Intracellular localization and induction of a dynamic RNA-editing event of macro-algal V-ATPase subunit A (VHA-A) in response to copper).

Short running title: Copper responsive RNA editing of VHA-A

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The nucleotide sequence data reported appear in the EMBL and GenBank nucleotide databases under the accession numbers AJ005834 *Fucus vesiculosus* MT, KC515393 (full length *fVHA-A*) and KC515394 (truncated variant *tVHA-A*) *Fucus vesiculosus V-ATPase A* and X98885 *Fucus vesiculosus actin* gene.

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Abstract

A V-ATPase subunit A protein *(VHA-A)* transcript together with a variant (C793 to U), which introduces a stop codon truncating the subunit immediately downstream of its ATP binding site, was identified within a *F. vesiculosus* cDNA from a heavy metal contaminated site. This is intriguing since the VHA-A subunit is the crucial catalytic subunit responsible for the hydrolysis of ATP that drives ion transport underlying heavy metal detoxification pathways. We employed a chemiluminescent hybridisation protection assay to directly quantify the proportion of both variants directly from mRNA whilst performing quantification of total transcript using Q-PCR. Polyclonal antisera raised against recombinant VHA-A facilitated simultaneous detection of parent and truncated VHA-A and revealed its cellular and subcellular localisation. By exploiting laboratory exposures and samples from an environmental copper gradient, we showed total *VHA-A* transcript and protein, together with levels of the truncated variant, were induced by copper. The absence of a genomic sequence representing the truncated variant suggest a RNA editing event causing the production of the truncated VHA-A. Based on these observations, we propose that RNA editing as a novel molecular process underpinning VHA-trafficking and intracellular sequestration of heavy metals under stress.

Keywords

Phaeophyta, macroalgae, copper, toxicity, V-ATPase, gene expression, protein location, RNA editing

Introduction

A role for the V-ATPases in dealing with cell stress is emerging (Dietz *et al.*, 2001). The enzyme has been shown to be essential for the survival of salt and osmotic stress in plants (Wang *et al.*, 2002) and *Saccharomyces cerevisiae* (Hamilton *et al.*, 2002) and is an oxidative stress responsive gene in *C. elegans* (Yanase & Ishi, 1999). It has also been identified as aiding resistance to elevated levels of metal in *S. cerevisiae* (Hamilton *et al.*, 2002), probably by increasing the ability of antiport mechanisms to sequester cations to the lumen of the vacuole (Gaxiola *et al.*, 2002)**.** It was therefore intriguing when a crucial component of the V-ATPase multi-subunit complex, VHA-A, was identified in a subtractive cDNA library indicating over-representation in a population of brown macro-algae, *Fucus vesiculosus*, resident to a heavy metal contaminated estuary where copper has been established as the major ecological driver.

The link between copper homeostasis and compartment acidification as modulated by the V-ATPase complex has been evidenced within organisms from a wide phylogenetic background suggesting a ubiquitously conserved process within biological systems. Knockouts of the Cu-ATPase gene in *Rhizobium* bacteria lead to a reduction in copper and acid tolerance (Reeve *et al.*, 2002) whilst vesicular copper bio-accumulation observed in yeast and oats is dependent on proton gradients generated by the vacuolar ATPase (Ramsay & Gadd, 1997, Salt & Wagner, 1993). The induced proton gradients are important for a number of intracellular processes implicated in copper accumulation including those mediated by Cu P-type ATPase (Madsen & Gitlin, 2008) and as well as providing the counter ion for cation transporters found in a wide spectrum of Eukaryotes, such as Nramp and DMT1 (Mackenzie & Hediger, 2004). The role of V-ATPase in copper detoxification, mitochondrial function and iron metabolism has been functionally demonstrated in *Saccharomyces cerevisiae* (Eide *et al.*, 1993) further establishing this complex as a component of cation trafficking pathways and, more explicitly, of copper. Elucidating the regulation mechanisms of V-ATPase activity

in response to environmental copper challenge is key to determining how this complex functions to maintain its role in essential cation homeostasis whilst facilitating metal detoxification.

The Vacuolar ATPases (V-ATPase) are ATP-driven proton pumps that were first isolated from yeast vacuoles (Kakinuma *et al.*, 1981). They are thought to share a common ancestor with F-ATPases and are found in virtually all eukaryotic cells. They play a pivotal role in the acidification of intracellular compartments (Forgac, 1999) and are widely associated with a variety of cellular membranes including various endomembranes such as the endoplasmic reticulum, the golgi apparatus, lysosomes, and vacuolar/tonoplast membranes (Dettmer *et al.*, 2006, Seidel *et al.*, 2008). V-ATPases have also been identified at the plasma membrane of certain human cell types where they function in such processes as pH homeostasis and coupled K^+ transport (Kawasaki-Nishi *et al.*, 2003, Kluge *et al.*, 2003, Toei *et al.*, 2010). In plants, V-ATPases have also been located on the plasma membrane in pea cotyledons and root cells of oat seedlings (Herman *et al.*, 1994, Robinson *et al.*, 1996).

The V-ATPase is a multi-subunit complex and consists of two distinct domains, the integral membrane V_0 domain and the V_1 cytosolic domain. The V_0 is embedded in the membrane and provides the pathway for proton translocation. The V_1 domain is hydrophilic and contains the catalytic site which binds and hydrolyses ATP. The energy released with the hydrolysis of ATP is used to pump protons (H^+) across a membrane. V-ATPase function may be controlled by the coupling and uncoupling of the V_1 and V_0 domains, by the regulation of ATP coupling and hydrolysis to the system and by the differential targeting to specific cellular membranes (Toei *et al.*, 2010). Such reversible dissociation has been shown to be coupled to the concentration of glucose in yeast (Kane 1995) but a recent study has suggested a different mechanism in *Arabidopsis* whereby the stability of the peripheral stalk is regulated but the complex did not dissociate (Schnitzer *et al.*, 2011). The V-ATPase is highly conserved between species and it is assumed that its mechanism of action is similar in different species (Aviezer-Hagai *et al.*, 2003, Nelson, 1999).

There are eight subunits in the V_1 domain, named A through to H. Subunit A of the V-ATPase (VHA-A) forms a catalytic head with a central cavity with subunit B to form the V_1 domain in a ratio of 3:3 (Sze *et al.*, 2002, Toei *et al.*, 2010). The ATP-hydrolysing domain of the complex occurs at the interface of the A and B subunits. VHA-A containing a glycine-rich motif and Walker consensus sequences (GXXXXGK(T/S)), common to ATP-binding proteins (Nelson & Klionsky, 1996). Structural studies of this subunit have been carried out by comparison with the homologous subunit β of the F-type ATPase (Stevens & Forgac, 1997). This method was able to elucidate the critical nature of the glycine-rich motif and the putative importance of several cysteine residues, for the functional activity of the V-ATPase complex (Tavakoli *et al.*, 2001). The primary sequence and crystal structure of an Archaeal A-type ATPase from *Pyrococcus horikoshii* has been characterised and shown to also retain many of these features (Maegawa *et al.*, 2006).

The identification of a transcript encoding the *VHA-A* from a site with an extended history of copper contamination and where we had previously established that the resident macro-algae could tolerant the significantly elevated levels of copper, raised questions as to the relationship between V-ATPase and metal stress. More significantly of our *VHA-A* clones, one displayed a mutation introducing a premature stop codon raising the possibility of a compromised function of both the VHA-A subunit and extended ATPase complexes. Therefore the aim of the current study was to exploit this well characterised environmental macro-algal model, where the expression of a key metal chaperone, metallothionein (FMT), and regulatory molecule 14-3-3 has been previously described (Morris *et al.*, 1999, Owen *et al.*, 2012), to investigate the molecular mechanisms behind metal-handling in the cell, focussing on the putative transcriptional control of *VHA-A* by environmental copper exposure.

Materials and Methods

Algal material

This study evaluated two different estuaries; the Fal estuary in Cornwall as it is downstream from a copper mine and suffers from significant copper pollution and the Severn Estuary, previously characterised as a control site with low metal contamination (Morris *et al.*, 1999, Owen *et al.*, 2012). From each estuary three sites were selected, which were accessible on foot and were known to contain populations of *Fucus vesiculosus*. These sites were, from the Severn estuary, Sully (OS grid 316750, 167250), Llantwit Major (OS grid 295000,165000) and Oxwich Bay (OS grid 255000, 185000). From the Fal estuary the locations were, Devoran (OS grid 175000, 350000), Flushing, (OS grid 180450, 33750) and Falmouth (OS grid 181250, 317500).

Eight samples were removed from different individuals for metal and gene expression analysis from 6 sites (within a week in June (2002)). Plastic containers were used to collect seawater samples (10 mls) from each site, 3 drops of conc. HNO_3 were added and stored 4^{o}C. Copper, zinc and iron concentrations in the water samples were determined by Inductively Coupled Plasma Mass Spectroscopy (ThermoElemental X-series ICP-MS) by the Elemental Analysis Unit, Department of Earth Sciences, Cardiff University. Metal content of *F. vesiculosus* samples was measured as described in Owen et al., (2012).

Laboratory Algal exposures

The samples (n=5) were collected in May (2002), 1 hour after high tide during daylight hours from Oxwich Bay, Wales, U.K., (O.S. grid reference 255000, 185000) and washed in sterile seawater. Copper sulphate was added as follows: 0 μg/l, 30 μg/l (0.47 μM) or 300 μg/l (4.7 μM) copper sulphate. Samples of algal material (~15 g wet weight) were taken at 0, 6, 11 days, snap frozen and stored at -70 ^{o}C. Water samples (10 mls) were taken during the experiment, 3 drops of concentrated

HNO_3 added and stored at 4 °C. A more detailed description of the collection and treatment of samples can be found in (Owen *et al.*, 2012).

RNA preparation

A sequential method of total RNA extraction using a CTAB (cetyl trimethylammonium bromide) method followed by a poly(T) magnetic bead separation protocol (Dynal, Oslo, Norway) was used as described in Morris *et al.* (1999).

Subtractive cDNA library construction

Poly(A)$^+$ RNA (800 ng) was reverse transcribed, restriction digested to give blunt ends, ligated to different adaptors, hybridised and then the resulting template used in two rounds of PCR amplification where only the cDNA molecules that are enriched in the metal exposed cDNA were exponentially amplified as detailed by the PCR Select cDNA subtraction (Clontech) protocol.

RACE library construction

The Marathon RACE library construction and the PCR amplification of the 5' and 3' ends of the *VHA-A* gene were performed according to Clontech instructions. The denaturation step was 95 °C for 30 s followed by an extension step at 68 °C for 2 minutes, 30 cycles. Primers used to amplify the 5' UTR region were the AP1 5' RACE primer and $^{5'}$ TTGGACAGGGATTGCGAAATAACGG $^{3'}$.

Isolation of Genomic DNA from *F. vesiculosus*

Fucus vesiculosus collected from Sully, Wales (ST152683) and frozen at -70° C was used for the isolation of DNA. The seaweed material was ground to a fine powder under liquid nitrogen, (approximately 3 g yielded sufficient DNA for further analysis). DNA extraction buffer (100 mM Tris HCl (pH 7.5),1.5 M NaCl, 2 % CTAB, 50 mM EDTA, 0.1 % PVPP, 0.2 % β-mercaptoethanol) containing SDS (0.01 % w/v) was added (4 mls per 1 g of ground seaweed). The homogenate was gently stirred and left to reach room temperature for 2 hours. The DNA was further extracted by the addition of

an equal volume of chloroform: isoamyl alcohol (24:1), which was mixed thoroughly and centrifuged at 3000 x *g* for 10 minutes. The aqueous layer was removed and the chloroform isoamyl alcohol washing procedure repeated twice further. DNA was precipitated from the final aqueous layer by the addition of 2/3 volumes of ice-cold isopropanol, which was gently mixed and then incubated at -20 ^{o}C for 3 hours. The DNA was pelleted by centrifugation at 3000 x *g* for 30 minutes at 4 ^{o}C. The pellet was washed in 70 % ethanol, centrifuged as before and allowed to air dry. The pelleted DNA was then resuspended in 1 ml of TE, pH 8.0 per gram of seaweed originally used and left overnight at 4 ^{o}C.

Remaining polysaccharides were precipitated by the addition of 1 g of CsCl per ml of TE, and centrifuged at 25 000 x *g* for 25 minutes. The supernatant was transferred to a fresh tube with the addition of 1 drop of Triton X-100 and 750 μg/ml of ethidium bromide and centrifuged at 200 000 x *g* for 16 hours. The ethidium bromide-containing band was visualized by UV light and extracted. The DNA containing sample was washed 3 times in salt saturated isopropanol (equal volumes of isopropanol and 5 M NaCl). DNA was precipitated by the addition of 2 volumes of TE pH 8.0, NaCl to a final concentration of 0.2 M and 2 volumes of cold ethanol and incubated overnight at -20 ^{o}C. The DNA was pelleted by centrifugation at 20,000 x *g* for 30 minutes. The pellet was resuspended in TE pH 8.0 and further precipitated with ethanol. The genomic DNA was stored at 4 ^{o}C until required. Before use in PCR amplification, the genomic DNA was diluted 100 fold in TE buffer.

Genomic DNA primers were designed against the GenBank sequences accession numbers *VHA-A* KC515393 and were as follows: *VHA-A forward* – $^{5'}$ CGGTGCTGGAGGTGGAGAACG $^{3'}$ *and VHA-A reverse* – $^{5'}$ CGGTTCCTTCTCTGCCTGGGG $^{3'.}$ Genomic DNA was used for PCR amplification utilising oligonucleotide primers *VHA-A* forward and reverse. Direct sequencing of a PCR product was carried out using the ABI Prism Dye™ Terminator cycle sequencing (Applied Biosystems Ltd.,). The amplified

fluorescently-labelled DNA fragments were separated, detected and sequence determined using an ABI 3100 Prism DNA Sequencer.

Protein Truncation test

In vitro transcription coupled translation was used to analyse the expression of the truncated isoform of the *VHA-A* gene *in vivo* (Supplementary Figure 1A&B). PCR amplification was performed on the RACE ready cDNA and the plasmid stock containing the thymine base truncated variant of the ATPase gene. The primers flanked the full length coding region but introduced a T7 RNA polymerase site immediately 5' to the start codon. This product was used as a template for *in vitro* coupled transcription/translation with the latter process performed in the presence of [^{35}S] methionine allowing us to visualise the protein products generated.

Production of the recombinant protein and generation of the antibody against VHA-A.

Detailed methodology for the production of the recombinant protein and generation of the antibody against VHA-A are described in detail in the supplementary information; including the construction of bacterial expression vector, recombinant expression and purification of truncated and full length VHA-A, production of recombinant VHA-A with S-Tag removed and production of antisera against VHA-A.

Western blot analysis

Frozen *F. vesiculosus* tissue, collected during the exposure was freeze-dried and ground to a fine powder using a pestle and mortar. Ground material (0.2 g) was used in 1 ml of Tri-reagent. Total protein was extracted from the Tri-reagent as directed by the manufacturer (Sigma, U.K.). The resulting pellet was resuspended in 20 µl of 1 M potassium carbonate and 20 µl of 2 x SDS loading buffer and left in a sonicating water bath overnight. A standard was also prepared containing a

combination of equal concentrations of tVHA-A and fVHA-A recombinant protein (S-Tag removed). All protein-containing solutions were quantified using a BCA assay. A control of standard protein solution (1 µg, total), and 100 µg of total protein extracted from *F. vesiculosus* were taken and resolved by 15 % SDS PAGE. Proteins were viewed by Coomassie blue staining. An equivalent SDS PAGE gel was utilised for Western blot analysis. An HRP conjugated secondary antibody was utilised and the Western blot was viewed using Super Signal West Pico® (Pierce, U.S.A) and exposure to autoradiography film.

Immuno-histochemistry of *Fucus vesiculosus* frond Sections

Intact algal material was collected from Sully, Wales, U.K. (ST152683) and washed with distilled water and cut into 5 mm strips. The tissue was then immediately frozen in liquid nitrogen before being stored at –80 ^{o}C. Frozen strips were mounted in Cryo-Jet™(BDH) and immersed in liquid nitrogen. Cryosections, nominally 15 µm thick, were cut on a rotary cryostat at -20 ^{o}C and mounted on Histobond™ glass microscope slides (R A Lamb U.K.). The sections were rehydrated in 3 changes of 0.1 M PBS (pH 7.4) containing 0.01 % Tween-20. The slides were then incubated with a blocking buffer containing 5 % swine serum (PBS-SS) for 30 minutes. The blocking buffer was removed and the sections were incubated with a 1:25 dilution of rabbit anti-VHA-A serum or preimmune serum in PBS-SS for 1 hour at room temperature. Sections were washed in 3 changes of PBS-Tween. A swine anti-rabbit IgG conjugated FITC (Dako Cytomation, U.K.) was diluted 1:50 in PBS-SS and applied to the section for 1 hour at room temperature. During all antibody incubations slides were kept in a moist chamber in the dark. Sections were washed in 3 changes of PBS-Tween and then mounted under a coverslip with Vectashield™ mountant containing propidium iodide (Vector Labs, U.S.A). Fluorescence images were acquired using a Molecular Dynamics Sarastro 2000 confocal laser

scanning system (Molecular Dynamics, California) based around a Nikon Optiphot II fluorescent microscope (Nikon UK Ltd). Specimens were examined using a Nikon Plan Apo x40 dry lens and scanned using a25mW air-cooled argon ion laser with appropriate excitation and emission filters for simultaneous recordings of FITC and propidium iodide (excitation 488nm, 30% laser attenuation, primary beam-splitter 510nm, secondary beam-splitter 595nm DRLP, detector filters 600nm EFLP and 530nm DF30). Series of optical sections (512 x 512 pixels) were taken through the sample at 1-2 microns using Molecular Dynamics Image Space software. Stacks of optical sections were reconstructed using a Maximum intensity algorithm and presented as red/green colour overlays.

Immunogold labelling of VHA-A

An immunogold labelling procedure was optimised for sub-cellular localisation of VHA-A. Intact algal material was collected from Sully, Wales, U.K. (ST152683) and washed with distilled water. The tips of the fronds were cut into 1 mm strips and incubated for 2 hours with continuous agitation in a cryo-protectant solution (10 mM PBS, 5 % sucrose and 10 % glycerol). Four different fixation methods were tested and 0.5 % osmium tetroxide, 0.5 % gluteraldehyde, 0.2 % uranyl acetate in methanol fixative was chosen as it retained cellular integrity most effectively. The algal strips were transferred to the fixative solution and fixed at low temperature in Lowicryl resin. The polymerised block was cut into 60 nm thick sections using a diamond knife on an Ultracut microtome and mounted on nickel-coated grids.

Sections on nickel grids were floated for 5 minutes on 25 µl droplets of 5 % sodium periodate solution, in order to remove the osmium tetroxide in the samples. The grids were then washed 4 times in distilled water before being transferred to a blocking solution (20 mM Tris buffer, pH 7.0, 0.2 % BSA) containing a 1:50 dilution of normal goat serum, for 30 minutes. Subsequently the grids were incubated for 12 hours at 4 ^{o}C in a primary antibody dilution (1:500 v/v in 20 mM Tris, pH 7.4, 0.2 % BSA and 0.02 % sodium azide). After washing in 20 mM Tris buffer, pH 7.0, 0.2 % BSA

the sections were transferred to the secondary antibody, anti-rabbit IgG (Amersham) gold conjugate diluted 1 in 50 in 20 mM Tris buffer, pH 8.0, 0.1 % BSA, 0.2 % tween 20. After 2 hours incubation in secondary antibody the grids were washed three times in 20 mM Tris buffer, pH 8.0, 0.1 % BSA, 0.2 % Tween-20 and then four times in de-ionised water.

The sections on the grids were counterstained in 2 % lead citrate for 5 minutes and thoroughly rinsed with double distilled water to remove excess stain before examination. Ultra structural examination was carried out using a Philips transmission electron microscope (Philips EM 208, Holland) operated at 80 kV accelerating voltage with variable magnifications. The immunogold reaction was recorded on KODAK 4489 film plates and printed as required.

Quantitative PCR

Reverse transcription was performed as according to the supplier's instructions (Promega, Southampton, U.K.) to generate cDNA from mRNA samples. Expression profiles of *FMT*, *VHA-A* and β-actin, a house keeping gene, were determined by Taqman Probe® real-time quantitative PCR using an ABI 7700 ™ . The amplification reactions consisted of 2.5 µl of template DNA, 900 nM primer of each primer, 200nM of probe, 0.4 mM dNTPs, 1 µM 6-carboxyl-X-rhodamine (ROX) and 1 unit of Taq DNA polymerase, in 20 mM Tris-HCl pH 8.3, 50 mM KCl and 4 mM $MgCl_{2.}$. The oligonucleotide primers and probe sequences were: metallothionein (GenBank: AJ005834) primers $^{5'}$CGTTCAAGTGTGCATGGGTTA$^{3'}$ & $^{5'}$GAGCTGTCGGTCTCCAACGT$^{3'}$ with probe $^{5'}$TGGATTTTTTTTTCTGTCTGATACCGCTCGAT$^{3'}$; actin (GenBank: X98885) primers $^{5'}$GCCCCAGTGAATCAGGAAAG$^{3'}$ & $^{5'}$CTAACCGCCGAGAACCAAAA$^{3'}$ with probe $^{5'}$TGTTGTGTCGATCGCGCCCACT$^{3'}$ and VHA-A primers $^{5'}$ACTCGAGTCCAAAAAGTCGTGTT$^{3'}$ & $^{5'}$TCTGATCTTGCCCGGTAGAAT$^{3'}$ with probe $^{5'}$ACCTCGTGAAAAGCGCGACTTTAGCGT$^{3'.}$

The TaqMan® real-time QPCR amplification reactions were as follows: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min followed by 40 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute. Standard curves

were generated in every run by using pGEM-T plasmids containing the target gene fragment (*VHA-A*, *FMT* or actin and diluting them at 10-fold intervals (1 ng/µl to 10 fg/µl) in 10 mM TrisHCl, 1 mM EDTA pH 8.0. The data was normalised against internal ROX fluorescence and C(t) values generated by the Opticon software. The actin data was used to adjust the VHA-A and FMT expression in each cDNA sample. For further details, see Owen *et al.* (2012).

Chemiluminescent assay

Three sites were chosen to determine if the C-to-U change was present in RNA transcripts and so post-transcriptional; Devoran, where the macroalgae had previously been shown to accumulate the highest levels of Cu, Fe and Zn (Owen *et al.*, 2012), and two sites, Restronguet Creek and Mylor Harbour, which are geographically close but where the metal levels (Cu, Zn, Fe) are known to be lower as the water becomes diluted further along the estuary (Bryan & Gibbs, 1983, Chaseley, 2003). Three individuals from each site were collected in March (2012) and RNA extracted as described above. Reverse complimentary oligonucleotides were designed against the *F. vesiculosus* beta actin or the *VHA-A* sequence over the 793 position to distinguish the unmodified and modified transcripts. Probe sequences were as follows: *F. vesiculosus* beta actin ^{5} GCGTGCGTAT#GCAGCTCGACTTTTTCC^{3}, unmodified *VHA-A* $^{5'}$GAACTTGGACAGGGATT#**G**CGAAATAACG^{3} and modified $^{5'}$GAACTTGGACAGGGATT#**A**CGAAATAACG^{3} where # designates the position of the linker and the single base difference is shown in bold. Oligonucleotides were labelled with acridinium-ester through an internal amine linker and purified using RP- HPLC based on the procedure described in (Nelson *et al.*, 1992). The labelled probes were diluted in buffer (100 mM lithium succinate, 2 mM EDTA, 2 mM EGTA, 10 % Lithium lauryl sulphate pH 4.8.) then hybridised at 65 ^{o}C to either DNA target or messenger RNA in buffer for 30 minutes. The final concentration of probes was 180 fmol for actin, 100 fmol for unmodified and 72.5 fmol for modified *VHA-A*. A second reagent was added (150 mM sodium tetraborate, 5% Triton X102 pH 8.5.) to hydrolyse any unbound probe for between 15 to 20 minutes. The temperature was reduced to 4 ^{o}C and remaining chemiluminescence was

read (2 s) using a Centro Luminometer LB960 with sequential injections of 32 mM hydrogen peroxide in 1 mM nitric acid followed by 1.5 M NaOH. A standard curve of DNA oligonucleotide targets were measured in parallel to allow quantification.

Results

Identification and Characterisation of the *VHA-A* from *Fucus vesiculosus*

A subtractive *F. vesiculosus* cDNA library was prepared from macroalgae collected from the Fal estuary from algae collected at Devoran (shown to be high in metal content and from Falmouth Beach as the control site (Morris *et al.*, 1999, Owen *et al.*, 2012)). Characterisation of this library led to the identification of a number of genes with relevance to metal handling pathways including two partial sequences of the V-ATPase subunit A gene (*VHA-A*). Utilising a RACE ready cDNA library, primers were designed which enabled the successful identification of the entire 1900 bp coding region. To ensure accurate sequence was determined, three separate recombinant clones were each sequenced three times. Homology analysis of the predicted protein sequence showed that it had 94% identity with a predicted protein from brown algae (*Ectocarpus siliculosus)* and 76% identity to V-ATPase A subunits from the plant fungal pathogen *Phytophthora infestans*, 64% identity to slime moulds and amphibians (*Dictyostelium discoideum* and *Xenopus Laevis)*, and 62% identity to higher plants and insects (*Arabidopsis thaliana* and *Drosophila Melanogaster)*. Alignment of the predicted protein sequence with previously characterised proteins revealed regions of greatest identity as the functionally important, glycine rich loop and catalytic site of the protein (Fig. 1).

However, within the predicted protein sequence, one of three recombinant clones analysed contained a single base difference at position 793. Cytidine (C) had been substituted with uridine (U), an alteration that resulted in the presence of a premature stop codon in the catalytic site of the protein (Fig. 1). The base substitution was also identified in the original sequence from the subtractive library. As a result, the genomic sequence was investigated. Genomic DNA was isolated from *F. vesiculosus* and utilised as a template for the isolation and amplification of the relative sequence. Only a single product was observed that contained a C at this position but did not contain any indication of a thymidine variant (Fig. 2). The presence of a premature stop codon in the location

of the C-to-U substitution would result in the disruption of this catalytic site. Furthermore, it is unlikely an enzyme complex co-ordinated with this subunit would function. Site directed mutagenesis studies in yeast have indicated that the preservation of the catalytic region of this subunit is absolutely critical for the functioning of the entire complex (Liu *et al.*, 1997).

To produce an initial line of evidence that the thymine variant leading to the truncated VHA-A protein was present within mRNA and was not a technical artefact, we performed a protein truncation test where the *VHA-A* sequence was amplified from cDNA with primers that flanked the full length coding region but introduced a T7 RNA polymerase site immediately 5' to the start codon. This product could then be used as a template for *in vitro* coupled transcription/translation. We also performed a parallel amplification and transcription/translation using the cloned *VHA-A* gene containing the thymine variant as a template. The resulting autoradiograph produced a dominant ~70KDa band, a myriad of marginally smaller protein, presumably generated from alternative internal ATG within the *VHA-A* sequence, together with a ~30KDa band identical molecular weight as that generated from the thymine variant (Supplementary Figure 1A & B).

Production and characterisation of VHA-A antisera

The production of an antibody was necessary in order to determine if there was evidence for the translation of both transcripts *in vivo*. To obtain antisera, we first had to generate a recombinant VHA-A antigen. This was achieved by expressing both full length and truncated VHA-A variants in *E. coli* using the pET29a expression vector engineered to produce N-terminal S-Tag fusions. We exploited the fact that the recombinant proteins thus produced formed into insoluble inclusion bodies to purify the recombinant products subsequently refolding them using a step dialysis methodology (see Material and Methods for details). The purification and analysis of the solubilised homogenous product is shown in Supplementary Fig. 2A-C). The refolded recombinant truncated VHA-A protein was selected

as an antigen for antibody production (see Material and Methods). Characterisation of the antigenic activity in the rabbit serum, was initially carried out using direct immobilised and competitive ELISA based antigen presentation which confirm the antisera displayed equivalent activity against both recombinant truncated and full length *Fucus* VHA-A (data not shown). Western blot analysis was used to confirm the ELISA results, this showed the antisera equally recognised both truncated and full length forms of the VHA-A protein (Supplementary Figure 3A & B). Since the antigen used was a recombinant VHA-A which contained an N-terminal S-Tag motif which would be absent from native proteins it was essential to assess whether there were polyclonal antibody binding sites associated with the VHA-A protein independent of the S-Tag. Thus we removed the S-Tag and purified the cleaved product confirming binding to both VHA-A variants by ELISA (data not shown) and Western blots analysis (Supplementary Fig. 3C & D).

Total protein was extracted from *F. vesiculosus* collected from a copper-polluted site at Devoran and resolved by SDS PAGE. Western blot analysis using the anti-VHA-A serum identified two significant protein products. The molecular masses of the products were approximately 70 kDa and 30 kDa (lane 4, Supplementary Fig. 4), the same protein masses as the full length (fVHA-A) and truncated form (tVHA-A) of the recombinant protein (lane 1, Fig. 3 & 4). The identification of a protein product of 30 kDa provided evidence that there is translation of the modified RNA variant.

Analysis of VHA-A protein content from environmental samples

To determine how common the presence of the VHA-A protein was, *F. vesiculosus* tissue was collected from 6 environmental sites within a week in June to ensure that there were no effects which could be attributed to seasonality. These sites were chosen to represent a gradient of metal content in the following order (low to high): Oxwich, Llantwit Major, Sully, Falmouth, Flushing, Devoran (Bryan & Gibbs, 1983, Smith *et al.*, 1984), (Morris *et al.*, 1999). The tissue was used to

extract total proteins which were resolved by SDS PAGE and analysed on a Western blot for the VHA-A protein. The Western blot demonstrates that the Llantwit Major, Flushing and Devoran sites have a larger quantity of the 70 kDa protein (Fig. 3). Notably, the results from samples extracted from two sites in the Fal estuary, Flushing and Devoran, have clearly more protein present, not only of the full length form but there is also the presence of the truncated form of the VHA-A protein (30 kDa) which was highest in the Devoran samples.

Time and dose induction of VHA-A protein content following copper exposure

To test whether the VHA-A protein could be induced by copper exposure specifically, we carried out an *in vitro* experiment. Using Western blot analysis, we assessed protein levels for any changes when algae were exposed in the laboratory to additional copper (none added, 30 and 300 µg/L) over a time course (0, 6, 11 days) (Fig. 4). Throughout the control time course, there was no detectable change in fVHA-A protein but protein extracted from *F. vesiculosus* exposed to an additional 30 µg/l of copper showed an increase in fVHA-A protein within the tissue over the duration of the exposure. The full length VHA-A protein was further increased in the protein extracted from *F. vesiculosus* exposed to 300 µg/l copper throughout the duration of the experiment, and after day 6 the tVHA-A protein was detected on the Western blot with an increase in concentration between days 6 and 11. These data indicate that the tVHA-A protein is induced by acute copper exposure.

Cellular and subcellular localisation of the VHA-A protein

Successful immune-detection of the VHA-A protein was achieved by sectioning of the tissue under cryogenic conditions and the use of a specific antibody raised against recombinant protein and a fluorescently labelled secondary antibody (Fig. 5). The VHA-A protein was seen to be expressed in all cell types. This indicated that the VHA-A protein identified by the polyclonal serum is not cell specific. Complex organisms have been shown to exhibit expression of different isoforms

of components of the VHA-A protein complex in a tissue or cell specific manner (Boesch *et al.*, 2003, Nishi & Forgac, 2000). These results indicate that either the VHA-A protein is ubiquitously expressed in *F. vesiculosus*, or that the polyclonal serum is unable to distinguish between different isoforms of the VHA-A protein. The VHA-A protein appears to be unevenly distributed in the cortex cells where the brightest staining was detected (see arrow, Fig. 5). These cells had more VHA-A protein throughout the interior of the cell in contrast to the larger medulla cells where the VHA-A protein appears to be concentrated around the periphery consistent with a plasma membrane/cell wall location for the protein. The meristoderm had the lowest levels of protein detectable. There is fluorescence of the cuticle in the control but it is much stronger in the presence of the antibody suggesting that there is VHA-A protein here.

The immuno-gold technique utilised a fixation method which compromised some of the structural integrity of the cells. However, the polyclonal serum was able to detect and localise VHA-A protein to different subcellular locations. Immunogold detection of VHA-A in the cells of *F. vesiculosus* revealed that the protein was localised throughout the cell wall, as identified in Figure 6 panel A and B, perhaps indicating a localisation in the plasma-membrane which could not be discriminated from the cell wall under these conditions. In plants, V-ATPases have been located on the plasma membrane in pea cotyledons and root cells of oat seedlings (Herman *et al.*, 1994, Robinson *et al.*, 1996). Metals ions, Fe and Cd, have previously been found concentrated in the cell walls of macroalgae (Bryan & Hummerstone, 1973, Lignell *et al.*, 1982), leading to a suggestion that this is a defensive mechanism, though a study by Smith et al, (1986) using X-ray probe microanalysis failed to find copper accumulated in the cell wall of *F. vesiculosus* from Devoran. Small electron dense structures called physodes accumulated significant levels of copper (Smith *et al.*, 1986) and cadmium (Lignell *et al.*, 1982) which was colocalised with sulphur suggesting binding to a chelator such as metallothionein, but we could not confirm the localisation of VHA-A within these structures with this methodology, as due to their dense nature immunogold staining would not be visible.

There was no detection of the VHA-A protein in the chloroplasts confirming the specificity of this anti-serum. Immunogold particles were also localised to intracellular vacuolar membranes as highlighted in panel C and D.

Localisation of gold particles was observed along lines of intracellular membranes; this is the characteristic subcellular location of the subunit A protein (Herman *et al.*, 1994, Kluge *et al.*, 2003). Evidence for its presence was also found in the cytosol. The VHA-A protein exists as part of the V_1 complex, when dissociated from the membrane bound V_0 complex in yeast, is also found in the cytosol (Kane, 1995). However, as no such dissociation was found in *Arabidopsis* (Schnitzer *et al.*, 2011), another explanation of these results may be *de novo* synthesis of V-ATPase complexes. These results indicate that the polyclonal serum is detecting the VHA-A protein, and the subcellular localisation in *F. vesiculosus* is consistent with studies in other organisms.

Quantitation of the full length and truncated *VHA-A* RNA transcript

To perform gene expression analysis on environmental *F. vesiculosus*, individual algae (n=8) collected from four sites (Sully, Falmouth, Flushing, Devoran), mRNA extracted and cDNA synthesised to prepare for the quantitative PCR analysis of *VHA-A* and metallothionein (MT) in relation to actin (Fig. 7). The primers were designed to amplify both the unmodified and modified C to U *VHA-A* variant (*fVHA-A* and *tVHA-A*). In conjunction, both water samples (Fig. 7) and the *F. vesiculosus* samples were analysed for metal ion content (Owen *et al.*, 2012). The metallothionein (*FMT*) transcript levels show no significant difference between the sites. However, the *VHA-A* shows a significant increase reflecting the same trend found when the copper levels in the water were measured. *VHA-A* transcripts are significantly higher in samples extracted from Devoran which correlates with the highest protein content in the environmental samples. Interestingly, there is no corresponding FMT peak at Devoran in response to the copper though there are significant levels of other metals found in the *F. vesiculosus* samples (Owen *et al.*, 2012) and environmental water

samples such as zinc and iron. However, the elevated transcription of *VHA-A* from Devoran suggests that some transcription may be influenced by copper exposure in the environment.

The laboratory exposure was repeated with the same concentrations of copper and the algal samples were harvested and prepared for transcript analysis for *FMT* and *VHA-A* in relation to actin (Fig. 8). The mean transcript value in relation to actin of the group of algae under control conditions at time 0 was used to divide all the sample data; the numbers, therefore, indicate change in transcript values (up or down) following copper exposure. Transcription of *VHA-A* at day 6 was 6-fold and 7.6-fold higher for algae exposed to an additional 30 µg/l and 300 µg/l copper, respectively, compared to control values. However, the individual variation of the values meant that this was not significantly different ($p>0.05$) from control. By day 11, algae exposed to an additional 30 µg/l copper showed *VHA-A* transcript levels 6.9 fold greater than those of control at time 0 but again, individual variation meant that this difference was not statistically significant. The algae exposed to 300 µg/l copper by day 11 showed a 21.5 fold increase in *VHA-A* transcripts and this was significantly different from the controls. This data suggests that *VHA-A* transcription is subject to a much greater individual variation than *FMT*, but is also influenced by copper exposure (Fig. 8).

To confirm whether the RNA transcript for the *VHA-A* had the cytidine or uridine present, a method capable of distinguishing the difference in a single base directly in the RNA was used (Nelson *et al.*, 1992, Thomas-Jones *et al.*, 2003a, Thomas-Jones *et al.*, 2003b). Three reverse complementary oligonucleotides were designed to the housekeeping gene, beta actin or *Fucus VHA-A* sequence with either a C or a U at position 793. These were labelled with a chemiluminescent molecule, acridinium-ester which when combined with a hydrolysis step to destroy the unbound probe, allows detection and quantitation of the RNA transcript. The specificity of the assay was confirmed and the assay was shown to be able to distinguish between the presence of a C or U (data not shown). Messenger RNA was extracted from macroalgae growing in three locations along the Fal Estuary (Devoran, Restrouguet Creek and Mylor Harbour). Devoran had the highest and Mylor Harbour the

lowest levels of heavy metal contamination confirmed by parallel analysis of the metal content of the seawater. These results confirm that the *F. vesiculosus* actin transcript is constant between the three sites (0.127 fmol ± 0.016, data not shown) whereas the unmodified *VHA-A* transcript increased significantly in the Devoran samples, in a similar fashion to the qPCR data (Fig. 9). The modified RNA transcript was detected only in samples from Devoran. When present, this transcript was detected at much lower fmol levels (~17**%** of the unmodified version). The existence of the modified RNA transcript confirms that the C to U substitution to be a posttranscriptional change.

Discussion

Our findings reveal a direct relationship between transcriptional control of the *VHA-A* gene and exposure to copper together with the first reported induction of a posttranscriptional modification or 'RNA editing' event by an environmental factor. Here we present convincing evidence that the transcript and protein levels of VHA-A are increased by the presence of copper, both in the environment and also in laboratory experiments. We have also shown that a truncated version of the protein is induced at the higher levels of copper, again both in the environment and in laboratory controlled experiments. As there is evidence for both the unmodified and modified forms of the RNA transcript in the seaweed exposed to high metal levels, we propose the mechanism behind the formation of the truncated enzyme is at the RNA level. This could possibly be an example of RNA editing by virtue of a C to U change in transcribed RNA which introduces a stop codon into the catalytic site of the enzyme; the genomic sequence of the macroalgae only shows the cytidine. The predicted protein presumably would not be able to bind or hydrolyse ATP and would be non-functional. One could speculate, on the basis of the crystal structure of the A-ATPase subunit A (Maegawa *et al.*, 2006), that truncation of the protein may reduce the interaction with VHA-D, the central core stalk, which then may reduce the required production of torque. The

disconnected V-ATPase sectors could cause proton leakage and so the breakdown of the proton motif force. These results are the first example to our knowledge of an inducible RNA editing event that correlates to environmental stress. The fact that the resulting protein is predicted to be non-functional is also unusual.

Whereas a number of the V-ATPase subunits are encoded by more than one gene (Sze *et al.*, 2002, Toei *et al.*, 2010), in the majority of animals and microorganisms investigated have been reported to have only one gene for subunit A (Hernando *et al.*, 1995). So far, only one sequence has been found in fungal organisms such as *Neurospora crassa* and *Saccharomyces cerevisiae* (Bowman *et al.*, 1988, Kane *et al.*, 1990). However, there is evidence for multiple genes in higher plants; for example, there are two genes for the A subunit identified in *Oryza sativa* (Hanitzsch *et al.*, 2007) and *Mesembryanthemum crystallinum* (Kluge *et al.*, 2003). A study looking at the conservation of an non-coding sequence within the V-ATPase A subunit genes found evidence for two genes in all land plants: *Cyathea crinitia; Equisetum arvense; Psilotum nudum; Ephedra altissima; Avena saliva; Daucus carota; Nicotiana tabacum; Lycopersicon esculentum; Magnolia virginiana; Hydrastis canadensis; Clematis ligusticifolia; Chenopodium rubrum* but only one in *Arabidopsis thaliana* (1 gene (Magnotta & Gogarten, 2002) (Sze et al., 2002) two isoforms from alternative splicing (information available on The Arabidopsis Information Resource (TAIR), accession numbers AT1G78900.1 and AT1G78900.2) or *Coleochaete scutata,* an green alga (Starke & Gogarten, 1993).

Our data suggests only one gene in *F. vesiculosus*. Amplification and sequencing the relevant section of the genome has only yielded the sequence containing cytidine not thymidine at this position. Repeated cloning of the complete *VHA-A* gene showed no variation in sequence with the exception of this one change. Closely related isogenes would be expected to contain additional variation in addition to this 'wobble' position. The full genome for *E. siliculosus*, a closely related brown alga, is available and a homology search for sequences yielded a single encoded VHA-A (CBN79008.1) with no evidence for a second gene (data not shown).

However, there is precedence for isoforms of the VHA-A formed by alternative splicing. There are two isoforms in Arabidopsis and chicken (A1 and A2). These latter splice variants are identical with the exception of a cassette (30 aa in A1 and 24aa in A2), which are formed from the splicing of the same gene and are both ubiquitously expressed (Hernando *et al.*, 1995). This difference removes the P-loop Walker consensus sequence required for ATP binding in isoform A2 and this has been shown to alter the function by removing the ability of the V_o and V_1 subunits to interact causing the failure of the complex to assemble and removing the ability to associate with the vacuolar membrane (Hernando *et al.*, 1999). Perhaps the introduction of the stop codon into the *F. vesiculosus* sequence has a similar effect by disrupting assembly of the V_o and V_1 subunits; however, the position of the stop codon in the *F. vesiculosus* protein occurs three amino acid downstream of the P-loop sequence which is presumably intact in the truncated protein. Presumably both mechanisms confer some advantage to the organism but the reason for either mechanism has yet to be elucidated.

Another example of subunit A isoforms occurs in *A. thaliana* where multiple polyadenylation sites have been shown; all four variants were shown to increase in response to stress (cold, salt and etiolation) and were translated into identically sized proteins as the transcripts differed only in the 3'UTR (Magnotta & Gogarten, 2002). In *S. cerevisiae*, the mechanism of protein splicing has been shown to produce the equivalent subunit;, an internal protein segment is removed and a ligation reaction re-joins the N and C terminal to form the functional protein (Chong *et al.*, 1996, Kane *et al.*, 1990). VHA-B has also been reported to be proteolytically processed in *Mesembryanthemum crystallinum* in the CAM state resulting in a 32KDa protein which contains the regulatory ATP binding site (Zhigang *et al.*, 1996). This suggests diverse mechanisms through which the function of the VHA-A protein is moderated outside gene duplication and the evolution through genetic drift of a second isogene.

There are an increasing number of examples of RNA editing in the literature. RNA editing is defined as a posttranscriptional change in the RNA transcript when compared to the genomic sequence and there are four recognised types of editing: U addition/deletion system in Trypanosomes where the phenomena was first discovered; C to U in mammals that insert a termination codon into the 3 known examples (including apolipoprotein B, NAT-1 and neurofibromatosis type-1 (NF-1) mRNA); an increasing number of A to I (inosine), which is then read as G, changes are being found in the nervous system of higher metazoans in ion channels and receptors and conversion of C to U in plant mitochondria which can introduce termination codons. A number of editing sites have been found in a large number of plant mitochondrial sequences including F_0-ATPase subunits *atp6, atp9* and *atpA*, some of which include the introduction of stop codons (Gray & Covello, 1993) (Hernould *et al.*, 1992, Schuster *et al.*, 1991, Wissinger *et al.*, 1992).

In the latter case and in the case of apolipoprotein B, the edited transcripts lead to proteins with a modified function and it is suggested that these changes allow a "fine-tuning" of protein function. Evidence for this has been discovered in tropical and Antarctic Octopus spp (Garrett & Rosenthal, 2012). Potassium channels, which are known to be sensitive to temperature changes, were found to have single nucleotide changes at the transcript level conferring functional differences at the protein level that represent adaptation to the cold environment. Another example of functional protein changes conferred by RNA editing is the regulation of Na^+ / K^+ ATPase transport in squid neurons (Colina *et al.*, 2010). The extensive scale at which RNA editing occurs has been hinted at by studies in humans (Li *et al.*, 2011, Peng *et al.*, 1994). Although the exact scale is to be confirmed, the studies suggest tens of thousands of sites, predominantly A to I, are changed post-transcriptionally. In humans, RNA editing is emerging as an important mechanism for adding variation to the proteome and changes in micro RNA sites have also been identified suggesting the possibility of additional mechanisms of transcriptional regulation (Peng *et al.*, 1994).

As noted above, two examples of RNA editing involve C to U conversion that can introduce a stop codon. The mechanism for the type of editing in humans has been elucidated for apolipoprotein B and requires single stranded RNA templates APOBECs (apolipoprotein B mRNA editing enzymes). However, whereas there is no evidence for the recognised consensus sequences (UUUN(A/U)U) in the *F. vesiculosus* cDNA sequence nor convincing evidence of a hairpin structure when the surrounding sequence is folded by Mfold (data not shown), there is a similar sequence (UUAUUU) upstream. However, the mechanism by which VHA-A transcripts are edited in *F. vesiculosus* remains to be elucidated.

In this study, we have demonstrated the transcriptional control of VHA-A by environmental copper and the induction of a RNA editing event which leads to a truncated protein, in a species previously shown to tolerate and accumulate high levels of copper. The laboratory induction of the truncated protein tantalisingly suggests a dose dependency in the amount of truncated protein produced. However, the downstream implications of this remain to be elucidated and to determine the molecular mechanism behind the RNA editing event in *F. vesiculosus* will require further study.

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Figure legends

Figure 1. VHA-A protein sequence alignment

Multalin protein alignment of the predicted ATPase sequence with previously characterised vacuolar ATPase sequences from the supergroup Chromalveolata to which brown algae belong. Amino acids shaded in black or grey according to sequence similarity, where black is 100% and grey is at least 50% similarity. The location of a premature stop codon is indicated with an arrow. The location of the glycine rich loop (Walker sequence) and catalytic region of the protein is highlighted with a box and bracket respectively. The accession numbers of the protein sequences shown are: *F. vesiculosus* KC515393; *E. siliculosus* CBN79008.1, *P.infestans* XP_002899949.1; *D. discoideum* XP_637351.1; *D. melanogasta* AAB02270; *X. laevis* NP_001080104; *A. thaliana* NP_178011.

Figure 2. Genomic DNA analysis, a section of the electrophoretogram of the genomic V-ATPase sequence

Genomic DNA was used for PCR amplification utilising VHA-A forward and reverse oligonucleotide primers. The PCR products were directly sequenced using ABI 3100 sequencer. The resulting sequence was analysed using Bioedit, paying particular attention to the location of the putative base substitution indicated by the arrow. The genomic sequence is clearly cytidine and not thymidine.

Figure 3. Analysis of VHA-A protein content from environmental samples

F. vesiculosus tissue was collected from six environmental sites (n=8) and protein was extracted. Total protein (100 µg) and 1 µg of standard mixture of purified tVHA-A and fVHA-A were resolved by 15 % SDS PAGE, viewed by Coomassie staining (Panel A) and an equivalent gel taken for Western blot analysis as shown in Panel B. Lane 1 contains the recombinant protein standards; Lane 2 is from Oxwich bay, Lane 3 from Llantwit Major, Lane 4 from Sully, Lane 5 from Falmouth, Lane 6 from Flushing, Lane 7 from Devoran. All protein masses are in kDa**.**

Figure 4. Time and dose induction of VHA-A protein content following copper exposure

F. vesiculosus plants was collected from Oxwich Bay and maintained in the laboratory in sea water in the presence of no additional copper (Panel A) or with the additional of 30 µg/l copper (Panel B) or 300 µg/l copper (Panel C). Fronds were removed and proteins extracted on day 0 (Lane 1), day 6 (Lane 2) and day 11 (Lane 4). Total protein from the fronds (100 µg), prepared as described in the material and methods section, and 1 µg of standard containing a mixture of tVHA-A (30 kDa) and fVHA-A (70 kDa) (Lane 1), were resolved by 15 % SDS PAGE, viewed by Coomassie staining and an equivalent gel processed for Western blot analysis with the anti-fVHA-A polyclonal antisera. All molecular masses are in kDa.

Figure 5. Immunofluorescent detection of VHA-A.

F. vesiculosus fronds were cryo-sectioned, incubated with either pre-immune serum or VHA-A antibody with a swine anti-rabbit IgG conjugated to FITC and mounted in Vecta shield® containing propidium iodide. Panel A shows the immunofluorescent pre immune serum control and Panel B shows the VHA-A. Anti-VHA-A rabbit serum was used at a 1:25 dilution to detect the cellular expression of VHA-A protein. The figure shows a composite view of the red and green fields, with green indicating the specific binding to VHA-A. The VHA-A protein is expressed in all cell types and most intensely in the cortex cells (indicated by the arrow). The scale bar is 20 µm. Panel C shows a diagrammatic representation of a histological cross-section through the thallus of *F. vesiculosus* (adapted from Lee, 1989).

Figure 6. Immunogold detection of VHA-A protein in *F. vesiculosus*

Tips of *F. vesiculosus* fronds were cut into 1 mm strips and fixed at low temperature in Lowicryl resin. After blocking, the samples were incubated in a primary antibody dilution (1:500). Incubation with the anti-gold conjugated rabbit secondary antibody, (1:50) was for 2 hours, the samples washed thoroughly and counterstained in 2 % lead citrate (5 mins). Ultra structural examination was carried

out using a Philips transmission electron microscope and the immunogold reaction was recorded on KODAK 4489 film plates. Panels A and B show the cell wall and Panel C shows an intracellular vacuolar membrane. Immunogold labelling was observed in the cell wall and the intracellular vacuolar membranes as indicated by the arrowheads. The scale bar is 300 nm.

Figure 7. Transcript analysis of environmentally exposed *F. vesiculosus*

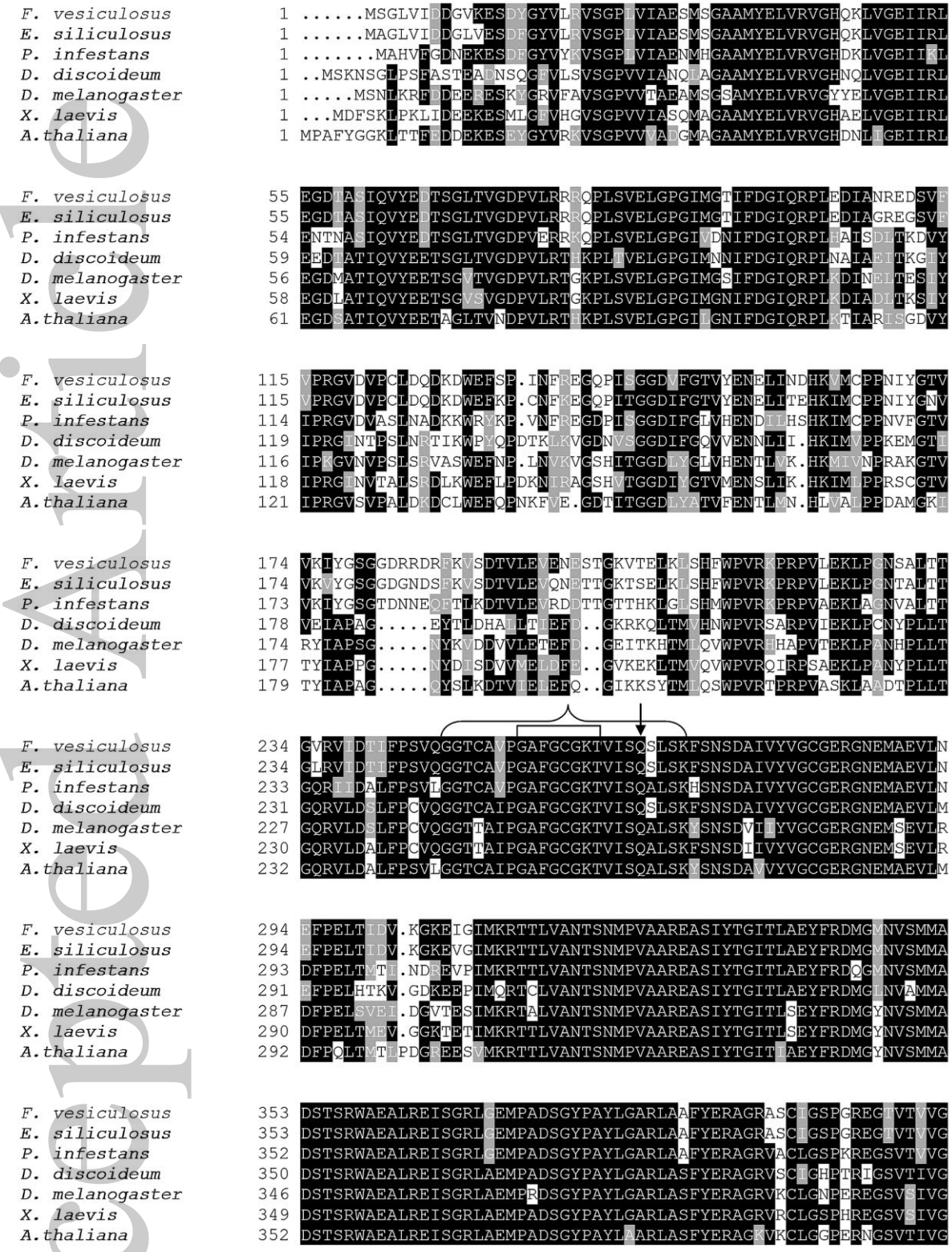
F. vesiculosus were collected from 4 environmental sites. Samples were prepared for gene transcription analysis and the resulting changes in the relative *VHA-A*/actin transcript ratios at each site are shown. Results show means ± SEMs (n=8). The transcript ratios were tested for normality using the Anderson-Darling test and then assessed for significant differences using the ANOVA, Tukey test. Groups were donated letters a, b or c with those sharing the same letter being not significantly different from each other. The environmental seawater samples were analysed by ICP-MS and the copper data (ppb) is shown (triangles).

Figure 8. Transcript analysis *F. vesiculosus* exposed to copper in the laboratory

F. vesiculosus from Oxwich Bay was incubated in seawater containing 3 different concentrations of copper for 0, 6 and 11 days. The resulting *VHA-A/*actin transcript ratios at each exposure over time are shown (means ± SEM, n = 5). The transcript ratios were tested for normality using the Anderson-Darling test and then assessed for significant differences using ANOVA (Tukey test). Groups were donated letters a, b or c and those sharing the same letter are not significantly different from each other (P>0.05). The corresponding tables in each panel are the mean values. The average relative expression of each gene was compared to a control set value set at 1 for time zero.

Figure 9. Analysis of the unmodified and modified *VHA-A* RNA transcripts using a Chemiluminescent Assay optimised to detect single base changes.

To investigate the gene expression of the unmodified and modified *VHA-A* in *F. vesiculosus,* three chemiluminescent assays were developed and optimised for actin, unmodified (C793) and modified *VHA-A* (U793) and shown to be specific for the intended transcripts. The limit of detection of the three assays was determined to be 0.01 fmol. Results show means ± standard deviations, n=3. Unmodified *VHA-A* (light grey bars) show significant up-regulation which was follows the trend of increasing copper concentrations towards the source of the estuary. The modified *VHA-A* (dark grey bars) was also detected in the Devoran samples. To assess variance, single factor ANOVA was performed and groups were donated letters a or b; those sharing the same letter are not significantly different from each other (P>0.05).



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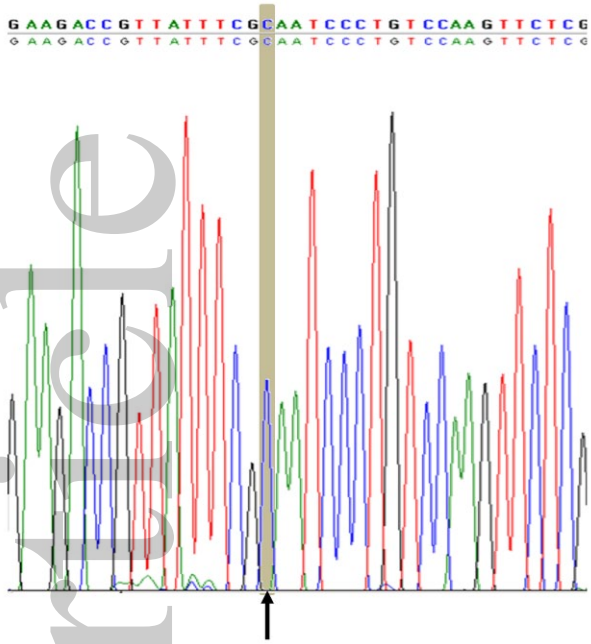

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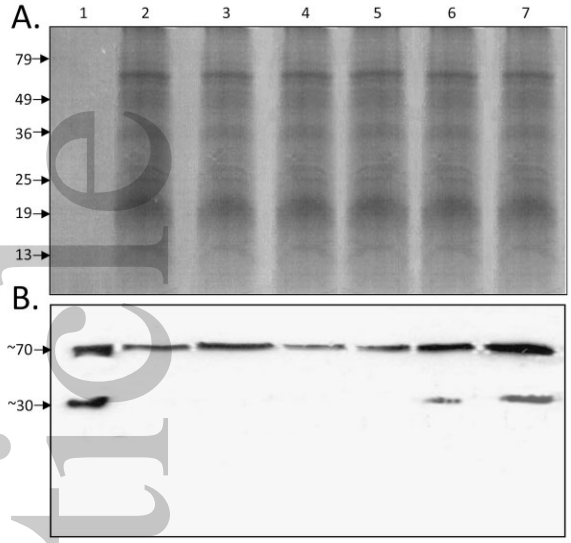

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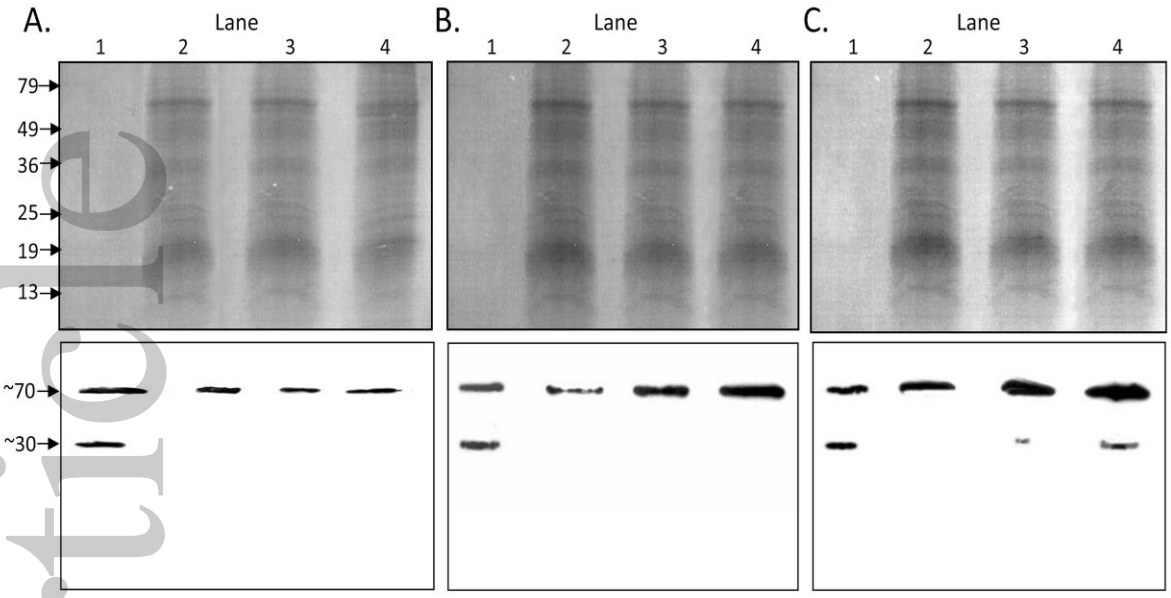

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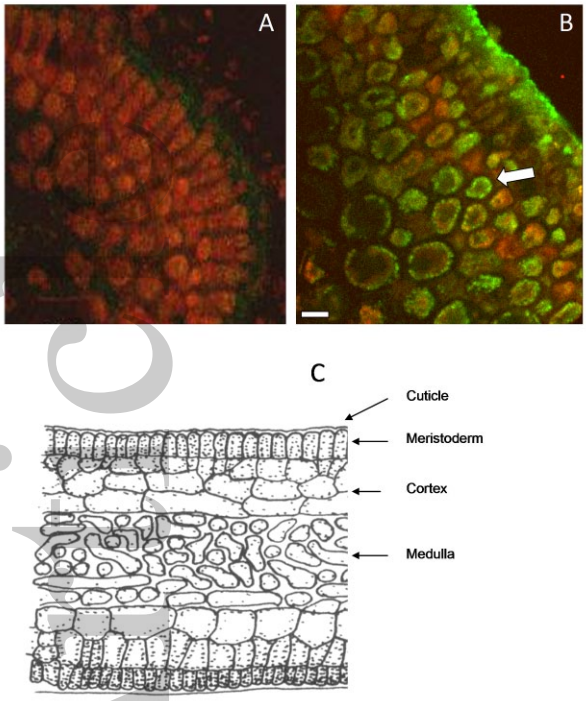

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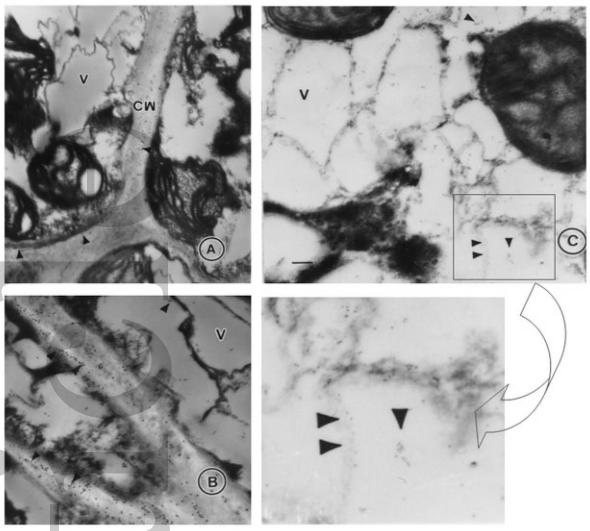

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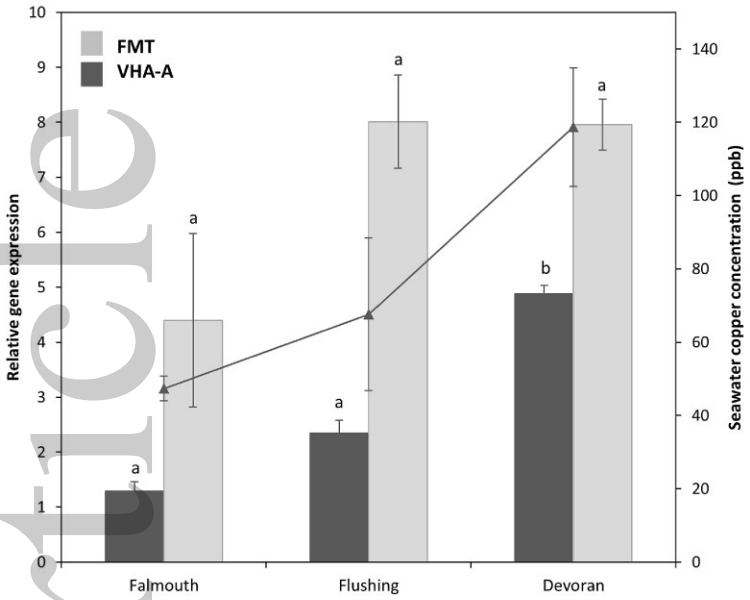

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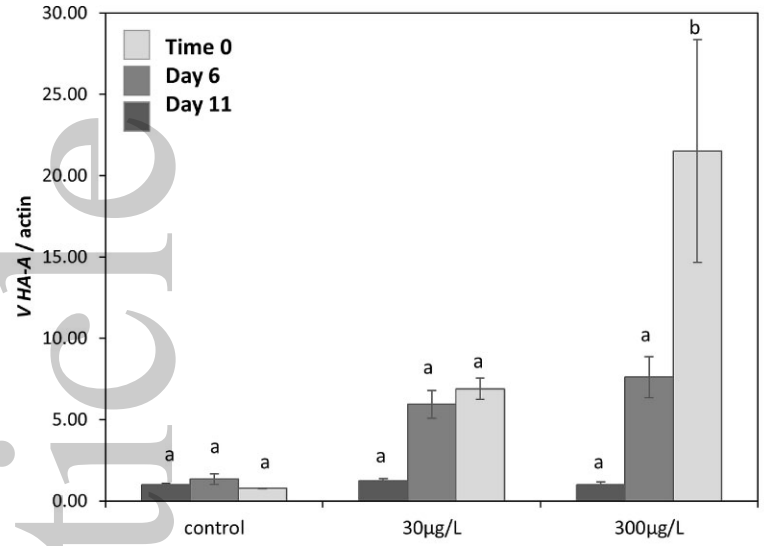

VHA-A	Control	+30 µg/L	+300 µg/L
Time 0	1.0	1.2	1.0
Day 6	1.3	6.0	7.6
Day 11	0.8	6.9	21.5

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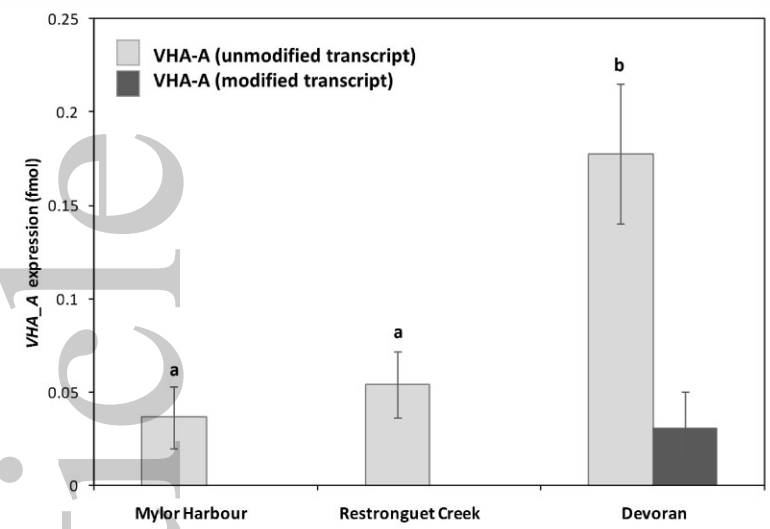

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