



# Kaolinite nanoclay-shielded dsRNA drenching for management of *Globodera pallida*: An environmentally friendly pest management approach

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## Abstract

*Globodera pallida*, an obligate sedentary endoparasite, is a major economic pest that causes substantial potato yield losses. This research aimed to study the effects of gene silencing of three FMRFamide-like peptides (FLPs) genes to reduce *G. pallida* infestation on potato plants by using kaolinite nanoclay as a carrier to deliver dsRNAs via drenching. A dsRNA dosage of 2.0 mg/ml silenced *flp-32c* by 89.5%, *flp-32p* by 94.6%, and *flp-2* by 94.3%. J2s incubated for 5 and 10 h showed no phenotypic changes. However, J2s of *G. pallida* efficiently uptake dsRNA of all targeted genes after 15 h of incubation. On the other hand, J2s that had been kept for 24 h had a rigid and straight appearance. Under fluorescence microscopy, all dsRNA-treated nematodes showed fluorescein isothiocyanate (FITC) signals in the mouth, nervous system, and digestive system. The untreated population of J2s did not show any FITC signals and was mobile as usual. The drenching of potato cultivar Kufri Jyoti with the dsRNA-kaolinite formulations induced deformation and premature death of J2s, compared with untreated J2s that entered J3 or J4 stages. This study validates that the nanocarrier-delivered RNAi system could be employed effectively to manage *G. pallida* infestations.

**Keywords** Exogenous dsRNA Delivery · FMRFamide-like peptides (FLPs) · *Globodera pallida* · Gene Silencing · Kaolinite · Potato

## Introduction

Plant-parasitic nematodes (PPNs) severely damage crops, resulting in significant annual losses (Bernard et al. 2017). PPNS are highly evolved pests with distinctive infection strategies. PPNS form feeding cells inside root cells by secreting effector proteins that can modify plant behaviour, suppress their immunity, and eventually promote nematode parasitism (Chen et al. 2018; Wen et al. 2021). Among various PPNS,

potato cyst nematodes (PCNs) are major quarantine pests (Hockland et al. 2012), hampering global potato production with an estimated yield loss of 9% (Turner and Rowe 2006). In India, PCNs were first detected in 1961 by Dr. F. G.W Jones in Tamil Nadu's Nilgiris hills. In the following years, PCNs have been reported from Tamil Nadu's Kodaikanal hills, Karnataka's adjoining hills, and Kerala's Idukki district. In recent years, several potato-growing areas in the northwestern Himalayas hills have been infested with PCNs. As a result, the Government of India (GOI) has prohibited the transportation of potato seeds from Himachal Pradesh, Uttarakhand, and Jammu & Kashmir to other states of the country (Gazette notification S.O. No. 5280 (E), dated 12th October 2018). PCNs include three pathotypes of *Globodera pallida* (Pa1-Pa3) and five pathotypes of *G. rostochiensis* (Ro1- Ro5) (Kort et al. 1977). In India, among all the pathotypes, Pa2 and Ro1 are the dominant ones in the Nilgiri and Kodaikanal hills (Prasad 2006). In the Kufri area of the Northern Himalayas, the Ro1, Ro4, and Ro5 pathotypes of *G. rostochiensis* and the Pa1 pathotype of *G. pallida* are the

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dominant ones (Unpublished data). Each cyst contains up to 100–500 eggs, which are easily dislodged when harvested. PCNs-infested plants exhibit stunted growth and reduced tuber yield, which can result in crop failure (Bairwa et al. 2022a). Nematicides like Fenamiphos, Ethoprophos, Cadusafos, Fosthiazate, Fluensulfone, and Nemathorin have been widely used to control PCNs; however, their adverse effects cannot be ignored (Damalas and Koutroubas 2016). Other options for managing PCNs include crop rotation, intercropping, trap cropping, deep ploughing, and host resistance (Bairwa et al. 2021). However, these cysts can survive in the soil for over twenty years even without the host crop; thus, it is extremely challenging to eliminate PCNs once they have been established (Gartner et al. 2021).

Understanding how nematodes successfully infect and parasitize a host can provide clues to halting physiological processes essential during their life cycle. Thus, targeting genes important for PCNs survival can control their spread. The availability of genome sequences of *G. pallida* with several effector proteins offers a valuable repository for identifying genes responsible for nematode parasitism (Cotton et al. 2014). As the nervous system of nematodes extensively relies on neuropeptides for communicating among nerve cells, any interference with the neuropeptide signaling system could lead to dysregulation of their typical behavior, offering an appealing strategy for nematode control. FMRFamide-like peptides (FLPs) are one of the largest and most diverse family of neuropeptides that binds to G-protein-coupled receptors or peptide-gated ion channels and modulate sensory and motor functions of nematodes (Peymen et al. 2014). Several FLPs genes have been characterized in *G. pallida* and silencing of any of these genes would lead to abnormal behavioral phenotypic change in J2s (Kimber et al. 2001, 2007; Atkinson et al. 2013).

RNA interference (RNAi) involves the interception and destruction of mRNA, leading to post-transcriptional silencing of genes, and thus helps in gaining insights into plant physiological processes, pathogen/pest resistance, and gene function (Fletcher et al. 2020; Niu et al. 2024). RNAi phenomenon was first discovered in *Caenorhabditis elegans*, a free-living model nematode, which showed inactivation of genes involved in parasitism and development through the degradation of mRNA (Fire et al. 1998). Since its first description in *C. elegans*, RNAi has been demonstrated in a wide range of organisms, including mammals (Qiu et al. 2017), plants (Kumar et al. 2023), fungi (Bhagta et al. 2023), viruses (Routhu et al. 2022), and insects (Laudani et al. 2017). Because PPNs are obligate parasites that require living hosts for feeding, delivering dsRNA to them has proven more challenging. For effective silencing of the targeted genes, it is crucial to deliver dsRNA to site-specific cells. Therefore, developing a long-term, reliable, and effective delivery method is a prerequisite. The advancement in

nanoparticle-mediated delivery dsRNA technology holds significant promise for RNAi-based pest control. Since nanoparticles encapsulate and shield dsRNA molecules from harsh environmental conditions and facilitate their translocation, they have been widely applied as carriers (Molesini et al. 2022; Sundaresha et al. 2022a). The nanoclay mineral kaolinite is composed of an octahedral sheet of alumina linked by oxygen atoms to a tetrahedral sheet of silica. Drugs and genes have been effectively delivered into cells using kaolinite as a carrier (Zhang et al. 2016).

Among various FLPs genes, *flp-32* [*flp-32c* (complete cds) and *flp-32p* (partial cds)] genes are expressed in the brain and ventral cord which encodes for AMRNALVR-Famide peptide (Atkinson et al. 2013), whereas *flp-2* is an arousing neuropeptide which encodes for KNKFEFIRFamide peptide (Kimber et al. 2001). As of yet, nanoclay-based dsRNA drenching has not been successfully employed to inhibit the infestation of *G. pallida* in potatoes. In the present study, we chose three pathogenicity genes, *flp-32c*, *flp-32p*, and *flp-2* to evaluate the silencing effect of drenching of kaolinite-shielded dsRNA on the potato crop.

## Materials and methods

### *G. pallida* juveniles (J2s) collection

The potato cultivar Kufri Jyoti was used to maintain the pure population of *G. pallida* in the glass house condition at the Indian Council of Agricultural Research-Central Potato Research Institute (ICAR-CPRI), Shimla, Himachal Pradesh, India. The *G. pallida* cysts were extracted from infested soil as per the methodology given by Fenwick (1940). Fenwick can used for the extraction of cysts from soil is based on the floating property of dried cysts. Dry cysts possess air pockets, enabling them to remain buoyant and float on top of the water surface. A sloping collar is present below the rim of Fenwick can. The air-dried soil samples were washed with a strong jet of water through the top sieve. The water was run until the overflow was clean. Heavy soil particles settled at the bottom whereas cysts and light soil debris rose to the top. A sieve of 840  $\mu\text{m}$  was placed over 250  $\mu\text{m}$  sieve to remove the debris and subsequently, the cysts were collected on a 250  $\mu\text{m}$  sieve. Cysts were surface sterilized and kept for hatching in the root leachates of the 4-week-old potato plant. Hatched J2s were collected, washed with nuclease-free water, and used for RNAi experiments.

### Selection of siRNA targets

The three pathogenicity genes, i.e., *flp-32c*, *flp-32p*, and *flp-2* that modulate sensory and motor functions in *G. pallida* were selected for silencing. The GenScript siRNA Target

Finder tool (<https://www.genscript.com/tools/sirna-target-finder>) was used to identify potential siRNA target regions and off-target sequences in each gene.

### dsRNA synthesis under in vitro conditions

200 *G. pallida* cysts were used for RNA isolation using Qiagen's RNeasy Mini Kit. RNA (1 µg) was used for cDNA synthesis using a High-Capacity Reverse Transcriptase cDNA Synthesis Kit from Applied Biosystems. PCR primers flanked by T7 promoter sequence were used for the amplification of selected regions of targeted genes (Table 1). The amplified fragments were cloned between NotI and XhoI into the L4404 vector carrying the T7 promoter. Ambion's MEGAscript® RNAi Kit was used for dsRNA synthesis by using the purified amplicons as a template (Sundaresha et al. 2022a).

### Induction of RNAi in *G. pallida* J2s via soaking

RNAi soaking was performed in accordance with Urwin et al. (2002). About 600 *G. pallida* J2s were soaked in 0.5, 1.0, 1.5, and 2.0 mg/ml dsRNA and M9 buffer containing 50 µl of 50 mM octopamine and 2 µl of fluorescein isothiocyanate (FITC) and then incubated for 5, 10, 15, and 24 h on a benchtop shaking incubator (NB-205, N-BIOTEK) at a speed of 10 rpm. J2s suspended in standard feeding components without dsRNA were kept as a control. FITC ingestion along with dsRNA was monitored, and photomicrographs of treated and untreated nematodes were captured using a fluorescence laser scanning microscope (Nikon Eclipse E600) with fluorescence set to default settings with excitation and emission wavelengths of 400 nm and 490 nm, respectively. The soaking experiment was carried out in four replicates.

### Large-scale synthesis of dsRNA for plant bioassay

HT115 (DE3 strain) expression cells of *Escherichia coli* were transformed with positive clones carrying dsRNA constructs of the selected genes. 0.5 mM isopropyl

β-D-1-thiogalactopyranoside (IPTG) was added to the T7 cell culture and kept in a shaking incubator at 37 °C for 4 h at 90 rpm to induce cell growth. Upon centrifugation at 4000 rpm at 4 °C for 15 min, the pellet was suspended in lysis buffer (10 mM Tris, 1 mM EDTA, and pH 7.5) (Huang and Lieberman 2013). After lysis, T7 cells expressing small RNA that carried a target gene of interest were subsequently mixed with a 1% sonified kaolinite nanoclay solution. The kaolinite nanoclay was obtained from M/s EICL Pvt Ltd in Thiruvananthapuram, Kerala, India.

### Plant bioassay with dsRNA molecules

Thirty potato plants of the *G. pallida*-susceptible cultivar Kufri Jyoti were grown using sterilized FYM and sandy loam soil (1:2) under glass house conditions for three weeks. dsRNA-treated 300 *G. pallida* J2s were inoculated in these plants. Untreated plants were used as controls. Plants were kept in a glass house for another 15 days and observed for nematode invasions. Plants were gently uprooted and roots were cleaned under running tap water. The roots were stained for 1–3 min with acid fuchsin lactophenol (0.1%), washed under running water, and then soaked in plain lactophenol to remove the stain (McBeth et al. 1941). Root sections in glass petri plates were observed under a stereo-zoom microscope and J2s inside the root segments were counted. The experiment was repeated three times. Statistical Analysis System (2011) (v9.3) software was used to analyze the data.

### qPCR of plant roots after inoculation with dsRNA-treated and untreated J2s

All three genes were analyzed by qPCR with Applied Biosystems' StepOne Real-Time PCR System. The RNeasy Mini Kit from Qiagen was used to isolate RNA from all treated and untreated plant samples. 1 µg of RNA was used for cDNA synthesis with Applied Biosystems' High-Capacity Reverse Transcriptase cDNA Synthesis Kit. qPCR was carried out in 20 µl containing 1 µl cDNA,

**Table 1** Primers used for amplification of targeted genes in *Globodera pallida*

Targeted genes	GenBank accession number	Primer sequence flanked by the T7 promoter sequence	GC (%)	Tm (°C)	Amplicon size (bp)
<i>flp-32c</i>	JQ685132	F-ATGGCCTCTCTGTGCGTTGTGCGGA-TAATACGACTCACTATAGG	58.30	64.7	372
		R-AGAATGGCACCGATGGTCAACTG-TAATACGACTCACTATAGG	54.20	61.3	
<i>flp-32p</i>	JQ685131	F-TTCTCGCTTCTTCTACGCCGTGC-TAATACGACTCACTATAGG	56.50	62.0	220
		R-GTCCATGCCAATGTGCGCAA-TAATACGACTCACTATAGG	57.10	62.1	
<i>flp-2</i>	AJ300488	F-ATGATCCAACAAATCCGACTGCA-TAATACGACTCACTATAGG	41.70	57.9	233
		R-GCATCTCTGTACGTTTGAAGGAGC-TAATACGACTCACTATAGG	33.90	58.8	

10 µl Applied Biosystems SYBR Green Master Mix, 1 µl of forward primer, 1 µl of reverse primer, and 7 µl of nuclease-free water. Details of the primers designed for the targeted genes with Primer3Plus software (<http://www.primer3plus.com/>) are given in Table 2. An analysis of the expression of three targeted genes was conducted using the elongation factor as a housekeeping gene. The housekeeping genes' average Ct values were compared to each gene's expression using ABI 7300 System Sequence Detection Software (v1.4). The  $2^{-\Delta\Delta CT}$  method was used to quantify gene transcript levels (Livak and Schmittgen 2001). Control samples were given a mean relative expression level of 1.0 and all lines were computed based on that value. Three biological replicates were averaged from the experimental data. For all tested lines, standard deviations were calculated with SDSRQ Manager software (Applied Biosystems, USA). A statistical analysis was conducted using GraphPad PRISM 9.0 on the dsRNA-treated and control plants.

### Plant bioassay with dsRNA-kaolinite formulations

A plant bioassay was conducted to test the efficacy of the dsRNA-kaolinite formulations against *G. pallida* infection. One medium-sized Kufri Jyoti tuber was planted in each pot (30 cm × 15 cm) containing sterilized FYM and soil (1:2). The soil was regularly irrigated to maintain optimal moisture levels. The dsRNA-kaolinite formulations of the selected genes were drenched into three-week-old plants. After 48 h of first drenching, J2s were inoculated into the plants followed by second drenching of formulations after seven days. Untreated pots were drenched with 1% kaolinite solution only. Each treatment was carried out five times. The roots were collected 15 days after inoculation, stained, and examined for nematode infestation as described above. Statistical

significance was determined by one-way analysis of variance by using Statistical Analysis System (2011) (v9.3).

### qPCR of plant roots after inoculation with kaolinite-shielded dsRNA-treated and untreated J2s

Following the manufacturer's instructions, the RNeasy Mini Kit (Qiagen) was used to extract total RNA from treated and untreated roots collected after 15 days. As described above, cDNA was synthesized from 1 µg of total RNA using an Applied Biosystems Kit (USA). The expression levels of each gene were calculated based on the average Ct values of the housekeeping gene actin of *Globodera* spp.

## Results

### Validation of dsRNA synthesis

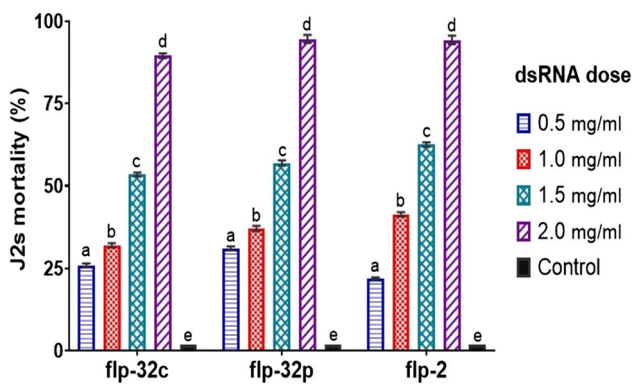
The siRNA targets (Fig. S1, Fig. S2, and Fig. S3) of the selected genes produced fragment sizes of 372 bp for *flp-32c* gene, 220 bp for *flp-32p* gene, and 233 bp for *flp-2* gene. Targeted genes were cloned into the L4404 vector and NotI and XhoI restriction enzymes were used to confirm that the targeted genes were incorporated into the vector (Fig. S4). dsRNA synthesis was validated by an intense smear pattern on the gel and a specific fragment size (Fig. S5).

### Soaking of *G. pallida* J2s in dsRNA solution using FITC as a marker

J2s were exposed to different dosages of dsRNA (0.5, 1.0, 1.5, and 2.0 mg/ml) with FITC and 50 mM octopamine and incubated for 5, 10, 15, and 24 h. 2.0 mg/ml of dsRNA after 15 h of incubation showed maximum silencing of *flp-32c*, *flp-32p*, and *flp-2* genes by 89.5%, 94.6%, and 94.3%, respectively (Fig. 1). J2s incubated for 5 and 10 h did not result in any phenotypic changes, while J2s kept for 24 h appeared rigid and straight, reflecting the movement being inhibited (data not shown). Under in vitro conditions, *flp-32c* and *flp-32p* dsRNA-treated J2s showed greater mobility than untreated controls; however, *flp-2* dsRNA-treated J2s displayed decreased mobility compared to untreated controls. *G. pallida* J2s in the untreated control remained mobile and active. All dsRNA-treated J2s exhibited FITC signals under fluorescence microscopy in the mouth, nervous system, and digestive system, whereas there was no FITC signal and normal mobility was observed in the untreated control population (Fig. 2).

**Table 2** qPCR primers used for expression analysis of targeted genes

Gene name	Primer sequence	GC (%)	Tm (°C)	Amplicon size (bp)
<i>flp-32c</i>	F-TATTCATGCCCGGACTTC	47.37	54.5	160
	R-AGTAAATGCGCACTGAGA AG	45.00	55.2	
<i>flp-32p</i>	F-TTCTTCTACGCCGTGCTT	50.00	53.6	153
	R-AAAAGCTTACCCGGACGA	50.00	53.6	
<i>flp-2</i>	F-GTCAAAACGAATGCACAG AA	40.00	53.2	161
	R-TCTCTGTACGTTGAAGGAG	45.00	55.2	
ACT	F-ATGTGYGAYGAR	42.86	55.9	250
	R-ATYTTYTCCATRTCRTCCCA	52.38	59.8	



**Fig. 1** Effect of different doses of dsRNA (0.5, 1.0, 1.5, and 2.0 mg/ml) on the silencing of *Globodera pallida* J2s in M9 buffer with 50  $\mu$ l of 50 mM octopamine and 2  $\mu$ l of fluorescein isothiocyanate (FITC) after 15 h of incubation. Standard feeding components without dsRNA acted as a control. Tukey's multiple comparison test indicates that mean values with the same letters on each bar are not significantly different ( $P < 0.05$ ). The error bars represent the mean  $\pm$  SEM

### Effect of dsRNA molecules on Kufri Jyoti plants

dsRNAs-treated J2s were inoculated in three-week-old plants. After 15 days of inoculation, we noted more infection points in *flp-32c* (73.9%) and *flp-32p* (76.8%) treated J2s than in untreated J2s (60.5%). Treated J2s were found deformed and prematurely dead inside the roots, compared to untreated J2s, which evolved to the J3 or J4

stage. Invasion studies of dsRNA of *flp-2*-treated J2s significantly reduced the infestation (49.5%) compared to the untreated J2s (60.5%) (Fig. 3a).

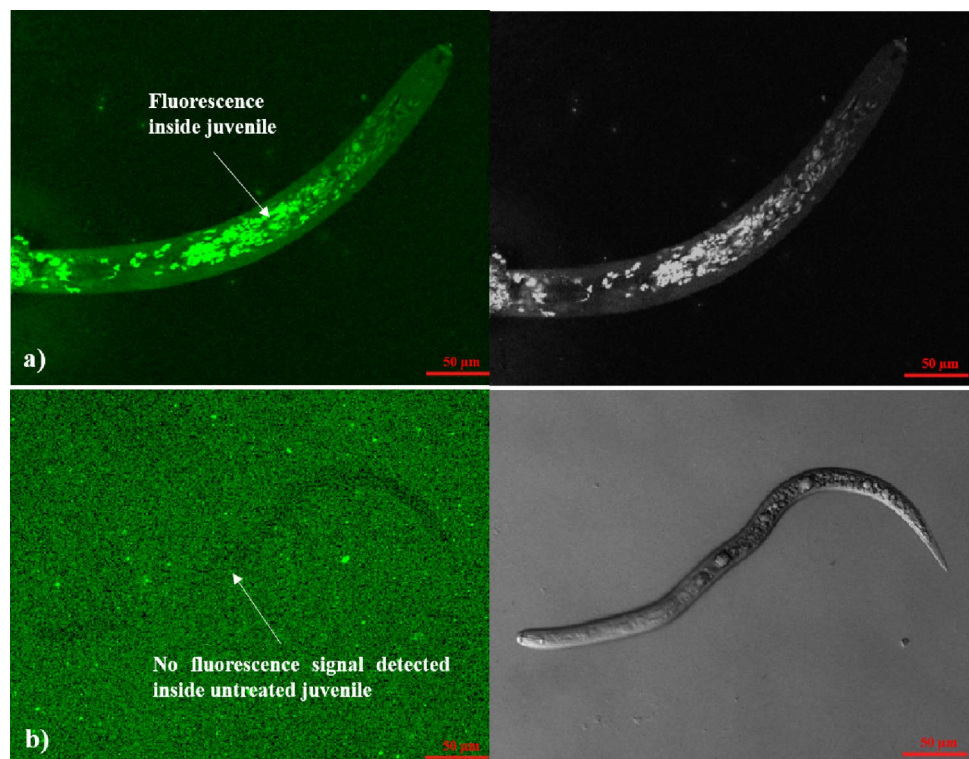
### qPCR of plant roots after inoculation with dsRNA-treated and untreated J2s

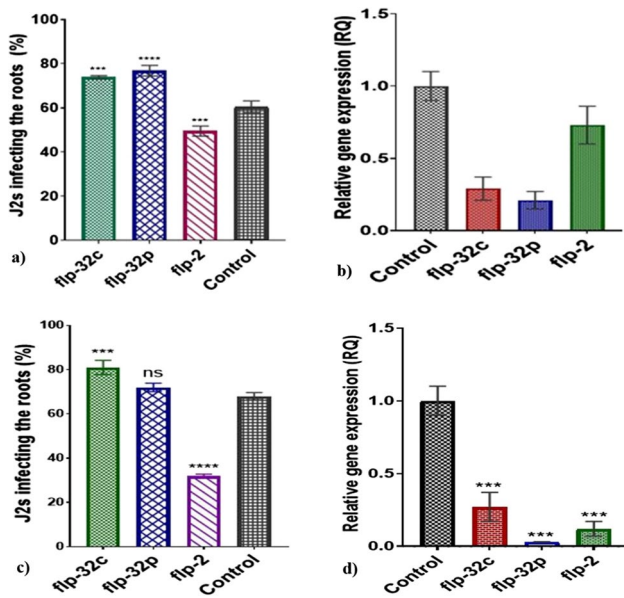
qPCR experiments showed a noticeable reduction in nematode transcripts in the roots treated with dsRNA of selected genes. The transcript levels of selected genes showed a significant decrease ranging from 0.21–0.73 in contrast to control root samples (Fig. 3b).

### Effect of dsRNA-kaolinite formulations on Kufri Jyoti plants

A total of 13.01 mg of dsRNA yielded from 1 ml of the lysed pellet was mixed with 1% sonified kaolinite. When the dsRNA-kaolinite formulations were drenched into the pot followed by inoculation of J2s, the infestation rate of J2s was increased for the *flp-32c* (81.0%) and *flp-32p* (72.0%) genes in comparison to the untreated plants (68.0%) ( $P < 0.001$ ). Whereas, as compared to the untreated control (68.0%), the *flp-2* (32.0%) dsRNA-kaolinite formulation-treated plant had less infestation ( $P < 0.0001$ ) (Fig. 3c).

**Fig. 2** a) Fluorescent microscopy for the confirmation of uptake of dsRNA labeled FITC by *Globodera pallida* J2 in 50 mM octopamine (inducer). The fluorescence was confined to the stylet, nerve ring, esophageal glands cells, and intestine (Scale bar = 50  $\mu$ m); and b) Untreated control showed no ingestion of FITC by *G. pallida* J2 soaked in the FITC + M9 solution without octopamine (inducer)





**Fig. 3** a) Effect of dsRNA of selected genes, i.e., *flp-32c*, *flp-32p*, and *flp-2* on percentage of J2s infecting the roots of potato cultivar Kufri Jyoti with control was compared using Dunnett's multiple comparison test (\*\*\*Significant at  $P < 0.001$ ; \*\*\*\*Significant at  $P < 0.0001$ ); b) Quantitative real-time PCR (qPCR) of expression of *flp-32c*, *flp-32p*, and *flp-2* genes in roots infested with dsRNA-treated J2s compared to average Ct values of the actin gene (endogenous control) using the  $2^{-\Delta\Delta CT}$  method; c) Effect of dsRNA-kaolinite formulations of selected genes, i.e., *flp-32c*, *flp-32p*, and *flp-2* on percentage of J2s infecting the roots of potato cultivar Kufri Jyoti with control was compared using Dunnett's multiple comparison test (\*\*\*Significant at  $P < 0.001$ ; \*\*\*\*Significant at  $P < 0.0001$ ; ns: non-significant); and d) Quantitative real-time PCR (qPCR) of expression of *flp-32c*, *flp-32p*, and *flp-2* genes compared to average Ct values of the actin gene (endogenous control) using the  $2^{-\Delta\Delta CT}$  method (\*\*\*Significant at  $P < 0.001$ ). Error bars represent the mean  $\pm$  SEM

### qPCR of plant roots after inoculation with kaolinite-shielded dsRNA-treated and untreated J2s.

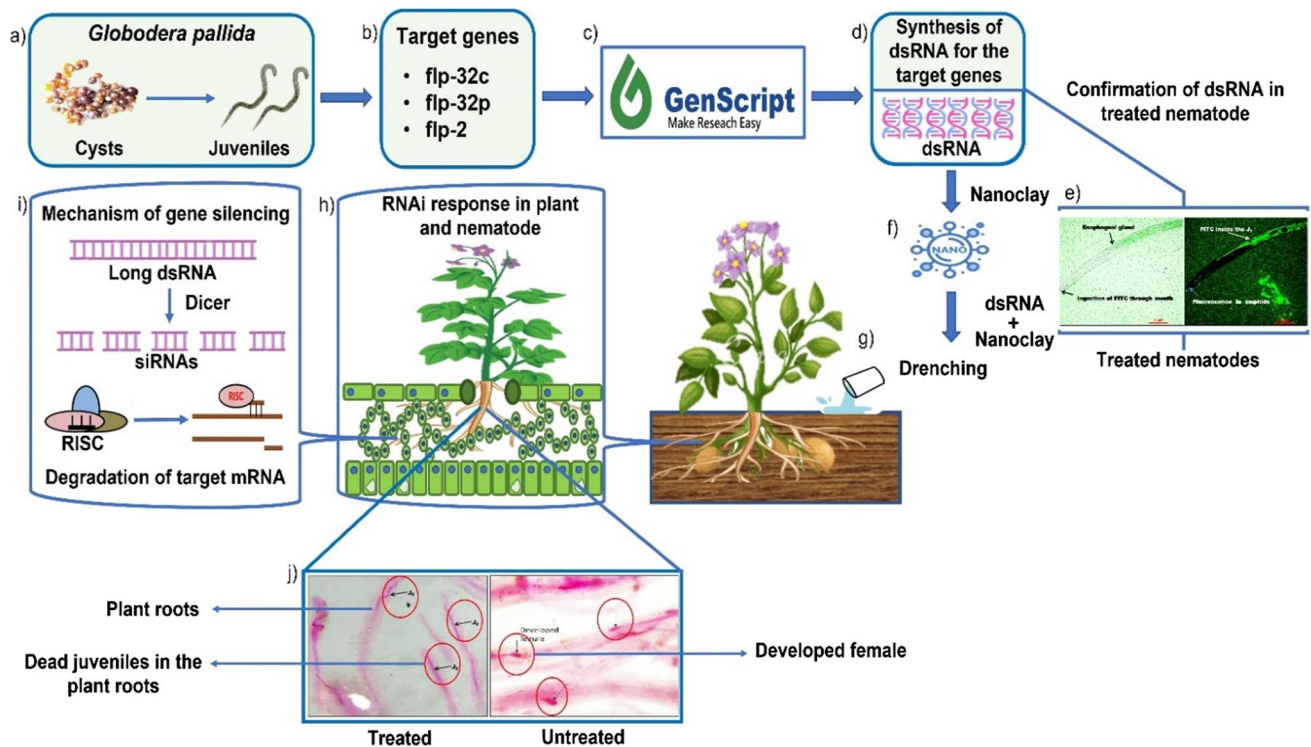
The qPCR results revealed that when drenched in the dsRNA-kaolinite formulations, the transcript levels of the selected genes were significantly decreased in the range of 0.03–0.27 compared to untreated plant samples (Fig. 3d).

## Discussion

In RNAi, sequence-specific dsRNAs are introduced into cells to disrupt mRNA functions through targeted degradation (Bairwa et al. 2022b; Sharma et al. 2022). Host-induced gene silencing (HIGS) is an intriguing approach for silencing genes in pathogens and pests by expressing RNAi constructs. However, stable transformants produced using RNAi constructs need to undergo genetically

modified organisms (GMOs) regulatory compliance policies to get approval for commercial use (Bawa and Anilakumar 2013). In contrast to GMOs, the exogenous application of dsRNA emerged as an environmentally friendly and publicly acceptable alternative method (Sundaresha et al. 2022a). It has been reported that plants can absorb and process topically applied dsRNAs. The exogenously applied RNA molecule spreads systemically and locally, enters pathogens, and induces RNAi-mediated resistance against plant pathogenic fungi, viruses, and pests (Konakalla et al. 2016; Dubrovina and Kiselev 2019). Spraying, spreading by brush or pipette, root drenching, mechanical inoculation, injection, and infiltration have been widely used for the exogenous application of dsRNAs onto the target cell to induce gene silencing (Das and Sherif 2020). PCNs pose a great threat to potato production and targeting genes important for their survival would play a role in the management of the specific nematode, as they would interrupt the nematode's ability to infect the host plant. FLPs, the nematode neuropeptides, are related to various physiological functions such as recognition of the host, sensory perception, locomotion, feeding, and reproduction and are prerequisites for successful parasitism (Kimber et al. 2007; Atkinson et al. 2013). In this study, we have selected three pathogenicity-related genes, i.e., *flp-32c*, *flp-32p*, and *flp-2* of *G. pallida* to evaluate the silencing effect of exogenous dsRNA.

For efficient silencing, the optimal concentration of dsRNA of the target genes was determined by exposing the J2s of *G. pallida* to different dosages of dsRNA (0.5, 1.0, 1.5, and 2.0 mg/ml). The dsRNA along with octopamine and FITC was delivered into the J2s using RNAi soaking method. The FITC-led dsRNA soaking at a dosage of 2.0 mg/ml inhibited the expression of *flp-32c*, *flp-32p*, and *flp-2* genes by 89.5%, 94.6%, and 94.3%, respectively, compared to the untreated control. Dalzell et al. (2009) have observed that increasing the concentration of dsRNA from 0.1 mg/ml to 1 mg/ml elicited the impaired phenotype and inhibited the motility in *G. pallida*. Niu et al. (2012) have also used a similar concentration (2 mg/ml) of dsRNA to treat the J2s of *Meloidogyne incognita* for silencing the *Rpn7* gene involved in their motility and infectivity, which is in congruence with our study. In the same way, Tan et al. (2013) used a 2 mg/ml concentration of dsRNA and observed a knockdown of the troponin C (*pat-10*) gene in *Pratylenchus thornei*. The dsRNA delivery using the soaking method has been widely used for pest control by many researchers (Bakhetia et al. 2005; Dalzell et al. 2009, 2010; Niu et al. 2012; Atkinson et al. 2013). The FITC served as a visual marker and accumulated more in the mouth, nervous system, and digestive system of *G. pallida* J2s. The neuroactive chemical octopamine facilitates the uptake of dsRNA. Urwin et al. (2002) have also used octopamine and FITC



**Fig. 4** A diagrammatic illustration of the silencing of pathogenicity genes in *Globodera pallida* through exogenous application of dsRNA-kaolinite formulation. **a)** Cysts and juveniles (J2s) of *Globodera pallida*; **b)** Selection of pathogenicity genes, viz., *flp-32c*, *flp-32p*, and *flp-2* genes; **c)** Identification of siRNA targets using the GenScript siRNA Target Finder tool; **d)** Synthesis of dsRNA for *flp-32c*, *flp-32p*, and *flp-2* genes; **e)** Uptake of dsRNA by *G. pallida* J2s; **f)** Use of kaolinite nanoclay particles as dsRNA carrier; **g)** Drenching of dsRNA-kaolinite formulation on *G. pallida*-infested plants; **h)** Penetration of dsRNA into the plant and nematode; **i)** Mechanism of gene silencing: Long dsRNA is cleaved by Dicer into siRNAs, which form a RISC complex to degrade target mRNA; **j)** Comparison of treated and untreated plant roots, showing dead juveniles in treated roots and a developed female in untreated roots

etration of *G. pallida* into the host plant; **i)** Upon uptake of dsRNA, RNase-III type endonuclease dicer rapidly cleaves them into siRNA. RNA-induced silencing complex (RISC) is formed when the guide strand is incorporated into an Argonaute (Ago) protein, while the passenger strand gets degraded. siRNA directs RISC to recognize and degrade complementary transcripts resulting in gene silencing; and **j)** Ingestion of dsRNA reduced the expression of targeted genes resulting in the mortality of J2s compared to untreated J2s

for uptake and visualization of dsRNA by J2s of *Heterodera glycines* and *G. pallida* nematodes.

J2s incubated in the dsRNA solutions of *flp-32c*, *flp-32p*, and *flp-2* for 5 and 10 h exhibited normal behavior, whereas the silencing effects were visible after 15 h. Following 24 h of soaking, J2s emerged straight and rigid with inhibited movement. In this study, the silencing effect of all three genes was observed after 15 h, however, the time course of silencing of two genes in the same tissue may vary. Rosso et al. (2005) found that the silencing of the *M. incognita* calreticulin gene could be seen in 16 h after soaking, while that of *M. incognita* polygalacturonase took 44 h. J2s with silenced *flp-32c* and *flp-32p* displayed an increase in mobility rate compared to untreated J2s of *G. pallida* after 15 h of incubation. The increased migration rates might have caused the knockdown phenotype to become more severe with time, thereby leading to the rapid depletion of energy reserves and eventually the death of J2s. In a similar study, *flp-32* silenced J2s of *G. pallida* showed an increased frequency of normal sinusoidal movement and migration rates compared

to controls (Atkinson et al. 2013). In contrast to the silencing of *flp-32c* and *flp-32p* genes, J2s with silenced *flp-2* gene exhibited decreased mobility compared to untreated controls. These results are in congruence with the study conducted by Dalzell et al. (2010). They have also found significant inhibition of migratory ability in J2s of *G. pallida* after soaking in *flp-12* (accession number AJ300488) siRNAs. Hence this showed that the dsRNA-based silencing of *flp-32c*, *flp-32p*, and *flp-2* plays an important role in the modulation of locomotory behavior of J2s of *G. pallida*.

Further on topical application of dsRNA in plant bioassay, more infection points were observed in *flp-32c* (73.9%) and *flp-32p* (76.8%) treated J2s compared to untreated J2s (60.5%) after 15 days of infection. However, compared to the untreated control (60.5%), J2s treated with *flp-2* showed a lower infestation (49.5%). This is due to the silencing of *flp-32c* and *flp-32p* genes which increased the mobility rate of J2s and thereby increased the infection points compared to untreated J2s of *G. pallida*. The qPCR studies showed that transcript levels of selected genes exhibited a significant

decrease ranging from 0.21 to 0.73 compared to the control root samples. Our study is in corroboration with Dalzell et al. (2010) and Atkinson et al. (2013) who have also reported a significant reduction in target transcripts of *flp* genes in treated nematodes compared to untreated nematodes. Thus, topical application of dsRNA in plant bioassay also downregulated the expression of selected pathogenicity genes.

However, the topical application of dsRNA has low persistence potential in soil due to its rapid degradation in soil and aquatic environments (Bachman et al. 2020). Therefore, for efficient dsRNA delivery, many biological barriers need to be addressed. These barriers include i) negative charge and high molecular weight of the molecule which make its absorption difficult into the cells; ii) degradation by gut enzymes; iii) targeting siRNA to the appropriate cell compartment; and iv) in vivo instability. Therefore, effective and biocompatible delivery mechanisms are required. Nanoparticles have been proven to be an efficient delivery vehicle for siRNAs into plant protoplasts as they exhibit advanced physiochemical characteristics such as small size, positive charge, and high surface area with excellent catalytic activity (Silva et al. 2010; Xu et al. 2023). Nanoparticles have facilitated the delivery of dsRNA to specific sites in organisms such as viruses (Mitter et al. 2017), fungi (Sundaresha et al. 2022b), and insects (Wang et al. 2023). Thus, to improve dsRNA delivery, we have used kaolinite nanoclay particles as a carrier for the drenching of potato plants and assessed its efficacy against J2s infestation. With dsRNA-kaolinite nanoclay formulations, the infestation rate of J2s was increased for the *flp-32c* (81.0%) and *flp-32p* (72.0%) genes compared to the untreated plants (68.0%). dsRNA-kaolinite formulation of *flp-2*-treated plant had less infestation (32.0%) compared to the untreated control (68.0%). Further, qPCR results revealed that transcript levels of the selected genes were significantly decreased in the range of 0.03–0.27 compared to untreated control plants. Zhang et al. (2022) have also used the nanocarrier-mediated dsRNA delivery method and suppressed the expression of genes involved in wing development of aphids. Recently, Wang et al. (2023) have studied the protective effect of nanoparticles on dsRNA and determined the stability, leaf absorption rate, and insect wall permeability of the nanoparticle-dsRNA complex. They have found it an easy and efficient tool for RNAi-based management of the invasive pest *Tuta absoluta*. This is the first study to demonstrate kaolinite-dsRNA-mediated silencing of pathogenicity genes in *G. pallida*; however, further research is also required for an in-depth understanding at the triggered biochemical and molecular level which can provide a strong basis for supporting sustainable agriculture. The overall illustration of the silencing of pathogenicity genes in *G. pallida* through exogenous application of the dsRNA-kaolinite formulation is shown in Fig. 4.

## Conclusion

In this study, we have silenced the *flp-32c*, *flp-32p*, and *flp-2* genes through exogenous application of dsRNA to reduce *G. pallida* infestation in potato plants. The dsRNA treatment downregulated the expression of these genes and modulated the locomotory behavior of J2s. Further, for efficient and targeted delivery of dsRNA, we have investigated the kaolinite nanoclay as a dsRNA carrier to inhibit the infestation of *G. pallida* in potatoes. This is the first report in which dsRNA-kaolinite formulations were successfully employed through drenching to manage *G. pallida* infestation in an important potato cultivar, Kufri Jyoti. The outcome of this study would be helpful in the development of sustainable and environmentally friendly biomolecules that would play a role in nematode management through drenching or root irrigation in potatoes.

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**Authors contribution** AB, SuS, and EPV conceptualized and designed the study. SS and BrS contributed reagents and materials. AB, SuS, and BD performed experiments. BS, KCN, and PHM analyzed the data. AB and BD did literature research and drafted the manuscript. BD and NS revised the manuscript. The submitted version of the manuscript has been approved by all authors.

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**Data availability** The manuscript contains all the data generated or analyzed during this study. Additional data is provided in supplementary files.

## Declarations

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this research paper.

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