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ORIGINAL PAPER

Cloning of *sft-4* and its influence on vitality and virulence of pine wood nematode, *Bursaphelenchus xylophilus*

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Abstract In our previous screening of the transcriptome of the causal agent of the devastating pine wilt disease, pine wood nematode (PWN, *Bursaphelenchus xylophilus*), after treatment with the nematicide fomepizole, Surfeit locus gene *sft-4*, which encodes a regulatory factor, was found to be downregulated. In situ hybridization results showed that the *sft-4* was continuously expressed from egg to adult and was especially high in the reproductive system. Here in a study of the effect of RNA interference (RNAi) of *sft-4* and recombinant SFT-4 on PWN activity, treatment with *sft-4* dsRNA inhibited feeding, reproduction, oviposition and egg hatching of PWN with the greatest inhibition on reproduction and oviposition, whereas recombinant SFT-4 had the opposite effect. In addition, RNAi of *sft-4* changed the female–male ratio and lifespan of PWN. In bioassays of PWNs, with RNAi of *sft-4* on seedlings and 2-year-old *Pinus thunbergii* trees, none of the treated plants developed symptoms during the monitoring period, indicating that virulence of PWNs was either significantly weakened. These results indicate that the influence of *sft-4* on PWN pathogenicity may be mainly through regulating reproductive function of PWN and its lifespan.

Keywords Black pine · Pinaceae · *Bursaphelenchus xylophilus* · *Sft-4* · In situ hybridization · RNAi · Pathogenicity

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Introduction

Pine wilt disease (PWD) caused by pine wood nematode (PWN, *Bursaphelenchus xylophilus*) causes serious ecological damage and economic losses worldwide (Takai et al. 2003; Akiba et al. 2012; Futai 2013; Li et al. 2021; Feng et al. 2022; Meng et al. 2022). At present, PWD management mainly relies on eliminating PWN or its insect vectors. Injecting nematicides against PWN into a tree trunk is considered one of the most effective and sustainable strategies to control PWD because it is less deleterious than spraying pesticides (Takai et al. 2003; Barbosa et al. 2012; Shanmugam et al. 2018; Liu et al. 2019; Lee et al. 2020). However, commonly used nematicides have single-action targets and their frequent use leads to resistance in the nematodes (Shanmugam et al. 2018; Liu et al. 2019). Hence, novel drug targets are greatly needed for developing promising nematicides against PWN.

The virulence of PWN is closely related to the spread of PWD (Wang et al. 2019). So far, many genes have been shown to be involved in the virulence of PWN. For example, cytochrome P450 gene has been shown to play a key role in the mechanism of low temperature tolerance in PWNs and be involved in regulating growth, development and longevity of nematodes (Wang et al. 2020). The pectate lyase gene is essential for successful invasion of their host plants by plant-parasitic nematodes. PWNs penetrate plant tissues

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by secreting pectate lyase, promoting feeding and migration in pine trees, eventually leading to host cell death and pine wilt. When pectate lyase gene expression was inhibited using RNAi, the reproduction and mobility of the PWN was inhibited and ultimately its pathogenicity or virulence was reduced in pine seedlings (Qiu et al. 2016). Inhibition of arginine kinase expression also decreases fertility and increases mortality of PWNs. These genes are thus potential targets for new nematicidal drugs against PWN.

We previously found that fomepizole, an inhibitor of alcohol dehydrogenase (ADH), rapidly killed PWNs (Wang 2019; Wang et al. 2019). By analyzing the transcriptome data of fomepizole-treated PWNs, we identified the differentially expressed gene Surfeit locus (*sft-4*) (Wang 2019). The *sft* family is widely present in the genome of animals and plays various important roles in organisms. Mashkevich et al. (1997) found that a homolog of mammalian *surf-1* in yeast encodes a mitochondrial membrane protein related to respiration. Mouse *surf-3* encodes ribosomal protein L7a (Giallongo et al. 1989). Homologs of *sft-4* are widespread in invertebrate genomes and encode an integral membrane protein associated with the endoplasmic reticulum (ER) (Armes and Fried 1996). However, reports of the function of *sft* genes in PWN are rare. In the present research, we explored the physiological effect of manipulations of *sft-4* on PWN to determine whether the gene is a potential drug target for PWN control.

Materials and methods

Experimental materials

PWNs were isolated from wilted black pines in Yantai, China using the Baermann funnel technique and cultured

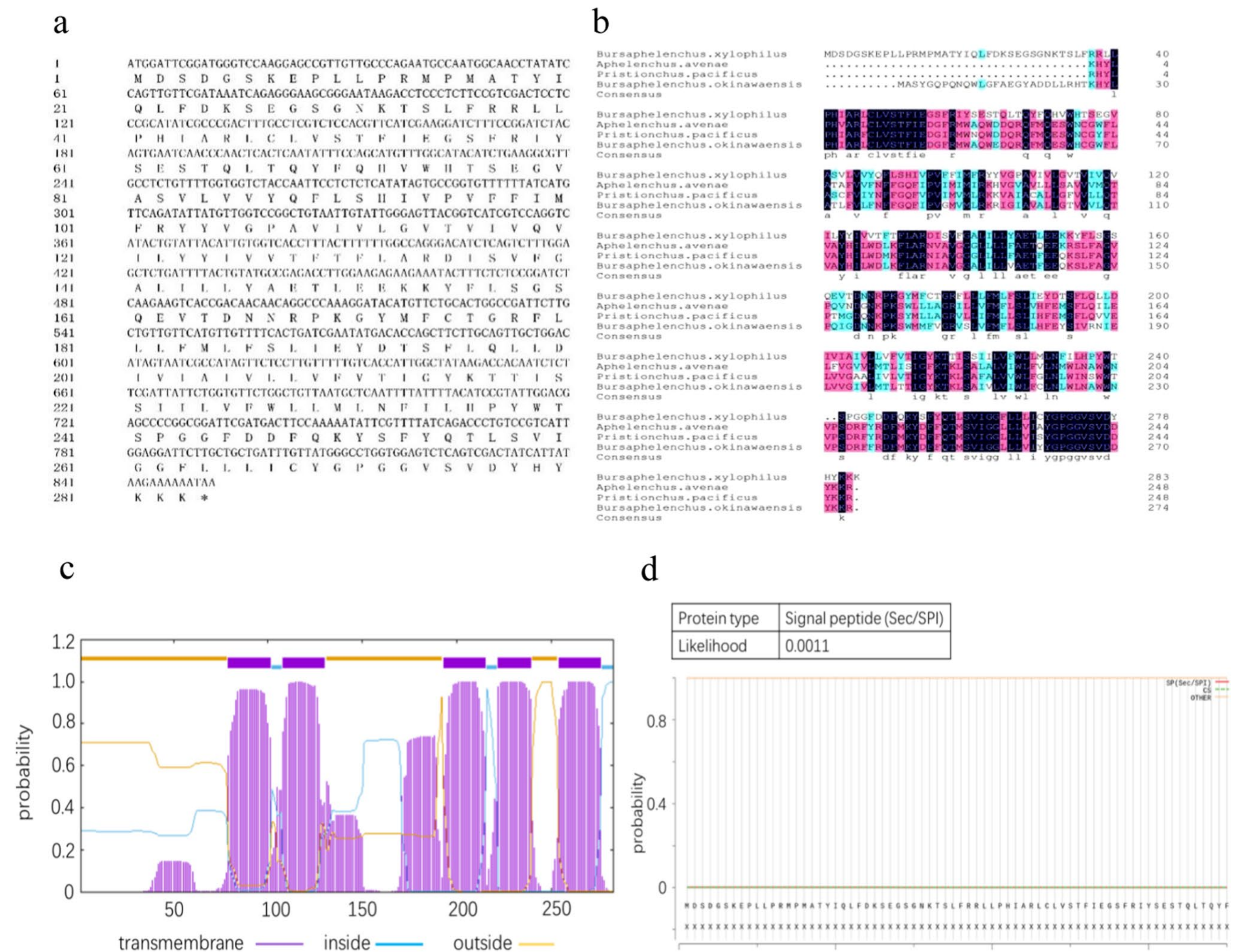

Fig. 1 Bioinformatic analysis of *sft-4*. **a** Nucleotide and deduced amino acid sequence of *sft-4* from pine wood nematodes (PWNs). **b** Alignment of the amino acid sequences of SFT-4 from PWNs, *Aphelenchus avenae*, *Pristionchus pacificus* and *Bursaphelenchus okinawaensis*. Colors indicate conserved amino acid residues. Predicted **c** SFT-4 signal peptide and **d** SFT-4 transmembrane structure

on *Botrytis cinerea* grown on potato dextrose agar (PDA) in the dark at 25 °C (Booth 1971; Viglierchio and Schmitt 1983; Guo et al. 2017).

Cloning of sft-4 and construction of expression vectors

Total RNA was extracted from the mixed-stage PWNs using Trizol reagent as reported by Chen et al. (2011) and used to synthesize cDNA with a reverse transcription kit (Takara, Dalian, China). The gene *sft-4* was amplified by polymerase chain reaction (PCR) using primers designed for the *sft-4* sequence (forward: 5′-GTTACGGTCATCGTCCAGGTCATAC-3′; reverse: 5′-CTTCTCTTCCAAGGTCTCGGCATAC-3′). The amplified products were ligated into pET-15b using T4-DNA ligase (Takara, Dalian, China) (Wang et al. 2022).

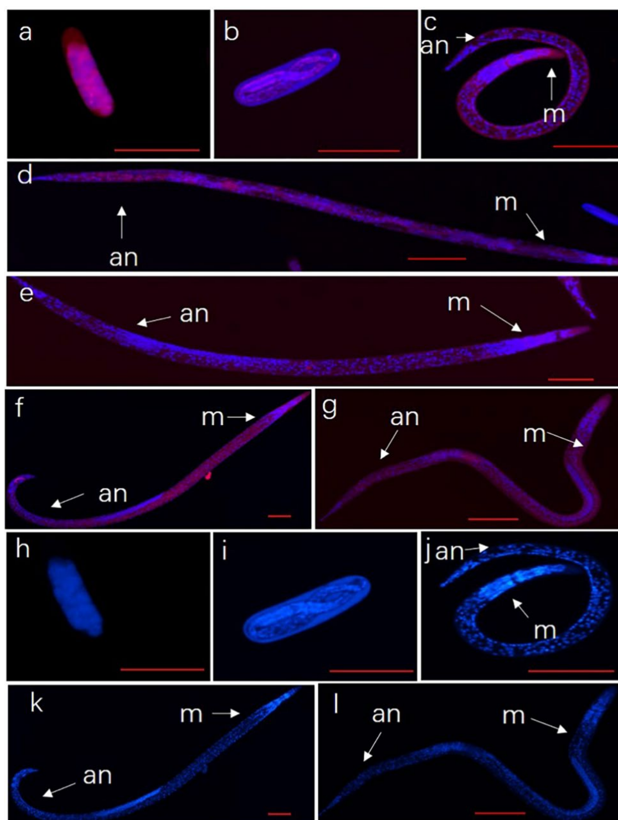

Fig. 2 Localization of *sft-4* mRNA in pine wilt nematodes (PWNs) using fluorescence in situ hybridization. Hybridization with red-fluorescence-labeled probe in **a** the egg **b** J1 juvenile, **c** J2 juvenile, **d** J3 juvenile, **e** J4 juvenile, **f** male adult, **g** female adult. Hybridization for negative controls in **h** egg, **i** J1 juvenile, **j** J2 juvenile, **k** male adult and **l** female adult. m, metacorpus; an, anus. Scale bar = 50 μm

Bioinformatics analysis of *sft-4* and SFT-4

The open reading frame (ORF) of sft-4 from PWN was analyzed using the ORF Finder program (NCBI, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The structure of PWN sft-4 was analyzed using WormBase ParaSite (Howe et al. 2016, 2017). Signal peptides in SFT-4 were predicted using the server SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP-5.0/), and transmembrane helices were predicted using the server TMHMM 2 (http://www.cbs.dtu.dk/services/TMHMM/).

Fluorescence in situ hybridization to localize *sft4* expression in PWNs at different stages

A sulfo-cyanine3-labeled probe (5′-Cy3-CGGAGACCGATTTCAACAGCCCGTCGACTGTCGACTTGAGCTTGG-3′) was designed based on the ORF sequence of PWN *sft-4* using Premier 5.0 software (PREMIER Biosoft International, San Francisco, CA, USA). The mixed-stage nematodes were fixed in 4% v/v RNase-free paraformaldehyde (PFA) aqueous solution at 5 °C for 16 h before using the WISH01 in situ hybridization kit (Gefan, Shanghai, China) and the manufacturer's protocol. Samples were examined for fluorescence at the excitation wavelength of 554 nm and emission wavelength of 568 nm using a fluorescence microscope (Nikon Eclipse Ci, Tokyo, Japan).

Expression and purification of recombinant SFT-4

Escherichia coli BL21 (DE3) (Takara, Dalian, China) was transformed with the expression vector pET-15b-*sft-4*, then plated on LB medium to select bacteria that express recombinant SFT-4. A single colony was then cultured in LB fermentation broth, after 4 h, 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the broth to induce expression of recombinant SFT-4 at 28 °C (Zhou et al. 2021). The bacteria cells were collected by centrifugation at 10,000 rpm for 20 min, then suspended in 20 mL binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 8.0), and lysed using an ultrasonic processor (400 W, 4 s, 15 s, 120 cycles), followed by centrifugation at 10,000*g* for 25 min. The recombinant SFT-4 was purified using Ni-NTA affinity chromatography (Xu et al. 2015).

Effects of recombinant SFT-4 on PWN vitality

PWNs were soaked in a solution of recombinant SFT-4 in Tris-HCl buffer (pH 8.0) and tested for locomotion (Zhou et al. 2021), feeding (Wang et al. 2019, 2022), reproduction (Xu et al. 2015; Meng et al. 2019; Zhou et al. 2021), oviposition and hatching (Wang et al. 2019, 2022; Zhou et al. 2021) as described previously. PWNs soaked in Tris-HCl

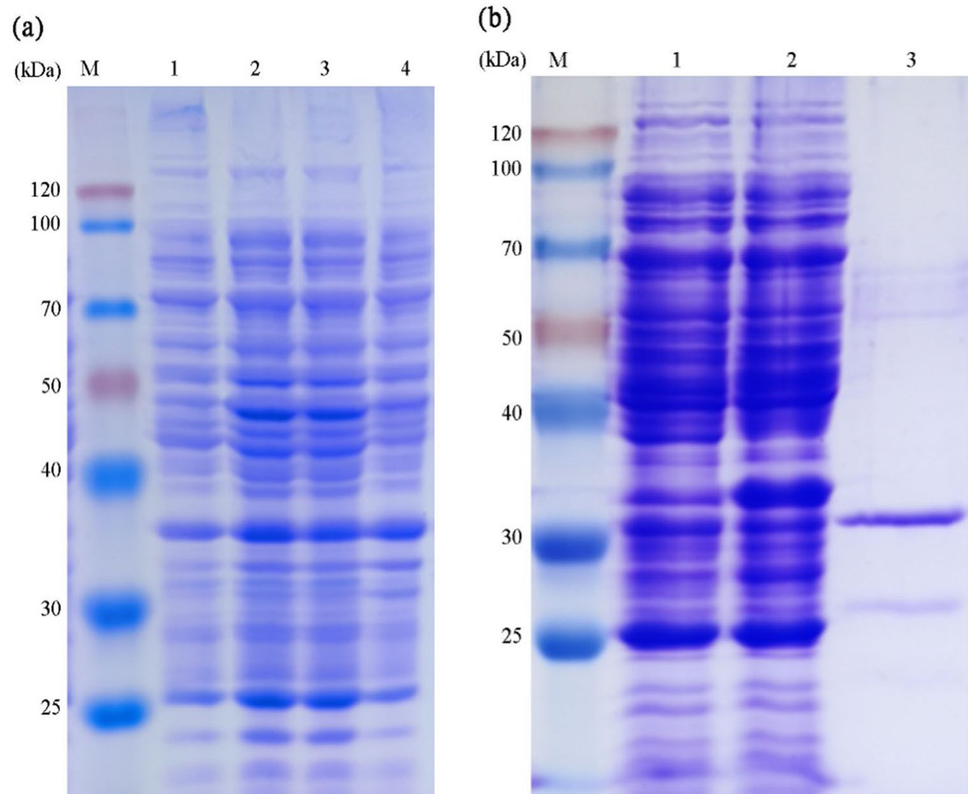

Fig. 3 SDS-PAGE analyses of purification and identification of recombinant SFT-4. **a** Isolated recombinant SFT-4. Lane 1: Total proteins of *E. coli* BL21 (DE3) harboring pET-15b-*sft-4*; after centrifugation at 10,000×*g* for 25 min, lanes 2, 3: supernatant; lane 4: pellet. **b** SDS-PAGE analysis of purified recombinant SFT-4. Lane 1: Total proteins of *E. coli* BL21 (DE3); Lane 2: total proteins of *E. coli* BL21 (DE3) harboring pET-15b-*sft-4*; Lane 3: purified recombinant protein

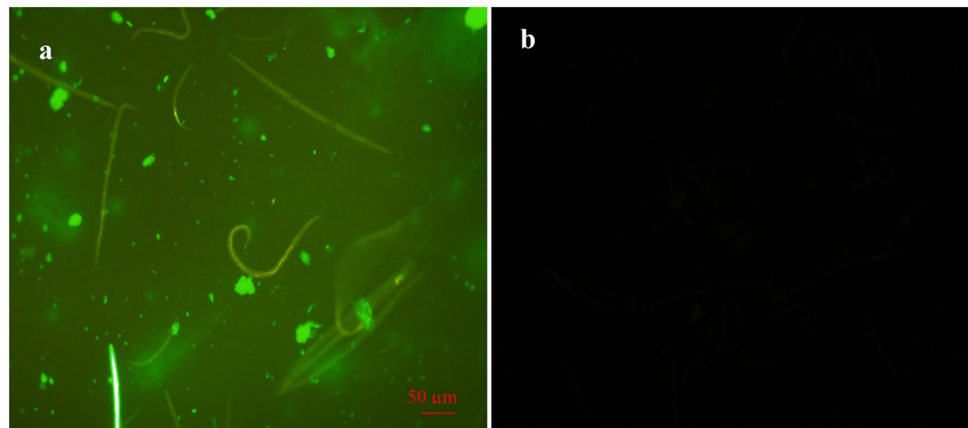

Fig. 4 GFP fluorescence in pine wilt nematodes (PWNs) under blue light after treatment with **a** GFP and no fluorescence after treatment with **b** sterilized water

buffer (pH 8.0) and BSA solution were used as the two controls, respectively. The BSA control was set to exclude the side effect of the protein. To determine whether recombinant SFT-4 can enter the body of PWN, the nematodes were soaked in purified GFP under the same conditions, then washed with sterilized water three times, and observed at the excitation wavelength of 488 nm and emission wavelength of 507 nm with a fluorescence microscope (Olympus IX73, Tokyo, Japan).

Synthesis of *sft-4* dsRNA and confirmation of RNAi

With pET-15b-*sft-4* as the template, three DNA fragments, P1 (537 bp), P2 (441 bp), P3 (366 bp), selected from the ORF of *sft-4* were PCR-amplified using T7-labeled gene-specific primers (F1: 5′-GATCACTAATACGACTCACTA TAGGGCTC CTCCCGCATATCGCCCGACTTTG-3′; R1: 5′-GATCACTAATACGACTCACTAT AGGGGGTCTTAT AGCCAATGGTGAC-3′; F2: 5′-GATCACTAATACGAC TCACT ATAGGGTTTTTGGCCAGGGACATCTCAG-3′; R2: 5′-GATCACTAATACGACT CACTATAGGGGTCGG ACTCCACCAG-3′; F3: 5′-GATCACTAATACGACTCA

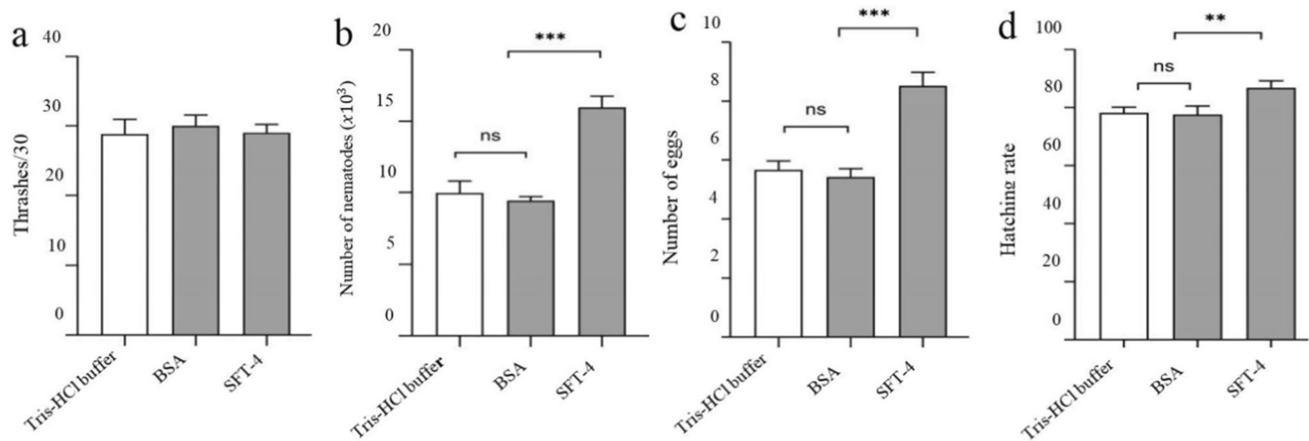

Fig. 5 Effect of recombinant SFT-4 on vitality of pine wilt nematodes (PWNs). **a** Mean number of head thrashes per 30 s, **b** the number of nematodes reproduced by the inoculated PWNs, and **c** egg hatching rate after treatment with recombinant SFT-4 (control treatments: Tris-HCl buffer, BSA). Means were tested for significant differences using Student's *t*-test; bars on means are standard errors; level of significance: $^{**}P < 0.005$, $^{***}P < 0.001$, ns, not significant

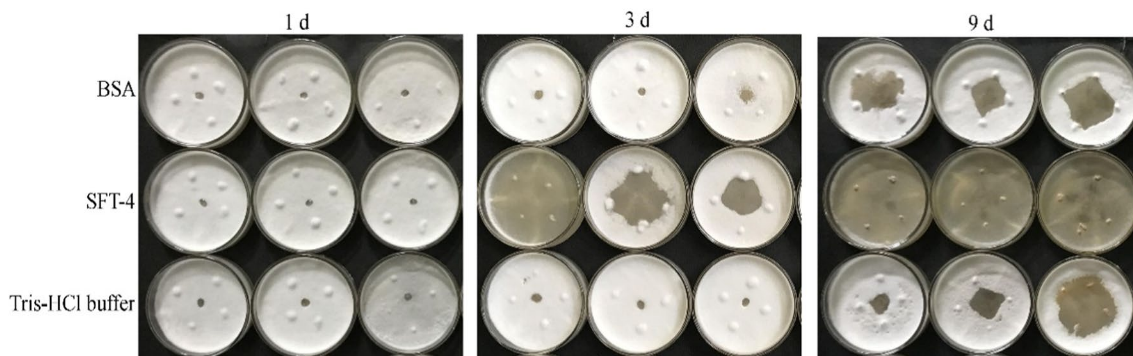

Fig. 6 Effects of recombinant SFT-4 on feeding of *Botrytis cinerea* in petri dishes by pine wilt nematodes (PWNs) at 1, 3 and 9 days after treatment. BSA and Tris-HCl served as control treatments

CT ATAGGGTCTCAAGAAGTCACCGACAAC-3′; R3: 5′-GATCACTAATACGACTC ACTATAGGGCTTATAATG ATAGTCGACTGAG-3′). A 323-bp DNA fragment of the *GFP* gene was amplified using a pair of T7-labeled gene-specific primers (forward: 5′-GATCACTAATACGACTCA CTATAGGGAACGGCCA CAAGTTCAGC-3′; reverse: 5′-GATCACTAATACGACTCACTATAGGGAAGTCGA TGCCCTTCAGC-3′) as the negative control. With the amplicons as templates, double-stranded RNA (dsRNA) for *sft-4* was synthesized using the MEGAscript RNAi Kit (Invitrogen, Vilnius, Lithuania) and the manufacturer's instructions. RNAi was carried out using the soak method previously described by Xu et al. (2015). Briefly, approximately 3000 PWNs were soaked in 50 μL of solution containing *sft-4* dsRNA (1.0 μg/μL) at 20 °C for 72 h. PWNs were soaked in 50 μL sterilized water and GFP dsRNA solution (1.0 μg/μL) and used as double negative controls. The efficiency of RNAi was assessed by qRT-PCR using forward primer 5′-GGTCATCGTCCAGGTCA TACT-3′ and reverse primer 5′-TTCCAAGGTCTCGGCATACA-3′). The gene for actin from PWN was used as an internal control using forward primer 5′-CTGCTGAGCGTGAAATCGT-3′ and reverse primer 5′-GTTGTAGGTGGTCTCGTGGA-3′). The data were analyzed using the $2-\Delta\Delta$Ct method. There were three biological replicates in this experiment.

Effects of *sft-4* dsRNA on PWN vitality and lifespan

The effects of *sft-4* dsRNA on PWN motility, reproduction, oviposition and egg hatching were determined as described in Sect. 2.6. Approximately 100 RNAi-treated PWNs were added to each well of three 48-well plates to evaluate the lifespan of nematodes. The experiment was done three times.

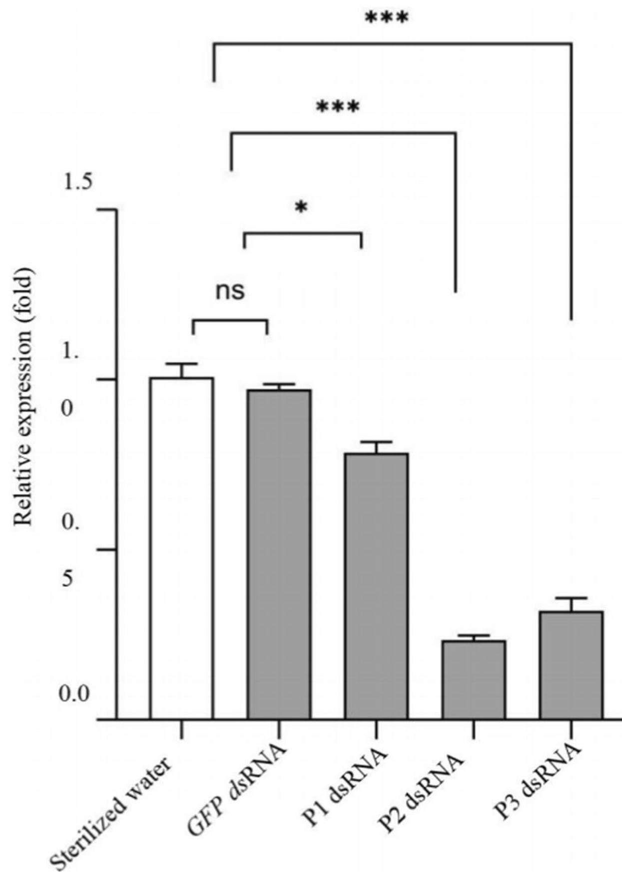

Fig. 7 qRT-PCR results for relative expression of *sft-4* in pine wilt nematodes (PWNs) after treatment with sft-4 dsRNA. RNAiP1, -P2, -P3 represent three synthesized RNA interfering fragments. Means were tested for significant differences using Student's *t*-test; bars on means are standard errors; level of significance: *$P<0.05$, ***$P<0.001$, ns, not significant

Effect of RNAi on pathogenicity of PWN

Approximately 100 30-day-old *Pinus thunbergii* seedlings and 1000 of 2-year-old *P. thunbergii* (about 1000 pieces) saplings were inoculated with RNAi-treated PWNs (Yu et al. 2012; Xu et al. 2015; Wang et al. 2020). PWNs treated with sterilized water and GFP dsRNA were used as double negative controls. The virulence of PWNs was determined after 15 days according to the wilting severity on seedlings (Yu et al. 2012).

Data analyses

All experiments were carried out in triplicate and means ± standard deviation (SD) calculated. Means among treatments were compared for significant differences using Student's *t*-tests in SPSS version 17.0 (SPSS, Chicago, IL, USA) and GraphPad (Boston, MA, USA) Prism 8. A significance level of $P<0.05$ was applied.

Results

Cloning and sequencing of *sft-4* coding gene in PWN

Sequence analysis of the *sft-4* PCR product showed an 852-bp ORF encoding a protein composed of 283 amino acids with a relative molecular weight of 31 kDa (Fig. 1a). The amino acid sequence of SFT-4 was 46%, 49%, and 46% homologous to that of *Aphelenchus avenae* (GenBank accession KAH7713954.1), *Pristionchus Pacificcus* (GenBank accession KAF8382798.1) and *Bursaphelenchus okanawaensis* (GenBank accession CAD5229971.1), respectively (Fig. 1b). The theoretical pI of PWN SFT-4 is 7.00, and its amino acid sequence was predicted to have five transmembrane helical structures (Fig. 1c) and no signal peptide sequence (Fig. 1d).

Localization of *sft-4* expression in PWN

Red hybridization signals for *sft-4* were found throughout the life cycle of PWN. Red fluorescence was detected nearly throughout the eggs and J1 nematodes (Fig. 2a, b). Signals in J2–J4 nematodes were mainly concentrated in the intestinal tract and the tail (Fig. 2c–e). In male and female adults, the signal was strong in the genital area, especially in the spicules of males (Fig. 2f, g).

Effect of recombinant SFT-4 on PWN

The relative molecular weight of the recombinant SFT-4 protein overexpressed in *E. coli* BL21 (DE3) after IPTG treatment was approximately 33 kDa as shown by SDS-PAGE analysis (Fig. 3), consistent with the predicted molecular

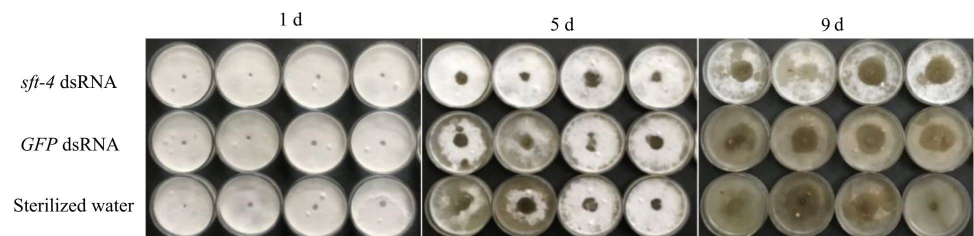

Fig. 8 Effect of treatment with *sft-4* dsRNA on feeding of *Botrytis cinerea* in petri dishes by pine wilt nematodes (PWNs) at 1, 5 and 9 days after treatment. Control treatments: *GFP* dsRNA and Sterilzed water

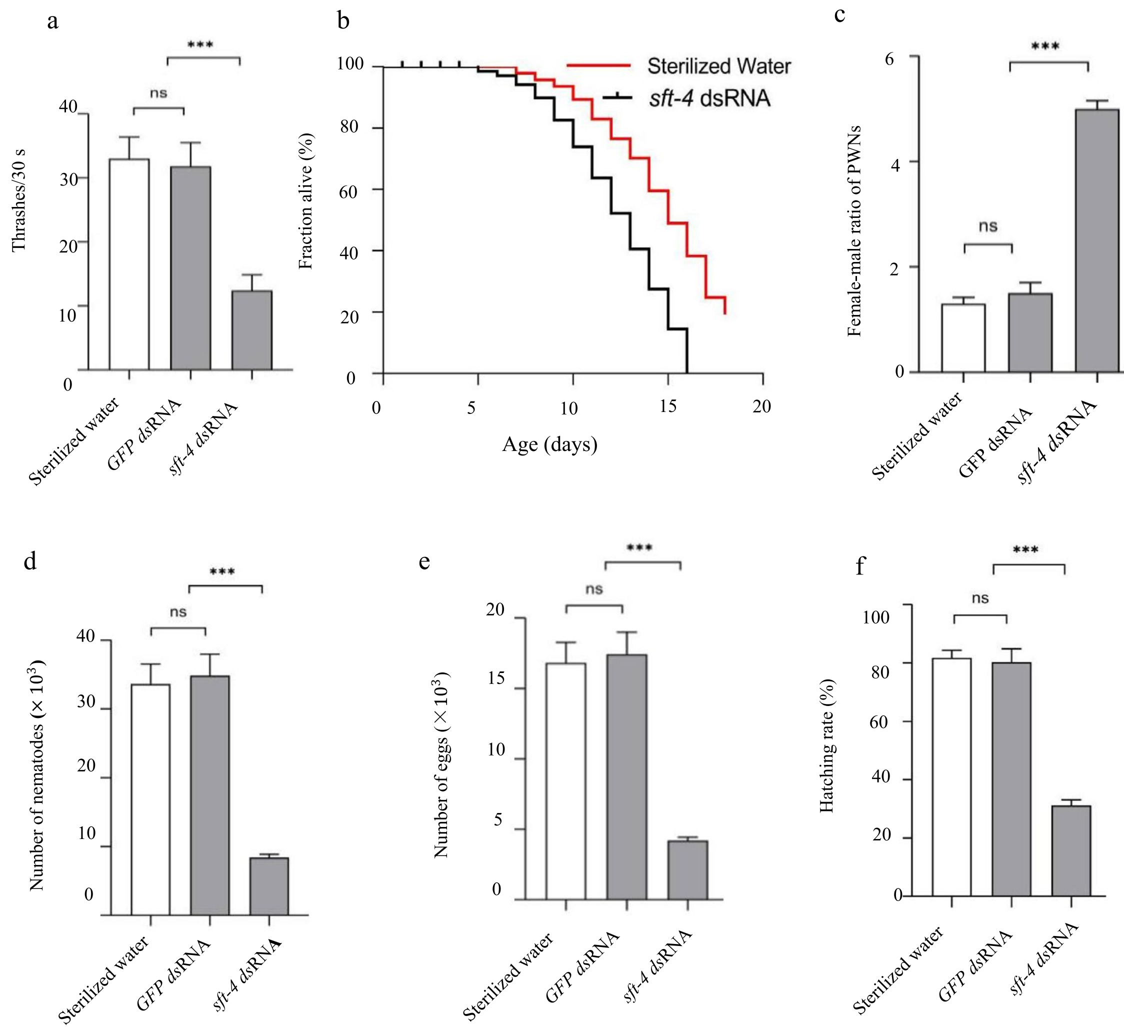

Fig. 9 Effects of treatment with *sft-4* dsRNA on movement (**a**), lifespan (**b**), female–male ratio (**c**), reproduction (**d**), oviposition (**e**) and percentage of eggs that hatched (**f**) of pine wilt nematodes (PWNs). Control treatments: *GFP* dsRNA-treated and Sterilzed water. Means were tested for significant differences using Student's *t*-test; bars on means are standard errors; level of significance: ***$P<0.001$, ns, not significant

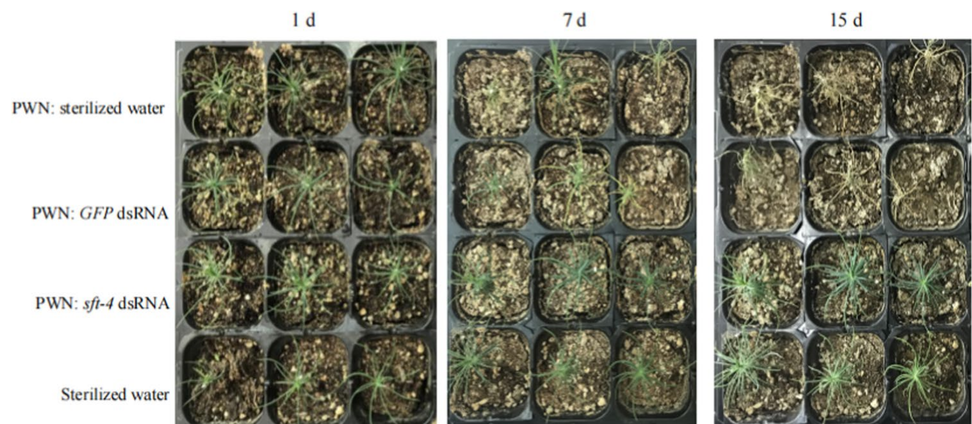

Fig. 10 Effect of *sft-4* dsRNA treatment of pine wilt nematodes (PWNs) on their pathogenicity on seedlings of *Pinus thunbergii*. Controls: Sterilized water-treated PWNs, *GFP* dsRNA-treated PWNs and Sterilized water

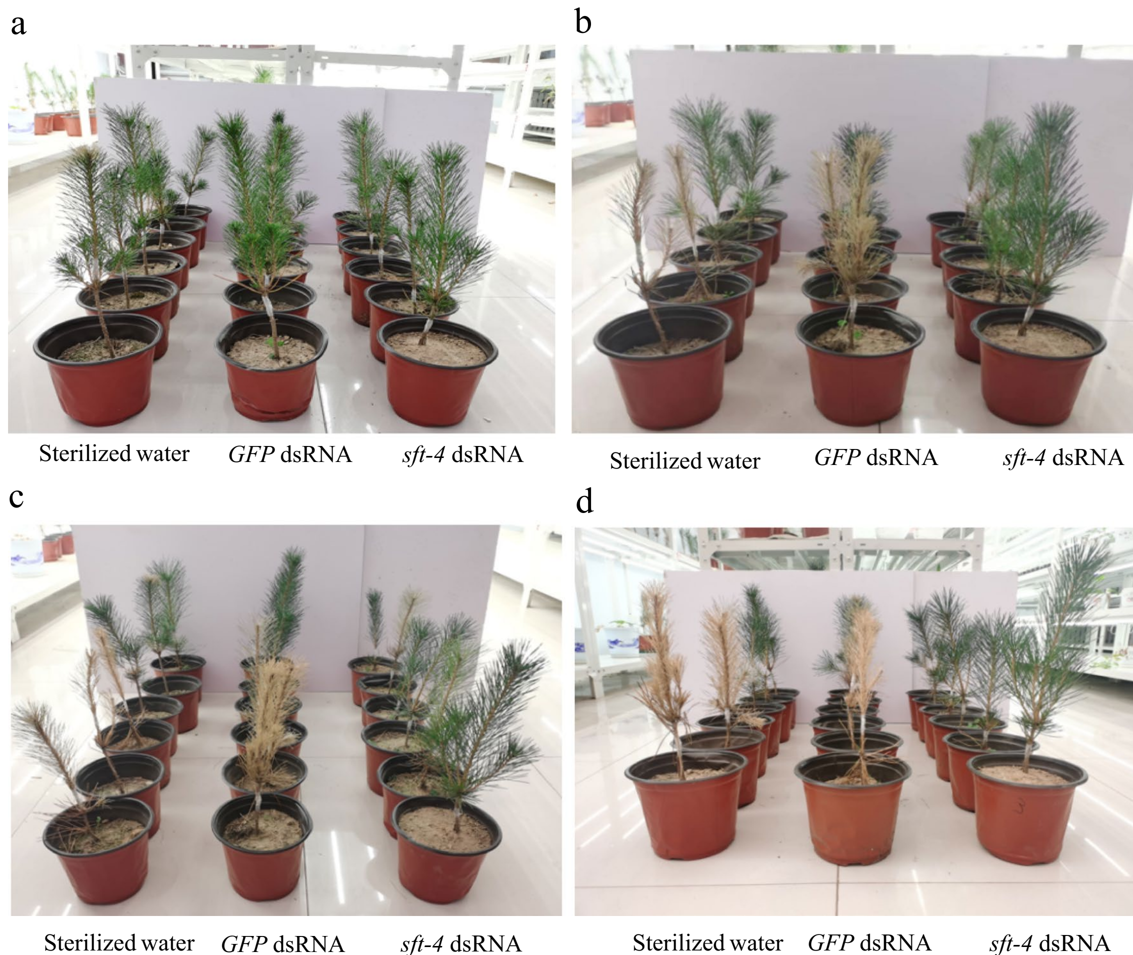

Fig. 11 Pathogenicity of PWNs treated with *sft-4* dsRNA on seedlings of *Pinus thunbergii* at different days post inoculation (dpi). **a** 1 dpi, **b** 14 dpi, **c** 30 dpi, **d** 40 dpi. Treatments: (1) Sterilized water control, (2) *GFP* dsRNA control, (3) *sft-4* dsRNA

size. The homogeneity of recombinant SFT-4 purified from the supernatant of the engineered bacterial lysate by Ni^{2+} affinity chromatography was verified by SDS-PAGE (Fig. 3).

Green fluorescence was detected in the GFP-treated PWNs, which indicated that SFT-4 can infiltrate PWN bodies (Fig. 4). The motility of SFT-4-treated PWNs was not significantly different from that of the controls (Fig. 5a) ($P > 0.05$), while the feeding rate (Fig. 6), reproductive ability (Fig. 5b), oviposition rate (Fig. 5c) and hatching rate (Fig. 5d) were significantly higher than for the controls ($P < 0.01$). These results indicated that recombinant SFT-4 may play an important role in regulating feeding and reproduction of PWN.

Effect of *sft-4* dsRNA on PWN

The qRT-PCR to analyze the expression level of *sft-4* after treatment with the three synthesized interfering fragments showed that fragment 2 inhibited expression the most (Fig. 7), so we selected fragment 2 for the subsequent *sft-4* RNAi experiment. Compared with the relative expression level for *sft-4* in the group treated with sterilized water, relative expression *sft-4* in PWN treated with *sft-4* dsRNA was strongly inhibited the expression (76.6% lower than in the control), and there was no significant effect in the *GFP* dsRNA-treated group (Fig. 7). Thus, treatments with *sft-4* dsRNA inhibited expression of *sft-4* in PWNs.

Based on the PWN feeding assays, the feeding rate of PWNs after RNAi was much lower than for those treated with sterilized water or *GFP* dsRNA (Fig. 8). The moving rate of PWN after RNAi was also significantly lower than for the negative controls, with 30 head thrashes per 30 s for PWN treated with *sft-4* dsRNA, 33 for sterilized water and *GFP* 32 dsRNA (Fig. 9a) ($P < 0.01$). The lifespan of PWNs after *sft-4* dsRNA treatment was also significantly shorter than for those treated with sterilized water. The *sft-4* dsRNA-treated PWNs reached 80% mortality in 12 days compared with 15 days for the control (Fig. 9b). In addition,

the female-male ratio of PWNs in RNAi group was five times higher than the 1.3 ratio after the *GFP* dsRNA treatment and 1.5 after the sterilized water treatment (Fig. 9c). Moreover, the reproductive ability, oviposition rate and egg hatchability for *sft-4* dsRN-treated PWNs were reduced significantly compared to that of the PWNs treated with sterilized water or *GFP* dsRNA (Fig. 9d). The PWNs treated with *sft-4* dsRN produced 4273 eggs, compared with 15,885 and 17,288 eggs after the treatments with sterilized water and *GFP* dsRNA, respectively (Fig. 9e) ($P < 0.01$), and the hatchability of these eggs for the three treatment groups was 31.22%, 82.98% and 80.26%, respectively (Fig. 9f) ($P < 0.01$). These results indicated that *sft-4* may play an important role in regulating PWN feeding, development, reproduction and lifespan.

In the seedlings inoculated with PWN from the four treatment groups, the virulence of the PWNs treated with *sft-4* dsRNA was significantly weaker than that of the control PWNs treated with sterilized water or *GFP* dsRNA. Specifically, the seedlings in the sterilized water group and *GFP* dsRNA group started wilting 7 days post inoculation (dpi), while the seedlings of RNAi group had no symptoms. On 15 dpi, the seedlings in both control groups had completely withered, whereas seedlings treated with *sft-4* dsRNA still had no wilting (Fig. 10). The 2-year-old saplings in the sterilized water group and *GFP* dsRNA group had begun to wilt by 14 dpi, and most had completely wilted by 40 dpi. In contrast, the *sft-4* dsRNA group never developed any symptoms. The results indicated that the *sft-4* dsRNA treatment of the PWNs either abolished pathogenicity or significantly decreased their virulence (Fig. 11) by influencing feeding, reproduction and mobility of the PWNs.

Discussion and conclusions

In the present study, we studied the role of *sft-4*, which we previously determined was significantly downregulated in PWNs after treatment with fomepizole, which inhibits ADH and rapidly kills PWNs (Wang et al. 2019). The gene *sft-4* and its homologues are widespread among organisms and play important regulatory roles in material transport and protein synthesis. In *Caenorhabditis elegans*, *sft-4* is crucial for the endoplasmic reticulum (ER) transport of vitellin VIT-2, which is one of the core proteins of vitellin and often used to detect the transportation of yolk proteins in *C. elegans* (Grant and Hirsh 1999; Belden and Barlowe 2001; Balklava et al. 2007; Saegusa et al. 2018). *Surf4*, a homologue of *sft-4* in mammalian cells, is involved in formation of the microsomal membrane and encodes a membrane protein (Reeves and Fried 1995). Several studies have indicated that *surf4* is involved in the movement of materials by the ER and Golgi (Belden and Barlowe 2001; Mitrovic et al. 2008; Saegusa et al. 2018). Based on our studies, *sft-4* overexpression in PWNs significantly increased the reproductive ability and oviposition, but RNAi of the gene significantly decreased reproduction and oviposition in PWNs with either weakened virulence or a loss of pathogenicity. In addition, our in situ hybridization analysis showed that *sft-4* was more strongly expressed in the reproductive system of adults. We also verified that *sft-4* affects PWN lifespan. Thus, *sft-4* is important for the functioning of the PWN reproductive system. Hence, we speculate that the *sft-4* might influence virulence or pathogenicity by regulating the reproduction and lifespan of the nematodes.

Overall, *sft-4* affected PWN vitality, reproduction, oviposition, female to male ratio and lifespan and significantly influenced PWN virulence. Therefore, *sft-4* is a potential drug target for developing a novel nematicide to control PWN.

Author contributions Conceptualization, SL, LW, RL, and GD; methodology, SL, LW, RL, CW, GD and QG; Software, SL; validation, RL, GD and QG; statistical analyses, SL; investigation, SL, LW, MC, WD; resources, RL, CW, GD and QG; data curation, SL, RL, GD and QG; writing original draft, SL; manuscript review and editing, RL, WD, GD and QG; visualization, SL, LW, MC; supervision, RL, GD and QG; project administration, RL, GD and QG; funding acquisition, RL and QG.

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