A polygalacturonase of animal origin isolated from the root-knot nematode *Meloidogyne incognita*[1]

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Abstract The first animal polygalacturonase (PG, EC 2.1.15) encoding cDNA, *Mi-pg-1*, was cloned from the plant parasitic nematode *Meloidogyne incognita*. The enzymatic activity of MI-PG-1 was confirmed after heterologous expression in *Escherichia coli*. The presence of a predicted signal peptide on the MI-PG-1 sequence together with the specific localization of the transcripts of the *Mi-pg-1* gene in the oesophageal glands of infective juveniles imply that MI-PG-1 could be secreted into plant tissues. The potential role of MI-PG-1 in parasitism is discussed. © **2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Nematode; Pectin; Polygalacturonase; Stylet secretion

1. Introduction

Plant cell walls are the first barrier encountered by most plant pathogens and have to be degraded in order to allow penetration and colonization. Therefore, the degradation of the main cell wall components such as pectin and cellulose is essential for phytoparasitic organisms. In order to degrade plant cell walls, plant pathogenic bacteria and fungi secrete an array of cell wall degrading enzymes including cellulases and pectinases [1,2]. Although degradation of the plant cell wall is necessary for many omnivorous and herbivorous animal species, most animals do not produce cell wall degrading enzymes and have to establish symbiotic relationships with micro-organisms for this to be achieved.

The microscopic plant parasitic cyst and root-knot nematodes were the first animals shown to express endogenous cellulases [3,4]. More recently, the first example of non-symbiotic pectin degradation by an animal was provided by the identification of a pectate lyase in the cyst nematode *Globodera rostochiensis* [5]. Genes encoding these enzymes are all specifically transcribed in the oesophageal gland cells of the nematode. These gland cells are responsible for the synthesis of secreted proteins that are thought to play a key role in the plant–nematode interaction. These proteins are secreted through the stylet, a specific protrusible structure developed by plant parasitic nematodes, into root tissues during parasitism.

While cellulolytic enzymes have been cloned from root-knot nematodes, no pectin degrading enzymes have been identified to date. Pectin is a major structural component of primary cell walls in non-woody tissues, along with cellulose, hemicellulose and proteins [6]. Pectin is a heteropolysaccharide with a backbone consisting of partially methylesterified galacturonic acid [7]. Pectic substances are major targets for degradation by plant pathogenic bacteria and fungi. These organisms secrete a variety of pectin degrading enzymes including pectin methylesterases, pectate lyases and polygalacturonases (PGs) that can be factors determining the virulence of the pathogen [8]. PGs catalyze the hydrolysis of the pectic polygalacturonic acid (PGA) and release oligogalacturonides. They are classified into two classes, endo-PGs and exo-PGs, according to their mode of action.

In this article we present the identification of a PG cDNA from the root-knot nematode *Meloidogyne incognita*. To our knowledge this is the first example of PG endogenously produced by any animal species. Homology analysis identified the *M. incognita* PG as an exo-PG. Transcription analysis showed that the *M. incognita* PG gene is specifically transcribed in the subventral oesophageal glands of infective second stage juveniles (J2s). Consequently, the MI-PG-1 protein could be secreted into plant tissues during parasitism in order to facilitate the penetration and intercellular migration of the nematode.

2. Materials and methods

2.1. Nematodes

M. incognita population Calissane, *M. arenaria* population St. Benoît and *M. javanica* population La Môle nematodes were bred on greenhouse-grown tomato *Lycopersicon esculentum* cv. St. Pierre and collected as described in [4]. *Caenorhabditis elegans* was grown in vitro on *Escherichia coli* OP50 [9].

2.2. Nucleic acids

M. incognita mRNAs were extracted as described in [10]. First strand cDNAs were synthesized using a poly(dT) (5′-TGA AGG TTC CAG AAT CGA TAG GAA TTC T_{16}G-3′) primer and Superscript II reverse transcriptase (Gibco BRL). Nematode and bacterial genomic DNA was extracted using the phenol/chloroform procedure described in [11]. Tomato genomic DNA was extracted using a DNeasy Plant kit (Qiagen).

2.3. Isolation of Mi-pg-1 cDNA

The 5′ end of the *Mi-pg-1* coding sequence (cds) was obtained by

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[1] The nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession number AY098646.

Abbreviations: J2s, second stage juveniles; PG, polygalacturonase; EST, expressed sequence tag; cds, coding sequence; PGA, polygalacturonic acid

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aligning the PG expressed sequence tags (ESTs) of *M. incognita* J2s using Cap software (http://www.infobiogen.fr/services/analyseq/cgi-bin/cap_in.pl). This sequence was also used to design the specific forward PolyF2 primer (5′-ATT ATG TGT TCC TCC GTT AG-3′). The 3′ end of the *Mi-pg-1* cds was obtained by PCR using the PolyF2 primer in combination with the T7 (5′-TAA TAC GAC TCA CTA TAG GGC GA-3′) primer on a *M. incognita* J2 cDNA library constructed in the Lambda ZAP Express vector (Stratagene).

2.4. Southern blot analysis

For each sample, 6 μg of genomic DNA was digested with 12 U of *Eco*RI or *Bam*HI (Roche Molecular Biochemicals). Digestion products were separated on a 1% agarose gel and transferred onto a Hybond N^+ membrane (Amersham Pharmacia Biotech). A specific *Mi-pg-1* probe was produced by RT-PCR on *M. incognita* J2 first strand cDNAs using the primers PolyF4 (5′-ATG AGT CAA ACA ATA ACA CCC CC-3′) and PolyR4 (5′-TCA CCA AGC ATC GTT AGT GCC AAA-3′). The probe was labeled with [^{32}P]dCTP using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech). The blot was hybridized overnight at 65°C as described in [4], washed at 65°C in 30 mM NaCl, 3 mM sodium tricitrate, 0.1% SDS and then in 15 mM NaCl, 1.5 mM sodium tricitrate, 0.1% SDS and set up for autoradiography.

2.5. In situ hybridization

In situ hybridization was performed on freshly hatched *M. incognita* J2s essentially as described in [4] except that the J2s were incubated 20 min in proteinase K (Roche Molecular Biochemicals) (2 mg/ml) and the hybridization was performed at 40°C. The *Mi-pg-1* sense and antisense DNA probes were amplified from *M. incognita* J2 first strand cDNAs using the PolyF2 and PolyR (5′-AAG GCT GAA GTT GAG GAA GA-3′) primers.

2.6. Heterologous expression in E. coli

The *Mi-pg-1* cds without the predicted signal peptide was obtained by RT-PCR on *M. incognita* J2 first strand cDNAs using the PolyF6 (5′-AGT CAA ACA ATA ACA CCC CCA-3′) and the PolyR6 (5′-CCA AGC ATC GTT AGT GCC AAA-3′) primers. The amplified cDNA fragment was cloned into the pBAD TOPO vector (Invitrogen). *E. coli* TOP10 cells were transformed and expression of the MI-PG-1 fusion protein was induced by addition of 0.002% L-(+)-arabinose. After induction, bacteria were lysed in lysis buffer (25 mM Tris pH 7, 40 μg/ml lysozyme (Roche Biochemicals), 70 μg/ml DNase I (Roche Biochemicals)) for 30 min. Expression of MI-PG-1 was checked by Western blot. 40 μg of total proteins of induced and non-induced *E. coli* were blotted. The recombinant MI-PG-1 protein was identified by probing the Western blot with a 1/5000 dilution of anti-V5 antibody coupled to horseradish peroxidase (Invitrogen). Total protein extracts were filtered on 0.45 μm sieves (Pall Corporation) and used for enzymatic assays.

2.7. Enzyme assays

PG activity was tested by cup plate assays on 0.8 mm agarose gels (1% agarose, 0.1% PGA from orange (Sigma), 0.1 M sodium acetate pH 5.0, 10 mM EDTA). 200 μg of total proteins were loaded on gels. Gels were then incubated at 37°C for 16 h. The hydrolysis of the PGA was visualized by staining the gel with 0.05% (w/v) ruthenium red (Sigma) for 20 min followed by rinsing with distilled water for 15 min. PG from *Rhizopus* sp. (Sigma) was used as a positive control while total proteins from *E. coli* TOP10 cells expressing LacZ β-galactosidase (Invitrogen) were used as a negative control.

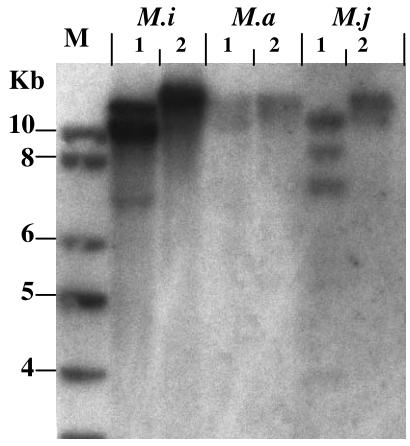

Fig. 1. Southern blot analysis of *Mi-pg-1*. A blot containing genomic DNA from *M. incognita* (*M.i*), *M. arenaria* (*M.a*) and *M. javanica* (*M.j*) digested with *Eco*RI (1) or *Bam*HI (2) was hybridized with a *Mi-pg-1* specific probe. M: molecular weight markers.

2.8. Phylogenetic analyses

The deduced protein sequence of MI-PG-1 was compared with protein sequences of exo- and endo-PGs from bacteria, plants and fungi in a phylogenetic analysis. In this analysis all signal peptides were removed. Multiple sequence alignment and unrooted tree construction were performed using ClustalW [12]. The significance of the results was assessed by bootstrap analysis [13] using 1000 bootstrap trials.

3. Results

3.1. Isolation of the M. incognita PG cDNA

The *M. incognita* J2 ESTs indexed in dBEST were screened for pectin degrading enzymes. Two sequences (AW829374 and AW829091) were identified as potentially encoding a PG. The alignment of these two ESTs allowed the identification of a 780 bp partial cds starting 39 bp before the putative ATG translation initiation codon. Based on this sequence the specific forward primer PolyF2 was designed. This primer was used to amplify the 3′ end of the PG cDNA from a *M. incognita* J2 cDNA library. The full-length *Mi-pg-1* cDNA could encode a 633 amino acid protein. Analysis using the BLASTX software (http://www.infobiogen.fr/services/

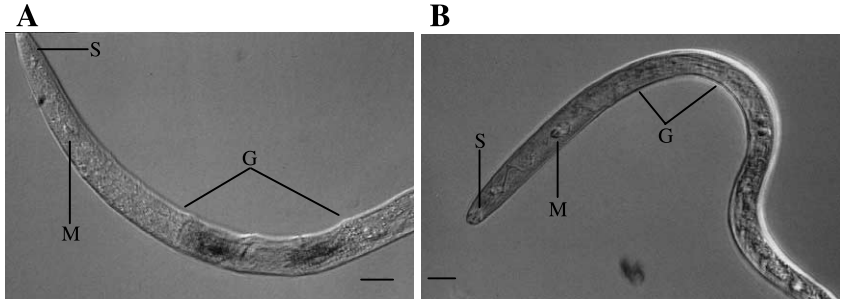

Fig. 2. Localization by in situ hybridization of the *Mi-pg-1* transcripts in the subventral oesophageal glands of *M. incognita* infective juveniles. J2 fragments were hybridized with antisense (A) or sense (B) *Mi-pg-1* digoxigenin-labeled DNA probes. G: subventral oesophageal gland region; S: stylet; M: metacorpus. Scale bars = 10 μm.

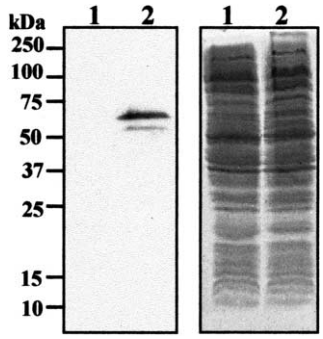

Fig. 3. Detection of recombinant MI-PG-1 expressed in *E. coli* by Western blotting. The lysate of induced (lane 1) and non-induced (lane 2) bacteria was tested. Left panel: Western blot; right panel: Coomassie blue-stained gel.

analyseq/cgi-bin/blast2_in.pl) showed that the MI-PG-1 deduced protein is highly similar to bacterial exo-PGs. 40% identity was observed with the exo-PG of *Ralstonia solanacearum* (GenBank accession number U60106). A predicted signal peptide of 29 amino acids was identified at the N-terminal of the deduced protein sequence using the PSORT II program (http://psort.nibb.ac.jp/form2.html).

3.2. Endogenous origin of Mi-pg-1

The nematode origin of the *Mi-pg-1* was confirmed by Southern blot analysis. A blot containing genomic DNA from the nematode species *C. elegans*, *M. incognita*, *M. javanica* and *M. arenaria* was made. As controls, the blot contained also genomic DNA from tomato and from the bacterium *Erwinia chrysanthemi*. The membrane was hybridized with a cDNA probe specific to *Mi-pg-1*. The probe hybridized specifically with DNA of all three *Meloidogyne* species, revealing three bands between 10 and 6 kb for the *Eco*RI digestion and one to two bands with the *Bam*HI digestion (Fig. 1). No signal was obtained with the other genomic DNAs (data not shown).

3.3. Mi-pg-1 expression analysis

The transcription of *Mi-pg-1* was studied in different developmental stages of *M. incognita* by RT-PCR. An amplification product of the expected 400 bp size was present specifically in freshly hatched J2s. No amplification was observed in unhatched larvae, adult males or adult females. As a control, the same PCR was repeated using *M. incognita* genomic DNA. A 600 bp amplification product was obtained suggesting the presence of an intron in the amplified region (data not shown). The tissue localization of the *Mi-pg-1* transcripts was analyzed in *M. incognita* J2s using in situ hybridization. Hybridization with the antisense *Mi-pg-1* probe gave a clear specific signal in the subventral oesophageal glands of the nematodes (Fig. 2A). No signal was observed with the control sense probe (Fig. 2B).

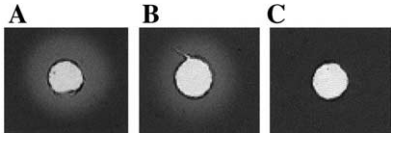

Fig. 4. Detection of PG activity on PGA cup plate assays. PG activity was indicated by clear haloes in commercial PG (A) and in total proteins from *E. coli* expressing MI-PG-1 (B). As a negative control, the same quantity of total proteins from bacteria expressing LacZ β-galactosidase was loaded on gel (C).

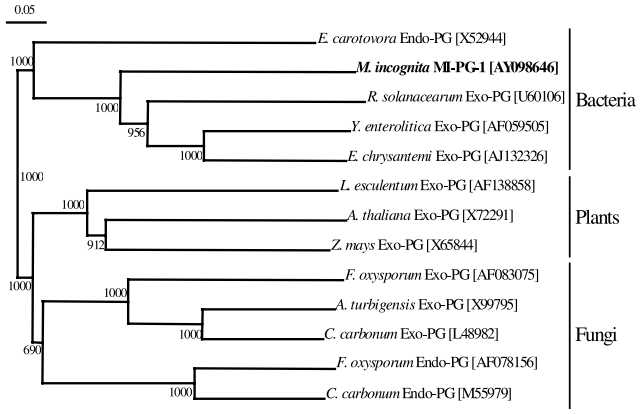

Fig. 5. Unrooted phylogenetic tree of PGs based on the protein sequences without signal peptides. GenBank accession numbers of PGs from *Erwinia carotovora*, *E. chrysanthemi*, *Ralstonia solanacearum*, *Yersinia enterolitica*, *L. esculentum*, *Arabidopsis thaliana*, *Zea mays*, *Fusarium oxysporum*, *Aspergillus niger* and *Cochliobonum carbonum* are indicated in brackets. Scale bar represents a genetic distance of 0.05 as the frequency of nucleotide substitutions in a pairwise comparison of two sequences according to Kimura's two-parameter method (1980) [20]. The bootstrap values are indicated at each node (1000 repetitions).

3.4. Heterologous expression and PG activity assays

The *Mi-pg-1* cds was cloned into the pBAD TOPO vector, which allowed production of the protein fused to the V5 epitope. Expression of the MI-PG-1 fusion protein in transformed bacteria was analyzed by Western blot. After cell lysis, a 70 kDa band was detected in the total proteins, similar in size to the 68.5 kDa predicted molecular weight for MI-PG-1 (Fig. 3). The enzymatic activity of the expressed MI-PG-1 protein was analyzed on ultra-thin agarose gels containing PGA. Total proteins of *E. coli* expressing MI-PG-1 were loaded on gels. The hydrolysis of PGA was indicated by clear haloes after staining the gel with ruthenium red with the commercial PG and with the MI-PG-1 samples (Fig. 4A, B). No PGA hydrolysis was observed with total proteins of *E. coli* expressing the LacZ β-galactosidase tested as a negative control (Fig. 4C). These data confirm MI-PG-1 as a functional PG.

3.5. Phylogenetic analysis

A phylogenetic tree generated from an alignment of the MI-PG-1 deduced protein sequence with exo- and endo-PGs from bacteria, plants and fungi is shown in Fig. 5. Prokaryotic and eukaryotic PGs form two distinct clusters. Surprisingly, MI-PG-1 is linked to the prokaryotic cluster. Moreover, while MI-PG-1 is assumed, on the basis of BLASTX analysis, to be an exo-PG, its protein sequence is placed closer to the bacterial endo-PGs than to eukaryotic exo-PGs by this analysis.

4. Discussion

In this article we describe the *Mi-pg-1* gene which encodes a functional PG from the root-knot nematode *M. incognita*. To

our knowledge, this is the first example of a PG of animal origin.

Mi-pg-1 encodes a 633 amino acid protein that is more similar to microbial exo-PGs than to eukaryotic enzymes. The presence of micro-organisms in root-knot nematodes has been investigated [14] and no bacterial symbionts have been reported in these nematodes to date. Moreover, several lines of evidence demonstrate the endogenous origin of *Mi-pg-1* as opposed to a symbiont or a contaminant origin. First, the polyadenylated 3′ end of the *Mi-pg-1* mRNA excludes a bacterial origin of *Mi-pg-1*. Second, in situ hybridization localized the transcripts of *Mi-pg-1* specifically in the subventral oesophageal glands of J2s. At the gene level, the nematode origin of *Mi-pg-1* was confirmed by (i) the specific hybridization of the *Mi-pg-1* probe to *Meloidogyne* genomic DNAs in Southern blot analysis and (ii) the presence of an intron in the genomic sequence of *Mi-pg-1*.

Phylogenetic analysis showed that MI-PG-1 is closer to prokaryotic PGs than to eukaryotic enzymes. Horizontal gene transfer from bacteria to root-knot nematodes has been proposed in the case of cellulases and polyglutamate synthase [15,16]. Although conclusive evidence is still lacking, the high similarity between MI-PG-1 and bacterial PGs provides additional evidence to support the hypothesis that nematode parasitism genes may have been acquired by gene transfer from micro-organisms.

Transcription analysis showed that *Mi-pg-1* is transcribed in mobile infective J2s of *M. incognita*. Moreover, in situ hybridization showed that active transcription of the gene occurs specifically in the subventral oesophageal glands of J2s. Oesophageal glands of plant parasitic nematodes are responsible for the synthesis of proteins that are thought to be secreted through the stylet during parasitism. The presence of a 29 amino acid predicted signal peptide at the N-terminal of MI-PG-1, together with the tissue localization of *Mi-pg-1* transcription in the oesophageal glands, provides good evidence that MI-PG-1 is present in stylet secretions. As suggested for cellulases, the *M. incognita* PG could play a role in weakening the cell walls of root tissue during penetration and intercellular migration of the parasite. BLASTX analysis suggested that MI-PG-1 is an exo-PG although biochemical evidence for this assignation is still lacking. Previous studies have shown that exo-PGs can degrade elicitor-active oligogalacturonides released by endo-PGs [17] and are usually not inhibited by plant PG-inhibiting proteins [18]. In this respect, the future analysis of MI-PG-1 enzymatic properties will be of particular interest.

The isolation of a pectin degrading enzyme from a root-knot nematode adds to the formal isolation of cellulose and hemicellulose degrading enzymes from this nematode [4,19]. Root-knot nematodes therefore possess the most diverse range of plant cell wall degrading tools of any animal studied to date.

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