HHS Public Access

Author manuscript

New Phytol. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:

New Phytol. 2018 January ; 217(2): 813–827. doi:10.1111/nph.14839.

A role for the gene regulatory module *miRNA172/TOE1/FT* in the feeding sites induced by *Meloidogyne javanica* in *Arabidopsis*

Fernando E. Díaz-Manzano[1,*], **Javier Cabrera**[1,*], **Juan-José Ripoll**[2], **Iván del Olmo**[3], **Mari Fe Andrés**[4], **Ana Cláudia Silva**[1], **Marta Barcala**[1], **María Sánchez**[1], **Virginia Ruíz-Ferrer**[1], **Janice de Almeida-Engler**[5], **Martin F. Yanofsky**[2], **Manuel Piñeiro**[3], **Jose Antonio Jarillo**[3], **Carmen Fenoll**[1], and **Carolina Escobar**[1]

[1]Universidad de Castilla-La Mancha. Facultad de Ciencias Ambientales y Bioquímica, Área de Fisiología Vegetal. Avda. Carlos III, s/n, 45071 Toledo, Spain

[2]Division of Biological Sciences, Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA 92093-0116 USA

[3]Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM) - Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Campus Montegancedo UPM, 28223 Pozuelo de Alarcón (Madrid), Spain

[4]Instituto de Ciencias Agrarias (ICA, CSIC), Protección Vegetal, Calle de Serrano 115, 28006 Madrid, Spain

[5]Institut National de la Recherche Agronomique (INRA) - University of Nice Sophia Antipolis, CNRS, UMR 1355-7254 Institut Sophia Agrobiotech, 06900 Sophia Antipolis, France

Summary

- Root knot nematodes (RKNs) penetrate into the root vascular cylinder triggering morphogenetic changes to induce galls, *de novo* formed ‘pseudo-organs’ containing several giant cells (GCs). Distinctive gene repression events observed in early gall/GCs development is thought to be mediated by post-transcriptional silencing *via*-miRNAs, a process far from being fully characterized.
- *Arabidopsis* backgrounds with altered activities based on target MIMICRY (*35S::MIM172*), 35S::TOE1-miR172-resistant (*35S::TOE1*^{R}) and mutant (*ft-10*) lines, were used for functional analysis of nematode infective and reproductive parameters. GUS-reporter lines, *MIR172A-E::GUS*, auxin (IAA) and auxin-inhibitor (PEO-IAA) treatments, as well as a *MIR172C*$^{AuxRE^{-}}$*::GUS* line with two mutated auxin responsive elements (*AuxREs*) were assayed for nematode-dependent gene expression.

Author for correspondence: Carolina Escobar, Tel: +34 925268800-5476, carolina.escobar@uclm.es.
*These authors contributed equally to this work.

Author contributions: Conceptualization: F.E.D-M., J.C., C.E.; methodology: F.E.D-M., J.C., J-J.R., I.d.O., M.F.A., A.C.S., M.B., M.S., V.R-F., J.d.A-E.; investigation: all authors; writing – original draft: F.E.D-M., J.C., C.E., J-J.R., M.P., J.A.J., C.F.; writing – review and editing: all authors; funding acquisition: C.E., C.F., J-J.R., M.F.Y., M.P., J.A.J.; resources: C.E., C.F., M.F.Y., M.P., J.A.J.; supervision: C.E.

Supporting Information: Additional Supporting Information may be found online in the Supporting Information tab for this article:

- *Arabidopsis* backgrounds with altered expression of either MIRNA172, *TARGET OF EAT1* (*TOE1*), and *FLOWERING LOCUS T* (*FT*), showed lower susceptibility to the RKNs and smaller feeding sites, galls/GCs. *MIR172C-D::GUS* showed restricted activity in galls/GCs that is regulated by auxins through auxin responsive factors. IAA induced their activity in galls while PEO-IAA treatment and mutations in *AuxRe* motifs abolished it.
- The results show that the activity of the regulatory module *miRNA172/TOE1/FT* plays an important role in correct GCs and gall development where miRNA172 is modulated by auxins.

Keywords

AP2; *Arabidopsis*; flowering; giant cells; *Meloidogyne* spp.; miRNA172; repression

Introduction

Plant parasitic nematodes that include two major groups, the root-knot nematodes (RKNs; *Meloidogyne* spp.) and the cyst nematodes (CNs; *Heterodera* spp. and *Globodera* spp.), constitute one of the major threats for plant productivity given the progressive ban of the most effective chemical nematicides (reviewed in Escobar *et al.*, 2015; European Parliament Directive 2009/128/EC). Juvenile RKNs invade the root through the elongation zone and then migrate intercellularly through the cortex to the root apex where they penetrate into the vascular cylinder and get established (reviewed in Escobar *et al.*, 2015). Once in the root vascular cylinder they select five to eight vascular cells to differentiate into their feeding cells, giant cells (GCs hereafter), as they present a much greater volume than adjacent cells in the vascular cylinder. The cells around the GCs proliferate and eventually a knot in the root, called gall is formed. This *de novo* structure is considered as a newly formed pseudo-organ in the roots (reviewed in Escobar *et al.*, 2015).

In the last decade, major advances have been made to understand the molecular mechanisms underlying the formation of the galls/GCs. Interestingly, genes previously defined to have key functions in plant developmental pathways also showed a role during galls/GCs development in *Arabidopsis* (reviewed in Cabrera *et al.*, 2015b). Some of them are transcription factors participating in root development, for example LBD16 or WRKY23 that are also crucial during the CNs and RKNs interaction in syncytia and gall and/or GCs development (Grunewald *et al.*, 2008; Cabrera *et al.*, 2014, respectively). Moreover, small signaling peptides such as CLAVATA3/ESR (CLE)-like or C-TERMINALLY ENCODED PEPTIDEs (CEP) peptides that participate in root and shoot apical meristem maintenance, vascular development and lateral root formation (Mohd-Radzman *et al.*, 2016; Yamaguchi *et al.*, 2016), were identified in RKNs secretions (Betsuyaku *et al.*, 2011; Mitchum *et al.*, 2012; Bobay *et al.*, 2013). The role of CLE-like peptides have been studied more deeply in CNs than in RKNs. One of the most recent studies indicates a clear link between nematode B-type CLE signaling and the WOX4-mediated cell proliferation pathway for feeding cell formation (Guo *et al.*, 2017). In this respect, 16D10 is a secretory peptide with a CLE-Like sequence identified in *Meloidogyne* spp., with a role during the RKN interaction (Huang *et*

al., 2006). Hence, one of the putative strategies used by RKNs to induce the formation of their specialized feeding sites is to hijack or interfere with established plant developmental pathways to induce the formation of their feeding sites (Cabrera *et al.*, 2015b).

Several transcriptomic analyses of galls and/or GCs induced by *Meloidogyne* spp. in a number of plant species have been performed, delivering extensive lists of misregulated genes (upregulated or downregulated) in the nematode galls and GCs (reviewed in Cabrera *et al.*, 2016a). Interestingly, a conspicuous trait that prevails in all these studies is the large number of significantly downregulated genes, particularly at early infection stages (Barcala *et al.*, 2010; Damiani *et al.*, 2012; Portillo *et al.*, 2013). Therefore, gene repression can be considered as a signature for proper nematode establishment and feeding site formation. In line with this, the overexpression of the repressed gene *TPX1* in tomato caused a reduction in the infectivity and in the size of the GCs (Portillo *et al.*, 2013). Similarly, in CNs, the overexpression of the repressed gene *RAP2.6* led to an enhanced resistance against *Heterodera schachtii* and to smaller feeding cells in *Arabidopsis* (Ali *et al.*, 2013). Bearing in mind the putative role of gene repression, changes in the sRNAs population in the feeding sites induced by RKNs and CNs have been studied by high-throughput sequencing (Hewezi *et al.*, 2008; Li *et al.*, 2012; Xu *et al.*, 2014; Zhao *et al.*, 2015; Cabrera *et al.*, 2016b). However, we have just started to delineate the functional role of sRNAs and their targets during the nematode infection. Evidence showed that miR396 and its *GRFs* target genes participate in the regulation of a large number of genes in the CNs nematode feeding cells (Hewezi *et al.*, 2012; Hewezi & Baum, 2015). Similarly, Hewezi *et al.* (2016) demonstrated that the miR827 and its target gene *NLA* (*NITROGEN LIMITATION ADAPTATION*) had a role in mediating the infectivity of *H. schachtii*. Furthermore, Arabidopsis loss of function lines for the module *miR390/TAS3/ARFs* displayed a decrease in the infectivity and smaller galls during RKN infection (Cabrera *et al.*, 2016b) and *in situ* localization of miR390 in tomato indicated accumulation of miR390 in galls/GCs (Díaz-Manzano *et al.*, 2016a) what suggests conserved roles in tomato. Additionally, miR319 and its target gene *TCP4* regulate the systemic defense response during RKN infection in tomato (Zhao *et al.*, 2015).

The riboregulator miRNA172 post-transcriptionally targets a small group of regulatory repressor genes (APETALA2 (AP2) and AP2-like transcription factors, such as TARGET OF EARLY ACTIVATION TAGGED 1 (TOE1), TOE2, and TOE3, SCHLAFMÜTZE (SMZ), and SCHNARCHZAPFEN (SNZ)) that encode transcription factors belonging to the evolutionarily conserved AP2/Ethylene Responsive Factor (ERF) plant family. This group of proteins comprises members of the AP2 class, ERF type (subgroup B1-B6), related to ABI3/VP1 (RAV), the DREB class (subgroup A1–A6), and others (Sakuma *et al.*, 2002). These proteins regulate various developmental and stress responsive pathways (Licausi *et al.*, 2013; Müller & Munné-Bosch, 2015) and bear at least one conserved AP2 domain of *c.* 60–70 amino acid residues, which recognizes particular DNA cis-elements (Nakano *et al.*, 2006). When overexpressed some AP2/ERF type transcription factors enhance biotic and abiotic stress tolerance (Jisha *et al.*, 2015; Mishra *et al.*, 2015).

Regarding the members of miRNA172-targeted AP2-like transcription factors, they have been involved in controlling plant aging, flowering time, tuber formation, fruit growth, and nodulation (Martin *et al.*, 2009; Zhu & Helliwell, 2010; Yan *et al.*, 2013; Wang *et al.*, 2014;

Ripoll *et al.*, 2015). Interestingly, miRNA172 is regulated by different auxin response factors (ARFs) during fruit development in Arabidopsis (Ripoll *et al.*, 2015). In Arabidopsis, AP2-like transcription factors act as flowering repressors and at least TOE1 (Zhang *et al.*, 2015) and SMZ (Mathieu *et al.*, 2009), negatively regulate FT expression as for example TOE1 binds to its promoter region and represses its expression in the vascular tissue of the leaves during the floral transition (Zhang *et al.*, 2015).

Hence, TOE1 functions in different developmental processes, but has also a role described only during a biotic interaction, that is, nodulation (Yanz *et al.*, 2013). In line with this, previous studies showed that the repression of a subclade within the AP2-like transcription factor family in Arabidopsis has a crucial functional role during the establishment and development of the feeding cells induced by the plant parasitic cyst nematode *H. schachtii* (Ali *et al.*, 2013). Moreover, TOE1 was downregulated in the transcriptomes of the RKNs GCs induced by *M. javanica* in Arabidopsis (Barcala *et al.*, 2010).

Our investigations show that the miRNA172-dependent regulatory module *miRNA172/TOE1/FT* is active and de-regulated in the feeding sites induced by *M. javanica* in *Arabidopsis* roots, and also participates in gall and GCs development. This study adds knowledge on how RKNs interfere with endogenous developmental pathways and contributes to a better understanding of the molecular signatures associated with feeding cells development.

Materials and Methods

Nematode population

Meloidogyne javanica Treub, (1885) was maintained *in vitro* on cucumber etiolated seedlings (*Cucumis sativus* cv Hoffmanns Giganta). Egg hatching was stimulated in sterile tap water 4 d before inoculation as described in Díaz-Manzano *et al.* (2016b).

Plant material, growth conditions and nematode inoculation

Arabidopsis thaliana (L.) Heynh Columbia-0 (Col-0) was the background of all the transgenic lines used. Reporter *GUS* promoter lines *MIR172A-E::GUS* and *MIR172C*$^{AuxRE-}$*::GUS* (Ripoll *et al.*, 2015) were used for the expression analyses. Four independent transgenic lines, homozygous and harbouring one T-DNA insertion for *35S:TOE1*^{R} and two independent lines for *MIM172* (Supporting Information Fig. S1a,b; Ripoll *et al.*, 2015) were selected for the infection tests with *M. javanica*.

For histochemical analysis, three independent experiments were performed each with 30 individual plants per line. Galls were hand-dissected at the selected infection stages and the histochemical analysis and GUS staining was carried out according to Cabrera *et al.* (2014). Roots from *MIR172C::GUS* and *MIR172D::GUS* plants were incubated for 2 d on medium containing 300 μM α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) as described in Cabrera *et al.* (2014).

For the infection tests *in vitro*, three to six independent infection tests, with at least 30 plants per experiment and line were performed using Col-0 (as control), *35S:TOE1*^{R}, *MIM172* and

ft-10 lines. Seeds were sterilized, grown vertically and homogeneously inoculated following the protocol set up in Olmo *et al.* (2017) at long day (LDs;16 h : 8 h, day : night) or short days (SDs;8 h : 16 h, day : night) regime.

For infection tests in soil, seedlings were planted singly into 15 ml clay pots containing steam-sterilized river sand. *Arabidopsis* plants were grown at 25 ± 2°C, 60% RH, at SDs for 2 months and inoculated with 200 J2 per pot. Nematode inoculum's was obtained as described in Andrés *et al.* (2012). Plants were watered as needed and fertilized with 1 ml of a 0.5% solution of 20-20-20 (N-P-K) every 15 d per pot. Each infection test was replicated at least three times with 10 plants per experiment. Reproductive parameters were evaluated 2 months after inoculation (i.e. 4 months after planting). The number of galls/egg masses per fresh root gram was counted from each root system. Egg production was obtained by extracting the eggs from the entire root system (Hussey & Barker, 1973).

Galls and GCs phenotyping

The volume or the estimation of the size of the GCs (n≥ 10 per line tested) induced by *M. javanica* in Col-0, *35S:TOE1*^{R}, *MIM172* and *ft-10* were obtained after 3D reconstruction following the method described in Cabrera *et al.* (2015a) by using the plugin TrakEM2 (Cardona *et al.*, 2012) for FIJI (Schindelin *et al.*, 2012). Gall diameters were measured from micrographs from each gall ($n \geq 20$ per line tested) by using the straight line and measurement tools from FIJI (Schindelin *et al.*, 2012).

Plant transgenic construction and selection

The 35S:TOE1^{R} constructs were generated by introducing three mutations in the miRNA172 pairing sequence of the *TOE1* transcription factor, avoiding alterations of the amino acid sequence of the protein. Specific oligos TOE1miR172 (Table S1; Fig. 1a) and the Quikchange lightning site-directed mutagenesis protocol (Agilent technologies) were used to generate the TOE1 miRNA172172 resistant version, which was fully sequenced and mobilized into the pGW2 vector under the control of a CaMV 35S promoter. This construct was transformed into *Arabidopsis* Col-0 plants, and several independent transgenic lines expressing the miRNA172-resistant version of TOE1 (*35S:TOE1*^{R}) were selected on hygromycin plates. These miRNA172-resistant lines were developed under the framework of the TRANSPLANTA project (Coego *et al.*, 2014).

Arabidopsis thaliana 35S:MIMICRY172-7 and *35S:MIMICRY172-23* plants (*MIM172*) are resistant to BASTA (Todesco *et al.*, 2010). For the homozygous lines selection, we performed a double check. A selection according to the expected late flowering phenotype (Fig. S1d,f) as compared to Col-0 and a selection based on BASTA resistance, with 120 mg l^{-1} (DL-PHOSPHINOTHRICIN, Duchefa® Biochemie, Ref.: P0159 0250 and Silwet-77 surfactant 500 µl l^{-1} (Lehle Seeds) as described by Bouchez *et al.* (1993). Plants were grown in soil (a mixture of peat substrate, Kekkilä PROJAR 70L 50/50, and vermiculite, 3 : 1) at 21°C, 60% humidity and 16 h : 8 h, light : darkness for 6 wk.

Lines *35S:TOE1*^{R} were grown on Murashige and Skoog (MS) agar medium supplemented with 1% sucrose plus hygromycin 20 µl l^{-1} (Sigma®, Ref.: H3274) and compared with Col-0 for selection.

Flowering time assays

Flowering time was scored as the total leaf number, including rosette and cauline leaves, just before the first flower opened (Martin-Trillo *et al.*, 2006).

RNA Isolation and quantitative PCR analysis

Total RNA was extracted from 25 hand-dissected root plant tissues (control root segments and/or galls from infected plants) using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. An amount (0.75 mg) RNA from each sample (control root segments and/or galls) was used for cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Foster City, California, USA) according to the company's guidelines (35 cycles). The cDNA was diluted up to 1/10 and 1 ng was used from each sample as a template for the subsequent qPCR transcript analysis for *TOE1* (TARGET OF *EAT* 1, AT2G28550); and 45 ng for *FT* (FLOWERING LOCUS T, AT1G65480) as its expression level is very low in most tissues.

Quantitative PCR analysis was done with SYBR-Green technology (SYBR Green Master Mix 2X, no ROX, Thermo Scientific, Waltham, MA, USA) in a LightCycler® 480 II machine (Roche, Indianapolis, USA). Quantification of the relative changes in gene expression levels was determined using the E-Method (Tellmann, 2006). In all cases, at least three independent experiments each with three technical replicates of each reaction were performed. *Arabidopsis thaliana* GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2 (*GAPC2*, AT1G13440) was used as internal control to normalize gene expression levels. Primers used for PCRs and qPCRs are described in Table S1.

Pharmacological treatments

For auxin treatments and *MIR172C-D::GUS* expression, plantlets were germinated and grown on 0.5 MS medium, as described in Chapman *et al.* (2012), transferred to media containing the corresponding auxin (IAA) concentration or DMSO (for the mock controls). The tissue was incubated for 30 min and total RNA was extracted using Trizol (Life Technologies) and treated for cDNA synthesis as previously described (Ripoll *et al.*, 2015). Five micrograms of total RNA was used for cDNA synthesis with an oligo (dT) primer and Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). After 1/10 dilution of the cDNA, 1 μl was used as a template for the subsequent qPCR reactions. Relative changes in gene expression levels were determined using the 2ΔΔ-CT method (Livak & Schmittgen, 2001) RNA levels were normalized to the constitutively expressed gene ACTIN2 as previously reported (Ripoll *et al.*, 2011). Each experiment was executed using three biological replicates. Primers used for this set of experiments can be found in Ripoll *et al.* (2015).

Data analysis

Data obtained were represented with histograms with mean values and/or percentages per line/treatment and ± SE. Statistical analysis of the infection and reproduction parameters; and GCs volumes and areas were performed using the *T*-test in the SPSS package (IBM, Armonk, NY, USA). The corresponding confidence intervals (CI) were calculated with a significance level of 5% ($P < 0.05$) which was indicated with an asterisk.

Moreover, data from q-PCRs were represented with histograms with pairing-fold change values (line/treatment vs control) and ± SE. Statistical analysis were performed using the *T*-test in the SPSS package (IBM, Armonk, NY, USA). The corresponding confidence intervals (CI) were calculated with a significance level of 5% (P <0.05) which was indicated with an asterisk.

Blast analysis

We searched for miRNA172 sequences described in tomato and pea on miRbase (http://www.mirbase.org), finding two sequences described in tomato (sly-miRNA172a and sly-miRNA172b). To find homologue sequences to *TOE1* from *Arabidopsis* in other crop species, we selected the miRNA172c *Arabidopsis* sequence (http://www.mirbase.org) and performed a blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) against tomato (*Solanum lycopersicum*; taxid: 4081) and pea (*Pisum sativum*; taxid: 3888). We obtained SlAP2d (NM_001247718.2) and AP2-like (AF325506.1) genes with a 100% cover query for tomato and pea, respectively.

Results

Repression of *TOE1* in galls and GCs induced by *M. javanica* in *Arabidopsis* is necessary for the correct progression of the infection and the development of the feeding sites

In the present study, we further investigated the role of *TOE1* (*RAP2.7*; At2g28550), during the formation of the GCs as it was downregulated in the transcriptomes of the GCs induced by *M. javanica* in *Arabidopsis* (Barcala *et al.*, 2010).

First, we corroborated by qPCR that *TOE1* expression was two-fold down-regulated in galls induced by *M. javanica* in *Arabidopsis* at 3 d post infection (dpi; Fig. 1b; $P < 0.05$), which is in line with the results obtained in previous microarray experiments (Barcala *et al.*, 2010). *TOE1* expression is regulated by miRNA172 during the floral transition (Zhu & Helliwell, 2010). Consistent with this observation and the downregulation of *TOE1*, pri-miRNA172d expression was clearly induced in the same microarrays of micro-dissected *Arabidopsis* GCs as compared to vascular control cells (Table S2; Barcala *et al.*, 2010). Hence, we generated four independent transgenic lines overexpressing a modified version of *TOE1* being resistant to the degradation by miRNA172 (*35S:TOE1*^{R}; Fig. 1a). All these lines (A12, E82, G73, D81) showed a noticeable increase in *TOE1* transcript abundance as compared to the Col-0 wild type (WT) line, although there were differences in the expression levels among lines (Fig. 1c; $P < 0.05$). All four *35S:TOE1*^{R} lines showed a conspicuous delay in flowering time ($P < 0.05$) under SD conditions, while two of them (D81 and A12) flowered significantly late under LDs (Fig. S1). When we challenged these *35S:TOE1*^{R} plants with juveniles of *M. javanica*, all independent lines showed a significant reduction in the percentage of galls per plant in the range of 15–44% (Fig. 1d). Remarkably, the flowering phenotype was correlated with the nematode resistant traits, as those lines with the strongest flowering phenotypes also showed the highest reduction in nematode infection rates (see lines A12 and D81 in Figs 1d, S1a,c,e). Our next step was to characterize in more detail the gall and GCs phenotypes in the *35S:TOE1*^{R} lines with the most extreme phenotypic alterations (Fig. 1e–g). Galls of *35S:TOE1*^{R} lines were *c.* 15% smaller than those formed in Col-0 at 14 dpi (Fig. 1e; $P <$

0.05). In addition, the GCs formed in these galls at 14 dpi were reduced in size to two-thirds compared to those formed in Col-0 galls at the same infection stage (Fig. 1f,g; $P < 0.05$), measured after 3D reconstruction following Cabrera *et al.* (2015a). Thus, we corroborated that downregulation of *TOE1* by miRNA172 in the GCs and galls induced by *M. javanica* in *Arabidopsis* is important for proper nematode establishment, and gall and GCs development.

FT, a defined target of TOE1, has a crucial role during gall development

It has been recently identified that the TOE1 regulatory protein directly binds to the promoter region of the gene *FT* and represses its expression in the vascular tissue of the leaves during the floral transition (Zhang *et al.*, 2015).

Because *TOE1* expression was repressed in 3 dpi galls induced by *M. javanica* in *Arabidopsis* (Fig. 1b) we checked by qPCR whether the expression levels of *FT* were altered after nematode infection in Col-0 roots (Fig. 2a). Interestingly, *FT* transcript levels were noticeable increased in the galls while they were almost undetectable in the uninfected Col-0 control roots (Fig. 2a; $P < 0.05$). By contrast, *FT* expression in *35S:TOE1*^{R} lines were barely detectable either in control roots or in galls, similarly to the uninfected control Col-0 roots (Fig. 2a). The levels of FT in control root tissue are extremely low, this might explain the large error bars in the *35S:TOE1*^{R} lines. This is consistent with *TOE1*^{R} repressing the expression of *FT* in galls, what suggests that the downregulation of *TOE1* in galls probably mediates the accumulation of *FT* transcripts in those cells.

To decipher the role of *FT* during the gall and GC formation we performed six independent infection tests using a previously characterized complete loss-of-function *FT* mutant allele (*ft-10*) of *Arabidopsis* that displayed a late flowering time phenotype (Yoo *et al.*, 2005). Mutant plants for *FT* showed a significant reduction in the number of galls, 24% less when compared to the WT plants (Fig. 2b), suggesting that *FT* function is required for the proper establishment of *M. javanica* in *Arabidopsis*. This idea is reinforced by the fact that the galls formed in the *ft-10* mutant plants were smaller than those formed in Col-0 at 14 dpi (Fig. 2c; $P < 0.05$).

MiRNA172 is upregulated in the galls induced by *M. javanica* in *Arabidopsis* and other crop species

While *TOE1* downstream regulation is at least partially mediating *FT* transcript accumulation, *TOE1* is regulated upstream by the action of the miRNA172 during *Arabidopsis* flowering (Aukerman & Sakai, 2003). As mentioned earlier, from the 83 pre-miRNAs that were detected in the microarray of developing GCs (Barcala *et al.*, 2010), the *pre-miRNA172d* was induced, being the only one differentially expressed in GCs as compared to non-infected vascular cells ($P < 0.05$; Table S2). To investigate the expression pattern of the gene family that generate the mature *miRNA172* in the feeding sites induced by *M. javanica* in *Arabidopsis*, we assayed the *GUS*-reporter lines for the promoters for all five miRNA172-encoding loci (*MIR172a*, *MIR172b*, *MIR172c*, *MIR172d* and *MIR172e*), fused to the coding sequence of the *GUS* marker gene (Ripoll *et al.*, 2015). In roots of uninfected plants, the promoters of the five precursors for the miRNA172 showed differential activity patterns. *MIR172A::GUS*, *MIR172B::GUS* and *MIR172E::GUS* were

activated along the vascular cylinder of the root (Fig. 3a,b,e), except in the elongation zone and root apex in the case of *MIR172A::GUS* and *MIR172E::GUS* (Fig. 3a1,e1). By contrast, the activity of the promoters for the precursors of *miRNA172c* and *miRNA172d* was not detectable along the vascular cylinder in the elongation or differentiation zones, being a specific signal only noticeable in the root tip (Fig. 3c,d,c1,d1). Hence, *MIR172C::GUS* and *MIR172D::GUS* showed a restricted staining pattern in root tips and both originate identical mature *miRNA172* (Table S3a).

Because pri-*miRNA172d* was induced in the transcriptome of 3 dpi GCs (Table S2), we infected these two lines *MIR172C::GUS* and *MIR172D::GUS* with similar expression patterns in roots, with RKNs to check their activation patterns during gall development (Fig. 4). At 4 dpi the *GUS* staining for both reporters was localized specifically in the vascular cylinder inside the galls induced by *M. javanica* in *Arabidopsis* (Fig. 4a,d). The signal remained visible and specific in the centre of the galls at early-medium stages of development (7 dpi; Fig. 4b,e). The number of galls with positive *GUS* signal decreased at 11dpi (Fig. 4h,j) and eventually, at 14 dpi, >90% of the galls in the lines *MIR172C::GUS* and *MIR172D::GUS* did not express *GUS* (Fig. 4c,f,h,j). Moreover, *MIR172C::GUS* and *MIR172D::GUS* were clearly expressed in the GCs induced by the nematode and in adjacent vascular cells inside the galls at 4 dpi as shown in semi-thin sections (Fig. 4g,i). These results indicate a specific induction of *MIR172C::GUS* and *MIR172D::GUS* within the galls and GCs in roots upon nematode infection. However, no specific *GUS* pattern in galls of *MIR172A::GUS, MIR172B::GUS* and *MIR172E::GUS* lines was observed as they are highly activated along the vascular cylinder of the non-infected roots and their expression pattern was similar within the galls (Fig. 3a,b,a1,b1; data not shown). Although, it cannot be ruled out that these three miRNAs also might contribute, to some extent, to the total amount of *miRNA172* molecules in the gall and GCs, no differences in the pattern of expression between uninfected roots and galls, or during the infection were observed (data not shown).

miRNA172 is highly conserved in the plant kingdom in angiosperms, gymnosperms, ferns, and across all tracheophytes (Luo *et al.*, 2013), that is, the sly-miRNA172a and sly-miRNA172b from tomato showed a 100% homology across 20 out of 21 nucleotides to miRNA172c from *Arabidopsis* (Table S3b). Although the sequence for miRNA172 from pea has not been yet identified, miRNA172 is described as an active molecular partner during nodulation in legumes, a process with some molecular similarities to gall formation (see discussion). Hence, we performed *in situ* hybridisation in galls of tomato and pea with the *Arabidopsis* miRNA172c probe. Our results confirm accumulation of miRNA172 in galls and particularly in GCs of tomato and pea at 7 dpi (Fig. S2c,f, respectively). No signal was observed in either non-infected roots or galls of tomato or pea with a negative control probe (Scramble; Fig. S2a,d,b,e, respectively). Interestingly, BLAST analysis of the miRNA172c from *Arabidopsis* against the tomato and pea available genomes identified putative AP2-like gene targets with a high complementarity to miRNA172, similar to *TOE1* in *Arabidopsis* (Fig. S2g). Those results suggest that induction of miRNA172 in galls is probably a mechanism conserved in these crop species.

MiRNA172 interferes with gall and GC development after nematode infection

To further investigate the putative role of miRNA172 in galls and GCs development, we tested two independent target mimicry lines for miRNA172 (*MIM172*; Franco-Zorrilla *et al.*, 2007; Fig. 5e) in which the function of this miRNA was impaired. The two mimicry lines largely reproduced the late flowering phenotype showed by the *35S:TOE1*^{R} plants (Fig. S1b), either at SD or LD (Fig. S1d,f), as expected when TOE1 is not repressed by miRNA172. When the mimicry lines *MIM172* were inoculated with *M. javanica*, both presented a conspicuous reduction in the infection level (at least 45%; Fig. 5a; $P < 0.05$), resembling again the phenotype displayed by the *35S:TOE1*^{R} lines after nematode infection (Fig. 1d). Moreover, the galls and GCs formed in the *MIM172* lines were smaller than those formed in Col-0 (Fig. 5b–d; $P < 0.05$). These results further support a role for miRNA172 during gall and GC development.

Auxin regulation of miRNA172 function

The importance of an auxin response maxima during the formation of the nematode feeding cells has been demonstrated (reviewed in Cabrera *et al.*, 2015b; Kyndt *et al.*, 2016). On the other hand, the ARFs orchestrate auxin responses, by targeting the cis-motifs called auxin response elements (AuxREs) to regulate gene expression (Chapman & Estelle, 2009). Interestingly, miRNA172 function is directly modulated by the auxin signaling pathway via the regulation of miRNA172c. The promoter of miRNA172c contains two canonical AuxREs and it is already known that the miRNA172 is regulated by different ARFs during fruit development in *Arabidopsis* (Ripoll *et al.*, 2015).

We analyzed the first 1000 bps of the promoter region upstream of *miRNA172b*, *miRNA172c* and *miRNA172d* loci, revealing the presence of canonical *AuxREs* (Table S3). To test whether auxin was influencing miRNA172c and miRNA172d expression in root tissues, we first treated seedlings with different auxin concentrations and measured transcript abundance for both loci. In both cases, auxin treatments increased transcript abundance when compared to mock treated samples (Fig. 3j). We next tested the activity of the *GUS* reporters for miRNA172c and miRNA172d after treatment with the auxin signaling inhibitor PEO-IAA. The signal for the reporters was largely abolished in the root zones where GUS activity was present in the mock treated roots (Fig. 3, compare c1, d1 to f, g, respectively). Moreover, when we challenged *MIR172C*$^{AuxRE-}$*::GUS* plants bearing two mutated AuxREs motifs (Ripoll *et al.*, 2015) with *M. javanica* juveniles, no induction was observed in the galls at 5 dpi as compared with the control reporter line *MIR172C::GUS* where a strong signal was detected in the center of the gall (Fig. 3h,i). Altogether, these results indicate that the miRNA172 is regulated by auxins both in uninfected roots and in the galls induced by *M. javanica* in *Arabidopsis*.

A role for miRNA172 and *TOE1* in the RKN–plant interaction at different day length regimes and plant developmental stages

The previously shown infection tests for the *miRNA172* and the *35S:TOE1*^{R} lines were carried out *in vitro* under LD photoperiods (16 h : 8 h, light : dark; Figs 1, 5). However, it has been reported that miRNA172 expression changes with the length of the light period, being higher under longer photoperiods (Jung *et al.*, 2007). Besides, the expression of

miRNA172 is regulated temporally, with no *miRNA172* transcripts detected 2 d after germination, and progressively more steady state transcript accumulation was seen with age (Aukerman & Sakai, 2003; Chuck *et al.*, 2007). With this in mind, we performed similar infection assays to those described above but under SD (8 h : 16 h, light : dark) and we also analyzed reproductive parameters in soil-grown plants under these conditions (Fig. 6). The reduction in the infection parameters for the *MIM172* and *35S:TOE1*^{R} plants was also observed and even enhanced in some cases under SD as compared to LD (Fig. 6a; $P < 0.05$). Interestingly, a reproductive parameter as the number of eggs per root weight was severely impaired in most of the lines grown in soil and infected *c.* 2 months after germination (Fig. 6b; $P < 0.05$) which indicate that the module miRNA172-TOE1 is crucial for nematode infection and also for reproduction. This is in agreement with the reduction of galls and GCs size observed in the *MIM172* and *35S:TOE1*^{R} lines in LDs (Figs 1, 5). These results confirmed that the miRNA172 performs an important function during the gall and GC development affecting also the reproductive cycle of the nematode. Therefore, the miRNA172 /TOE1 module play a role in galls and GCs development under different photoperiods and plant developmental stages, yet it seems to be independent of the day light regime.

Discussion

RKNs constitute a major problem for the agriculture, exacerbated in recent years due to the ban of effective but contaminant chemical nematicides (Directives 91/414/EEC or 2009/128/EU). *Meloidogyne* spp. nematodes establish an obligate and endoparasitic interaction with a broad spectrum of valuable agronomic crops by inducing their feeding cells, GCs, inside the gall, a *de novo* developed structure (pseudo-organ) within the host roots (Escobar *et al.*, 2015). Increasing knowledge of the molecular mechanisms orchestrating GCs and galls formation could assist in the development of new biotechnological tools against the RKNs (Fosu-Nyarko & Jones, 2015). Our results reveal the activation in the nematode feeding sites (GCs and galls) of a well-documented gene regulatory module (miRNA172/AP2-like), implicated in other developmental processes directing *de novo* emerging organs in plants like flowers (Aukerman & Sakai, 2003), tubers (Martin *et al.*, 2009), fruit (Ripoll *et al.*, 2015) or nodules (Yan *et al.*, 2013; Wang *et al.*, 2014).

The riboregulator miRNA172 regulates the abundance of a number of AP2-like transcription factors, like TOE1, at both transcriptional and translational levels (Chen, 2004). Yet, in our transcriptomic study of microdissected GCs compared to non-infected cells, the only flowering related AP2-like gene repressed was *TOE1* (Barcala *et al.*, 2010). Therefore, an increase in the level of expression of *miRNA172* in the cell would result in a reduction of *TOE1* transcript abundance and/or protein accumulation. Our expression analyses by q-PCR confirmed *TOE1* down-regulation in galls (Fig. 1) while the promoters of the precursor genes for the *miRNA172c* and *miRNA172d* (Fig. 4) were strongly activated in galls and GCs induced by *M. javanica* in *Arabidopsis*. As mentioned, this is in agreement with previous results of microarrays experiments of laser-capture microdissected GCs at 3 dpi in *Arabidopsis* (Barcala *et al.*, 2010; Table S2). Our previous work showed a massive and conserved down-regulation of genes in the transcriptome of early developing GCs in *Arabidopsis* and tomato (Barcala *et al.*, 2010; Portillo *et al.*, 2013) also evident in galls

(Jammes *et al.*, 2005; Barcala *et al.*, 2010). The repression of *TOE1* expression seems to have a role for proper nematode establishment as infection was reduced in overexpressing lines resistant to the miRNA172 mediated silencing (Fig. 1). Similarly, *TPX1,* a peroxidase coding gene was repressed in tomato GCs and in *Arabidopsis* GCs (Portillo *et al.*, 2013). Overexpression of *TPX1* in the plant conferred not only a higher resistance to the infection but impaired nematode feeding site development, showing smaller GCs. Likewise, *RAP2.6*, a transcription factor containing an AP2 domain, was down-regulated in syncytia induced by *H. schachtii* in *Arabidopsis* (Szakasits *et al.*, 2009) and its overexpression enhanced the resistance against the nematode, resulting in smaller syncytia (Ali *et al.*, 2013).

In our study we have observed lower infection and reproductive levels in genetic backgrounds with either impaired miRNA172 function or misexpressed *TOE1*-miRNA172-resistant (*MIM172* and *35S:TOE1*^{R}, respectively). Moreover, in those lines the growth of galls and GCs was dramatically impaired when compared to the corresponding controls (Figs 1, 5, 6). These results suggest a role for the repression of *TOE1* in the morphogenetic processes leading to gall/GCs development. Our results are in line with previous publications showing that AP2-like target repression by miR172 is essential for correct tuberization and fruit morphogenesis (Licausi *et al.*, 2013; Ripoll *et al.*, 2015). This is in accordance with previous studies showing the interference caused by the nematodes in different plant developmental pathways, hijacking regulatory circuits to use them for their own benefit (reviewed in Cabrera *et al.*, 2015b).

MiRNA172 regulates the expression of a small group of AP2-like transcription factors during flowering, including *TOE1* (Zhu & Helliwell, 2010). In this respect, we demonstrated the activation of the promoters of the precursor genes for miRNA172c and miRNA172d at early infection stages within the galls, with a high specific pattern in GCs and the adjacent vascular cell layers (Fig. 4) as well as the accumulation of miRNA172 homologues to *Arabidopsis* miRNA172c in galls from tomato and pea (Fig. S2). These observations are in line with a role for miRNA172 in the nematode feeding site. The regulation of TOE1 by miRNA172 is reinforced by the fact that mimicry lines for miRNA172 showed late flowering phenotype either at SDs or LDs similar to *35S:TOE1*^{R} lines (Fig. S1) and a significant reduction in the infection parameters and size of the galls and GCs (Figs 1, 5), somehow mimicking the phenotype observed for *35S:TOE1*^{R} also during nematode infection.

The relevance of the down-regulation of a gene for the proper nematode feeding site development has been recently demonstrated, that is, miRNA827 was induced and its target gene *NLA*, repressed, being necessary for the correct establishment of CNs feeding sites (Hewezi *et al.*, 2016). Moreover, *ARF3* is downregulated by the induction of the auxin responsive miRNA390 in *Arabidopsis* (Cabrera *et al.*, 2016b) and mutants for the miRNA390 activity showed a decrease in the infection parameters and a reduction in the galls size (Cabrera *et al.*, 2016b). This latter example suggests a connection between the hormone auxin and the regulation of genes by miRNAs in galls. Here we demonstrated the regulation of miRNA172 by auxins in RKN-induced galls by two independent assays, one after exposure to a TIR inhibitor, PEO-IAA, and the second by mutating two AuxREs in the miRNA172 promoter (*MIR172C*$^{AuxRE-}$ *::GUS*). In both cases the specific activation of

MIR172C::GUS in galls disappeared (Fig. 3). The regulation of miRNA172 expression by auxins has also been demonstrated during the *Arabidopsis* fruit development, where *miRNA172* is induced by different auxin response factors (*ARF6* and *ARF8*) and targets the expression of *AP2* (*TOE3)*, mediating in this way the proper fruit growth (Ripoll *et al.*, 2015). The presence of RKNs in the roots alters the auxin levels, showing a high accumulation during early stages of feeding site development and it is a key signal for the formation of GCs and galls (Karczmarek *et al.*, 2004; Kyndt *et al.*, 2013; Cabrera *et al.*, 2014). Auxins also drive fruit development and flowering (Ripoll *et al.*, 2011; 2015). Moreover, nodulation also shows some parallels with gall formation (Mathesius, 2003), such as the accumulation of auxins at early stages (Grunewald *et al.*, 2009). In addition, the regulatory module miRNA172-AP2 also plays a role during the formation of nodules in legumes (Yan *et al.*, 2013; Wang *et al.*, 2014). In parallel, miRNA172c expression induced in the nodules negatively regulates an AP2-like transcription factor, *NNC1*, allowing in this way the proper formation of the new organ (Wang *et al.*, 2014). Interestingly, *MIM172* mimicry lines showed abnormal phenotype of the GCs, as they were smaller than in wild type plants (Fig. 5). Similarly, during fruit development in *MIM172* lines smaller valve cells are formed (Ripoll *et al.*, 2015). All these suggest common regulatory networks mediated by miRNA172 in apparently distant processes of plant development and during biotic interactions.

Zhang *et al.* (2015) showed that TOE1 binds the *FT* promoter in leaves and that the expression levels of *FT* increase in an overexpressing line for the miRNA172 and in the double mutant line *toe1/toe2*, suggesting that *TOE1* acts as a negative regulator of *FT* expression. We showed that *FT* transcripts accumulate in galls formed by *M. javanica* in *Arabidopsis* roots (Fig. 2). These data together with the fact that the phenotype of MIMR172 lines as well as of *35S:TOE1*^{R} lines was maintained at different day length regimes, suggest, that RKNs might induce local changes in FT abundance. Accordingly, mutant *ft-10* lines showed a decrease in the infection by RKNs and in the gall size (Fig. 2). FT is a mobile molecule broadly characterized as a positive regulator of flowering (Turck *et al.*, 2008). This data in addition to the down-regulation of *TOE1* (Fig. 1) and the up-regulation of *miRNA172* (Fig. 4) supports a scenario in which the expression of these three genes could be coordinated in nematode feeding sites (Fig. 7b). Belowground, *FT* orthologous genes have been identified to positively regulate tuber formation in potato (Navarro *et al.*, 2011) or bulb formation in onion (Lee *et al.*, 2013). These results correlate well with the up-regulation and positive role demonstrated for *miRNA172* repressing the expression of a *TOE1* relative, the AP2-like transcription factor *RAP1,* during the tuber formation in potato (Martin *et al.*, 2009). Thus, taking into consideration all of the above, it is reasonable to postulate that all those developmental programs (tuber formation, nodule formation, gall development and fruit growth) share a common gene regulatory architecture mediated by miRNA172.

Our data support a model where the *miRNA172/TOE/FT* regulatory module is shared by seemingly unrelated and distant processes such as flowering in the aerial parts and responses to pathogens in roots (Fig. 7). Hence, the initial cells leading to *de novo* formation of GCs might share some characteristics. For instance, the establishment of floral organ founder cells precedes an auxin response maxima, providing local competence for G1-S cell cycle

progression (Chandler, 2011; Seeliger *et al.*, 2016). In this respect, although the founder cells of RKN feeding cells, GCs, are still undetermined, an auxin maxima occurs early during nematode establishment (Karczmarek *et al.*, 2004; Cabrera *et al.*, 2014; Kyndt *et al.*, 2016) preceding successive mitosis and endoreduplication events during their differentiation (De Almeida-Engler *et al.*, 2015; Coelho *et al.*, 2017).

In conclusion, here we show the participation of the regulatory module *miRNA172/TOE1/FT* in GC and gall development induced by *M. javanica* in *Arabidopsis* (Fig. 7). Our data show that the activity of this module plays an important role during RKN parasitism, as several genotypes affected in the activities of this module show lower susceptibility to nematode infection and smaller galls/GCs. The regulation of miRNA172 by auxin response factors together with previous results highlights the key role of auxin signaling during early GC and gall development, strongly suggesting that this regulatory module is in turn regulated by auxins during feeding cells development. Moreover, *FT*, a gene encoding a mobile molecule that positively regulates flowering, is induced in galls and its loss of function compromised nematode infection levels. Further studies should be performed to understand these common regulatory networks. Yet, common regulatory molecular partners at the cellular level should be controlling different organogenetic processes triggered either by developmental cues, or by biotic interactions, as plant/nematodes, probably coordinated by internal hormonal signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank to Dr G. Engler for their technical advices during *in situ* procedure. We thank Mr Iñaki Velasco, Mr Jose Perez and Ms Ana Bombín for his technical help. Work supported by the Spanish Government (grants AGL2013-48787; AGL2016-75287-R to C.E., CSD2007-057 and PCIN-2013-053 to C.F. and BIO2013-43098R and BIO2016-77559R to M.P. and J.A.J.; FPU-AP2009-1577 fellowship to F.E.D-M.) and by the Castilla-La Mancha government (PEII-2014-020-P to C.F. and Cátedra Fundación Enresa UCLM grant to F.E.D-M.). J.C. is supported by a Cytema-Santander contract from UCLM. NIH (grant 1R01GM112976-01A1) and the Paul D. Saltman Endowed Chair in Science Education (J-J.R. and M.F.Y.).

References

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T. FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science. 2005; 309(5737):1052–1056. [PubMed: 16099979]

Ali MA, Abbas A, Kreil DP, Bohlmann H. Overexpression of the transcription factor RAP2.6 leads to enhanced callose deposition in syncytia and enhanced resistance against the beet cyst nematode *Heterodera schachtii* in *Arabidopsis* roots. BMC Plant Biol. 2013; 13:47. [PubMed: 23510309]

Andrés MF, González-Coloma A, Sanz J, Burillo J, Sainz P. Nematicidal activity of essential oils: a review. Phytochemistry Reviews. 2012; 11(4):371–390.

Aukerman MJ, Sakai H. Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell. 2003; 15(11):2730–2741. [PubMed: 14555699]

Barcala M, Garcia A, Cabrera J, Casson S, Lindsey K, Favery B, Garcia-Casado G, Solano R, Fenoll C, Escobar C. Early transcriptomic events in microdissected *Arabidopsis* nematode-induced giant cells. Plant J. 2010; 61(4):698–712. [PubMed: 20003167]

Betsuyaku S, Takahashi F, Kinoshita A, Miwa H, Shinozaki K, Fukuda H, Sawa S. Mitogen-activated protein kinase regulated by the CLAVATA receptors contributes to shoot apical meristem homeostasis. Plant and Cell Physiology. 2011; 52(1):14–29. [PubMed: 20965998]

Bobay BG, DiGennaro P, Scholl E, Imin N, Djordjevic MA, Mck Bird D. Solution NMR studies of the plant peptide hormone CEP inform function. FEBS Letters. 2013; 587(24):3979–3985. [PubMed: 24211833]

Bouche F, D'Aloia M, Tocquin P, Lobet G, Detry N, Perilleux C. Integrating roots into a whole plant network of flowering time genes in *Arabidopsis thaliana*. Scientific Reports. 2016; 6:29042. [PubMed: 27352932]

Bouchez D, Camilleri C, Caboche M. A binary vector based on Basta resistance for *in planta* transformation of *Arabidopsis thaliana*. *Comptes Rendus de l'Académie des Sciences*. Sciences de la Vie. 1993; 316(10):1188–1193.

Cabrera J, Barcala M, Fenoll C, Escobar C. The power of omics to identify plant susceptibility factors and to study resistance to root-knot nematodes. Current Issues in Molecular Biology. 2016a; 19:53. [PubMed: 26363962]

Cabrera J, Barcala M, Garcia A, Rio-Machin A, Medina C, Jaubert-Possamai S, Favery B, Maizel A, Ruiz-Ferrer V, Fenoll C, et al. Differentially expressed small RNAs in *Arabidopsis* galls formed by *Meloidogyne javanica*: a functional role for miR390 and its TAS3-derived tasiRNAs. New Phytol. 2016b; 209(4):1625–1640. [PubMed: 26542733]

Cabrera J, Díaz-Manzano FE, Barcala M, Arganda-Carreras I, de Almeida-Engler J, Engler G, Fenoll C, Escobar C. Phenotyping nematode feeding sites: three-dimensional reconstruction and volumetric measurements of giant cells induced by root-knot nematodes in *Arabidopsis*. New Phytologist. 2015a; 206(2):868–880. [PubMed: 25613856]

Cabrera, J., Díaz-Manzano, FE., Fenoll, C., Escobar, C. Chapter seven – Developmental pathways mediated by hormones in nematode feeding sites. In: Escobar, C., Fenoll, C., editors. Advances in botanical research. Elsevier Academic Press; 2015b. p. 167-188.

Cabrera J, Diaz-Manzano FE, Sanchez M, Rosso MN, Melillo T, Goh T, Fukaki H, Cabello S, Hofmann J, Fenoll C, et al. A role for LATERAL ORGAN BOUNDARIES-DOMAIN 16 during the interaction *Arabidopsis–Meloidogyne* spp. provides a molecular link between lateral root and root-knot nematode feeding site development. New Phytol. 2014; 203(2):632–645. [PubMed: 24803293]

Cardona A, Saalfeld S, Schindelin J, Arganda-Carreras I, Preibisch S, Longair M, Tomancak P, Hartenstein V, Douglas RJ. TrakEM2 software for neural circuit reconstruction. PLoS ONE. 2012; 7(6):e38011. [PubMed: 22723842]

Chandler JW. Founder cell specification. Trends in Plant Science. 2011; 16(11):607–613. [PubMed: 21924666]

Chapman EJ, Estelle M. Mechanism of auxin-regulated gene expression in plants. Annual Review of Genetics. 2009; 43:265–285.

Chapman EJ, Greenham K, Castillejo C, Sartor R, Bialy A, Sun TP, Estelle M. Hypocotyl transcriptome reveals auxin regulation of growth-promoting genes through GA-dependent and-independent pathways. PLoS ONE. 2012; 7(5):e36210. [PubMed: 22590525]

Chen X. A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. Science. 2004; 303:2022–2025. [PubMed: 12893888]

Chuck G, Meeley R, Irish E, Sakai H, Hake S. The maize *tasselseed4* microRNA controls sex determination and meristem cell fate by targeting *Tasselseed6*/indeterminate *spikelet1*. Nature Genetics. 2007; 39(12):1517–1521. [PubMed: 18026103]

Coego A, Brizuela E, Castillejo P, Ruíz S, Koncz C, Del Pozo JC, Piñeiro M, Jarillo JA, Paz-Ares J, León J. The TRANSPLANTA collection of *Arabidopsis* lines: a resource for functional analysis of transcription factors based on their conditional overexpression. Plant Journal. 2014; 77(6):944–953. [PubMed: 24456507]

Coelho RR, Vieira P, de Souza Júnior JDA, Martin-Jimenez C, De Veylder L, Cazareth J, Engler G, Grossi-de-Sa MF, de Almeida-Engler J. Exploiting cell cycle inhibitor genes of the KRP family to control root-knot nematode induced feeding sites in plants. Plant, Cell & Environment. 2017; 40(7):1174–1188.

Damiani I, Baldacci-Cresp F, Hopkins J, Andrio E, Balzergue S, Lecomte P, Puppo A, Abad P, Favery B, Hérouart D. Plant genes involved in harbouring symbiotic rhizobia or pathogenic nematodes. New Phytologist. 2012; 194(2):511–522. [PubMed: 22360638]

De Almeida-Engler, J., Vieira, P., Rodiuc, N., Grossi-de-Sa, MF., Gilbert, E. Chapter four – The plant cell cycle machinery: usurped and modulated by plant-parasitic nematodes. In: Escobar, C., Fenoll, C., editors. Advances in botanical research. Elsevier Academic Press; 2015. p. 91-118.

Díaz-Manzano FE, Barcala M, Engler G, Fenoll C, de Almeida-Engler J, Escobar C. A reliable protocol for *in situ* microRNAs detection in feeding sites induced by root-knot nematodes. Front Plant Sci. 2016a; 7:966. [PubMed: 27458466]

Díaz-Manzano FE, Olmo R, Cabrera J, Barcala M, Escobar C, Fenoll C. Long-term *in vitro* system for maintenance and amplification of root-knot nematodes in *Cucumis sativus* roots. Front Plant Sci. 2016b; 7:124. [PubMed: 26941745]

[accessed 5 April 2017] Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. [WWW document] URL http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3Al13002a

[accessed 5 April 2017] Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. [WWW document] URL http://eur-lex.europa.eu/legal-ontent/EN/TXT/?uri=CELEX%3A32009L0128

Escobar, C., Barcala, M., Cabrera, J., Fenoll, C. Chapter one – Overview of root-knot nematodes and giant cells. In: Escobar, C., Fenoll, C., editors. Advances in botanical research. Elsevier Academic Press; 2015. p. 1-32.

Fosu-Nyarko, J., Jones, MGK. Chapter fourteen - Application of biotechnology for nematode control in crop plants. In: Escobar, C., Fenoll, C., editors. Advances in botanical research. Elsevier Academic Press; 2015. p. 339-376.

Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, Garcia JA, Paz-Ares J. Target mimicry provides a new mechanism for regulation of microRNA activity. Nature Genetics. 2007; 39(8):1033–1037. [PubMed: 17643101]

Grunewald W, van Noorden G, Van Isterdael G, Beeckman T, Gheysen G, Mathesius U. Manipulation of auxin transport in plant roots during Rhizobium symbiosis and nematode parasitism. Plant Cell. 2009; 21(9):2553–2562. [PubMed: 19789282]

Guo X, Wang J, Gardner M, Fukuda H, Kondo Y, Etchells JP, Wang X, Mitchum MG. Identification of cyst nematode B-type CLE peptides and modulation of the vascular stem cell pathway for feeding cell formation. PLoS Pathogens. 2017; 13(2):e1006142. [PubMed: 28158306]

Hewezi T, Piya S, Qi M, Balasubramaniam M, Rice JH, Baum TJ. *Arabidopsis* miR827 mediates post-transcriptional gene silencing of its ubiquitin E3 ligase target gene in the syncytium of the cyst nematode *Heterodera schachtii* to enhance susceptibility. Plant Journal. 2016; 88(2):179–192. [PubMed: 27304416]

Hewezi, T., Baum, TJ. Chapter nine – Gene silencing in nematode feeding sites. In: Escobar, C., Fenoll, C., editors. Advances in Botanical Research. Elsevier Academic Press; 2015. p. 221-239.

Hewezi T, Howe P, Maier TR, Baum TJ. *Arabidopsis* small RNAs and their targets during cyst nematode parasitism. Molecular Plant–Microbe Interactions. 2008; 21(12):1622–1634. [PubMed: 18986258]

Hewezi T, Maier TR, Nettleton D, Baum TJ. The *Arabidopsis* microRNA396-GRF1/GRF3 regulatory module acts as a developmental regulator in the reprogramming of root cells during cyst nematode infection. Plant Physiology. 2012; 159(1):321–335. [PubMed: 22419826]

Huang G, Allen R, Davis EL, Baum TJ, Hussey RS. Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proceedings of the National Academy of Sciences, USA. 2006; 103(39):14302–14306.

Hussey RS, Barker KR. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Reporter. 1973; 57:1025–1028.

Jammes F, Lecomte P, de Almeida-Engler J, Bitton F, Martin-Magniette ML, Renou JP, Abad P, Favery B. Genome-wide expression profiling of the host response to root-knot nematode infection in *Arabidopsis*. Plant Journal. 2005; 44(3):447–458. [PubMed: 16236154]

Jisha V, Dampanaboina L, Vadassery J, Mithofer A, Kappara S, Ramanan R. Overexpression of an AP2/ERF Type Transcription Factor OsEREBP1 Confers Biotic and Abiotic Stress Tolerance in Rice. PLoS ONE. 2015; 10(6):e0127831. [PubMed: 26035591]

Jung JH, Seo YH, Seo PJ, Reyes JL, Yun J, Chua NH, Park CM. The *GIGANTEA*-regulated microRNA172 mediates photoperiodic flowering independent of *CONSTANS* in *Arabidopsis*. Plant Cell. 2007; 19(9):2736–2748. [PubMed: 17890372]

Karczmarek A, Overmars H, Helder J, Goverse A. Feeding cell development by cyst and root-knot nematodes involves a similar early, local and transient activation of a specific auxin-inducible promoter element. Mol Plant Pathol. 2004; 5(4):343–346. [PubMed: 20565601]

Kyndt T, Goverse A, Haegeman A, Warmerdam S, Wanjau C, Jahani M, Engler G, de Almeida-Engler J, Gheysen G. Redirection of auxin flow in *Arabidopsis thaliana* roots after infection by root-knot nematodes. Journal of Experimental Botany. 2016; 67(15):4559–4570. [PubMed: 27312670]

Kyndt T, Vieira P, Gheysen G, de Almeida-Engler J. Nematode feeding sites: unique organs in plant roots. Planta. 2013; 238(5):807–818. [PubMed: 23824525]

Lee R, Baldwin S, Kenel F, McCallum J, Macknight R. FLOWERING LOCUS T genes control onion bulb formation and flowering. Nat Commun. 2013; 4:2884. [PubMed: 24300952]

Li X, Wang X, Zhang S, Liu D, Duan Y, Dong W. Identification of soybean microRNAs involved in soybean cyst nematode infection by deep sequencing. PLoS ONE. 2012; 7(6):e39650. [PubMed: 22802924]

Licausi F, Ohme-Takagi M, Perata P. APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytol. 2013; 199(3):639–649. [PubMed: 24010138]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods. 2001; 25(4):402–408. [PubMed: 11846609]

Luo Y, Guo Z, Li L. Evolutionary conservation of microRNA regulatory programs in plant flower development. Developmental Biology. 2013; 380(2):133–144. [PubMed: 23707900]

Martin A, Adam H, Diaz-Mendoza M, Zurczak M, Gonzalez-Schain ND, Suarez-Lopez P. Graft-transmissible induction of potato tuberization by the microRNA miR172. Development. 2009; 136(17):2873–2881. [PubMed: 19666819]

Martin-Trillo M, Lázaro A, Poethig RS, Gómez-Mena C, Piñeiro MA, Martinez-Zapater JM, Jarillo JA. EARLY IN SHORT DAYS 1 (ESD1) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates FLC accumulation in *Arabidopsis*. Development. 2006; 133(7):1241–1252. [PubMed: 16495307]

Mathesius U. Conservation and divergence of signalling pathways between roots and soil microbes – the Rhizobium–legume symbiosis compared to the development of lateral roots, mycorrhizal interactions and nematode-induced galls. Plant and Soil. 2003; 255(1):105–119.

Mishra S, Phukan UJ, Tripathi V, Singh DK, Luqman S, Shukla RK. PsAP2 an AP2/ERF family transcription factor from *Papaver somniferum* enhances abiotic and biotic stress tolerance in transgenic tobacco. Plant Mol Biol. 2015; 89(1-2):173–186. [PubMed: 26319514]

Mitchum MG, Wang X, Wang J, Davis EL. Role of nematode peptides and other small molecules in plant parasitism. Annual Review of Phytopathology. 2012; 50:175–195.

Mohd-Radzman NA, Laffont C, Ivanovici A, Patel N, Reid DE, Stougaard J, Frugier F, Imin N, Djordjevic MA. Different pathways act downstream of the peptide receptor CRA2 to regulate lateral root and nodule development. Plant Physiology. 2016; 171(4):2536–2548. [PubMed: 27342310]

Müller M, Munne-Bosch S. Ethylene response factors: a key regulatory hub in hormone and stress signaling. Plant Physiol. 2015; 169(1):32–41. [PubMed: 26103991]

Navarro C, Abelenda JA, Cruz-Oro E, Cuellar CA, Tamaki S, Silva J, Shimamoto K, Prat S. Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. Nature. 2011; 478(7367):119–122. [PubMed: 21947007]

Nakano T, Suzuki K, Fujimura T, Shinshi H. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiology. 2006; 140(2):411–432. [PubMed: 16407444]

Olmo R, Silva AC, Díaz-Manzano FE, Cabrera J, Fenoll C, Escobar C. A standardized method to assess infection rates of root-knot and cyst nematodes in Arabidopsis thaliana mutants with

alterations in root development related to auxin and cytokinin signaling. Auxins and Cytokinins in Plant Biology: Methods and Protocols. 2017:73–81.

Portillo M, Cabrera J, Lindsey K, Topping J, Andres MF, Emiliozzi M, Oliveros JC, Garcia-Casado G, Solano R, Koltai H, et al. Distinct and conserved transcriptomic changes during nematode-induced giant cell development in tomato compared with *Arabidopsis*: a functional role for gene repression. New Phytol. 2013; 197(4):1276–1290. [PubMed: 23373862]

Ripoll JJ, Bailey LJ, Mai QA, Wu SL, Hon CT, Chapman EJ, Ditta GS, Estelle M, Yanofsky MF. MicroRNA regulation of fruit growth. Nature Plants. 2015; 1:15036. [PubMed: 27247036]

Ripoll JJ, Roeder AH, Ditta GS, Yanofsky MF. A novel role for the floral homeotic gene *APETALA2* during *Arabidopsis* fruit development. Development. 2011; 138(23):5167–5176. [PubMed: 22031547]

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012; 9(7):676–682. [PubMed: 22743772]

Seeliger I, Frerichs A, Glowa D, Velo L, Comelli P, Chandler JW, Werr W. The AP2-type transcription factors DORNRÖSCHEN and DORNRÖSCHEN-LIKE promote G1/S transition. Molecular Genetics and Genomics. 2016; 291(5):1835–1849. [PubMed: 27277595]

Szakasits D, Heinen P, Wieczorek K, Hofmann J, Wagner F, Kreil DP, Sykacek P, Grundler FM, Bohlmann H. The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis* roots. Plant J. 2009; 57(5):771–784. [PubMed: 18980640]

Tellmann G. The E-Method: a highly accurate technique for gene-expression analysis. Nature Methods Application Notes. 2006:i–ii.

Todesco M, Rubio-Somoza I, Paz-Ares J, Weigel D. A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. PLoS Genet. 2010; 6(7):e1001031. [PubMed: 20661442]

Turck F, Fornara F, Coupland G. Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol. 2008; 59:573–594. [PubMed: 18444908]

Van Helden J. Regulatory sequence analysis tools. Nucleic Acids Research. 2003; 31(13):3593–3596. [PubMed: 12824373]

Wang Y, Wang L, Zou Y, Chen L, Cai Z, Zhang S, Zhao F, Tian Y, Jiang Q, Ferguson BJ, et al. Soybean miR172c targets the repressive AP2 transcription factor NNC1 to activate ENOD40 expression and regulate nodule initiation. Plant Cell. 2014; 26(12):4782–4801. [PubMed: 25549672]

Xu M, Li Y, Zhang Q, Xu T, Qiu L, Fan Y, Wang L. Novel miRNA and phasiRNA biogenesis networks in soybean roots from two sister lines that are resistant and susceptible to SCN race 4. PLoS ONE. 2014; 9(10):e110051. [PubMed: 25356812]

Yamaguchi YL, Ishida T, Sawa S. CLE peptides and their signaling pathways in plant development. J Exp Bot. 2016; 67(16):4813–4826. [PubMed: 27229733]

Yan Z, Hossain MS, Wang J, Valdes-Lopez O, Liang Y, Libault M, Qiu L, Stacey G. MiR172 regulates soybean nodulation. Mol Plant–Microbe Interact. 2013; 26(12):1371–1377. [PubMed: 23980625]

Yoo SK, Chung KS, Kim J, Lee JH, Hong SM, Yoo SJ, Yoo SY, Lee JS, Ahn JH. *CONSTANS* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to promote flowering in Arabidopsis. Plant Physiology. 2005; 139(2):770–778. [PubMed: 16183837]

Zhang B, Wang L, Zeng L, Zhang C, Ma H. *Arabidopsis* TOE proteins convey a photoperiodic signal to antagonize CONSTANS and regulate flowering time. Genes Dev. 2015; 29(9):975–987. [PubMed: 25934507]

Zhao W, Li Z, Fan J, Hu C, Yang R, Qi X, Chen H, Zhao F, Wang S. Identification of jasmonic acid-associated microRNAs and characterization of the regulatory roles of the miR319/TCP4 module under root-knot nematode stress in tomato. Journal of Experimental Botany. 2015; 66(15):4653–4667. [PubMed: 26002970]

Zhu QH, Helliwell CA. Regulation of flowering time and floral patterning by miR172. J Exp Bot. 2010; 62(2):487–495. [PubMed: 20952628]

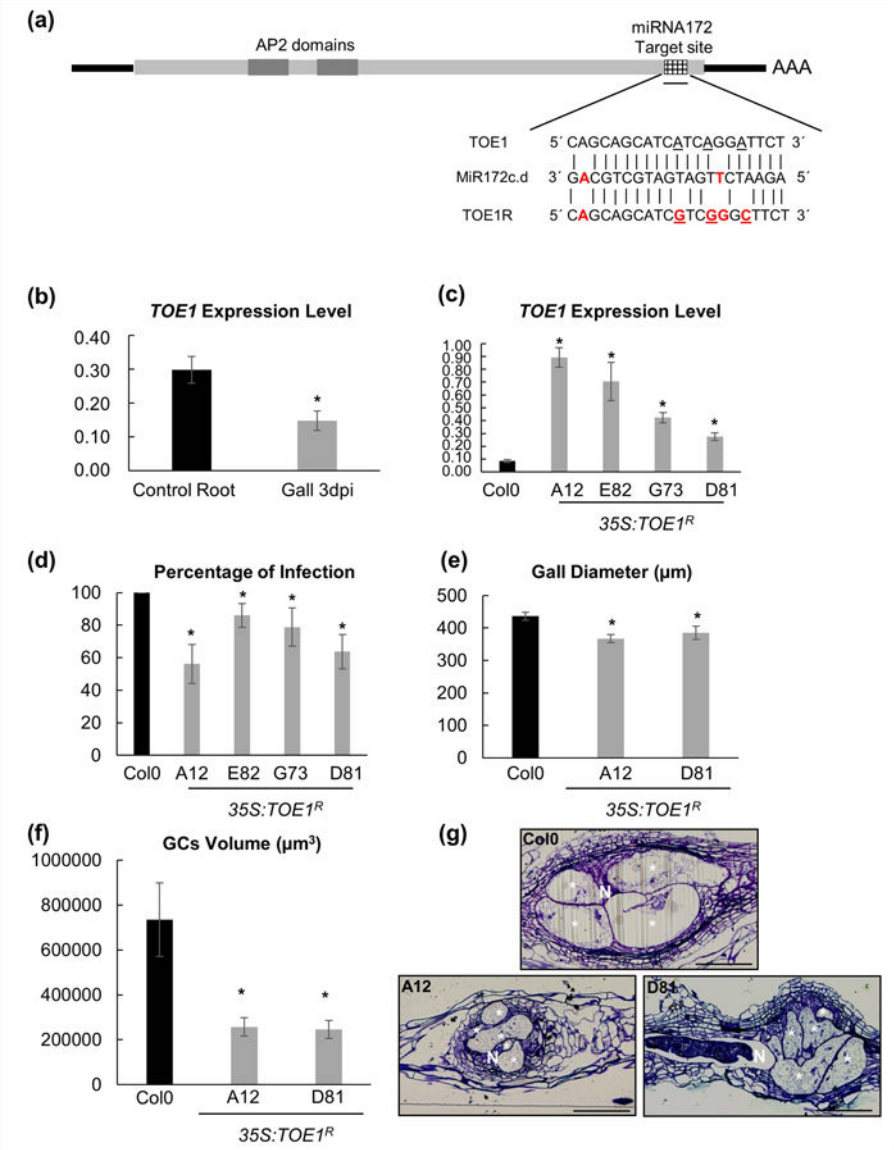

Fig. 1.

TARGET OF EAT1 (*TOE1*) is involved in the root-knot nematode infection. (a) Schematic representation of miRNA712c-d (*GACGTCGTAGTAGTTCTAAGA*) target sequence for *TOE1* (*CAGCAGCATCATCAGGATTCT*) and *TOE1* resistant (*CAGCAGCATCGTCGGGCTTCT*). Red bold text, nonmatching base pairs; underlined text, mutated TOE1 resistant base pairs respect to TOE1. (b) Q-PCR analysis of *TOE1* abundance in galls as compared to control uninfected root segments. *TOE1* decreased two-fold in galls induced by *Meloidogyne javanica*. (c) Q-PCRs of *35S:TOE1*^{R} lines of uninfected roots. The four lines showed higher *TOE1* transcript abundance compared to their endogenous control Columbia 0 (Col-0). (d) *In vitro* infection tests of four independent *35S:TOE1*^{R} lines challenged with *M. javanica*. The percentage of galls per main root was lower in all *TOE1* lines than in Col-0. (e) Galls diameter of *35S:TOE1*^{R}-A12 and *35S:TOE1*^{R}-D81. Both lines showed a smaller gall diameter than their control Col-0 at 14 d

post infection (dpi) ($P < 0.05$). (f) Giant cells (GCs) volume in *35S:TOE1*^{R}-A12 and *35S:TOE1*^{R}-D81 lines was smaller than that of Col-0 at 14 dpi ($P < 0.05$). (g) Representative pictures of *35S:TOE1*^{R} lines and Col-0 at 7 dpi Araldite® gall sections (2 μm). Statistical analysis was performed with three independent experiments per line using *T*-test, significant differences with Col-0 or corresponding controls are indicated by asterisks, $P < 0.05$; values are means ± SE. N, nematodes; bars, 100 μm. GCs are labelled with a white asterisk.

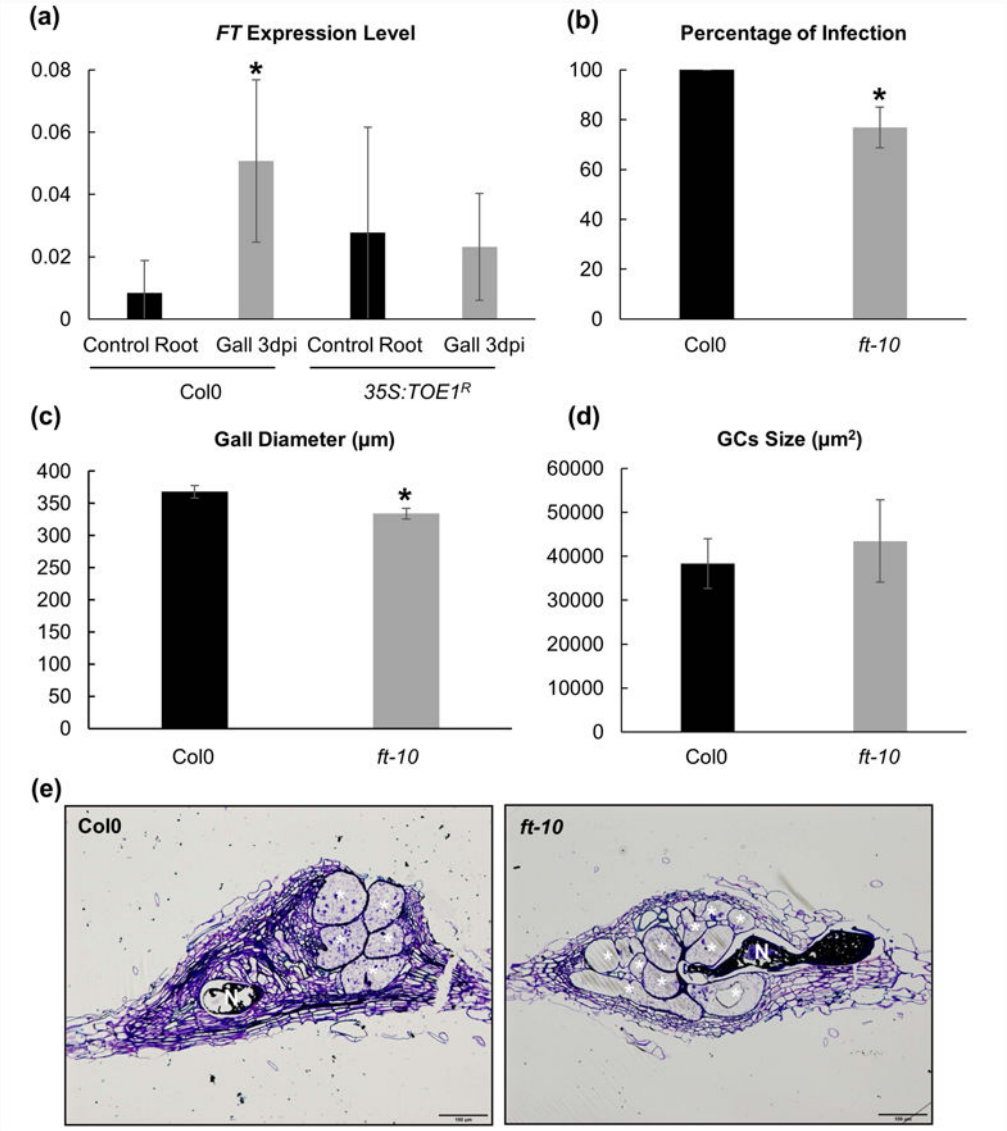

Fig. 2.

FLOWERING LOCUS T (*FT*) is functional during root-knot nematode interaction. (a) q-PCR analysis of the *FT* transcript in galls at 3 d post infection (dpi) compared to uninfected roots in background Col-0 and *35S:TOE1*^{R}-A12. *FT* was induced five-fold in galls formed by *Meloidogyne javanica* in the ecotype Col-0 as compared to uninfected control root samples, but not in *35S:TOE1*^{R} lines. (b) *In vitro* infection tests of *ft-10* mutant line. The percentage of galls per main root was significantly lower in the *ft-10* line than in the ecotype Col-0. (c) Galls diameter of *ft-10* mutant line showing smaller values than their control Col-0 at 14 dpi ($P < 0.05$). (d) Giant cells (GCs) volume measurement compared to Col-0 at 14 dpi. The volume occupied by the pool of GCs within the gall showed no significant differences between *ft-10* and Col-O. (e) Representative images of Araldite® gall sections (2 µm) of *ft-10* line and Col-0 at 14 dpi. Asterisks, significant differences (*T*-student; $P < 0.05$)

from three independent experiments. Values are means ±S E. N, nematodes; bars, 100 μm. GCs are labelled with a white asterisk.

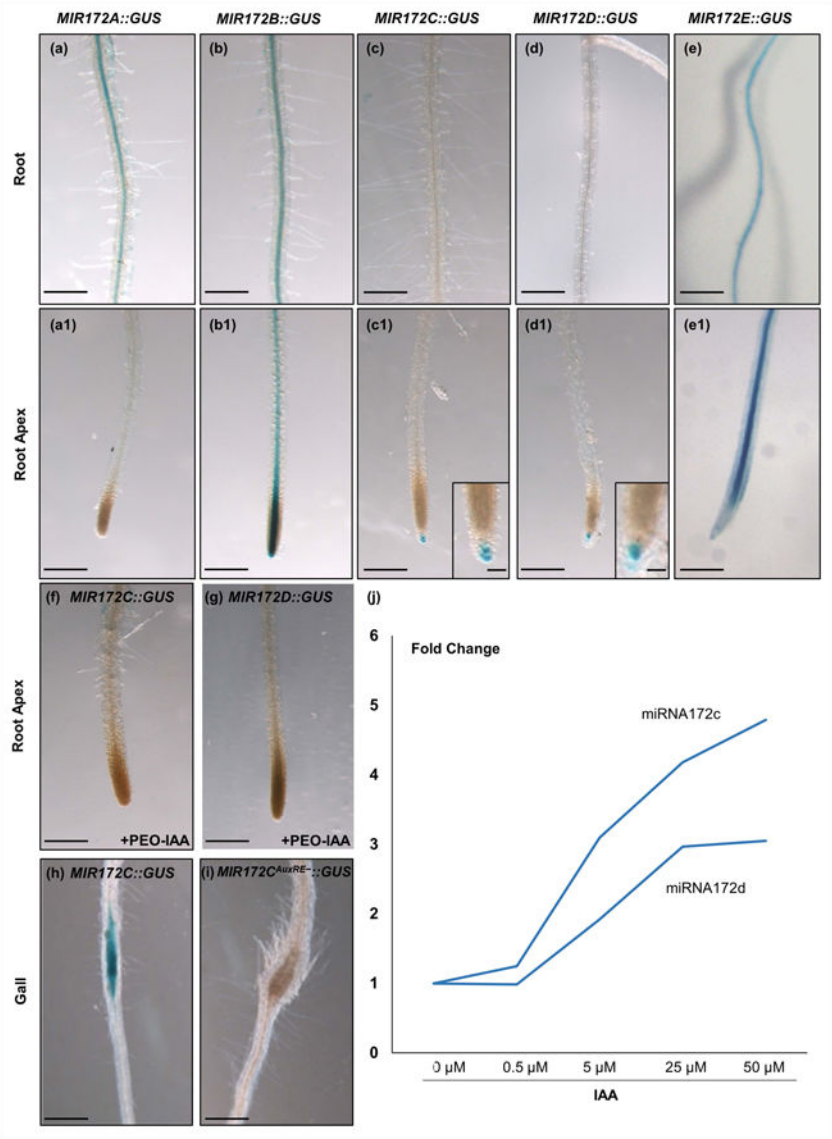

Fig. 3.

MIR172::GUS constructs are active in feeding sites and activity of miRNA172 promoter is modulated by auxin. (a–e) Basal GUS activity in roots and (a1–e1) zoom in detailed image of the elongation zone and root apex. Only C and D lines showed specific staining in the root apex (c1 and d1, respectively). The lines A (a, a1), B (b, b1) and E (e, e1) presented either no signal or it was centred in the vascular cylinder of the root. (f, g) GUS expression of *MIR172C::GUS* and *D* lines under auxin-inhibitor (PEO-IAA) treatment showing no GUS signal. (h, i) *MIMR172C::GUS* galls induced by *Meloidogyne javanica* at 5 d post infection showing restricted GUS signal in the center of the gall (h); by contrast, the *MIR172C*$^{AuxRE-}$*::GUS* line with two mutated *AuxRe* elements displayed no GUS signal (i). (j) Expression changes of MIR172 C and D after treatments with exogenous auxin (up to 50 µM). Values are means ± SE. Bars, 200 µm (except in magnifications, 50 µm).

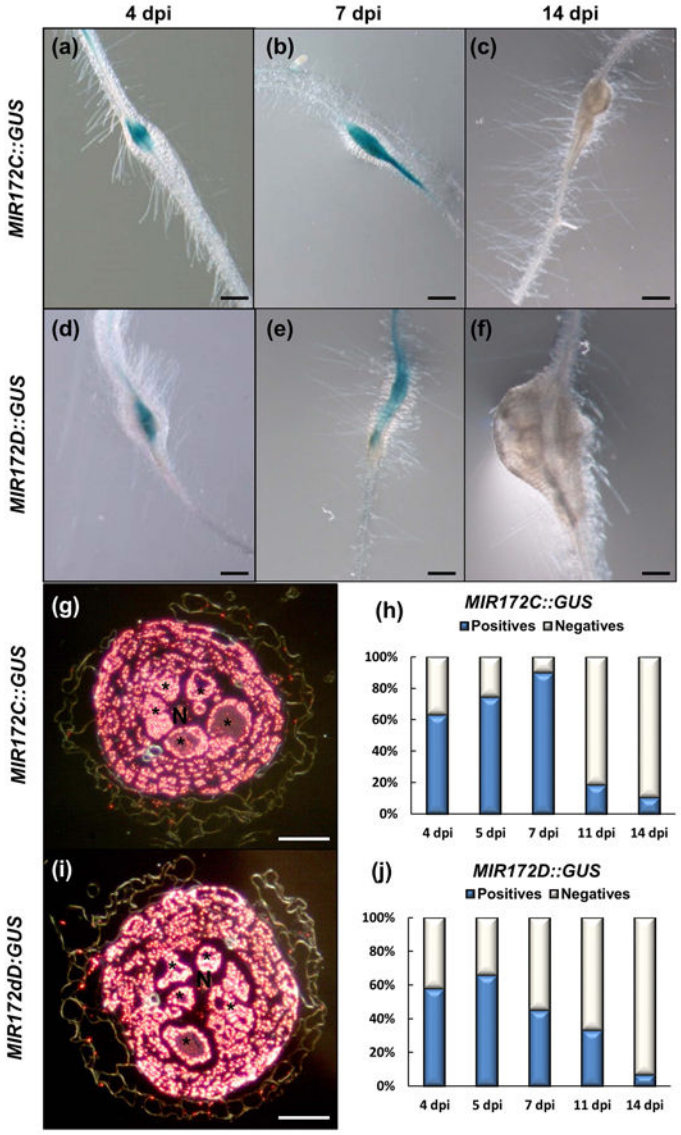

Fig. 4.

A timeline of *miRNA172c* and *miRNA172d* promoters indicated early activation patterns after root-knot nematode infection. (a–f) Representative pictures of GUS assays at 4, 7 and 14 d post infection (dpi), respectively of (a–c) *MIR172C ::GUS* and (d–f) *MIR172D ::GUS* lines in *Arabidopsis* galls induced by *Meloidogyne javanica.* (g, i) Dark field images of Araldite® cross sections of GUS stained galls at 7 dpi showing signal in the giant cells and adjacent cell layers within the vascular cylinder. Asterisks, giant cells; N, nematode. (h, j) Percentage of blue galls for (h) *MIR172C ::GUS* and (j) *MIR172D ::GUS*. Bars: (a–f) 200 μm; (g–i) 50 μm.

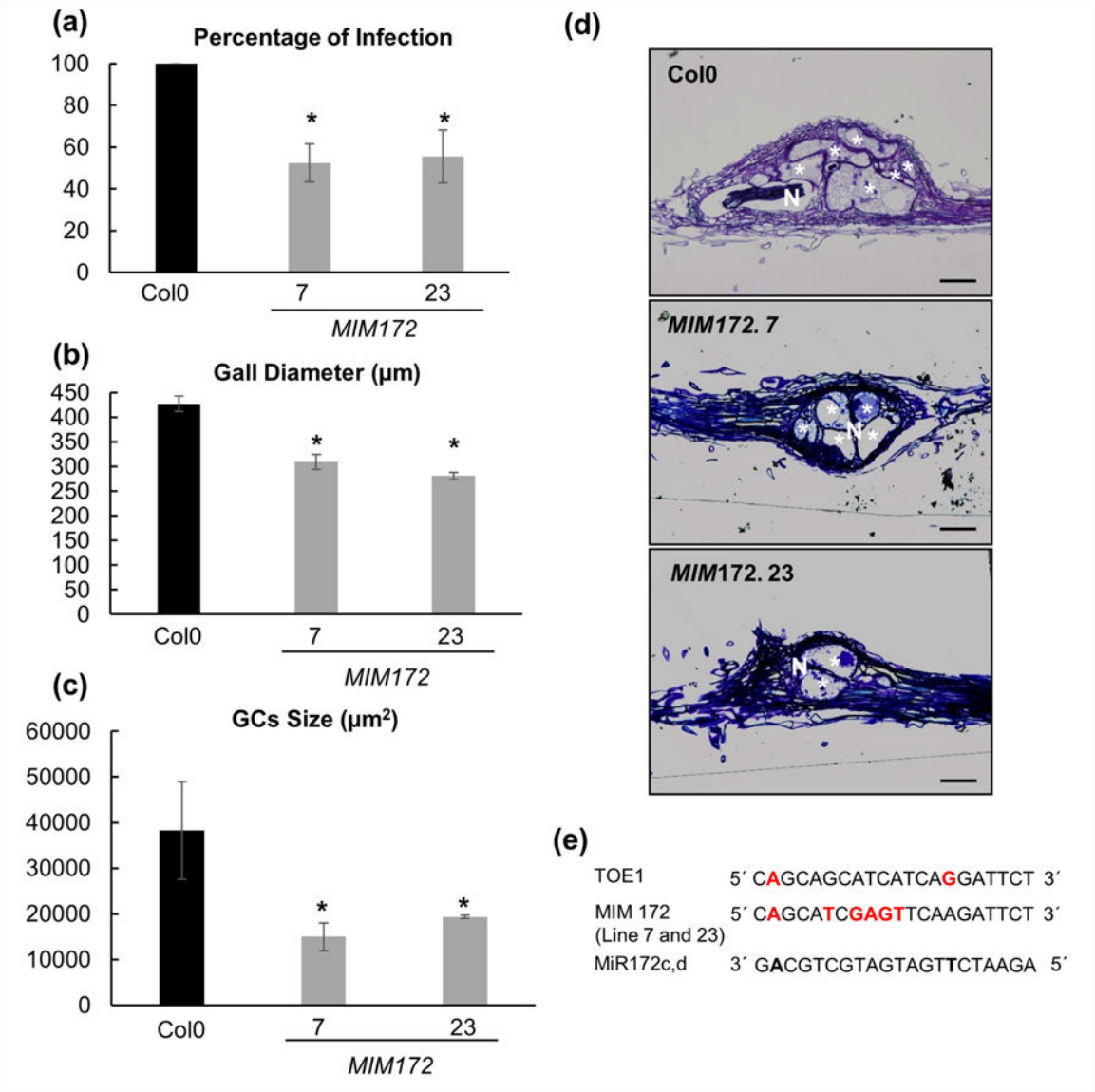

Fig. 5.

Target mimicry lines for miR172 show increased resistance to root-nematode infection. (a) *In vitro* infection tests of two independent mimicry (*MIM*) lines 7 and 23 showing significant differences ($P < 0.05$) between *MIM* lines and Col-0, as the percentage of galls is 40–50% lower in MIM lines as compared to controls. (b) Galls diameter of *MIM* lines and (c) volume of giant cells (GCs), were significantly lower than in Col-0 at 21 d post infection (dpi). (d) Representative pictures of Araldite® gall sections (2 µm) of *MIM* lines and Col-0 at 21 dpi. (e) Schematic comparison of miRNA172c-d, MIMICRY (MIM172) and miR172-TOE1 target sequences. In red bold, nonmatching base pairs with respect to miR172c-d. Asterisks, significant differences (*T*-Student; $P < 0.05$). Values are means ± SE. N, nematode; bars, 100 µm. GCs are labelled with a white asterisk.

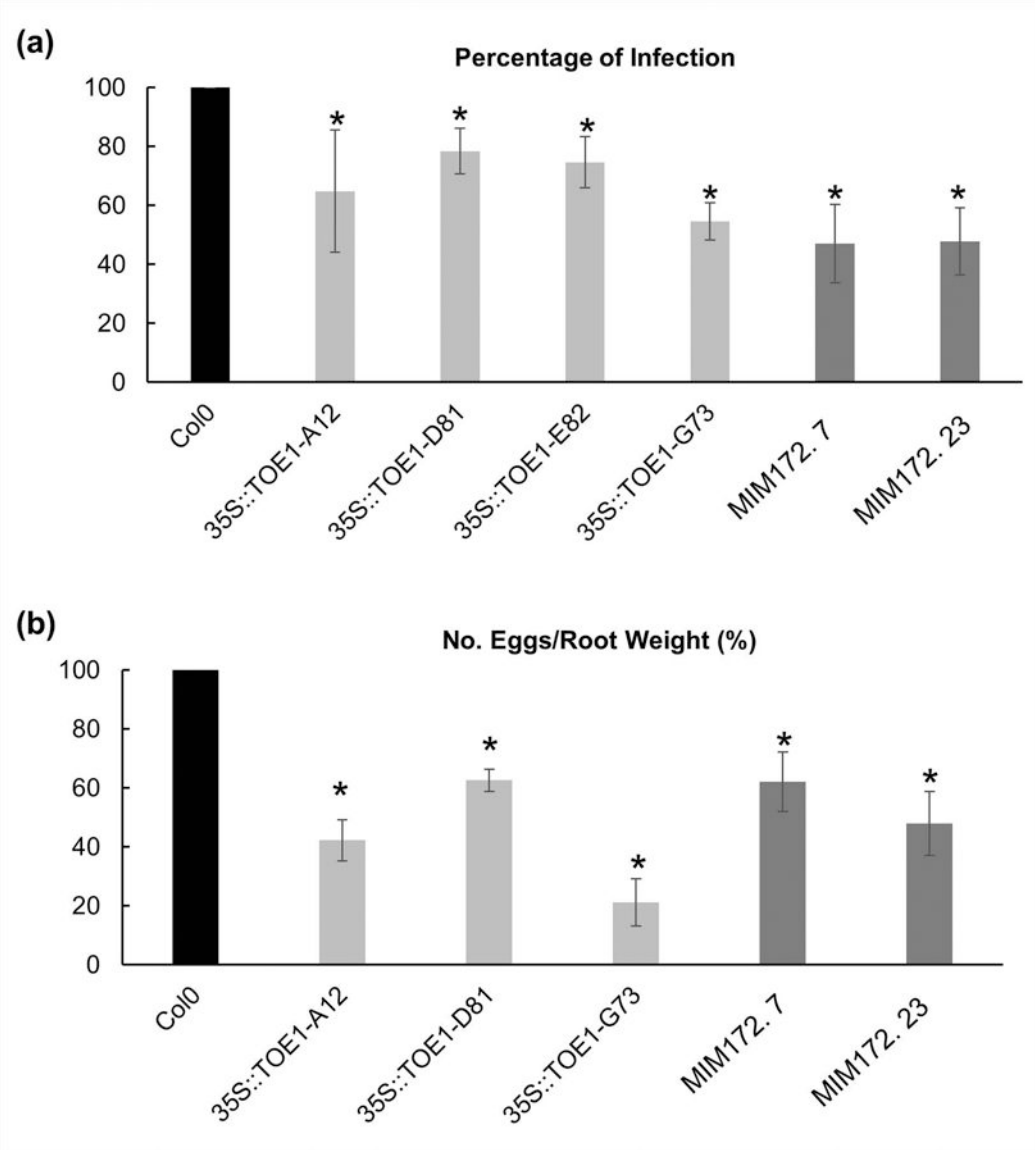

Fig. 6.

TOE1-miRNA172 resistant lines, *35S:TOE1*^{R} and target mimicry lines for miRNA172 show resistance to root-nematode infection under short days. (a) *In vitro* grown analysis of *35S:TOE1*^{R} lines A12, D81, E82 and G73 (in light grey); and mimicry (*MIM*) lines 7 and 23 (dark grey). The percentage of galls per main root was significantly lower in all lines studied than ecotype Col-0 (black). At least 80 plants per line were analyzed. (b) Soil reproduction tests for *35S:TOE1*^{R} lines A12, D81 and G73; and *MIM* lines 7 and 23. The percentage of number of eggs per root weight was lower in all lines than in Col-0 ($P < 0.05$). At least 30 plants per line were analyzed. Asterisks, significant differences (*T*-Student; $P < 0.05$). Three independent experiments were performed for each treatment. Values are means ± SE.

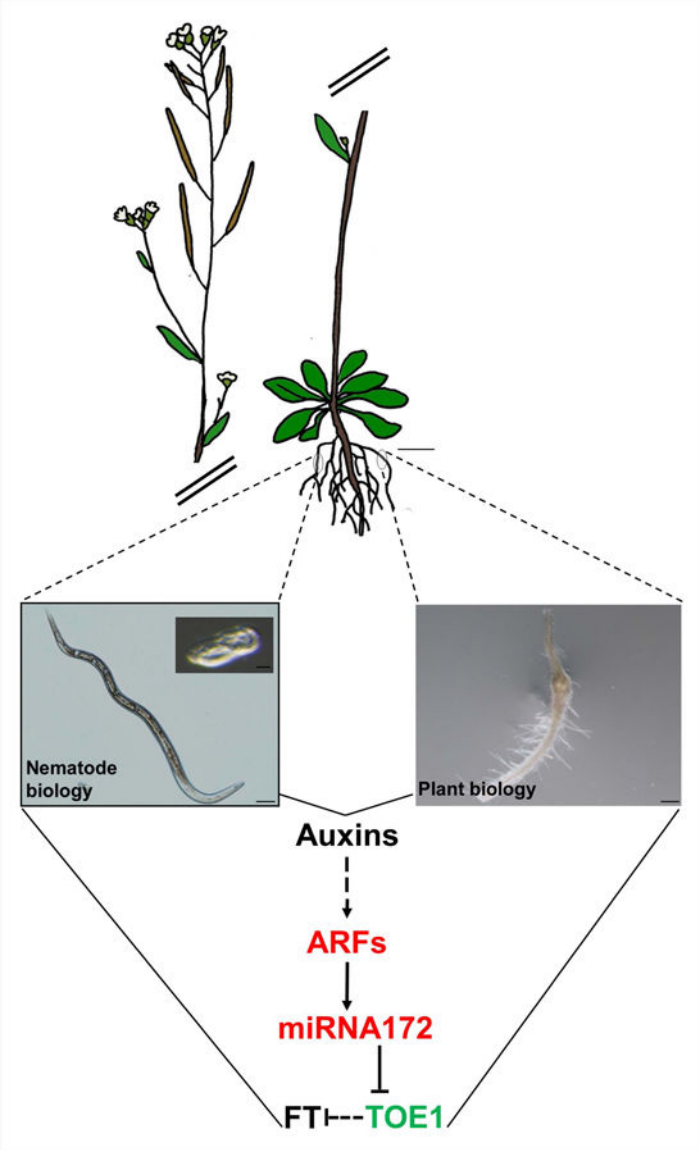

Fig. 7.

Schematic model of the miRNA172/TOE1/FT function and regulation during the plant–nematode interaction. As a result of the cross talk between *Arabidopsis thaliana* and *Meloidogyne* spp. hormonal signaling pathways such as auxins should be altered. Auxins are a positive signal for miRNA172d and c accumulation through auxin response factors (ARFs) activation that induce the miRNA172c promoter, and probably through intermediate partners still not characterized. miRNA172 mediates gene silencing of *TOE1* that strongly correlates with the accumulation of *FT* transcripts, thus *TOE1* should regulates *FT* either directly or indirectly through other partners, also still not known. Bars: egg picture, 20 μm; nematode, 50 μm; gall, 200 μm; *Arabidopsis*, 1 cm. Red text, induced genes in the microarray of microdissected giant cells (GCs) according to Barcala *et al.* (2010); green text, repressed; black text, nondifferentially expressed.