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Calreticulin is required for responding to stress, foraging, and fertility in the white-tip nematode, *Aphelenchoides besseyi*

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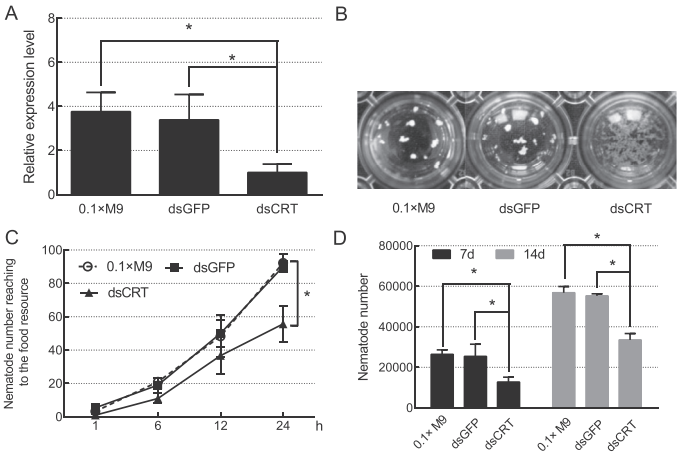
HIGHLIGHTS

- CRT from *A. besseyi* is largely expressed in oesophageal gland and gonad cells of the female nematodes.
- AbCRT-1 is induced by a moderate osmotic stress but suppressed in the extreme desiccation condition.
- AbCRT-1 is involved in aggregation, foraging and reproduction of *A. besseyi*.

GRAPHICAL ABSTRACT



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ABSTRACT

Calreticulin (CRT) regulates a wide array of cellular responses in physiological and pathological processes. A full-length cDNA-encoding CRT protein, namely AbCRT-1, was isolated from *Aphelenchoides besseyi*, an ectoparasitic plant nematode and the agent of white tip disease of rice. The deduced amino acid sequence of AbCRT-1 was highly homologous with other nematode CRTs, and showed the closest evolutionary relationship with BxCRT-1. *In-situ* hybridization showed that AbCRT-1 is specifically located in the oesophageal gland and gonads of *A. besseyi*, suggesting its potential role in parasitism and reproduction. Quantity real-time PCR analysis showed that AbCRT-1 is highly expressed in female nematodes but poorly expressed in eggs, juveniles, and male nematodes. Exposing the nematode to relatively low osmotic stress promotes the transcription of AbCRT-1 whereas extreme desiccation suppresses the transcription significantly. Nematodes in which AbCRT-1 mRNA level had been knocked down by soaking them in AbCRT-1 dsRNA solution distributed randomly and did not aggregate temporally, with a decreased capacity of food discernment. Thus the affected nematodes were markedly less fecund. These results demonstrate that AbCRT-1 is required in *A. besseyi* for responding to stress, foraging, and fertility.

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1. Introduction

Phytonematodes have evolved complicated parasitism strategies to support their growth and development. They pierce the cell walls of host plants with a protrusible stylet to access the cell contents. The stylet, which is hollow, also serves as a pipeline to introduce into the host cells the effectors secreted by the nematode's oesophageal glands (Mitchum et al., 2013). Advances in the identification and functional analysis of nematode effector proteins suggest that effectors play a key role in the nematode–host interaction including modification of the cell wall, molecular mimicry, and suppression of resistance (Mitchum et al., 2013; Rosso et al., 2012).

Calreticulins (CRTs) are chaperone calcium-binding proteins that are highly conserved in plants and animals and are increasingly believed to have a multifunctional role in development and in the suppression of pathology-related signals including gene expression, cell adhesion, and immune regulation by means of calcium binding or direct interaction with signalling proteins (Gold et al., 2010; Obeid et al., 2007). In species that parasitize humans and other animals, CRTs emerge as key modulators of several immunological aspects of the relationship between the parasites and their vertebrate hosts. For instance, *Trypanosoma cruzi*, the agent of Chagas' disease in human, can translocate CRT from the ER to the surface, and inhibit both the classical and lectin complement pathways. Moreover, TcCRT can bind and inactivate first complement component C1, thus helping the parasite to evade from its hosts' immune response, and to promote infectivity (Ramirez et al., 2012).

Although not well understood, CRTs in plant parasitic nematodes may interfere with Ca^{2+}-mediated immune signalling in host plants (Goverse and Smant, 2014). Mi-CRT was the first identified CRT from the stylet secretions of the root knot nematode *Meloidogyne incognita*, and was mainly expressed in the subventral oesophageal glands of the nematode at stage J2 and in the dorsal gland at the sedentary stages (Jaubert et al., 2002). The secreted Mi-CRT is transferred into the apoplast of infected plant cells and accumulated along cell walls of the giant cells that make up the feeding site (Jaouannet et al., 2013; Jaubert et al., 2005) . In addition, transgenic *Arabidopsis thaliana* overexpressing Mi-CRT was shown to be more susceptible to *M. incognita*, and the transgenic form was thus linked to a reduction of defence-related genes and deposition of callose after treatment with PAMP elf18. These findings indicate that Mi-CRT serves as an immune suppressor – it suppresses the plant's basic defence mechanisms during pathogenic or parasitic interactions (Jaouannet et al., 2013).

BxCRT-1, from *Bursaphelenchus xylophilus*, which causes pine wilt, was found to be highly homologous to the animal parasite *Haemonchus contortus* (71% identity) but not as much to *M. incognita* (68.2% identity). The single-copy gene, determined by Southern blotting, is transcribed mainly in adult nematodes and less so in juvenile nematodes. BxCRT-1-silenced *B. xylophilus* was less fecund *in vitro* than normal nematodes of the species, suggesting that BxCRT-1 has an important role in the development of *B. xylophilus* (Li et al., 2011).

The white-tip nematode *Aphelenchoides besseyi*, which is among the top ten plant parasitic nematodes, lowers rice yields by 12%–20%; the loss worldwide is estimated at $16 billion (Jones et al., 2013). The ecto-parasite infects above-ground parts, eventually causing the characteristic white tip of the flag leaf, small grains, and erect panicles (EPPO, 2004, Feng et al., 2014a, 2014b). As a seed-borne nematode, *A. besseyi* is known to survive anhydrobiotically under the husk of infected rice seeds for several years (Tenente et al., 1994).

More recently, *A. besseyi* was subjected to transcriptomic analysis using the latest sequencing technology (Wang et al., 2014). The 13 unique potential effectors identified from 41 candidate effector homologues included a CRT gene, which was also discovered in an independent transcriptomic data of *A. besseyi* (Kikuchi et al., 2014). However, the CRT gene in *A. besseyi* is yet to be identified completely.

The present study had the following objectives: (1) clone and sequence calreticulin in plant pathogenic *A. besseyi* (AbCRT-1) using RACE-PCR; (2) examine the expression of AbCRT-1 at different developmental stages; and (3) probe its effects on nematode adaptation to stress in the form of desiccation and on the development of the nematode population.

2. Materials and methods

2.1. Nematode

A. besseyi was initially isolated from infected rice seeds and multiplied for 3 weeks at 25 °C on *Botrytis cinerea* growing on potato dextrose agar (PDA). A sample of *A. besseyi* containing different stages of the nematode was collected by rinsing the substrate with deionized water and filtering the suspension through a metal sieve (Endecotts, London; pore size 25 μm). The nematodes were cleaned by washing them first with sucrose solution (30% w/v) and then with deionized sterile water containing penicillin (100 units/mL), streptomycin (100 mg/mL), and amphotericin (0.25 mg/mL), followed by centrifuging the suspension at a low speed.

2.2. AbCRT-1 gene discovery

Given that the calreticulin gene BxCRT from the pine wilt nematode *B. xylophilus*, a species closely related to *A. besseyi* (van Megen et al., 2009), has been cloned and sequenced and that the data are available with the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), we ran a Blastp (protein–protein BLAST) search of the database using the protein sequence BxCRT (accession no. ADD82420) as a query. The thorough search showed that CRTs are evolutionarily conserved in all extant nematode species. After alignment of the hypothetical protein sequences of the CRT from the search results, we designed the degenerate primers CRTdeg-F/R targeting the conservative proteins sequence, using a consensus-degenerate hybrid oligonucleotide primers (CODEHOP) designer (http://blocks.fhcrc.org/codehop.html) as described by Rose et al. (2003).

For the preparation of whole RNA of *A. besseyi*, a 100 μL sample of active nematodes was frozen in liquid nitrogen for 30 s and quickly homogenized using a disposable homogenization pestle on ice. The RNA was then extracted using TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA) as specified in the product manual. For genomic DNA (gDNA), the standard procedure was used (Aljanabi and Martinez, 1997). The quantity and the quality of the RNA and gDNA were measured with Nano-drop 2000 spectrophotometer (Thermo Scientific, West Palm Beach, Florida, USA) under OD 260/280 and 1.2% agarose gel electrophoresis.

For the first-strand cDNA synthesis, the reaction mixture containing 6 μg of RNA, 3 μL of Oligo dT (14 nM), 400 units of M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA), and 17 μL RNase-free water were incubated at 70 °C for 5 min, placed on ice for 1 min, and 80 units of Riblock Rnase inhibitor (Thermo Scientific, USA) and 2 μL of dNTP (10 mM) were added to the mixture. The reaction tubes were incubated at 70 °C for 10 min.

Segments of AbCRT-1 were obtained using the degenerated primers CRT CRTdeg-F and CRTdeg-R by PCR amplification in a 25 μL reaction mixture as described earlier (Staheli et al., 2011). Based on the sequence, gene-specific primers were designed by the Primer3 program (http://primer3.ut.ee/). The 3′ and 5′ terminal fragments were amplified using the SMART™ RACE cDNA amplification kit (Clontech Laboratories, Inc., USA). For the 3′ end, AbCRTgsp-F3 and