300 μM luminol (Merck KGaA), 20 μg/mL horseradish peroxidase (HRP), and either 100 nM flg22 (GenScript, Nanjing, China) or a control solution (water) was applied to the leaf discs. The chemiluminescence emitted was recorded for a duration of 45 min using a Photek camera (HRPCS5, Photek, East Sussex, UK).

Prediction of miRNAs targeting *MgTTL1* and luciferase reporter assays

The miRNAs of *M. graminicola* (Tian et al. 2023) targeting the *MgTTL1* sequence were identified using three prediction tools—RNAhybrid, TargetScan, and miRanda—with their standard settings. Notably, both TargetScan and miRanda indicated a potential interaction at a conserved site between *MgTTL1* and *mgr-mir-228*, located in the 3′ UTR of *MgTTL1* mRNA, starting at position 2153 (Fig. 4a).

To confirm this interaction, luciferase assays were carried out as described previously (Tian et al. 2023). Briefly, the 3′ UTR of *MgTTL1* and a variant with mutations in the *mgr-mir-228* seed sequence binding region were inserted into the pMIR-REPORT miRNA Expression Reporter Vector (Invitrogen, Carlsbad, CA, USA) (Fig. 4a). This mutant construct was used as a negative control in the following experiments. HEK293 cells were transfected with these constructs along with *mgr-mir-228* mimics or a non-targeting miRCURY LNA miRNA mimic (used as a negative control) employing Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The interaction was quantified 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega Madison, WI, USA).

qRT-PCR of *mgr-mir-228*

To further examine the expression profile of *mgr-mir-228*, total miRNAs were isolated from J2 and par-J3/J4s nematodes (5 dpi) using the High Pure miRNA Isolation Kit (Roche, Mannheim, Germany). Subsequently, cDNAs were synthesized following the protocol provided by the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The universal real-time qPCR primer included in this kit was used, along with miRNA-specific forward primers (refer to Additional file 2: Table S1). The qPCR procedure was conducted as previously described (Tian et al. 2023).

Application of *mgr-mir-228* mimics in *M. graminicola*

Mgr-mir-228 mimics were synthesized by Sangon Biotech (Shanghai, China). The soaking of mimics was conducted as outlined previously with some modifications (Rosso et al. 2005; Huang et al. 2006). In brief, 25,000 newly hatched J2s of *M. graminicola* were submerged in an miRNA solution (containing 0.1 mg/mL mimics or a negative control mimic, 3 mM spermidine, 50 mM octopamine, 0.05% gelatin, and adjusted with 0.25 × M9 buffer) for 36 h at room temperature in darkness on a rotator. Subsequently, the J2s were rinsed three times with DEPC water to eliminate any remaining miRNA solution.

To quantify the presence of *mgr-mir-228*, miRNAs were extracted from 5,000 treated J2s, and qRT-PCR of *mgr-mir-228* was conducted as described earlier. To evaluate the expression level of *MgTTL1* in treated *M. graminicola*, qRT-PCR was carried out using primers listed in Additional file 2: Table S1. Additionally, to assess the reproductive capability of the treated *M. graminicola*, an infection assay was performed and the MF was calculated.

Statistical analysis

All statistical analyses were performed using IBM's SPSS Statistics 20.0 software. The data, which followed a normal distribution, were reported as mean values ± standard deviation (SD). For data analysis, one-way ANOVA was utilized, accompanied by Tukey's test for post-hoc comparisons, with a significance level set at $P < 0.05$.

Abbreviations

ANOVA	Analysis of variance
dpi	Days post-infection
dsRNA	Double-stranded RNA
ETI	Effector-triggered immunity
HRP	Horseradish peroxidase
J2s	Second-stage juveniles
KBM	King's B medium
MF	Multiplication factor
NCBI	National Center for Biotechnology Information
NOS	Nopaline synthase
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PF-127	Pluronic F-127
pI	Isoelectric point
PPNs	Plant-parasitic nematodes
PTI	Pathogen-associated molecular pattern-triggered immunity
Pto	*Pseudomonas syringae* Pv. *tomato*
qRT-PCR	Quantitative reverse transcription PCR
RACE	Rapid amplification of cDNA ends
RKNs	Root-knot nematodes
RNAi	RNA interference
ROS	Reactive oxygen species
SCN	Soybean cyst nematode
SD	Standard deviation
ssDNA	Single-stranded DNA
TTL	Transthyretin-like
UTR	Untranslated region

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-025-00328-2.

Additional file1: Figure S1. Molecular phylogenetic analysis of *TTL* genes based on TTL-deduced amino acid sequences. **Figure S2.** Structural prediction of MgTTL1. **Figure S3.** Impact of MgTTL1 overexpression on *M. graminicola* infection in soybean roots. **Figure S4.** Sequence similarity analysis of promoter and 5′UTR regions (2 kb) in *R. similis* and *M. graminicola TTL1* gene. **Figure S5.** Staining photos used for calculating multiplication factor.

Additional file2: Table S1. List of primers used in this study. **Table S2.** Accession numbers of genes or proteins used in this study.

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Author contributions
ZT and SH designed the experiment and wrote the manuscript; ZT, YC, MZ, LW, and QL (Qiannan Liu) performed experiments and data analysis; QL (Qinghong Li) performed the Arabidopsis transformation; BL, JZ, XG, and KZ improved the manuscript. All authors have read and agreed to the published version of the manuscript.

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Not applicable.

Declarations

Ethics approval and consent to participate
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Competing interests
The authors declare that they have no competing interests.

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