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A putative double role of a chitinase in a cnidarian: pattern formation and immunity

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Abstract

Chitinases are enzymes that degrade chitin, the second most abundant polymer in nature. They are ubiquitous among living organisms where they play a role in development, food-digestion and innate immunity. We have cloned and characterized the first cnidarian chitinase cDNA from the hydroid *Hydractinia*. The *Hydractinia* chitinase exhibits a typical secreted family 18 hydrolases primary structure. In situ hybridization and RT-PCR experiments showed that it is exclusively expressed in ectodermal tissues of the animal, only following metamorphosis while undetectable in embryonic and larval stages. Most prominent expression was observed in the stolonal compartment of colonies, structures that are covered by a chitinous periderm. Chitinase mRNA was detected in new branching points along stolons and in hyperplastic stolons indicating a role of the enzyme in pattern formation and allorecognition. It was also expressed in polyps where it was mostly restricted to their basal portion. This expression pattern suggests that *HyChit1* also fulfills a role in host defense, probably against fungal and nematode pathogens. Endodermal expression of *HyChit1* has never been observed, suggesting that the enzyme does not participate in food-digestion.
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1. Introduction

Chitin, a water-insoluble homopolymer of β-(1,4)-*N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant polymer in nature after cellulose. It is synthesized by arthropods, nematodes, mollusks, fungi and other organisms [1]. The general function of chitin in animals is to provide mechanical rigidity to extracellular structures and to form a physical barrier against invading microorganisms (e.g. insect cuticle).

Chitinases are enzymes that degrade chitin by catalyzing the hydrolysis of the GlcNAc linkages to produce oligosaccharides. Chitinases are found not only in chitin-containing taxa, but also in a variety of organisms that are not composed of chitin such as prokaryotes, plants, and vertebrates. In chitinous organisms, the functions of chitinases are mainly related to growth and pattern formation (e.g. insect molting). Non-chitinous organisms, in contrast, express chitinases for the purposes of nutrient supply or for immunity against chitinous pathogens (e.g. Ref. [2]).

Abbreviations: EST, expressed sequence tags; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR.

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Chitinases have a potential interest in biological pest control and innate immunity studies. Transgenic plants expressing chitinases show better resistance against several pathogenic fungal and insect infections. Also, viral and bacterial pathogens of pest insects, which have been transformed to express chitinases, show increased pathogenicity [2]. Chitinases are part of the innate immune response of many organisms, plants and animals as well. They function by degrading chitineus parts of potential pathogens. Chitinases are expressed by e.g. fungal-infected wheat [3], human macrophages [4,5] and in bovine liver [6]. A direct evidence for their function in innate immunity in mammals has not been provided yet (see also Ref. [4]). The lack of endogenous substrate in the mammalian serum, however, supports this assumption.

Based on sequence similarities, chitinases have been grouped in families 18 and 19 of glycosyl hydrolases [7–9]. Chitinases of the family 19 of glycosyl hydrolases are mostly plant chitinases. Chitinases of the family 18 of glycosyl hydrolases include plant, fungal, bacterial and animal chitinases. Site directed mutagenesis experiments [10] and crystallographic data [11,12] in this family have shown that a conserved glutamate is involved in the catalytic mechanism and probably acts as a proton donor. This glutamate is the last residue of this active site signature.

The evolution of chitinases in animals and their function in innate immunity are not yet fully understood [4,13] and this problem may be addressed by the analysis of chitinases from basal metazoans. Here we report the cloning and characterization the first full-length chitinase cDNA from a cnidarian. The expression pattern of this gene, as demonstrated by in situ hybridization assays, suggests a double role of this chitinase in the hydroid *Hydractinia echinata*.

2. Materials and methods

2.1. Animals

Hydractinia[1], a colonial marine hydroid, is a common inhabitant of shallow water communities in the North Atlantic. This animal is a popular model organism, mostly for developmental biology and comparative immunology studies [14]. Animals are mostly found encrusting gastropod shells inhabited by hermit crabs. The colonies are composed of a dense network of gastrovascular tubes, termed stolons, from which polyps emerge. The stolons are covered by a chitinous periderm that has to be modified during growth to enable stolon plasticity (e.g. anastomosis). The life cycle of *Hydractinia* starts after fertilization of the egg, which occurs in the water column. The embryos develop into a ciliated, non-feeding planula larva within 3 days. The larvae may be induced to metamorphose by a variety of natural and artificial agents [14,15]. Metamorphosis lasts about 24 h, resulting in a feeding primary polyp with 2–3 short stolons. The stolons grow and new polyps bud, forming a new colony through asexual reproduction.

H. echinata colonies were sampled at the Island of Sylt, North Sea and shipped to the laboratory at Heidelberg. They were cultured in artificial seawater at 18 °C under a 14:10 light–dark regime. Animals were fed daily with >3-days old *Artemia salina* nauplii. Under these conditions they spawned several times per week. Fertilized eggs were collected by Pasteur pipettes and kept in artificial seawater where they developed into metamorphosis-competent planula larvae within 3 days. Metamorphosis was induced by a 3 h pulse treatment of 116 mM CsCl in seawater [16]. Induced larvae were positioned on glass coverslips where they completed metamorphosis within 24 h. Several induced larvae were positioned at a distance of 1–2 mm from each other. As they metamorphosed and grew into allogeneic contact, hyperplastic stolons (sensu [17]) developed in incompatible pairs.

2.2. Full-length cDNA clone of HyChit1

A cDNA fragment of 347 bp, displaying significant sequence similarity to chitinases was obtained from an EST sequencing project (accession No. BU237873). The full-length cDNA sequence was obtained by RACE-PCR using the SMART RACE kit (Clontech) according to the manufacturer's protocol. Colony total RNA was used to prepare the 5′ and 3′ cDNA templates. As Forward primer for the 3′ RACE we used 5′-tgggctcttgatctcgatga-3′, and the primer

[1] Most studies on *Hydractinia* were carried out on the European *H. echinata* and its American congener, *H. symbiolongicarpus*. In this paper we use the generic *Hydractinia* for simplicity, referring to both species.

5′-ctcctagtgccttggatactgctga-3′ was used as a reverse primer for the 5′ RACE. PCR fragments were cloned into a pGEM-T vector (Promega) and sequenced using T7 and SP6 primers.

2.3. Sequence analysis

The protein sequences of 10 family 18 chitinases from different organisms were obtained from public databases as follows: *Homo sapience* (Genbank AAG60019); *Mus musculus* (Genbank AAF31644); *Danio rerio* (Genbank AAH46004); *Caenorhabditis elegans* (Genbank NM_076187); *Penaeus* (TrEMBL Q26042); *Drosophila melanogaster* (Swiss-Prot O17420); *Arabidopsis thaliana* (GenBank AB006065); *Glycin max* (Swiss-Prot O48642); *Saccharomyces sereviciae* (Swiss-Prot P29029); *Bacillus cereus*. (Genbank AB041931). Only the catalytic domains were aligned using Clustal X (1.82) and further processed using the program GeneDoc.

2.4. Digoxygenin-labeled RNA probe synthesis

A pGEM-T vector containing a 296 bp fragment of the 3′ coding region (position 1135–1429 in the transcript) was linearized by *Not* I and *Nco* I restriction enzymes to create templates for sense and antisense RNA probes, respectively, and phenol: chloroform extracted. The digested plasmids were then used as a template for a digoxygenin-labeled in vitro RNA probe synthesis according to the manufacturer's protocol (Roche). Sense and antisense probes were evaluated on a formaldehyde agarose gel and kept at −70 °C until used.

2.5. Northern blotting

For Northern blotting, colony total RNA was isolated using acid guanidinium thiocyanate [18]. Four microgram of RNA was loaded onto a 1.2%, formaldehyde agarose gel and blotted onto a nylon membrane (Hybond-N, Amersham). It was then hybridized overnight at 60 °C with 80 ng/ml of the probes and visualized by anti-digoxygenin, alkaline phosphatase conjugated antibodies and NBT/BCIP substrate.

2.6. In situ hybridization

In situ hybridization was conducted as described [19] with the same probes used for the Northern analysis. Larvae were induced to metamorphose as described above. Thereafter, they were positioned on glass coverslips where they underwent metamorphosis within 24 h. Metamorphosed animals were cultured for up to 3 wk before being fixed for in situ hybridization analysis. They were either starved for 3 days, or fed 3 h before fixation for studying normal expression and expression of putative inducible digestive enzymes, respectively. Hybridization and detection were performed under the same conditions as for Northern blot analysis.

2.7. RT-PCR

For RT-PCR, 2 μg of total RNA were reverse transcribed using PowerScript reverse transcriptase (Life Technologies) in a 20 μl RT reaction. Two microliters of 1:10 diluted cDNA were subjected to 25 cycles of PCR. Primers used were: forward, 5′-tgggctcttgatctcgatga-3′, and reverse 5′-gcacatgtatcag-caggaca-3′, corresponding to the chitinase sequence. Total RNA was extracted from the following developmental stages as described above: planula larvae, 3, 9, 18 h post metamorphosis induction, and primary polyps. An actin PCR was performed as control using the following primers: forward 5′-aaacccttttccaac-catcctt-3′, and reverse: 5′-tgggccagattcatcgtattct-3. PCR products were resolved on a 1.2% agarose gel and stained by ethidium bromide.

3. Results

RACE-PCR (5′ and 3′) on the chitinase EST cDNA fragment revealed the 1467 bp full-length clone of the *Hydractinia* chitinase cDNA, which we have termed *HyChit1* for Hydroid Chitinase 1. The nucleotide sequence has been deposited at EMBL-bank under accession number AJ634589. Northern blot analysis confirmed the size of the full-length transcript (Fig. 1). The cDNA contained an open reading frame of 1395 bp, corresponding to a protein of 464 amino acid residues with a predicted molecular mass of approximately 51 kDa. The protein has a putative signal

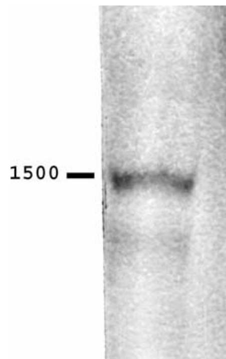

Fig. 1. Northern blot analysis of *Hy-chit1*. A single band is visible at 1.5 kb.

peptide of 18 amino acids at the N-terminus (predicted molecular mass of secreted protein 49 kDa) and a family 18 chitinase-like domain between positions 21-370. A chitinase catalytic active site is located between positions 135-143 with the highly conserved, proton-donor Glu-residue at its C-terminus. A putative polyadenylation signal (AATAAA) starts at position 1441 (Fig. 2). Fig. 3 shows an alignment of the most conserved region of family 18 chitinases from several organisms. Hychit1 shows a typical sequence for this protein family, especially at the catalytic active site.

RT-PCR on various *Hydractinia* developmental stages revealed that *HyChit1* is mostly expressed in post metamorphic stages (Fig. 4). The expression was almost undetectable in all embryonic stages analyzed (see Section 2) and in planula larvae. It started several hours after the induction to metamorphose (CsCl treatment) and reached a maximum in growing primary polyps. Actin control PCR confirmed equal cDNA load among all stages.

Based on the RT-PCR results, we have conducted in situ hybridization analysis only on post-metamorphic animals. Fig. 5 shows the results of the in situ hybridization analysis. *HyChit1* is exclusively expressed in ectodermal tissue, mostly in stolon ectoderms. The expression along the stolons was not always uniform (Figs. 5a and c). In some cases, it was restricted to distinct portions of the stolons (Figs. 5c and e). Stolon tips, the growing, path finding organ, never expressed *HyChit1*. Expression in polyp ectoderm was occasionally observed, always at a lower position, close to the stolon plate (Fig. 5b). Staining of other parts of polyps (e.g. hypostome, tentacles or upper parts of polyps) has never been observed. High levels of *HyChit1* mRNA was also detected in putative future branching points in stolon flanks (Fig. 5e), where a new stolon tip is formed. High levels of *HyChit1* mRNA were detected in hyperplastic stolons (*sensu* [17]). These structures are used during allogeneic rejection of histoincompatible colonies. They are formed following contact to an incompatible conspecific [17,20]. Animals that had been fed 3 h prior to fixation showed the same expression pattern as those that had starved 3 days before being processed. A specific staining of endodermal tissues was never observed. No staining was observed in samples hybridized with sense probes (Fig. 5f).

4. Discussion

We have identified and characterized the first chitinase full-length cDNA from a cnidarian. The predicted amino acid sequence showed a typical secreted family 18 glycosyl hydrolases chitinase. It included a signal sequence at the N terminal, and a catalytic domain, containing the highly conserved consensus sequence of the active site signature including the glutamate residue that functions as a proton donor (Fig. 2).

Hydractinia is a chitinous animal. Within colonies, polyps are interconnected by a network of gastrovascular tubes, the stolons, which are covered by a chitinous layer, the periderm. This stolonal network, resembling the vertebrate blood capillary system in many functional aspects [21], is highly dynamic. The stolons grow by subterminal elongation [22]. The stolon tip, a locomotory organ, is the path-finding unit during growth and is not covered by periderm. Therefore, the initiation of a secondary, lateral stolon tip requires the local removal of periderm at the site of tip formation. In *Hydractinia* stolons, two types of lateral tip induction are known: intrinsic and extrinsic. The former type occurs at more or less regular distances along the stolon flank, induced by an unknown factor or spontaneous. Such an induction never occurs at a distance of <400 μm from an existing growing tip due to the inhibitory field stolon tips maintain, repressing the induction of lateral, new

```
   1 ACAGTTTCTTGGTGAGGTAGCTGGAAAGAGAACTTCACAAAATGATCAAGCTTACAACTG
                                              M  I  K  L  T  T          6
  61 CCATCTTCCTGACTTTGTGCGCCGTTGCGGCCGCCAAAAATTATGTTCGTGTATGTTATT
     A  I  F  L  T  L  C  A  V  A  A  A  K  N  Y  V  R  V  C  Y        26
 121 ACACAAATTGGGCGCAATACCGCCCTCCGCCAATGAAGTTTTTCCCTGAGAACTTAGATC
     Y  T  N  W  A  Q  Y  R  P  P  P  M  K  F  F  P  E  N  L  D        46
 181 CACTGTTATGTACACATGTTGTCTACTCGTTCGCTAAGATTGGTAGAGGTCACACTTTAC
     P  L  L  C  T  H  V  V  Y  S  F  A  K  I  G  R  G  H  T  L        66
 241 AAATGTACGAATGGAATGACGATAAGATGTACCCACGAATGATGGCTTTGAAGCAACAAA
     Q  M  Y  E  W  N  D  D  K  M  Y  P  R  M  M  A  L  K  Q  Q        86
 301 ATCCAGCTTTGAAAGTTTTGTTGGCTGTCGGAGGATGGAACCACGAGAATGGAGGCACCA
     N  P  A  L  K  V  L  L  A  V  G  G  W  N  H  E  N  G  G  T       106
 361 GTAAATTCTCCGTTATGGTGAACTCAGATTCCAACCGAAAAGCTTTTATAGATTCTTCAG
     S  K  F  S  V  M  V  N  S  D  S  N  R  K  A  F  I  D  S  S       126
 421 TCGCTCTTTTAAGAAAATGGGGATTCGACGGTTTGGATTTGGATTGGGAATACCCTGGTG
     V  A  L  L  R  K  W  G  F  D  G  L  D  L  D  W  E  Y  P  G       146
 481 GTCGCGGTAATTCCCCTGCAGGTGATAAACAAAGGTTTACTCAACTGTGTCGAGAATTAA
     G  R  G  N  S  P  A  G  D  K  Q  R  F  T  Q  L  C  R  E  L       166
 541 TCGAAGCTTTTGACAAAGATGCTGCAGAGAAACAAAAACCAAGATTGATGCTCACTGCTG
     I  E  A  F  D  K  D  A  A  E  K  Q  K  P  R  L  M  L  T  A       186
 601 CTGTTGCTGCTGGATTCAAAACCATCGATGGAGGATATGAAATTCAAAAGATTGCAAAAT
     A  V  A  A  G  F  K  T  I  D  G  G  Y  E  I  Q  K  I  A  K       206
 661 ATCTAGACATTTTGAATTTTGATGGCTTTTTGATTTACACGGTAACTGGGGAGAAGATCT
     Y  L  D  I  L  N  F  D  G  F  L  I  Y  T  V  T  G  E  K  I       226
 721 CTGGCCATCACACTGCATGGGATTTTGATGGAGCTCCTGGCGATGACAGAAACAAACTTA
     S  G  H  H  T  A  W  D  F  D  G  A  P  G  D  D  R  N  K  L       246
 781 CTGTAACCTATGCTGTTGATTATTGGATCAAAGGAGGTTTTCCAGCCAACAAAATCGCTC
     T  V  T  Y  A  V  D  Y  W  I  K  G  G  F  P  A  N  K  I  A       266
 841 TTGGTATGGGTACCTACGGACGCGCTTTTAAACTCAAAGATGCCAGCAACAACGGCTTAG
     L  G  M  G  T  Y  G  R  A  F  K  L  K  D  A  S  N  N  G  L       286
 901 GTGCGCCTAAAGCTGATTGGCAAAAGCCACCTAAAGGACAGTTCACACGAGAAGCCGGTT
     G  A  P  K  A  D  W  Q  K  P  P  K  G  Q  F  T  R  E  A  G       306
 961 TCTTGTCTTATTATGAAATTTGTAAAATGGGTTTGACAGTTGTGAAGGACAATGCAGTAA
     F  L  S  Y  Y  E  I  C  K  M  G  L  T  V  V  K  D  N  A  V       326
1021 AATCTCCATACGGTTACAAAGGTCAAGATTGGATTGGTTATGACGATCAAGAAAGTTTGG
     K  S  P  Y  G  Y  K  G  Q  D  W  I  G  Y  D  D  Q  E  S  L       346
1081 TTCATAAAGTCAACACATTAATCAAAGGCAAGGGACTTATGGGAGCTATGTTTTGGGCTC
     V  H  K  V  N  T  L  I  K  G  K  G  L  M  G  A  M  F  W  A       366
1141 TTGACCTCGATGATTTCAGAGGTGTATGCGGTGAAGGAAAATATCCATTAATCTCAGCAG
     L  D  L  D  D  F  R  G  V  C  G  E  G  K  Y  P  L  I  S  A       386
1201 TATCCAAGGCACTAGGAGGATACACTCCACCACCAGAACCCACTCATGGACCAAGACCAC
     V  S  K  A  L  G  G  Y  T  P  P  P  E  P  T  H  G  P  R  P       406
1261 CAAGTAAAGCACCAACAAAGGCTCCTTCAAGAGGTCCAACCAACAAACCGGTTACAAGCG
     P  S  K  A  P  T  K  A  P  S  R  G  P  T  N  K  P  V  T  S       426
1321 GACCAGGCGGTAAATGTCACGCCATCGGTGTTTGGAAAGGCAACGCTAATATGGATTCCT
     G  P  G  G  K  C  H  A  I  G  V  W  K  G  N  A  N  M  D  S       446
1380 GGTGTGTTGCTAACTGTGCACGAAATAATTGTCCTGCTGATACATGTGCATGTTAAAGA
     W  C  V  A  N  C  A  R  N  N  C  P  A  D  T  C  A  C  *          464
1441 AATAAATAAATAAAGTTTCAAATATTACAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 2. Nucleotide and predicted amino acid sequences of *HyChit1*. The catalytic domain is underlined, putative signal peptide is double underlined, active site signature is boxed, putative polyadenylation sequence is written in boldface. Stop codon is marked by asterisk.

tips at a close vicinity [21]. The second mode of stolon induction is extrinsic, by the approaching of a growing tip to the flanks of another stolon. A, yet biochemically uncharacterized, diffusible factor termed stolon inducing factor (SIF) was isolated from stolon tips and found to induce the formation of new stolon tips on the lateral side of encountered stolons [22,23]. In intrinsic tip induction, it is clear

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Homo sapiens : QLKTLLAIGGWNF---GTAPFTAMVSTPENRQTFITSVIK---FLRQYEFDGLDFDWEYPGSRG--SPPQDKHLFTV : 136
Mus musculus : KLKTLLAIGGWNF---GTAPFTTMVSTSQNRQTFITSVIK---FLRQYGFDGLDLDWEYPGSRG--SPPQDKHLFTV : 136
Danio rerio  : NLKTLLAVGGWNF---GTTQFSSMVSTPQNRQTFIQSSIT---FLRTHGFDGLDLDWEYPGSRG--SPPEDKQRFTL : 137
Hydractia    : ALKVLLAVGGWNHENGGTSKFSVMVNSDSNRKAFIDSSVA---LLRKWGFDGLDLDWEYPGGRGN-SPAGDKQRFTQ : 141
C. elegans   : QLKTLLSFGGWSF---GTALFQGMAASSASRKVFIDSAIT---FVRTWGFDGIDIDWEYPSGAT------DMANYVA : 140
Penaeus      : DMKTNIAVGGWAE---GGRKYSQMVMVAERRASFIRSVVQ---LLTDYGFDGLDLDWEYPGATDRGGQYADKDNFLK : 143
Drosophila   : GAKVTVAIGGWNDS--AGDKYSRLVRNPEARSRFIRNVLD---FIEEYNFDGLDLDWEYPVCWQVDCKKGTAEEKIG : 142
Arabidopsis  : GIKVMLSLGGGIGNYSIGSREDAKVIADYLWNNFLGGKSSS-RPLGDAVLDGIDFNIELGSPQH------------- : 133
Glycin max   : GVKVFLSLGGAKGTYSLCSPEDAKEVANYLYQNFLSGKPG---PLGSVTLEGIDFDIELGSNLY------------- : 130
Saccharomyces: GKKVLLSLGGASGSYLFSDDSQAETFAQTLWDTFGEGTGASERPFDSAVVDGFDFDIENNNEVG------------- : 137
Bacillus     : GKKVVLSIGGQNG--------VVLLPDNASKQRFINSIQS---LIDKYGFDGIDIDLESGIYLNG----NDTNFKNP : 127
                 K  L  GG                       F                G D D E
```

Fig. 3. Alignment of a portion of the catalytic domain of chitinases showing conserved amino acids.

that chitinase has to be expressed by the cells at the site of the future stolon tip. Indeed, Fig. 5e shows a strong staining at a putative new tip development site. In extrinsic tip induction, chitinase could either be expressed locally (i.e. similar to intrinsic induction), but also by the inducing, approaching tip. Since this type of stolon induction occurs over distances of up to 100 μm [21], it is unlikely that chitinase secreted by the inducing stolon tip would diffuse and dissolve the periderm of a remote stolon. A seawater column of 100 μm would render this ineffective due to the dilution effect. Our result, in which no *HyChit1* expression has ever been observed in stolon tips, also support the local chitinase expression in extrinsic tip induction.

The ectodermal expression of *HyChit1* along stolons was irregular (Figs. 5a, c and e). In addition to the establishment of new stolon tips, requiring the complete removal of the periderm at the site of the future tip, described above, partial digestion of the periderm along the stolons may also be necessary. As stolons grow and increase in diameter, the periderm size has to be adjusted, similar to molting in insects. Growth of the stolons in *Hydractinia* is not continuous, and the irregular *HyChit1* mRNA levels along the stolons might be the reflection of this.

We have also recorded a strong *HyChit1* expression in hyperplastic stolons (Fig. 5d). Hyperplastic stolons are organs specialized for allorejection of genetically incompatible conspecifics [17,20,24, 25]. When approaching an incompatible counterpart, the stolons of both contact partners attract specialized migrating stinging cells, which accumulate near their tips [26]. The stolons swell and lift up from the substratum, forming a front of highly armed devices. The nematocysts discharge, inflicting tissue death on the allogeneic partner. Acquiring a hyperplastic nature obliges the complete removal of periderm from those stolons engaged in allogeneic rejection. The strong *HyChit1* expression in hyperplastic stolons (Fig. 5d) corresponds well to this assumption.

Bearing in mind that *Hydractinia* mostly feeds on small crustaceans, one could assign HyChit1 an additional function, namely food digestion. We have tested this hypothesis by subjecting fed animals to in situ hybridization assays with *HyChit1* RNA as probe. Contrary to our expectations, no difference in the spatial expression pattern could be observed between fed and starved animals (not shown). If HyChit1 was indeed used also as a digestive enzyme, we would expect its mRNA to be expressed by the endoderm of fed animals. This was not the case. We may therefore speculate that at least one additional chitinase is encoded by the *Hydractinia* genome. This putative gene is probably significantly different in sequence from *HyChit1*, as its mRNA did not cross-hybridize to our probe in Northern blot. Alternatively, its size may not significantly differ from the *HyChit1*'s transcript, which could also explain the single band on the Northern blot.

The occasional *HyChit1* expression observed in polyps' ectoderm deserves attention. Polyps are never

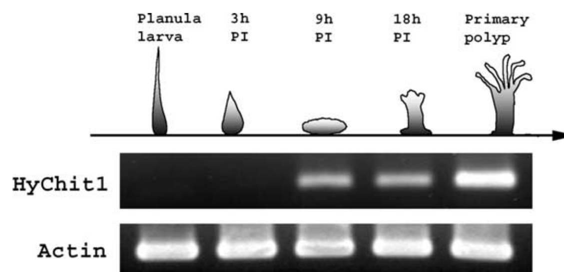

Fig. 4. RT PCR of *HyChit1*. Expression in the following stages is shown: Planula larva, 3 h post metamorphosis induction, 9 h post metamorphosis induction, 18 h post metamorphosis induction, primary polyp. PI = post metamorphosis induction.

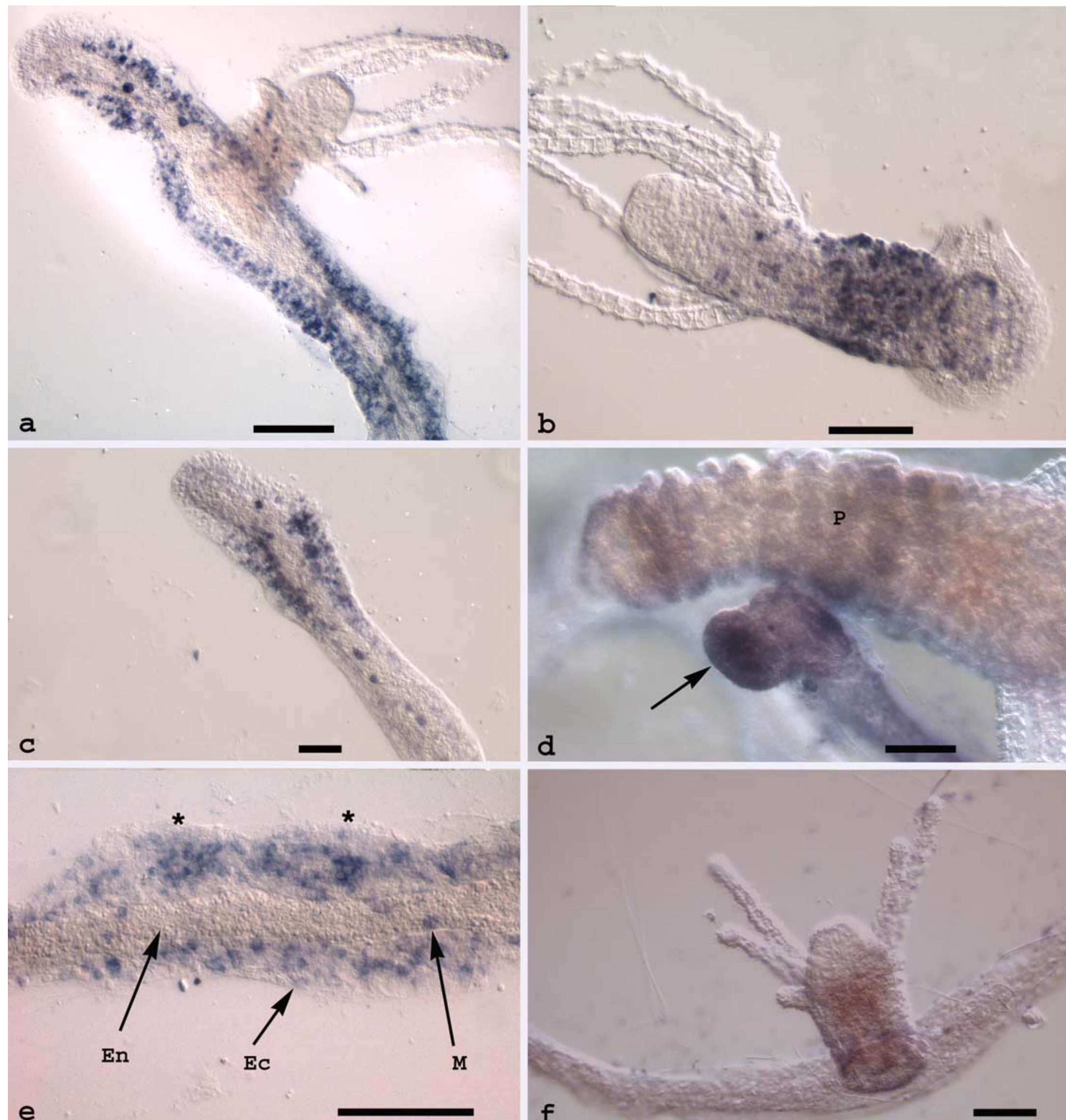

Fig. 5. In situ hybridization of *HyChit1*. (a) A 2-days old primary polyp. Expression is restricted to stolon ectoderm, absent from polyp and stolon tip. (b) A 2-days old primary polyp. Expression in this sample was also observed in the lower part of the polyp (see text). (c) A stolon of a 2-days old primary polyp. Expression is restricted to a part of the stolon just proximal to the growing tip. (d) A hyperplastic stolon strongly expressing *HyChit1* (arrow). p = polyp, the stolons of which are outside the focal plane. (e) A higher magnification of a stolon, indicating the tissue structure and the expression of *HyChit1* at sites of putative future stolon tips (asterisks). Ec = ectoderm, En = endoderm, M = mesoglea. (f) Sense control showing no staining. Scale bars 100 μm.

covered by periderm and a developmental function for chitinases is therefore excluded here. Ectodermal expression of chitinase in polyps could reflect the third function assigned to chitinases—immunity. *Hydractinia* polyps are directly exposed to the surrounding seawater. There is no physical barrier that could prevent parasites or pathogens from direct access to the ectoderm of polyps. Such parasites may include non-chitinous organisms such as bacteria and viruses, but also chitinous ones like fungi and nematodes.

Expression of chitinase could inhibit the growth of the latter and protect the host hydroid. The irregular expression of *HyChit1* in polyps suggests that the gene is not constitutively expressed. Exposure to a chitinous pathogen may be the inducing agent, similar to chitinase expression in some plants [3]. Staining of *HyChit1* mRNA was always restricted to the basal portion of polyps, while absent from other parts (Fig. 5b). This expression pattern corresponds well to the predicted immunological function of the enzyme, as pathogen infection is likely to originate from the substratum, rather than from the water column. Further, we cannot exclude that *HyChit1* expression in stolons also fulfills an immunological function by inhibiting the growth of chitinous parasites between periderm and stolon ectoderm. It is interesting to note in this regard that in an EST database of another hydroid, the solitary freshwater polyp, *Hydra magnipapillata* (http://mpc.uci.edu/hampson/public_html/blast/jfjp/), we have found several ESTs showing sequence similarity to chitinases. Since *Hydra*, unlike *Hydractinia*, does not contain chitin, chitinases expressed by this organism do not have an endogenous substrate and function either as digestive enzymes or for immunity. Characterization of chitinase genes in *Hydra* could shed light on this question.

Taken together, our findings further strengthen the existing database on chitinases in the animal kingdom, closing an important gap at the base of the Metazoa. Out of three possible functions one could assign to chitinases, namely pattern formation, food digestion and immunity, *HyChit1* fulfills a role in pattern formation, and likely also in immunity. A function for this enzyme in digestion has been excluded in this study. This is, to the best of our knowledge, the first evidence for a double function ever assigned to a chitinase, though this may not be unique. Further research is required to characterize the role chitinases plays in immunity in *Hydractinia* and other organisms.

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