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Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi

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Abstract

A single chitin synthase gene is responsible for chitin production in the eggshells of the plant parasitic nematode *Meloidogyne artiellia*. In this paper we describe a functional analysis of this gene using RNAi as well as further analysis of two similar genes from the free-living nematode *Caenorhabditis elegans*. The parasitic life-style of *M. artiellia* required the development of a novel method for delivery of dsRNA to nematode eggs that may be of utility in other experimental systems. *C. elegans* chitin synthase genes were silenced by feeding nematodes bacteria expressing appropriate chitin synthase dsRNA from a plasmid vector, while *M. artiellia* egg masses were soaked in dsRNA solution. The results obtained demonstrated that the synthesis of chitin continues to take place in nematode eggs within the egg sac in the parasitic nematode, and that the removal of this activity affects egg development in both *C. elegans* and *M. artiellia*. The method described here provides a new way of investigating gene function in plant parasitic nematodes allowing the validity of potential target genes for novel control methods to be assessed. Furthermore, since intact egg cells within the gelatinous matrix of *M. artiellia* are permeable to dsRNA, eggs of other nematodes may also be similarly permeable to dsRNA and therefore amenable to use with dsRNAi.

Keywords: Gene silencing; Chitin synthase; Parasitic nematodes

1. Introduction

RNA-mediated interference (RNAi) is a phenomenon in which introduction of double-stranded RNA results in the specific suppression of the protein encoded by the target gene, through degradation of the endogenous mRNA. This phenomenon was first described in *Caenorhabditis elegans* (Fire et al., 1998) but has since been reported to occur in a variety of organisms including zebrafish (Wargelius et al., 1999), planaria (Sanchez Alvarado and Newmark, 1999), *Hydra* (Lohmann et al., 1999), fungi (Romano and Macino, 1992), *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999) mammals (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000) and plants (Napoli et al., 1990).

Messenger RNA degradation mediated by complementary double-stranded RNA occurs in a series of reactions. The dsRNA is first recognized by an RNase III family member, named Dicer in *Drosophila* (Ketting et al., 2001) and cleaved into small silencing RNAs (siRNAs; Elbashir et al., 2001). The siRNA is thought to form a multicomponent nuclease complex called the RNA-induced silencing complex (RISC; Hammond et al., 2000; Bernstein et al., 2001). The target mRNA recognized by RISC is cleaved in the region complementary to the siRNA and rapidly degraded. In *C.*

Abbreviations: Ct, threshold cycle; dsRNA, double-stranded RNA; RISC, RNA-induced silencing complex; siRNA, short interfering RNA.

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elegans, injecting dsRNA into the body cavity produces specific and robust interference with gene expression in the progeny of the injected individual. Alternative methods of delivering dsRNA to *C. elegans* have also been used, including soaking of worms in a solution of dsRNA (Maeda et al., 2001) or feeding worms with bacteria expressing the dsRNA from a construct cloned into a standard plasmid vector (Kamath et al., 2001). Although the efficiency of inhibition is usually lower using these feeding methods than with microinjection, the ease with which these protocols can be used mean that they are often the method of choice when performing studies of gene function in *C. elegans*.

Although RNAi can be performed routinely in *C. elegans*, using this technique with parasitic nematodes is more problematic. RNAi has been used with animal parasites (Montalvetti et al., 2003; Skelly et al., 2003), but there are fewer reports of its use with plant parasitic nematodes. A method for induction of feeding in second stage juveniles of cyst nematodes has been described which allowed uptake of dsRNA from solution and led to RNAi in these nematodes (Urwin et al., 2002). However, no reports of the use of RNAi to analyze gene function in other plant parasitic nematodes or its use with other nematode stages are present in the literature. Development of RNAi for use with plant parasitic nematodes is important as it permits functional analysis of nematode genes and allows the roles of various gene products to be assessed by examination of the effects of their removal on nematode viability. Such information is helpful in prioritising potential targets for new control methods.

Plant parasitic nematodes are devastating pathogens of plants that cause considerable yield losses in food and fibre crops each year. They have a life cycle consisting of five developmental stages. The first and second juvenile stages (J1 and J2) occur in the egg. Motile J2s hatch from eggs in the soil and locate host plants by following gradients of chemical cues (Dusenbery, 1997). Following invasion of the host plant roots, a permanent feeding site is established within the root and the nematodes feed, grow and moult three more times to the adult stages. Adult males emerge from the root while the females remain in the roots and lay eggs at various stages of development into a gelatinous matrix which extrudes from the root. The eggs are surrounded by an eggshell whose strength is provided by a chitinous layer (Bird and Self, 1995).

We previously described a chitin synthase from *Meloidogyne artiellia* (Veronico et al., 2001). Here we describe functional analysis of this gene using RNAi, the first such analysis from any root knot nematode, as well as further analysis of two similar genes from *C. elegans*. In addition, the parasitic life-style of *M. artiellia* required the development of a method for delivery of dsRNA to nematode eggs that may be of utility in other parasitic nematodes.

2. Materials and methods

2.1. Biological material

M. artiellia was cultured on wheat plants as previously described (De Giorgi et al., 1997). Egg masses, bearing eggs containing nematodes at varying stages of development, were collected and used immediately or stored at −80 °C until use. *C. elegans* (N2 strain) was maintained on lawns of OP50 *E. coli* using standard protocols (Sulston et al., 1998).

2.2. Chitin synthase assays

Fresh *M. artiellia* eggs suspended in TM buffer (50 mM Tris–HCl, pH 7.5 and 2.5 mM $MgCl_2$) were broken mechanically by vortexing four times for 30 s in the presence of glass beads (425–600 μm in diameter). Samples were cooled on ice between the vortexing steps. After a short centrifugation to remove insoluble material and glass beads, the supernatant (crude egg extract) was used for enzyme assays. The protein content of crude extracts was determined using a Bio-Rad protein assay kit.

Chitin synthase assays were performed using 80 μg of protein. The crude extract was incubated for 10 min at 30 °C with trypsin and a twofold excess by weight of soybean trypsin inhibitor was then added (Orlean, 1987). Control incubations at 30 °C, without trypsin, were performed in parallel. The chitin synthase assay was performed as described (Leighton et al., 1981) in a reaction mixture containing 25 mM Tris, pH 8.0; 50 mM $MgCl_2$; 1mM UDP-*N*-acetylglucosamine; 50 mM *N*-acetylglucosamine; 0.075 μCi of [^{3}H]UDP-*N*-acetylglucosamine ([6-^{3}H] glucosamine, 32.5 μCi/nmol) in a final volume of 150 μl. Reactions were started by adding crude extract to the assay mixture and were incubated for 90 min at 28 °C. The reaction was terminated by the addition of cold trichloroacetic acid to a final concentration of 5%. The reaction mixture was then collected on nitrocellulose filter paper which was washed with trichloroacetic acid and dried. Incorporated radiolabel was counted in a liquid scintillation spectrometer.

2.3. Eggshell chitin staining

M. artiellia eggs were collected in water and incubated in Calcofluor white (Sigma) at a concentration of 0.1mg/ml for 5 min. The eggs were then washed twice with water and observed under the fluorescence lamp (365 nm–397 nm) of an Axioplan 2 microscope (Zeiss).

Pictures were taken digitally with an Axiocam CCD camera fitted with an Axiovision 3.1 Processor.

2.4. DNA sequences

The following sequences were used in this study: *C. elegans* chitin synthase genes, accession numbers Z72516