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RESEARCH ARTICLE

Identification and characterisation of a functional aquaporin water channel (*Anomala cuprea* DRIP) in a coleopteran insect

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SUMMARY

Water transport across the plasma membrane depends on the presence of the water channel aquaporin (AQP), which mediates the bulk movement of water through osmotic and pressure gradients. In terrestrial insects, which are solid and/or plant feeders, the entrance and exit of water is primarily executed along the alimentary tract, where the hindgut, particularly the rectum, is the major site of water conservation. A cDNA encoding the homologue of the water-specific *Drosophila* AQP [*Drosophila* integral protein (DRIP)] was identified through the RT-PCR of RNA isolated from the rectum of the cupreous chafer larvae, *Anomala cuprea*, a humus and plant root feeder. This gene (*Anocu* AQP1) has a predicted molecular mass of 26.471 kDa, similar to the DRIP clade of insect AQPs characterised from caterpillars, flies and several liquid-feeding insects. When expressed in *Xenopus laevis* oocytes, *Anocu* AQP1 showed the hallmarks of aquaporin-mediated water transport but no glycerol or urea permeability, and the reversible inhibition of elevated water transport through 1 mmol l^{-1} $HgCl_2$. This is the first experimental demonstration of the presence of a water-specific AQP, namely DRIP, in the Coleoptera. The genome of the model beetle *Tribolium castaneum* contains six putative AQP sequences, one of which (Trica-1a, XP_972862) showed the highest similarity to *Anocu* AQP1 (~60% amino acid identity). *Anocu* AQP1 is predominantly expressed in the rectum. Using a specific antibody raised against DRIP in the silkworm *Bombyx mori* (AQP-Bom1), *Anocu* AQP1 was localised to the apical plasma membrane of rectal epithelial cells, and lacking in the midgut and gastric caecal epithelia. Based on the BeetleBase prediction, there are three putative AQPs (Trica-3a, 3b, 3c: XP_970728, 970912, 970791) that are homologous to *B. mori* aquaglyceroporin [AQP-Bom2 (GLP)]. The immunocytochemical studies using the specific anti-peptide antibody against AQP-Bom2 revealed the presence of the GLP homologue at the apical plasma membrane of enterocytes in the midgut and gastric caeca. Thus, DRIP (*Anocu* AQP1) and the putative GLP share epithelial fluid-transporting roles along the alimentary tract in cupreous chafer larvae.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/14/2564/DC1

Key words: *Drosophila* integral protein, DRIP, aquaglyceroporin, rectum, midgut, gastric caeca, excretion, coleopteran AQP.

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INTRODUCTION

Water handling is a fundamental and essential daily task for many terrestrial animals confronted with harsh, stressful environments and posed with the threat of desiccation. The movement of water into and out of cells is a key feature of numerous physiological functions in living organisms. The current knowledge of the molecular aspects of water regulation has greatly increased over the last two decades. Water transport across the plasma membranes is mediated through a water channel, called aquaporin (AQP), which regulates rapid water fluxes between cellular and tissue compartments. These points are widely distributed in different species, and many homologues have been identified in animal tissues (Agre, 2006; Carbrey and Agre, 2010). The AQP family is functionally divided into two subgroups: water-selective (orthodox AQP) and water-permeable, which includes other small permeable non-electrolyte solutes, such as glycerol and urea, often referred to as ‘aquaglyceroporin’ (GLP) (Rojek et al., 2008). Despite functional differences between orthodox AQP and GLP, these molecules share a common protein structure comprising six transmembrane domains (I–VI) connected by five loops (A–E), including two signature Asn-Pro-Ala (NPA) motifs at the loops B and E to restrict proton conductance in the channel (Wspalz et al., 2010). The discovery of AQP has increased the current understanding of the epithelial and cellular mechanisms underlying osmoregulation.

Since the reviews of Campbell et al. (Campbell et al., 2008) and Spring et al. (Spring et al., 2009), the identification and functional characterisation of insect AQPs have received increasing attention. Based on bioinformatic predictions, reflecting the paucity of data concerning the cellular and physiological roles of these proteins, insect AQPs were classified into four clusters (Kambara et al., 2009; Goto et al., 2011), including *Drosophila* integral protein (DRIP) (Kaufmann et al., 2005), *Pyrocoelia rufa* integral protein (PRIP) (Lee et al., 2001) and big brain protein (BIB) subfamilies (Campbell et al., 2008). ‘Group 1’ comprises the well-characterised DRIP and PRIP subfamilies, which are apparently water-specific. ‘Group 2’ contains BIB, which exhibits no water permeability and does not function in water balance and osmoregulation. ‘Group 3’ is a heterogeneous clade, and most of the members are still predicted genes. Only three of the Group 3 AQPs are functional GLPs, which have been experimentally demonstrated to transport glycerol and

are structurally distinct from the vertebrate GLPs (Kataoka et al., 2009a; Kataoka et al., 2009b; Wallace et al., 2012). 'Group 4' comprises unorthodox AQPs with unusual NPA boxes (Ishibashi, 2006; Ishibashi et al., 2011) that still require experimental determination in insects.

The physiological role of specific AQPs in insect osmoregulation has been determined in liquid feeders, such as the green leafhopper (Le Cahérec et al., 1996), the buffalo fly (Elvin et al., 1999), two mosquitoes (Pietrantonio et al., 2000; Liu et al., 2011), the pea aphid (Shakesby et al., 2009) and the whitefly (Mathew et al., 2011). These Group 1 AQPs are localised to the alimentary tract (midgut and hindgut) and Malpighian tubules (MTs; insect kidney) for osmoregulation in diuresis and excretion through several transporters and channels across each epithelium.

Advances in the functional characterisation of AQPs in the vectorial fluid transport of liquid-feeding insects have led to the identification of two distinct water-specific AQPs, namely DRIP (AQP-Bom1) and PRIP (AQP-Bom3), in the hindgut of the silkworm *Bombyx mori* (Kataoka et al., 2009a; Azuma et al., 2012). The DRIP is distributed at the apical plasma membrane in fluid-transporting epithelia [colon, rectum and cryptonephric MT (cMT)] for water entry, and as an exit channel, the PRIP is constitutively expressed at the basal plasma membrane in the same cells. The expression and distribution of these channels provide a route for water retrieval from the gut lumen to the haemocoel, and the transcellular movement of water across the excretory epithelia enables the gluttonous caterpillar to endure water loss and body desiccation and explains why solid/plant feeders are normally robust to drought and starvation compared with liquid feeders (Azuma et al., 2012).

A similar physiological role might be established in the coleopteran hindgut, as the hindgut, particularly the rectum, is considered to be a major site of water conservation in beetle larva. Although six AQP homologues have been predicted through genomic database mining on the red flour beetle, *Tribolium castaneum*, a model coleopteran insect (Campbell et al., 2008), to our knowledge, there is only one report on AQP characterisation in the Coleoptera, in larvae of the firefly *Pyrocoelia rufa* (Lee et al., 2001). The results of this study led to the identification of the first original PRIP member, but there are no reports on either DRIP or GLP-type AQPs from coleopteran insects. In the present study, we chose a plant-root-feeding insect, the cupreous chafer, *Anomala cuprea* (Coleoptera: Scarabaeidae), and characterised a DRIP-type AQP (*Anocu* AQP1), which was highly homologous to the predicted DRIP from *T. castaneum* (Trica-1a, XP_972862). Furthermore, we examined another putative AQP at the protein level using specific anti-peptide antibodies against several *Bombyx* AQPs.

MATERIALS AND METHODS

Insects and tissues

The larvae of the cupreous chafer, *Anomala cuprea* (Hope), in the laboratory culture were kindly provided from Dr Wataru Mitsuhashi (National Institute of Agrobiological Sciences, Tsukuba, Japan). The larvae were reared in the laboratory at 20–25°C for a few weeks using the humus and fresh carrot chips. The final (third) instar larvae (feeding phase) were used in all experiments. Larvae were anaesthetised on ice for 15 min and dissected before the removal of tissues. The midgut (including gastric caeca), MTs, colon and rectum [Fig. 1; cf. Plate 1 in Berberet and Helms (Berberet and Helms, 1972)] were collected and rinsed with diethylpyrocarbonate (DEPC)-treated phosphate buffered saline (Dulbecco's PBS without $CaCl_2$ and $MgCl_2$) following previous methods (Kataoka et al., 2009a; Kataoka et al., 2009b). Collected tissues from five larvae (except for MTs from 20 larvae) were weighed (0.05–0.15 g wet weight), immediately frozen in liquid nitrogen and stored at −80°C until further use for RNA extraction. All procedures described below for the RNA studies were performed under RNase-free conditions at room temperature unless otherwise specified. For the protein studies, the tissues were collected in a similar manner using isotonic homogenisation buffer (0.3 mol l^{-1} mannitol, 5 mmol l^{-1} EDTA, 10 mmol l^{-1} HEPES-NaOH, pH 7.5, without DEPC treatment), as used in a previous study (Azuma et al., 2012). The collected tissues were weighed and stored at −30°C until further use.

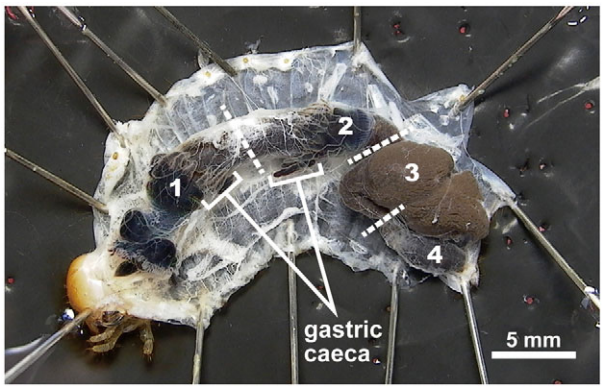

Fig. 1. Larval anatomy of the cupreous chafer, *Anomala cuprea*. For the RNA extraction, the alimentary tract was divided into four regions: 1, anterior midgut; 2, posterior midgut; 3, colon; and 4, rectum. The gastric caeca are situated at the anterior and posterior regions of the midgut. Note that the Malpighian tubules of these larvae are fibrous and whitish or hyaline, running along the gut.

Design of primers and PCR cloning of the full-length cDNA

Poly(A) RNA was prepared from several tissues using the guanidinium-phenol-chloroform extraction method, followed by further purification with the QuickPrep *Micro* mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK) as described in Kataoka et al. (Kataoka et al., 2009a). cDNA was reverse transcribed from the mRNA using the anchored-oligo $(dT)_{18}$ primer (Transcriptor First-Strand cDNA Synthesis Kit, Roche Diagnostics, Mannheim, Germany). The synthesised first-strand cDNA was used as a template for PCR amplification of partial AQP cDNA fragments encoding a central domain between two highly conserved NPA motifs, using a set of degenerate primers: forward, 5′-GGD KGH CAC ATY AAY CCV GCS GTS AC-3′; and reverse, 5′-CC GAA WGW NCK RGC BGG RTT CAT RCT-3′ (supplementary material Table S1). The primers were respectively designed on the basis of the nucleotide sequences corresponding to the coding regions between the two NPA motifs in the four insect AQPs from *Cicadella viridis* (AQPcic) (Le Cahérec et al., 1996), *Haematobia irritans exigua* (AqpBF1) (Elvin et al., 1999), *Aedes aegypti* (AeaAQP) (Pietrantonio et al., 2000) and *Bombyx mori* (AQP-Bom1) (Kataoka et al., 2009a). Using these degenerate primers, we successfully cloned a DRIP-type AQP from *Coptotermes formosanus* (CfAQP1) (Kambara et al., 2009) and *Grapholita molesta* (AQP-Gra1) (Kataoka et al., 2009b). The PCR amplification was performed with an initial step at 95°C for 3 min, followed by 30 cycles at 94°C for 30 s, 45°C for 30 s and 72°C for 30 s, with a final incubation at 72°C for 10 min. The resulting 381 bp product was purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden,

Germany) and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The plasmid DNA was purified, and both strands of the DNA were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and a DNA sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). According to the partial-length cDNA information on a candidate of *A. cuprea* AQP, the 5′- and 3′-RACE (rapid amplification of cDNA ends) DNA fragments were produced from the rectum of mRNA using the SMART RACE cDNA Amplification Kit (BD Biosciences, Clontech, Palo Alto, CA, USA). For 5′ and 3′ RACE-PCR analyses, the gene-specific primers (forward, 5′-GGT AAT CTC GGT GCA ACT GCT CC-3′; reverse, 5′-C ACT TAA ATG GCA GGC GGT AAT AGC-3′) (supplementary material Table S1) were designed based on the central region sequence of an AQP-like cDNA fragment. The longest open reading frame corresponding to the candidate was confirmed through joining the three overlapping PCR-derived fragments, including the central fragment (381 bp).

A Kyte–Doolittle hydropathy profile of the deduced amino acid sequence was analysed using the GENETYX-Windows version 10 software (Genetyx Corporation, Tokyo, Japan) at a 10-residue window. Phosphorylation and glycosylation sites were predicted using the CBS Prediction Server (http://www.cbs.dtu.dk/index.shtml). The amino acid identities between *Anocu* AQP1 and other insect AQPs were analysed using the BLAST programme at the National Centre for Biotechnology Information (http://blast.nbci.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed using the neighbour-joining method with 1000 bootstrap replicates using the software MEGA 5 (http://www.megasoftware.net).

cRNA synthesis and AQP protein expression studies in *Xenopus laevis* oocytes

The entire coding region for *Anocu* AQP1 including the 5′-untranslated sequence (59 bp upstream sequences; see supplementary material Fig. S1) was amplified using the specific primer sets containing a *Bgl*II restriction site (supplementary material Table S1) according to previous reports (Kataoka et al., 2009a; Kataoka et al., 2009b; Kikawada et al., 2008). After digestion with *Bgl*II, the amplified products were subcloned into the *Bgl*II sites of the pT7XβG-2 vector, which was kindly provided by Dr Takahiro Kikawada (National Institute of Agrobiological Sciences, Tsukuba, Japan). The capped RNA (cRNA) was synthesised *in vitro* using the mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions.

Osmotic water permeability (P_f; $\times 10^{-4}$ cm s^{-1}) through *Anocu* AQP1 was measured from the time course of oocyte swelling as described previously (Kataoka et al., 2009a; Kataoka et al., 2009b). Briefly, 5 ng of the cRNA or water, as a control, were injected into a *Xenopus* oocyte. Following injection, the oocytes were incubated in modified Barth's saline (MBS) [0.33 mmol l^{-1} $Ca(NO_3)_2$, 0.41 mmol l^{-1} $CaCl_2$, 88 mmol l^{-1} NaCl, 1 mmol l^{-1} KCl, 2.4 mmol l^{-1} $NaHCO_3$, 0.82 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} HEPES (pH 7.5) and 10 μg ml^{-1} penicillin and streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA)] at 15°C for 3 days. Subsequently, the oocytes were incubated in threefold-diluted (with distilled water) MBS. Images of the swelling oocyte silhouette were taken every 15 s using a CCD camera (DP-25, Olympus, Tokyo, Japan) attached to an Olympus SZX10 stereomicroscope. P_f was determined using a previously described method (Kataoka et al., 2009a; Kataoka et al., 2009b). The oocyte volume was calculated based on the cross-sectional area of a single cell using ImageJ 1.46e (http://rsbweb.nih.gov/ij/). To examine the effect of mercury on P_f, the oocytes were pre-incubated in MBS containing 1 mmol l^{-1} $HgCl_2$ for 10 min prior to the swelling assay, which was also performed in the presence of $HgCl_2$. To confirm the reversible effect of mercury chloride inhibition, the oocytes were rinsed with MBS containing 5 mmol l^{-1} 2-mercaptoethanol (three times, 5 min each) and subjected to the swelling assay in the absence of $HgCl_2$.

Immunocytochemistry with anti-peptide antibodies against *Bombyx mori* AQPs

All procedures were performed according to previous studies (Miyake and Azuma, 2008; Azuma et al., 2012). Polyclonal rabbit antibodies were commercially raised against a synthetic peptide corresponding to part of the most hydrophilic loop D region of AQP-Bom1 (DRIP) and AQP-Bom2 (GLP). These peptides have a negligible similarity to each other and the specificity of each antibody has been previously described (Azuma et al., 2012). Using the *Bombyx* DRIP antibody (IgG fraction, 1:500 dilution with 3% bovine serum albumin in PBS), the immunoblotting analyses of the rectum showed two major bands of 25 and 27 kDa and the non-specific signal at 35 kDa, and the latter band also appeared using the *Bombyx* GLP antibody (supplementary material Fig. S2). The humus within the rectal extracts might produce some non-specific immunoreactions. We also used the AQP-Bom3 (PRIP) antibody, which was raised against the C-terminal peptide sequence (Azuma et al., 2012). However, we failed to detect reliable signals from *A. cuprea* using this antibody (data not shown).

The tissues used for immunocytochemistry were quickly dissected in PBS, immediately placed in Bouin's fixative and fixed on ice for 4–6 h depending on the tissue mass. The specimen was divided into the anterior midgut (with gastric caeca), the posterior midgut (with gastric caeca), the colon and the rectum (with the MT) in a manner similar to that used for the RNA extraction (see Fig. 1). We could not completely remove the gut contents (humus), particularly from the colon and rectum. The tissue was dehydrated and embedded in paraffin (Histosec pastilles, Merck, Darmstadt, Germany). The sections (~5 μm) were first incubated with 10% normal goat serum in PBS (10% NGS) at room temperature for 2–4 h, followed by incubation with anti-AQP-Bom1 or anti-Bom2 antiserum (diluted at 1:1000 or 1:2000 with 10% NGS) at 4°C overnight in a humid chamber. For the control staining, we used the preimmune serum (1:2000 dilution with 10% NGS) instead of the above-mentioned *Bombyx* antisera. After rinsing four times (10 min each) with PBS, the sections were incubated with Alexa Fluor 555 goat anti-rabbit IgG (H^+L) (Molecular Probes, Eugene, OR, USA; 1:1000 with PBS) for 2–3 h at room temperature. After rinsing four times (10 min each) with PBS, the sections were placed into a few drops of ProLong Gold antifade with DAPI (Molecular Probes) and coverslipped.

To examine the immuncytochemistry in the midgut, the affinity-purified IgG fractions from each *Bombyx* antiserum were applied to the sections after dilution to 1:100, 1:200 or 1:500 with 10% NGS to significantly reduce the background staining. After rinsing in PBS, the sections were successively incubated with a goat anti-rabbit biotin-conjugated antibody at room temperature for 2–3 h, followed by incubation with an avidin/peroxidase reagent (Vectastain elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and a final incubation with the substrate (0.01% 3,3′-diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide) until sufficient colour development was attained (at room temperature, up to 10 min). The tissue sections were observed under a microscope (Olympus BX51) equipped with a differential

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Anocu AQP1  -MSKDMK------------------AAESYVGLSELTASTNLWRALCAEFIGTFLLVLI    40
Trica-1a    MANKEIK------------------GADSVLGLSEITENKAIWRMLVAEFIGTFLLVVI    41
AeaAQP      -MTESAG--------------------VKQLVGVADITENRNIWRMLVAEFLGTFFLVSI    39
PregAQP1    -MVEKVD--------------------MSAMVGVKDITDNKKIWRQLLAELIGTFFLVVI    39
CfAQP1      -MVKPTG--------------------ARALLGLHDITDNRNIWRMLSAEFLGTFFLVLV    39
AqpBF1      -MVEKLD--------------------MSAVVGVKDITDNKKIWRQLMAELIGTFFLVVI    39
AQP-Gra1    MSAKNTQNVEAATPAPEEPATAKPLLETSSILGLSDVTDNKLIWRQLLAELVGTFLLTSI    60
AQP-Bom1    MATKTTE--------------------KTSSIIGLSDVTDNKLIWRQLVAELVGTFLLTSI    41
AQPcic      -MAADKS-----------------VDNTKKIIGIDDITDTKTIWRCLAAELIGTLLLVLI    42
BtAQP1      -MEDISSSGEEIS-----------MKAISKVIGVPDIRDGPTLTKCIVAEFVGTLLLVLI    48
DRIP        -MVEKTE--------------------MSKFVGVADITENKKIWRMLLGELVGTFFLIFV    39
                                         :*: ::     : : : .*::**::*  :

Anocu AQP1  GCGTILQFKPTAAPSIVAISLAFGLIVATLAQAIGHVSGGHVNPAVTLSLFVTGDCKLIR   100
Trica-1a    GCGSIVLS---EEVVIVRIGATFGITVPTLAQSIGHVSGGHINPAVTLSLFATGNIKLLR    98
AeaAQP      GIGSTMGWGGDYAPTMTQIAFTFGLVVATLAQAFGHVSGGHINPAVTIGLMITADISILK    99
PregAQP1    GVGSCTGGA-DWTPSIPQIAFTFGLTVATLAQAIGHISGGHINPAVTLGFLIVGEMSIIK    98
CfAQP1      GCSSITPLA--GRSSVIQIAFTFGLAVATIAQAVGHVSGGHINPAVTCGLIVSGHVSILK    97
AqpBF1      GVGSCTGGS-EWSPSIPQIAFTFGLTVATLAQAIGHISGGHINPAVTVGFLIVGEMSIIK    98
AQP-Gra1    GVASCVSITDAKVNSIVSIALTFGLLVGTIVQTIGHVSGGHINPAVTLGLLAAGEIKIIK   120
AQP-Bom1    GVAACITINASTAPHTTSIALCFGLLVGSIVQGIGHVSGGHINPAVTAGLFAAGDIKLLK   101
AQPcic      GTGSCTGVQISEGDVVVRIALTFGFIIATMVQCIGHVSGGHINPAVTCGLLVTGHISILK   102
BtAQP1      GCMSVAFVHQDNFVDVVKIAMAFGLIIASMVQAIGHVSGGHINPAVTCGLAVSGHVSIIK   108
DRIP        GVGSTTSG------SVPQIAFTFGLTVATIAQGLGHLSGGHINPAVTLGFLIVGEISILK    93
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Anocu AQP1  SCLYIVVQHLGAMAGSAMLMVATPSD-FQG--NLGATAPHADLAPAQAFFYEAVLTFLLC   157
Trica-1a    ALLFIAVQHIGAVGGSAVLRLMVPEE-KEH--SLGITNIGPGITDVQGFLMEIILTFLLL   155
AeaAQP      GAFYIVSQHVGAIAGAALIKAATPSD-VIG--GLGVTGIDPRLTAGQGVMMEALITFILV   156
PregAQP1    SALYIAVQHVGAIAGAAVIKVGVSEA-VGGN-ELGVSAYAASLTVGQAVLIEALITFILV   156
CfAQP1      AVFYIAVQHIGAVAGAAVLQALTPAE-AED--SLGMTTINALVTPVQAFFMEALITFVLV   154
AqpBF1      SVLYIAVQHVGAIAGAAVIKVGVSEA-VSGL-DLGVSSFSSTLTVGQAVLIEALITFILV   156
AQP-Gra1    SLFYIVVQHLGAVAGAAFIRLAVPDV-EVKSDGFGMTLPGKNVTDGQAVLIEALITFVLV   179
AQP-Bom1    AIFYIVVQHLGAVAGAAFIRLAIPAD-SIG--GFGMTLPGPGVTEAQAVLVEALITFVLV   158
AQPcic      AIFYIIVQHVGAIAGSAILKVITPAE---FRGTLCMTSLAPGVTPPMGFLVEACITFVLI   159
BtAQP1      GMLYIVAQHLGAICGAIILNEITPKTGYTAAGNLGVTTLSTGVSDLQGVAIEALITFVLL   168
DRIP        AAFYIIVQHVGAIAGAAVIKVALDGV-AGG--DLGVSSFDPSLNCAQAVLIEALITFILV   150
            . ::*  * :**: *: .:              :  :     :    ..  *  :**:*

Anocu AQP1  FVIHGVHDAKRKDIKGSAPLAIGLAITCHLSGIKYTGSSINPARSFGPAVIKN-NWHNH   216
Trica-1a    FIIHAVHDPRRKDIKGSAPLAIGLAVTACHLCGIPYSGSSVNPARSFGPAVIMD-SWENH   214
AeaAQP      FVVHGVHDNRRSDIKGSAPLAIGLSITAGHLSAIKYTGASMNPARSFGPAVVMG-NWTDQ   215
PregAQP1    FVVKGVHDPGRTDIKGSAPLAVGLSIVAGHLCAIKLTGASMNPARSFGPAVVQN-MWEDH   215
CfAQP1      LTVEAVHDERRTDVKGSAPLAIGLSIATCHLAAINYTGASMNPARSFGPAVMTG-MWNHH   213
AqpBF1      VVVKGVHDPGRTDIKGSAPLAVGLSIAAGHLCAIKLTGASMNPARSFGPAVVQN-MWIDH   215
AQP-Gra1    MVVNGVHDGRRTDVKGSAPLAIGLSITACHAACIPFTGSSMNPARSFGPALVMG-YWASH   238
AQP-Bom1    MVVMGVHDPQRNDLKGSAPLAIGLSITACHAAVIPFTGSSMNPARTFGPALVIG-NWTSQ   217
AQPcic      LLVQSVHDDRRKNLGNAAPVAVGLAITCCHLAAIKYTGSSMNPARSFGPAVNGDDNWANH   219
BtAQP1      LVVQSVHDGKRTDIKGSIGVAIGFAIACCHLAAIKYTGASMNPARSLGPAFVSG-IWDKH   227
DRIP        FVVKAVHDPGRQDIKGSAPLAVGLAIAAGHLCAIKLSGASMNPARSFGPAVVQG-VWTYH   209
            . : .*.*  * :: .:  :*:*:::.  * . *  :*:*:****::***.  .  *  :

Anocu AQP1  WIYWAGPIIGGLVAGLIYKFIFKVPKD-EED-SMDF          250
Trica-1a    WVYWAGPLLGGVLAGLIYKYLFKTRKS-DVD-SYDF  59.4%   248
AeaAQP      WVYWVGPIVGGILAGAVYRLFFKVRKGDEE--SYDF  56.4%   249
PregAQP1    WVYWVGPFAGAAVAALLYKFVFKVRKGDDEANSYDF  53.3%   251
CfAQP1      WVYWAGPIVGGVVAGAVYSFIFRARKGEDETSSYDF  52.0%   249
AqpBF1      WVYWVGPIVGAIVAALLYKFVFKVRKGDDEANSYDF  51.6%   251
AQP-Gra1    WVYWVGPITGGVIAGLVYRYVFRIGKTGESG-SYDF  50.3%   273
AQP-Bom1    WVYWVGPVVGSVVAGLLYKFALRIKKAGDTG-SYDF  50.0%   252
AQPcic      WVYWAGPIVGGVVAGITYRALFRARKPEEEASSYDF  49.7%   255
BtAQP1      WVYWAGPILGGVTASLLYAITFKAKKRSDE-SSYDF  48.8%   262
DRIP        WVYWVGPIAGGLLAGIIYRLIFKVRKGDDETDSYDF  46.9%   245
            *:**.**. *.  *.  *   ::  *  :   * **
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Fig. 2. Multiple amino acid sequence alignments of the cupreous chafer, *Anomala cuprea*, aquaporin (*Anocu* AQP1). *Anocu* AQP1 is closely related to the predicted AQP deduced from the genomic sequence in *Tribolium castaneum* (XP_972862.1; Trica-1a) and shares significant sequence similarity with the well-characterised insect DRIPs, including *Aedes aegypti* (AF218314 or AAF64037.1; AeaAQP), *Phormia regina* (AB713909: PregAQP1), *Coptotermes formosanus* (AB433197: CfAQP1), *Haematobia irritans exigua* (AAA96783.1 or U51638; AqpBF1), *Grapholita molesta* (AB469882: AQP-Gra1), *Bombyx mori* (AB178640; AQP-Bom1), *Cicadella viridis* (X97159 or CAA65799.1; AQPcic), *Bemisia tabaci* (EU127479.1; BtAQP1) and *Drosophila melanogaster* (ABA81817.1) as the original DRIP. The percentages represent the amino acid sequence similarity of each sequence to that of *Anocu* AQP1. The two NPA motifs are indicated by bold horizontal lines. Selectivity filter 'aromatic-arginine' regions (ar/R) are highlighted with boxes. Within these insect DRIPs, '*' indicates residue identity, ':' indicates conserved residues and '.' indicates semi-conserved residues. Note that the candidate cysteine residues for mercury sensitivity are well conserved among insect DRIPs (not only at dotted boxes but also near His residues in the ar/R).

interference contrast device, and recorded with a DP-70 digital camera and cellSens imaging software (version 1.4.1, Olympus).

RESULTS

cDNA cloning and sequence characterisation of *Anomala cuprea* AQP

Using degenerate oligonucleotide primers corresponding to sequences highly conserved in two NPA motifs of insect AQPs, we obtained one strong PCR product of the expected size (~380 bp) using the first-strand cDNA obtained from the rectum as a template. The cloned DNA fragment was sequenced, and BLAST analysis revealed high similarity with the DRIP-type AQPs as a candidate of *A. cuprea* AQP (data not shown). Consecutive 5′- and 3′-RACE experiments were performed to obtain the full-length cDNA, *Anocu* AQP1 (DDBJ/EMBL/GenBank accession no. AB741517). The *Anocu* AQP1 cDNA comprises 1428 bp in length with an open reading frame of 250 amino acids (nucleotide positions 276–1025), which encodes a polypeptide with a calculated molecular mass of 26.471 kDa. According to the hydropathy analysis, *Anocu* AQP1 contains six putative transmembrane domains, five connecting loops (A–E), cytoplasmic N- and C-terminal domains, and two highly conserved hydrophobic NPA repeats (NPA boxes) at loops B and E, all of which are typical AQP features (Agre, 2006; Campbell et al., 2008; Carbrey and Agre, 2010). *Anocu* AQP1 also contained a consensus sequence for casein kinase II phosphorylation at Thr-123. No potential *N*-linked glycosylation site was detected in *Anocu* AQP1 (supplementary material Fig. S1).

BLAST search comparisons of the coding sequence of *Anocu* AQP1 revealed high amino acid identity (>50%) with the DRIP members in insect AQPs, and among them, *Anocu* AQP1 was closely related to a predicted AQP (Trica-1a; XP_972862) from *T. castaneum* (Fig. 2). The amino acid alignment of *Anocu* AQP1 with the 10 DRIPs showed the typical aromatic/arginine (ar/R) constriction residues (Phe, His and Arg) for water selectivity. Although the cysteine residue upstream of the second NPA motif for the mercury-sensitive site in vertebrate AQPs (Preston et al., 1993) was lacking, the remaining candidate cysteine residues were well conserved among DRIPs, such as those in the PRIP subfamily (Azuma et al., 2012).

Water permeability of *Anocu* AQP1 upon expression in *Xenopus laevis* oocytes

The water-transporting function of *Anocu* AQP1 was evaluated through expression in *Xenopus* oocytes. Changes in the osmotic volume were measured in control oocytes and those injected with the synthesised cRNA encoding *Anocu* AQP1. After transfer to a hypo-osmotic solution, the volume increased more rapidly in oocytes expressing *Anocu* AQP1 than in the controls (Fig. 3A). The

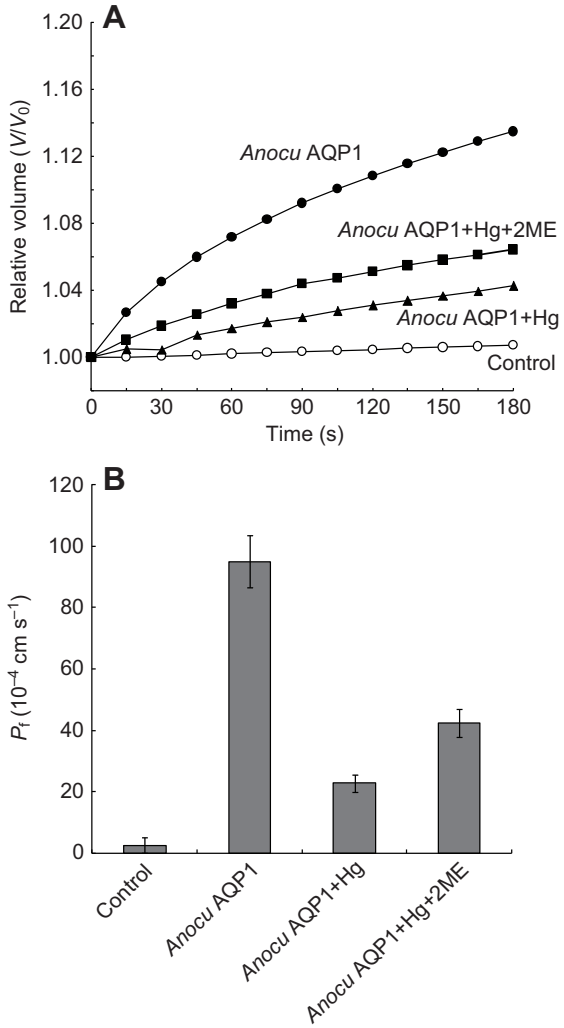


Fig. 3. Functional expression of the cupreous chafer, *Anomala cuprea*, aquaporin (*Anocu* AQP1) in *Xenopus* oocytes. (A) Representative time course data and Hg^{2+} inhibition of the osmotic swelling of oocytes expressing *Anocu* AQP1. Changes in the osmotic oocyte volume (V/V_0) in control oocytes, *Anocu* AQP1 cRNA-injected oocytes (*Anocu* AQP1), those after 1.0 mmol l^{-1} $HgCl_2$ treatment and those with $HgCl_2$ treatment followed by the administration of 5 mmol l^{-1} 2-mercaptoethanol. (B) Osmotic water permeability (P_f) in different experimental groups in expressing *Anocu* AQP1. Values represent means ± s.e.m. obtained after measuring 5–10 oocytes.

P_f values were ~40-fold higher in *Anocu* AQP1-expressing oocytes ($95.0\times10^{-4}\pm8.6\times10^{-4}$ cm s^{-1}) than in the controls (Fig. 3B). Mercury chloride inhibits the function of AQPs by combining the mercury-sensitive cysteine residues to block the aqueous pore (Preston et al., 1993; Jung et al., 1994). The increase in P_f mediated through *Anocu* AQP1 expression was inhibited after pre-treatment with 1 mmol l^{-1} $HgCl_2$, and this inhibition was partially reversed through 5 mmol l^{-1} 2-mercaptoethanol treatment following incubation in $HgCl_2$ (Fig. 3B). It was not possible to fully reverse the effects of mercury using 2-mercaptoethanol treatment. These results provide functional evidence of *Anocu* AQP1 as a water channel. Moreover, we analysed glycerol and urea permeability in an iso-osmotic swelling assay using 140 mmol l^{-1} inwardly directed gradients of each solute. However, similar to the DRIPs in other insects (Kataoka et al., 2009a; Kataoka et al., 2009b; Ishida et al., 2012), there was no appreciable transport of glycerol and urea in *Anocu* AQP1-expressing oocytes (data not shown).

Localisation of *Anocu* AQP1 in the rectum of *Anomala cuprea* larvae

The higher identity in the loop D region among DRIPs (Fig. 2) prompted us to characterise the *Anocu* AQP1 protein at the cellular level using the *Bombyx* DRIP antibody. Immunoblotting analysis of the rectal extracts of *A. cuprea* showed the polypeptide bands at the predicted molecular mass with 25 and 27 kDa (supplementary material Fig. S2). Positive immunostaining was distributed along the apical surface of the rectal epithelium (Fig. 4A). This antibody showed clear staining at the apical plasma membrane of the rectal epithelial cells of *B. mori* larvae (Fig. 4B). There was no significant staining at the rectal epithelium with the preimmune rabbit serum (Fig. 4C), nor with the antiserum against the *Bombyx* GLP (Fig. 4D). We observed no cryptonephric structure around the rectum of *A. cuprea* larvae [Fig. 1; cf. Areekul (Areekul, 1957) and Berberet and Helms (Berberet and Helms, 1972)]. The normal (not cryptonephric) MT along the rectum (Fig. 4A) and the colon (data not shown) showed no significant reaction with the *Bombyx* DRIP antibody. In contrast, we observed the strong immunofluorescence at the apical and basal membranes of MT epithelium using the *Bombyx* GLP antibody (Fig. 4D). The MT along the rectum appears to lack in immunoreactivity with the *Bombyx* DRIP antibody (Fig. 4A). This was a clear difference from the MT of the house cricket, *Acheta domesticus*, where the DRIP-like AQP showed the apical and basal distributions (Spring et al., 2007).

Aquaglyceroporin homologue in the midgut and gastric caeca of *Anomala cuprea* larvae

Based on comparisons of *Anocu* AQP1 with the six predicted *Tribolium* AQPs [cf. fig. 6 in Campbell et al. (Campbell et al., 2008)] and three previously characterised *Bombyx* AQPs (including the original DRIP and PRIP), we constructed a phylogenetic tree of the related members assessed in the present study (Fig. 5A). The six *Tribolium* AQPs were categorised in the DRIP (Trica-1a), PRIP (Trica-1b), BIB (Trica-2) and Group 3 (Trica-3a, 3b, 3c) subfamilies. Because the *Bombyx* GLP (AQP-Bom2) is structurally most similar to Trica-3a (XP_970728) (Kataoka et al., 2009a), we tentatively referred to Group 3 AQPs as GLP. The peptide sequence of the loop D domain for DRIP or GLP and the C-terminal peptide sequence for PRIP were aligned and the conserved regions between the *Bombyx* and the coleopteran AQPs were depicted (Fig. 5B).

Immunocytochemistry using the *Bombyx* DRIP antibody showed specific labelling at the rectal epithelium (Fig. 4A), potentially reflecting a high degree of conservation in the epitope domains among DRIPs. There was more divergence among the GLP subfamily (Group 3 members), such as the three predicted GLPs (Trica-3a, 3b, 3c) in *T. castaneum*. However, *Bombyx* GLP antibody could probe the presence of a certain GLP-like AQP at the MT of *A. cuprea* (Fig. 4D). We further explored the localisation of the GLP homologue in the midgut of *A. cuprea* using the *Bombyx* GLP antibody, as *Bombyx* GLP is primarily expressed in the midgut and MT (not cryptonephric) of silkworm larvae (Kataoka et al., 2009a; Azuma et al., 2012).

Copious staining was detected at the apical surface of the midgut (Fig. 6). Three types of enterocytes were discernible on the apical surface: long, tall cells with flat surfaces (Fig. 6A), columnar cells with spherical blebs (Fig. 6B), and columnar cells with brush-borders (Fig. 6C). These structures with the positive signals were observed in the anterior and posterior regions of the midgut. The MTs along

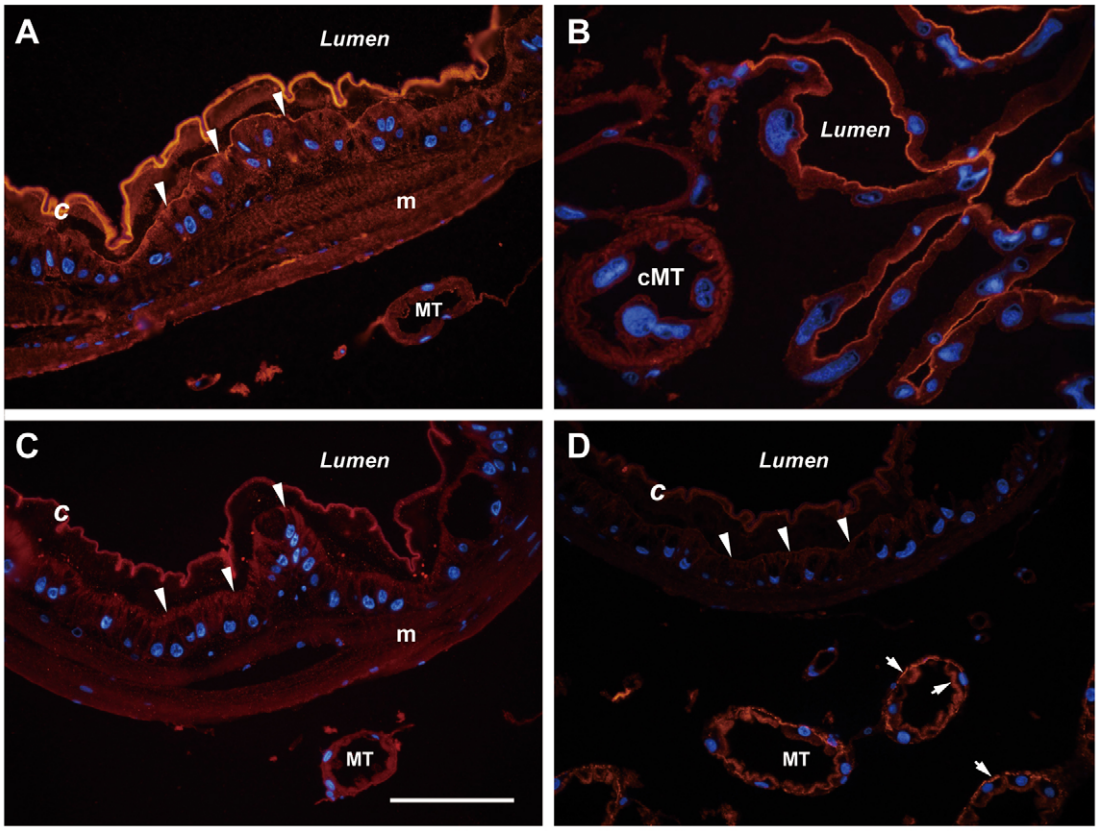


Fig. 4. The water-specific AQP (DRIP) localisation in the larval rectum of *Anomala cuprea* (A,C,D) and *Bombyx mori* (B). (A) The apical surface distribution (arrowheads) was observed using the *Bombyx* DRIP (AQP-Bom1) antiserum. The cuticle along the rectal epithelia showed non-specific fluorescence. Note that the Malpighian tubule (MT) along the rectum was negative. (B) In *B. mori*, this antibody showed clear staining at the apical plasma membrane of rectal epithelial cells. Note that the inner cryptonephric MT (cMT) is negative (cf. Azuma et al., 2012). (C) Control staining using normal rabbit serum. (D) Control staining using AQP-Bom2 (GLP) antiserum. There was no significant fluorescence in the rectal epithelia, including the cuticle. In contrast, immunolocalisation with the *Bombyx* GLP antibody was detected on the apical and basal surface (arrows) of the MT epithelia along the rectum. m, muscle layer; c, cuticle. Scale bar, 100 μm (applies to all panels).

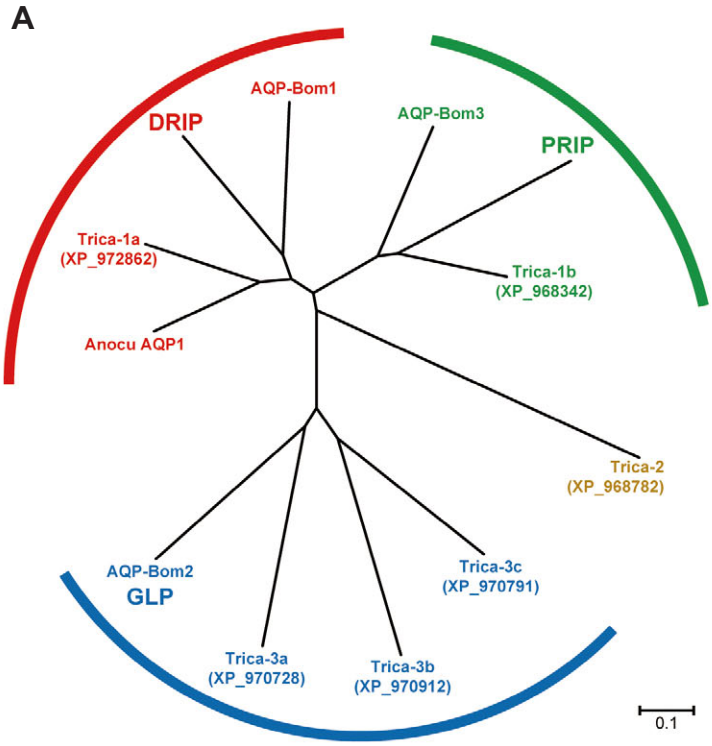
the midgut (see Fig. 1) had no significant signals (data not shown). Furthermore, specific staining was clearly observed at the apical surface of gastric caeca (Fig. 6C). The DRIP immunostaining (Fig. 6D) was faint, similar to that obtained with the IgG fraction from the preimmune serum (data not shown). The columnar cell apical membrane of the posterior midgut in *B. mori* clearly showed specific staining with this antibody (Fig. 6E). These observations suggest that GLP-like AQP(s), other than *Anocu* AQP1 (DRIP), play roles in the molecular functions of the midgut region. A putative *Tribolium* PRIP (Trica-1b) with a high identity to *Bombyx* PRIP at the C terminus (Fig. 5B) was recognised, but the PRIP homologue was not detected in the midgut, MT, colon or rectum of *A. cuprea* larvae using the *Bombyx* PRIP antibody (data not shown).

DISCUSSION

Anocu AQP1, a water-transporting aquaporin in the rectum of *Anomala cuprea*

The first demonstration of coleopteran AQP, the original PRIP, was reported in the firefly *P. rufa* (Lee et al., 2001). To our knowledge, the present study is the first to functionally characterise a DRIP-type AQP in coleopteran insects. Using heterologous expression in *Xenopus* oocytes, we demonstrate that *Anocu* AQP1 selectively transports water (Fig. 3). Currently, the functionally characterised DRIPs from several insect species listed in Fig. 2 (including *Anocu* AQP1) are unambiguously selective for water and show no permeability for glycerol and urea. The DRIP and PRIP subfamilies as water-specific orthodox AQPs are placed into one major clade of Group 1 AQPs in insects (Kambara et al., 2009; Goto et al., 2011), and the members of this clade have been steadily accumulating since the Campbell et al. (Campbell et al., 2008) review of these channels.

In homopteran insects, as liquid/plant feeders, the DRIPs are typically associated with fluid homeostasis through the movement of water across tissues for excretory purposes to maintain the osmotic potential. A specialised filter chamber or alimentary structure with a similar function is an indispensable organ for water balance in an insect body (Le Cahérec et al., 1997; Shakesby et al., 2009; Mathew et al., 2011). We also have identified and characterised several DRIPs from two caterpillars (Kataoka et al., 2009a; Kataoka et al., 2009b), the termite (Kambara et al., 2009), the blowfly (Ishida et al., 2012) and the chafer examined in the present study. Therefore, it is conceivable that DRIP, as a water-specific AQP, is ubiquitously distributed across Insecta, and the loop D domain is common among the members in the DRIP subfamily (Fig. 5B). This high identity in the epitope region of *Anocu* AQP1 with *Bombyx* DRIP (AQP-Bom1) facilitated the detection of specific AQP protein expression at the apical plasma membrane in the rectal epithelia of *A. cuprea* larvae (Fig. 4A). The apical plasma membrane localisation of *Anocu* AQP1 is consistent with that of *Bombyx* DRIP in the hindgut



B

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AQP-Bom1          163GVCDPQRNDLKGSAPLAIG181
Anocu AQP1        172GVCDAKRKDIKGSAPLAIG180
Trica-1a          160AVCDPRRKDIKGSAPLAIG178
DRIP              155AVSDPGRQDIKGSAPLAVG173
Aedae-1a(AeaAQP)  161GVCDNRRSDIKGSAPLAIG179
                     .*.*  *.*:*******:*

AQP-Bom3          253GDEKELKRLDGGKLDDMA270
Trica-1b          251ADEKELKRLDVNR-DEP-266
PRIP              253TDEKELQRLAGGKQDESM270
                      *****:**  .: *:

AQP-Bom2 GLP      175CACWDPVNKNKQDSVPVKF193
Trica-3a          151CAVWDKRNETKADSVPLRF169
Trica-3b          159CAVWDARNKTKTDSTPIRF177
Trica-3c          199CGVWDCRNNTKHDSVPLRF217
Aedae-3a(midgut)  185CGLWDPRNSKMGEGTPVKF203
Aedae-3b(caeca)   152CGVWDPRNSKLHDSVPLKF170
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Fig. 5. Phylogenetic analysis of the cupreous chafer, *Anomala cuprea*, aquaporin (*Anocu* AQP1) with the characterised *Bombyx* AQPs and predicted *Tribolium* AQPs. (A) Phylogenetic tree of the insect AQPs examined in the present study using the neighbour-joining method. *Anocu* AQP1 was categorised in the DRIP subfamily with AQP-Bom1 (AB178640), DRIP (ABA81817.1) and Trica-1a (XP_972862). The remaining putative *Tribolium* AQPs have been annotated as Trica-1b (PRIP subfamily) with AQP-Bom3 (AB458833) and PRIP (AF420308), and Trica-2 (BIB subfamily) and Trica-3a, 3b, 3c (GLP subfamily) with AQP-Bom2 (AB245966). The scale bar represents 10% divergence. (B) Polyclonal rabbit antibodies were raised against the amino acid sequences of the loop D domain for AQP-Bom1 (in red) or Bom2 (in blue) and the C-terminal sequences for AQP-Bom3 (in green). The sequence identities of these AQPs with the respective *Tribolium* AQPs and *Anocu* AQP1, DRIP and PRIP are in colour and bold (DRIP in red, PRIP in green, GLP in blue). The number denotes the amino acid residue in each insect AQP. For further comparison, *Aedes aegypti* DRIP (AeaAQP: AF218314) and two putative *Aedes* GLPs (Aedae-3a: XP_001650168, Aedae-3b: XP_001650169) are aligned. Note that only AQP-Bom2 has been experimentally demonstrated to transport glycerol (Kataoka et al., 2009a) among the members listed here.

(Fig. 4B) (Azuma et al., 2012). Therefore, it is highly probable that the *Anocu* AQP1 (DRIP) is working as a water gate at the rectum of *A. cuprea* larvae.

Efficient and massive water movement may need the other water-specific AQP, i.e. PRIP, because highly fluid-transporting cells separate two compartments with a polarised plasma membrane. The *Bombyx* PRIP is localised on the basal plasma membrane of colonic and rectal epithelial cells, which confers high-capacity water flow transcellularly through the epithelial membranes for water retrieval function in the cryptonephric rectal complex, producing dry silkworm larvae faeces (Azuma et al., 2012). Such a physiological adaptation for high recovery of water has long been studied and argued in another coleopteran insect, the mealworm, *Tenebrio molitor* (Bradley, 1985; O'Donnell and Machin, 1991), and probably in *T. castaneum* as a stored insect pest. In contrast, the cupreous chafer, a scarabaeid larva, produces soft and wet faeces, potentially reflecting a lack of the cryptonephric anatomy in the rectum of *A. cuprea* larvae as non-tenebrionid beetles (cf. Fig. 1). Although *Anomala* PRIP has not been confirmed, considerable identity in the C-terminal peptide sequence of *T. castaneum* PRIP (Trica-1b; predicted) with that of *Bombyx* PRIP (Fig. 5B) prompted an immunocytochemical analysis using the *Bombyx* PRIP antibody. However, we could not obtain any reliable data supporting a putative PRIP at the rectal epithelia of *A. cuprea* larvae (data not shown), suggesting that non-tenebrionid beetles, such as *A. cuprea*, do not express the PRIP-type AQP in the rectum and that DRIP/PRIP coexpression for the vectorial water transport present in silkworms is not present in the fluid-transporting epithelia of *A. cuprea* larvae. This result might reflect that fact that these insects feed on the humus and survive in wet soil. The molecular physiological adaptations with a structural tissue organisation like that of the cryptonephric rectal complex may vary among the Coleoptera.

Aquaglyceroporin-like AQP in the midgut of *Anomala cuprea*

One of the first insect GLPs to be functionally characterized was AQP-Bom2 from *B. mori* larvae, expressed in the larval midgut and MT (not cryptonephric MT). AQP-Bom2 (GLP) showed sequence similarity with a different group of insect AQPs from the DRIP and PRIP subfamilies (see Fig. 5; Kataoka et al., 2009a; Kambara et al., 2009) and is classified into the structurally miscellaneous AQPs in the Group 3 (or GLP-like) clade, which appears as the sister group of DRIP, PRIP and BIB, are functionally heterogeneous (Herraiz et al., 2011). Recently, a second AQP gene (*ApAQP2*) from the pea aphid, *Acyrthosiphon pisum*, has been identified and categorised into Group 3, in which the members bear novel structures, including irregular NPA boxes (Wallace et al., 2012). Despite potential as a source of great insight into the structure and function of vertebrate GLPs, insect GLPs remain poorly characterised to date.

The members of the Group 3 subfamily include many predicted genes with higher diversity; however, we have observed similarities

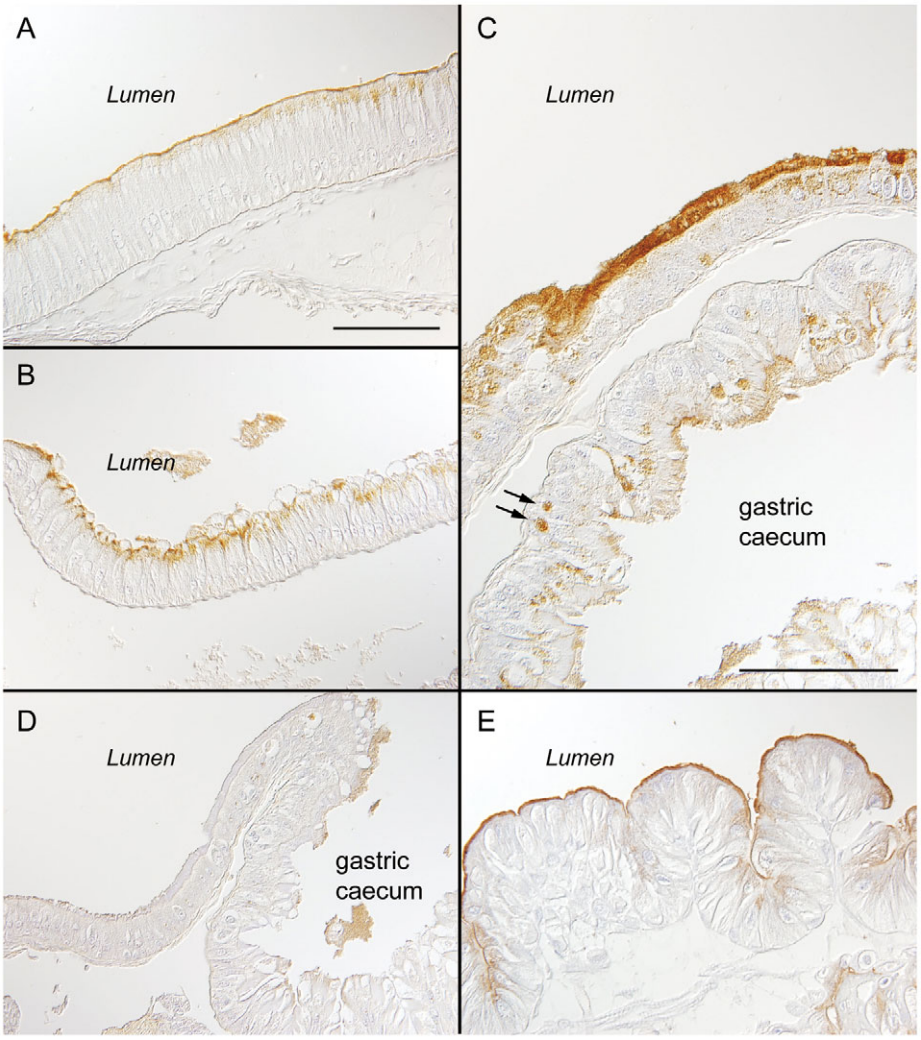

Fig. 6. Aquaglyceroporin homologue in the midgut and gastric caeca of *Anomala cuprea* larvae. Immunolocalisation with *Bombyx* GLP is almost exclusively present at the apical surface of the midgut epithelia. (A) Long, tall cells with flat surfaces. (B) Columnar cells with spherical blebs. Note that significant staining was observed in the cytoplasmic area underneath the apical plasma membrane (in A and B). (C) Columnar cells with brush-borders. The gastric caeca also showed positive signals, some of which were present in the pocket regions of the caecal epithelium (arrows). (D) Control staining using the *Bombyx* DRIP antibody. There was no positive staining in the midgut or gastric caecal epithelia. The contents (fixed humus, seen in B and D) in the lumen often stain as a brown colour. (E) In *B. mori* midgut epithelia (the posterior region), the *Bombyx* GLP antibody specifically stains the columnar cell apical membranes. Scale bars, (A,C) 100 µm. The scale bar in A applies to B, D and E.

in the loop D domain between AQP-Bom2 (GLP) and the predicted GLPs in *T. castaneum* (Trica-3a, 3b, 3c; Fig. 5). Immunocytochemistry using the *Bombyx* GLP antibody strongly suggests that the GLP-like protein is abundantly expressed at the apical plasma membranes of enterocytes in the midgut and gastric caeca of *A. cuprea* (Fig. 6). Although there is no information concerning a candidate gene for Group 3 AQPs in *A. cuprea*, it is reasonable to expect the existence of GLP-like AQP(s) with higher similarity to those in *T. castaneum*. Notably, the homologous genes in *Aedes aegypti* of Group 3 (*Aedae*-3a, 3b mRNA) are predominantly expressed in the midgut and gastric caeca of mosquito larvae (Marusalin et al., 2012). The corresponding loop D domain of *A. aegypti* also showed fair similarity (see Fig. 5B). Taken together, these data suggest that the Group 3 subfamily is primarily distributed in the absorptive, digestive and/or excretory region of the insect midgut, as previously reported in silkworm larvae (Kataoka et al., 2009a). It would be fascinating to determine whether the GLP-like AQPs in solid/plant feeders play a physiological role in transporting non-charged solutes, such as ammonia, urea and certain polyols, at the midgut with a much broader function. In contrast, the pea aphid GLP (*ApAQP2*; Group 3 member) is not expressed in the alimentary tract, but rather in the fat bodies of insects and the symbiont bacteria *Buchnera* (Wallace et al., 2012), potentially reflecting the unique dietary and nutritional habitat of these phloem sap feeders. The molecular and functional characterisation of the GLP-like AQPs and DRIPs in *A. cuprea* will provide further insight into water balance and fluid homeostasis throughout the alimentary tract of the chafer larvae that are present in the soil.

Water permeability in an insect gut: a perspective

Insects develop an open circulatory system, and their body plans are generally simple. Therefore, the fluid-transporting epithelia as a barrier strictly establish the osmotic gradients to regulate many solutes and water between the gut lumen and the circulating haemolymph. Evidence that AQP shows a ubiquitous tissue distribution in insects is accumulating steadily. The hindgut is a principal organ for water conservation, being composed of highly water-permeable epithelia enriched in Group 1 AQPs (DRIPs and PRIPs). Each AQP is separately observed in the cryptonephric rectal complex of *B. mori* (Azuma et al., 2012). At present, we have no evidence that some AQP molecules localise at the basal (haemocoel) side of the midgut epithelia (cf. Fig. 6E from *B. mori*). This implies that the midgut is less water-permeable than the hindgut (colon and rectum) and/or appears to transport preferentially other solutes such as glycerol, urea, etc., only at the apical (luminal) membranes. Water movement might be strictly regulated across the midgut epithelium. Although the physiological reality of Group 3 AQPs awaits further investigation in other insect species, Ishibashi et al. (Ishibashi et al., 2011) pointed to a different evolution of insect functional GLPs.

The actual water permeability of midgut *in vivo* and functional determinations of solute specificity in GLP members are of great interest for future research.

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AUTHOR CONTRIBUTIONS

T.N., S.M. and M.A. designed the research; T.N., S.M. and S.K. conducted the experiments; T.N., S.M. and M.A. interpreted and analysed the data; and T.N., S.M. and M.A. wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

No competing interests declared.

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