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Research paper

Silencing of a *Meloidogyne incognita* selenium-binding protein alters the cuticular adhesion of *Pasteuria penetrans* endospores

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ABSTRACT

Pasteuria penetrans is an endospore forming hyperparasitic bacterium of the plant-pathogenic root-knot nematode, *Meloidogyne incognita*. For successful parasitization, the first step is adherence of bacterial endospores onto the cuticle surface of nematode juveniles. The knowledge of molecular intricacies involved during this adherence is sparse. Here, we identified a *M. incognita* selenium-binding protein (*Mi-SeBP-1*) differentially expressed during the initial interaction of *M. incognita* and *P. penetrans*, and show that it is involved in modulating parasitic adhesion of bacterial endospores onto nematode cuticle. Selenium-binding proteins (SeBPs) are selenium associated proteins important for growth regulation, tumor prevention and modulation of oxidation/reduction in cells. Although reported to be present in several nematodes, the function of SeBPs is not known in Phylum Nematoda. *In situ* hybridization assay localized the *Mi-SeBP-1* mRNA to the hypodermal cells. RNAi-mediated silencing of *Mi-SeBP-1* significantly increased the adherence of *P. penetrans* endospores to the nematode juvenile cuticle. Silencing of *Mi-SeBP-1* did not change the nematode's ability to parasitize plants and reproduction potential within the host. These results suggest that *M. incognita Mi-SeBP-1* might be involved in altering the attachment of microbial pathogens on the nematode cuticle, but is not involved in nematode-host plant interaction. This is the first report for a function of SeBP in Phylum Nematoda.

1. Introduction

Selenium (Se) is an essential trace nutrient for majority of living organisms such as archaea, protozoa, bacteria and animals (Behne and Kyriakopoulos, 2001). Se is absorbed and accumulated in plants and subsequently circulated along the food chain (Schild et al., 2014). Although not absolutely essential for land plants, at low concentration Se helps the plants withstand abiotic and biotic stresses (Pilon-Smits et al., 2009). The selenium-containing proteins have been classified into three groups, *viz*., specific selenoproteins, non-specific selenium-containing proteins and selenium-binding proteins (Behne and Kyriakopoulos, 2001). Unlike the first two groups of selenium-containing proteins where Se is translationally incorporated into the protein specifically or non-specifically, the selenium-binding proteins (SeBPs or SBPs) are proteins to which Se is bound to the protein molecule (Behne and Kyriakopoulos, 2001). Secondary structure prediction of SeBPs revealed that these proteins are not transmembrane proteins and their size is conserved in different plants and animals with the amino acid identity spanning through the length of polypeptide (Flemetakis et al., 2002). SeBPs are largely known to be involved in tumor prevention (Ishii et al., 1996), growth regulation (Bansal et al., 1989) and intra-Golgi protein transport (Porat et al., 2000). However, the knowledge on the physiological function of SeBPs in living organisms is scant, and no information is available on the role of these proteins in Phylum Nematoda.

Root-knot nematodes (RKNs; *Meloidogyne* spp.) are reckoned as top-priority nematode pests and are responsible for extensive crop losses (Jones et al., 2013). *Pasteuria penetrans* (Thorne) Sayre and Starr, a Gram-positive, endospore forming bacterium is a hyperparasite of the RKN, and represents a typical naturally coevolved pathogen-hyperparasite system (Davies et al., 2011). The soil dwelling bacterial endospores attach to the cuticle surface of the migrating pre-parasitic juveniles (J2 s), germinate *via* germtube and proliferate into enormous number of endospores leading to death of the adult females (Davies et al., 2011). The attachment of endospores onto the J2 cuticle is considered to be the first and most crucial step of infection and the cuticle surface coat plays a pivotal role in facilitating the attachment (Davies and Danks, 1992; Spiegel and McClure, 1995).

Nematode cuticle is an extracellular matrix secreted by hypodermis

Abbreviations: SeBP, Selenium-binding protein; FAR, Fatty acid and retinol binding protein; ROS, Reactive oxygen species; Mi, *Meloidogyne incognita*; *C. elegans*, *Caenorhabditis elegans*; RKN, Root-knot nematode

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and acts as an exoskeleton (Lee, 2002). Apart from maintaining morphological integrity, the cuticle also protects the nematodes from pathogenic microorganisms (Davies and Curtis, 2011). The outermost layer of the cuticle (epicuticle) is overlaid with the surface coat, and is formed from modification of various non-structural hypodermal secretions. The surface coat moieties undergo continuous shedding and replenishment, and helps the nematodes interact with microbes and external environment (Davies and Curtis, 2011). The analysis of cuticular chemistry has revealed the presence of several components that collectively contribute to the interaction with microorganisms including *P. penetrans* (Blaxter and Robertson, 1998; Davies, 2009). Underlying the cuticle, the syncytial hypodermis also responds to pathogen attack in *Caenorhabditis elegans*, and injury in the hypodermal cells provokes rapid induction of antimicrobial peptide gene expression (Taffoni and Pujol, 2015). Recently, it was discovered that a *M. incognita* fatty acid and retinol binding protein (Mi-FAR-1) protects the nematode from *Pasteuria* endospore adhesion (Phani et al., 2017). Additionally, collagen and glycoprotein like molecules are also presumed to be involved in the endospore adhesion on nematode cuticle (Davies and Danks, 1992; Davies and Opperman, 2006; Phani et al., 2018). However, our understanding of the factors involved in interaction between *M. incognita* and *P. penetrans* is preliminary.

In an attempt to ascertain the genes involved in the *M. incognita – P. penetrans* interaction, we identified a selenium-binding protein coding transcript which was differentially expressed (upregulated) in the endospore encumbered nematode J2 s (our unpublished results). Since the role of SeBPs has been suggested in immune response in few invertebrates, for example scallop and scleractinian coral (Song et al., 2006; Vidal-Dupiol et al., 2011), we hypothesized that the *M. incognita* SeBP (hereafter Mi-SeBP-1) could be involved in protecting the nematodes against bacterial pathogens. Here we show that Mi-SeBP-1 protects the nematode from *P. penetrans* endospore adhesion. To the best of our knowledge, this is the first report on the functional role for a nematode SeBP.

2. Materials and methods

2.1. Nematode population

The pure culture of an Indian isolate of *M. incognita* (Kofoid & White) Chitwood race 1 was multiplied on tomato plant (*Solanum lycopersicum* L. cv. Pusa Ruby) in a glasshouse at ICAR-Indian Agricultural Research Institute, New Delhi, India. Eggmass were hand-picked from infected roots and hatched *via* modified Baermann's assembly (Whitehead and Hemming, 1965). Freshly hatched juveniles (J2 s) were used for experimental purpose.

2.2. Cloning and characterization of Mi-SeBP-1 of M. incognita

The candidate selenium-binding protein was identified as a differentially expressed transcript in the *Pasteuria* endospore encumbered juveniles in an RNA-Seq experiment comparing transcriptomes of encumbered juveniles to non-encumbered juveniles of *M. incognita* (our unpublished results). Briefly, the RNA-Seq experiment was carried out to identify the nematode genes involved in the initial stage of *M. incognita* and *P. penetrans* interaction. The transcriptional response of *M. incognita* was investigated at eight hour post attachment of *P. penetrans* endospores. Total RNA was extracted from the endospore encumbered J2 s incubated for 8 h on a slowly moving rotator. The freshly hatched J2 s incubated in M9 buffer for 8 h served as control. RNA-Seq was performed using Illumina platform and the differentially expressed genes were identified. The selenium-binding protein coding transcript was found to be upregulated in *M. incognita* J2 s at 8 h post endospore attachment. Specific primers (SBP_F and SBP_R) (Table 1) were designed to amplify the sequence from *M. incognita* cDNA. Total RNA (500 ng) was extracted from J2 s with NucleoSpin® RNA kit (Macherey-Nagel, Germany) according to manufacturer's protocol and cDNA was synthesized as described earlier (Phani et al., 2017). The amplified product was cloned into pGEM-T Easy vector (Promega, USA) and re-sequenced *via* Sanger sequencing for confirmation. The sequence, thus obtained, was analyzed for presence of selenium-binding protein moiety using NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterPro protein sequence search tool (https://www.ebi.ac.uk/interpro/). The sequence of the *M. incognita* SeBP coding gene was named as *Mi-SeBP-1* and submitted to GenBank (acc. no. MG744257). Sequence homology comparisons were done by using BLASTx against non-redundant protein (nr) databases of NCBI, WormBase Parasite (http://www.wormbase.org) and UniProt (http://www.uniprot.org). The putative selenium-binding protein sequences of other nematodes were retrieved and used to reconstruct the phylogenetic relationship using MEGA6 (Tamura et al., 2013). The phylogenetic tree was constructed using maximum likelihood method, bootstrapped 1000 times and SeBP sequence of *Danio rerio* (AAH56590) was used as an out-group.

2.3. In situ hybridization

Digoxigenin (DIG)-labeled sense and antisense cDNA probes (Rosche, Germany) were designed with specific primers (SISH_F and SISH_R; Table 1) to localize the mRNA expression site of *Mi-SeBP-1* by *in situ* hybridization (Kimber et al., 2002). Nematode fixation, permeabilization, probe hybridization and signal detection were carried out as described previously (Kimber et al., 2002; Phani et al., 2017). Specimens were examined under a Zeiss Axiocam M2 m compound microscope and images were captured by AxioVision software. The experiment was repeated thrice.

2.4. dsRNA synthesis and in vitro RNAi

Double stranded RNA (dsRNA) for *Mi-SeBP-1* was synthesized as described earlier (Phani et al., 2017). Approximately 1500 freshly hatched J2 s were soaked in a solution containing 0.1 mg ml^{-1} target dsRNA for *in vitro* RNAi following standard methodology (Urwin et al., 2002). The soaking was continued for 18 h in dark on a slowly moving rotator at 28 °C. After incubation, the dsRNA treated J2 s were thoroughly washed, total RNA was extracted and level of transcript suppression was assessed by qRT PCR (Phani et al., 2017) with three biological and three technical replicates. dsRNA of an unrelated gene (*gfp*, HF675000) was used as non-native negative control. J2 s incubated in dsGFP and in soaking buffer without dsRNA were used as controls. All soaking experiments were repeated thrice. Primer details are provided in Table 1.

The effect of *in vitro* knockdown of *Mi-SeBP-1* on transcriptional profile of five other genes was analyzed for understanding the coexpression. *Mi-far-1* (MF510388; encoding fatty acid and retinol binding protein), *Mi-muc-1* (MG579969; encoding mucin-like protein), *hsp-20* (heat shock protein 20) and genes encoding ubiquitin and aspartic protease were selected as they were differentially expressed in the transcriptome dataset and are known to be involved in bacterial adhesion on nematode cuticle or nematode immune responses (Tort et al., 1999; Cohen and Troemel, 2015; Pérez-Morales and Espinoza, 2015; Phani et al., 2017, 2018). Additionally, sequences were checked for the presence of short stretches of similar sequences in their targeted dsRNAs and homology was assessed as described (Naito et al., 2005; Shivakumara et al., 2016). In addition, the sequences were aligned to assess their homology, and searched for any possibility of unintended off-target effect with processed siRNAs from the existing database by dsCheck software (Naito et al., 2005). qRT PCR was conducted with three biological and three technical replicates. Primer details are given in Table 1.