Immunolocalisation of Secreted-Excreted Products of *Meloidogyne* spp. Using Polyclonal and Monoclonal Antibodies

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ABSTRACT

Molecules expressed at the surface cuticle (SC) of plant parasitic nematodes represent the primary plant-nematode interface, and together with secreted-excreted (S-E) products are probably the first signals perceived by the host. These molecules, which are released into plant tissue, probably play important roles in the host-parasite interactions. Characterisation of these antigens will help in the identification of nematode targets useful for novel control strategies, which interfere with the nematode infection of plants. Three monoclonal (MAbs) and three polyclonal (PAbs) antibodies produced to S-E products of *Meloidogyne* spp. and *Heterodera avenae* were used to examine their reactivity towards *M. incognita* and/or *M. arenaria* second stage juveniles and adult females. The three PAbS showed cross-reactivity with *M. incognita* and *M. arenaria*. Antibody Roth-PC 373 strongly recognised molecules present in the SC, amphids and intestine, antibody Roth-PC 389 recognised the nematode amphids and metacorpus, while antibody Roth-PC 419 bound to molecules present in the subventral glands. Reactivity of the MAbs was tested only against *M. arenaria*. Monoclonal antibody Roth-MAb T116C1.1 showed intense reactivity with molecules present in the amphidial and phasmidial glands. Monoclonal antibodies Roth-MAb T46.2 and T42D.2 labeled the nematode amphids and molecules present in the nematode oesophagus (metacorpus), respectively.

Additional keywords: root-knot nematodes, surface coat, amphid, subventral and phasmidial glands, oesophagus.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are highly polyphagous plant sedentary endoparasites, which cause major crop losses in agriculture worldwide (Sasser, 1980; Sasser and Freckman, 1987). Plant-parasitic nematodes have diverse parasitic relationships with their host plants in order to obtain the nutrients necessary for their development and reproduction. Nematode species that are evolutionarily advanced become sedentary and feed from a single cell or a group of cells for prolonged periods of time (Hussey et al., 2002). *Meloidogyne* spp establish a permanent feeding site in the differentiation zone of the root by inducing nuclear division without cytokinesis in host cells (Williamson &
Gleason, 2003), modulating complex changes in plant cell gene expression, physiology, morphology and function (Gheysen and Fenoll, 2002). These nematodes spend most of their life cycle inside the roots, where they undergo distinct changes in morphology; they pass through three consecutive moults in developing from a second stage juvenile (J2) to a globose sedentary adult female (Bird & Bird, 1998). The change in morphology is accompanied by biochemical and ultra-structural changes in the surface cuticle (SC). The cuticle is a complex structure that is involved in the motility, maintenance of morphology and interactions with the external environment (Blaxter & Robertson, 1998). Molecules expressed at the SC of these parasitic nematodes represent the primary host-parasite interface, and together with secreted-excreted products are probably the first signals perceived by the host (Lopez de Mendonça et al., 1999). Nematode surfaces have a coat which contains different carbohydrates probably in the form of glycoproteins (Gems & Maizels, 1996). Among the nematode’s secretory products, stilet secretions are believed to play a role in the penetration and migration through root tissue, modification and maintenance of root cells as feeding sites, formation of feeding tubes, and digestion of host cell contents to facilitate nutrient acquisition by the nematode (Hussey, 1989). These secretions are produced by two subventral and one dorsal oesophageal gland cells and are secreted through the stylet into the plant tissue during parasitism (Davis et al., 2004). Over the last decade, research has focused on the secretions of plant parasitic nematodes, in particular those of the cuticle, amphids and oesophageal glands, which are considered to be involved in several aspects of the infection process (Fioretti et al., 2001). In this present study, different polyclonal and monoclonal antibodies raised against some secreted-excreted (SE) products of Meloidogyne spp. and Heterodera avenae (Curtis, 1996; Curtis et al., 1997; Sharon et al., 2002) were used to characterise in vitro binding to the SC, amphids, subventral glands, phasmidial glands, metacorpus and intestine of J2 and adults females of M. incognita and the J2 of M. arenaria.

MATERIALS AND METHODS

Nematodes

Second stage juveniles (J2) and females of M. incognita race 1 from North Carolina State University and J2 of M. arenaria from the University of Coimbra were obtained from infected tomato (Lycopersicon esculentum Mill.) plants cv. Tiny Tim. Egg masses were collected as described by Hussey & Barker (1973) and adult females were dissected from infected tomato roots.

Antibodies

Polyclonal antibodies (PAs) were previously raised in rabbits: Roth-PC 373 (live pre-parasitic J2 and SC extract of M. incognita), Roth-PC 389 (SE products from M. incognita) (Sharon et al., 2002) and Roth-PC 419 (SE products from M. arenaria) produced as described in Curtis (1996). The monoclonal antibodies (MAbs) were previously raised in mice (Curtis, 1996) to plant parasitic nematodes: Roth-MAb T116C1.1 and Roth-MAb T42D.2 were prepared using homogenate of pre-parasitic J2 and SC extract of live pre-parasitic J2 of M. incognita; Roth-MAb T46.2 was prepared using H. avenae cyst homogenate as described in Curtis et al. (1997).

Indirect immunofluorescence for nematode pieces and cryosections

Freshly hatched M. incognita and M. arenaria J2 were pelleted by centrifugation and used to prepare nematode pieces and cryosections (30 µm), respectively. These were prepared and processed as described by De Boer et al. (1996) and Curtis (1996). The immunolabelling procedure utilizing monoclonal and polyclonal antibodies described above was done according to Fioretti et al. (2001) and Sharon et al. (2002).

Enzyme Linked Immunosorbent Assay

Microtitre-enzyme linked immunosorbent assay (Elisa) plates (Nunc-immunoplate) were coated overnight at 4 °C with 50 µl (10 µg/ml) of nematode antigens and the ELISA test was performed as described by Curtis et al. (1996).

SDS-PAGE and Western blotting

One dimensional SDS-PAGE was carried out following the methodology described by Laemmli (1970). Antigen samples of M. incognita and M. arenaria J2 were prepared as described in Fioretti et al. (2001). Western blotting of proteins was performed using standard protocol (Sambrook et al., 1989) utilising the antibodies Roth-PC 373, Roth-PC 389, Roth-MAb T116C1.1 and Roth-MAb T42D.2.

RESULTS

Immunofluorescence studies

In the immunolocalisation assays, all the antibodies used in this study bound to the amphids. Two out of three PAs reacted with the SC and other structures. The three PAs (Roth-PC 373, Roth-PC 389 and Roth-PC 419) tested showed cross-reactivity with pre-parasitic J2 of M. incognita and M. arenaria. Immunofluorescence with J2 of M. incognita showed that Roth-PC 373 reacted with SC and amphids (Figure 1a), Roth-PC 419 reacted with the subventral glands (Figure 1b) and amphids, and Roth-PC 389 reacted with amphids (data not shown). Moreover, Roth-PC 373 labelled with the SC, amphidial pores (Figure 1c) and intestine in J2 of M. arenaria (Figure 1d). Antibody Roth-PC 389 labelled amphids and the metacorpus, and Roth-PC 419 labelled amphids, SC and intestine (data not shown). Strong reactivity, especially in the amphids was also detected when MAbs were probed in
immunofluorescence tests with J2 of *M. arenaria*. Also, Roth-MAb T46.2 reacted with the metacorpus, Roth-MAb T116C1.1 reacted with the phasmidal glands and Roth-MAb T42D.2 labelled the oesophagus (data not shown). None of the MAbs tested showed reactivity with the SC of *M. arenaria*. No immunofluorescence was observed with the negative control, which consisted of the medium (20D) used to grow the cell lines secreting antibodies.

**Elisa assay**

All monoclonal and polyclonal antibodies tested reacted with antigens present in the homogenates of J2 of *M. incognita* and *M. arenaria* using indirect Elisa assay. Antigens were also present in the homogenate of females, but in lower concentration, except for the antibody Roth-PC 373. This antibody strongly recognised antigens in *M. incognita* females (Figure 2a). The antibodies Roth-PC 389 and Roth-MAb T46.2 weakly recognised antigens present in all the nematode samples tested (Figure 2a, b). Boiling nematode homogenates for 10 min, prior to the Elisa did not affect their reactivity with the antibodies.

**Analysis by Western Blots**

Antibodies Roth-PC 373, Roth-PC 389, Roth-MAb T116C1.1 and Roth-MAb T42D.2 were tested by Western blot using *M. incognita* and *M. arenaria* J2 homogenates. Roth-PC 373 labelled a broader range of proteins in the homogenate of *M. incognita*. Common bands were also present in homogenate of *M. arenaria*, with apparent molecular weights of 20 kDa and 37 kDa (Figure 3, line 1 and 2). Roth-PC 389 recognised a series of bands between <14 kDa and >94 kDa in *M. incognita*, and two common bands were observed in *M. arenaria*, with molecular weights of approximately 33 kDa and 64 kDa (data not shown). The MAbs Roth-MAb T116C1.1 and Roth-MAb T42D.2 labelled a single protein band of approximately 94 kDa in *M. incognita* (Figure 3, line 3 and 4). In contrast, no reactivity was observed with *M. arenaria*.

**DISCUSSION**

All polyclonal and monoclonal antibodies analysed reacted with antigens present in the amphids of both nematode species *M. incognita* and *M. arenaria*, indicating these amphidial antigens cross react in both species of *Meloidogyne* tested. The monoclonal antibody, Roth-MAb T116C1.1, recognised antigens present in amphidial and phasmidal glands of *M. arenaria*. The amphids and phasmids are sensory organs, which appear to be chemosensory because they are associated with openings in the nematode cuticle which allow enclosed neurons to contact the external environment (Freitas et al., 2001). The nematodes tested possess two bilaterally symmetrical amphids and phasmids, located in the nematode head and tail, respectively. They may be involved in host recognition and have been implicated in aiding the nematode to locate its host. Studies reported by Sharon et al. (2002) also showed cross-reactivity with *M. javanica* of several antibodies, which had been raised against *M. incognita*.

Two of the three PAbs, Roth-PC 373 and Roth-PC 419, bound to the amphids and also bound to SC and the intestine of *M. incognita* and *M. arenaria*, respectively. Cross-reactive immunodominant epitopes present in the amphids and cuticle of plant parasitic nematodes may be involved in physiological mechanisms shared by these nematodes. Moreover, the nematode cuticle selectively regulates the flow of fluids through the body wall and could be a source of secreted compounds recognized as signal molecules by plants (Abad et al., 2003). This inhibitory effect of antibodies on nematode movement and infection of plants has been demonstrated by Sharon et al. (2002) and Fioretti et al. (2003), implicating amphidial and cuticular antigens as good targets for devising novel nematode control strategies.

The antibody Roth-PC 419 also recognised *M. incognita* subventral glands and *M. arenaria* intestine. Oesophageal gland secretions are considered to contain products of the parasitism genes (Hussey et al., 2002). These secreted products can be involved in the degradation of plant cell walls, as cellulases (Rosso et al., 1999), pectate lyase (De Boer et al., 2002; Popeijus et al., 2000) and chorismate mutase (Lambert et al., 1999). Most efforts in analysing nematode secretions have focused on proteins secreted from amphids and oesophageal glands.

The antibodies were used in indirect Elisa to investigate the stage specificity of the molecules they recognize. Antibodies Roth-PC 389, PC 419, MAb T46.2,
FIG. 2 - Indirect Elisa of homogenates, untreated and heat treated, of *Meloidogyne arenaria* (Ma) and *M. incognita* (Mi) J2. A– Polyclonal antibodies; B– Monoclonal antibodies. 1- Ma/J2 nt; 2- Ma/J2 t; 3- Mi/J2 nt; 4- Mi/J2 t; 5- Mi/F nt; 6- Mi/F t; 7- Ma/J2 nt; 8- Ma/J2 t; 9- Mi/J2 nt; 10- Mi/J2 t; 11- Mi/F nt; 12- Mi/F t; 13- Ma/J2 nt; 14- Ma/J2 t; 15- Mi/J2 nt; 16- Mi/J2 t; 17- Mi/F nt; 18- Mi/F t. J2: second stage Juvenile; F: Females; nt: untreated; t: treated.

MAb T116C1.1 and MAb T42D.2 reacted strongly with the J2 and poorly with the adult females, indicating that these antibodies are recognising epitopes which are produced in the early stages of the nematode life cycle. In contrast, Roth-PC 373 showed strong reactivity towards antigens present in the J2 and females, indicating that these molecules might play a role in both stages of nematode development. Furthermore, the antibodies tested may be recognizing carbohydrate epitopes; the nematode amphid and cuticle surface secretions are formed by different carbohydrates, probably glycoproteins (Gems & Maizels, 1996). Sugar residues were identified on both the surface coat and amphids of some species of nematodes (Robertson *et al*., 1989).

Western blot analysis, using the antibody Roth-PC 373, revealed antigen bands in *M. incognita* and *M. arenaria* J2, with the same molecular weights, which indicate that identical protein epitopes are present in the two different species of nematodes studied and might also be present in different stages of the life-cycle of theses nematodes. The Roth-PC 389 labelled a series of bands between <14 and >94 kDa in *M. incognita* homogenate and two common bands in *M. arenaria* homogenate. The MAb Roth-MAb T116C1.1 and Roth-MAb T42D.2 recognised one single band in homogenates of J2 of *M. incognita* with similar molecular weights. These antigens may represent dominant epitopes presents in the amphids, metacorpus or intestine.

Many antigens involved in parasitism of plant-parasitic and animal-parasitic nematodes have been found in the range of 14-94 kDa (Lopez de Mendonça *et al*., 1999).

The identification of molecules secreted by plant nematodes *in planta* might represent potential targets for the development of novel alternatives to control economically important plant parasitic nematodes. The expression in plants of antibodies affecting the infection of nematodes in the roots is a promising alternative to reduce crops losses.

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