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 HgCl₂. 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
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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 transcellular movement of water across the excretory epithelia enabling the glutonous 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 larvae. 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 Pyrocotila 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 CaCl2 and MgCl2) 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.

**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 RNA 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′-CCG 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 (AqAQP) (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-Gr1) (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, 54°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′-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 hydrophathy 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 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 (598 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 pT7XJG-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 mACHINE T7 Kit (Ambion, Austin, TX, USA) following the manufacturer’s instructions.

Osmotic water permeability (*P*$_{\text{f}}$; ×10$^{-4}$ cm$^{-1}$ 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 μg 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*$_{\text{f}}$ 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*$_{\text{f}}$, 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 immunocytochemistry 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
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. AB713909). 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 water-transporting function of Anocu_AQP1 was evaluated through expression in Xenopus laevis 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 increase in sensitive cysteine residues to block the aqueous pore (Preston 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 GLP 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 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...
Aquaporin in the gut of chafer larvae

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.
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. 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.
Aquaporin in the gut of chafer larvae 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). 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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REFERENCES


Fig. S1. Nucleotide and deduced amino acid sequences of *Anomala cuprea* aquaporin protein (*Anocu* AQP1; accession no. AB741517). The initiation (ATG) and stop (*) codons are double-underlined. The six putative transmembrane domains deduced from the hydropathy analysis (see lower panel) are bold-underlined (I–VI). The consensus NPA motifs are indicated in grey and the putative polyadenylation signals (AATAAA) are indicated in red. The circle indicates a phosphorylation site for casein kinase II (Thr-123).

Lower panel: The hydrophobicity profile of *Anocu* AQP1 protein, calculated using Kyte–Doolittle with a window size of 10. The six transmembrane domains are indicated (I–VI), and two NPA motifs are marked with arrowheads.
Fig. S2. Western blot analyses of *Anomala cuprea* aquaporin protein using (A) the *Bombyx* DRIP and (B) *Bombyx* GLP antibodies. The IgG fraction was diluted (1:500) with 3% BSA dissolved in PBS. The SDS-PAGE was performed under the presence of 2 mol l⁻¹ urea in the stacking gels (4%) and 4 mol l⁻¹ urea in the separating gels (12%). The 35 kDa band (*) was non-specific, because it was detected with both antibodies.

### Supplemental Table S1: Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer direction</th>
<th>Sequence (5’ – 3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anocu</em> AQP1 cloning</td>
<td>Forward</td>
<td>GGD KGH CAC ATY AAY CCV GCS GTS AC</td>
<td>Degenerate PCR</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CC GAA WGW NCK RGC BGG RTT CAT RCT</td>
<td></td>
</tr>
<tr>
<td><em>Anocu</em> AQP1 RACE</td>
<td>Forward</td>
<td>GGT AAT CTC GGT GCA ACT GCT CC</td>
<td>3’-RACE cloning</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>C ACT TAA ATG GCA GGC GGT AAT AGC</td>
<td>5’-RACE cloning</td>
</tr>
<tr>
<td><em>Anocu</em> AQP1</td>
<td>Forward</td>
<td>GGA AGA TCT GGA CAT TTC ACT TTT CTG</td>
<td><em>Anocu</em> AQP1 subcloning</td>
</tr>
<tr>
<td>Xenopus</td>
<td>Reverse</td>
<td>GGA AGA TCT ATT TTA AAA ATC CAT TGA ATC</td>
<td>into pT7XβG2; <em>Bgl</em>II site</td>
</tr>
</tbody>
</table>

* The sequence of each primer is given with the nucleotides proceeding in the 5’ to 3’ direction. The underlined nucleotides represent restriction sites engineered into the primer sequences to facilitate cloning.

*b* The experiments utilizing each of the primers in the table are indicated.