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Host-delivered RNAi-mediated root-knot nematode resistance in *Arabidopsis* by targeting *splicing factor* and *integrase* genes

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Abstract Root-knot nematodes (RKNs) are one of the most important biotic factors limiting crop productivity in many crop plants. The major RKN control strategies include development of resistant cultivars, application of nematicides and crop rotation, but each has its own limitations. In recent years, RNA interference (RNAi) has become a powerful approach for developing nematode resistance. The two housekeeping genes, *splicing factor* and *integrase*, of *Meloidogyne incognita* were targeted for engineering nematode resistance using a host-delivered RNAi (HD-RNAi) approach. *Splicing factor* and *integrase* genes are essential for nematode development as they are involved in RNA metabolism. Stable homozygous transgenic *Arabidopsis* lines expressing dsRNA for both genes were generated. In RNAi lines of *splicing factor* gene, the number of galls, females and egg masses was reduced by 71.4, 74.5 and 86.6%, respectively, as compared with the empty vector controls. Similarly, in RNAi lines of the *integrase* gene, the number of galls, females and egg masses was reduced up to 59.5, 66.8 and 63.4%, respectively, compared with the empty vector controls. Expression analysis revealed a reduction in mRNA abundance of both targeted genes in female nematodes feeding on transgenic plants expressing dsRNA constructs. The silencing of housekeeping genes in the nematodes through HD-RNAi significantly reduced root-knot nematode infectivity and suggests that they will be useful in developing RKN resistance in crop plants.

Keywords *Arabidopsis thaliana* · Host-delivered RNA interference (HD-RNAi) · *Integrase* gene · Nematode resistance · Root-knot nematode · *Splicing factor* gene

Introduction

Root-knot nematodes (RKNs), classified in the genus *Meloidogyne*, are commonly considered sedentary parasites of the plants. Approximately 100 species of the *Meloidogyne* are distributed worldwide having a wide host range. The most widespread species are *M. incognita, M. javanica, M. arenaria* and *M. hapla*. RKNs are among the most destructive plant pathogens producing some of the most devastating symptoms that account for an estimated worldwide crop loss of hundreds of billions of dollars per year (Abad et al. 2008; Chitwood 2003; Elling 2013).

In the past few years, several strategies have been used to control RKN infection, including cultural, chemical and biological control. In tomato, the *Mi-1* gene confers genetic resistance to three species of RKN (Vos et al. 1998), but this resistance breaks down under high temperatures

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(Williamson 1998). However, recently, resistance broken by short-term heat stress was reported to recover over time (Carvalho et al. 2015). Despite the effectiveness of the various strategies against nematodes, each has drawbacks; hence, a novel approach is required to control these parasites. RNA interference (RNAi) is a process in which dsRNA that corresponds to a particular gene is processed by Dicer to generate siRNAs, that in turn further activate the silencing complex (RISC) and recognize target mRNA and either degrade or prevent its translation into proteins. In 1998, Fire et al. reported silencing in *Caenorhabditis elegans* Maupas, using dsRNA and suggested it as a potential tool to study gene function by suppressing expression.

The RNAi mechanism can be used against nematode infection through a process called host-delivered RNAi (HD-RNAi); dsRNA corresponding to a specific nematode gene is expressed through host-plant mediated siRNA production. RNAi is triggered on the basis of homology between siRNA and the target mRNA upon nematode feeding.

The target gene can be chosen on the basis of its functional role in vital processes such as motility, feeding, development, mRNA metabolism, neurotransmission and different nematode effectors. Selection of the target gene is an important aspect of HD-RNAi that plays a key role in avoiding off-target effects. The criteria for effective target gene selection are that: (1) target gene sequences be discrete enough to ensure that the RNAi is nematode specific; and that (2) the target gene be conserved in various plant-damaging RKN species but absent in nontarget species such as chordates, plants, annelids, insect pollinators and mollusks (Danchin et al. 2013).

Numerous reports illustrate the success of RNAi against RKN infection (Bakhetia et al. 2008; Dinh et al. 2014a, b; Dong et al. 2016; Dutta et al. 2015; Fairbairn et al. 2007; Huang et al. 2006; Ibrahim et al. 2011; Jaouannet at al. 2013; Klink et al. 2009; Li et al. 2010; Lourenço-Tessutti et al. 2015; Niu et al. 2012, 2016; Papolu et al. 2013; Rosso et al. 2009; Sindhu et al. 2009; Steeves at al. 2006; Xue et al. 2013; Yadav et al. 2006). Varying levels of resistance have been demonstrated in different studies, and genes involved in nematode parasitism, development and reproduction have been targeted in a wide variety of plant systems including *Arabidopsis*, tobacco, tomato and soybean (Tamilarasan and Rajam 2013).

Yadav et al. (2006) generated tobacco transgenic lines expressing dsRNA for the *splicing factor* and *integrase* genes of *M. incognita* that showed great potential as RNAi targets. *Splicing factor* and *integrase* genes target mRNA metabolism and impair the development of *M. incognita*. Gall formation and the number of females feeding on transgenic plants expressing dsRNA were significantly reduced. However, these two genes have not been evaluated in *Arabidopsis* plants, which is the major model plant for evaluating nematode resistance using the HD-RNAi approach. The use of *Arabidopsis* in the majority of the studies so far prompted us to evaluate these genes, which are very effective in inducing resistance in tobacco. Therefore, the present study was designed to generate and evaluate the stable transgenic *Arabidopsis* lines expressing dsRNA of *splicing factor* and *integrase* genes in terms of RKN resistance.

Materials and methods

Designing the RNAi vector

The *splicing factor* and *integrase* gene constructs were used (Yadav et al. 2006). For the expression of dsRNA in transgenic *Arabidopsis* plants, a 349 and 624 bp fragment of the *splicing factor* and *integrase* gene, respectively, were amplified in sense and anti-sense direction using gene-specific primers and cloned in pBC06 vector. The pBC06 was driven by the CaMV35S promoter, placed upstream of the intron of the *Arabidopsis* MADS-box gene (Y12776) flanked by two multiple cloning sites.

Maintenance of pure culture of root-knot nematode in tomato

A pure culture of root-knot nematode, *M. incognita* (Kofoid and White) Chitwood race 1, maintained on tomato plants, was obtained from Dr. Anil Sirohi, Division of Nematology, Indian Agricultural Research Institute (IARI), New Delhi in March 2008 and maintained on tomato (*Solanum lycopersicum* L.) plants in a greenhouse of IARI, New Delhi. Originally, the nematodes were collected from the brinjal field of the Division of Vegetable Science, ICAR-IARI, Pusa Campus, New Delhi during August 2006. For assessing culture purity, a single egg mass and its corresponding female were harvested and gently washed with tap water. The nematode was identified using sequence-characterized amplified region (SCAR) primers (Zijlstra et al. 2000) and the perineal pattern on the female (Eisenback et al. 1985). After confirmation of the species as *M. incognita*, a single egg mass was allowed to hatch, and second-stage juveniles (J2s) were used to generate a pure culture on tomato plants. Two-week-old tomato seedlings, grown in cocopeat, vermiculite and sand (1:1:1), were infected with a pure culture of *M. incognita* (Atamian et al. 2012). After 6–7 weeks, egg masses were hand-picked and allowed to hatch at 28 °C in petri plates containing 10–15 mL of sterile water.

Development of transgenic plants containing *splicing factor* and *integrase genes*

Arabidopsis thaliana (Col-0) plants were transformed with *splicing factor* and *integrase* RNAi constructs using the floral dip method (Clough and Bent 1998). T_1 seeds were screened on a plate of MS medium containing kanamycin (50 µg/mL). Kanamycin-resistant plants were transferred to autoclaved soilrite and grown in a greenhouse at 22 °C with 16 h light/8 h dark. T_2 seeds were used to raise T_3 homozygous lines of transgenic plants. RNAi transgenic plant roots, shoots and leaves were compared with the wild type to check for any phenotypic variations that might incidentally modify the infection of nematodes.

Nematode infection assay of transgenic *Arabidopsis* lines

Arabidopsis thaliana seeds were surface-sterilized (2 min in 70% ethanol and 7 min in 0.1% mercuric chloride + 0.1% SDS) and washed three times with sterile distilled water to remove residual solutions and vernalized for 72 h at 4 °C before germination on Gamborg's B-5 medium. Plates were maintained at 22 °C under a 16 h light/8 h dark. After 18 days of growth, wild-type and transgenic plants were uprooted and transferred to a 24-well tray, with one plant per well, of the vermiculite, cocopeat and sand mix. The trays were sealed with cling film and allowed to grow in a growth chamber with 16 h light/8 h dark. After 1 week, the roots of each plant were inoculated with freshly hatched 1000 second-stage juveniles (J2s) of *M. incognita* (Atamian et al. 2012). After 6–7 weeks post inoculation, the plants were uprooted and washed with tap water to remove any growth mixture. The galls, females and egg masses on each plant root were counted and used as a measure of nematode vulnerability; means were calculated from 10 to 15 replicates per RNAi line. Images were captured using a Nikon DS-Fi2 camera attached to a microscope (Eclipse 80i and stereomicroscope SMZ1000, Tokyo, Japan), and the area and diameter of nematode females were recorded using microscope software.

RNA isolation and gene expression analysis of RNAi females

Expression of *M. incognita* genes were analyzed using semi-qRT-PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The RNA integrity was checked by formaldehyde gel electrophoresis with ethidium bromide dye. A Nanodrop Spectrophotometer 8000 (Thermo Scientific, Wilmington, DE, USA) was used to calculate purity ratios and quantify total RNA. Roughly 600 ng RNA was used to synthesize cDNA with a Protoscript M-MuLV first strand synthesis kit (NEB, Massachusetts, USA) and oligo d $(T)_{23}$VN primers. cDNA was normalized and re-quantified before semi-qRT-PCR. RT primers were designed from the cDNA sequence of selected genes using the online Primer3 portal (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000). PCR was carried out on a G-Storm, GS1 Thermal Cycler (Gene Technologies, Somerset, UK) with the following cycling conditions: initial melting temperature of 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min; and a final extension of 10 min at 72 °C. The PCR products were electrophoretically separated in an agarose gel and visualized using the G:Box gel documentation system (Syngene, UK).

Results

Infection assay of dsRNA-expressing transgenic *Arabidopsis* line

For the nematode infection assay, three independent, homozygous T_3*Arabidopsis* RNAi lines (*integrase*: E1, E2, E3; *splicing factor*: SF-E1, SF-E2 and SF-E3) were inoculated with 1000 J2s of *M. incognita* per plant. For each transgenic event, 10 replicates were used for the analysis, and each experiment was repeated twice. The response of these lines against the RKN was evaluated at 45 days post inoculation (dpi) by determining the number of gall, female and egg mass and compared with control *Arabidopsis* plants harboring the empty vector (Fig. 1). In the case of *splicing factor* RNAi lines (SF-E1, SF-E2 and SF-E3), significant reduction was observed in the number of gall (71.6–74.5%), female (68.1–71.4%) and egg mass (81.8–86.6%) compared with the empty vector control (Fig. 2a). The *integrase* gene RNAi lines had 63.3–66.8%, fewer galls, 55.5–59.5% fewer females and 49.2–63.4% fewer egg mass compared with the vector control (Fig. 2b). Altogether, transgenic lines of both genes had significantly reduced number of galls, females and egg masses, indicating a deleterious effect of silencing of these target genes on the growth and development of nematodes.

Morphological analysis of nematode female feeding on dsRNA-expressing transgenic *Arabidopsis* plants

For assessing the effect of host-delivered RNAi (HD-RNAi) on RKN, females were isolated from transgenic plants expressing dsRNA of *splicing factor* and *integrase* genes and stained with acid fuchsin. Females feeding on transgenic lines expressing *splicing factor* dsRNA or expressing *integrase* dsRNA were very small and distorted in shape compared with females isolated from

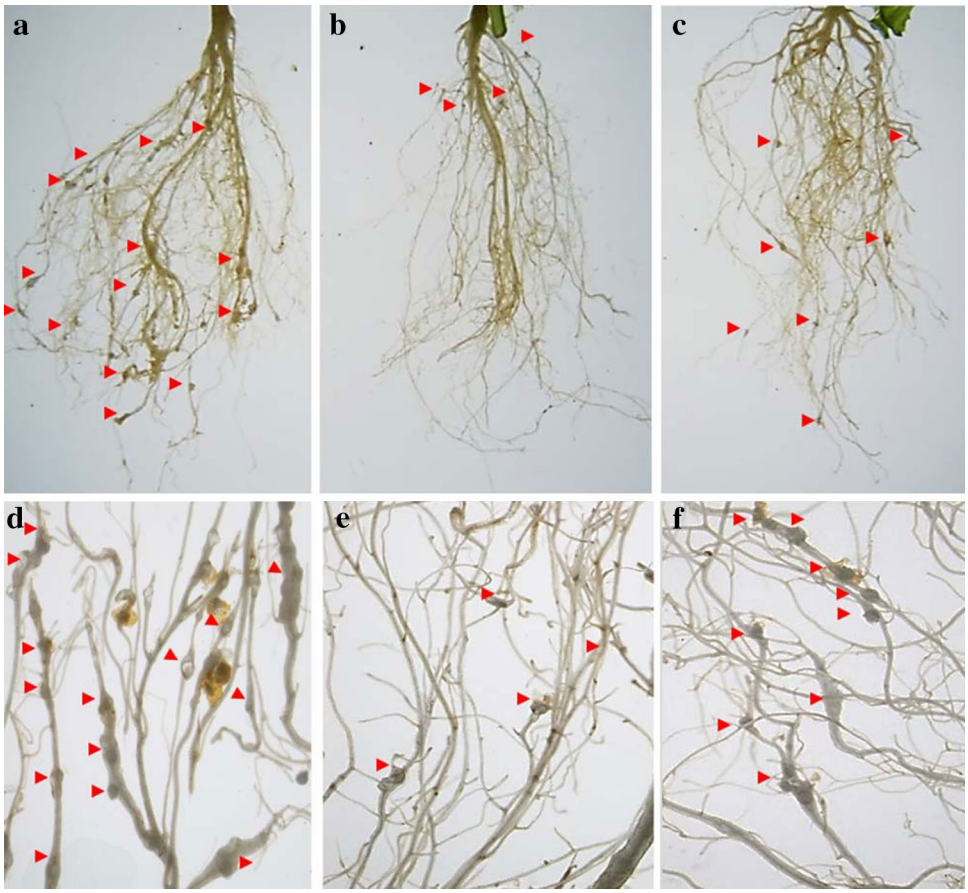

Fig. 1 Root-knot nematode infection assay in roots of *Arabidopsis* plants. **a** Empty-vector-transformed plant (control) showing numerous galls compared with the **b** transgenic line expressing *splicing factor* dsRNA (SF-RNAi) and **c** transgenic line expressing *integrase* dsRNA (Int-RNAi). **d–f** Enlargement of control, SF-RNAi and Int-RNAi plants. *Arrowheads* indicate galls

control plants (Fig. 3a–c). Females isolated from both the RNAi lines also had smaller surface area and diameter than did females feeding on control plants (Table 1).

Transcript downregulation in *M. incognita* females feeding on RNAi plants

At 45 dpi, females were isolated from the dsRNA expressing RNAi line of *splicing factor* and *integrase* genes. We isolated three batches (each contained 20 female nematodes) of nematodes for each gene construct along with their corresponding control females. Females were frozen in liquid nitrogen and used for RNA isolation. Semi-qRT-PCR was used to quantify the transcript level in the females feeding on the RNAi lines and control plants. Actin was used as an internal control gene to normalize the samples. For each of the RNAi lines, the expression of the respective gene was significantly downregulated in females extracted from transgenic plants compared with the control (Fig. 4a, b).

Discussion

Root-knot nematodes infect nearly all vascular plants and is a major problem in a wide variety of crops. In the decade since work on developing resistance against RKN using RNAi began, RNAi has become a potent tool for controlling infection by plant parasitic nematodes and implemented successfully in *C. elegans* to analyze the function of important genes (Fire et al. 1998; Kamath et al. 2003). There have also been several reports in different crops on the successful reduction of nematode infection, up to 90% in certain cases, using housekeeping genes, such as secretory genes, kinases, transcription factor, neuropeptides, peptides and genes involved in metabolism. In this study *splicing factor* and *integrase* genes, involved in nematode metabolism and development, were targeted using HD-RNAi approach.

Although our RNAi lines targeting *splicing factor* and *integrase* genes of nematode significantly reduced (49–86%) the number of galls, females and egg masses compared with the empty vector controls, Yadav et al. (2006) reported up to 90% reduction for *M. incognita* using the same genes in transgenic tobacco RNAi lines. Clearly, the resistance of tobacco and *Arabidopsis* vary greatly,

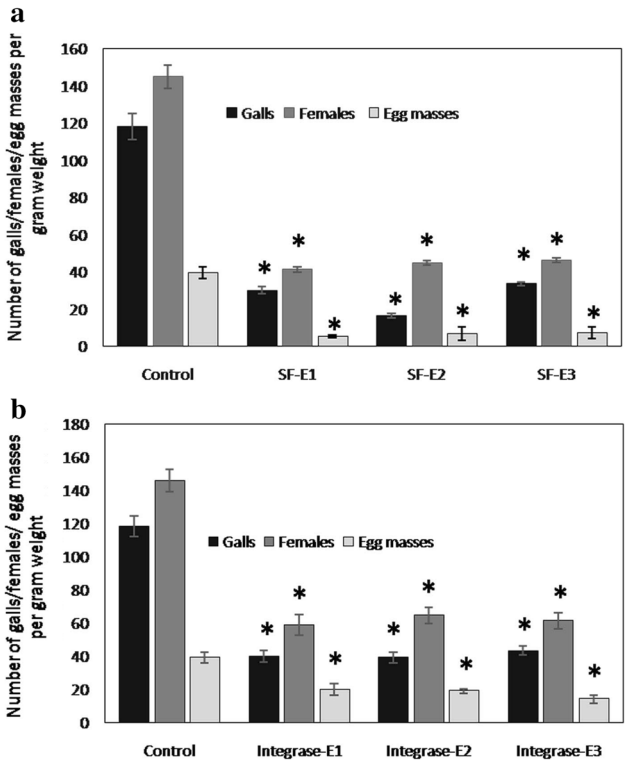

Fig. 2 RNAi lines with a reduction in number of galls, females and egg masses relative to the control. Three *independent lines* were evaluated for infection. **a***Splicing factor* RNAi lines (SF-E1, SF-E2 and SF-E3). **b***Integrase* RNAi lines (*integrase*-E1, *integrase*-E2 and *integrase*-E3). *Each bar* represents the mean ± SE ($n = 10$). An *asterisk* indicates statistically significant differences in one-way ANOVA and Tukey test ($p \leq 0.05$)

Table 1 Area and diameter of *Meloidogyne incognita* females isolated from control and RNAi lines

dsRNA construct	Area (μm^2)	Diameter (μm)
Control	124,834^a	398^b
dsRNA::*splicing factor*	16,744^c	138^d
dsRNA::*integrase*	63,112^e	275^f

Ten replicates were used for each treatment; *different letters* (*a, b, c, d, e* and *f*) indicate statistically significant differences in a one-way ANOVA and Tukey test ($p \leq 0.05$)

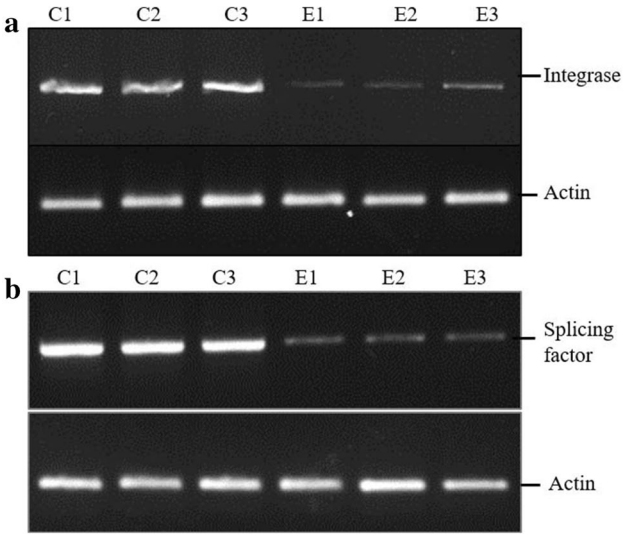

Fig. 4 Semi-quantitative RT-PCR analysis of targeted genes in root-knot nematode females feeding on control and RNAi plants. **a** Expression of *integrase* gene. **b** Expression of *splicing factor* gene. E1, E2 and E3 are independent transgenic lines of *integrase* and *splicing factor* RNAi lines, and C1, C2 and C3 are control plants containing empty vector. Actin was used as a housekeeping gene

underlying the uniqueness of each plant system and variation in their responses. However, it also brings forth the point that good candidate genes for RNAi, once identified, will be an immensely useful resource and the judicious use of the genes singly or in combination hold a key to generating nematode-resistant crops.

The root-knot nematode females isolated from the dsRNA expressing *Arabidopsis* lines of *splicing factor* and *integrase* genes were also smaller and their morphology distorted compared with the controls, similar to the elongated, transparent and developmentally weak females from

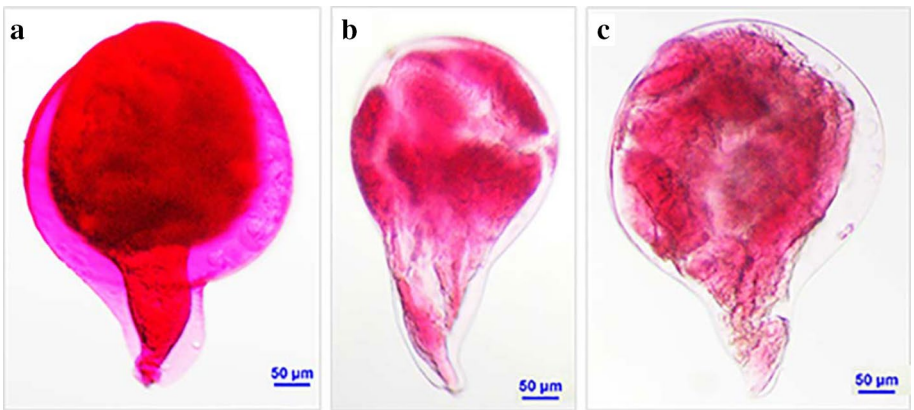

Fig. 3 Size and shape of females of root-knot nematode feeding on *Arabidopsis* control plants and transgenic lines expressing dsRNA at 45 dpi. **a** Females from control plants were large with a normal shape compared with **b** small females with an aberrant phenotype from the dsRNA::*splicing factor* transgenic line and **c** dsRNA::*integrase* transgenic line

the transgenic tobacco lines (Yadav et al. 2006). The use of these genes to control nematodes in agricultural crops appears to be a viable approach, and pyramiding of both genes may improve field control of nematodes. However, the host plant is a dynamic system, and each will behave in a unique manner in terms of resistance. Numerous candidate genes need to be evaluated with extensive molecular and infection analysis and select the best to develop nematode resistance in crop plants.

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