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RESEARCH ARTICLE

Light exposure enhances urea absorption in the fluted giant clam, *Tridacna squamosa*, and up-regulates the protein abundance of a light-dependent urea active transporter, DUR3-like, in its ctenidium

Christabel Y. L. Chan[1], Kum C. Hiong[1], Mel V. Boo[1], Celine Y. L. Choo[1], Wai P. Wong[1], Shit F. Chew[2] and Yuen K. Ip[1,3,*]

ABSTRACT

Giant clams live in nutrient-poor reef waters of the Indo-Pacific and rely on symbiotic dinoflagellates (*Symbiodinium* spp., also known as zooxanthellae) for nutrients. As the symbionts are nitrogen deficient, the host clam has to absorb exogenous nitrogen and supply it to them. This study aimed to demonstrate light-enhanced urea absorption in the fluted giant clam, *Tridacna squamosa*, and to clone and characterize the urea active transporter DUR3-like from its ctenidium (gill). The results indicate that *T. squamosa* absorbs exogenous urea, and the rate of urea uptake in the light was significantly higher than that in darkness. The *DUR3-like* coding sequence obtained from its ctenidium comprised 2346 bp, encoding a protein of 782 amino acids and 87.0 kDa. *DUR3-like* was expressed strongly in the ctenidium, outer mantle and kidney. Twelve hours of exposure to light had no significant effect on the transcript level of ctenidial *DUR3-like*. However, between 3 and 12 h of light exposure, DUR3-like protein abundance increased progressively in the ctenidium, and became significantly greater than that in the control at 12 h. DUR3-like had an apical localization in the epithelia of the ctenidial filaments and tertiary water channels. Taken together, these results indicate that DUR3-like might participate in light-enhanced urea absorption in the ctenidium of *T. squamosa*. When made available to the symbiotic zooxanthellae that are known to possess urease, the absorbed urea can be metabolized to NH_3 and CO_2 to support amino acid synthesis and photosynthesis, respectively, during insolation.

KEY WORDS: Amino acid, Ammonia, Calcification, Nitrogen, *Symbiodinium*, Zooxanthellae

INTRODUCTION

Tropical waters are often referred to as 'deserts', characterized by poor nutrient content due mainly to the lack of overturn (de Goeij et al., 2013). To overcome the scarcity of nutrients, specialized tropical marine invertebrates, such as hard corals and giant clams, live in symbiosis with symbiotic dinoflagellates of the genus *Symbiodinium*, which are also known as zooxanthellae (Trench, 1987). Giant clams (Phylum: Mollusca, Family: Cardiidae, Subfamily: Tridacninae, Genus: *Tridacna* or *Hippopus*) are common inhabitants of coral reefs in the tropical Indo-Pacific. The host clam harbors symbiotic zooxanthellae (*Symbiodinium* clade A, C and D; LaJuenesse et al., 2004; Takabayashi et al., 2004; Hernawan, 2008; Lee et al., 2015) which live extracellularly in a branched tubular system surrounded by hemolymph (Norton et al., 1992). Zooxanthellae reside mainly inside the tiny tertiary tubules located below the surface of the fleshy and colorful outer mantle (Norton et al., 1992; Hiong et al., 2017b), where they conduct photosynthesis when light is available. More than 95% of the photosynthates produced by the zooxanthellae is donated to the host, accounting for a high percentage of the clam's energy requirements (Fisher et al., 1985; Klumpp et al., 1992). The donation of photosynthates from the symbionts to the host closes the nutrient gap in tropical waters. For that reason, giant clams can increase the rate of shell formation during insolation (Sano et al., 2012; Ip et al., 2017a) and maintain a high growth rate in nutrient-deficient tropical waters with the availability of sunlight (Lucas et al., 1989). Because of photosynthesis in the symbiotic zooxanthellae, attention has been devoted previously to inorganic carbon assimilation in giant clams (Rees et al., 1994; Baillie and Yellowlees, 1998; Leggat et al., 2002, 2005; Yellowlees et al., 2008). However, the growth of giant clams requires not only carbon but also nitrogen. While symbiotic zooxanthellae can fix molecular CO_2 into organic compounds (e.g. glucose and glycerol), they are not known to fix N_2; hence, they must obtain nitrogen from the host because of the lack of direct access to the ambient seawater. The *a priori* assumption is that the host clam absorbs inorganic and organic nitrogen from the ambient seawater and supplies them to its symbionts; however, there is currently a dearth of information on the molecular mechanisms of nitrogen uptake in the host clam.

Living organisms need nitrogen, which is a basic component of nucleic acids and amino acids that make up all proteins. The degradation of amino acids produces ammonia, which must be removed because of its toxicity (Campbell, 1991). Most free-living aquatic animals excrete ammonia as the major nitrogenous waste and are regarded as ammonotelic (Ip and Chew, 2010; Chew and Ip, 2014). By contrast, symbiotic cnidarians and giant clams can absorb and assimilate exogenous ammonia during insolation (Muscatine et al., 1979; Wilkerson and Muscatine, 1984; Wilkerson and Trench, 1986; Miller and Yellowlees, 1989). Zooxanthellae isolated from giant clams are nitrogen deficient and absorb ammonia and nitrate from the external medium (Wilkerson and Trench, 1986). For intact giant clam–zooxanthellae associations, the addition of inorganic

[1]Department of Biological Sciences, National University of Singapore, Kent Ridge, Singapore 117543, Republic of Singapore. [2]Natural Sciences and Science Education, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Republic of Singapore. [3]The Tropical Marine Science Institute, National University of Singapore, Kent Ridge, Singapore 119227, Republic of Singapore.

*Author for correspondence (dbsipyk@nus.edu.sg)

Y.K.I., 0000-0001-9124-7911

nitrogen to the ambient seawater enhances photosynthesis in the symbionts (Summons et al., 1986) and augments the growth rate of the host (Onate and Naguit, 1989; Hastie et al., 1992). Overall, the assimilation and recycling of nitrogen contribute to the success of symbiotic cnidarians and giant clams in nutrient-poor waters in the tropics.

Symbiotic invertebrates have access to multiple sources of nitrogen, as they can capture prey and absorb dissolved nutrients directly from the environment. In seawater, dissolved inorganic nitrogen is available in the form of ammonium, nitrite and nitrate, while dissolved organic nitrogen is available as urea and amino acids. Nitrate is one of the main N sources for phytoplankton; its concentration ranges between undetectable and 50 µmol N l^{-1} in oceanic waters, and up to 500 µmol N l^{-1} in coastal waters (Collos and Berges, 2003). Ammonium is generally present in small quantities (undetectable to 2 µmol N l^{-1}), except in polluted areas (up to 600 µmol N l^{-1}). Urea, with the chemical formula of $CO(NH_2)_2$, contains two nitrogen atoms, and is therefore a good nitrogen source for many species of phytoplankton. Its concentration ranges from undetectable to 1 µmol N l^{-1} in oceanic waters (Bronk, 2002; Painter et al., 2008) and up to 25 µmol N l^{-1} in coastal waters (Solomon et al., 2010). In reef environments, urea concentrations vary from <0.2 µmol N l^{-1} (Wafar et al., 1986) to 2.0 µmol N l^{-1} (Beauregard, 2004). Some lower organisms possess urease, an enzyme that hydrolyzes urea into ammonia and carbon dioxide, and can therefore utilize exogenous urea as a source of nitrogen. Although urea is available at seemingly low concentrations around coral reefs, it represents a significant amount of nitrogen in the seawater (Crandall and Teece, 2012). Urea in reef waters is derived primarily from bottom sediments and fishes schooling around seagrass beds. It has been established that hard corals can absorb urea from the external medium, and the rate of urea absorption can be enhanced by light (Grover et al., 2006). While both the host and zooxanthellae can absorb urea, the rate of urea uptake in the coral–zooxanthellae association (1028 µg urea mg^{-1} protein h^{-1}) is higher than that in the isolated zooxanthellae (728 µg urea mg^{-1} protein h^{-1}) (Barnes and Crossland, 1976). At present, no information is available on exogenous urea uptake in giant clams, although it is logical to hypothesize that they can do so in order to satisfy the nutritional requirement of the nitrogen-deficient symbionts.

It has been established that giant clams absorb and assimilate exogenous ammonia in the presence of light (Wilkerson and Trench, 1986; Fitt et al., 1993a). In fact, instead of excreting ammonia, giant clams have the ability to deplete seawater of inorganic nitrogen (Wilkerson and Trench, 1986). The rate of ammonia absorption in *Tridacna derasa* is 5–18 times faster in light than in darkness (Fitt et al., 1993b). Furthermore, the addition of ammonia and nitrate to the external medium augments pigmentation and division of zooxanthellae, and enhances the growth rate in giant clams (Hastie et al., 1992; Fitt et al., 1993a; Belda et al., 1993). It has been suggested that the major site of ammonia uptake and assimilation in giant clams is the ctenidium (gill), which is basically a respiratory organ located inside the mantle cavity (Rees et al., 1994; Hiong et al., 2017a). Recently, a *Glutamine Synthetase* (*GS*) gene of host (clam) origin has been cloned and characterized from the ctenidium of the fluted giant clam, *Tridacna squamosa* (Hiong et al., 2017a). Light exposure leads to significant increases in the expression levels of this ctenidial *GS* gene and GS protein, indicating an increase in the assimilation of the absorbed ammonia to glutamine. In addition, the ctenidium of *T. squamosa* apparently also participates in proton excretion and inorganic

carbon uptake, as light exposure also upregulates the gene and protein expression levels of ctenidial vacuolar-type H$^+$-ATPase subunit A (ATP6V1A; Ip et al. 2018), Na$^+$/H$^+$ Exchanger 3 (NHE3)-like (Hiong et al., 2017b) and Dual Domain Carbonic Anhydrase (DDCA; Koh et al., 2018). Hence, it is logical to hypothesize that the ctenidium could also take part in the absorption of urea from the external medium.

The hydrophobic phospholipid bilayer of biological membranes has a relatively low permeability to the highly hydrophilic urea molecule (Goodman, 2002). Nevertheless, transmembrane urea movement can be augmented by urea transporters, including facilitated urea transporters (UTs) and urea active (energy-dependent) transporters, which are found in all living organisms (Bankir, 2014). To date, several UTs have been cloned (Sands, 2002), and a few urea active transporters that can raise the concentration of intracellular urea above that of the medium have also been identified (Bankir, 2014). The most well established urea active transporter is DUR3, which has been characterized in bacteria (Navarathna et al., 2011), yeast (ElBerry et al., 1993), fungi (Morel et al., 2008; Abreu et al., 2010) and plants (Liu et al., 2003; Kojima et al., 2007; Wang et al., 2012). Considering the low concentrations of urea in reef waters, it is logical to hypothesize that the ctenidia of giant clams would express some sort of urea active transporter to augment urea absorption.

Therefore, the first objective of this study was to demonstrate light-enhanced urea uptake in *T. squamosa* by determining the rate of urea absorption in darkness or in light. The second objective was to clone and characterize a homolog of *DUR3* (*DUR3-like*) from the ctenidium of *T. squamosa*. The identity of *DUR3-like* and its host (animal) origin was confirmed by sequence similarity analysis. The gene expression of *DUR3-like* in various organs and tissues was examined to verify the ctenidium as the main site of expression. In addition, the effects of light and dark exposure on the expression levels of *DUR3-like* gene and DUR3-like protein in the ctenidium were determined to test the hypothesis that light would exert an up-regulatory effect to support light-enhanced urea uptake. Finally, immunofluorescence microscopy was performed to confirm that DUR3-like was localized to the apical membrane of the ctenidial epithelial cells, where it could engage in urea absorption from the ambient seawater.

MATERIALS AND METHODS
Giant clams
Adult *T. squamosa* Lamarck 1819 weighing 500±180 g (*N*=30) were purchased from Xanh Tuoi Tropical Fish, Ltd (Ho Chi Minh City, Vietnam), and maintained in the laboratory as described by Ip et al. (2015), but at 26±1°C. Research on giant clams did not require any institutional approval (National University of Singapore Institutional Animal Care and Use Committee).

Urea uptake experiments
Clams were maintained on a 12 h light: 12 h dark regime. For the urea uptake experiments, 10 individuals of *T. squamosa* were randomly selected and individually transferred in complete darkness to a clear container containing 8 volumes (volume:mass of clam) of artificial seawater with optimal aeration. The clams were acclimatized in darkness for 2 h prior to the addition of urea at a concentration of 50 µmol l^{-1}. The initial concentration of 50 µmol l^{-1} was higher than the urea concentration reported for reef waters (~2.2 µmol l^{-1}; Crandall and Teece, 2012), but was required to sustain the continued uptake of urea by the giant clam over a 6 h experimental period. Two minutes after the addition of

urea (time 0), 5 giant clams were exposed to light (80–85 µmol m^{-2} s^{-1}) while the remaining 5 giant clams were kept in darkness (control). Water was sampled at 0, 3, 4 and 6 h for urea assay. Preliminary experiments indicated that an initial 3 h period was needed to achieve a substantially detectable decrease in urea concentration in the external medium. The experiment was stopped at the 6th hour because the urea concentration would have decreased by ∼50%. Containers with only urea in artificial seawater were set up as blanks to verify the constancy of the urea concentration during the experimental period. Water samples were kept at 4°C, and urea analysis was performed within 1 week, following the method of Jow et al. (1999). The decrease in urea concentration in the external medium was expressed as percentage change with reference to the initial urea concentration, which displayed some variation (48–53 µmol l^{-1}), and the rate of urea absorption by the giant clam was expressed as µmol urea g^{-1} h^{-1}.

Experimental conditions for tissue sampling

In order to simulate the conditions in their natural habitat, parallel controls were not adopted in this study so that no giant clam was exposed to >12 h of darkness. Giant clams were anesthetized with 0.2% phenoxyethanol before they were killed. For molecular work, 5 giant clams were killed for tissue sampling at the end of a 12 h dark period (controls; $N=5$); another 15 giant clams ($N=5$ for each time point) were killed for tissue sampling after 3, 6 or 12 h of light exposure. Samples of the ctenidium, outer mantle, inner mantle, foot muscle, adductor muscle, kidney and hepatopancreas were dissected, blotted dry, frozen with aluminium tongs precooled in liquid nitrogen, and kept at −80°C until analysis. Separately, ctenidium samples from 4 other giant clams that had been exposed to light for 12 h and anesthetized in 0.2% phenoxyethanol were collected for immunofluorescence microscopy.

Gene sequencing and sequence analyses

Total RNA was extracted from the tissues of *T. squamosa* using TRI Reagent™ (Sigma-Aldrich, St Louis, MO, USA), purified by the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and quantified by a Shimadzu BioSpec-nano spectrophotometer (Shimadzu Corporation, Tokyo, Japan). RNA integrity was examined electrophoretically, and RNA was then used for cDNA synthesis using a RevertAid™ first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

A pair of primers (forward: 5′-GAYGARCAYAACCTRGAC-AC-3′; reverse: 5′-AWACAATACACCARGTYTTG-3′), designed based on the conserved regions of *Crassostrea gigas* DUR3 (XM_011436019.1), *Aplysia californica* DUR3-like (XM_013086007.1), *Octopus bimaculoides* DUR3-like (XM_014928571.1) and *Lingula anatine* DUR3-like (XM_013532760.1), was used to obtain a partial *DUR3-like* sequence from the ctenidium of *T. squamosa*. PCR and cloning were performed according to the methods described in Hiong et al. (2017a,b) with minor modifications. The cycling conditions were 94°C (3 min), followed by 35 cycles of 94°C (30 s), 55°C (30 s), 72°C (1.5 min) and 1 cycle of final extension at 72°C (10 min). Analyses of multiple clones of *DUR3-like* fragments did not reveal the presence of isoforms. Using 5′ and 3′ RACE (SMARTer™ RACE cDNA amplification kit, Clontech Laboratories, Mountain View, CA, USA) and a set of specific primers (forward: 5′-CAGTTACCGCAGTCAAGCTAACGCTC-3′; reverse: 5′-ACGACCTTGCTGCCAGATTGTCCA-3′), the complete cDNA sequence of *DUR3-like* was obtained. Sample preparation and sequencing were performed according to the methods of Hiong et al. (2017a,b). Sequences were assembled using BioEdit

version 7.2.5 (Hall, 1999), and the cDNA sequence of *DUR3-like* (accession number MF073181) was deposited in GenBank.

The *DUR3-like* nucleotide sequence was translated into the DUR3-like amino acid sequence using the ExPASy Proteomic server (http://web.expasy.org/translate/). The transmembrane regions (TMs) were identified using MEMSAT3 and MEMSAT-SVM provided by the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/). DUR3-like of *T. squamosa* was aligned and compared with selected DUR3 or DUR3-like sequences from various animals using BioEdit. A sequence similarity table was generated to confirm the identity of DUR3-like from *T. squamosa*.

Gene expression of *DUR3-like* in various tissues/organs

The mRNA expression of *DUR3-like* in various tissues/organs of *T. squamosa* was examined through PCR using a set of gene-specific primers (forward: 5′-GCCTTATCTACGGTATTGTGCTC-3′; reverse: 5′-TAGAAGACTTAGACTCCGCCCT-3′). The PCR reaction was performed in a total volume of 10 µl using DreamTaq polymerase (Thermo Fisher Scientific) with the following cycling conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were separated by 1% agarose gel electrophoresis.

Determination of transcript level by quantitative real-time PCR (qPCR)

A StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) was used to perform the absolute quantification of *DUR3-like* transcripts by qPCR following the methods of Hiong et al. (2017a,b). Using the RevertAid™ first-strand cDNA synthesis kit, cDNA (4 µg) was synthesized from total RNA with random hexamer primers. The specific qPCR primers used were forward: 5′-ATTATCCTCTGCTGTCCGCC-3′ and reverse: 5′-CATTCCCG-CTCCTCATCGT-3′. The amplification efficiency was 98.1%. Although we performed absolute quantification of *DUR3-like* transcripts, a pair of specific qPCR primers (forward: 5′-GTGCC-AAAGGATGTCAATGTC-3′; reverse: 5′-CTTAGCCATATCTC-CGCCTG-3′) was designed to quantify the transcript level of α-tubulin as the reference gene; the aim was to demonstrate the constant transcript level of the reference gene throughout the 12 h of light exposure as compared with the control.

Antibodies and immunoblotting

Based on the epitope sequence of LRQNRAESKSSREM that corresponded to residues 769–782 of DUR3-like of *T. squamosa*, a rabbit polyclonal anti-DUR3-like antibody was custom-made by GenScript (Piscataway, NJ, USA). The anti-α-tubulin 12G10 antibody was produced by the Developmental Studies Hybridoma Bank of the Department of Biological Sciences in the University of Iowa.

For immunoblotting, protein extraction and SDS-PAGE were performed according to the methods of Hiong et al. (2017b) with minor modifications. The samples were not heated before electrophoresis. Proteins (100 µg) were separated by SDS-PAGE, and then transferred electrophoretically onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were first blocked at 25°C with Pierce Fast Blocking Buffer (Thermo Fisher Scientific) for 15 min, then with Superblock® Blocking Buffer (Thermo Fisher Scientific) for another 15 min. Immunoblotting was performed with Pierce Fast Western Blot kit, SuperSignal® West Pico Substrate (Thermo Fisher Scientific). The optimized

concentration of the anti-DUR3-like antibody was 2.5 µg ml^{-1}, and that of the anti-α-tubulin antibody was 0.05 µg ml^{-1}. The identity of the DUR3-like band was validated by a peptide competition test, whereby the anti-DUR3-like antibody (25 µg) was incubated with the immunizing peptide (125 µg) provided by GenScript for 1 h at 25°C. Bands were visualized by chemiluminescence using X-ray films (CL-XPosure™ Film, Thermo Fisher Scientific). The immunoblot images were digitized and quantified densitometrically following the methods of Hiong et al. (2017b). The protein abundance of DUR3-like was presented as the optical density of the DUR3-like band normalized with that of the α-tubulin band.

Immunofluorescence microscopy

The subcellular localization of DUR3-like in the ctenidial epithelial cells was performed by immunofluorescence microscopy as described previously (Hiong et al., 2017b), using the custom-made anti-DUR3-like antibody (2.5 µg ml^{-1}) and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (2.5 µg ml^{-1}; Life Technologies Corporation, Carlsbad, CA, USA). To validate the specificity of the anti-DUR3-like antibody, a peptide competition test was performed by incubating the anti-DUR3-like antibody with the immunizing peptide as described in 'Antibodies and immunoblotting', above. Images were examined using an Olympus BX60 epifluorescence microscope and DP73 digital camera (Olympus Corporation, Tokyo, Japan), and were acquired under optimal exposure settings (300–500 ms) using cellSens software (Olympus Corporation). Differential interference contrast (DIC) images were obtained to define gross tissue structure and tissue orientation.

Statistics

Results were statistically analyzed using SPSS Statistics software, version 19 (IBM Corporation, Armonk, NY, USA). The homogeneity of variance was examined by Levene's test. Differences among means were evaluated by one-way analysis of variance (ANOVA). Depending on the homogeneity of variance, the *post hoc* test used was either Tukey's or Dunnett's T3 test. Statistical significance was set at $P<0.05$.

RESULTS

Rates of urea absorption in darkness or in light

The urea concentration in seawater without giant clams remained unchanged for 6 h, but that in containers with *T. squamosa* exposed to darkness or light decreased almost linearly with time (Fig. 1A). These results indicate that *T. squamosa* could absorb urea from the external medium, and the rate of urea absorption during the first 3 h was significantly higher (~1.6-fold; $P<0.05$) in light than in darkness (Fig. 1B). The lack of significant changes in the rate of urea absorption at 4 and 6 h could be attributable to the resulting decreases in urea concentration in the external medium. Overall, the average rate of urea absorption over the entire 6 h experimental period in clams exposed to light (0.045±0.013 µmol urea g^{-1} h^{-1}, $N=5$) was significantly higher than that in clams exposed to darkness (0.029±0.005 µmol urea g^{-1} h^{-1}, $N=5$).

Nucleotide sequence, deduced amino acid sequence and molecular characterization of *DUR3-like*/DUR3-like

The complete cDNA coding sequence (2346 bp) of *DUR3-like* obtained from the ctenidium of *T. squamosa* has been deposited in GenBank (accession no. MF073181). The sequence coded for a protein of 782 amino acid residues with an estimated molecular
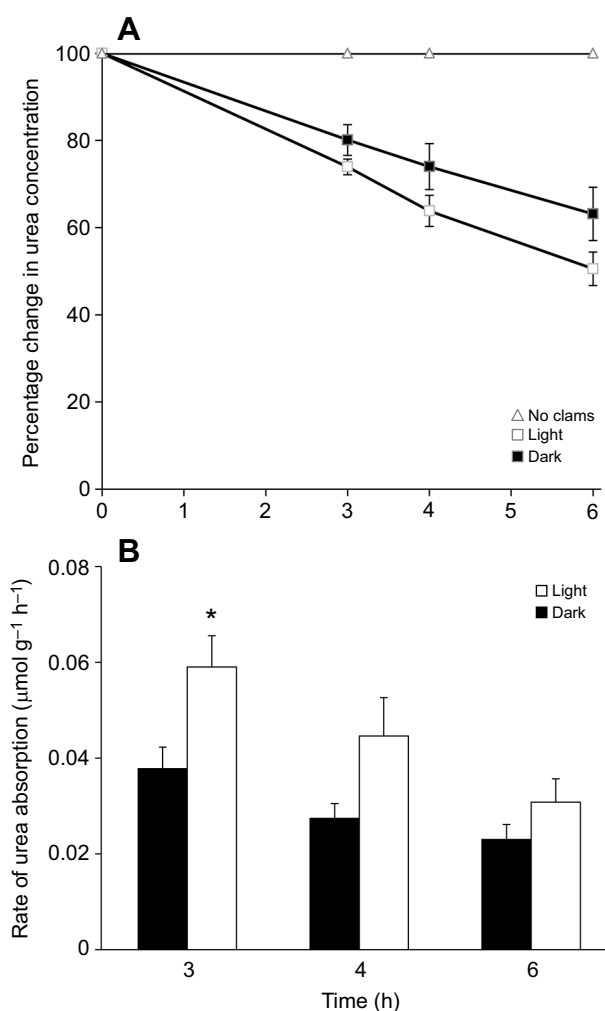

Fig. 1. Effects of light on the absorption of urea in *Tridacna squamosa*. (A) The percentage change in urea concentration in seawater without clams, or with clams, exposed to light or darkness (control) for 6 h. (B) The rate of urea absorption in *T. squamosa* during 6 h of exposure to darkness (control) or light. Results represent means±s.e.m. ($N=5$). *Significantly different from clams kept in darkness ($P<0.05$).

mass of 87.0 kDa. The deduced DUR3-like of *T. squamosa* had the highest similarity with sequences of urea active transporter from mollusk species (56.1–69.5%), followed by those of echinoderms and chordates (42.4–55.1%; Table 1). However, it had low similarity with urea active transporter from plants (38.2–46.9%), and displayed the lowest similarity to Urea Active Transporter A and Urea Active Transporter B of the single-celled alga *Chlamydomonas reinhardtii* (38.2–40.6%). These results confirmed that the DUR3-like obtained from the ctenidium of *T. squamosa* had a host (animal) origin.

A multiple sequence alignment of DUR3-like from *T. squamosa* with sequences of urea active transporter from several organisms obtained from GenBank (DUR3 of *Aspergillus nidulans*, DUR3-like of *Pyropia yezoensis* and the predicted urea–proton symporter DUR3-like of *Crassostrea gigas*) revealed a number of highly conserved amino acid residues (Fig. 2). Notably, residues (W131, T180, D335, Y440 and W555; numbered according to DUR3-like of *T. squamosa* in Fig. 2) involved in urea binding, and possibly translocation of urea in DUR3 of *A. nidulans*, were conserved in DUR3-like of *T. squamosa* (Sanguinetti et al., 2014). Residues involved in protein folding and structure (G216, P757, G146 and

Table 1. Percentage similarity between the deduced amino acid sequence of DUR3-like from *Tridacna squamosa* and sequences of urea active transporters from other species obtained from GenBank

Phylum	Species (accession number)	Protein	Similarity (%)
Mollusks	*Crassostrea gigas* (XP_011447729.1)	Predicted urea-proton symporter DUR3-like	69.5
	Crassostrea gigas (EKC42095.1)	Putative urea active transporter 1	60.7
	Aplysia californica (XP_012934693.1)	Solute carrier 5- and 6-like	57.9
	Octopus bimaculoides (XP_014784057.1)	Urea-proton symporter DUR3-like	56.1
Echinoderms	*Strongylocentrotus purpuratus* (XP_011662595.1)	Urea-proton symporter DUR3	55.1
Brachiopods	*Lingula anatina* (XP_013388214.1)	Urea-proton symporter DUR3-like	55.8
Hemichordata	*Saccoglossus kowalevskii* (XP_006823326.1)	Urea-proton symporter DUR3-like	54.7
Chordata	*Branchiostoma belcheri* (XP_019622723.1)	Urea-proton symporter DUR3-like	51.9
	Branchiostoma floridae (XP_002611485.1)	Solute carrier 5- and 6-like	45.6
Arthropods	*Limulus polyphemus* (XP_013780777.1)	Solute carrier 5-like	42.4
Plantae	*Chondrus crispus* (XP_005716890.1)	Urea active transporter-like protein 1	46.9
	Nelumbo nucifera (XP_010255736.1)	Predicted urea-proton symporter DUR3	42.2
	Brassica rapa (XP_009128785.1)	Predicted urea-proton symporter DUR3	41.5
	Chlamydomonas reinhardtii (EDO97038.1)	Urea active transporter A	40.6
	Chlamydomonas reinhardtii (EDO97039.1)	Urea active transporter B	38.2

Sequences are arranged in a descending order of similarity.

R188) were also highly conserved. DUR3-like of *T. squamosa* had 15 TMs according to the PSIRED MEMSAT-SVM server (Nugent and Jones, 2009). An analysis using the Conserved Domain Database (Marchler-Bauer et al., 2005) indicated that DUR3-like from *T. squamosa* had conserved domains corresponding to the sodium solute carrier 5- and 6-like families, with five predicted sodium-binding residues (S127, L130, A422, S425 and T426).

Gene expression of *DUR3-like* in various tissues/organs
In *T. squamosa*, the strongest expression of *DUR3-like* was detected in the ctenidium, followed by the kidney and outer mantle (Fig. 3). In comparison, *DUR3-like* was weakly expressed in the inner mantle, adductor muscle and hepatopancreas, and undetectable in the foot muscle (Fig. 3).

Effects of light on expression levels of *DUR3-like*/DUR3-like in the ctenidium
The transcript level of *DUR3-like* in the ctenidium of *T. squamosa* remained statistically unchanged throughout the 12 h of light exposure, despite being slightly higher at 3 h compared with the control (Fig. 4). There was no significant change in the transcript level of α-tubulin throughout the 12 h of light exposure (results not shown).

Immunoblotting revealed a band of interest at 95 kDa, which was close to the estimated molecular mass of 87 kDa (Fig. 5A). Results from the peptide competition assay supported the identity of this band as DUR3-like. Between 3 and 12 h of light exposure, there was a progressive increase in the protein abundance of ctenidial DUR3-like, which became significantly higher (by ∼8-fold) than the control value at 12 h (Fig. 5B).

Subcellular localization of DUR3-like in the ctenidium
DUR3-like was immunolocalized to the apical epithelium of the ctenidial filaments of *T. squamosa* (Fig. 6A). In addition, almost all the epithelial cells surrounding the tertiary water channels displayed apical DUR3-like immunofluorescence (Fig. 6B). The validity of the immunofluorescence labeling of DUR3-like was validated by the peptide competition test (Fig. 7).

DISCUSSION
Light-enhanced urea absorption in *T. squamosa*
In giant clams, the host clam absorbs inorganic nitrogen, mainly as ammonia, and supplies it to the symbiotic zooxanthellae, which are

nitrogen deficient (Wilkerson and Trench, 1986). In addition to ammonia, urea can also act as a source of nitrogen for some symbiotic invertebrates (Barnes and Crossland, 1976; Grover et al., 2006). Our results reveal for the first time that *T. squamosa* can absorb urea from the external medium, and the rate of urea absorption was higher in light than in darkness. Hence, exogenous urea may be an important source of organic nitrogen to the giant clam–zooxanthellae association, and the host clam must logically possess some sort of urea active transporter because of the low concentration of exogenous urea.

Only a few urea active transporters have been cloned, mainly from unicellular organisms (ElBerry et al., 1993; Mills et al., 1998; Valladares et al., 2002). Although the vectorial transport of urea across epithelia of higher organisms has been well characterized functionally (Katz et al., 1981; Beyer and Gelarden, 1988; Sands et al., 1996b; Zanin et al., 2014), none of the related urea active transporters have been identified. In marine elasmobranchs, which retain urea for osmoregulatory purposes, active urea absorption is known to occur in the intestine and kidney (Bankir, 2014), but the associated urea active transporters have not been cloned or characterized. In mammals, active urea transport may be involved in urea secretion in the proximal tubule of the kidney (Sands et al., 1996a; Bankir, 2014), but again no urea active transporter has been cloned. Functional characterization of these transport processes indicates that, in some cases, urea transport is Na$^+$ dependent.

Molecular characterization of DUR3-like from *T. squamosa*
This is the first report on the complete coding sequence and characterization of DUR3-like from an invertebrate. DUR3-like of *T. squamosa* consisted of 15 predicted TMs, differing from members of the UT family, which are characterized by 10 TMs (Levin and Zhou, 2014). Unlike the urea-binding ABC transporters and Bra proteins of bacteria (Hoshino and Kose, 1990; Valladares et al., 2002), DUR3-like of *T. squamosa* did not contain any ATP-binding motifs. DUR3-like of *T. squamosa* was confirmed to be a urea transporter, as residues W131, D335 and W555 are known to be involved in urea binding and recognition in DUR3 of *Aspergillus nidulans* (Sanguinetti et al., 2014). Residues W131 and D335 were located in the transmembrane regions, while W555 was found in a small extracellular region. These urea-binding residues could be involved in different stages of the translocation process, whereby W555 could bind to an exogenous urea molecule and channel it into

Fig. 2. A multiple sequence alignment of *T. squamosa* DUR3-like with *Aspergillus nidulans* DUR3 (ACZ62639.1), *Pyropia yezoensis* DUR3-like (BAU04114.1) and *Crassostrea gigas* DUR3-like (XM_020074166.1). Identical or similar residues are indicated by shading. Asterisks indicate residues involved in urea binding. Open triangles indicate residues involved in protein structure and folding. Filled triangles indicate residues involved in the Na⁺-binding site. The transmembrane regions (TM1 to TM15) predicted from PSIPRED using the MEMSAT-SVM server are marked by red boxes. The region corresponding to the solute carrier 5- and 6-like families as predicted by the Conserved Domain Database of the National Center for Biotechnology Information (NCBI) is underlined.

the transporter pore. Furthermore, it has been established that residues T180 and W555 are involved in substrate (urea) selectivity, while W555 can also function as an extracellular filter gate (Sanguinetti et al., 2014).

Based on the Conserved Domain Database (Marchler-Bauer et al., 2005), DUR3-like of *T. squamosa* contained characteristic

domains of the solute carrier families 5 (SLC5) and 6 (SLC6). In human, members of SLC5 are known to be Na⁺/glucose cotransporters, while those of SLC6 are described as Na⁺- and Cl⁻-dependent/solute symporters (Turk and Wright, 2004). Although the sequences selected for alignment with DUR3-like of *T. squamosa* (Fig. 2) had been characterized as urea/H⁺ symporters
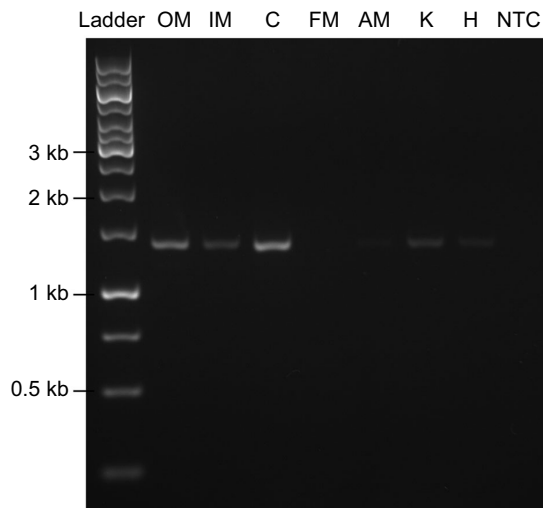
Fig. 3. mRNA expression of *DUR3-like* in *T. squamosa* kept in darkness for 12 h. OM, outer mantle; IM, inner mantle; C, ctenidium; FM, foot muscle; AM, adductor muscle; K, kidney; H, hepatopancreas; NTC, no-template control.

(Sanguinetti et al., 2014; Kakinuma et al., 2016), they also contained conserved domains of SLC5 and SLC6 members. In fact, similar to DUR3-like of *T. squamosa*, all of them apparently contained Na^+-binding sites according to the Conserved Domain Database. Specifically, the Na^+-binding site of DUR3-like of *T. squamosa* comprised residues S127, L130, A422, S425 and T426 (Fig. 2). Hence, DUR3-like of *T. squamosa* could actually be a secondary active transporter using the Na^+ motive force to drive the active uptake of urea (Jung, 2002). Furthermore, residues R188 and G216 involved in maintaining the structure of DUR3-like of *T. squamosa* in the plasma membrane are also highly conserved in the Na^+/solute symporter family of transporters (Sanguinetti et al., 2014). Taken together, DUR3-like of *T. squamosa* could be a secondary active transporter that utilizes the electrochemical potential gradient of Na^+ to drive the active uptake of urea.
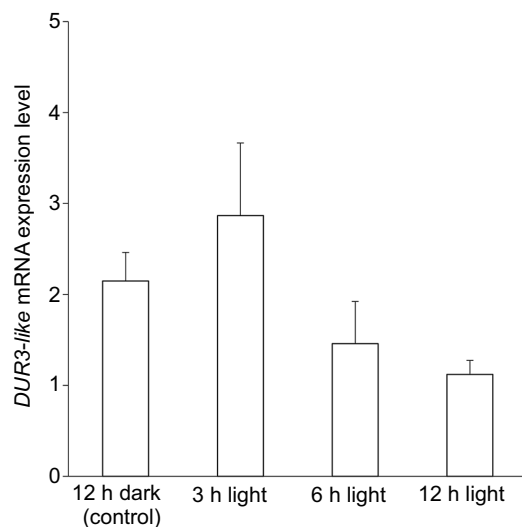

Fig. 4. mRNA expression level of *DUR3-like* in the ctenidium of *T. squamosa* kept in darkness for 12 h (control) or exposed to light for 3, 6 or 12 h. Data are expressed as $\times 10^2$ copies of transcripts per ng of total RNA. Results represent means±s.e.m. (*N*=4).
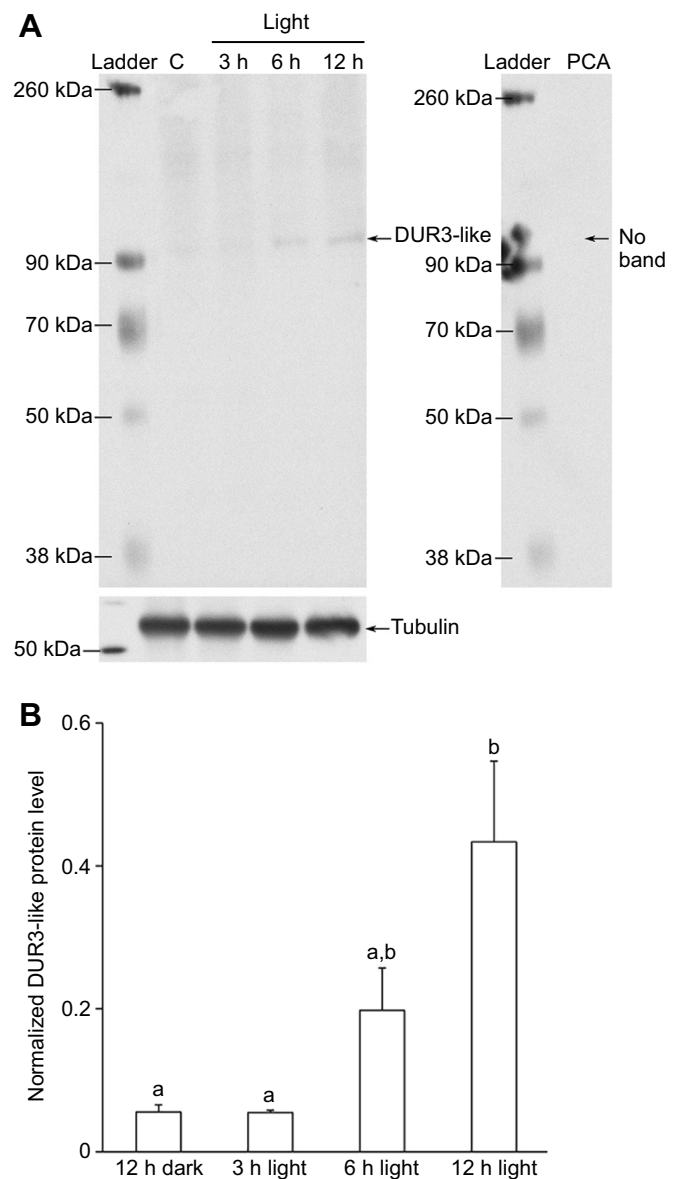

Fig. 5. Protein abundance of DUR3-like in the ctenidium of *T. squamosa* kept in darkness for 12 h (control) or exposed to light for 3, 6 or 12 h. (A) Left, example immunoblot of DUR3-like and tubulin. C, control (12 h dark). Right, immunoblot of DUR3-like (12 h light exposure) using antibody neutralized with the immunizing peptide (peptide competition assay, PCA). (B) DUR3-like protein level. The optical density of the DUR3-like band for a 100 µg protein load was normalized to tubulin. The results represent means ±s.e.m. (*N*=4). Means not sharing the same letter are significantly different from each other (*P*<0.05).

DUR3-like of *T. squamosa* is expressed strongly in the ctenidium, where it is localized apically in the ctenidial epithelium

A priori, the pattern of gene expression of *DUR3-like* in various tissues/organs might provide clues to the physiological functions of DUR3-like in *T. squamosa*. In *T. squamosa*, *DUR3-like* was strongly expressed in the ctenidium. The ctenidium has a large surface area to volume ratio as it consists of many filaments and tertiary water channels (Norton and Jones, 1992), and is known to take part in the absorption of ammonia and phosphate (Fitt et al., 1993a; Rees et al., 1994). Hence, it is probable that the ctenidial
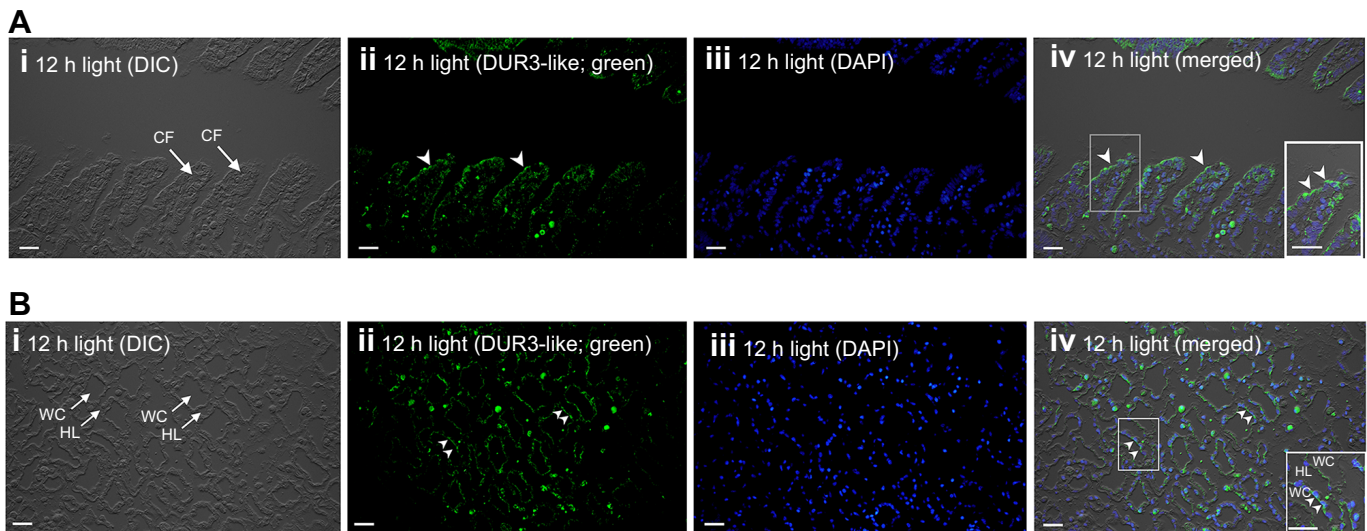
Fig. 6. Immunofluorescence localization of DUR3-like in *T. squamosa*. (A) Immunofluorescence localization of DUR3-like in the ctenidial filaments (CFs) of the ctenidium of *T. squamosa* exposed to 12 h of light. (i) The structure of the CFs is revealed through differential interference contrast (DIC) microscopy. (ii, iii) The green immunofluorescence indicates the presence of DUR3-like (ii) while blue represents DAPI nuclear staining (iii). (iv) The composite image of ii and iii overlaid with the DIC image. Arrowheads mark DUR3-like immunostaining of the apical membrane of the epithelial cells of the CF. Reproducible results were obtained from four individual clams. Scale bars: 20 μm. (B) Immunofluorescence localization of DUR3-like in the tertiary water channels (WCs) of the ctenidium of *T. squamosa* exposed to 12 h of light. (i) The lattice formation of WCs in the ctenidium is demonstrated in the DIC image. (ii, iii) The green immunofluorescence indicates the presence of DUR3-like (ii) while blue represents DAPI nuclear staining (iii). (iv) The composite image of ii and iii overlaid with the DIC. Arrowheads mark DUR3-like immunostaining of the apical membrane of the epithelial cells lining the WC. HL, hemolymph. Reproducible results were obtained from four individual clams. Scale bars: 20 μm.

DUR3-like participates in the uptake of exogenous urea. Indeed, the apical localization of DUR3-like in the epithelial cells of the ctenidial filaments and water channels confirms that it is positioned to transport urea between the ambient seawater and the cytoplasm of the ctenidial epithelial cells.

Expression of DUR3-like in the ctenidium is light dependent

Light exposure had no significant effect on the transcript level of *DUR3-like* but led to a significant increase in the protein abundance

of DUR3-like in the ctenidium of *T. squamosa*. Hence, unlike ctenidial GS (Hiong et al., 2017a) and NHE3-like (Hiong et al., 2017b), DUR3-like was regulated predominantly at the translational level. The increase in protein abundance of DUR3-like in response to light corroborates the phenomenon of light-enhanced urea absorption in *T. squamosa*. Besides DUR3-like, several other transporters/enzymes of *T. squamosa* also display light-dependent gene and protein expression. They include GS (Hiong et al., 2017a), NHE3-like (Hiong et al., 2017b) and DDCA (Koh et al., 2018) in
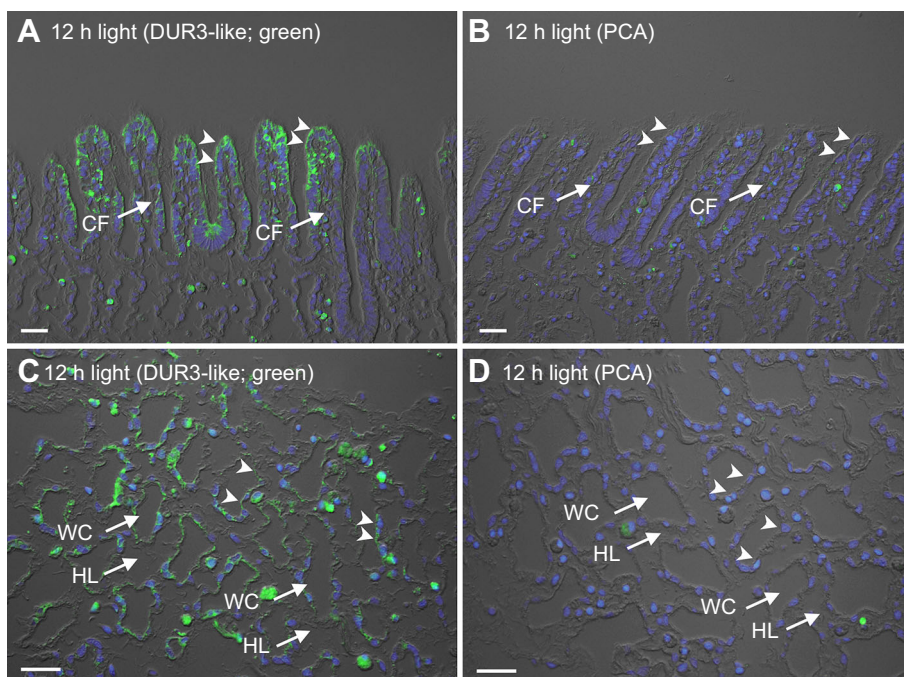

Fig. 7. The validation of DUR3-like immunostaining of the ctenidium of *T. squamosa* by a PCA. DUR3-like immunofluorescence in ctenidial filaments (CFs) and tertiary water channels (WCs) exposed to 12 h of light using the normal anti-DUR3-like antibody (A,C) or anti-DUR3-like antibody neutralized with the immunizing peptide (PCA in B,D). DUR3-like immunofluorescence is shown in green and DAPI nucleic staining is shown in blue in the DIC image. Arrowheads in A mark DUR3-like immunostaining of the apical membrane of the epithelial cells in the CFs as compared with the lack of DUR3-like immunostaining with the PCA in B. Arrowheads in C mark DUR3-immunostaining of the apical membrane of the epithelial cells surrounding the WCs as compared with the lack of DUR3-like-immunostaining with the PCA in D. HL, hemolymph. Scale bars: 20 μm.

the ctenidium, the plasma membrane Ca^{2+}-ATPase (Ip et al., 2017a) and the Na^+/K^+-ATPase α-subunit (Boo et al., 2017) in the inner mantle, and carbonic anhydrase 2-like (Ip et al., 2017b) in the outer and inner mantle. Overall, it would appear that *T. squamosa* has acquired a general light-dependent mechanism to coordinate the expression levels of a variety of enzymes and transporters in relation to various diurnally light-dependent physiological processes, which include inorganic carbon absorption, ammonia absorption and assimilation, and calcification. However, daily changes in the protein expression level of DUR3-like are energy intensive. So, why would *T. squamosa* depend on transcriptional and/or translational changes to regulate DUR3-like and other transporters/enzymes in response to light? The reason could be related to the autotrophic nature of *T. squamosa* as a clam–zooxanthellae association. Notably, the host's daily energy and growth requirements can be satisfied fully by the photosynthates donated by its symbionts (Muscatine et al., 1983; Fisher et al., 1985; Edmunds and Davies, 1986; Davies, 1991; Klumpp et al., 1992; Klumpp and Griffith, 1994; Hawkins and Klumpp, 1995).

What happens to the urea absorbed by the host clam?

Animals, with the exception of *Aplysia californica* (Pedrozo et al., 1996), do not possess enzymes to catabolize urea. Hence, the urea absorbed by *T. squamosa* is probably transported through the hemolymph and tubular fluid to the symbiotic zooxanthellae, which reside mainly in the extensible outer mantle. In fact, urea is an excellent source of nitrogen for many free-living algae (Naylor, 1970), and DUR3 is known to be expressed in the green alga *Chlamydomonas reinhardtii* (de Michele et al., 2012). Algae can metabolize urea, and they express two different types of urea-degrading enzymes: urease and ATP–urea amidolyase. Urease releases NH_3 and CO_2 from urea in a one-step reaction, while urea amidolyase degrades urea to NH_3 and CO_2 through a two-step process (Bekheet and Syrett, 1977; Solomon and Gilbert, 2008). However, these two enzymes are not known to be present in the same algal species (Al-Houty and Syrett, 1983). While all members of Chlorophyceae contain ATP–urea amidolyase, members of other algal classes contain urease (Leftley and Syrett, 1973).

Analyses of the two available *Symbiodinium* genome databases for three different clades (http://palumbi.stanford.edu/data/, Ladner et al., 2012; http://smic.reefgenomics.org/download, Aranda et al., 2016) confirm that all consist of *Dur3* and *Urease*, but not *ATP–Urea Amidolyase*. Hence, it is probable that symbiotic zooxanthellae in *T. squamosa* can absorb urea from the tubular fluid through their own algal *Dur3*, and then hydrolyze urea to NH_3 and CO_2 by urease. In the light, the CO_2 released can be utilized by ribulose-1,5-bisphosphate carboxylase/oxygenase in the plastids during photosynthesis. Separately, NH_3 can combine with H^+ to form NH_4^+, and NH_4^+ can enter the glutamate synthase cycle (van den Heuvel et al., 2004), which is present in the zooxanthellae of *T. squamosa* (Fam et al., 2018), for the synthesis of glutamate and other amino acids. Subsequently, some of the carbohydrates and amino acids produced by the zooxanthellae can be donated to the host to support its growth and metabolism.

Why would *T. squamosa* evolve uniquely to absorb urea and express DUR3-like in its ctenidium?

Urea is normally an excretory nitrogenous waste in animals, and most animals possess transporters to facilitate urea excretion. As animals cannot metabolize urea, there is no good reason for aquatic animals to absorb it from the external medium. However, *T. squamosa* is capable of light-enhanced urea absorption, and its

ctenidium expresses a DUR3-like of animal origin. This uncommon phenomenon could be a result of the selective advantage of symbiosis in the giant clam–zooxanthellae association. The degradation of the absorbed urea and the utilization of the resulting NH_3 and CO_2 are only feasible through the collaboration between the host clam and its symbiotic zooxanthellae, as the latter possess the enzyme for urea degradation. Unlike inorganic ammonia, each mole of urea comprises two moles of nitrogen and one mole of carbon. Upon degradation, urea can provide not only NH_3 to support amino acid metabolism but also CO_2 to sustain photosynthesis in the symbiotic zooxanthellae. Hence, despite urea being present at relatively low concentrations in tropical reef waters (Crandall and Teece, 2012), it would be advantageous to symbiotic invertebrates, including giant clams and hard corals, to acquire mechanisms (e.g. urea active transporters) to absorb exogenous urea. More importantly, these mechanisms must display light-dependent properties to enable the host to respond to light in synchrony with the photosynthetic activity of its symbionts.

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