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Double-stranded RNA-mediated interference of dumpy genes in *Bursaphelenchus xylophilus* by feeding on filamentous fungal transformants

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ABSTRACT

RNA interference (RNAi) is a valuable tool for studying gene function in vivo and provides a functional genomics platform in a wide variety of organisms. The pinewood nematode, *Bursaphelenchus xylophilus*, is a prominent invasive plant-parasitic nematode and has become a serious worldwide threat to forest ecosystems. Presently, the complete genome sequence of *B. xylophilus* has been published, and research involving genome-wide functional analyses is likely to increase. In this study, we describe the construction of an effective silencing vector, pDH-RH, which contains a transcriptional unit for a hairpin loop structure. Utilising this vector, double-stranded (ds)RNAs with sequences homologous to the target genes can be expressed in a transformed filamentous fungus via *Agrobacterium tumefaciens*-mediated transformation technology, and can subsequently induce the knockdown of target gene mRNA expression in *B. xylophilus* by allowing the nematode to feed on the fungal transformants. Four dumpy genes (*Bx-dpy-2*, *4*, *10* and *11*) were used as targets to detect RNAi efficiency. By allowing the nematode to feed on target gene-transformed *Fusarium oxysporum* strains, target transcripts were knocked down 34–87% compared with those feeding on the wild-type strain as determined by real-time quantitative PCR (RT-qPCR). Morphological RNAi phenotypes were observed, displaying obviously reduced body length; weak dumpy or small (short and thin) body size; or general abnormalities. Moreover, compensatory regulation and non-specific silencing of *dpy* genes were found in *B. xylophilus*. Our results indicate that RNAi delivery by feeding in *B. xylophilus* is a successful technique. This platform may provide a new opportunity for undertaking RNAi-based, genome-wide gene functional studies in vitro in *B. xylophilus*. Moreover, as *B. xylophilus* feeds on endophytic fungi when a host has died, RNAi feeding technology will offer the prospect for developing a novel control strategy for the nematode. Furthermore, this platform may also be applicable to other parasitic nematodes that have a facultative, fungivorous habit.

1. Introduction

The pinewood nematode, *Bursaphelenchus xylophilus*, is a notorious, invasive, plant-parasitic nematode that is responsible for the disastrous forest epidemic of pine wilt disease and has resulted in immense economic losses and negative ecological consequences in regions where it has been introduced. Currently, it is considered a serious worldwide threat to forest ecosystems (Webster and Mota, 2008). As the whole genome DNA sequence of *B. xylophilus* has been published (Kikuchi et al., 2011), and multiple transcriptome and secretome datasets are available (Kikuchi et al., 2007; Kang et al., 2009; Yan et al., 2012; Shinya et al., 2013), genome-wide functional analyses are becoming very popular.

RNA interference (RNAi) is a reverse-genetic technique that permits the ablation of mRNA by the introduction of complementary, double-stranded RNA (dsRNA) through cellular mechanisms. Since it was first described in the free-living nematode, *Caenorhabditis elegans* (Fire et al., 1998), RNAi has become a valuable tool for studying gene function. It has provided a functional genomic platform in a wide variety of organisms (Dalzell et al., 2010). Currently, RNAi is an established experimental technique

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used in plant-parasitic nematodes, mainly in root-knot nematodes and cyst nematodes (Urwin et al., 2002; Yadav et al., 2006; Dalzell et al., 2009, 2010; Charlton et al., 2010). Attempts have been made to employ RNAi technology for functional gene analyses in *B. xylophilus*. By immersing worms in a concentrated dsRNA solution, RNAi phenotypes have been observed in *B. xylophilus* (Park et al., 2008; Cheng et al., 2010; Kang et al., 2011; Li et al., 2011). However, the efficiency of RNAi as a result of soaking is not as high as that following microinjection or electroporation in *B. xylophilus* (Park et al., 2008), and potential pitfalls were found when using the soaking method (Geldhof et al., 2007; Lilley et al., 2012). RNAi delivery by feeding is a convenient and inexpensive method used in *C. elegans*, performed by feeding worms dsRNA-expressing bacteria, and has become the preferred method for genome-wide RNAi screening (Min et al., 2010). In plant-parasitic nematodes, dsRNA can also be delivered through plants and has been shown to result in obvious phenotypic changes in root-knot and cyst nematodes that fed on the transgenic plants (Dutta et al., 2015; Lilley et al., 2012). In contrast to root-knot and cyst nematodes, the nematode *B. xylophilus* can be cultured on a filamentous fungus in vitro, which could present an opportunity to perform RNAi by feeding the nematode transgenic fungi. At the present time, transgenic technology involving filamentous fungi has advanced via *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Sugui et al., 2005). Therefore, RNAi feeding in *B. xylophilus* should be possible by culturing the nematode on dsRNA-expressing fungi.

Previous studies in *C. elegans* have demonstrated that collagens, encoded by a multi-gene family, are structural proteins required for normal body morphology, locomotion and larval development (Johnstone, 2000; McMahon et al., 2003; Page and Johnstone, 2007). Mutations in individual collagen genes and their biosynthetic pathway components can result in a range of defects from abnormal morphology (dumpy, blister and roller) to embryonic and larval death (Johnstone, 2000; Page and Johnstone, 2007). In *C. elegans*, it was reported that mutants of cuticle collagen genes (*dpy-2*, *3*, *4*, *6*, *7*, *8*, *9* or *10*) are dumpy (short and fat), have no cuticular annuli and experience severe morphological changes (Levy et al., 1993; McMahon et al., 2003; Simmer et al., 2003). Mutants of the *dpy-11* gene, a gene also required for sensory organ morphogenesis, displayed similar symptoms, with dumpy bodies observed in all larval and adult stages (Ko and Chow, 2002). As the dumpy phenotype is easily observed, dumpy genes are good candidates for use as target genes for the assessment of the effects of RNAi. We also speculate that *dpy* genes would perhaps be suitable for an assay of RNAi efficiency in other nematodes.

In this paper, we describe an RNAi feeding technique in the plant-parasitic nematode, *B. xylophilus*. A binary vector was used to construct silencing vectors. Utilising ATMT technology, a well-established method previously used to transfer genes to a wide variety of plants and fungi (Mullins et al., 2001), silencing vectors were transformed into the filamentous fungus, *Fusarium oxysporum*. To obtain obvious RNAi phenotypes, four cuticle collagen genes from *B. xylophilus* were used as RNAi targets. By feeding the nematode the fungal transformants, dsRNA was delivered and RNAi silencing effects were triggered. We assayed RNAi efficiency and observed the resulting RNAi phenotypes. The RNAi feeding technique will provide an opportunity for a genome-wide analysis of gene function in *B. xylophilus* in vitro, especially given that the acquisition of mutants is technically difficult in *B. xylophilus*. This technology also provides the prospect for the development of a novel control strategy for the parasitic nematode. We postulate that this method could be used in other parasitic nematodes with a facultative fungivorous habit.

2. Materials and methods

2.1. Biological materials

The nematode, *B. xylophilus* (strain ZJSS), was cultured on a fungal mat of *Botrytis cinerea* or *F. oxysporum*, grown on potato dextrose agar (PDA) plates at 25 °C. The fungus *F. oxysporum* f. sp. *conglutinans* wild-type Fox-A8 strain was used for the construction of the genetic transformation system. A *gfp*-expressed transformant of *F. oxysporum* Fox-A8 strain, which could express the GFP, was used for tracking the nutrient digestion process in the nematode. The *A. tumefaciens* strain AGL1 was used for bacteria-mediated fungal transformation.

2.2. RNA isolation, cDNA synthesis and dumpy gene acquisition

Protein sequences of *C. elegans* and other nematode dumpy genes were acquired from National Center for Biotechnology Information (NCBI), USA. Using those dumpy genes as the query sequences, a BLASTp search was performed against our dataset of the *B. xylophilus* genome (named BxCN, unpublished data). The DNA sequences encoding the proteins with the highest percentage similarity were further researched by searching the published *B. xylophilus* genome in NCBI (GCA_000231135.1) using BLASTn. Subsequently, primers were designed for PCR or reverse transcription PCR (RT-PCR) amplification and sequencing to obtained the whole genomic DNA and cDNA. Four dumpy genes (*dpy-2*, *4*, *10*, and *11*) were chosen for this study.

Freshly cultured nematodes were isolated using Baermann's funnel technique (Viglierchio and Schmitt, 1983) and washed with double distilled water (DDW) four to five times. *Bursaphelenchus xylophilus* total RNA (from mixed stages) was extracted using a method combining TRIzol and the NucleoSpin RNA II Kit (Macherey–Nagel, Germany) (Zhang et al., 2015). First strand cDNA was synthesised using the PrimeScript II 1st strand cDNA synthesis kit (TaKaRa, Dalian, China), following the manufacturer's protocols. The full-length cDNA sequences of dumpy genes were amplified, sequenced and subsequently used for RNAi experiments.

2.3. Silencing vector construction

The vector used for *Agrobacterium*-mediated filamentous fungi transformation, pDH-RH, was constructed from the binary plasmid pDHt/SK, containing a hygromycin B resistance gene (*hph*) flanked by polycloning site sequences in the *Agrobacterium*-mediated transfer DNA (T-DNA) fragment (Supplementary Fig. S1A). A 1984 bp fragment from another generic gene-silencing vector, pSilent-1, containing a transcriptional unit for hairpin RNA (hpRNA) expression with a spacer from a *cutinase* (*CUT*) gene intron from the rice blast fungus *Magnaporthe oryzae* (Supplementary Fig. S1B), previously used in RNA silencing studies in filamentous fungi (Nakayashiki et al., 2005; Zhong et al., 2012; Schumann et al., 2013), was inserted into the pDHt/SK plasmid containing *Kpn*I/*Bst*XI restriction sites. Both pDHt/SK and pSilent-1 plasmids were a gift from Dr. X.Z. Liu (Institute of Microbiology, Chinese Academy of Sciences). Details of the vector construction procedure are as follows: firstly, we synthesised a DNA fragment composed of an *Aspergillus nidulans* trpC promoter (PtrpC), 5′-polycloning sites (*Xho*I, *Sna*BI, *Hin*dIII, *Xba*I), an intron, 3′-polycloning sites (*Stu*I, *Spe*I, *Bgl*II, *Apa*I), and the *A. nidulans* trpC terminator (TtrpC), incorporating the *Kpn*I and *Bst*XI restriction sites at the 5′- and 3′-ends, respectively (Supplementary Fig. S1C). This artificial fragment was then inserted into the *Kpn*I/*Bst*XI-restricted plasmid, pDHt/SK. Secondly, the hygromycin-resistant marker involved in the