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ProPhenolOxidase in *Daphnia magna*: cDNA sequencing and expression in relation to resistance to pathogens

Pierrick Labbé *, Tom J. Little

University of Edinburgh, IEB, Ashworth Laboratory, Kings Buildings, Edinburgh EH9 3JU, UK

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ABSTRACT

Invertebrates utilise the innate immune system when defending against pathogenic attack. However, except for some effectors as proPhenolOxidase (proPO), the innate immune response is less well understood outside model insect species, and its role in natural host–pathogen systems is generally not well documented. We have therefore initiated studies on the immune response of the crustacean *Daphnia* when exposed to the specialist endobacterial pathogen, *Pasteuria ramosa*. This study was focused on the proPO gene of *Daphnia magna*. *D. magna* possesses a single copy of proPO (as does its congener, *D. pulex*), but there was some evidence of alternative splicing. Analyses of sequence similarity in a range of arthropod taxa suggested that the proPO gene in *Daphnia* was as dissimilar to other crustaceans as it was to insects, while analysis on intraspecific variation indicated that the gene is highly conserved. ProPO was found to be significantly up-regulated within 1–4 h following exposure to the bacteria. This is the first evidence of a *Daphnia* immune response, and our observations raise the possibility that the PhenolOxidase (PO) cascade is involved in the defence against pathogenic gram-positive bacteria.

1. Introduction

In host–pathogen interactions, hosts defend themselves through various strategies, including behavioural modification, physical barriers that prevent pathogen invasion, and finally their immune system (e.g. for insects, see review in Ref. [1]). In invertebrates, this last defence is the innate immune system only (in contrast to vertebrates which have both an innate and a well-defined acquired immune system), which is now well characterised in some model species (see review in Ref. [2]), particularly *Drosophila melanogaster* (see review in Ref. [3]). Three main innate immune system components have been identified: (i) receptors, which recognise pathogen associated molecular patterns (PAMPs), (ii) regulators, which are implied in signalling pathways (e.g. the Toll and imd pathways) and (iii) effectors, which directly inhibit pathogen growth or survival [4,5].

One effector that has received considerable attention in different species of invertebrates (worms, insects and crustaceans) is PhenolOxidase (PO) (see [6]). Activation (via wounding or receptors that detect PAMPs) of the PO-cascade leads to the cleavage of a zymogen, proPhenolOxidase (proPO), which becomes active PO. Once PO is active, it will launch a cascade, including the production of highly reactive and toxic quinone intermediates, and ending with the production of melanin. Melanin will be deposited on intruders, preventing or reducing their growth [6]. Most studies have focused on PO activity in response to pathogen challenge, but several studies have also shown that proPO expression was up-regulated following a pathogen or immunostimulant challenge [6–12].

Daphnia magna, a planktonic crustacean found in temperate freshwater ponds, is one of the few non-insect arthropod models for the study of host–pathogen interactions (see review in Ref. [13]). Field studies of natural *Daphnia* populations have documented a diverse pathogen fauna, including bacteria, fungus or microsporidians that can have dramatic effects on *Daphnia* biology (including sterilization, see review in Ref. [13]). The best studied of these pathogens is the gram-positive bacterium *Pasteuria ramosa*, which is highly amenable to laboratory experimentation. Through experimentation, much is known about the conditions that favour *Pasteuria* infection of *D. magna* [13], and resistance has been shown to depend on current environmental conditions, conditions in the maternal generation, as well as genetic variation in both the host and the pathogen (genotype-by-genotype interactions [13–18]). However, while *Daphnia* have been extensively characterised with respect to whole organism, phenotypic responses to parasitism,

* Corresponding author. Tel.: +44 131 650 7287.
E-mail addresses: Pierrick.Labbe@ed.ac.uk (P. Labbé), tlittle1@staffmail.ed.ac.uk (T.J. Little).

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little is known about their immune response or the genetic basis of resistance to pathogens.

It has been shown that PO activity was increased after wounding of *D. magna*, and that PO activity differs between populations [19]. However, no sign of melanisation following *P. ramosa* infection are visible under the microscope (P. Labbé, pers. obs.). To further investigate the role of PO in resistance to pathogens in *D. magna*, we used the recently sequenced genome of a related species, *D. pulex* (http://daphnia.cgb.indiana.edu/ [20]), to help gain the full cDNA sequence of *D. magna* proPO, and compared the protein encoded by this cDNA to the proPO sequences of other arthropod taxa. We also obtained polymorphsim data by sequencing a large part of the coding region in 14 different clones of *D. magna* originating from various locations in Europe. Finally, we examined proPO expression in *D. magna* challenged by *P. ramosa*.

2. Material and methods

2.1. Daphnia clones and P. ramosa strain

D. magna is a crustacean cladoceran filter-feeding zooplankter that reproduces by cyclical parthenogenesis. *P. ramosa* is a gram-positive bacterium that is an obligate, spore-forming endopathogen of *D. magna*. Hosts become infected with *P. ramosa* by filtering transmission spores present in the water or sediments at the pond bottom. Infection causes host castration and gigantism, as well as premature death. Within the host, *P. ramosa* goes through a developmental process that culminates in the formation of spores. Host death is essential for transmission, mature spores being released from the remains of dead infected hosts. *P. ramosa* spores are horizontally transmitted only, i.e. there is no evidence of transovarial infection [21].

We used a total of 14 clones originating from various places. Six clones (GG3, GG4, GG7, GG8, GG13, and GG15) and the strain of pathogen (Sp1) were originally collected from a population near Gaazerfeld, Germany [14]. Four clones (KA51, KA5, KA24 and KA47) originate from the Kaimes pond near Leitholm in the Scottish Borders (Desiree Allen, personal collection [22–24]). Two clones (BelD1 and BelD3) originate from Belgium (Oude Meren, Abdij van't Parkl, Heverlee [25]). The last two clones used are the reference clones Mullinb3, collected in Munich, Germany, and Xinb1, collected in Finland (Tom Little and Dieter Ebert, personal collection). All were maintained in the laboratory in a state of clonal reproduction (host) or frozen (pathogen).

2.2. Sequences used

To design degenerate primers, we used a model of the proPO cDNA from *D. pulex* (Crustacea, Cladocera [20]) and several sequences collected from various public libraries: (i) Insects: (a) Lepidopteran: *Helicoverpa armigera* (GenBank ABU98653), *Ostrinia furnacalis* (GenBank ABC59699), *Manduca sexta* (EMBL CAL25133), *Hyphantria cunea* (GenBank AAC34251); (b) Dipteran: *Anopheles gambiae* (Genbank AAC27383), *Musca domestica* (Genbank AAR84669), *Aedes aegypti* (GenBank AAG02219), *Anopheles stephensi* (GenBank AAC69182), *Culex quinquefasciatus* (REFSEQ XP_001867412), *Drosophila melanogaster* (REFSEQ NP_476812); (c) Coleopteran: *Tenebrio molitor* (DDBJ BAA75470); (d) Hymenopteran: *Apis mellifera* (GenBank AAO72539); (ii) Crustaceans, Decapoda: *Marsupenaeus japonicus* (DDBJ BAB70485), *Procambarus clarkii* (GenBank ABR12412), *Litopenaeus vanname* (GenBank AAW51360), *Scylla serrata* (GenBank ABD90511), *Macrobrachium rosenbergii* (GenBank ABA60740), *Pacifastacus leniusculus* (EMBL CAA58471), *Homarus americanus* (GenBank AAT73697), *Penaeus semisulcatus* (GenBank AAM77690), *Penaeus monodon* (GenBank AAD45201), *Cancer magister* (GenBank ABB59713), *Homarus gammarus* (EMBL CAE46724), *Fenneropenaeus chinensis* (DDBJ BAF98646). These sequences were also used to draw an interspecific phylogeny.

2.3. D. magna proPO cDNA sequencing

2.3.1. RNA isolation, RT-PCR and sequencing

Four 5-day-old *Daphnia* were pooled in 200 μL RNAlater™ (Ambion). RNA was extracted using the RNAeasy midi Kit (Qiagen), according to manufacturer's instructions. The RNA was further purified with RNAse-Free DNAse (Promega). Two microliters RNA was reverse-transcribed into cDNA using the Promega Reverse Transcription System kit according to manufacturer instructions. cDNA was diluted 5-fold by adding 80 μL of H_2O to each tube. The purity of the cDNA was checked with PCR, using specific primers (pPO 2.5F 5′ CATATCACGACTGGGACGAA 3′/pPO 3.1R 3′ CTTGCCAGCCAGTGATAATGAG 5′) to amplify a fragment encompassing an intron, to distinguish between DNA and cDNA products.

The first sequences for proPO cDNA in *D. magna*, were acquired using degenerate primers using the *D. pulex* sequence and designed in well-conserved regions. The 5′ and 3′ ends of the cDNA were acquired by RACE-PCR using the GeneRacer™ Core kit (Invitrogen) according to manufacturer specifications. The 5′ end showed a mix of sequences, so we cloned the gel purified RACE-PCR product (Gel purification Kit, Qiagen) using the TOPO TA cloning kit (Invitrogen) according to manufacturer recommendations.

2.3.2. Sequence polymorphism in D. magna ProPO

Once the complete cDNA sequence was obtained, a set of sequencing primers was designed to PCR amplify the complete coding cDNA of a range of *D. magna* genotypes. Using the Primer3 online software (http://www.bioinformatics.nl/cgi-bin/primer3-plus/primer3plus.cgi), six pairs of primers were used: SeqpPO F0 5′ GTTGATTTACTCTTGTTCTCGTGTC 3′/SeqpPO R0 3′ CTTATTTTC CACGACCCTGTT 5′; SeqpPO F1 5′ TCCAACAGATTTGTCTTCAAAACC 3′/SeqpPO R1 3′ TGTTGATGCCCAAGTCTTCT 5′; SeqpPO F2 5′ AAGATCTTTAAGGATGCCCCAACT 3′/SeqpPO R2 3′ GGCACCGTTAG-CATTTGTAAGTTT 5′; SeqpPO F3 5′ AGTTGAAGCGACAGGAGAAAT-CAT 3′/SeqpPO R3 3′ ACTTTCTTGCCAGCCAGTGATAAT 5′; SeqpPO F4 5′ CATTCGGAAAACATGGGAGTTATG 3′/SeqpPO R4 3′ GCAAC-CACAGAAATTGGTCATTCT 5′; SeqpPO F5 5′ AGGTCGCC-CATTTTCTTTCAG 3′/SeqpPO R5 3′ TATAGCTCAAGAGA GCGTCATTGG 5′. These primers amplify overlapping ~500–700 pb fragments.

Six independent PCR were performed (30 cycles, 92 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min) using Bioline *Taq* DNA polymerase Kit, according to manufacturer recommendations. The fragments were assembled using the Geneious Pro 4.0.2 software (Drummond AJ, et al., 2008 available at www.geneious.com).

The PCR products were either purified from agarose gels (Gel purification kit, Qiagen) or by taking 3 μL of PCR product to which was added 1 μL of Shrimp Alkaline Phosphatase (SAP, Promega) and 0.074 μL of Exonuclease I (NewEngland Biolabs) and 1 μL of ddH_2O. This mix was then incubated 40 min at 37 °C, and then 15 min at 80 °C. The sequencing mix consisted of 2 μL of purified PCR product with 0.7 μL of primer solution (two reactions per PCR products, one with each primer used to amplify it), 2.5 μL of BigDye® 5X Buffer, 1.5 μL of BigDye® Terminator mix (Applied Biosystems) and 3.5 μL of ddH_2O (25 cycles, 95 °C for 30 s, 50 °C for 20 s and 60 °C for 2 min). Sequences were analysed with an ABI3730 sequencer.

2.4. Alignments and phylogenetic analyses

Interspecific protein and intraspecific cDNA alignments were computed using the online software Multalin (http://bioinfo.gen-

Table 1
cDNA sequence variation between *D. magna* clones. Each variable site is indicated with its position relative to the first nucleotide of the first Methionine. GG3 cDNA is used as the reference sequence, a dash (–) indicates no differences with the reference. R, M and Y correspond to heterozygote sites with respectively A and G, A and C, and C and T. The non-synonymous mutations are indicated with an asterisks (*), and the corresponding amino-acid changes are indicated.

Clone	Position													
			1	1	1	1	1	1	1	1	1	1	1	2
	4	4	1	3	3	3	3	4	4	5	5	6	8	1
	0	2	0	1	3	3	6	3	4	2	9	3	4	2
	8	0	4	4	1	6	2	1	3	5	3	5	2	5
GG3	A	C	T	T	C	T	A	T	C	A	A	A	T	C
GG4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GG7	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GG8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GG13	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GG15	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mullinb3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KA51	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KA5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KA24	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KA47	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xnb1	R	M	Y	Y	–	Y	R	–	Y	–	–	–	–	Y
BelD1	G	M	C	C	Y	C	R	C	Y	R	R	G	Y	Y
BelD3	G	M	C	C	T	C	G	C	T	G	G	G	C	T
					*	*				*				*
					A → V	S → P				I → V				Q → Stop

otoul.fr/multalin/multalin.html [26]) and a Kimura distance tree was built using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-e.htm [27]).

2.5. proPO expression

2.5.1. Exposure protocol

ProPO expression was analysed in the *D. magna* clone GG4. Before exposing hosts to pathogen spores, host maternal lines were raised in standard conditions for at least 3 generations to equilibrate maternal effects [16]. Twelve independent replicates of four female *Daphnia* of each genotype were maintained in jars containing 60 mL of artificial medium [28], fed 3.5×10^6 chemostat grown *Chlorella* sp. algae cells per *Daphnia* per day, and maintained within 20 °C temperature-controlled incubators with a light: dark cycle of 12:12 h. Medium was changed with every clutch or every 4 or 5 days regardless of a clutch being present.

Second clutches were collected from the twelve maternal lines on the same day (all *Daphnia* were less than 24 h old). These 'day 1' individuals were randomly distributed in 39 jars, with four *Daphnia* per jar, filled with 60 mL of artificial medium (Aachener Daphnien Medium [28]). They were fed 3.5×10^6 cells chemostat grown *Chlorella* sp. algae per *Daphnia* per day. On day 4, the *Daphnia* were transferred to new jars with medium only, for a short starvation prior to the exposure treatment, to promote filter-feeding during pathogen exposure. The exposure was initiated on day 5.

On day 5, jars were split in two groups: (i) 18 "exposed" jars received a solution with *P. ramosa* Sp1 spores (from crushed, infected *Daphnia*), and (ii) 18 "not-exposed" jars received a sham-solution consisting of crushed *Daphnia* only. The four *Daphnia* from each jar were transferred to 1.5 mL Eppendorf tubes, with 1 mL medium. Two hundred microliters of a *P. ramosa* Sp1 solution (~100 000 spores per *Daphnia*) were added to the "exposed" samples, and an equal volume of sham-solution was added to the "not-exposed". A small volume of algae (300 μL, $\sim 1 \times 10^6$ *Chlorella* algae cells) was also added to promote filter feeding. The exposure lasted for 2 h at 20 °C, the tubes being mixed every 20 min, five times by inversion. After these 2 h, the *Daphnia* of each tube were removed and placed in a new jar containing 60 mL of medium with 3.5×10^6 *Chlorella* cells per *Daphnia* at 20 °C. Three jars for each treatment were randomly collected 1 h, 2 h, 6 h, 12 h, 24 h and 48 h after the end of the exposure treatment. Additionally, three jars were also collected at time 0 as controls, just before starting the infection. For each time-point, the four *Daphnia* were transferred to one Eppendorf tube with 200 μL of RNAlater™ (Ambion), and stored at −20 °C for later extraction. Three replicates of each treatment were kept in rearing conditions until day 16, to estimate infection success.

2.5.2. RT-QPCR

RNA was extracted and purified from each sample and transformed into cDNA as described above. Relative RT-QPCR was done using the Roche LightCycler® 480. A ~100 bp fragment of *actin*, a house keeping reference gene, was amplified using primers from Heckmann et al. [29]. A 101 bp fragment of Dmag-proPO cDNA was then amplified separately using specific primers (pPO-QF2 5′ GCCGGATCACTTTTTAGTGC 3′/pPO-QR2 3′ CATTAAC-GACGCGATCCTCT 5′). For each gene, we added 1 μL of cDNA and 0.5 μL of each primer to 8 μL of SYBR Green I Master mix (Roche). Cycling conditions were as follows: 95 °C, 5 min followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 10 s. Quantification of proPO relative to *actin* was performed using the Roche LightCycler® 480 software, using the maximum secondary derivative method.

2.5.3. Statistical analysis

The data were fitted to the general linear model (GLM): Log(Activity) = Exposure + Time + Exposure:Time, where Exposure:Time represents the interaction between Exposure, a categorical variable with two levels (exposed/not-exposed), and Time, a continuous variable (hours). Log-transformation of the response variable ensured the normal distribution of residuals. The initial model was simplified according to Crawley (2007). Models were compared using *F*-tests. Analysis was performed using the R package (http://www.r-project.org/).

3. Results and discussion

As a potentially important invertebrate immunity gene, proPO has been sequenced in a variety of organisms. Here, we used the

```
5′ 1    GTTGATTTACTCTTGTTCTCGTGTCACGATGTCGGATTTGGAGCTTCAACAACGTCAAGTTTTTAACCTTTTCGAGCGGGTTTCTCAGCC
   1                             M  S  D  L  E  L  Q  Q  R  Q  V  L  N  L  F  E  R**V  S  Q  P

  91    ATTTTCACCCAAACCGAACATTACTTTTGAATTCCCAAATTTTGTGTCTCCAACAGATTTGTCTTCAAAACCGGGTTTAGCCACCCCTGC
  22      F  S  P  K  P  N  I  T  F  E  F  P  N  F  V  S  P  T  D  L  S  S  K  P  G  L  A  T  P  A

 181    TAGTGCACCTACTGATGGCTTGAATATCCCAGAATTAGACCTGGTAAAGACGATCCCACGCGGAAGATTATTCTCGAACTTTCATCCAGG
  52      S  A  P  T  D  G  L  N  I  P  E  L  D  L  V  K  T  I  P  R  G  R  L  F  S  N  F  H  P  G

 271    CCACCGTGTAGCCGCCTACACACTTGTGAAGATCTTTAAGGATGCCCCAACTGTGGCGGACATGTTGAATTTGGCTGCCCATCCCGATGT
  82      H  R  V  A  A  A  Y  T  L  V  K  I  F  K  D  A  P  T  V  A  D  M  L  N  L  A  A  H  P  D  V

 361    ACACGACGCTGTAAACGAACACATTTTCGTATATGCTTTGTCTGCTGCCCTCATTCAAAGAAAAGATGCTCGCTCATTACGCCTTCCCCC
 112      H  D  A  V  N  E  H  I  F  V  Y  A  L  S  A  A  L  I  Q  R  K  D  A  R  S  L  R  L  P  P

 451    CATCTATGAAATTTTTCCGGGAAAATTTTTTGAGACTAAAGTGCGAAGCGAAGCACAAATCAATGTTCAACAAAAAAAGGAGCCTGCCAC
 142      I  Y  E  I  F  P  G  K  F  F  E  T  K  V  R  S  E  A  Q  I  N  V  Q  Q  K  K  E  P  A  T

 541    TCAAGAACCCATCATCATCGATAAAAACTTTGCAGCTACGAACAGGGTCGTGGAAAATAAGGTGTCATATTTCCGAGAAGACTTGGGCAT
 172      Q  E  P  I  I  I  D  K  N  F  A  A  T  N  R  V  V  E  N  K  V  S  Y  F  R  E  D  L  G  I

 631    CAACAGCCATCATTGGCATTGGCATTTAATTTTTCCAGTTGAAGCGACAGGAGAAATCATCAATCCACCAGATCGTCGGGGCGAACTTTT
 202      N  S  H  H  W  H  W  H  L  I  F  P  V  E  A  T  G  E  I  I  N  P  P  D  R  R  G  E  L  F

 721    CTATTACATGCACCAACAAATTTTGGCTCGTTATGATGCTGAAAGAATAGCTAATGGTCTGGCCCGCGTAACGTCATATCACGACTGGGA
 232      Y  Y  M  H  Q  Q  I  L  A  R  Y  D  A  E  R  I  A  N  G  L  A  R  V  T  S  Y  H  D  W  D

 811    CGAACCCATTATGGAAGCCTACTTTCCGAAACTTACAAATGCTAACGGTGCCATTCATTGGGCATCTCGTCCGGCTGGGCTCGTTCTCAA
 262      E  P  I  M  E  A  Y  F  P  K  L  T  N  A  N  G  A  I  H  W  A  S  R  P  A  G  L  V  L  K

 901    GGACATAAATATTCCCGACGAAGGCGTCGAACTTGGAATCGAATTTCAAATTAATAATCTAGAACTGTGGCGCACCCGCATTCTTCACGC
 292      D  I  N  I  P  D  E  G  V  E  L  G  I  E  F  Q  I  N  N  L  E  L  W  R  T  R  I  L  H  A

 991    TATACACAAAGGATCGGTAGCAAAGGCGAATGGCGAAATCATCTATTTAGATGAAACAACGGGCACAAACACCTTAGGAGAGCTCATCGA
 322      I  H  K  G  S  V  A  K  A  N  G  E  I  I  Y  L  D  E  T  T  G  T  N  T  L  G  E  L  I  E

1081    AGCGTCGCAGTGGTCTGCCGACAAGTTGTTTTACGGTGATCTGCACAATTTTGGACACATTGCAGTGTGTTATATTCACGATCCTGACCG
 352      A  S  Q  W  S  A  D  K  L  F  Y  G  D  L  H  N  F  G  H  I  A  V  C  Y  I  H  D  P  D  R

1171    CAGCCATTCGGAAAACATGGGAGTTATGGGCGATTCGGTGACGGCCATGCGTGACCCCATCTTTTACCGCTGGCACAAGTTCATCGACTC
 382      S  H  S  E  N  M  G  V  M  G  D  S  V  T  A  M  R  D  P  I  F  Y  R  W  H  K  F  I  D  S

1261    ACTTTTTCAGCAATTCAAAGCCACTTTAAATCCCTATACTGCTCAACAGTTGACTCTGGAAGGAATTACAGTGAACAATGTTGAAGTACG
 412      L  F  Q  Q  F  K  A  T  L  N  P  Y  T  A  Q  Q  L  T  L  E  G  I  T  V  N  N  V  E  V  R

1351    CCGCAACGCGACTTCTGATAATCCCCGGTCAAATCCACCAAAAAATCTCATTATCACTGGCTGGCAAGAAAGTATTGTGGAGCTGGACCG
 442      R  N  A  T  S  D  N  P  R  S  N  P  P  K  N  L  I  I  T  G  W  Q  E  S  I  V  E  L  D  R

1441    CGGTTTGGATTTTTCAAGTTTGCGTCCCGTCCAAGCACGAGTGACGCACCTGCAGCACGAAGATTTTTCCTATCGAATACAGGTGACCAA
 472      G  L  D  F  S  S  L  R  P  V  Q  A  R  V  T  H  L  Q  H  E  D  F  S  Y  R  I  Q  V  T  N

1531    TTCGACAAAAACTACTCAAGAAATTGTGTTCCGAATTTTTCTGGCACCGATCGCGGACGAGGCAGGTCGCCCATTTTCTTTCAGGGAACA
 502      S  T  K  T  T  Q  E  I  V  F  R  I  F  L  A  P  I  A  D  E  A  G  R  P  F  S  F  R  E  Q

1621    ACGTCTTCTAATGATCGAACTGGACAAATTTGTCGTCAGAGTAACCCCTGGAGCCAACATGGTCAAGAGAAAATCGGACGATTCTTCCGT
 532      R  L  L  M  I  E  L  D  K  F  V  V  R  V  T  P  G  A  N  M  V  K  R  K  S  D  D  S  S  V

1711    GACGATCCCGTTCGAACGTACGTTTAGAGATCTGGAAGCAGCTGTTCCCGCAACACCACTCGCTCCAGGAGGTGTCCCAACCGACTTGCC
 562      T  I  P  F  E  R  T  F  R  D  L  E  A  A  V  P  A  T  P  L  A  P  G  G  V  P  T  D  L  P

1801    GACCGACAGAATGACCAATTTCTGTGGTTGCGGTTGGCCGGATCACTTTTTAGTGCCGCGAGGTGCGCCTAACCCAGGAATGCCCTTCAC
 592      T  D  R  M  T  N  F  C  G  C  G  W  P  D  H  F  L  V  P  R  G  A  P  N  P  G  M  P  F  T

1891    GCTTTTCGTTATGGCCACCAGCTGGAAAGAGGATCGCGTCGTTAATGGTGAGGACTCTAAAAAACTCTGCCGCAATGCAGCCTCTTATTG
 622      L  F  V  M  A  T  S  W  K  E  D  R  V  V  N  G  E  D  S  K  K  L  C  R  N  A  A  S  Y  C

1981    CGGGATATTGGACGAAAAGTATCCCGACAAGAGACCGATGGGCTTTCCATTTGATCGCCCCCCTGATAAAATGATAACCACATTGGCGAA
 652      G  I  L  D  E  K  Y  P  D  K  R  P  M  G  F  P  F  D  R  P  P  D  K  M  I  T  T  L  A  K

2071    ATTTATTGAAAAGTCGCCTAATATCAAAACCACAGAGATCCGCATTCAGTTTGAAGATCGCATCATCCGTCGATTTCCCCAACAATAATT
 682      F  I  E  K  S  P  N  I  K  T  T  E  I  R  I  Q  F  E  D  R  I  I  R  R  F  P  Q  Q stop

2161    GGAACAGTCATGCTTTCTGTTTCCGTTTTCCACCTTCTTTTACTCAGCCAATGACGCTCTCTTGAGCTATAGTACTTAACGTTTATCGCA
2251    CAGAAAAGGAACAACAGGAATTGTCTGCTCTTAATTTTCATTTCCTGAAAGCGGATGTTGTGATACTTCCTTAATCCGTACTAATTGAAA
2341    AGCATTTCAAGATACTTGCATTCTTCGCTTTGCTTGGGGGAGCTTGATCCTATAAAGATCAGATTTCTTTTGCCATTTACTACAACACAG
2431    TCTACCAAACTTTTCTTTGGATAACTGCCTCTTTTCTCAAGGCTATGGTGCCCAACAATCCCCCTCAATTGCTGCACAATAACATTCATT
2521    TGATTCTTTTTCTCCCAATTCTTTTCATTGCTTCCCTTTTTAGAAATACATCTGGAACTGAAAAGCGAAAAAAAAAAAAAAAAAA 3′
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Fig. 1. Nucleotide (above) and deduced amino acid (below) sequence of *D. magna* proPO. The nucleotide sequence is numbered from the first base at the 5′ end of the longer transcript. The first methionine (**M**) is numbered on the first deduced amino acid of the longer transcript. The double asterisk (**) indicates a putative cleavage site for zymogen activation by ppA (see text). The six histidine residues within the Cu (A) binding site: His 205, His 209 and His 235 and the Cu (B) binding site: His 366, His 370 and His 406 are shown in bold letters (**H**). The thiol-ester-like motif is shown with a double underline (**GCGWPDHF**).

recently sequenced and partially annotated genome of *Daphnia pulex* [20] to sequence proPO in *D. magna*, which has been the subject of extensive investigations of host–pathogen interaction. Only one copy of ProPO was found in the *D. pulex* genome, and we also found no evidence of multiple copies in *D. magna*. We assembled a total of 2607 bp of cDNA for Dmag-proPO. The nucleotide sequence was found to contain a 28 bp 5′ untranslated region (UTR), a 449 bp 3′ UTR containing the poly-A tail, and an open-reading frame of 2130 bp corresponding to a deduced protein of 709 amino-acids (Fig. 1). The calculated molecular mass is 80 641 and the estimated p*I* is 6.28. The sequence of Dmag-proPO was deposited in NCBI GenBank under the accession number FJ381649 (Table 1).

All arthropod proPO genes contain highly conserved features, in particular two sites that code for copper-binding sites, and a thiol-ester-like motif [8–11,30]. These three features were highly conserved in Dmag-proPO: the six histidine residues for the copper binding site A were found in positions 205, 209 and 235, and for the copper binding site B in positions 366, 370 and 406; the thiol-ester like motif was found as GCGWPDHF in positions 600–607 (the positions are given from the first Met amino-acid, Figs. 1 and 2). A putative cleavage site for proPO activation by the proPO–activating enzyme (ppA [6]) is proposed between Arg17 and Val18 due to close proximity with cleavage sites of other species' proPO in the protein alignment. However, this proposed cleavage site is speculative, as it does not align with other species' sites. No signal

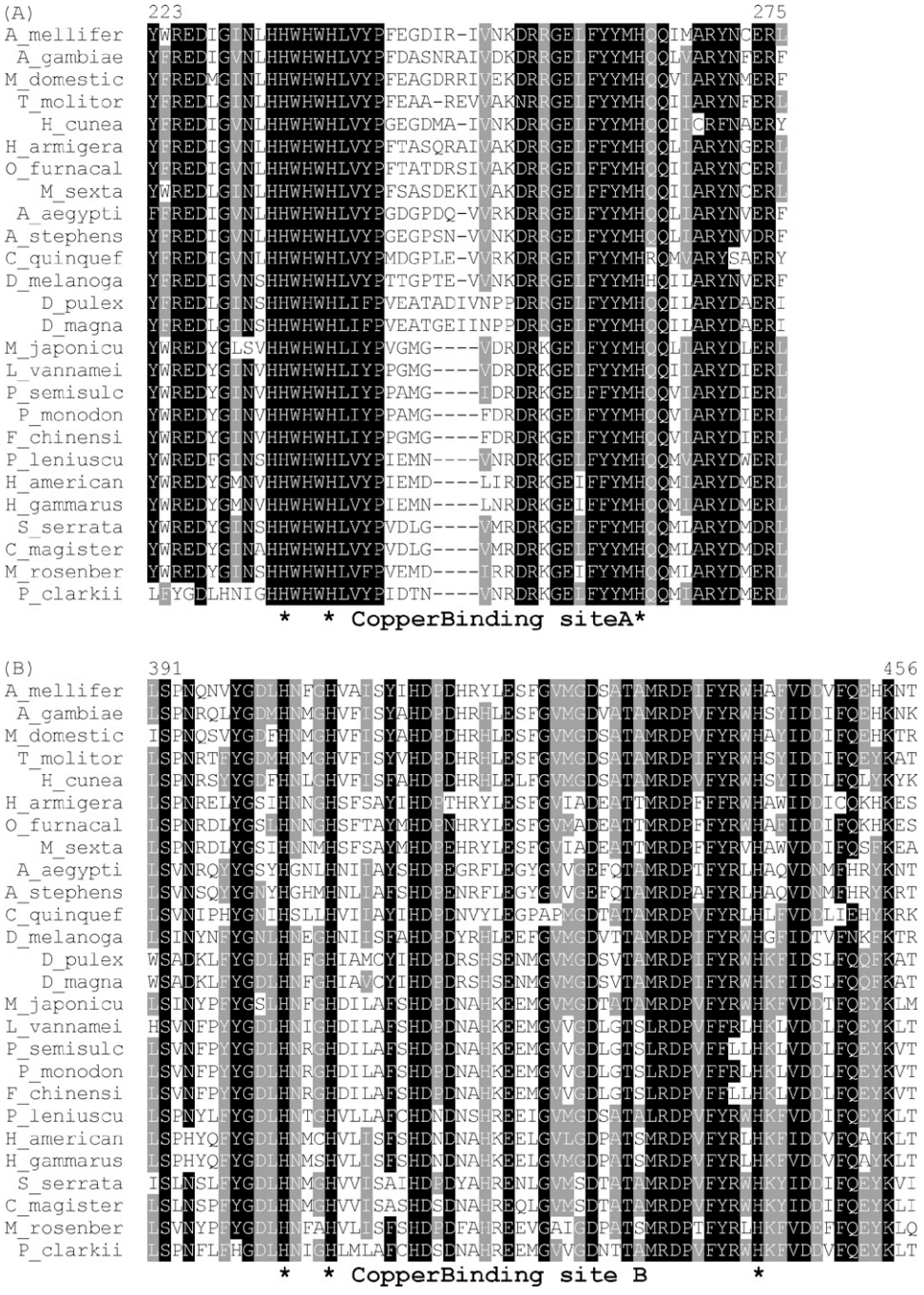

Fig. 2. Multiple alignment for conserved motives of arthropod proPO amino acid sequences. (A) copper-binding site A; (B) copper-binding site B, (C) thiol-ester-like motif. Identities are blackened, similarities are shaded. Asterisks (*) indicate conserved histidines or the thiol-ester-like motif.

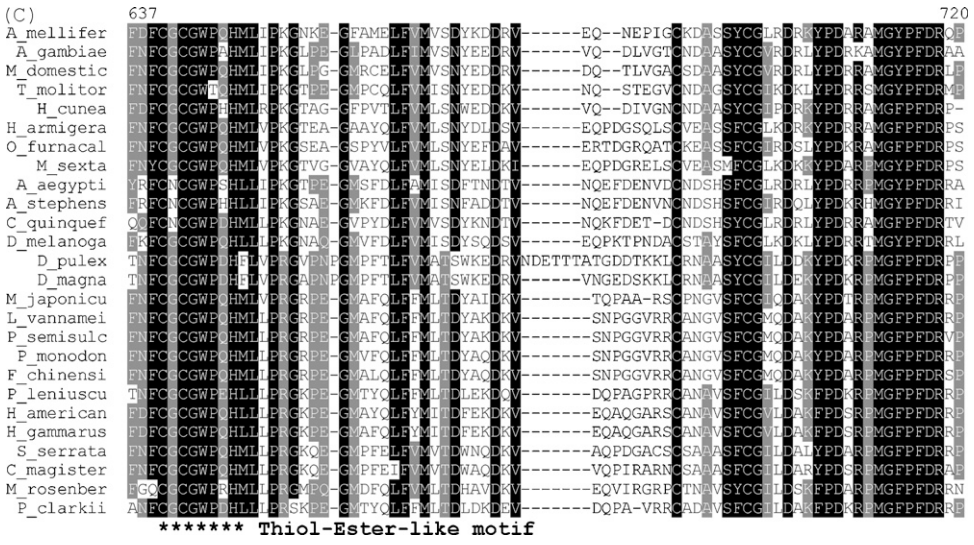

Fig. 2. (*Continued*).

peptide was detected using the online software SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/ [31]).

Using the NCBI BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Dmag-proPO was shown to have similarity with other arthropods (Fig. 3) ranging from 54% to 61%. Notably, *Daphnia* proPO is not more similar to decapoda crustaceans (from 55% to 59%) than to insects (from 54% to 61%). This is confirmed by the midway position of both *D. pulex* and *D. magna* proPO sequences in the proPO protein Kimura distance tree (Fig. 3).

To assess intraspecific variability, we sequenced 2020 pb of the cDNA of 14 different clones (from the 36th amino-acid to the stop codon). We did not sequence the entire coding region because it appeared that several transcripts were present in all clones, differing only in the very beginning of the sequences. Some of these were sequenced and showed insertions of ~20 bp, which probably corresponded to alternative splicing events, as all indels can be found in the DNA sequence and all 14 clones seem to display several variants in the beginning of the cDNA sequence (data not shown), perhaps due to different expression localizations [32]. Overall, proPO sequence appeared remarkably conserved as we found only 14 variable sites, with 11 clones displaying identical sequences. Of the 14 variable sites, only four carried non-synonymous mutations, among which two do not change the polarity of the amino-acid (A1359V and I1553V), one changes the amino-acid into a stop codon (Q2153Stop, but this amino-acid is the penultimate of the protein), and the last one changes a polar amino-acid into a non-polar one (S1364P). No variability was found among the Gaazerfeld clones ("GG" clones) despite the great variability they display in terms of resistance to *P. ramosa* infection (GG3 and GG4 are relatively susceptible, whereas GG7 and GG13 are relatively resistant [14]). Thus, if proPO is involved in resistance to this pathogen, the differences among clones is not due to protein differences but rather to expression level variation.

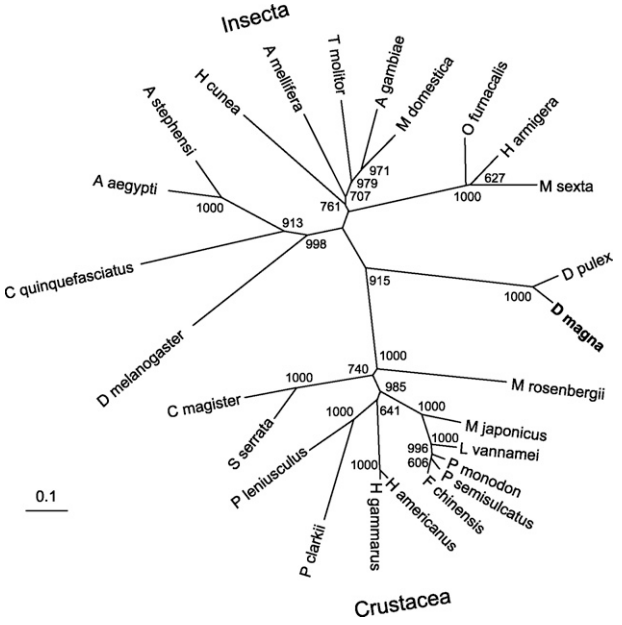

Fig. 3. Kimura distance tree for proPO proteins from various arthropods. See text for the reference sequences of the various organisms. *D. magna* is in bold. The scale for 0.1 amino-acid substitutions per site is indicated.

In our exposure experiment, *D. magna* individuals were exposed to *P. ramosa* spores for 2 h. This generated a high level of infection (8 of 11 individuals infected after 16 days for the exposed treatment, 0 on 12 for the 'not-exposed' treatment), in accordance with previous experiments [14]. We found a significant increase in the expression of proPO for individuals exposed to the pathogen with an approximately 2-fold induction in the 2 h following the exposure. After 6 h, however, no difference in expression was found between exposed and not-exposed *Daphnia* (Exposure:Time interaction, p-value = 0.012, Fig. 4). This is the first evidence of a *D. magna* immune response to *P. ramosa*.

With respect to the rapid up-regulation of proPO transcription, *Daphnia* does not appear special, as increased expression of proPO in response to pathogens or immunostimulants ranging from 1 to 12 h after exposure has been found in several studies of crustaceans [7–12], sometimes coupled with and increased PO activity [10,11]. The increase in proPO expression is usually higher than the 2-fold found in our study, however a similar scale of induction has been found in crayfish [11]. Moreover, our measure was taken on the whole organism (thus decreasing the relative quantity of proPO RNA compared to actin RNA), whereas in most studies the increase in proPO is measured in haemocytes only [7–11]. In most cases, proPO induction is short-lived [10,11], although a few studies have indicated longer-lasting increases in expression after immune challenge [8,9,12]. A clear link between susceptibility to a pathogen and proPO expression has been found in the crayfish *Pacifastacus leniusculus*: silencing the proPO gene using RNAi resulted in increased bacterial growth and host mortality,

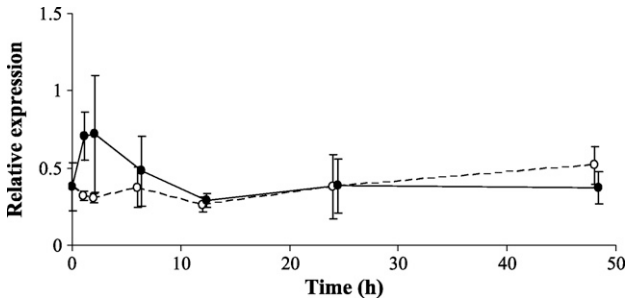

Fig. 4. Relative proPO expression following pathogen exposure in the GG4 *D. magna* clone. Expression of proPO relative to *actin* is presented for exposed (full black circles, solid line) and not-exposed (white circles, dotted line) treatments. Each point corresponds to the mean of 3 independent replicates, the error bars shown are standard error. The black circles have been slightly shifted horizontally for easier reading.

whereas silencing of an inhibitor of the proPO-activating cascade resulted in increased survival and enhanced bacterial clearance [7]. However, despite many such studies pointing towards a role of PO in the invertebrate innate immune response to pathogens, there is still some debate on its overall effect, notably due to the potentially harmful effects of the quinones intermediates released during the production of melanin [32]. Moreover, PO is also implied in non-immune related functions, as indicated by the high number of proPO genes in some insects (e.g. nine genes in the mosquito *A. gambiae* [6]).

In summary, we showed that the proPO gene of *D. magna* possesses all the features commonly found in invertebrates, although the existence and localization of the cleavage site remain to be confirmed. It seems also that several transcripts are produced by the same gene, with most differences apparent at the beginning of the sequence. At the intraspecific level, the sequence is conserved, suggesting a high degree of purifying selection level. We did not find any evidence of mutations that could explain previously observed differences in resistance phenotypes [14]. Nevertheless, proPO may be linked to pathogen resistance as its expression is enhanced shortly after exposure to the gram-positive bacterial pathogen *P. ramosa*. This is an important observation: the *D. magna*–*P. ramosa* system is relatively well characterised in terms of phenotypic variation, virulence and field epidemiology, but the genes and immunological mechanisms that mediate this pathogenic interaction have not been established. Our data are thus a first step towards using knowledge of defence mechanisms to understand the evolution and coevolution of this natural host–pathogen system.

Acknowledgments

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