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Symbiotic *Hydra* express a plant-like peroxidase gene during oogenesis

Matthias Habetha and Thomas C. G. Bosch*

Zoological Institute, Christian-Albrechts-University Kiel, Olshausenstrasse 40, 24098 Kiel, Germany

*Author for correspondence (e-mail: tbusch@zoologie.uni-kiel.de)

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Summary

Symbiotic associations accompanied by gene exchange between the symbionts form the phylogenetic origin of eukaryotic cells and, therefore, had significant impact on species diversity and evolutionary novelty. Among the phylogenetically oldest metazoan animals known to form symbiotic relationships are the Cnidaria. In the Cnidarian *Hydra viridis*, symbiotic algae of the genus *Chlorella* are located in endodermal epithelial cells and impact sexual differentiation. When screening for *Hydra viridis* genes that are differentially expressed during symbiosis, we found a gene, *HvAPX1*, coding for a plant-related ascorbate peroxidase. HvAPX1 is expressed exclusively during oogenesis and in contrast to all known ascorbate peroxidase genes in plants does not contain introns. No member of this gene family has previously been identified from a member of the animal kingdom. We discuss the origin of the HvAPX1 gene and propose that it may have been transferred horizontally following an endosymbiotic event early in evolution of the *Hydra* lineage as an RNA or cDNA intermediate.

Supplementary material available online at
http://jeb.biologists.org/cgi/content/full/208/11/2157/DC1

Key words: *Hydra viridis*, *Chlorella* sp., symbiosis, horizontal gene transfer, oogenesis, ascorbate peroxidase.

Introduction

There is increasing evidence that symbiotic associations played a major role in the generation of diversity and organismic evolution (Margulis and Sagan, 2002; Timmis et al., 2004). The raw genetic material for species diversification is thought to be provided by gene transfers that have occurred from symbionts to host nuclei (Ochman and Moran, 2001; Bushman, 2002; Itoh et al., 2002; Margulis and Sagan, 2002; Martin et al., 2002; Palenik, 2002). The phylogenetically oldest eumetazoan phylum known to form symbiotic relationships with unicellular algae are the Cnidaria. Among them, the freshwater polyp *Hydra viridis* forms a stable symbiosis with intracellular green algae of the genus *Chlorella* (Lenhoff and Muscatine, 1963). The symbionts are located in endodermal epithelial cells. Each alga is enclosed by an individual vacuolar membrane (O'Brien, 1982) resembling a plastid of eukaryotic origin similar to the complex plastids of chlorarachniophytes at an evolutionary early stage of symbiogenesis. Proliferation of symbiont and host is tightly correlated (McAuley, 1985). The photosynthetic symbionts provide nutrients for the polyps enabling *Hydra* to survive extended periods of starvation (Lenhoff and Muscatine, 1963; Thorington and Margulis, 1981). Symbiotic *Chlorella* is unable to grow outside the host, indicating a loss of autonomy during establishment of the intimate symbiotic interactions with *Hydra* (Huss et al., 1993/94; Habetha et al., 2003). During sexual reproduction of the host, *Chlorella* algae are translocated into the oocyte, giving rise to a new symbiont population in the hatching embryo (Thorington et al., 1979; Campbell, 1990).

Very little is known about the molecular basis that enables *Chlorella* to survive and proliferate within the polyp's vacuoles and controls the interaction between both partners. As described previously (Habetha et al., 2003), aposymbiotic *Hydra viridis* polyps deprived of their endosymbionts grow normally when fed under laboratory conditions but show dramatically reduced number of ovaries compared to symbiotic ones; testes formation appears not to be affected by the absence of algae. Thus, symbiotic algae have severe impact on sexual reproduction in *Hydra viridis* by promoting oogenesis.

To understand the underlying genetic machinery we screened *Hydra viridis* for symbiosis-related genes using an unbiased approach based on cDNA representational difference analysis. Here, we show that one of these genes, *HvAPX1*, encodes an ascorbate peroxidase that is expressed exclusively during oogenesis. Sequence comparison shows that the gene is most closely related to plant peroxidases. Since the *HvAPX1* gene, in contrast to orthologous genes in plants, does not contain introns, we discuss the possibility that during metazoan evolution it was translocated from a plant symbiont to the *Hydra* genome.

Materials and methods

Animals, algae and culture

Experiments were carried out with the Australian *Hydra viridis* L., strain A99. The strain was obtained from Dr Richard Campbell, University of California, Irvine, USA, and maintained at 18°C using standard conditions described previously (Habetha et al., 2003). Aposymbiotic polyps were obtained by photobleaching of 20 symbiotic polyps (Pardy, 1983), which were used as a starter population for a permanent aposymbiotic mass culture. Symbiotic *Chlorella* sp. was isolated from polyps as described by Muscatine (Muscatine, 1983) with two additional washing steps of the algae in 0.5% SDS. A free-living closely related (Friedl, 1997) *Chlorella* was also used, *Chlorella vulgaris* strain 211-11b, which was obtained from W. Reisser, Leipzig University, Germany. *Chlorella* 211-11b was cultured in Bristol's solution as described previously (Habetha et al., 2003).

Nucleic acid preparation

For *Hydra* samples, total RNA was isolated by use of the Trizol reagent (Invitrogen, Karlsruhe, Germany). After lysis of *Hydra* tissue, symbiotic algae were removed by repeated centrifugation. As shown by RT-PCR, algae could be removed quantitatively (see Fig. S1 in supplementary material). The supernatants were subjected to the purification procedure according to manufacturer's (Invitrogen) protocol. *Chlorella* RNA was obtained with the RNeasy kit (Qiagen, Hilden, Germany) from isolated symbiotic or pelleted free-living algae from liquid mass cultures. Genomic DNA from *Hydra* was prepared using standard phenol/chloroform extraction and from *Chlorella* with the DNeasy plant kit (Qiagen).

Subtraction of cDNA by representational difference analysis

Representational difference analysis (RDA) was used to identify genes with different expression in symbiotic and aposymbiotic *Hydra*. We prepared RNA samples from two sources: (i) synchronized mass cultures containing asexual, testes-bearing and hermaphroditic *H. viridis* polyps without algae (aposymbiotic) and (ii) with normal levels of algae. Double stranded cDNA was generated using the Super Script System (Gibco-BRL, Gaithersburg, USA). The three steps of RDA, (a) PCR generation of amplicons representative of the starting populations of RNA molecules being compared, (b) the two-step subtractive hybridization leading to the enrichment of amplified fragments of differentially expressed genes and the sequential depletion of sequences common to both populations; and (c) the purification, cloning and sequencing of the resulting difference products were performed as described (Hubank and Schatz, 1994). Resulting DNA fragments were cloned, amplified by PCR and spotted on nylon membranes for further analysis.

Isolation of ascorbate peroxidase genes from Chlorella

To be able to include sequence from the *Chlorella* symbionts in the phylogenetic analysis, we cloned a cDNA fragment of *Chlorella* ascorbate peroxidase from both symbiotic and free living *Chlorella* (strain 211-11b) using PCR and primer set 5′-TTCGTCT(GC)G(GC)(AG)TGGCACG-3′ and 5′-CCTGG-AAGTAGG(AG)GTTGTC(AG)AA-3′. These primers cover conserved regions of ascorbate peroxidase genes in various plant species including *Chlamydomonas* sp*?*. The resulting PCR products were extended by 5′- and 3′-RACE to fragments of 1080 bp (symbiotic *Chlorella*) and 1055 bp (free living *Chlorella*). Comparison of the two *Chlorella* ascorbate peroxidase-encoding fragments revealed an identity of 98% at the cDNA level within 1000 bp of overlapping sequence. Both fragments include the plant peroxidase domain and show high similarity to known ascorbate peroxidases (e.g. expect value=5e-81 to ascorbate peroxidase from wheat; *Triticum aestivum*).

Polymerase chain reaction amplifications

All PCRs were carried out with Taq polymerase (Amersham, Braunschweig, Germany). For comparing the peroxidase domains between cDNA and genomic DNA of *Chlorella* and *Hydra* the following primer were used: 5′-ATGCCGGCA-CGTACAGTGTTG-3′ and 5′-CTTGAGCCACTCTACGG-TCCAA-3′ (for *Chlorella*); 5′-ATGATTGCTGGTACAGT-TCGA-3′ and 5′-CGTTATCAAGTATACTCGTTGG-3′ (for *Hydra*). To amplify the complete coding region of HvAPX1, we used the primer pair 5′-GTACAATGGTACCAAATAG-AGT-3′ and 5′-GAGGTAAATTAAAATATGTCTTCTG-3′.

Expression analysis by RT-PCR

Nucleic acid isolation and cDNA cloning were carried out as described previously (Weinzinger et al., 1994). The HvAPX1 fragment, corresponding to nucleotides 277–515 of the full-length cDNA of HvAPX1 was amplified with the primer set 5′-AAAACCAGGCAATGCTGGCTT-3′, 5′-CT-CTTGGTATATCAGAACTATCAA-3′. *Hydra viridis* actin was amplified with 5′-CAATTTATGAAGGTTATGCTC-TTC-3′ and 5′-TATTTCCTTTCAGGTGGAGCAATA-3′.

Northern blot analysis

RNA was separated on a 1.3% agarose gel containing 2% formaldehyde and transferred onto a nylon membrane (Hybond N). The hybridization probe of 239 bp was amplified with the same primers used for expression analysis of HvAPX1 by RT-PCR, gel purified and labelled with [α-^{32}P]dCTP. Hybridization was performed in hybridization solution (50% formamide, 10 mmol l^{-1} Tris–HCl, pH 7.5, 1% SDS, 1× Denhardt's solution, 5× SSC, 10% dextran sulphate) at 42°C overnight. Stringent washing was performed: 2× 10 min at room temperature in 2× SSC/0.1% SDS and 2× 20 min in 0.2× SSC/0.1% SDS at 65°C.

In situ *hybridization*

Whole-mount *in situ* hybridization was performed following standard procedures (Endl et al., 1999) using 239 bp digoxigenin-labelled RNA probes corresponding to nucleotides 277–515 of the full-length cDNA. For *in situ* hybridization of

single cell preparations, polyps were washed with 0.2× PBS and 0.5× PBS. To obtain single cell suspensions, trypsinization was carried out with a standard 10× trypsin cell culture solution (Gibco-BRL) diluted to 0.5× with 0.5× PBS. After washing in chilled 1× PBS suspended cells were settled in a 1:1 mixture of 1× PBS and 1× HBSS (Hanks' balanced salt solution; Gibco-BRL) and fixed on poly-L-lysine-coated glass slides with formaldehyde. Slides with immobilized cells were subjected to the standard *in situ* hybridization protocol as used for whole mounts. Proteinase K treatment was for 2 min.

Peroxidase activity staining

Regions of peroxidase activity in polyps were stained as described previously (Hoffmeister and Schaller, 1985) with the following modifications. Polyps were fixed in 4% paraformaldehyde dissolved in 0.1 mmol l^{-1} sodium phosphate buffer, pH 7.0. After fixation, the animals were washed in 0.1 mmol l^{-1} sodium phosphate buffer, pH 7.0, containing 5% sucrose. The animals were then incubated for 15 min at room temperature in a solution containing an insoluble substrate for the peroxidase, 20 mmol l^{-1} 3,3′-diaminobenzidine (Sigma, Taufkirchen, Germany) and 0.003% (v/v) hydrogen peroxide in PBS containing 0.1% Tween 20. Staining was stopped by removing the solution and repeated washings in water and finally in ethanol prior to embedding. L-ascorbate stock solution was made at 100 mmol l^{-1} in 100 mmol l^{-1} sodium phosphate buffer, pH 7.0.

Bioinformatics

For determination of the plant peroxidase domain a BLAST protein/protein search on the NCBI server was used (http://www.ncbi.nlm.nih.gov/BLAST). Alignment of multiple sequences of the ascorbate peroxidase (APX) family was performed using the ClustalW program (http://www.ebi.ac.uk/clustalw). Evolutionary relationships were analysed using the neighbor-joining method (Saitou and Nei, 1987) and viewed as a phylogenetic tree. Sequences were analysed using DNAMAN 3.0 (Lynnon BioSoft, Quebec, Canada) and the TreeView dendrogram production software. Tree descriptions were generated using the neighbour-joining algorithm in DNAMAN.

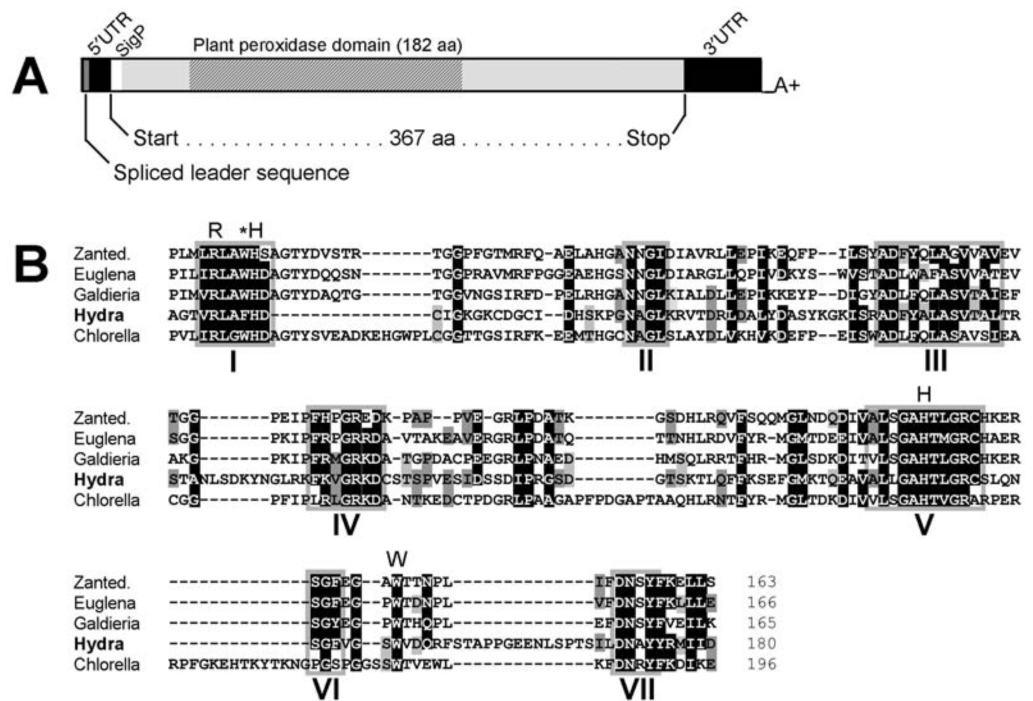

Fig. 1. (A) Schematic diagram of the *Hydra viridis* HvAPX1 mRNA depicting the region coding for the plant peroxidase domain and other putative structural elements. UTR, untranslated region; SigP, ER signal peptide. (B) Multiple amino acid sequence comparison of the plant peroxidase domain (amino acids 48-229) of HvAPX1 with the plant ascorbate peroxidases from *Zantedischia aethiopica*, AAC08576.1, (Magnoliaceae), *Euglena gracilis*, BAC05484.1 (Euglenida), *Galdieria partita*, BAC41199.1 (Rhodophyceae). NCBI BLAST search ranked these plant peroxidases highest in amino acid scores. Additionally ascorbate peroxidase from symbiotic *Chlorella,* which is in the aligned region identical to free living *Chlorella* 211-11b is shown. Comparison was carried out with ClustalW under standard parameter settings. Similarities between HvAPX1 and the other sequences are shaded. Residues that are conserved between *Hydra* and in at least three other sequences are shown in white capitals on a black background. Residues conserved between *Hydra* and two or one of the other sequences are shaded dark or light grey respectively. The number of conserved residues between HvAPX1 and the aligned sequences are as follows: *Hydra*/*Zantedischia*=74 (41%), the expect as given by NCBI BLAST analysis is 2e-09; *Hydra*/*Euglena*=73 (41%), expect=1e-08; *Hydra/Galdieria*=70 (39%), expect=1e-08; *Hydra/Chlorella*=65 (36%), expect=0.83. Five amino acids implicated in the redox activity of plant ascorbate peroxidases are located within the plant peroxidase domain of HvAPX1 (indicated by the letters R and H and one asterisk above the aligned blocks). Seven boxes (I–VII) of high similarity between HvAPX1 and all ascorbate peroxidases aligned by NCBI BLASTp are framed in grey.

Results

Nonbiased screening for symbiosis-related genes in Hydra viridis *leads to identification of a plant-like putative ascorbate peroxidase*

Although physiological and morphological features of the *Hydra/Chlorella* symbiosis are well described, the underlying genetic machinery is not understood at all. To identify genes involved in symbiosis in *Hydra viridis*, we compared pools of mRNA from symbiotic and aposymbiotic polyps using representational difference analysis (RDA; Hubank and Schatz, 1994). So far we found six differentially expressed genes. All of them were expressed exclusively during oogenesis in agreement with our observation that oogenesis in *Hydra viridis* is strongly promoted by *Chlorella* symbionts (Habetha et al., 2003). One of these was represented in a cDNA of about 1300 bases coding for an ORF of 367 amino acids (Fig. 1A, see also Figs S2 and S3 in supplementary material). The predicted protein has a molecular mass of 41 kDa. A stretch of 17 bases within the 5′ untranslated region not far from the 5′ end was identified as part of a so called spliced leader sequence, shown previously to be present on mRNAs in green *Hydra* (N. A. Stover and R. E. Steele, personal communication). In addition, the *Hydra* gene contains a putative ER translocation signal (see Fig. 1A, see also Fig. S2 in supplementary material). A BLAST search comparison of the *Hydra viridis* gene revealed a plant-specific peroxidase domain with highest amino acid sequence identity scores to ascorbate peroxidases of *Euglena gracilis* (Euglenida), *Galderia partita* (Rhodophyceae), *Zantedischia aethiopica* (Magnoliaceae) followed by other mostly monocotyledonous plants. *Chlorella* APX, isolated as described in Materials and methods, shows less similarity than the above mentioned APXs. The superfamily of plant peroxidases comprises three classes, which are characterized by their heme prosthetic group and a specific conserved domain of about 200 amino acids, which differs between the subfamilies. Class I peroxidases contain catalase peroxidases, (APXs) and cytochrome c peroxidases. The predicted *Hydra viridis* protein contains six motifs within the peroxidase domain that is very highly conserved among plant ascorbate peroxidases. The search also indicated (Fig. 1B) strong conservation of amino acids known to be of structural and functional relevance in the redox activity of plant ascorbate peroxidases (Wilkinson et al., 2002).

The completion of the *Caenorhabditis elegans*, *Drosophila melanogaster, Ciona savignyi,* mouse and human genome sequencing projects allows a comparison of peroxidase-related proteins from these organisms. By BLAST searches, the *Hydra* ascorbate peroxidase-related amino acid sequence could not be aligned with the translated genomes of these animals, nor with any animal amino acid sequence. Thus, the results strongly indicate that the predicted amino acid sequence of the *Hydra viridis* gene shares common sequence characteristics with plant ascorbate peroxidases and is distinct from animal peroxidases.

In order to determine the phylogenetic relationship of this unusual *Hydra viridis* protein within the ascorbate peroxidase family, we performed a genetic distance analysis. Fig. 2 illustrates the presumptive phylogenetic distances between the *Hydra* putative ascorbate peroxidases and ascorbate peroxidases of photosynthetic organisms from different phyla, including *Trypanosoma*, which may have lost an ancient chloroplast (Hannaert et al., 2003). The analysis shows that the sequences encoded by the HvAPX1 group together with those of plants and plant-like organisms. The phylogenetic comparison also supports the view that the *Hydra* ascorbate peroxidase-encoding sequence is distinct from the previously described putative peroxidases PPOD1 and PPOD2 from *Hydra vulgaris* (Hoffmeister-Ullerich et al., 2002) and other heme-containing peroxidases, such as catalases, found in animals.

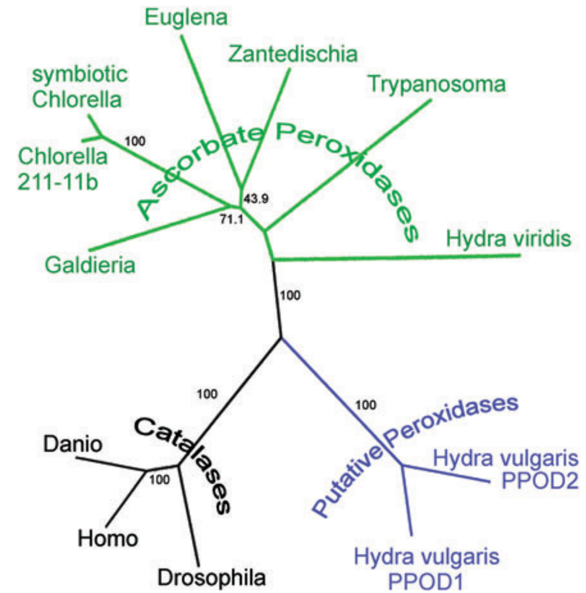

Fig. 2. Unrooted distance tree, depicting the relationships between *Hydra viridis* ascorbate peroxidase 1 and ascorbate peroxidase sequences from different protozoans including *Trypanosoma cruzi* (CAD30023.1), free living *Chlorella vulgaris* 211-11b, and symbiotic *Chlorella* from *Hydra viridis* strain A99. Related catalase sequences are from *Danio rerio* (Q9PT92), *Homo sapiens* (P04040), *Drosophila melanogaster* (NP_536731.1) and 'putative' peroxidases PPOD1 (AAK56445.1) and PPOD2 (AAK56446.1) from *Hydra vulgaris*. Numbers on branches indicate bootstrap values.

HvAPX1 expression is restricted to oogenesis

Initial RDA screening suggested that the HvAPX1 gene is upregulated in symbiotic polyps compared to aposymbiotic polyps. This was confirmed by RT-PCR using cDNAs from randomly selected aposymbiotic and symbiotic *Hydra viridis* from our mass culture (Fig. 3A). To analyse whether gametogenesis affects expression of HvAPX1, northern blot analysis was performed with RNA from three sources: manually selected aposymbiotic and symbiotic polyps without any sign of sexual differentiation, aposymbiotic and symbiotic polyps carrying testes, and hermaphroditic aposymbiotic and

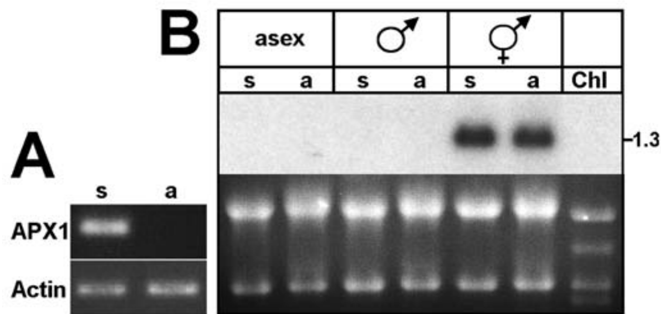

Fig. 3. Expression of HvAPX1 in symbiotic and aposymbiotic polyps. (A) RT-PCR. The quantity of the cDNA template was equalized using *Hydra viridis* actin specific primers. (B) Northern blot hybridisation of 8 µg total RNA from asexual, testes-bearing and hermaphroditic polyps as well as 4 µg of total RNA from symbiotic *Chlorella* with radiolabelled HvAPX1 revealing a transcript of about 1300 bases. Lower panel shows the amount of ribosomal RNA within the blotted samples. s, symbiotic polyps; a, aposymbiotic polyps; Chl, symbiotic *Chlorella*.

symbiotic polyps having both testes and ovaries (Fig. 3B). The analysis with RNA from aposymbiotic and symbiotic *Hydra viridis* polyps revealed the presence of a 1.3 kb HvAPX1 transcript only in animals undergoing oogenesis. The northern analysis provided no evidence for the presence of HvAPX1 transcripts in asexual animals and polyps undergoing spermatogenesis. In polyps undergoing oogenesis, HvAPX1 transcripts can be detected in both symbiotic and aposymbiotic polyps (Fig. 3B). The differential accumulation of HvAPX1 transcripts in symbiotic polyps *versus* aposymbiotic polyps shown in Fig. 3A most probably is due to the fact that RNA for this RT-PCR experiment was isolated from two pools of randomly picked polyps from the mass culture. In such cultures aposymbiotic polyps develop ovaries only very rarely (Habetha et al., 2003).

The spatiotemporal expression pattern of HvAPX1 was visualized using whole-mount and single cell *in situ* hybridization. As shown in Fig. 4, HvAPX1 expression is restricted to the ovary. The first HvAPX1-expressing cells are detectable at an early stage of oogenesis, (Fig. 4A). At this stage the oocyte becomes determined and starts phagocytosing the endocytes (Technau et al., 2003). During later stages, when the oocyte incorporates numerous endocytes, the number of HvAPX1-expressing cells rapidly increases (Fig. 4B), and finally results in a strongly stained oocyte (Fig. 4C). Transcript level rapidly decreases after the onset of embryogenesis (data not shown). To determine in which cell types HvAPX1 transcripts are localized, we performed *in situ* hybridization on suspended cells. As shown in Fig. 4D,E, HvAPX1 is expressed in interstitial cells/oogonia, as well as in the oocyte itself.

In contrast to other sex-related *Hydra* genes such as *CnOtx* (Miller et al., 2003) and *CnNos1* (Mochizuki et al., 2000), HvAPX1 is expressed exclusively during oocyte formation and is not detectable in any other developmental processes such as testes formation or budding.

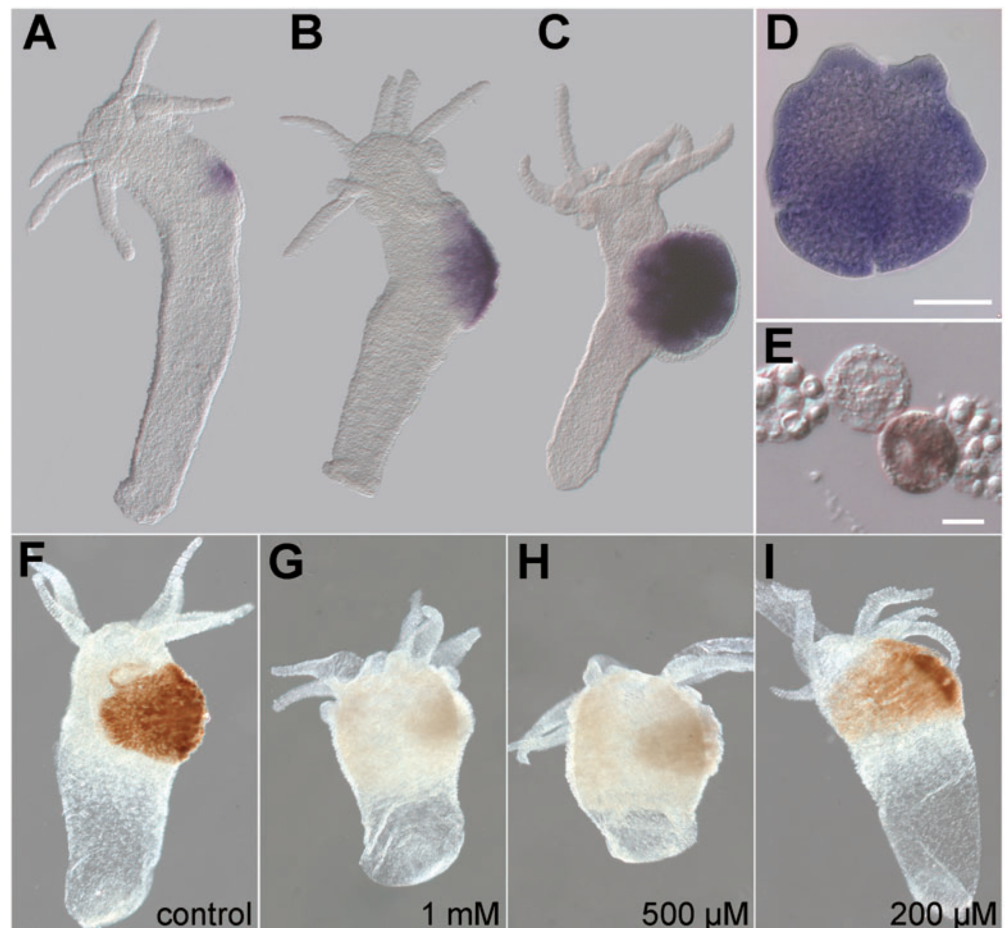

Fig. 4. Spatiotemporal expression of HvAPX1 and ascorbate-dependent peroxidase activity in hermaphroditic *H. viridis*. (A–C) Whole-mount *in situ* hybridisation. (D,E) Cellular *in situ* hybridisation. (D) Early oocyte; (E) cells from the ovary region showing HvAPX1 expression in one of the interstitial cells. (F–I) Peroxidase activity in the absence (F) or presence of ascorbate as competing substrate at 1 mmol l^{-1} (G), 500 µmol l^{-1} (H), and 200 µmol l^{-1} (I). Scale bars: 100 µm (D) and 5 µm (E).

HvAPX1 in contrast to all known ascorbate peroxidases lacks introns

Plant genes encoding ascorbate peroxidases contain several introns, are subjected to alternative mRNA splicing, and produce isoenzymes controlled by tissue-specific or developmental signals (Shigeoka et al., 2002). To check for the presence of introns in both the *Chlorella* and *Hydra viridis* ascorbate peroxidase genes, we performed RT-PCR and PCR on genomic DNA using primers located at the beginning and end of the coding regions, as well as primers flanking the peroxidase domain. Fig. 5 shows that amplification of the *Chlorella* peroxidase domain results in a smaller fragment from cDNA and a larger one from genomic DNA. This indicates that in agreement with other plant ascorbate peroxidase genes, the peroxidase domain in the *Chlorella* gene is interrupted by intronic sequence of about 500 bp. In contrast, when using *Hydra* cDNA and genomic DNA as template, PCR amplifications resulted in an identical product of about 500 bp (lanes 3,4) indicating that this region does not contain any intron. Moreover, when using a primer pair flanking the complete coding region of HvAPX1, identical fragments of about 1100 bp were obtained independently of cDNA or genomic DNA being used as template (lanes 5,6). Thus, the *Hydra* HvAPX1 gene appears to lack introns. The absence of introns was surprising, not only because all known plant ascorbate peroxidase genes contain introns, but also because all *Hydra* genes characterized so far at the genomic level were found to contain introns (M.H. and T.C.G.B., personal observation). Thus, HvAPX appears to have a rather unique gene structure.

Peroxidase activity in Hydra viridis *ovaries is sensitive to ascorbate*

To test for peroxidase activity in developing ovaries of *Hydra*, we analysed *in situ* oxidation of the substrate diaminobenzidine in the presence of hydrogen peroxide. As shown in Fig. 4F, peroxidase activity is restricted to the ovary of *Hydra*. Addition of ascorbate in various concentrations (1000, 500 and 200 μmol l^{-1}) strongly and in a concentration-dependent manner reduces the level of peroxidase activity (Fig. 4G–I) suggesting that ascorbate functions as a competing substrate. Thus, since ovary-specific oxidation of diaminobenzidine can be blocked with ascorbate, and since the ascorbate peroxidase-encoding gene HvAPX1 is expressed exclusively in this area, HvAPX1 may use ascorbic acid as a substrate.

Discussion

We discovered that the freshwater polyp *Hydra viridis* expresses a plant-like ascorbate peroxidase gene during oogenesis. The results raise several interesting questions related to the origin and the function of the gene in *Hydra*.

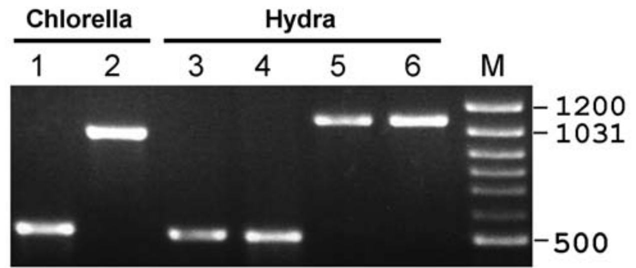

Fig. 5. HvAPX1 in contrast to plant ascorbate peroxidases lacks introns. RT PCR (lanes 1,3,5) and genomic PCR amplification (lanes 2,4,6) of *Chlorella vulgaris* 211-11b and *Hydra viridis* ascorbate peroxidase. PCR was carried out with one set of primers against the *Chlorella* APX peroxidase domain (lanes 1,2), another set against the homologous region of HvAPX1 (lanes 3,4), and a third pair of primers flanking the complete open reading frame of HvAPX1 (lanes 5,6). PCR with primers flanking the peroxidase domain resulted in fragment sizes of 527 bp (lane 1) from *Chlorella* cDNA and of about 1000 bp from *Chlorella* genomic DNA as well as 523 bp (lane 3) and 1123 bp (lane 5) from *Hydra* cDNA and equally sized genomic fragments (lanes 4,6). M, size markers.

How does a plant-related gene become incorporated into the Hydra *nucleus?*

Sequence comparison shows that HvAPX1 is closely related to plant peroxidases and cannot be aligned with peroxidase-encoding genes from animals. At least three different evolutionary scenarios appear possible.

First, HvAPX1 may have evolved from ancient precursors related either to APX enzymes found in some protists or to cytochrome c peroxidases, which are present in plants but also known from some fungi. The fact that such proteins have not yet been reported from animals may be because of a deletion in the animal lineage after divergence of the cnidarians. This idea would be supported by finding of APX-related sequences in genomes of basal metazoans, such as sponges, and of choanoflagellates which diverged before appearance of the cnidarians. However, despite a relatively large body of databanks, there is no evidence for the presence of APX-related genes in these taxa. Interestingly, and supporting the view that ascorbate peroxidases are restricted to plant-related organisms, phylogenetic analyses based on mitochondrial sequences propose that APX-containing trypanosomes group together with plants but not with animals (Löytynoja and Milinkovitch, 2001).

Second, it has been proposed that the cnidarian-specific nematocytes originated from free-living protists such as microsporidians whose genomes were acquired early during jellyfish evolution (Shostak and Kolluri, 1995; Margulis and Sagan, 2002). If so, the origin of the *Hydra APX* gene may be the result of this ancient fusion event.

Third, the HvAPX1 precursor was acquired early in evolution from an organism not belonging to the animal lineage. The mechanisms by which genetic material is transported within the cell are largely a matter of speculation;

possibilities that have been proposed for organelle gene transfer include bulk DNA recombination and mRNA or cDNA (possibly virus-mediated) intermediates (Henze and Martin, 2001; Martin, 2003). Given the presence of several introns in all ascorbate peroxidase genes known in plants (e.g. Shigeoka et al., 2002), the conspicuous absence of introns in HvAPX1 is at least consistent with the view that this gene was translocated from an ancestral photosynthetic endosymbiont to the *Hydra* genome *via* mRNA or cDNA intermediates. Who was the donor? Since genome or EST data on basal organism are rare, a detailed phylogenetic analysis of ascorbate peroxidases and related proteins is not possible yet. However, the *Chlorella* enzyme is less similar to HvAPX1 than those of other photosynthetic organisms such as *Euglena gracilis* or *Galdieria partita* (Figs 1, 2). This makes it unlikely that symbiotic *Chlorella* was the donor of this gene. Although the taxonomy of the genus *Hydra* is complicated and still not settled, in a phylogenetic analysis of the genus based on nuclear ribosomal DNA (D. Martinez, personal communication) *H. viridis* appears as the basal species. Performing TBLASTN searches of the *Hydra magnipapillata*-expressed sequencing tag (EST) database (www.hydrabase.org), using the *Hydra viridis* HvAPX1 amino acid sequence as the query sequence, resulted in identification of two non-overlapping *Hydra magnipapillata* ESTs with high similarity to HvAPX1 (tac33e05.y1 with expect value 7e-51; and taa92e12.x1 with expect value 8e-45) and one of which contains the plant peroxidase domain (data not shown). Thus, the gene is present and expressed not only in green but also in non-symbiotic species. Moreover, since the EST library was prepared from asexual *Hydra magnipapillata* polyps, APX, in species other than *Hydra viridis* appears not to be restricted to oogenesis and may have taken on a different function. A symbiotic relationship with algae is, under natural conditions, observed only within *Hydra viridis*; transient symbiosis, however, can experimentally be induced in non-symbiotic *Hydra* (Rahat and Reich, 1984; Rahat, 1985; Rahat and Reich, 1986) indicating that the ability to form a symbiosis is a common feature of the *Hydra* group. A symbiotic polyp, therefore, may best represent the common ancestor.

A putative transfer of an *APX* gene may have happened early in evolution from an ancient symbiont that got lost and was replaced in *Hydra viridis* by *Chlorella*. This view is supported by (i) the fact that *Hydra viridis* accepts other algae than *Chlorella*, at least transiently, as symbionts (Pool, 1979; M.H., personal observation); and (ii) an increasing number of reports indicating that loss of symbionts seems to have been common during evolution (e.g. Hannaert et al., 2003; for a review, see Cavalier-Smith, 2002). Recent evidence for loss of symbionts accompanied by subsequent colonization with secondary symbionts as a response to dramatic changes in the ecosystem comes from reef corals (Buddemeier and Fautin, 1993; Baker, 2001; Baker et al., 2004). Thus, since the oldest known fossil cnidarians were found in marine sediments, the early evolution of the *Hydra* group from marine to freshwater habitants may have been accompanied by an exchange of endosymbionts.

What is the possible function of HvAPX1 in Hydra viridis *oogenesis?*

Peroxidase activity is known to coincide with oogenesis and embryogenesis in *Hydra* (Technau et al., 2003). During egg formation and embryo development, cells of the interstitial cell lineage aggregate and proliferate so that the ovary is visible as a swelling on the upper body column. These interstitial cells become highly motile and invade the cytoplasm of the oocyte. It can be assumed that this process requires signalling, which induces and controls the cell movements and incorporation into the egg, as well as local interaction with the surface of the oocyte during ingestion. There is evidence that reactive oxygen species (ROS) are involved in cell signalling, proliferation and pattern formation in Cnidaria (Blackstone, 2003). Thus, HvAPX1 may take part in the regulation of the processes during ovary formation and oogenesis by balancing the redox state at an optimal level. Alternatively, since high doses of reactive oxygen species (ROS), as well as lack of antioxidants, have severe negative impact on gametes in human and rats (e.g. Aziz et al., 2004), HvAPX1 may act as a ROS-scavenger, protecting the developing egg from oxidative damage. Similarly, Technau and co-workers suggest that peroxidase activity during oogenesis and embryogenesis may protect the incorporated cells in the oocyte from rapid apoptotic degradation (Technau et al., 2003).

Transfer of genes from endosymbionts to hosts is well known from mitochondrial and plastidal genes, resulting in a centralization of the genetic material within the nucleus. Moreover, recent data show that genes originally belonging to the symbiont can be subject to secondary transfer events between different species (e.g. Bergthorsson et al., 2003; Won and Renner, 2003). It is intriguing to speculate that transferred genes are involved in oocyte formation to ensure the survival of the symbiont. During later stages of symbiogenesis the function of this gene may even be transferred to a secondary symbiont.

Taken together, the work described here represents the first step in determining the molecular changes associated with symbiosis in *Hydra*. The observations may have far-reaching evolutionary implications and provide evidence that the evolution of genomes in basal metazoa may be much more dynamic than previously thought.

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