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鎘離子誘發類金屬硫蛋白於裂足海葵上的表現

Cadmium induced expression of putative metallothioneins in sea anemones, *Aiptasia pulchella*

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本論文係董于瑄君（R97B41021）在國立臺灣大學生命科學院動物學研究所完成之碩士學位論文，於民國九十九年七月六日承下列考試委員審查通過及口試及格，特此證明

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台大動物所 董于瑄

中華民國九十九年八月十一日

Abstract

Metallothioneins (MTs) are cysteine rich, heat stable proteins with low molecular weight. They have been demonstrated that can chelate various divalent heavy metals. In addition to mediate homeostasis of essential metals Zn and Cu, MTs can bind with the nonessential metals, like Cd and Hg, to reduce their toxicities, which is recognized as a mechanism of detoxification. According to previous studies, MTs have been identified in a wide range of organisms, from bacteria to mammals. In invertebrate, MTs were proven existing in molluscs, crustaceans and annelids, but not in sea anemones. Sea anemone, *Aiptasia pulchella*, was chosen as the experimental organism in this study, because they are easy to be cultured in a laboratory system and have a life cycle with asexual reproduction, which reduce inter-individual variation. After exposed to cadmium, obvious morphological changes can be observed such as the shed tissues accumulated higher level of cadmium than normal tissues. From the results of immunoblotting, three heat stable, low molecular MT-like proteins were detected. Furthermore, the results of immunohistochemistry study revealed that the MTs expressed mainly in ectoderm and were induced after cadmium treatment. Although the exact MT sequence on *A. pulchella* was not obtained by using RT-PCR with primer combinations based on the immunobloting and immunohistochemistry results, the MT molecules were expressed in the rational location and their expression

levels were induced after cadmium exposure. Therefore, it is strongly believed that MT molecules exist in *A. pulchella*.

摘要

金屬硫蛋白 (Metallothionein, MT) 是一含有大量cysteine並且耐熱的小分子量蛋白質。這類蛋白質已經證實具有螯合二價重金屬的功用，除了可以調控必需重金屬鋅或銅的含量外，也可以在生物體內螯合非必需的重金屬如鎘或汞等，以減輕毒性，作為解毒機制的一環。從文獻知道，現存大部分物種從細菌到哺乳類動物皆發現有此類蛋白質，包括軟體、節肢及環節動物身上皆已証實有金屬硫蛋白的存在，然而於刺絲胞動物門的海葵至今是否存有金屬硫蛋白仍尚未被證實。本實驗中的動物，海葵*Aiptasia pulchella* 容易飼養並具有無性生殖的特性，可在實驗的過程中減少個體差異。經由實驗結果得知重金屬鎘除了會對海葵造成外觀上的傷害，也會進一步造成海葵部分累積較高量重金屬的組織脫落。鎘處理過的*A. pulchella*以immunoblotting可以偵測到三個耐熱的低分子量蛋白質，再以免疫組織染色可以更進一步看到金屬硫蛋白主要出現在海葵組織外胚層的部分，在重金屬鎘離子處理後明顯看到其被誘發表現的現象。雖然利用相近物種設計而來的核酸引子無法以RT-PCR在海葵身上找到確切的金屬硫蛋白序列，但是依據immunoblotting及免疫組織染色的結果，偵測到的蛋白質其分子量大小，耐熱特性以及表現模式，因此應可確認在海葵體內存在著金屬硫蛋白。

Contents

1. Introduction

1.1 Heavy metals and cadmium

Over the past decades, the term “heavy metals” has been widely used, but the definition is still ambiguity. Many different definitions have been proposed- some based on density, some on atomic number or atomic weight, and some on chemical properties or toxicity. Heavy metals are often a group of metals and semimetals (metalloids) associated with contamination and potential toxicity or ecotoxicity in the past (Duffus, 2002). However, recently heavy metals are sometimes identified as a member of elements that exhibit metallic properties, which mainly include the transition metals, some metalloids, lanthanides, and actinides (Rand et al, 1995; Harrison and Waites, 1998). Pioneer works on heavy metals began in 1928, but most researches were focused on the essential trace metals such as zinc (Zn) in the food (Wu, 2005). Bryan (1976) was the first to notice that the danger of heavy metals did not derive from the existence of heavy metals but the criteria of adequacy and deleterious excess. Afterwards, the studies of heavy metals have been expanded to other aspects, such as their distribution, pollution, as well as the toxicity and adverse effects on organisms (Kotsonis and Klaassen, 1977; Williams et al., 1986). In the past decades, a large amount of pollutants including heavy metals discharged into the river

and the ocean. The aquatic organisms that live in polluted areas suffered from the impacts of contaminants. Ever since 1980s, many studies have been focused on monitoring and detecting the distribution of heavy metals in aquatic animals to reveal the impacts of heavy metals on ecosystems and the aquacultural systems.

Cadmium (Cd) is a non-essential heavy metal. It was discovered and identified by Stromeyer (1817) as an impurity in zinc carbonates (Weeks, 1968). Cadmium is a natural element in earth's crust and is usually found as a mineral combined with other elements such as oxygen, chlorine, or sulfur (Cuypers et al., 2010). In the past, anthropogenic and industrial activities have led to high emissions of Cd into the environments at concentrations significantly exceeding those originated from natural sources (Nriagu and Pacyna, 1988; Vangronsveld et al., 1995). Furthermore, cadmium is a common by-product of mining and smelting of zinc (Zn) and lead (Pb) (Park et al., 2001; Wu, 2005).

1.2 Cadmium pollution and toxicity

Cadmium is a very important metal with many applications, such as electroplating and galvanizing, color pigment stabilizer for paints and plastics, and the cathodic material for nickel-cadmium batteries. All of these applications increase the possibility of Cd entering the environment in unusual ways, that cause adverse effects

to ecosystems, organisms, and humans (Wu, 2005). Since cadmium can hardly secreted and metabolized such as the biological half life of cadmium in mussels is about 5~6 months (Viarengo et al., 1985), the risk of environmental exposure is constantly increasing because of bioaccumulation via the food chain (Kim et al., 2005).

When the cadmium waste liquid produced by anthropogenic and industrial activities was discharged into the environment, organisms will suffer harsh impact on physiology and induce the pathological changes in different organs (Jarup and Akesson, 2009; Jarup et al., 1998; Nawrot et al., 2008). The toxicity induced by cadmium is derived from increasing the production of reactive oxygen species (ROS) (Gupta et al., 2004; Thevenod and Friedmann, 1999)

Toxicological experiments have proved that cadmium can cause several kinds of diseases including pulmonary disease, nephrotoxicity, skeletal effects, hypertension and cardiovascular effects, neurologic disorders, and carcinogenicity and itai-itai disease on human (Almedia and Stearns, 1998; Park et al., 2001). Moreover, cadmium is also regarded as a carcinogen in human that may induce pancreatic, prostate and renal cancers (Schwartz and Reis, 2000; Waalkes, 2003).

1.3 Protection mechanisms in animals

Since heavy metals cause severe toxicities to animals, many protection mechanisms have been discovered including autophagy, mucus secretion and cytosolic proteins. Animals suffered heavy metals may autotomize the terminal or caudal portion of their body. For example, sublethal mercury induced physiological changes in *Sparganophilus pearsei* (Oligochaeta), resulting in the constriction and posterior loss of caudal segments. The segments become sphere-like shapes with a "rosary-bead" appearance (Vidal and Horne, 2003). The autotomy has been considered as a detoxification mechanism via accumulating high amount heavy metal in the periphery tissues (Vidal and Horne, 2003). Dhainaut-Courtois (1988) showed that when *Tubifex tubifex* (Oligochaete) were subjected to the heavy metals, they would secrete mucus on their body surface and this representing a detoxification mechanism (Bouche et al., 2000). Cytosolic proteins such as metallothioneins can bind heavy metals and sequestrate the metals to reduce the toxicity in the cytosol (Steinebach and Wolterbeek, 1994).

1.4 Characteristics of metallothioneins

Metallothioneins (MT) were first reported by Margoshes and Vallee (1957), and they showed that MT could bind with cadmium in equine kidney cortex (Margoshes

and Vallee, 1957). The name “metallo-thionien” was given based on its capacity of metal binding and cysteine content (Miles et al., 2000). MTs have been defined as non-enzymatic proteins with low molecular weight (ranging from 500 to 14000 Da), high cysteine content, no aromatic amino acids and heat stability (Amiard et al., 2006). Cysteines account for 33% of the 61 constitutive amino acids of mammalian MTs (Binz and Kagi, 1999). The alignment of Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys sequences where X and Y are amino acids other than cysteine, is the criterion to distinct among different structural MT classes and to form many isoforms of the same metallothioneins (Amiard et al., 2006).

As noted by Templeton and Cherian (1991), the property of MTs is dominated by the chemistry of the thiol group (–SH) of cysteine residues. MTs contained two domains, α domain and β domain, which can chelate different number of heavy metals by the cysteine residues (Otvos and Armitage, 1980). For example, the α domain chelates four cadmium ions while β domain chelates three cadmium ions only (Winge et al., 1981).

Given the metal-binding capacity of MTs, they are considered to play a role in the homeostatic control for essential metals (Cu, Zn), which are ready to fulfill enzymatic and other cellular processes (Brouwer et al., 1989; Roesijadi et al., 1996; Viarengo

and Nott, 1993). In addition, metallothioneins can bind non-essential metals (Cd, Hg) within cells, and so restrict their toxic potential (Roesijadi, 1992; Roesijadi et al., 1996; Zaroogian and Jackim, 2000). The affinity of the protein *in vitro* decreases in the hierarchical sequence $Hg^{2+} > Cu^{+}$, Ag^{+}, $Bi^{3+} >> Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+}$ (Vasak, 1991). When non-essential heavy metals get into the cells, they might compete with essential metals for the binding site on metallothioneins. Finally, the toxicity will decrease due to the effects of replacement.

Although MTs were first discovered to be functional as metal binding, they were also found to be induced after hormone administration or oxyradical-generating compound exposure (Hamer, 1986; Kagi, 1993). Regardless which kind of the inducible materials, MTs play a role to protect the tissues or organs in an organism (Sato and Bremner, 1993; Viarengo et al., 2000).

1.5 Previous studies of vertebrate and invertebrate metallothioneins

Since metallothioneins were discovered in equine kidney cortex by Margoshes and Vallee (1957), more and more creatures have been examined whether MT or MT-like molecules exist in their tissues or organs. MT or MT-like proteins have been reported in many vertebrates including several species of fish (Olsson et al., 1998; Roeva et al., 1999) and in aquatic invertebrates (Roesijadi and Fowler, 1991), mainly molluscs

(Isani et al., 2000; Langston et al., 1998) and crustaceans (Barka and Pavillon, 2000; Engel and Brouwer, 1993; Roesijadi, 1992).

In past five decades, many isoforms and functions of metallothioneins have been reported. In mammals, at least four isoforms with different characteristics have been demonstrated. MT-I and MT-II can be induced by different heavy metals in different organs (Binz and Kagi, 1999; Chapman et al., 1999). These MTs are synthesized primarily in the liver and kidneys. In addition, MT-III is expressed specifically in brain, and MT-IV is expressed exclusively in stratified squamous epithelium (Davis and Cousins, 2000; Quaife et al., 1994). The polymorphism of MTs also coexists in invertebrate. The MTs in molluscs are also classified into two multicomponent gene families- MT-10 and MT-20 (Barsyte et al., 1999; Frazier, 1986; Mackay et al., 1993). It has been shown that MT-10 is constitutively expressed, and MT-20 could be induced by heavy metals or oxidative stresses (Dondero et al., 2005). In invertebrates, MTs have been investigated in many and several phyla. MT or MT-like proteins have been found in sponges (Berthet et al., 2005; Philp, 1999; Schroder et al., 2000), molluscs (Isani et al., 2000; Langston et al., 1998; Roesijadi and Klerks, 1989; Stone et al., 1986), crustaceans (Barka and Pavillon, 2000; Engel and Brouwer, 1993; Roesijadi, 1992), polychaetes (Eriksen et al., 1990) and echinoderms (Riek et al., 1999). Fowler (1987) classified these MTs into three classes. The metallothioneins in

class I were homologous with horse MT. The rest of the MTs with no homology with horse MT belong to class II. Class III consists of non-proteinaceous MTs also known as phytochelatins. Afterwards, Binz and Kägi (1999) classified MTs into 15 families based on taxonomic status and the patterns of distribution of Cystein residues among the MT sequences.

In spite of many studies on MTs in invertebrates, very few studies of MTs were conducted on Cnidaria. Andersen et al. (1988) used gel-permeation rechromatography and considered that MTs did not exist in the Coelenterates. However, a putative coral MT gene fragment was reported by using EST technique (Morgan et al., 2005). Based these two studies, the existence of MTs in Cnidaria is still unclear. Comparing with fishes with high mobility, animals with limited mobility or sessile animals have the advantage to be a biomarker for studying heavy metal pollutions (Wu, 2005). Proteins that associated with heavy metal resistance and susceptible to rapid fluctuations in these animals are suitable for detecting heavy metals pollution. Therefore, the expression level of MTs in animals is a potential target to be monitored (Wu, 2005).

1.6 Cnidarians and *Aiptasia pulchella*

Cnidarians are diploblastic animals evolved at very early stage. The cnidarins possess complex cellular organelles known as cnidae and they have the polyp and/or

medusa stages in their life cycle (Grassi et al., 1995). Most members of Cnidaria are predominantly found in marine. Modem cnidarians are generally classified into four classes: Hydrozoa (hydra), Scyphozoa (jellyfish), Cubozoa (box jellyfish), and Anthozoa (sea anemones and corals).

Aiptasia pulchella (Carlgren, 1943) is belongs to the Family Aiptasiidae, Order Actiniaria, Class Anthozoa. *A. pulchella* was found firstly in the North Western Pacific east to Hawaii (Carlgren, 1943) and now has widely distributed in the tropical and subtropical Pacific Ocean (Wang et al., 2008). So it is also known as "tropic sea anemone." *A. pulchella* possess an elongate, smooth stalk and long, smooth tentacles without any kind of projections. The color of *A. pulchella* primary comes from the symbiosis algae, zooxanthellae (Wicksten, 1989). Asexual reproduction of *A. pulchella* is mainly by pedal laceration. A tiny segment of tissues detached from the foot can quickly develop into a new anemone. This is the way of *A. pulchella* to form large cloned colonies. For this reason, *A. pulchella* with same genome was chosed to reduce inter-individual variation in this study.

1.7 Objective

The main goal of this study is to demonstrate whether the metallothionein or metallothionein-like proteins are expressed in *Aiptasia pulchella*. Immunoblotting,

immunocytochemistry and RT-PCR methods are applied to confirm the existence of MTs in the sea anemone, *Aiptasia pulchella*.

2. Materials and Methods

2.1 Animals

The salinity of the seawater used to culture *Aiptasia pulchella* was maintained roughly around 30~31‰. And those *A. pulchell*a were exposed to ambient sunlight, and the water temperature was maintained at 26 °C to 27 °C with a cooling machine (*Tung Fa Aquarium Co., Ltd*). The animals were fed with newly hatched brine shrimp nauplii twice per week.

2.2 Cadmium exposure

$CdCl_2 \cdot H_2O$ (0.0538 gm) was dissolved in 100 ml artificial seawater with salinity 31‰ and pH 8.27 as 300 mg Cd/L stock solution. The stock solution was stored at 4 ºC until used. The sea anemones were removed from the aquacultural tanks and kept in flashing bottle for acclimation. After the animals adhere to the wall, they were treated with different cadmium concentrations of artificial sea water and maintained at 26 °C in an incubator during the whole exposure process.

2.3 Measurement of cadmium bioaccumulation

The sea anemones were collected from flashing bottle after exposed to cadmium solution for 48 hrs. The surface water of the sea anemones was removed by tissue

papers. Each sample contained three anemones in microtubes was dried by oven and weighted by micro balance (*AE50, Mettler*). Afterwards, 1 ml HNO_3 was added to each sample and heated at 70°C overnight before measuring cadmium concentration by a polarized Zeeman atomic absorption spectrophotometer (*Hitachi model Z-8100, Tokyo, Japan*). Metal concentration analysis was based on the *Standard Guide for heavy Metal Analysis by Atomic Absorption Spectrophotometer*.

2.4 RNA extraction and reverse transcription

Total RNA was purified from whole sea anemones by acid guanidinium thiocyanate-phenolchloroform extraction method (Chomczynski and Sacchi, 1987) using the Trizol Reagent (Sigma-Aldrich). Extracted RNA was resuspended with DEPC water. The RT-PCR was carried out according to the manufacturer instructions (SuperScript® III, invitrogen). First, 13 μl mixed solution A in a PCR tube, made of 5 μg RNA, 1 μl dNTP (10 μM), 5 μl oligo-dT (10 μM) and DEPC water, was heated at 65°C for 5 min and incubated on ice for at least 1 min. Mixed solution B contained 0.1M DTT 1 μl, 5X first-strand buffer 4 μl, 0.1% DEPC water 1 μl and SuperScript™ III RT 1 μl was added in the previous mixed solution A. The tube was incubated at 50°C for 60 min and 70°C for 15 min to finish the reverse transcription process.

2.5 Polymerase chain reaction (PCR)

In the molecular study, it was considered at first that metallothionein should be obtained by the RT-PCR method with the primers combinations. Six primers combinations were designed from the metallothionein sequences identified in other animals such as coral (*Acropora cervicornis*), hydra (*Hydra magnipapillata*), Placozoa (*Trichoplax adhaerens*), and human (*Homo sapiens*). PCR sample was made of 1× PCR buffer, 0.2 mM dNTP mix, 0.4 μM forward primer, 0.4 μM reverse primer, 0.2 μL *Taq* DNA polymerase, 4 μL cDNA obtained from reverse transcription process, and 35.8 μL deionized water. The sample was mixed adequately and then performed 35 cycles about 94°C denaturation step for 30 sec, 44.8 °C annealing step for 1 min, and 72°C extension step for 30 sec. And the sample was heated at 72°C for 10 min to ensure that any remaining single-stranded DNA was fully extended. The sequences were analyzed by *chromas*, *CLC sequence viewer*, *Clustal W*, *BLAST of NCBI*.

2.6 Partial purification of Metallothionein

Sea anemones exposed to 0.5 mg/L cadmium solution for 24 hrs in the flashing bottle were removed and soaked by tissue papers. The animals were homogenized by gagte mortar and pestle in liquid nitrogen. Then the sample powder was placed in eppendorf tube with 30 μl extraction buffer (1mM DTT, 0.02 M Tris-HCl, pH 7.5,

0.01M NaCl). The homogenates were centrifuged at 8500 rpm at 4°C for 10 minutes to remove zooxanthellae and unbroken cells. Each supernatant was then transferred to a new eppendorf tube and adjusted its protein concentration. Each tube contained consistent 100 μg proteins. Since metallothioneins are heat-stable proteins, homogenates were furthermore heated at 90°C for 10 minutes and centrifuged at 16000 rpm, 4°C for 20 minutes (Ford and Graham, 1991) to remove denatured proteins, cellular debris and larger organelles. The heat-stable supernatants were frozen at - 80°C for further analyses.

2.7 SDS-PAGE and immunoblotting

The heat-stable supernatants were mixed with 2X sample buffer (0.25M Tris-HCl, pH 6.8, 4% SDS, 20 mM EDTA, 30% glycerol, 0.2% (w/v) Bromophenol blue, 2% β-mercaptoethnol). After boiled for 10 minutes, 50 μg aliquots of protein were loaded in each lane and then separated by 15% SDS-PAGE. The SDS-PAGE was proceed at 200 V for 2 hours in XCell *SureLock*™ Mini Cell *(Invitrogen)* at 4°C. After the electrophoresis, half gel was stained with Coomassie blue and the other was proceed the western blotting to PVDF membrane (*Millipore* pore size: 0.45μm) by XCell II™ Blot Module (*Invitrogen*) at 25 V, 401 mV for 1 hr at 4°C. The PVDF membrane was then blocked with 5% BSA in TBSt (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH

7.4) for 1 hr. After washed twice with TBSt for 10 minutes, the PVDF membrane was cut into two pieces at 30 kDa and soaked in different primary antibody solution. The upper piece was incubated overnight with rabbit polyclonal antibodies against human actin (*sc1616-R, Santa Cruz*) 1:8000 dilution in TBSt at 4°C, and the bottom one was incubated overnight with rabbit anti human metallothionein (*sc-11377, Santa Cruz*) 1:500 dilution in TBSt at 4°C. The PVDF membrane was washed four times with TBSt for 5 min each time. And the two pieces of PVDF membrane were both incubated with 1:1000 dilution of Alkaline Phophatase-conjugated goat anti-rabbit IgG (*AP132A, Chemicon*) at 4°C overnight. The PVDF membranes were washed four times with TBSt for 5 minutes each time. Both pieces of membranes were detected by LumiGLO® chemiluminescence (*KPL*) and exposed to medical X-ray film (*Fuji, Japan*) for 3 minutes.

2.8 Immunohistochemistry (IHC)

The sea anemones exposed to 0.25 mg/L cadmium for 0, 24, 48, 72 hrs (time-dependent) and 0, 0.25, 0.5, 1.0 mg/L cadmium for 24 hrs (dose-dependent) in the flashing bottle were removed and anesthetized by magnesium chloride for 10 min. The animals were then fixed in 10% formalin at 4°C overnight, and infiltrated in 30% sucrose solution at 4°C overnight. After full infiltration, the animals were submerged

into O.C.T in microtube and rapid cooled with liquid nitrogen. The frozen samples were stored at -80°C until cryosection. Sections about 20 μm were adhered on the slide which was coated with poly-L-Lysine. The samples were stored at -20°C until immunohistochemistry process. For antigen retrieval, slides were incubated in 0.01M citric acid (pH= 6.0) at 95°C for 5~10 min. After citric acid solution were returned to 25°C, the slides were taken out and dried out at dry box. The sections were ringed by liquid-repellent slide marker pen (*Daido Sangyo Co., Ltd*). The samples were washed twice by PBSt and incubated with blocking solution (2% BSA and 10% normal goat serum in PBSt) at 25°C for 1 hr. After blocking procedure, the samples were washed twice by PBSt and incubated overnight with anti-human metallothionein (*sc-11377, Santa Cruz*) 1:40 dilution in PBSt at 4°C. The samples were washed four times with PBSt for 5 min each time and incubated away from light with secondary antibody solution (Dylight^{TM} 488-conjugated AffiniPure goat anti-rabbit IgG) 1:500 dilution in PBSt at 25°C for 1 hr. Then the sections were washed four times with PBSt for 5 minutes each time and stained with DAPI solution at 25°C for 10 minutes. The sections were washed with deionized water twice for 5 minutes each time and mounted with fluoromounting solution (Fluoromount-G ^{TM}, *SouthernBiotech, US*). The slides were stored in a black box to avoid bleaching until they were analyzed by fluorescence microscopy (*Zeiss AxioCam*). Then the fluorescence density was

measured by *MetaMorph offline* software.

2.9 Statistic

Statistical analysis was performed with randomized complete block design (RCBD); Duncan's new multiple range test to determine the treatment effects on cadium accumulation after exposure to cadmium. Statistical analysis was performed with student's t test to present the treatment effects on metallothionein induction in the immunohistochemistry experiment. All results are reported as the mean ± standard error (SE).

3. Results

3.1 Morphological changes in *A. pulchella* during cadmium treatment

The sea anemones, *A. pulchella* were acclimated in artificial sea water without cadmium as the control group (Figure 1A). After exposed to 0.25 mg/L cadmium sea water for 48 hours, the tentacles and body column of *A. pulchella* withdrew and some dark brown tissues (red arrow) were shed (Figure 1B). When the cadmium-exposed *A. pulchella* were moved back to the artificial sea water without cadmium for 48 hours, their appearance and morphology of tentacles and body column were completely recovered (Figure 1C).

3.2 Bioaccumulation of cadmium in *A. pulchella*

As sea anemone, *A. pulchella*, was exposed to 0.25 mg/L Cd for 48 hrs, dark brown shed tissues were observed (Figure 1B). After the cadmium treatment, an intact sea anemone was separated for shed tissues, tentacles, and body columns. The sea anemone in cadmium-free artificial sea water (Figure 1A) was treated as the baseline control. The accumulated concentrations (mean ± SE) of cadmium in different tissues were then measured by an atomic absorption spectrum (*HITACHIi, z-6100*). The cadmium concentration of the shed tissues was 13.08 ± 6.12 μg/g, the body columns was 8.73 ± 5.67 μg/g, the tentacles was 8.06 ± 5.05 μg/g, and normal tissues is 0 ±

0.01 μg/g. Compared to the normal tissues group, cadmium-treated groups showed significant difference (t-test, $p<0.01$) (Figure 2). Similar significant difference (RCBD, $p<0.01$) was also observed between the shed tissue group and other cadmium-treated groups (body and tentacles). But no significant difference was found between the tentacles and the body columns.

3.3 Molecular analyses of metallothionein genes on *A. pulchella*

The putative metallothionein sequence in *A. pulchella* was tried to obtain by using RT-PCR technique. Six primer combinations (Table 1) were designed from Placozoa (*Trichoplax adhaerens*), coral (*Acropora cervicornis*), hydra (*Hydra magnipapillata*) and human (*Homo sapiens*). The corals like *A. pulchella* also belong to Phylum Cnidaria, however they are separated to different Orders. And the Phylum Placozoa is the closest phylum in phylogenetic tree. According to previous studies (Kortschak, 2003; Sullivan, 2006), more than 10% of the Cnidaria ESTs match stronger to the databases of human homologs than in the Drosophila or Caenorhabditis genomes. So, primers 11 and 12 were designed to obtain the putative MT sequences in *A. pulchella*. The quality of every cDNA sample was confirmed by using 16S universal primers.

Using the primers from Phylum Placozoa, two clone sequences (DNA sequence 1 and 2) were obtained (Figure 3). Both of them can be blasted to the MT of *Trichoplax*

adhaerens (Phylum Placozoa) because of about 25 bps in the sequence (Tables 2 and 3). But more than one stop codons were observed from the translation frames. Thus, the two putative sequences could not be confirmed at this stage. Using primers designed from MT of human, no sequence could be obtained. In addition, the sequences obtained from primers designed from corals and hydra could not be blasted to any MT-like protein. Unfortunately, the exact MT sequence on *A. pulchella* was not obtained by the method of RT-PCR and primer combinations.

3.4 Detection of metallothioneins in *A. pulchella* by immunoblotting

Due to the heat stable property of metallothionein, the extracted protein samples from *A. pulchella* were heated at 90 ºC for 10 min to eliminate the non-heat stable proteins before protein electrophoresis. After Western blotting, the SDS-PAGE protein samples were recognized by a rabbit anti-human metallothionein antibody which has been used to detect metallothioneins in several invertebrates. Based on the immunoblotting result, three bands were obtained between 11~17 kDa which are the predicted sizes of metallothionein. The expression pattern of metallothionein-like protein was different (Figure 4A): the 1st band was mainly expressed in the samples of sea anemone's tentacles, whereas the 3rd band was predominately expressed in the samples of sea anemone's body column. The 2nd band was generally expressed in the

samples of tentacles and body column. The metallothionein expression patterns were different between the tentacles and body column. In tentacles, the expression of 1st band was higher in the cadmium treated group than that in the control group (Figure 4C). But in the body column, the expression of 2nd and 3rd band was slightly higher in the cadmium treated than that in the control group (Figure 4D). The protein samples were also detected with a rabbit anti-shrimp heat shock protein 21 antibodies, but no band was shown between 11~17 kDa. In this study, actin was served as an internal control for equal loading.

3.5 Location of metallothioneins in *A. pulchella*

A. pulchella is a diploblastic organism. The tissue of *A. pulchella* can be easily separated to ectodermal, gastrodemal and mesoglea parts (Figure 5A, 5B). The location of MTs in *A. pulchella* could be examined by using immunohistochemistry (IHC) experiment. The sea anemones were proceeded cryosection in accordance with the schematic diagram (Figure 5C).

After exposed to cadmium, the intensity of fluorescence increased the particles detected by fluorescence-conjugated antibody in the tentacles' section. In the negative control group, no obvious particles were observed. The amount of detected particles distributed unevenly, while most particles were localized in the ectoderm layer.

Compared to the control group, the average amount of particles significantly increased after different cadmium exposures (Figure 6A). According to the counting of particles, the fluorescence intensity was performed as 10^{-3} dots per μm^2, and it showed that the number of particles increased significantly after exposure to cadmium for different time period (Student's t-test, $p<0.05$) (Figure 6B). In addition, compared to the control group, the average amount of particles significantly increased as exposed to higher concentrations of cadmium (Figure 7A). It also showed that the number of particles increased significantly in the groups of exposure to 0.25 and 0.5 mg/L cadmium for 24 hrs (Student's t-test, $p<0.05$) (Figure 7B) although no significant difference was observed between the control and 1.0 mg/L group.

The location of particles was further checked by using confocal microscopy (*Zeiss LSM 780*) (Figure 8A). The fluorescence was observed mainly in the cytoplasma. And the symbiotic algae was detected by emission ultra red, and found mostly at gastrodermis (Figure 8B). This result confirmed that the symbiotic alage did not interfere the analysis of MTs in *A. pulchella*.

4. Discussion

It is well known that cadmium can cause severe toxicity to aquatic animals (Taylor, 1983). The sessile animals such as sea anemones are usually forced to suffer cadmium contaminants in the sea side. Those animals have to possess certain detoxification reactions to protect themselves. Metallothioneins have been shown to serve as a detoxification mechanism in several organisms (Amiard, 2006; Hogstrand and Haux, 1991). However, the evidence of MTs existing in sea anemones is still conflicting (Andersen et al., 1988; Morgan et al., 2005).

The morphology of *A. pulchella* can be altered apparently after exposure to cadmium (Huang, 2007). Following the increased concentrations and exposure period, the animals became shrunk and darker. In addition, the phenomenon of tissue shed from Cd-exposed sea anemones was first reported. Pedal laceration is a common way of asexual reproduction in sea anemones (Lin et al., 1992). The period of growth and differentiation from a fragment tissue to a functional individual is about one week. In this study, the shed tissue could not differentiate to normal individual and the appearance was not similar to the reproductive fragments. Therefore it indicates that the shed tissues were not from the regular pedal laceration. Due to high cadmium accumulation, the shed fragments should be considered as a process of autotomy for

detoxification. The reactions of autotomy have been reported in many other aquatic invertebrates such as limbs in crustaceans (Smith, 1995), in polychaetes and earthworms and arms in sea stars (Lawrence, 2010). In crustaceans and echinoderms, the autotomy is mainly related with regeneration (Smith, 1995; Venuti and Edds, 1986). The relationships between autotomy and heavy metal accumulation have been studied extensively in aquatic oligochaetes. For example, autotomy occurred in both *Sparganophilus pearsei* (Vidal and Horne, 2003) and *Tubifex tubifex* (Bouche et al., 2000) after exposure to either cadmium or mercury. Such kind of autotomy has been considered as a mechanism of detoxification, because harmful toxins usually were accumulated in their caudal segments. In this experiment, the Cd-exposed *A. pulchella* was found to shed some dark-brown tissues. According to atomic absorption spectrophotometer results, those shed tissues contain higher Cd concentration than the normal tissues. Because the shed tissues contained higher cadmium concentration were only observed in higher dosage treatment or at longer exposure time periods, the shed tissue under heavy metal treatment should be regarded as a detoxification mechanism in *A. pulchella*. This is the first documentation of this mechanism in sea anemone. The sea anemones exposed to 0.25 mg/L for 48 hrs could recover its appearance after returned to the artificial sea water without cadmium. It is demonstrated that the treatment in this study did not exceed the physiological capacity

of *A. pulchella*.

According to the metallothionein sequences from the gene bank, metalothioneins are not conserved and only partial homology among animal phyla. Probably, it is why most of the primer combinations designed from closely related species in this study did not obtain the sequence fragment. Only two of the obtained sequences could be blasted to the MTs in NCBI database. However, the obtained amino acid sequence includes more than one stop codon. Therefore, the obtained sequences might not be metallothioneins. It is uncertain why the MT sequences among different Orders in Phylum Cnidaria are not conserved. Actually, similar phenomenon has also been observed in earthworms (phylum Annelida) and bivalves (phylum Mollusca) by using *Clustal X* software. In both earthworms and bivalves, MTs show extreme sequence diversity among different Orders. This phenomenon implied that either this gene suffered from relatively low selection pressure or there are some unclear mechanisms responsible for such high diversity. It seems that the non conserved MT gene in *A. pulchella* could not be obtained by utilizing the RT-PCR technique and primer combinations. Another possible interpretation is that MTs are not homologous genes. Therefore, all MTs might be the products of convergent evolution and the DNA sequences were not highly conserved. Therefore, using RT-PCR with primers of closely related animals is not a feasible way to obtain the MT sequences in sea

anemone.

Several heat stable and low molecular weight proteins in *A. pulchella* were detected by anti-human metallothionein antibody. These detected proteins possess the characteristics of MTs. After cadmium treatment, the proteins were induced and expressed in different patterns among different body portions of *A. pulchella*. Therefore, the proteins detected by anti-human metallothionein antibody are highly probably MTs. In this study, heat shock protein antibodies were also used to confirm that the proteins between 11~17 kDa detected by anti-human MT antibodies were not belong to heat shock proteins. In addition, in immunohistochemistry study, MTs detected by the same antibody were induced after cadmium treatment. This induced expression patterns are both dosage-dependent and time-dependent. After cadmium treatment, the metallothioneins detected in the ectoderm increased significantly and presented the saturation phenomenon at higher dosage or after longer exposure period. No matter in immunoblotting or in immunohistochemistry study, cadmium induced MTs expression in *A. pulchella*.

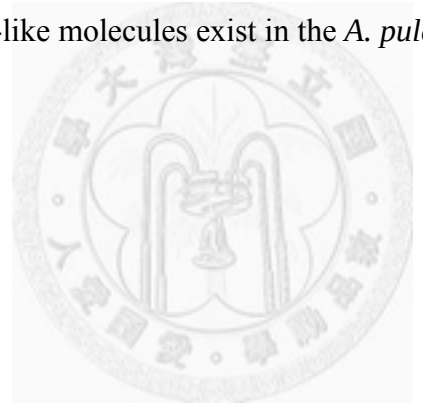
The immunohistochemistry results showed that the MTs were predominantly in the ectoderm of *A. pulchella*. However, MTs in other animals are not only expressed in ectoderm. Several studies about the localization of MTs have been performed in sea

urchin, *Strongylocentrotus purpuratus* (Angerer et al., 1986; Nemer et al., 1984; Olsson et al., 1990). During the development of sea urchin, the gene expression of metallothioneins A (MT-A) was expressed endogenously and was restrictedly in ectoderm, while other isoform, MT-B, was elicited primarily by heavy-metal induction (Nemer et al., 1984). Recently, there is no report on MTs localization in cnidarian. But a few studies on metal localization in cnidarian might offer some hints. Zinc and copper were detected in the nematocytes and in other ectodermal cells (Gupta, 1984). Although Gupta's study did not detect the existence of cadmium, there were evidences showing that cadmium can compeet the binding site of zinc. In addition, an X-ray spectral analysis (EDAX) also demonstrated that high concentration of divalent cations (Mg^{2+} and Ca^{2+}) existed in the isolated undischarged nematocysts in the ectoderm of cnidarian (Weber et al., 1987). For this reason, it is deduced that the cadmium is possible to exist in ectoderm of cnidarian. This inference is supported by the result of the localization for MTs detected by immunohistochemistry in this study.

Besides the localization of MTs, the ectoderm was thicken after cadmium treatment. When the morphology was examined, the tentacles become dramatically darker, shorter, and tougher. This discovery was also described by Grant *et al*. (2010). In *Zoanthus robustus* (Phylum Cnidaria), the external lamina of the ectoderm was

thicker in the copper exposure group than in the control group (Grant et al., 2010). But the possible reason is still not clear.

Andersen *et al.* (1988) reported that metallothioneins did not exist in cnidarian. Although the existence of MTs in *A. pulchella* was not confirmed by genetic methods in this study, the MT-like molecules were expressed in the rational location and their induced expression after cadmium exposure was positively confirmed by the immunobloting and immunohistochemistry methods. Base on these results, it is strongly believed that MT-like molecules exist in the *A. pulchella*.



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1A.

1B.

1C

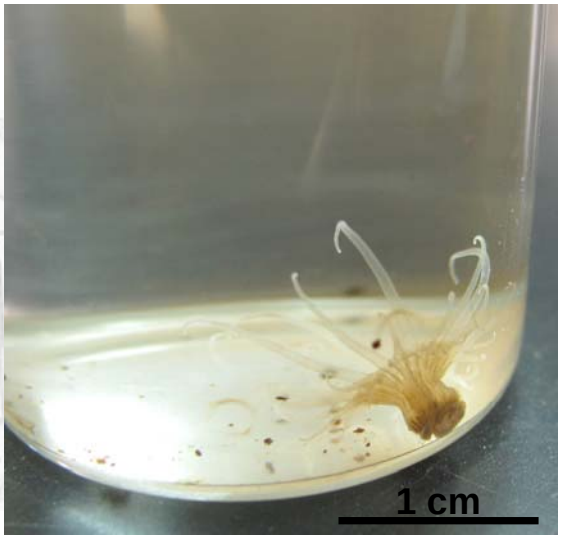


Fig. 1. Morphological changes in *A. pulchella* during cadmium treatment

(A) *A. pulchella* was exposed to artificial sea water without the cadmium. (B)After 48 hours of 0.25 mg/L cadmium exposure, the tentacles of *A. pulchella* were withdrawn and dark brown shed tissues (red arrow) were observed. (C) After 48 hours of 0.25 mg/L cadmium exposure, *A. pulchella* were changed into artificial sea water without cadmium and incubated for additional 48 hours. The appearance of *A. pulchella* was recovered.

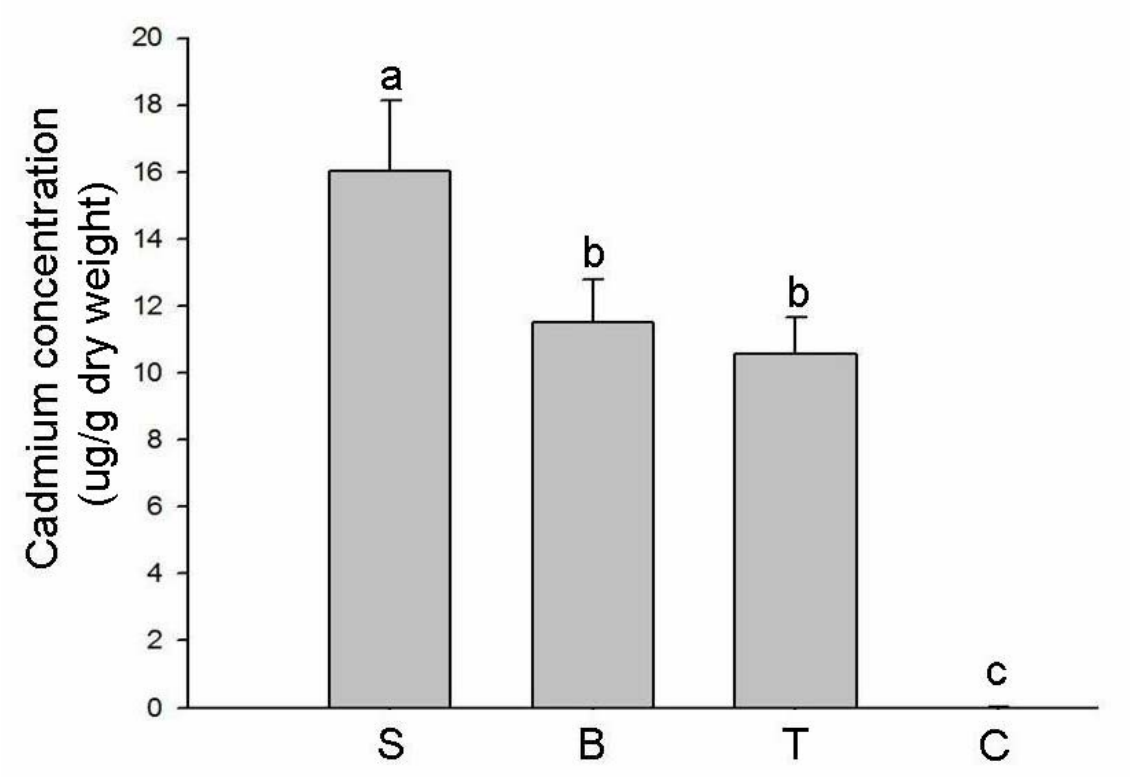


Fig. 2. Bioaccumulation of cadmium in *A. pulchella*

Extent of Cd accumulation in *A. pulchella* tissues μg/g Cd in dry weight tissues following exposure to 0.25 mg/L cadmium for 48 hours was expressed as means (± SE). Significant differences (RCBD and Duncan's Multiple Range Test, $p<0.05$) were observed between cadmium-treated groups and the control group normal tissue. The cadmium concentration in the shed tissue was significantly higher than in the body or tentacles, whereas no significant difference was observed between the bodies and the tentacles. S: shedding tissue, B: body, T: tentacles, C: control (normal tissues without cadmium treatment).

3A

DNA sequence 1

```
001    AGTTGAACCA AATAGTTACC AAACAATTAC CATGAAAAAC
041    ATCAAGCAAA TAGAAATGGA AATCGTAACT GTTCGTCAAT
081    AAAGTATTAC CAAAAATATA CGGCAAGTGT CCTAAAGGAT
121    GCA
```

Deduced amino acid sequence

```
001    S*TK*LPNNY HEKHQANRNG NRNCSSIKYY QIIYGKCPKG
041    C
```

3B

DNA sequence 2

```
001    GGATCTTGTA CACAACATCA CTAATCCTCT TCATAACAAG
041    ATATGGGCCC TCCCACGGAA GCTGTAAGCA AGGGGAAACT
081    CCCTTCTTCC TTTTTGGGTT GTAAAACCAA ACTGGTGCCC
121    CTCGATCATA CGTTTTCTGA TTAAGTCGTT GATTGTACTT
161    TCTCTTCTGC CTGTCACTTT GGATTGTTAA GTGCTCTCTT
201    GCGTAGCCAT GCACCTTACG GATCTTATCC TGTAAGGAAC
241    GTGCATAGTC GCAAGTGTCC TAAAGGATGC AA
```

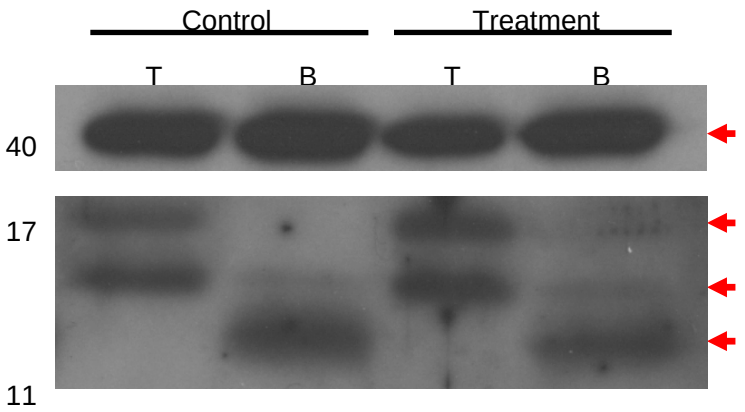
Deduced amino acid sequence

```
001    GSCTQHH*SS S*QDMGPPTE AVSKGKLPSS FLGCKTKLVP
041    LDHTFSD*VV DCTFSSACHF GLLSALLRSH APYGSYPVRN
081    VHSRKCPKGC
```

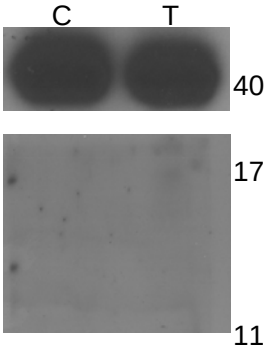
Fig. 3. The putative sequences cloned by RT-PCR.

The two putative sequences (3A and 3B) were cloned by the primer 3 and 4 in Table 1. The two sequences were blast to the metallothionein of *Trichoplax adhaerens*. The matched sequences were underlined. The numbers were marked as the length of sequence. The words in italic type were one of deduced protein sequence frame.

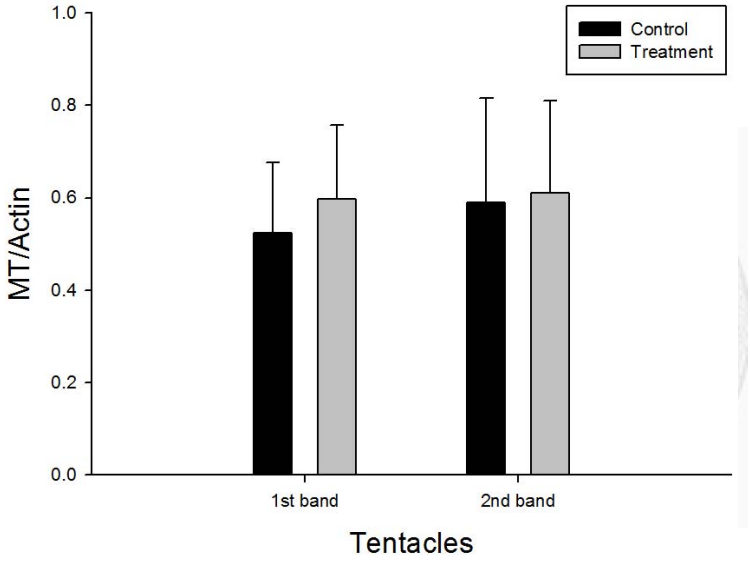
4A


Control
Treatment
T
B
T
B
40
17
11
actin
1st band
2nd band
3rd band

4B


C
T
40
17
11

4C


Control
Treatment
MT/Actin
1.0
0.8
0.6
0.4
0.2
0.0
1st band
2nd band
Tentacles

4D

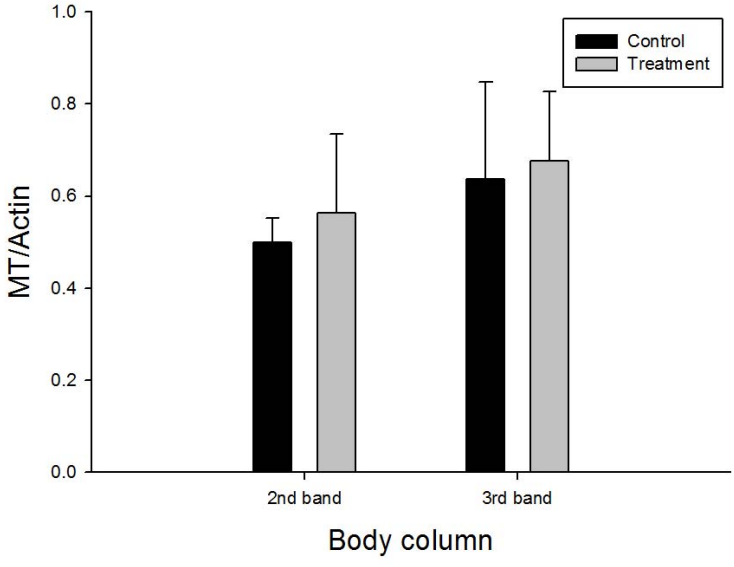
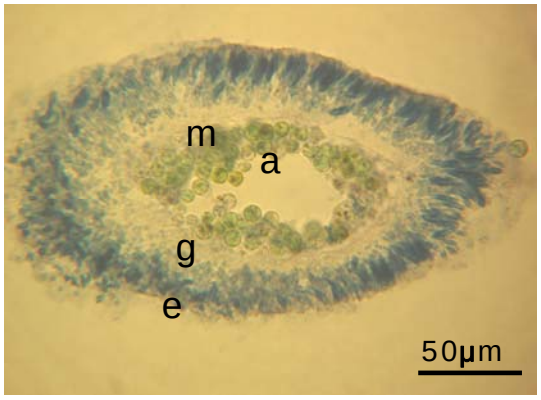

Control
Treatment
MT/Actin
1.0
0.8
0.6
0.4
0.2
0.0
2nd band
3rd band
Body column

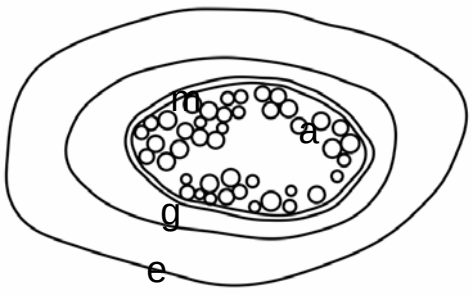
Fig. 4. **MT-like proteins with low molecular weight in *A. pulchella*.**

Proteins of *A. pulchella* were detected by immunoblotting. (A) MT-like proteins (1^{st}, 2^{nd}, 3^{rd} band) were observed between 11~17 kDa by anti-human MT antibodies. Actin was measured as a control for equal loading (Treatment: 0.25 mg Cd/L in artificial sea water for 24 hr; Control: artificial sea water; B: body column, T: tentacles). (B) Proteins could not been observed between 11~17 kDa by anti-shrimp heat shock protein 21 antibodies. Actin was also measured as a control for equal loading (C: control, T: treatment). (C) Relative quantification of protein bands (1^{st} and 2^{nd} band) of tentacles was performed using *Analysis* software. (D) Relative quantification of protein bands (2^{nd} and 3^{rd} band) of body column was performed using *Analysis* software.

5A.



5B



5C

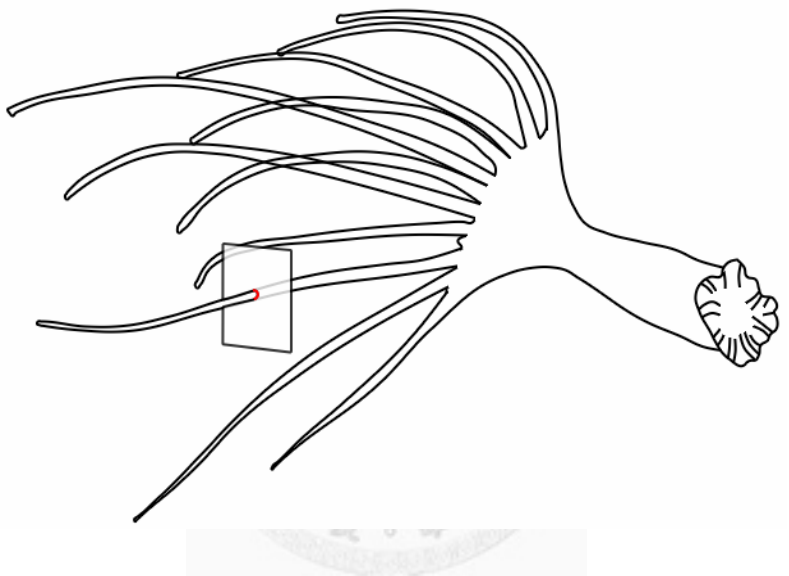
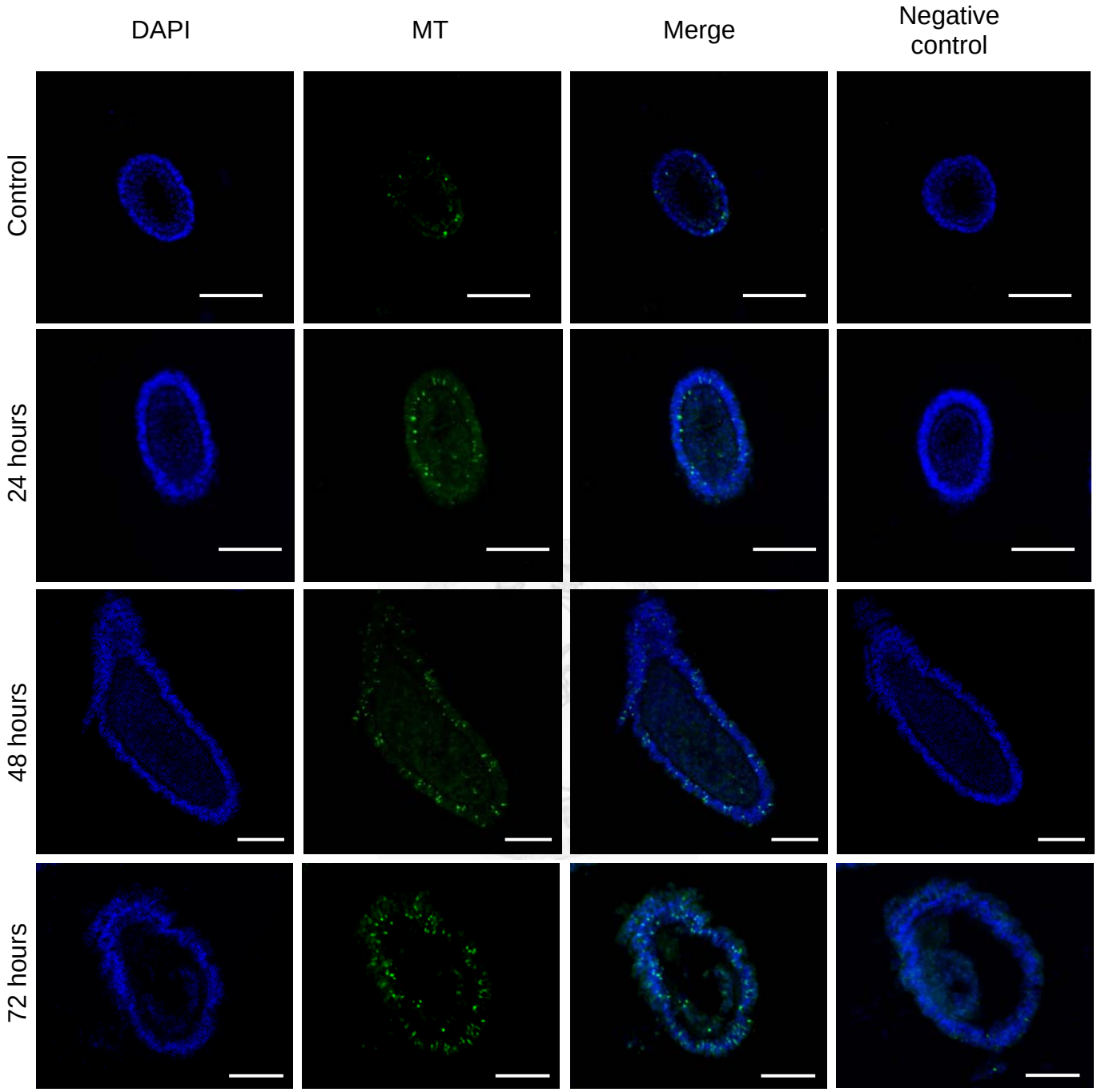


Fig. 5. The diagram of immunohistochemistry study of metallothionein location

Microscopic photograph (A) and schematic diagram (B) of the cross section of tentacle in *A. pulchella* shows three tissue layers: e: ectoderm, m: mesoglea, g: gastrodermis a: algae cells. (C) The plane of section by cryosection is in compliance with the schematic diagram.

6A


DAPI
MT
Merge
Negative control
Control
24 hours
48 hours
72 hours

6B

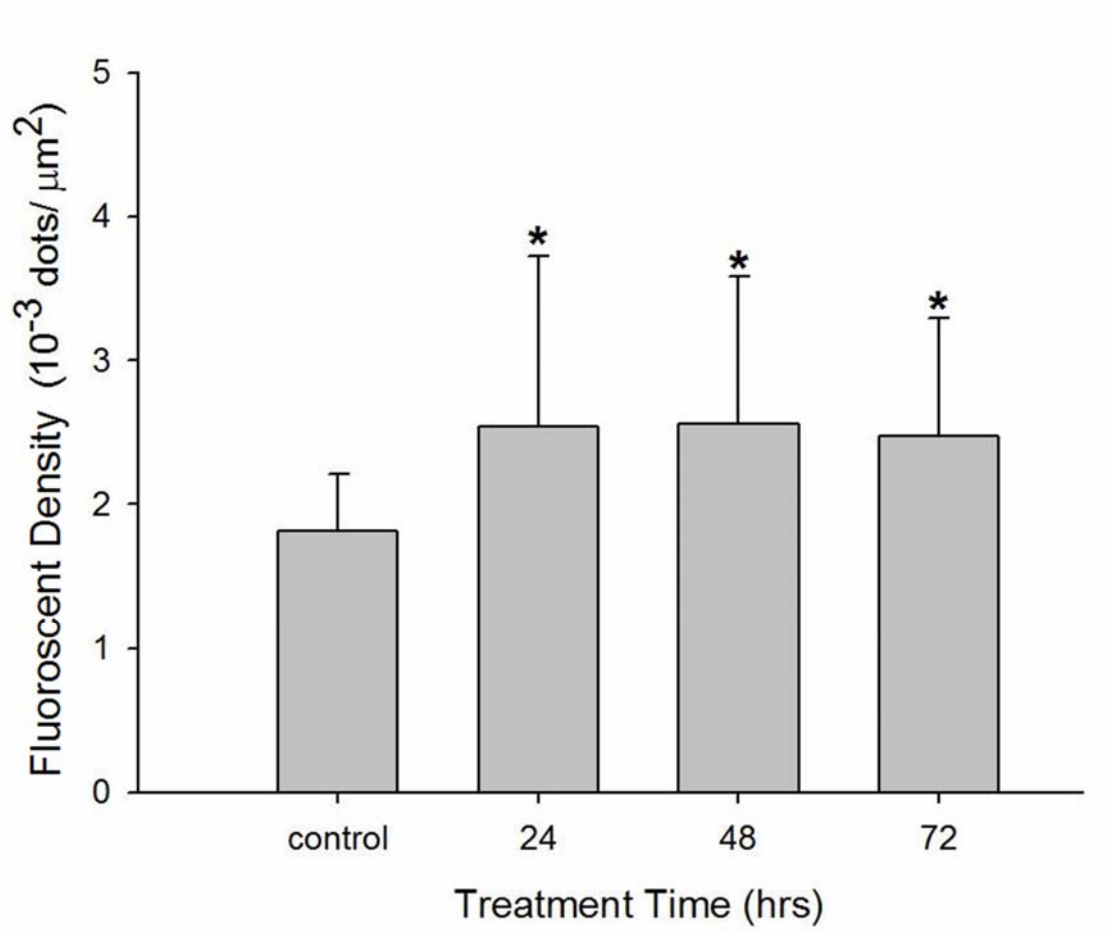
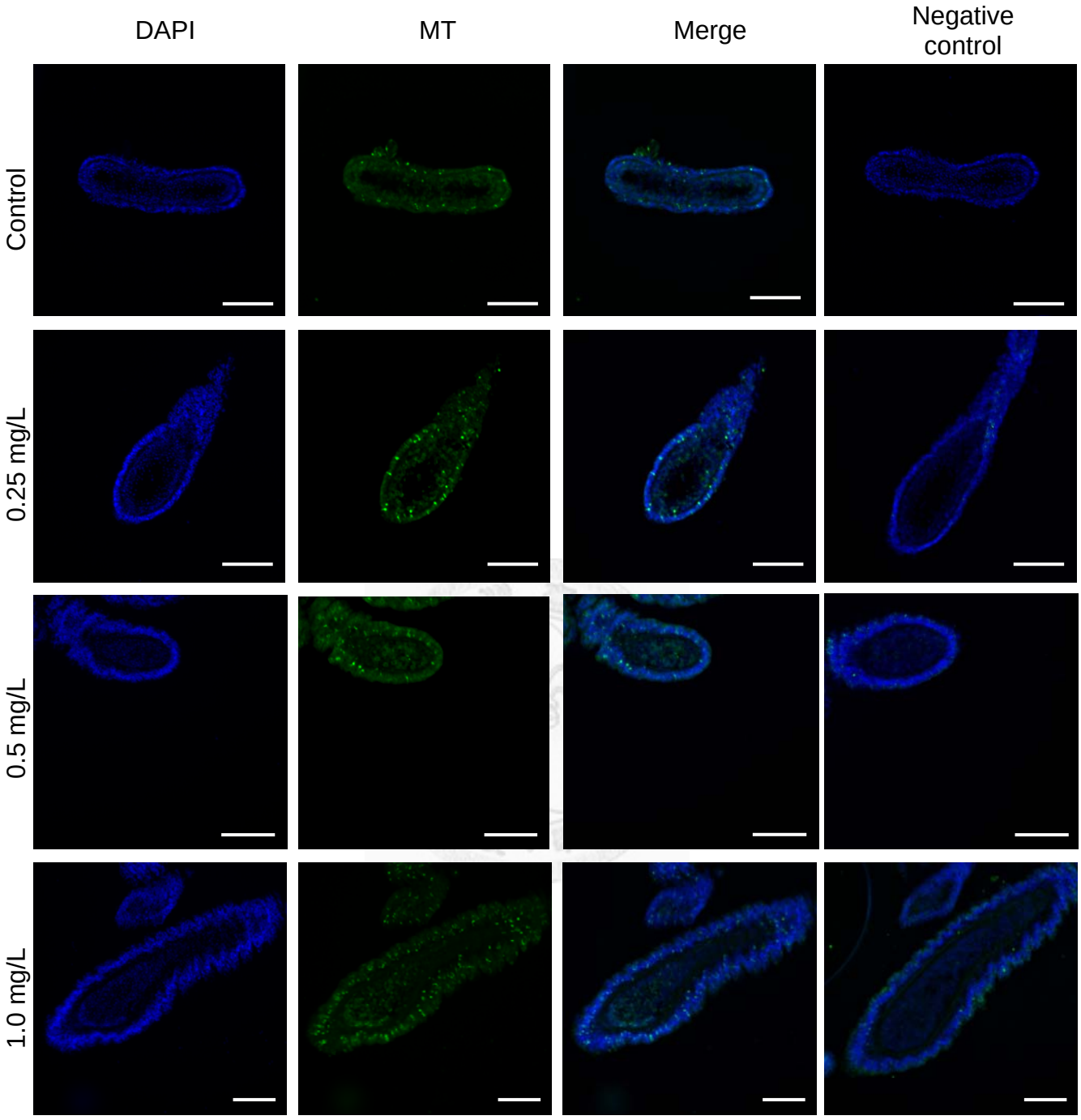


Fig. 6. Metallothionein induction in *A. pulchella* at different time periods.

(A) The sea anemones exposed to 0.25 mg/L cadmium for 0, 24, 48, or 72 hrs were then detected by polyclonal rabbit anti-human metallothioneins, and secondary antibody (Dylight™ 488-conjugated AffiniPure goat anti-rabbit IgG). Negative control was incubated with secondary antibody only. Those cryosection were stained by DAPI as nuclear counter stain. The images were obtained from 20 μm cryosection of the tentacles of sea anemones by using a fluorescence microscope. In the negative control group, no obvious particles were detected. The green particles in the ectoderm were considered as the location of metallothioneins, which showed significant induction after the sea anemones were treated with cadmium. Bar = 100 μm. (B) The amount of the detected fluorescent particles was analyzed by *Metamorph* software. The quantified result was showed that the number of particles increased significantly after exposure to cadmium after 24 hrs (student's t-test, $p<0.05$).

7A



7B

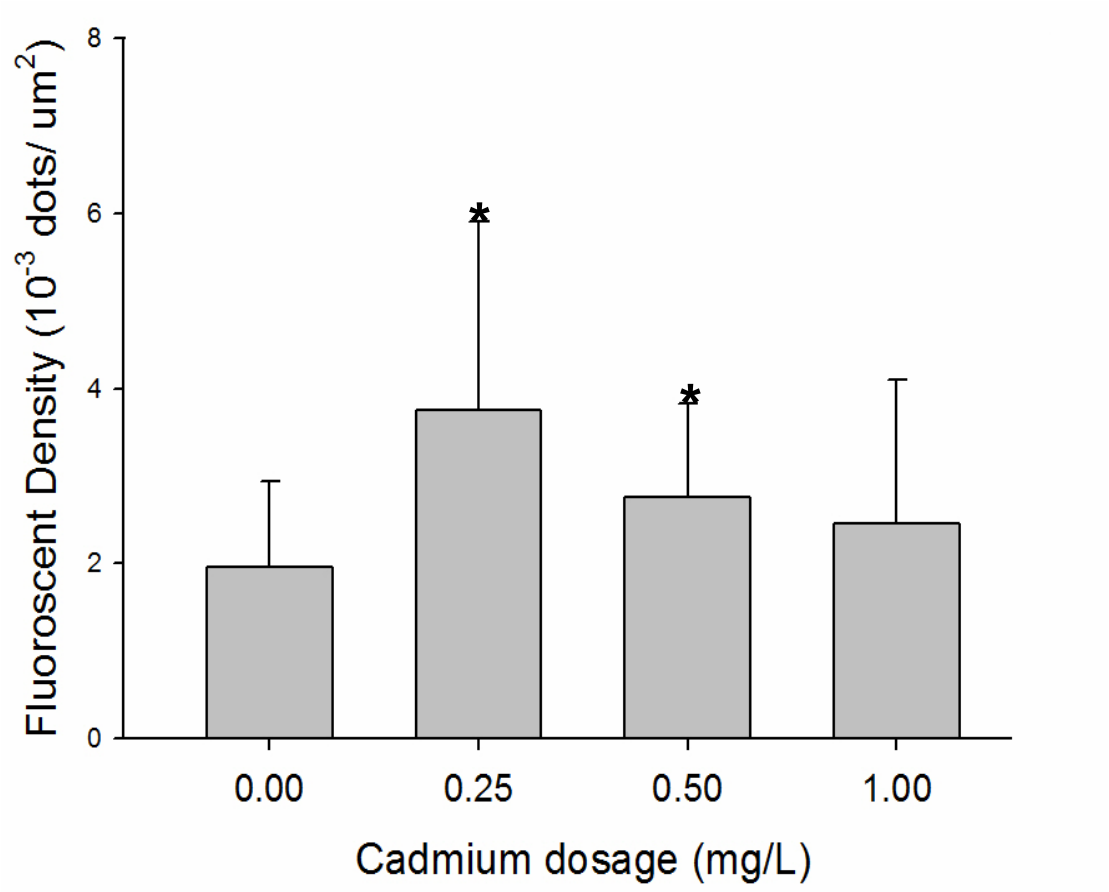
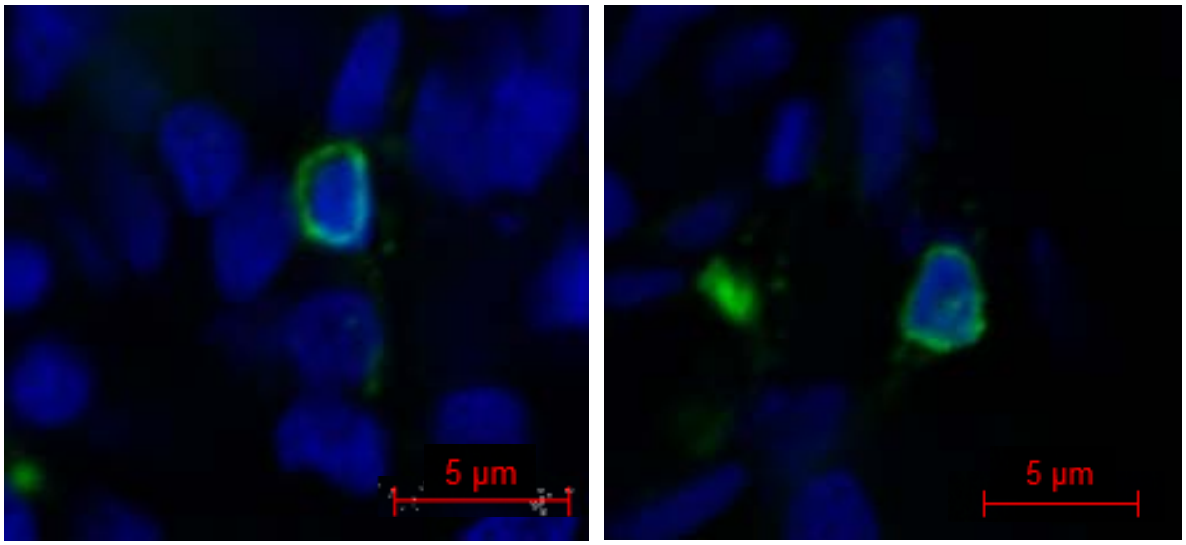


Fig. 7. Metallothionein induction in *A. pulchella* after exposed to different cadmium dosages.

(A) The sea anemones exposed to 0, 0.25, 0.5, or 1.0 mg/L cadmium for 24 hrs were then detected by polyclonal rabbit anti-human metallothioneins, secondary antibody (Dylight™ 488-conjugated AffiniPure goat anti-rabbit IgG). Negative control was incubated with the secondary antibody only. Those cryosection were stained by DAPI as nuclear counter stain. The images were obtained from 20 μm cryosection of the sea anemone tentacles by using a fluorescence microscope. In the negative control group, no obvious particles were detected. The green particles in the ectoderm were considered as the location of metallothioneins, which showed significant induction after the sea anemones were treated with cadmium. Bar = 100 μm. (D) The amount of the detected fluorescent particles was analyzed by *MetaMorph offline* software. The analyses result was showed that the number of particles increased significantly after exposure to cadmium. Significant difference was observed between control and 0.25 and 0.5 mg/L groups (Student's t-test, $p<0.05$), but no significant difference was observed between control and 1.0 mg/L group.

8A



8B

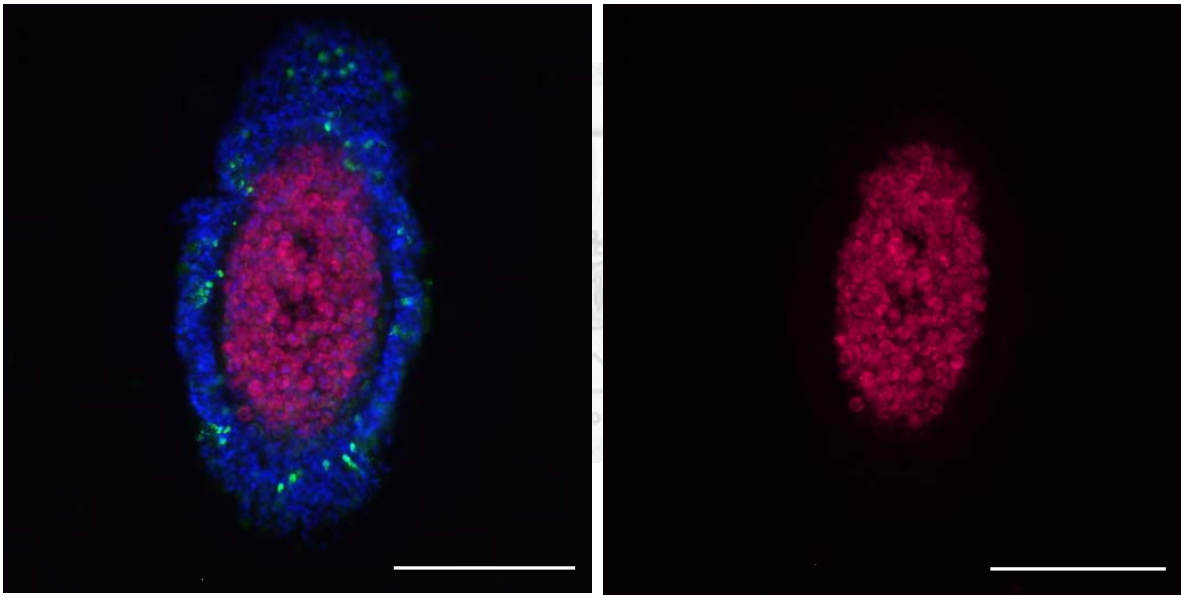


Fig. 8. The localization of metallothioneins in *A. pulchella* cells.

(A) By confocol microscopy, the detected fluorescent particles (green) were localized around the nucleus (blue), which were stained with DAPI. The detected particles were localized in the cytoplasm, not in the nucleus. (B) When the symbiotic algae were excited by infrared, they emitted light. It was clear that the algae were only localized in gastroderm of sea anemones. The detected fluroscence particles of metallothioneins localized in the symbiosis algae position were not taken into statistics. Bar: 100 μm. Red: symbiosis algae; Blue: DAPI; Green: metallothioneins.

Table 1. The primers utilized in the cloning of putative metallothionein sequences.

The Primer 1 and 2 were used to confirm the species in the studies and the quality of RNA extraction. Other primers were design from the sequences in the NCBI database. Primers were designed by the tool of "Primer 3' and "Primer Blast (NCBI)".

Primer	Sequence	Target gene	Template sequence
Primer 1	5'- CGCGGTACCCTGACCGTGTG-3'	16S	Various
Primer 2	5'- TCTCAGGTCGCCCCAACCAA-3'		
Primer 3	5'-CTGTTGCTGTTCACGTCGTC-3'	Metallothionein, placozoa	*Trichoplax adhaerens* mRNA for metallothionein (AJ581011)
Primer 4	5'-TGCATCCTTTAGGACACTTGC-3'		
Primer 5	5'-AGCCCTTGTAATTGCATTGA-3'	Metallothionein, coral, cnidaria	Mt1 Coral stress responses, copper exposure *Acropora cervicornis* cDNA similar to Metallothionein homolog, mRNA sequence (DR681654)
Primer 6	5'-CGAACAACTGGAGTCACATTTA-3'		
Primer 7	5'- CCACCGATAACGCCGTGTTTTCCA-3'	Metallothionein, hydra, cnidaria	Hydra EST UCI 6 *Hydra magnipapillata* cDNA similar to TR:Q9U623 Q9U623 METALLOTHIONEIN, mRNA sequence (CV565233, CV564390, CF656454)
Primer 8	5'- TGCACTCAGCGAAGCAACCGT-3'		
Primer 9	5'-TGCACTCAGCGAAGCAACCGT-3'	Metallothionein, hydra, cnidaria	Hydra EST UCI 6 *Hydra magnipapillata* cDNA similar to TR:Q9U623 Q9U623 METALLOTHIONEIN, mRNA sequence (CV565631, CV564777, CV286374, CF656147, CB887917)
Primer 10	5'-CGCAGGGATTACCACCGATAACGC-3'		
Primer 11	5'- ATCCCAACTGCTCCTGCGCC-3'	Metallothionein, *Homo sapiens*	*Homo sapiens* metallothionein 2A (MT2A), mRNA (NM_005953)
Primer 12	5'- TGGGCACACTTGGCACAGCC-3'		

Table 2. The blast results of putative sequence 1 (Figure 3A) by NCBI BLAST.

These twelve genes were the blast results by importing the putative sequence 1 (Fig. 3A) on the NCBI BLAST. The primers (prime 3 and 4 in Table 1) used for obtaining the putative sequence are designed from the *Trichoplax adhaerens* mRNA for metallothionein.

Blast gene	Score	Query coverage	E value	Max identity
Podospora anserina S mat+ genomic DNA chromosome 1, supercontig 5	48.2	27%	0.009	91%
Arabidopsis thaliana chromosome 1 BAC F5D21 genomic sequence, complete sequence	46.4	53%	0.031	76%
Trichodesmium erythraeum IMS101, complete genome	42.8	30%	0.38	84%
Mus musculus chromosome 15, clone RP24-468P6, complete sequence	42.8	32%	0.38	85%
Oryza sativa Japonica Group genomic DNA, chromosome 6, BAC clone:OSJNBa0007O20	42.8	27%	0.38	88%
Butyrivibrio fibrisolvens 16/4 draft genome	41.0	24%	1.3	90%
Ustilago maydis strain SRX3 large subunit ribosomal RNA gene, partial sequence	41.0	25%	1.3	87%
Zebrafish DNA sequence from clone ZFOS-2548D7 in linkage group 18 Contains a novel gene	41.0	26%	1.3	88%
Mouse DNA sequence from clone RP24-426H7 on chromosome 17, complete sequence	41.0	25%	1.3	87%
Populus trichocarpa clone POP003-F15, complete sequence	41.0	21%	1.3	92%
Trichoplax adhaerens expressed hypothetical protein, mRNA	39.2	16%	4.7	100%
Trichoplax adhaerens mRNA for metallothionein (mt gene)	39.2	16%	4.7	100%

Table 3. The blast results of putative sequence 2 (Figure 3B) by NCBI BLAST.

These twelve genes were the blast results by importing the putative sequence 1 (Fig. 3B) on the NCBI BLAST. The primers (prime 3 and 4 in Table 1) used for obtaining the putative sequence are designed from the *Trichoplax adhaerens* mRNA for metallothionein.

Blast gene	Score	Query coverage	E value	Max identity
Mus musculus BAC clone RP23-277P7 from chromosome 15, complete sequence	42.8	15%	1.0	82%
Trichoplax adhaerens expressed hypothetical protein, mRNA	41.0	8%	3.5	100%
Uncultured bacterium clone Ax2_636 16S small subunit ribosomal RNA gene, partial sequence	41.0	12%	3.5	86%
Pongo abelii BAC clone CH276-238F10 from chromosome unknown, complete sequence	41.0	12%	3.5	88%
Paramecium tetraurelia hypothetical protein	41.0	15%	3.5	82%
Drosophila melanogaster chromosome 2R, complete sequence	41.0	8%	3.5	100%
Mus musculus 6 BAC RP23-205F19	41.0	13%	3.5	83%
Uncultured bacterium clone X445 16S ribosomal RNA gene, partial sequence	41.0	12%	3.5	86%
Mus musculus 6 BAC RP23-205F19	41.0	9%	3.5	96%
Desulfotalea psychrophila LSv54 chromosome	41.0	12%	3.5	88%
Drosophila melanogaster, chromosome 2R, region 60F-60F, BAC clone	41.0	8%	3.5	100%
Trichoplax adhaerens mRNA for metallothionein (mt gene)	41.0	8%	3.5	100%