

Possible involvement of Tachylectin-2-like lectin from *Acropora tenuis* in the process of *Symbiodinium* acquisition

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Abstract Most reef-building corals in tropical and subtropical areas symbiose with microalgae from the genus *Symbiodinium* (dinoflagellate) and depend on the photosynthate produced by the microalgae. The majority of corals acquire *Symbiodinium* from the surrounding environment through horizontal transfer, but the molecular mechanisms involved in the acquisition of *Symbiodinium* remain unknown. It has been hypothesized that carbohydrate-binding proteins, or lectins, of the host coral recognize cell surface carbohydrates of *Symbiodinium* in the process of acquiring symbionts. Thus, we examined the molecular mechanisms involving lectins and carbohydrates using model organism *Acropora tenuis*, a common reef-building coral, and *Symbiodinium* culture strains. Juvenile polyps acquire more cells of *Symbiodinium* strain NBRC102920 at 72–96 h of metamorphosis induction than in any other period. Glycosidase treatment of *Symbiodinium* inhibited the acquisition of *Symbiodinium* by juvenile coral polyps. The presence of carbohydrates D-galactose,

N-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine at 10 mM also tended to decrease *Symbiodinium* acquisition. We isolated two *N*-acetyl-D-galactosamine binding lectins with apparent molecular masses of 14.6 and 29.0 kDa from *A. tenuis*, and de novo sequencing and cDNA cloning showed that the 29.0 kDa protein is Tachylectin-2-like lectin (AtTL-2). The anti-Tachylectin-2 antibody is suggested to bind specifically to AtTL-2. The antibody also inhibited binding of AtTL-2 to *N*-acetyl-D-galactosamine-resin and the acquisition of *Symbiodinium* by juvenile *A. tenuis* polyps. Based on these results, AtTL-2 is likely involved in the process of *Symbiodinium* acquisition.

Keywords *N*-acetyl-D-galactosamine · *Acropora tenuis* · Coral · Lectin · *Symbiodinium* · Symbiosis

Introduction

Reef-building corals thrive in tropical and subtropical areas and grow in symbiosis with dinoflagellates in the genus *Symbiodinium*. *Symbiodinium* have been found in numerous hosts, including a jellyfish, a clam, and a sea slug [1]. Since these dinoflagellates have very similar morphologies, *Symbiodinium* were classified into phylogenetic clades A through I based on analyses of ribosomal DNA (rDNA) and chloroplast 23S rDNA [2–5]. *Symbiodinium* strains isolated and cultured from hosts exhibited diurnal changes in morphology, changing from a flagellated motile form to a non-motile coccoid form [6]. The morphology of the coccoid cells is similar to that of *Symbiodinium* found within the host. The symbiosis between corals and *Symbiodinium* is essential for the survival of coral, as more than 90 % of the symbiont photosynthate is used by the coral [7]. Bleached corals, which have either lost their *Symbiodinium*, or which

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have *Symbiodinium* that are losing pigments, eventually die [8, 9].

Corals acquire *Symbiodinium* from the surrounding environment (horizontal transmission) or directly from the parent (vertical transmission); about 85 % of corals acquire *Symbiodinium* by horizontal transmission [10]. The molecular mechanisms involved in the acquisition of *Symbiodinium* remain unknown, but some cues for their acquisition have been identified. *Symbiodinium* modified by digestive enzymes, such as glycosidase and proteinase, or lectin treatments, showed reduced acquisition rates by hosts *Aiptasia pulchella* and *Fungia scutaria* [11, 12]. The results of these studies suggest that carbohydrates on the *Symbiodinium* cell surface play an important role in symbiont acquisition by the host.

Lectins, which are carbohydrate-binding proteins that are widely distributed in organisms from viruses to vertebrates [13], are thought to be involved in non-self recognition, and the participation of lectin in symbiosis has been reported for organisms, such as legumes, sponges, and nematodes [14–16]. It is, therefore, plausible that coral lectins also participate in the acquisition of *Symbiodinium*. Given the considerable variety of lectins in corals [17–21], it is not clear whether coral lectins play a role in symbiosis.

Interestingly, the galactose-binding lectin SLL-2 purified from coral *Simularia lochmodes* [22–24] and CcL purified from coral *Ctenactis echinata* [25] can arrest some *Symbiodinium* strains in the coccoid form. Moreover, the lectin gene *Pdc-Lectin* in coral *Pocillopora domicornis* was down-regulated 6 days before a bleaching event [26], and a C-type lectin, Millectin, in coral *Acropora millepora* bound to bacterial pathogens and *Symbiodinium* [27]. Although these reports suggest that the lectin is involved in the maintenance of symbiosis between *Symbiodinium* and its coral host, a coral lectin specifically involved in the acquisition of *Symbiodinium* has not been reported to date.

The coral, *A. tenuis* has been used as a model organism in previous coral-algal symbiosis studies based on its ability to acquire several *Symbiodinium* strains [28]. The planula larvae of *A. tenuis* live for more than 1 month [29, 30], and they can be chemically induced to metamorphose into juvenile polyps [31]. Therefore, in the present study, we sought to establish a model system for *Symbiodinium* acquisition by *A. tenuis* polyps, and to identify the lectin(s) involved in the acquisition of symbiotic algae.

Materials and methods

Materials

Larvae of *A. tenuis* that were either naturally spawned from 2008 to 2011 on Aka Island or Sesoko Island, Okinawa or

artificially spawned using a modification of Hayashibara's method [32] on 20 May 2013 on Ishigaki Island, Okinawa, were collected and maintained in artificial sea water (ASW) MARINE ART SF-1 (Osaka Yakken, Osaka, Japan) at 25 °C. Larvae were grown to the juvenile polyp stage and used for *Symbiodinium* acquisition experiments. Additionally, some of *A. tenuis* colonies collected at Ishigaki Island and Sesoko Island, Okinawa were kept at –20 °C until further extraction.

Symbiodinium strains CCMP1633 (clade B), CCMP2467 (clade A type A1), and CCMP2556 (clade D type D1–4) were purchased from the National Center for Marine Algae and Microbiota (East Boothbay, Maine, USA). CS-156 (clade F) and CS-161 (clade A type A3) were purchased from the Commonwealth Scientific and Industrial Research Organisation (Tasmania, Australia). NBRC102920 (clade A type A3) was purchased from the National Institute of Technology and Evaluation (Tokyo, Japan). GTP-A6-Sy (clade A type A2 relative) and AJIS2-C2 (clade A type A1) were originally isolated by Yamashita and Koike [33]. All *Symbiodinium* strains were cultured in IMK medium for marine microalgae (Wako Pure Chemical Industry, Osaka, Japan) at 25 °C with light at 80 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (12:12 h light:dark cycle).

All reagents not otherwise specified were purchased from Wako Pure Chemical Industry.

Optimization of the conditions for *Symbiodinium* acquisition by metamorphosed juvenile polyps

Planula larvae and juvenile polyps acquire *Symbiodinium*, but naturally metamorphosed polyps sometimes do not harbor any *Symbiodinium* [34]. Because of the ease of observation, we used them for the acquisition experiment. Since *Symbiodinium* acquisition activity may change during the period after metamorphosis induction in planula larvae, *Symbiodinium* acquisition activity was examined over 6 days after metamorphosis. Ten planula larvae of *A. tenuis* were placed individually in the wells of eight-well chambered coverglass (Nunc, Rochester, NY, USA), incubated in 100 μl of ASW with 1.0 μM Hydra-derived neuropeptide, Hym-248 (EPLPIGLW-amide), at 25 °C for 24 h to induce larval settlement and metamorphosis [30], and each chamber was replenished with 400 μl of ASW. After 0, 24, 48, 72, 96, and 120 h, a group of juvenile polyps ($n = 5\text{--}9$) were added with 500 μl of ASW containing 1,000 cells of *Symbiodinium* strain NBRC102920 having a motility percentage greater than 50 %. The individuals in each treatment were incubated for an additional 24 h at 25 °C with light at 80 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (12:12 h light:dark cycle). A series of optical sections (20 μl thickness) of juvenile polyps were recorded by confocal microscopy (LSM510Meta, Carl Zeiss, Oberkochen,

Germany) with in vivo chlorophyll *a* fluorescence (excitation, 480 nm; emission, 680 nm). The number of *Symbiodinium* cells within the juvenile polyps was manually counted using these images.

Selection of a *Symbiodinium* strain suitable for the acquisition by juvenile polyps

Planula larvae of *A. tenuis* were metamorphosed to juvenile polyps using Hym-248 as described above. At 72 h after the addition of Hym-248, 500 μ l of ASW containing 2,500 cells of one of the *Symbiodinium* strains, having a motile percentage greater than 50 %, was added, and the chambers were incubated at 25 °C for 24 h. The number of *Symbiodinium* cells within each juvenile polyp was counted as described above except that the detector gain was adjusted for each *Symbiodinium* strain.

Inhibition of *Symbiodinium* acquisition due to glycosidase treatment of *Symbiodinium*

Symbiodinium cells were pretreated with two glycosidases to examine the effect of surface glycan on *Symbiodinium* acquisition. To 1,000 cells of *Symbiodinium* strain NBRC102920 in 0.5 ml ASW, 10 μ g of glycosidase mix from *Turbo cornutus* (Seikagaku Corp., Tokyo, Japan), which randomly digests non-reducing terminal oligosaccharides or 1 U of Glycopeptidase F (Roche Diagnosis K. K., Tokyo, Japan), which digests *N*-glycans of glycoproteins, was added [22], and the mixture was incubated for 4 h and centrifuged at 3,000 \times *g* for 5 min. The supernatant was removed, and the pellet was washed with ASW and incubated for 24 h to recover motile cells. Following the method above, 500 cells of glycosidase-treated *Symbiodinium* cells were added to juvenile polyps, incubated for 24 h, and acquisition was analyzed.

Inhibition of *Symbiodinium* acquisition due to carbohydrates

The inhibitive effect on *Symbiodinium* acquisition of the presence of carbohydrates, which are known inhibitors of lectins, was examined. Juvenile polyps at 72 h after the addition of Hym-248 were incubated in ASW at 25 °C for 1 h with the following carbohydrates at a concentration of 10 mM: L-fucose (Fuc), D-galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-neuraminic acid (NANA), and sucrose (Suc). Then, 2,500 cells of *Symbiodinium* strain NBRC102920 were added to the juvenile polyps and incubated at 25 °C for 6 h. The numbers of *Symbiodinium* cells within the juvenile polyps were counted using images taken by confocal microscopy.

Preparation of *A. tenuis* crude extract

A portion of each *A. tenuis* colony was crushed, mixed with five volumes of extraction buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5), and centrifuged at 21,500 \times *g* at 4 °C for 10 min. The supernatant was combined with one-fifth weight of polyvinylpyrrolidone, which had been washed with ultrapure water and dried, and the mixture was incubated at 4 °C for 30 min with mixing. After centrifugation at 21,500 \times *g* at 4 °C for 1 min, the supernatant was collected and stored at –20 °C for further analysis.

Separation of the GalNAc-binding lectin

GalNAc-Sepharose 6B resin was prepared as previously described [24]. Then, 0.1 ml of GalNAc-Sepharose 6B resin was put into a 1.5 ml tube and washed three times with 1 ml of extraction buffer. The pelleted resin was combined with 0.2 ml of crude extract of *A. tenuis* tissue and incubated at 4 °C overnight with rotation using a Mini Disk Rotor BC-710I (BIO CRAFT, Tokyo, Japan). The tube was centrifuged at 380 \times *g* and 4 °C for 1 min, and the supernatant was removed. The resin was washed three times with ten volumes of the extraction buffer supplemented with 100 μ l of 0.2 M GalNAc, and then incubated at 4 °C for 30 min. After centrifugation at 380 \times *g* and 4 °C for 1 min, lectin was obtained as the supernatant.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The crude extract of *A. tenuis* and each of the fractions obtained by affinity separation were treated by reduction-alkylation as follows: 10 μ l of sample buffer (20 % glycerol, 4 % SDS, 125 mM Tris-HCl, pH 6.8, 0.01 % bromophenol blue) and 4 μ l of 1 M dithiothreitol were added to 10 μ l of each protein sample, and incubated at 65 °C for 20 min. After cooling to room temperature, 2.5 μ l of 1 M iodoacetamide was added to the sample solution, and the mixture was incubated at room temperature for 20 min in the dark. Finally, the sample solution was neutralized by the addition of 3 μ l of 1 M Tris-HCl, pH 9.5. SDS-PAGE was performed on 15 % polyacrylamide gels, according to Laemmli [35]. SDS-PAGE standard Broad Range (Bio Rad Laboratories, Hercules, CA, USA) was used as the protein standards. The gel was stained by zinc reverse staining [36].

De novo sequencing of GalNAc-binding proteins by MALDI-TOF/MS

GalNAc-binding proteins were separated by SDS-PAGE and stained using Oriole Fluorescent Gel Stain (Bio-Rad

Laboratories). The protein bands were excised and destained three times with 0.1 ml of 50 % methanol containing 16 mM Tris-HCl, pH 8.0. The gel was washed with 0.1 ml acetonitrile and then dried for 20 min at room temperature. After the addition of 10 μ l of 5 μ g ml⁻¹ trypsin (proteomics grade, Sigma-Aldrich, St. Louis, MO, USA) in 50 mM NH₄HCO₃, the mixture was incubated at 37 °C overnight. Digested peptides were extracted with 10 μ l of 49 % acetonitrile containing 0.1 % TFA by sonication for 10 min in ice water, and the supernatant was dried using the Centrifugal Evaporator (CE1D, Hitachi Koki, Tokyo, Japan).

Guanidination and sulfonation were performed according to Beardsley et al. [37] and Chen et al. [38] with some modifications. The tube containing dried peptides was added with 12 μ l of *O*-MIU mix (1.5 μ l of 1 mg μ l⁻¹ *O*-methylisourea hemisulfate, 5.5 μ l of 7 M NH₄OH, 5.0 μ l of water) and incubated at 65 °C for 15 min. The peptides were bound to ZipTip C18 pipette tips (Millipore, Bedford, MA, USA) and incubated with 10 μ l of 10 mg ml⁻¹ 4-sulfophenyl isothiocyanate in 20 mM NaHCO₃ at 55 °C for 30 min. Modified peptides were eluted from the ZipTip C18 using 80 % acetonitrile containing 0.1 % TFA and

mixed with 10 mg ml⁻¹ MassPREP MALDI Matrix CHCA (Waters, Milford, MA, USA) in 50 % acetonitrile containing 0.1 % TFA. The mixture was deposited on an MTP 384 target plate (ground steel T F, Bruker Daltonics, Bremen, Germany) and dried. Mass spectrometry was performed using a MALDI-TOF/MS Autoflex III (Bruker Daltonics). The mass spectra were acquired in the reflectron mode. The standard peptides used are angiotensin II (Sigma-Aldrich) and insulin B chain (Sigma-Aldrich). Among the obtained peaks, sulfonated peaks were analyzed by MS/MS analysis using LIFT. The mass spectra were analyzed by open source mass spectrometry tool mMass [39].

Sequence similarity search

The obtained partial amino acid sequences were compared with sequences in a publicly available putative protein database of *A. digitifera* (*Acropora digitifera* Genome, Ver 1.1; http://marinegenomics.oist.jp/genomes/viewer?project_id=3¤t_assembly_version=oist_v1.1) by FASTS analysis [40]. The similarity search of the obtained proteins was performed using BLASTP [41].

29.0kDa_protein	1	-----NJJFGVTAGK-----
adi_v1.08085	1	MDIKFHLCLLFSVFGAL--ACTPTFENLLFGVTGGKLYLRTPPOVSSDNWLGSATETGTA
A.millepora	1	MDIKFHLCLLFSVFGAL--ACTPTICNLLFGVTGGKLYSRTPPOVSSDNWLGSATETGTA
A.tenuis	1	--IKFHLCLLFSVFGAL--ACNPTSENLLFGVTAGKLYSRTPPOVSSDNWLGSATETGTA
AtTL-2	1	MDIKFHLCLLFSVFGAL--ACTPTCENLLFGVTAGKLYSRTPPOVARDNWLGSATETGTA
Tachylectin-2	1	M--KF--LVVVLGFVIGFLKDGITVGGESMLRQVYQDKFYQCHYPOKKNNDNWLARATLIGKG
		↓
29.0kDa_protein	11	-----VXXXGWHVFK-----
adi_v1.08085	59	GWNDFKFLFFHPDGTLYGLVNDKFKYKGPPESSSAEDWIAHATLIGTAGWNAPOQLFFDPP
A.millepora	59	GWNDFKFLFFHPDGTLYGLVNDKFKYKGPPESSSAEDWIAHATLIGTAGWNAPOQLFFDPP
A.tenuis	57	GWNDFKFLFFHPDGTLYGLVNDKFKYKGPPESSSAEDWIAHATLIGTAGWNAPOQLFFDPP
AtTL-2	59	GWNDFKFLFFHPDGTLYGLVNDKFKYKGPPESSSAEDWIAHATLIGTAGWNAPOQLFFDPP
Tachylectin-2	58	GWSNFKFLFLSFGGELYGLVNDKFKYKGT--PPTHNDNMMGRAKKLGNGGWNPQQLFFDPP
		↓
29.0kDa_protein	11	-----VXXXGWHVFK-----
adi_v1.08085	119	EGVLYGVHNDKFKYKRSPTTFFSDNWIGSATKVGSGGWHVFKFLFFDPOGILYGVANGKFKY
A.millepora	119	EGVLYGVHNDKFKYKRSPTTFFSDNWIGSATKVGSGGWHVFKFLFFDPOGILYGVANGKFKY
A.tenuis	117	EGVLYGVHNDKFKYKRSPTTFFSDNWIGSATKVGSGGWHVFKFLFFDPOGILYGVANGKFKY
AtTL-2	119	EGVLYGVHNDKFKYKRSPTTFFSDNWIGSATKVGSGGWHVFKFLFFDPOGILYGVANGKFKY
Tachylectin-2	117	NGVLYAVSKDRDKYKASPPQSDTDNWIARATEIGSGGWSGFKFLFFHPNGLVLYAVHGGQFFY
		↓
29.0kDa_protein	179	-----VXXXGWHVFK-----
adi_v1.08085	179	KRNPPPTHASDNWLGSSSTLIGSAGWNGFOLFPMADGELYGVHSAKFYKRSAPAHGGDDWL
A.millepora	179	KRNPPPTHASDNWLGSSSTLIGSAGWNGFOLFPMADGELYGVHSAKFYKRSAPAHGGDDWL
A.tenuis	177	KRSPPPTHASDNWLGSSSTLIGTAGWDGQFLFFMADGELYGVHSAKFYKRSAPAHGGDDWL
AtTL-2	179	KRNPPPTHASDNWLGSSSTLIGTAGWDGQFLFFMADGELYGVHSAKFYKRSAPAHGGDDWL
Tachylectin-2	177	KALPPVSNQDNWLARATKIGQGGWDTFRFLFPSSVCTLFGVQGGKFKYEDYPPSYAHDDWL
		↓
29.0kDa_protein	239	-----VXXXGWHVFK-----
adi_v1.08085	239	GSABEIGTGGWQVFKYLMSP-----
A.millepora	239	GSABEIGTGGWQVFKYLMSPGQPPGQR--
A.tenuis	237	GSABEIGTGGWQVFKYLMSPGQPPSRDQR*
AtTL-2	239	GSSEIIGTGGWQVFKYLMSPGQPPGQR*
Tachylectin-2	237	ARAKLIGNGGWDDFRFLFF-----

Fig. 1 Comparison of peptide sequences derived from *Acropora tenuis* 29.0 kDa protein, Tachylectin-2 and three related species of genus *Acropora* and AtTL-2. Black boxes and gray boxes indicate identical and similar amino acids, respectively. J indicates I or L and X indicates an undetermined amino acid. Thin lines indicate the regions used to design the PCR primers. Thick lines indicate regions used to design the gene-specific primers. An arrow indicates the starting position of a mature protein of Tachylectin-2. Each doublet indicates a repetitive region. Sequences used were *A. tenuis*, a

29.0 kDa protein with an identical amino acid sequence determined by de novo sequencing, *A. digitifera* Tachylectin-2 (adi_v1.08085) with the same deduced amino acid sequence of *A. millepora* Tachylectin-2 (GenBank accession no. EZ038328), and *A. tenuis* Tachylectin-2 (http://www.bio.utexas.edu/research/matz_lab/matzlab/Data.html, isotig20551 and isotig22486), AtTL-2 (DDBJ accession no. AB972924) and Tachylectin-2 from *Tachypleus tridentatus* (UniProtKB accession no. Q27084)

cDNA cloning of Tachylectin-2-like cDNA

Total RNA was extracted from fresh adult *A. tenuis* using RNAiso PLUS (TaKaRa Bio, Otsu, Japan). mRNA was purified from the total RNA using a Poly (A) Purist MAG Kit (Ambion, Austin, TX, USA). Based on the amino acid sequence alignment of Tachylectin-2 homologue of corals (Fig. 1), the PCR primers were designed from the homologous regions at residues 64–69 (5'-AAATTCCTGTTCTTYCACCC-3') and residues 232–238 (5'-CAACAGTYATCACYCCRT-3'). Using these primers, PCR was performed with Advantage 2 Polymerase Mix (Clontech, Palo Alto, CA, USA) with the following cycle condition: (94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min) × 2 cycles, (94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min) × 2 cycles, (94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min) × 2 cycles, (94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min) × 2 cycles, and (94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min) × 20 cycles. The amplified PCR products were directly sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Two gene-specific primers were designed based on the obtained sequences (forward primer, 5'-ATCGC CCACGCCACCCTTATTG-3' and reverse primer, 5'-AGTTGGGGCTGATCGCTTGTA-3'), and RACE was performed with a SMARTer RACE cDNA Amplification Kit (Clontech) according to manufacturer protocol. The cycle conditions were as follows: (94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min) × 5 cycles, (94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min) × 5 cycles, and (94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min) × 20 cycles. The RACE products were cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced with a DNA sequencer (ABI3130, Applied Biosystems). The determined nucleotide sequence of AtTL-2 cDNA was deposited to DDBJ with Accession no. AB972924.

Amino acid sequence alignment of Tachylectin-2 homologues

The deduced amino acid sequence of AtTL-2 was aligned with that of Tachylectin-2 from *Acopora* and *Tachypleus tridentatus* using ClustalW [42], and this alignment was colored with BoxShade 3.21 (BoxShade Server; http://www.ch.embnet.org/software/BOX_form.html).

Dot blotting using the anti-Tachylectin-2 antibody

The crude extract of *A. tenuis* and each of the fractions obtained by affinity separation (30 µl) were applied to a polyvinylidene difluoride membrane Fluorotrans W (Pall, Port Washington, NY, USA) using a Bio-Dot apparatus (Bio-Rad Laboratories). Binding of antibodies was

performed with a SNAP i.d. (Millipore) according to the manufacturer's protocol. Briefly, the membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated with 0.1 µg ml⁻¹ of anti-Tachylectin-2 antibodies raised from Tachylectin-2 (courtesy of Dr. Kawabata) [43] in Can Get Signal Solution 1 (TOYOBO, Osaka, Japan) for 10 min. After washing with PBS-T (0.1 % Tween-20 in phosphate-buffered saline), the membrane was incubated with 1.0 µg ml⁻¹ of horseradish peroxidase (HRP)-labeled anti-rabbit IgG antibodies (Wako Pure Chemical Industry) in Can Get Signal Solution 2 (TOYOBO) for 10 min and then washed with PBS-T. Detection was performed using Luminata Forte Western HRP Substrate (Millipore).

Inhibition of carbohydrate binding activity of AtTL-2 by anti-Tachylectin-2 antibodies

One microliter of anti-Tachylectin-2 antibodies (0.1 mg ml⁻¹) or the extraction buffer was added to 60 µl of the crude extract of *A. tenuis* (0.37 mg ml⁻¹) and the mixture was incubated at 4 °C for 1 h. Using the mixture, lectins contained were partially purified according to the separation method for the GalNAc-binding lectin.

Inhibition of *Symbiodinium* acquisition by anti-Tachylectin-2 antibodies

The involvement of AtTL-2 for *Symbiodinium* acquisition was examined. Rabbit IgG or anti-Tachylectin-2 antibodies (1.5 µg ml⁻¹) was added in ASW to juvenile polyps at 72 h after the addition of Hym-248 and incubated at 25 °C for 1 h before the addition of 2,500 cells of *Symbiodinium* strain NBRC102920 followed by an additional incubation at 25 °C for 6 h. The numbers of *Symbiodinium* cells within the juvenile polyps were counted on images obtained using confocal microscopy.

Statistical analysis

Results of *Symbiodinium* acquisition tests were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple comparison test with GraphPad Prism 4 for Macintosh (GraphPad software, CA, USA).

Results

Establishment of an experimental model for *Symbiodinium* acquisition by juvenile polyps

The number of *Symbiodinium* cells acquired by polyps after Hym-248 treatment was greatest at 72 h (11.7 ± 2.0 cells/

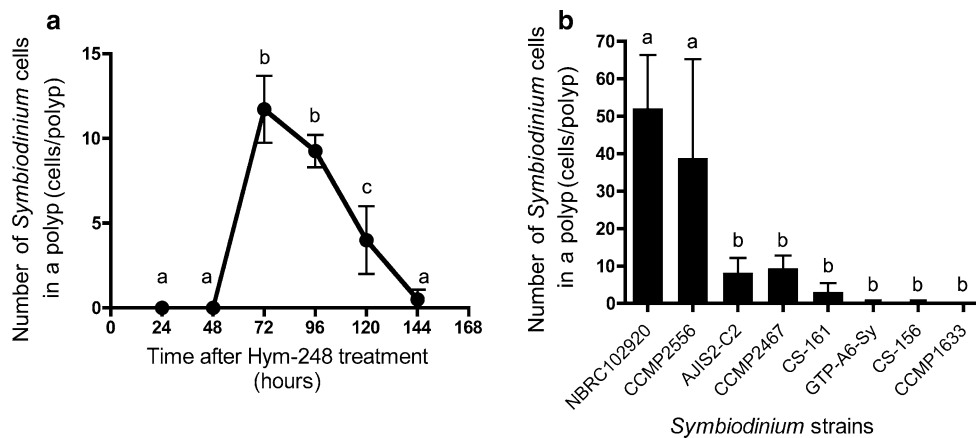


Fig. 2 Optimal experimental conditions for *Symbiodinium* acquisition by juvenile polyps. **a** Juvenile polyps treated with Hym-248 were incubated for 24, 48, 72, 96, 120, and 144 h, and then were incubated in the ASW with *Symbiodinium* strain NBRC102920 for 24 h. *Symbiodinium* cells within juvenile polyps were counted using *Symbiodinium* chlorophyll *a* fluorescence with confocal microscopy. Values are mean \pm SD ($n = 5-9$). Different letters indicate significant dif-

ferences between incubated times after Hym-248 treatment ($P < 0.05$, Tukey's multiple range test). **b** Juvenile polyps were incubated with each *Symbiodinium* strain for 24 h. *Symbiodinium* cells within juvenile polyps were counted. Values are mean \pm SD ($n = 5$). Different letters indicate significant differences between *Symbiodinium* strains ($P < 0.01$, Tukey's multiple range test)

polyp) and 96 h (9.3 ± 1.0 cells/polyp) and decreased thereafter (Fig. 2a). Acquisition activity was greatest for NBRC102920 (52.2 ± 14.2 cells/polyp) and CCMP2556 (38.8 ± 26.4 cells/polyp), followed by CCMP2467 (9.4 ± 3.4 cells/polyp), AJIS2-C2 (8.2 ± 3.9 cells/polyp), CS-161 (3.1 ± 2.3 cells/polyp), GTP-A6-Sy (0.3 ± 0.5 cells/polyp), CS-156 (0.3 ± 0.5 cells/polyp), and CCMP1633 (0 cells/polyp) (Fig. 2b). The number of *Symbiodinium* cells in juvenile polyps differed among *Symbiodinium* belonging to these clades: NBRC102920 (clade A), CCMP2556 (clade D), CS-156 (clade F), and CCMP1633 (clade B). Taken together, the optimal acquisition model was determined as the introduction of *Symbiodinium* strain NBRC102920 to juvenile polyps at 72 h after treatment with Hym-248, and this procedure was used in subsequent acquisition experiments.

Effect of glycosidase treatment and carbohydrates on *Symbiodinium* acquisition by juvenile polyps

To test the hypothesis of whether lectin, a carbohydrate binding protein, is involved in the recognition process between *A. tenuis* and *Symbiodinium*, we examined the effect of glycosidase treatment of *Symbiodinium* on its acquisition by juvenile polyps (Fig. 3a). The uptake of *Symbiodinium* by juvenile polyps was inhibited by glycopeptidase F-treatment (10.3 ± 11.6 cells/polyp) and by glycosidase mix treatment (20.0 ± 5.7 cells/polyp) (Fig. 3a). There were significant differences between the control (no treatment) and glycosidase treatments ($P < 0.05$, Dunnett's multiple range test). These glycosidase treatments did not affect *Symbiodinium* motility.

Next, the effect of carbohydrates on *Symbiodinium* acquisition by juvenile polyps was examined. One-way ANOVA revealed that *Symbiodinium* acquisition was significantly different between different carbohydrates; however, Tukey's multiple comparison test did not show significant differences in the number of *Symbiodinium* acquired among different carbohydrates ($P > 0.05$). The number of *Symbiodinium* acquired by juvenile polyps (38.3 ± 21.0 cells/polyp) tended to decrease after exposure to Gal (19.3 ± 10.3 cells/polyp), GlcNAc (22.2 ± 9.0 cells/polyp) and GalNAc (16.9 ± 12.6 cells/polyp) (Fig. 3b). Thus, it is plausible that polyps have Gal-, GlcNAc-, and GalNAc-binding lectins that are involved in *Symbiodinium* acquisition. Based on the report of the isolation of GalNAc-binding lectin from other coral species [24], we attempted to purify GalNAc-binding lectin from *A. tenuis*.

Identification of GalNAc-binding lectin

In the purification of GalNAc-binding lectin from *A. tenuis* crude extract using GalNAc-Sepharose 6B resin, the eluted fraction contained proteins of sizes 14.6 and 29.0 kDa as shown by SDS-PAGE (Fig. 4). On de novo sequencing using MALDI-TOF/MS, three trypsin-digested amino acid fragments of the 14.6 kDa protein (m/z 1378.77, 1463.86, and 1913.16) had sequences of EFEN (I/L) VSGVK, YDQW (I/L) (I/L) ASPR, and HVNTV (I/L) AR, and two trypsin-digested fragments of the 29.0 kDa protein (m/z 1330.86 and 1866.19) had sequences of VXXXGWHVFK and N (I/L) (I/L) FGVTAGK, where X indicates undetermined amino acid residues. Comparison of these partial

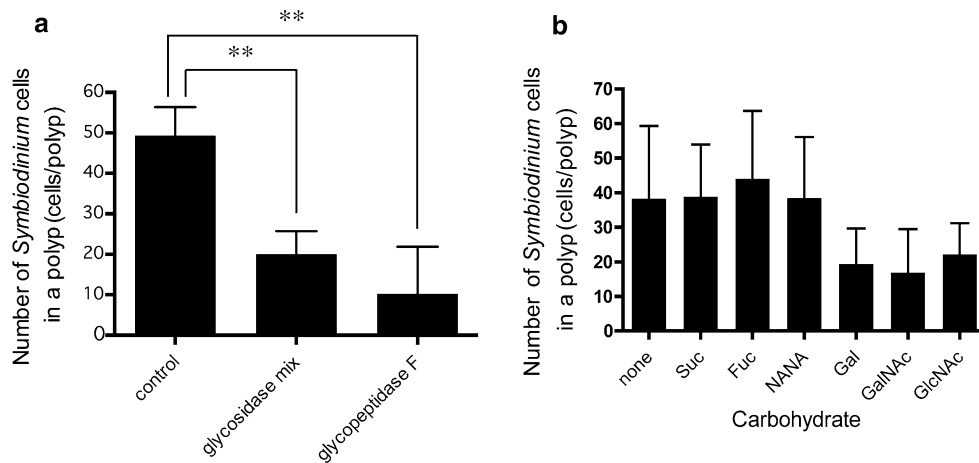


Fig. 3 Inhibition test of *Symbiodinium* acquisition activity by glycosidase treatment of *Symbiodinium* and the presence of carbohydrates. **a** Juvenile polyps were incubated with *Symbiodinium* strain NBRC102920 after treatment with the glycosidase mix, glycopeptidase F and no treatment. After 24 h, the number of *Symbiodinium* per polyp was counted. Values are mean \pm SD ($n = 5$). Treatments

that differ from the control (no treatment) by one-way ANOVA and Dunnett's multiple comparison test at $P < 0.01$ are indicated by **. **b** Juvenile polyps were incubated with different carbohydrates for 1 h followed by 6 h incubation with *Symbiodinium*. After 6 h, the number of *Symbiodinium* cells per juvenile polyps was counted using in vivo chlorophyll *a* fluorescence. Values are mean \pm SD ($n = 5$)

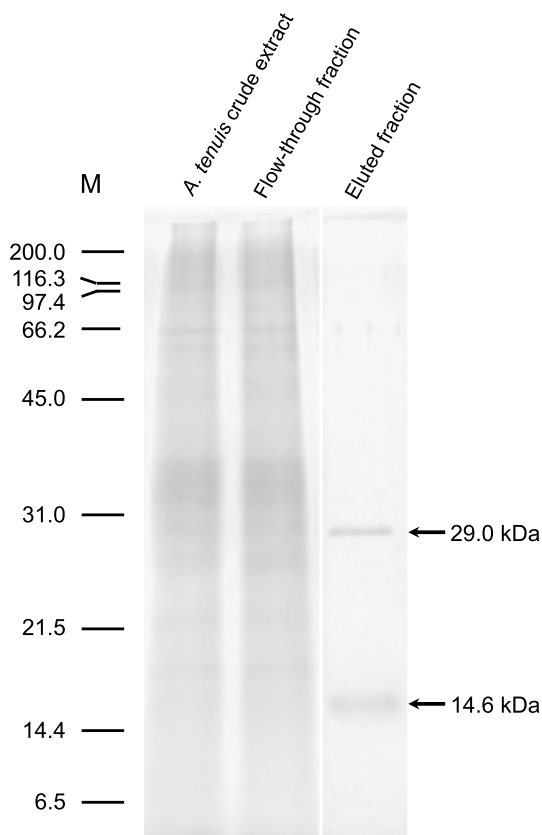


Fig. 4 SDS-PAGE of GalNAc affinity purified lectin. The GalNAc-binding lectins were purified using GalNAc-Sepharose 6B resin. The *Acropora tenuis* crude extract, flow-through fraction and eluted fraction were analyzed by SDS-PAGE. *M* indicates protein standard

amino acid sequences with those in a public putative protein database of *A. digitifera* by FASTS analysis showed the sequences of the 14.6 kDa protein from *A. tenuis* to be similar to a region corresponding to adi_v1.13780 of *A. digitifera* putative protein (E -value 6.7×10^{-6}). The N-terminal amino acid sequence of adi_v1.13780 (20–99 aa) showed similarity to tripartite motif-containing protein 2 (424–499 aa) of the Pacific oyster *Crassostrea gigas*, which contains a neuraminidase motif. The sequence of the 29.0 kDa protein from *A. tenuis* showed similarity to a corresponding region of adi_v1.08085 of *A. digitifera* putative protein (E -value 7.6×10^{-8}) and to GalNAc- and GlcNAc-binding lectin Tachylectin-2 TL-2 (corresponding peptide region: 47.7 % identity, Fig. 1), one of the horseshoe crab lectins [44]. Therefore, the 29.0 kDa protein was termed AtTL-2.

Determination of the cDNA sequence of AtTL-2

AtTL-2 cDNA was cloned using the RACE method. The nucleotide sequence of AtTL-2 open reading frame was 801 bp, and the deduced amino acid sequence of AtTL-2 coincided with that obtained by de novo sequencing at regions of 25–34 and 150–159 aa (Fig. 1). The percent identities of AtTL-2 with the deduced amino acid sequence of Tachylectin-2 and related proteins from *A. tenuis*, *A. digitifera*, and *A. millepora* were 48.1, 92.1, 89.5, and 91.4 %, respectively (Fig. 1).

The structure of Tachylectin-2 is composed of a five-bladed β -propeller structure with five tandem repeats

(49–68 % identity) [44, 45], and AtTL-2 also consists of five tandem repeats (43–66 % identity) (Fig. 1). The partial amino acid sequence obtained by de novo sequencing of a peak at m/z 1866.19 was located close to the starting position of mature Tachylectin-2 (Val20) [44] (Fig. 1). As the trypsin digests of AtTL-2 had a peak at m/z 1866.19, which corresponded to the molecular weight of TPTCENLLFGVTAGK (m/z 1866.13) in the deduced amino acid sequences, Thr20 of AtTL-2 is likely a starting position of the mature protein.

Specificity and neutralization activity of the anti-Tachylectin-2 antibody

To examine AtTL-2 function in the acquisition of *Symbiodinium* by juvenile polyps, it is instructive to use an antibody in experimental tests. We tested whether the anti-Tachylectin-2 antibody specifically recognizes AtTL-2. This antibody did not react with fractions separated by GalNAc-Sepharose chromatography that were shown to contain AtTL-2 by western analysis but did react with AtTL-2 on dot blotting. Among the fractions separated by GalNAc-Sepharose 6B chromatography, anti-Tachylectin-2 antibodies reacted with the crude extract of *A. tenuis* and the eluted fraction, which contained AtTL-2, but it did not react with the flow-through fraction, which did not contain AtTL-2 (Fig. 5). These findings suggest that anti-Tachylectin-2 antibodies recognize AtTL-2.

Some antibodies have the capability to inhibit the activity of proteins. Therefore, we investigated whether anti-Tachylectin-2 antibodies inhibit the binding activity of AtTL-2. When *A. tenuis* crude extract was separated on GalNAc-Sepharose 6B resin, the eluted fraction contained two proteins of sizes 14.6 and 29.0 kDa, similar to those shown in Fig. 4 (Fig. 6). The fraction also contained contaminant bands ranging in size from 50 to 66 kDa, which also appeared in sample buffer without proteins. On the other hand, when *A. tenuis* crude extract preincubated with anti-Tachylectin-2 antibodies was separated on GalNAc-Sepharose 6B resin, only the AtTL-2 of size 29.0 kDa disappeared from the eluted fraction (Fig. 6), indicating that anti-Tachylectin-2 antibodies inhibited the carbohydrate-binding activity of AtTL-2. Moreover, this result also shows that anti-Tachylectin-2 antibodies specifically recognized AtTL-2.

Participation of AtTL-2 in acquisition of *Symbiodinium* by juvenile polyps

We examined the effect of anti-Tachylectin-2 antibodies on the acquisition of *Symbiodinium* by juvenile polyps. The number of *Symbiodinium* acquired by polyps in the presence of anti-Tachylectin-2 antibodies (4.8 ± 3.8 cells/

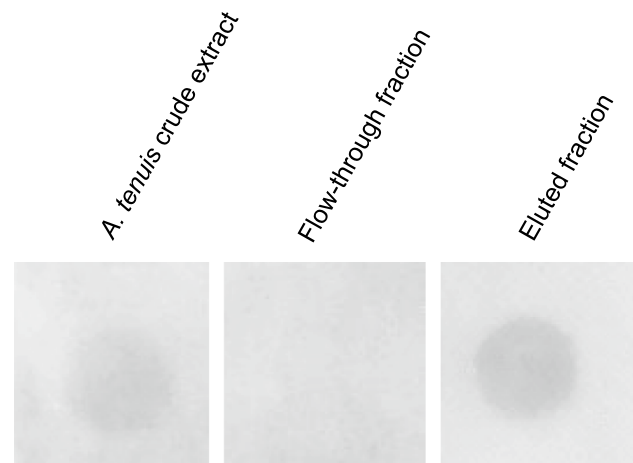


Fig. 5 Binding of anti-Tachylectin-2 antibodies to AtTL-2. *A. tenuis* crude extract, flow-through fraction and eluted fraction were dot-blotted to a PVDF membrane. AtTL-2 was detected using anti-Tachylectin-2 antibodies as the primary antibody

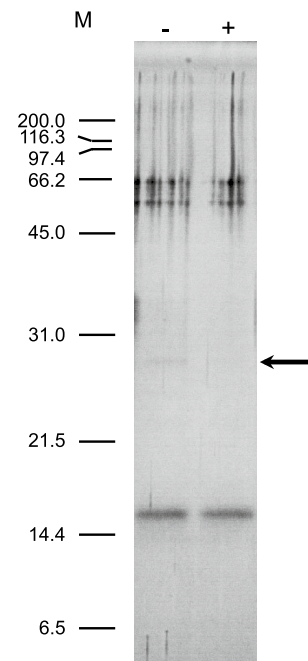


Fig. 6 Inhibition of the carbohydrate-binding activity of AtTL-2 by anti-Tachylectin-2 antibodies. Crude extract of *A. tenuis* was mixed with or without anti-Tachylectin-2 antibodies and incubated at 4 °C for 1 h. These mixtures were subjected to batch GalNAc affinity chromatography. The eluted fractions for treatment without any antibody (–) or with anti-Tachylectin-2 antibodies (+) were analyzed by SDS-PAGE. *M* indicates protein standards. An arrow indicates the region containing AtTL-2

polyp, $P < 0.05$, Dunnett's multiple range test) was significantly reduced to less than 23 % of that without any antibodies (20.5 ± 8.4 cells/polyp) (Fig. 7).

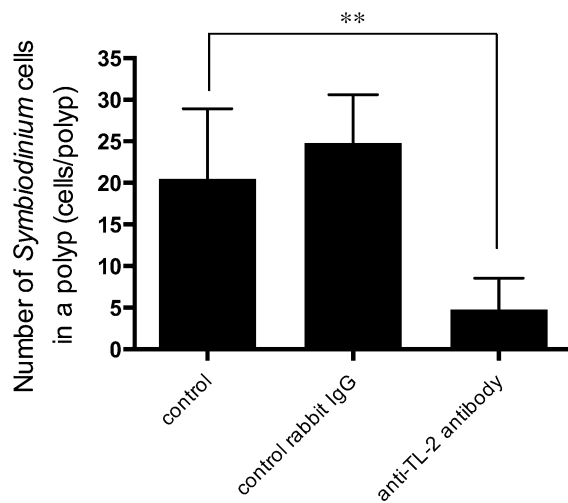


Fig. 7 Inhibition of *Symbiodinium* acquisition activity by anti-Tachylectin-2 (TL-2) antibodies. Juvenile polyps were incubated without any antibodies, with control rabbit IgG, or with anti-TL-2 antibodies for 1 h. Then, polyps were incubated with *Symbiodinium* cells for 6 h. The number of *Symbiodinium* cells in juvenile polyp was counted using *in vivo* chlorophyll *a* fluorescence. Values are mean \pm SD ($n = 5$). The treatment with antibodies that differs from the control (treatment with no antibodies) by one-way ANOVA with Dunnett's multiple comparison test at $P < 0.01$ are indicated by **

Discussion

Acquisition of *Symbiodinium* strains NBRC102920 (clade A) and CCMP2556 (clade D) by juvenile *A. tenuis* polyps was highest, followed by the acquisition of strains CCMP2467, AJIS2-C2 and CS-161 (clade A) (Fig. 1b). This finding corroborates previous studies showing that *Symbiodinium* from different clades have physiologically different function [46–48]. A previous study by Yamashita et al. [34, 49] also showed that polyps of *Acropora* corals in natural environments mainly acquired *Symbiodinium* from clade A and/or D. Although a few clade C *Symbiodinium* were found in *Acropora* juvenile polyps in natural environments [49, 50], no strains from clade C were included in the present study due to the immobility of these strains in our laboratory culturing conditions. The *Symbiodinium* acquisition model developed here is expected to reflect the selectivity observed in natural environments. *Symbiodinium* acquisition decreased with an increase in rearing period (data not shown), and this may have led to the production of controls with different values for *Symbiodinium* acquisition (Figs. 2a, b, 3a, b).

Each *Symbiodinium* genetic clade was further divided into many types [51, 52]. Interestingly, differences in acquisition were observed even among closely related *Symbiodinium* in clade A: NBRC102920 (type A3), CCMP2467 (type A1), AJIS2-C2 (type A1), CS-161 (type A3), and

GTP-A6-Sy (type A2 relative) (Fig. 2b). Similarly, varying densities of strains from clade A were also found in larvae of *A. tenuis* [49]. The glycan profile of the *Symbiodinium* cell surface does not appear to depend on the clade assignment of *Symbiodinium* [53], suggesting that glycosylation patterns on the surface of *Symbiodinium* cells are more important than clade assignment in the acquisition of *Symbiodinium* by corals.

Lectin and glycosidase treatments targeting the cell surface of *Symbiodinium* inhibited the acquisition of *Symbiodinium* by the hosts *Aiptasia pulchella* and *Fungia scutaria* [11, 12]. In the present study, the acquisition of *Symbiodinium* by *A. tenuis* polyps was also inhibited by glycosidase treatment of *Symbiodinium* (Fig. 3a), suggesting that particular carbohydrate chains on the *Symbiodinium* cell surface play an important role in the acquisition process.

Based on the observed decrease of *Symbiodinium* acquisition by GalNAc, a GalNAc-binding lectin was separated from *A. tenuis* crude extract using GalNAc-Sepharose 6B resin (Fig. 4). The eluate contained two proteins with molecular masses of 14.6 and 29.0 kDa. The 29.0 kDa protein (AtTL-2) showed high similarity with Tachylectin-2. Tachylectin-2, one of the lectins from the horseshoe crab *T. tridentatus*, binds to GalNAc and GlcNAc [44]. In addition, Tachylectin-2 agglutinates bacteria, and the hemagglutinating activity is inhibited by some lipopolysaccharides and lipoteichoic acids [43, 54]. It has been proposed that Tachylectin-2 is involved in the immunity of horseshoe crab, such as in opsonization or in the blocking of adhesion of pathogens to the host cells [45]. Since some lectins have functions that are related not only to immune system functioning, but also to symbiosis [55], it is possible that AtTL-2 plays a role in the symbiosis between *A. tenuis* and *Symbiodinium*. Recently, the distribution of several coral lectins was determined [23, 26, 56], but their roles in symbiotic relationships remain to be clarified. Based on the inhibition of *Symbiodinium* acquisition by *A. tenuis* polyps by anti-Tachylectin-2 antibodies (Fig. 7), our results first clarified the important role of coral lectins in symbiosis; that is, AtTL-2 is involved in the acquisition step of symbiosis establishment between corals and *Symbiodinium*.

Tachylectin-2 homologues have been found in the corals *Montastrea feveolata*, *Oculina diffusa*, *O. robusta*, *O. varicosa*, *Acropora palmata*, *A. digitifera*, and *A. millepora* [57–60], but the function of Tachylectin-2 homologues in corals remains unknown. Our study revealed that AtTL-2, a Tachylectin-2 homologue, plays a role in the acquisition of *Symbiodinium*. AtTL-2 may bind to *Symbiodinium* and promote the acquisition of *Symbiodinium* by *A. tenuis*. Future studies will investigate the localization of AtTL-2 and the effect of AtTL-2 on *Symbiodinium* in order to clarify the role of AtTL-2 in symbiosis.

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