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Characterisation of proteins differentially present in the plasma of *Biomphalaria glabrata* susceptible or resistant to *Echinostoma caproni*

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Abstract

Snail immune responses towards a trematode infection are known to rely on both plasmatic and cellular host factors. As an approach to further investigate the suspected involvement of plasmatic factors in *Biomphalaria glabrata* resistance/susceptibility to *Echinostoma caproni*, we compared protein patterns of plasma collected from susceptible and resistant snails. This proteomic approach revealed that 13 plasmatic proteins exhibited significant differences in their apparent representativity. The genes corresponding to five of them were characterised by a combination of mass spectrometry and molecular cloning. They encode two isoforms of a glycolytic enzyme, two isoforms of a calcium binding protein and an inhibitor of cysteine protease. Furthermore, we investigated gene expression in parasite-exposed or -unexposed snails as well as in various tissues by quantitative PCR. This study showed that: (i) differential representation of plasma proteins between the snail strains was correlated with a differential level of transcripts; (ii) expression of these genes after parasite exposure was differentially regulated in the two strains; and (iii) these genes were expressed predominantly in the albumen gland.

Keywords: *Biomphalaria glabrata*; *Echinostoma caproni*; Host-parasite interactions; Proteomic; Susceptibility; Albumen gland

1. Introduction

Snail-trematode immunobiological interactions have been studied on the fresh-water snail *Biomphalaria glabrata* following infection by the human blood fluke *Schistosoma mansoni* and by the echinostome species, *Echinostoma paraensei* and *Echinostoma caproni*. In contrast to *S. mansoni*, these two echinostomes are known to strongly interfere with the snail immune response via their excretory-secretory (ES) products (Loker and Adema, 1995; Humbert and Coustau, 2001). Analysis of interactions between *E. paraensei* ES products and *B. glabrata* plasmatic factors led to the identification of the numerous fibrinogen-related proteins (FREPs) (Adema et al., 1997; Leonard et al., 2001; Zhang et al., 2001, 2004; Zhang and Loker, 2003). At least some of these extremely diversified molecules are capable of binding and precipitating *E. paraensei* ES products, suggesting that they may play an important role in snail-trematode molecular interactions. In addition to the discovery of FREPs, previous studies provided evidence that plasmatic factors may play an important role in the outcome of a trematode infection. In vivo comparison of *E. caproni* development in *B. glabrata* snails selected for their susceptibility or resistance (Langand et al., 1998) revealed that, in resistant snails, sporocysts were abnormally developed and degenerated regardless of their level of encapsulation (Ataev and Coustau, 1999). These results suggested that, in addition to cellular factors, humoral factors are actively involved in the molecular processes underlying resistance or susceptibility to *E. caproni* (Ataev and Coustau, 1999).

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Except for the presence of FREPs, virtually nothing is known on the composition of plasma, and plasmatic factors remain uncharacterised.

In the present study, we addressed the question of potential constitutive differences between plasma factors of resistant and susceptible snails. We used, for the first time in this model, a proteomic approach aimed at: (i) identifying proteins differentially represented in the plasmas of susceptible and resistant snails; (ii) characterizing the genes encoding these proteins; and (iii) analyzing their expression p.i. and in a range of tissues.

2. Material and methods

2.1. Biomphalaria glabrata strains and plasma preparation

Biomphalaria glabrata snails used in this study belonged either to the unselected Bg.Bra stock originating from Brazil (Guillou et al., 2004), or to the EAF-S or CB-R strains selected, respectively, for their susceptibility or resistance to *E. caproni* (Langand et al., 1998; Humbert and Coustau, 2001). At the time of the study, the percentages of adult snails susceptible to *E. caproni* were: Bg.Bra = 70%, EAF-S = 98% and CB-R = 1%.

Hemolymph was extracted from the headfoot of 1,000 uninfected EAF-S and 1,000 uninfected CB-R snails according to standard procedure (Sminia and Barendsen, 1980) and immediately placed on ice. Approximately 100 ml of hemolymph were recovered from each strain and centrifuged at $800 \times g$ for 10 min at 4 °C. The supernatant (plasma) was collected, centrifuged at $20{,}000 \times g$ for 20 min at 4 °C and filtered using a stirred ultrafiltration cell with a cut-off 500 kDa (Millipore). The filtered plasma was then stored at −80 °C until use.

2.2. Experimental infection

The parasite *E. caproni* (Jeyarasingam et al., 1972) was maintained in Bg.Bra snails and mice (SWISS OF1 stock) as described previously (Trouvé et al., 1996). For one experiment, adult EAF-S and CB-R snails (measuring 9–14 mm in shell diameter) were exposed to *E. caproni* infection according to previously described procedures (Guillou et al., 2004). Ten individuals from each strain were collected at 3, 6, 12, 24, 48 and 96 h post-exposure and frozen in liquid nitrogen until RNA extraction.

2.3. Two-dimensional gel electrophoresis

2.3.1. Plasmatic protein extracts

Plasmatic proteins were precipitated by adding four volumes of a solution containing 10% trichloroacetic acid, 0.07% β-mercaptoethanol in acetone to 3 or 9 ml of plasma (for analytical or preparative electrophoresis, respectively). After 1 h at −20 °C, proteins were pelleted by centrifugating at $15{,}000 \times g$ for 30 min at 4 °C. Four volumes of acetone containing 0.07% β-mercaptoethanol were added to the pellet and incubated 1 h at −20 °C. After centrifugation ($15{,}000 \times g$ for 30 min at 4 °C), the pellet was air-dried and incubated 90 min at room temperature in 350 µl of 'lysis' buffer (9 M urea, 2% Triton X-100 and 10 mM Dithiothreitol (DTT) (Wu et al., 2000).

2.3.2. Isoelectrofocusing (IEF)

IEF for the 2D gel electrophoresis was performed using the IEFCell system™ (Biorad). Immobilised pH 3–10 non-linear gradient (IPG) strips were rehydrated with the 350 µl of protein extracts to which 0.2% v/v Bio-lyte (pH 3–10:pH 5–8:pH 7–9, 1:1:2, Biorad) were added. Passive rehydration was performed for 5 h at 20 °C, followed by an active rehydration at 50 V for 14 h at 20 °C. Isoelectrofocusing was raising gradually until 8,000 V and running until 90,000 V h at 20 °C. Strips were then incubated twice with 130 mM DTT in equilibration buffer (6 M urea, 375 mM Tris–HCl, 2% SDS, 20% glycerol, 0.02% Coomassie blue G-250, pH 8.8) for 15 min according to Wu (Wu et al., 2000). Proteins were then carbamidomethylated for 15 min (135 mM iodoacetamide in equilibration buffer).

2.3.3. SDS-PAGE

IPG strips™ were transferred on a 12% polyacrylamide gel containing 0.8% of cross-linker piperazine diacrylamide in a 0.5% agarose low melting point gel. Electrophoresis were performed in Tris/Glycine/SDS buffer according to the conditions defined by O'Farrell (O'Farrell, 1975). Proteins were separated 1 h at 30 V and then at 200 V until Coomassie blue has reached the bottom of the gel. After electrophoresis, gels were silver stained according to a modified protocol of Morrissey (Morrissey, 1981) or Coomassie blue stained according to Neuhoff (Neuhoff et al., 1988).

2.3.4. 2D image analysis

Three gels per condition were analyzed using the Progenesis™ v1.5 software program (Nonlinear dynamics, Newcastle upon Tyne, UK). Analysis protocol included spot detection and filtering, whole image warping on a reference gel, background subtraction, average gel creation, spot matching and volume normalization against total volume of all protein spots present in the gel. Each analysis step was manually validated. Statistical significance was assessed using a Student's *t*-test.

2.4. Protein identification

2.4.1. In-gel digestion for mass spectrometry

Proteins of interest were excised and gel pieces were washed in 25 mM ammonium bicarbonate/50% acetonitrile. After drying, gel pieces were placed for 30 min on ice in 50 µl of protease solution (sequence grade modified trypsin, Promega, at 0.02 mg/ml in 25 mM ammonium bicarbonate).

Excess protease solution was then removed and replaced by 50 µl of 25 mM ammonium bicarbonate. Digestion was performed at 30 °C overnight. Peptide extraction was performed twice for 15 min with 50% acetonitrile/1% trifluoroacetic acid (TFA) for further Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-ToF MS) analysis or with 50% acetonitrile/1% formic acid for further ESI-MS-MS (Electro Spray Ionization-Mass spectrometry-Mass spectrometry) analysis. Trypsin digests were then lyophilised in a SpeedVac concentrator (Savant) and resuspended in 5 µl of 0.1% TFA or 0.1% formic acid.

2.4.2. MALDI-ToF MS

MALDI-ToF MS analysis of trypsin digests was performed on a Voyager DE Pro™ (Applied Biosystems) in reflector mode at an accelerating voltage of 20 kV. One microliter of trypsin digests was spotted on 1 µl of dried α-cyano-4-hydroxycinnamic acid (HCCA, 15 mg/ml in acetone) and covered with 1 µl of HCCA (10 mg/ml in 70% acetonitrile/0.1% TFA). About 300 laser shots were accumulated to obtain the final spectrum. Peptide masses were determined after peak smoothing and internal calibration using the two autolysis trypsin fragment $[M+H]^+ = 2211.1$ and 842.51 Da.

2.4.3. ESI-MS-MS analysis

After desalting by on reversed-phase C18 Zip-Tip microcolumn (Millipore, Bedford, MA, USA), samples were loaded into nanoESspray capillaries (Protana, Odense, Denmark) using a 5 µl on-column syringe. Capillaries were inserted into an Applied Biosystems Q-STAR Pulsar™ using an Ion spray source (nanoESI-Q-ToF). Doubly-charged peptides were selected, fragmented by N_2 collision and analysed by ESI-MS-MS. The sequences obtained were manually assigned to search against SWISSPROT, NCBI and GenPept and the division dbEST of GenBank (ESTs of *B. glabrata*) using MS-Pattern (http://prospector.ucsf.edu/ucsfhtml4.0/mspattern.htm).

2.5. RNA extraction

RNA extraction, and reverse transcription were performed from whole body *B. glabrata* according to previously described procedures (Guillou et al., 2004).

2.6. Gene characterization

First-strand cDNAs were prepared from total RNAs with oligo $d(T)_{17}$ for primer using the Superscript II™ (Invitrogen). The 3′ portion of the cDNAs were amplified by PCR using an oligo $d(T)_{17}$ and the different degenerate primers designed from the tags obtained by ESI-MS-MS (Table 1). PCR was performed in 50 µl mixtures containing 2 µl of first-strand cDNA using the DyNAzymeEXT™ kit (Finnzymes). Primer concentration of 2 and 0.2 µM were used for the degenerate primers and the oligo $d(T)_{17}$, respectively. In order to clone the 5′- or 3′-ends of the cDNAs, we used the GeneRacer™ Kit (Invitrogen).

The PCR products were ligated into the pCR2.1-TOPO™ vector according to the manufacturer's instructions (Invitrogen). Dideoxy sequencing reactions of the recombinant plasmids were sequenced using a dideoxy-dye-terminator method (CEQ™ DTCS-Quick Start kit, Beckman Coulter) and a CEQ™ 8000 apparatus (Beckman Coulter). M13 forward and reverse primers were used for sequencing. Sequences were obtained using the CEQ™ 8000 sequence analysis software.

2.7. Real-time PCR

Real-time PCR analysis of gene expression was performed on total RNAs extracted from parasite-exposed

Table 1
Sequences obtained by ESI-MS-MS and degenerated primers designed for cDNA cloning

Spot	Sequences	Degenerate primers
1	[PND]S[FM]Y[QK][QK][QK]G…T[IL]ND…[IL][FM]AVK HATS[QK]V–	
3	[IL][FM]DEVGD [QK][QK][FM]DSGWR	HTIWTBGAYGARGTIGGIGAY
4	YNEGNAYENK	GGIAAYGCITAYGARAAYAAR
6	DVDGDGN[IL]EPR	GAYGTIGAYGGIGAYGGIAAY
7	DVDGDGN[IL]EPR	GAYGTIGAYGGIGAYGGIAAY
8	[IL][FM][DA/W][IL]DY	
31	[IL][QK]W[FM][QK]YDDSAR	MARTGGTTYMARTAYGAYGAY
43	ns	
48	V[IL]TP[IL]ATAVK [IL]DG[IL][IL]VDER [FM]VNW[QK]AAA[IL]K	YTIGAYGGIYTIATHGTIGAYGA TTYGTIAAYTGGCARGCIGC
51	ns	
58	[FM][IL]NW[QK]ADA[IL]K DSG[F*M*/][IL]VTVGSWNPK YSYYDT[IL]R [IL]DG[FM][IL]VDWR	TTYHTIAAYTGGCARGCIGA
137	ns	
138	ns	

ns, no sequence obtained by ESI-MS-MS; [ ], ambiguous amino acids are in square brackets; M, oxydised methionine; wobble, M = A + C, R = A + G, W = A + T, Y = C + T, H = A + C + T, B = G + T + C, I, inosine.

Table 2
Specific primers used for real time PCR

Gene	Primer name and sequence
RiboS19	R19F1: TTCTGTTGCTCGCCAC
	R19R1: CCTGTATTTGCATCCTGTT
SPOT1	S1F1: CCATGTTGAAGATTGCCCTAG
	SR1: CACCATTCAAGTCAGCGGCG
SPOT58	DisS58F1: GCACTCAGCAGGAGACTAT
	DisS58R2: GACCATTCCAATTGTAATTTTTG
SPOT48	DisS48F1: GCATTCTGCTGGAGATTTC
	DisS48R2: TTACACCATTAGAAGTTAAGC
SPOT6	DisS6F2: ACACTAACGGAGACAATCA
	DisS6R2: CTGATTGGTCAAGGAATCC
SPOT7	DisS7F2: CGCCGACGGTGACGG
	DisS7R2: GGACTGGTCAAGATGGTT

EAF-S and CB-R snails. RNA extraction, reverse transcription, PCR and relative quantitation were performed according to previously described procedures (Guillou et al., 2004) with a Light cycler™ apparatus (Roche Molecular Biochemicals, Germany). Specific primers for real-time quantitative PCR (Table 2) were edited using the Light Cycler Probe Design™ Software version 1.0.

3. Results

3.1. Differentially represented proteins in plasma from susceptible and resistant strains

The 2D electrophoretic profiles of plasmatic proteins of CB-R and EAF-S strains is illustrated in Fig. 1A. Image analysis revealed 197 plasmatic proteins on the gels. Among these spots, we selected all spots showing a differential representation greater than 2.5-fold. Statistical analysis revealed that differences observed for the following 13 spots—1, 3, 4, 6, 7, 8, 31, 43, 48, 51, 58, 137 and 138—were significant ($P<0.05$). Ten of these proteins were more represented in the plasma of susceptible snails and three were more represented in the plasma of resistant snails (Fig. 1B and C).

In order to identify these proteins, we first performed an in-gel digestion with trypsin. Trypsic digest were then analysed by ESI-MS-MS to obtain sequence tags for these proteins (Table 1). These sequences were compared to databases using MS-Pattern software. Sequence tags obtained for spot 1 matched with four ESTs of *B. glabrata*. These ESTs (accession numbers: AA547743, AA547744, AA547745 and AA547746) correspond to the 5′ portion of cDNAs displaying high similarities to molecules belonging to the cystatin family of protease inhibitors. For example, AA547745 displayed an *E*-value of 1×10^{-5} for a cystatin of *Drosophila melanogaster* (accession number: S12913). In order to better characterise the gene encoding this protein, RT-PCR experiments were performed using primers designed from the ESTs (see Fig. 2). From the PCR product sequenced, we designed forward primers which were used to obtained the 3′ portion of the cDNA. This cDNA is 459 bp in length and encodes a 117 amino acids precursor (Fig. 2). The deduced amino acids sequence begins with a 18 residue signal peptide. The cleavage site for signal peptidase is most likely located after the serine residue preceding the glutamine in position 19 as predicted by the SignalP 3.0 server (http://www.cbs.dtu.dk/servicesSignalP/) and PSORT II prediction program (http://psort.nibb.ac.jp/form2.html). This signal peptide is directly flanked by a carboxyl-terminal 99 amino acid sequence. This protein has a calculated monoisotopic mass of 10,509.26 Da and a theoretical pI of 6.74, which are in good agreement with the position of the protein in the gel. Theoretical masses of peptides generated by the PeptideMass software (http://www.expasy.org/tools/peptide-mass.html) were compared to the experimental peptide mass fingerprint of spot 1 obtained by MALDI-ToF mass spectrometry. Four common fragments were identified covering 49% of the protein (signal peptide excluded). Combined results from cDNA cloning and mass spectrometry therefore confirmed that the spot 1 is a novel *B. glabrata* protein belonging to the type-2 cystatin family (Bg type-2 cystatin, GenBank accession number AY678119).

The sequence tags obtained from the other spots were insufficient to identify the other proteins using the same approach. Consequently, tags obtained from spots 3, 4, 6, 7, 31, 48 and 58 were used to design the degenerate forward primers shown in Table 1. These primers and a $d(T)_{17}$ primer were used in RT-PCR experiments to amplify cDNA fragments corresponding to the 3′ portion of the genes encoding these different proteins. After cloning and sequencing, reverse primers were designed from these sequence and 5′-RACE PCR experiments were performed to obtain the complete open reading frame. cDNAs corresponding to the spots 6, 7, 48 and 58 were characterised.

cDNAs corresponding to the proteins 6 and 7 (GenBank accession numbers: AY678123 and AY678120) encode precursors displaying high degrees of similarities (Fig. 3). They encode two isoforms containing four EF-hand domains (detected by RPS-BLAST program, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) characteristic of Calcium Binding Proteins (CaBPs). These precursors are highly similar to other CaBPs like calmodulins for example. Furthermore, these proteins are probably secreted as predicted by the PSORT II prediction program (http://psort.nibb.ac.jp/form2.html) and which is in agreement with the plasmatic location of the proteins. These two proteins were named Bg CaBP 1 (spot 6) and 2 (spot 7).

cDNAs corresponding to the proteins 48 and 58 also encode two highly similar isoforms (Fig. 4). Confrontation of the deduced amino acids sequence to the databank reveal high similarities to an endo-1,4-β-mannanase of the bivalve

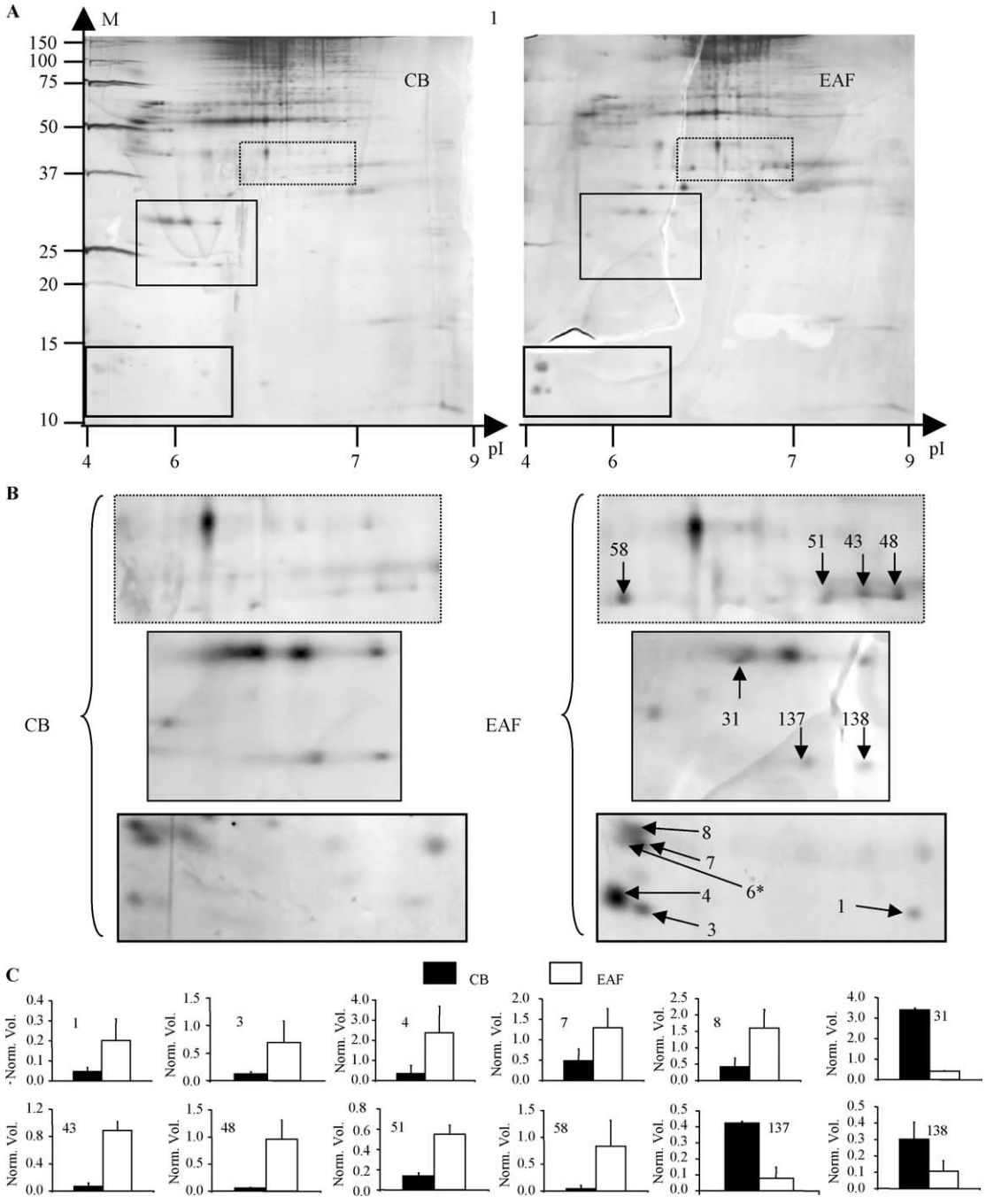

Fig. 1. Comparison of plasmatic protein patterns from uninfected susceptible and resistant snails. (A) Silver-stained 2D gels of plasmatic proteins from *Biomphalaria glabrata* resistant (CB-R) and susceptible (EAF-S) snails. Molecular mass (M) calibration is indicated on the left of the gel and pI calibration on the bottom of the gel. The pI calibration was done with pI standards run in the same conditions on a separate gel. Areas containing differentially represented proteins are boxed. (B) Enlarged views of the areas of interest. Positions of differentially represented proteins are indicated by arrows. * Spot 6 was not detected on CB-R gels. (C) Normalised volume of plasmatic proteins differentially represented between CB-R and EAF-S quantified with Progenesis™ software. The figure shows the average values obtained from three gels for each condition. * Significant differences when using the Student *t*-test ($P<0.05$).

Mytilus edulis (*E*-value $=1\times10^{-106}$, Swiss-Prot accession number Q8WPJ2, Xu et al., 2002). These two proteins were named endo-1,4-β-mannanase 1 and 2 (GenBank accession numbers: AY678121 (S58) and AY678122 (S48)). Note that S58 is also probably secreted as predicted by the PSORT II prediction program.

3.2. Differential plasmatic representation of the proteins is correlated to a differential transcription level between the two strains

The expression ratio of each gene to ribosomal protein (S19) appears substantially higher in susceptible snails

Fig. 2. cDNA (complete coding sequence and 3′ untranslated region) corresponding to the protein 1. The deduced amino acids sequence is shown in upper case letters. The precursor displays a signal sequence highlighted in pale grey. The domain shared by all cysteine protease inhibitors is boxed. The amino acids shared by type 2 cystatins are highlighted in dark grey. Fragments of the protein identified by analysis of mass spectrometry data are underlined (mass fingerprint and tags obtained by MALDI-ToF and ESI-MS-MS, respectively). Polyadenylation signal is double-underlined. The primers designed from the ESTs are indicated in italic and bold.

(Fig. 5A). These results are in agreement with the differential representation of the corresponding protein quantified using the Progenesis™ software (Fig. 5B). Therefore, differences in plasmatic protein content appear correlated with differences in expression ratios of the corresponding genes.

3.3. Effect of infection on expression ratios

Expression ratio of the different genes were analysed in susceptible and resistant snails at different times (0, 3, 6, 12, 24, 48 and 96 h) post-exposure to *E. caproni* (Fig. 6). Expression ratios of all genes are substantially lower in uninfected resistant than in uninfected susceptible snails. A global analysis of expression ratios during the kinetics of infection reveals a significant increase of transcript levels for the different genes in resistant snails. Indeed, transcripts corresponding to proteins 1, 6, 7, 48 and 58 are 2.8, 16.9, 6.5, 5.4 and 4.8 times more abundant at different times after infection of resistant snails, respectively. In contrast, in susceptible snails, significant decreases are observed for all the genes following parasite exposure.

3.4. Comparison of expression ratios in various snail tissues

To investigate tissue transcription of the genes encoding proteins 1, 6, 7, 48 and 58, quantitative PCR analyses were carried out using total RNA from the various snail tissues representative of the entire snail (Fig. 7). For the five genes, the highest expression ratios were observed in the albumen gland. Weak amounts of transcripts were detected in other tissues, particularly in hemocytes and head-foot for the gene encoding protein 1 and in head-foot for the gene encoding protein 7.

4. Discussion

Although, the importance of both humoral and cellular factors in determining the success or failure of a trematode infection has been highlighted for a number of years, past molecular studies mainly focused on the cellular effectors (Miller et al., 2001; Raghavan et al., 2003). Except for the highly diversified family of plasmatic fibrinogen-related proteins (Adema et al., 1997; Zhang et al., 2004), little is currently known on humoral factors.

As an approach to further investigate the suspected involvement of plasmatic factors in *B. glabrata* resistance/susceptibility to *E. caproni*, we compared protein patterns of plasma collected from susceptible and resistant snails. This proteomic approach revealed that 13 out of the 197 plasmatic proteins exhibited significant differences in their apparent representativity.

Characterization of the cDNA corresponding to the spot 1 has been possible by direct comparison of tags obtained by

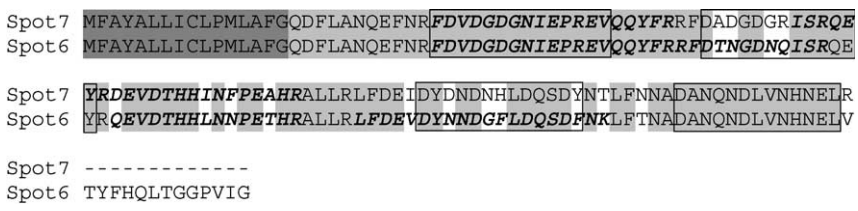

Fig. 3. Deduced amino acid sequences and alignment of protein 6 and 7 precursors (complete and partial CDS, respectively). Conserved amino acids are highlighted in grey. Signal sequences are highlighted in dark grey. The four EF-hand domains are boxed. Theoretical trypsic fragments matching with the peptide mass fingerprint obtained after trypsic digestion of these proteins are represented in italic and bold. These matching fragments cover 39 and 56% of the protein 7 and 6, respectively (signal peptide excluded).

```
Spot58    MKTLITGFLVVLCTLKLVCARLAVSGNQFTYNGQRIFLSGGNLPWIQYAYDFGDHQWDSR
Spot48    ------------------------------------------------------------

Spot58    KGTFENQLTQLKNAGGNSIRLWVHIQGESTPAFDGNGYVTAPDHQGTLINDFKDMLDIAQ
Spot48    ------------------------------------------------------------

Spot58    RHNILVFPTLWNAAVDQDNSHRLDGFIVDWRKLQSYIDKALVPLASAVRGHPALGAWDIM
Spot48    ----------------------LDGLIVDERKLQSYIDKVLTPLATAVKGHPALGAWDIM

Spot58    NEPEGMINTDISNWDRCYDSTALKNSGAGWAGKKYSYYDTLRFINWQADAIKNVDSGFLV
Spot48    NEPEGMINPDIGNSDRCYDATALKNSGAGWAAKKYGYHDIIRFVNWQAAAIKHVDPGFLV

Spot58    TVGSWNPKSNTDQFGFVDHYSDNCLVKLGKPNGKLDFYQFHTYSYQGNFDNVSPFKHSAG
Spot48    TVGAWNPKSNTDRFGFVDHYSDACLLKGGKPNGKLDFYQVHSYSYQGNFDNVSPFKHSAG

Spot58    DYGTGKPIVVGEFWEQDGGGMNIDQLFDYVYNHGYAGAWSWDLMAHGDNQRGGISHIKNY
Spot48    DFGTGKPIVVGEFWEQDGGGMNINQLFEYVYNHGYAGAWSWDLQAHGANQRGGISHIKGL

Spot58    NWNGQIGINL
Spot48    TSNGVIPINV
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Fig. 4. Deduced amino acid sequences and alignment of partial and complete CDS of proteins 48 and 58, respectively. Conserved amino acids are highlighted in pale grey. Signal sequence of protein 58 is highlighted in dark grey. Theoretical trypsic fragments matching the peptide mass fingerprint obtained after tryptic digestion of these proteins are represented in italic and bold. These matching fragments cover 48 and 45% of the proteins 48 and 58, respectively.

ESI-MS-MS to databases. This cDNA has been identified as encoding a type-2 cystatin. Cystatins are cysteine protease inhibitors that have been reported in all *phyla* of living organisms (Abrahamson et al., 2003). They control activity of cathepsins, themselves involved in various biological processes, including immune processes (Abrahamson et al., 2003). Some parasites such as the nematodes *Brugia malayi*, *Onchocerca volvulus*, *Acanthoceheilonema vitea* or *Nippostrongylus brasiliensis* are known to secrete cystatins that inhibit hosts immune responses (Hartmann and Lucius, 2003). Whether the *B. glabrata* cystatin characterised here is involved in regulation of immune processes remains to be elucidated. However, its higher representativity in the plasma from susceptible snails is intriguing as it could reflect a constitutively stronger down-regulation of immunity facilitating parasite establishment in the first hours following infection.

cDNAs corresponding to the spots 6 and 7 are isoforms of a CaBP of the EF-hand type. CaBP play a key role in calcium signaling and homeostasis. Because the CaBP identified here are extracellular proteins located in the plasma, it is more likely that they participate in calcium homeostasis. However, links between differential representation of these proteins and susceptibility/resistance may exist. A first possibility is that these proteins are involved in regulation of adhesion processes. Cellular adhesion is often calcium-dependant and may be regulated by calcium-dependant molecules such as C-lectins in *B. glabrata* (Duclermortier et al., 1999). Because adhesive processes are optimal for a given concentration range of extracellular calcium (Johansson and Soderhall, 1988), differences in some extracellular CaBP content may result in differences in extracellular calcium concentration and therefore in differential regulation of adhesive processes. A second possibility is that these CaBP are antiparasitic. Such activity has been shown for the human plasmatic CaBP, Calgranuline C. This protein: (i) is one of the initiation factors of *S. mansoni*-induced granulomas (Yang et al., 1997); (ii) is involved in

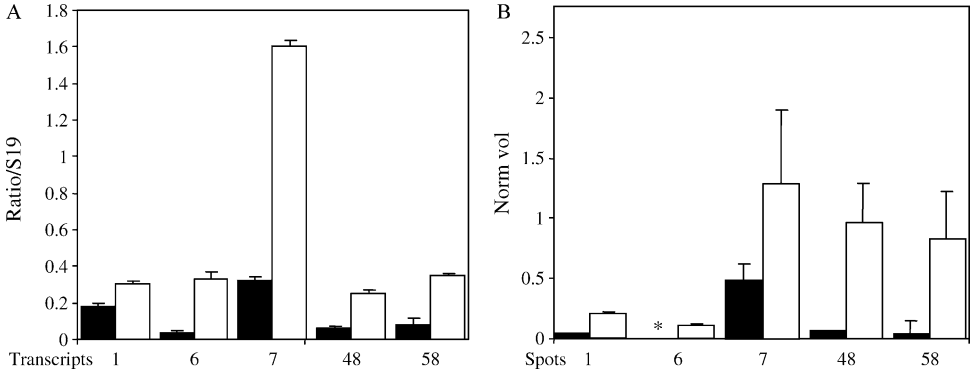

Fig. 5. Differential abundance of transcripts (A) and the corresponding plasmatic proteins (B) in the resistant CB-R (dark boxes) and susceptible EAF-S (light boxes) uninfected snails. (A) Transcript ratios were determined using real-time quantitative PCR and are expressed relative to S19 (ratio/S19). Each histogram is the average value of triplicates. (B) Normalised volumes (volume of each spot/all spot volumes added) of plasmatic proteins calculated with the Progenesis™ software. Histograms represent the average values from three gels. *, not detected.

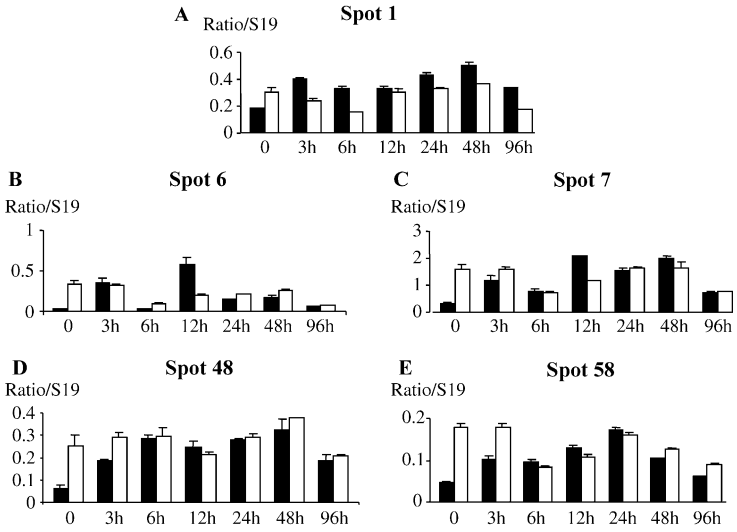

Fig. 6. Ratios of transcripts corresponding to spots 1 (A), 6 (B), 7 (C), 48 (D) and 58 (E) in the resistant CB-R (dark boxes) and susceptible EAF-S (light boxes) infected snails at different times post-exposure. Ratios were determined using real-time quantitative PCR and are expressed relatively to S19 expression levels (ratio/S19). Each histogram is the average value of triplicates ±SD.

the inflammatory response against *O. volvulus* (Marti et al., 1996) and; (iii) shows a filaricidal action against *B. malayi* (Gottsch et al., 1999). In our model, the massive induction of the gene encoding protein 6 in resistant snails 12 h p.i. could directly participate to the elimination of this parasite.

Two other cDNAs corresponding to spots 48 and 58 encode two isoforms of an endo-1,4-β-mannanase which displays significant similarities with a mannanase from *Mytilus edulis* (Xu et al., 2002). This type of molecule randomly cleaves within the β-1,4-mannan main chain of galactomannan, glucomannan, galactoglucomannan and mannan. It has been purified from a range of plants, fungi, and bacteria, and has been reported in gastropod snails such as *Pomacea insularus* and *Littorina brevicula* (Yamaura et al., 1997; Yamaura et al., 1996). Mannanases could be involved in the *B. glabrata/E. caproni* interactions as carbohydrates are known to participate to helminth immune-evasion strategies (Harnett and Harnett, 2001; Haslam et al., 2001; Khoo and Dell, 2001). For example, previous results suggested that a glycosylated polypeptide was at least partly responsible for the immunosuppressive activity of *E. caproni* ES products on *B. glabrata* hemocytes (Humbert and Coustau, 2001). Such glycosidases could

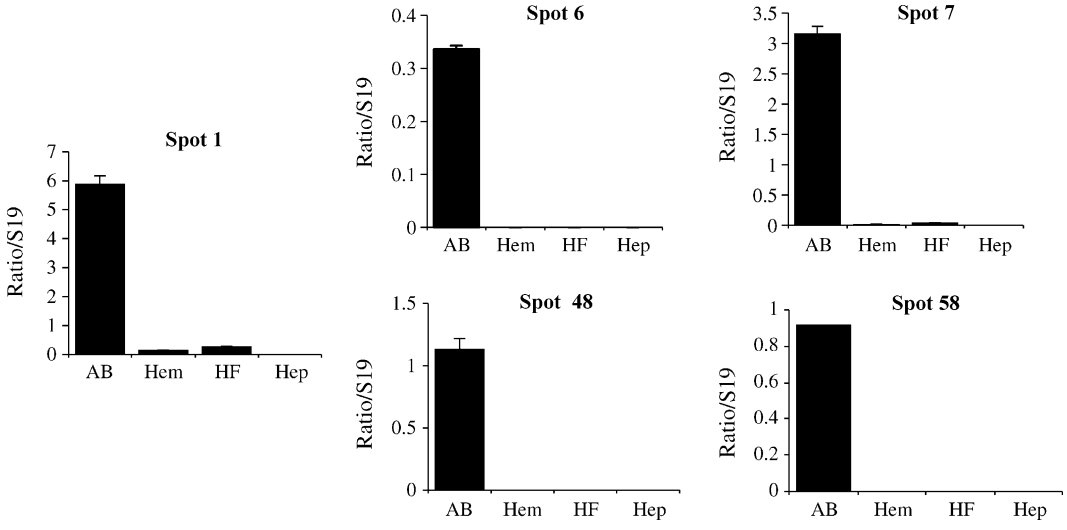

Fig. 7. Ratios of transcripts corresponding to spots 1, 6, 7, 48 and 58 in tissues of *Biomphalaria glabrata*: AB, albumen gland; Hem, hemocytes; HF, head foot; Hep, hepato-pancreas. Ratios were determined using real-time quantitative PCR and are shown relative to S19 levels (ratio/S19). Each histogram is the average value of triplicates ±SD.

therefore modify the structure of parasite molecules (whether secreted or surface-exposed) and their interactions with host factors. This hypothesis may be supported by previous observations showing that *S. mansoni*-susceptible *B. glabrata* exhibit a higher β-D-mannosidase activity than resistant snails (Zelck, 1999).

Expression studies of these five candidate genes showed that the transcript content of all candidates are stable in susceptible snails following infection, while they increase significantly in exposed resistant snails. For the gene corresponding to protein 6, we note a significant increase of transcripts level especially 12 h p.i. whereas transcription is quite stable in susceptible snails.

As high transcript levels of these genes are observed in the albumen gland, all the differences observed between susceptible and resistant snails could be linked to a differential regulation of genes expressed by this organ. In molluscs, the albumen gland is considered as an accessory sexual gland involved in the synthesis of various compounds like galactogen necessary for egg development (Wijdenes et al., 1983). Nevertheless, this organ is also involved in the production of defense effectors like agglutinins in planorbid snails (Michelson and Dubois, 1977; Stein and Basch, 1979; Boswell and Bayne, 1984) or antimicrobial proteins in oposthobranchs gastropods (Iijima et al., 1994; Takamatsu et al., 1995). Our results further support the idea that the albumen gland may play a significant role in snail immune response.

In conclusion, this first proteomic approach has been successful in identifying secreted proteins that were differentially represented in the plasma from *E. caproni*-susceptible and—resistant *B. glabrata*. Molecular characterization of the corresponding genes provided informative data on the potential functions of these proteins. According to these predicted functions, various assumptions can be made on their potential involvement in susceptibility/resistance processes. Future functional studies are needed to clarify the role of these genes in the host-parasite interaction. Interestingly, our results also showed that these genes are mainly expressed in the albumen gland and raise the question of the potential importance of this organ in processes underlying susceptibility/resistance to *E. caproni*. In order to elucidate the role of the albumen gland in defense and/or resistance processes, future studies will focus on genes expressed by this organ in susceptible and resistant snails after infection.

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