Identification and Characterization of a Selenoprotein EhSEP1, Thioredoxin Reductase, in *Emiliania huxleyi*

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Abbreviations

2D-PAGE: two dimensional PAGE

CBB: coomassie brilliant blue

cDNA: complementary DNA

cpm: count per minutes

DI: deiodinase

DNA: deoxyribonucleic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic) acid

EDTA: ethylenediaminetetraacetic acid

ER: endoplasmic reticulum

ESM: Erd-Schreiber's medium

Fd: ferredoxin

FTR: ferredoxin-thioredoxin reductase

GPX: glutathione peroxidase

GSH: glutathione

GS-Se^-: glutathione-conjugated selenide

GS-SeH: selenoglutathione

GS-SeO_3^-: glutathione-conjugated selenite

GS-Se-SG: selenodiglutathione

HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

HPLC: high performance liquid chromatography

LEP: lysyl endopeptidase

MA: Marine Art SF

mRNA: messenger RNA

MSe^-: methyl selenol

MsrB: Methionine-R-sulfoxide reductase B

NADPH: nicotinamide adenine dinucleotide phosphate reduced form

NTRC: NADPH thioredoxin reductase

NS: natural seawater

OD_{750}: optical density at 750 nm

ORF: open reading frame

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

PDI: protein disulfide isomerase

PHGPX: phospholipid hydroperoxide glutathione peroxidase

pK: dissociation exponent

PVDF: polyvinyliden difluoride

RACE: rapid amplification of cDNA ends

RI: radioisotope

RNA: ribonucleic acid

RT: reverse transcription

SDS: sodium dodecyl sulfate

Sec: selenocysteine

SECIS: selenocysteine insertion sequence

Sel T: selenoprotein T

Sel U: selenoprotein U

Se-Met: selenomethionine

tRNA: transfer RNA

TR: thioredoxin reductase

TRX: thioredoxin

UTR: untranslated region

Abstract

A coccolithopholid *Emiliania huxleyi* is a unicellular marine haptophyte alga that has unique $CaCO_3$ crystals on the cell surface. A huge bloom of the species sometimes occurs in the ocean and transports the great amount of CO_2 into the sea floor. Such algal bloom strongly affects the global carbon cycle through the change in primary productivity. Studies on regulatory factors of the algal growth are very important to find a key for controlling algal bloom. *E. huxleyi* is known to require essentially a nanomolar level of selenium (Se) for its growth and the cell has an ability to concentrate Se up to micromolar level in the cells. Such high biological potentials of *E. huxleyi* may be related to the ability of this alga to efficiently use nutrients, including Se. Previously, it was demonstrated that *E. huxleyi* efficiently absorb environmental nanomolar level of selenite via an active transport process and then immediately metabolize it into low molecular compounds (LMCs) to detoxify and accumulate, and was also used for the synthesis of Se-containing proteins that were synthesized *de novo* via the translation process (Obata *et al. plant cell physiol.* 45: 1434-1441, 2004). These findings suggested that *E. huxleyi* adapted for environmental Se concentration, namely an active transport acts efficiently in Se-limited condition and LMCs are produced to obtain Se for the synthesis of selenoproteins in Se-sufficient condition. To understand the regulatory mechanism of metabolism by Se, selenoproteins were searched using ^{75}Se-radio-labeling technique in a coccolithophorid, *Emiliania huxleyi* and the presence of at least six selenoproteins (EhSEP1-6) including a protein disulfide isomerase (PDI)-like protein that is EhSEP2, the most abundant selenoprotein in this alga, were found (Obata and Shiraiwa *J. Biol. Chem.* 280: 18462–18468, 2005).

In this study, I focused on a second abundant selenoprotein, EhSEP1, in the *E. huxleyi* cells and analyzed its molecular properties and the regulatory pattern of gene expression by Se. The cDNA sequence of EhSEP1 consists of 1950 base pairs encoding a putative product of 495 amino acids with a calculated molecular mass of 52.2 kDa. The nucleotide and amino acid sequences of EhSEP1 showed strong similarities to those of the enzyme thioredoxin reductase (TR) 1 in the public databases. The EhSEP1 protein contains redox-active cysteine residues in the putative FAD-binding domain of the pyridine nucleotide-disulfide oxidoreductase class-1 domain, a dimerization domain, and a C-terminal Gly-Cys-Sec (selenocysteine)-Gly sequence that is known to function as an additional redox center. In the 3' untranslated region of EhSEP1 cDNA, I found a selenocysteine insertion sequence (SECIS) that is similar to the SECIS found previously in animals. The expression of *EhSEP1* showed almost the same pattern under both Se-sufficient and Se-deficient conditions. TR activity was kept very low level in cells grown under Se-deficient conditions, but gradually increased four-fold within *ca.* 70 h when cells were transferred to the medium containing 10 nM selenite.

In this study, I revealed a part of Se utilization strategies in *E. huxleyi*. Namely, this alga efficiently incorporates the environmental low levels of Se through the high-affinity transport mechanism of selenite ion. The mechanism is thought to be useful to supply enough Se on the requirement for the synthesis of selenoproteins in Se-limited environment. Se may contribute to the continuous growth or the increase in cell viability by the stimulation of the activity of selenoenzymes that mostly function in oxidoreduction reactions, such as EhSEP1 and EhSEP2 possessing TR and PDI activities, respectively. Further studies on Se metabolism

in coccolithophorids such as *E. huxleyi* will also be required to fully elucidate how and why these organisms induce selenoproteins and have developed the strategy of Se utilization.

Introduction

Selenium (Se) is the homologous elements with sulfur so that its property is analogous to sulfur (S). This suggests that Se may play important roles in many functions of living cells since S is the essential elements in all organisms. The relative proportion of Se in the environment is very low. In the Earth's crust, sea water and stream water, Se is present in a concentration of 0.05 – 0.09 mg/kg, 0.45 μg/kg and 0.2 μg/kg, respectively (Rezanka and Sigler 2008). In the environment, Se is present in elemental form or in the form of selenide (Se^{2+}), selenite (SeO_3^{2-}), or selenate (SeO_4^{2-}). In soils, the forms depend strongly on the redox-potential conditions, with the lower oxidation forms predominating in anaerobic conditions and acidic soils, while the higher oxidation forms are favored in alkaline and aerobic conditions. Trace amounts of selenium present in water usually as selenate or selenite and this is due to a result of geochemical processes.

Se is an essential trace element for many organisms including mammals, but is very toxic at high concentration. The major biological form of Se is represented by the analogue of cysteine that is known as selenocysteine (Sec). In comparison with the thiol in cysteine, the selenol in selenocysteine is highly reactive. The main biological function of Sec is associated with its incorporation into proteins that is generally called selenoproteins. The presence of Sec in the active sites of these selenoproteins confers a more catalytic advantage than cysteine residues. This difference is probably due to the lower p*K*a of the selenol compared with the thiol. Sec reversibly changes its redox state during catalysis. Due to the nature of Sec, most selenoproteins function as oxidoreductases. In all organisms that are known to possess

selenoproteins, Sec is co-translationally incorporated into proteins at the site of the UGA codon, which is usually used as a stop codon, on mRNA through a specific Sec insertion mechanism. This mechanism includes a *cis*-acting mRNA structure known as the Sec insertion sequence (SECIS), and *trans*-acting factors consisting of Sec tRNA, a selenocysteyl-tRNA-specific elongation factor, and an SECIS binding protein 2 (Hatfield and Gladyshev 2002). In eukaryotes, SECIS is located in the 3'-untranslated region (UTR) of selenoprotein mRNAs. Eukaryotic SECIS RNA folding is categorized into two slightly different secondary structures, form 1 and 2, and the non-Watson–Crick quartet is highly conserved in both forms (Krol 2002).

Inorganic and organic Se compounds can be utilized as nutritional and supplemental sources. In animals, Se is generally ingested through organic selenoconpounds. These are divided into two categories: 1) selenocystenyl and selenomethionyl residues in selenoproteins and common proteins; 2) monomeric Se-methylated selenoamino acids (selenomethionine and Se-methylselenocysteine) and a Se-methylated dipeptide (γ-gultamyl-Se-methylselenocysteine) as the forms of accumulation. Ingested organic selenocompounds are metabolized to selenide for the synthesis of selenoproteins or selenosugar to excrete (Suzuki 2005). Although most of the selenium is excreted in the form of selenosugar in the urine, excess selenium ingestion causes the excretion of selenium in the form of trimethylselenonium (Suzuki *et al.* 2006) (Fig. 1).

In photosynthetic organisms, a large number of the analysis of selenium metabolism had been done in land plants. Nevertheless, neither selenoprotein

genes nor any of the components of the Sec insertion mechanism were found in the genomes of the plant *Arabidopsis thaliana* (Novoselov *et al.* 2002). Therefore the nutritional essentiality of selenium in land plants is not recognized yet.

There are some land plants that accumulate Se more than thousand times (Pickering *et al.* 2003) and the mechanism of Se accumulation is well known (Ellis and Salt 2003). The first step of Se accumulation is its uptake from environment. Se is directly taken up by plants from the soil as forms of SeO_4^{2-} or SeO_3^{2-}. The uptake of SeO_4^{2-} is competed with sulfate. Thus SeO_4^{2-} is thought to be taken up by the mediation of sulfur transporter (Zayed *et al.* 1998, Arvy 1993). The second step is detoxification to accumulate high levels of selenium. Selenate is metabolized through sulfur metabolic pathway such as sulfate. The non-proteinic amino-acid, methylselenocysteine (MSeC) which is produced from the methylation of selenocysteine is the predominant organic Se detoxification form in plants (Trelease *et al.* 1960). Other compounds, such as dimethylselenide and dimethyldiselenide, are known as volatile forms to reduce selenium toxicity (de Souza *et al.* 1998, 2000) (Fig. 2).

In marine, selenium is present in the form of SeO_4^{2-}, SeO_3^{2-} and organic selenium (3 : 1 : 1, by concentration) at euphotic zone (Cutter and Bruland 1984). There are some algae that required selenium for their growth. When selenium was added to the culture, growth-stimulating effect was observed in a diatom *Thalassiosira pseudonana* (Price *et al.* 1987), haptophyceae *Chysochromulina breviturrita* and *Emiliania huxleyi* (Wehr and Brown 1985, Danbara and Shiraiwa 1999) and dinofagellates *Gymnodinium catenatum*

and *Alexandrium minutum* (Doblin *et al.* 1999, 2000). And the growth-stimulating activity was higher when SeO_3^{2-} was added than the addition of SeO_4^{2-} (Hu *et al.* 1996). Nevertheless there is little knowledge about the Se metabolism in algae. In the diatom *Thalassiosira pseudonana*, 50 percent of Se was accumulated in proteins and a Sec-containing glutathione peroxidase-like protein was experimentally identified (Price and Harrison 1988). In addition, 70 percent of Se was accumulated in low molecular weight compounds and a protein disulfide isomerase-like protein was identified in the haptophyte alga *Emiliania huxleyi* (Obata *et al.* 2004, Obata and Shiraiwa 2005). It is very interesting why selenite shows higher growth-stimulating effect, how selenite is absorbed by the cells, which kinds of Se containing compounds algae possess, and so on.

More than 40 selenoprotein families have been identified in diverse organisms, including bacteria, archaea, and eukaryotes, by metabolic labeling with ^{75}Se and bioinformatics approaches (Kryukov *et al.* 2003, Kryukov and Gladyshev 2004). A number of advanced studies on Se-containing proteins have been performed, mostly in humans and other mammals. Significant biological effects of Se have been shown to be due to the action of various selenoproteins that contain Se in the form of Sec (Brow and Arthur 2001, Kim *et al.* 2006). In these proteins, Sec functions as the catalytic residue because of its strong nuclephilicity and low p*K*a (Stadtman 1996). All selenoproteins are clustered into three groups: 1) Sec is located in the N-terminal regions and those selenoproteins often exhibit thioredoxin or thioredoxin-like foldings. This group is most abundant; 2) Sec is located in the C-terminal sequences

and these selenoproteins have been described only in eukaryotes. The most famous selenoprotein in this group is thioredoxin reductase; 3) Selenoproteins utilize Sec to coordinate redox metals (molybdenum, tungsten and nickel) in the active site (Gladyshev *et al.* 1994, Graentzdoerffer *et al.* 2003, Valente *et al.* 2005). This selenoprotein group is found only in prokaryotes. Although there are many selenoproteins of which functions are still not known, some selenoproteins are known to function as oxidoreductases. Recent results from proteomic characterization of selenoproteins in some organisms provided many clues about the utilization of selenium (Kryukov *et al.* 2003, Castellano *et al.* 2001, Taskov *et al.* 2005, Lobanov *et al.* 2006, Cassgo *et al.* 2006, Lobanov *et al.* 2007, Kryukov and Gladyshev 2004, Zhang *et al.* 2005). Further study on identification and functions of selenoprotein genes will help the elucidation of biological and biomedical effects of selenium.

Neither selenoprotein genes nor any components of the Sec insertion mechanism were found in the genomes of the plant *Arabidopsis thaliana* and the yeast *Saccharomyces cerevisiae* (Novoselov *et al.* 2002). The lack of selenoproteins in both organisms raised intriguing questions about the general role of selenoproteins in organisms and the strategy for Se utilization adopted during evolution. Little information on selenoproteins in Se-requiring photosynthetic organisms is available. Some selenoproteins have been discovered by bioinformatics approaches in photosynthetic organisms, such as the diatom *Thalassiosira pseudonana* and the green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Lobanov *et al.* 2007). Two selenoproteins were experimentally identified, *i.e.*, a Sec-containing glutathione peroxidase in

Thalassiosira pseudonana (Price and Harrison 1988) and a protein disulfide isomerase-like protein in the haptophyte alga *Emiliania huxleyi* (Obata and Shiraiwa 2005). On the other hand, ten selenoproteins were identified in the unicellular green alga, *Chlamydomonas reinhardtii*, although Se is not essential for growth of this alga (Novoselov *et al.* 2002). Therefore, characterization and identification of selenoproteins in Se-requiring photosynthetic organisms will provide information to help elucidate the importance of Se and changes in Se utilization strategies during evolution.

Unicellar marine algae coccolithophorids are classified into four orders (Parke and Dixon 1976), such as Prymnesiales, Isochrysidales, Coccosphaerales and Pavlovales, based on the length of the haptonema and whether or not the cells are covered with unique $CaCO_3$ crystals, called coccoliths. *Emiliania huxleyi* is the most abundant coccolithophorid and widespread all over the world. *E. huxleyi* is known to form huge blooms in the ocean and is consequently expected to affect the air-sea interchange of CO_2. It is thought that such algal bloom strongly affects the global carbon cycle due to the effect of great amount of calcification (Robertson *et al.* 1994). In addition, this alga produces the great deal of ß-dimethylsulfoniopropionate (DMSP) (Turner *et al.* 1988) as an osmoregulant. DMSP is leached from dead cells and degraded to dimethylsulfide (DMS) that forms cocktail of sulphur dioxide, methanesulphonic acid and other acidic compounds within submicron aerosols (Burkill *et al.* 2002). This cocktail influences various climatically important processes, so the proliferation of *E. huxleyi* is also thought to affect meteorological changes. Thus, changes in this algal growth rates and bloom

frequencies in the ocean affect the global environment especially the concentration of atmospheric CO_2 (Martínez *et al.* 2007). Therefore, analysis of the factors that regulate the growth status of algae is fundamental to understanding how the global carbon cycle can be controlled.

It was reported previously that *E. huxleyi* requires nanomolar levels of Se for growth and the selenite ion is the predominant species used by this alga (Danbara and Shiraiwa 1999). It is postulated that the biological potentials of *E. huxleyi* may be related to efficient utilization of various nutrients, including Se. Selenite absorbed by the cells is incorporated into low-molecular weight selenocompounds, as reported in land plants (Sors *et al.* 2005), and may also be utilized in the synthesis of Se-containing proteins *via* the translation process (Obata *et al.* 2004). It was also found that *E. huxleyi* possesses at least six selenoproteins (EhSEP1–6), one of which, EhSEP2, is homologous to protein disulfide isomerase (Obata and Shiraiwa 2005). These results suggest that *E. huxleyi* possesses both the land plant-type Se metabolic system and the Sec insertion mechanism for selenoprotein synthesis. Therefore, the identification and molecular characterization of as yet unidentified selenoproteins in *E. huxleyi* are very important to confirm experimentally how Se functions in protein molecules.

In this study, I found that EhSEP1 is highly homologous to animal thioredoxin reductase (TR) 1. In this selenoprotein, the form 2 SECIS is located in the 3' UTR region and Sec is encoded by a penultimate TGA codon located within the C-terminal tetrapeptide active site, as in animal TRs. EhSEP1 requires Se for stimulating its activity but not for the regulation of

its induction at the transcriptional level in *E. huxleyi*.

Materials and Methods

Cell growth

The coccolithophorid, *Emiliania huxleyi* (Lohman) Hay & Mohler (NIES 837) (Haptophyceae), was obtained from the algal culture collection of the National Institute for Environmental Studies (NIES), Tsukuba, Japan. The cells were grown in MA-ESM medium containing synthetic seawater, Marine Art SF (initially from Senju Pharmaceutical, Osaka, Japan and later from Osaka Yakken, Osaka, Japan) and additional micronutrients, and Erd-Schreiber medium (ESM) containing 10 nM disodium selenite, instead of soil extract, according to the method of Danbara and Shiraiwa (1999). The culture was continuously illuminated at 100 μmol photons · m^{-2} · s^{-1} by fluorescent light and maintained at 20°C.

Databases

All genomic, expressed sequence tag (EST), and predicted protein sequences involving *E. huxleyi* were obtained from NCBI (http://www.ncbi.nlm.nih.gov/), except for the genomes of *Ostreococcus lucimarinus* CCE9901, *Thalassiosira pseudonana*, and *Cyanidioschyzon merolae*. The information of genomes of *O. lucimarinus* CCE9901 and *T. pseudonana* was obtained from the Joint Genome Institute (http://www.jgi.doe.gov/). The information of genome of *C. merolae* was obtained from the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/).

Preparation of ^{75}Se-labeled proteins

For preparation of ^{75}Se-labeled proteins, 1 nM (final concentration)

^{75}Se-selenite (7.5 TBq/mmol; University of Missouri, Columbia, MO) was added to the cultures and cells were grown for one week.

The ^{75}Se-labeled cells were harvested by centrifugation (3,500×*g* for 10 min at 4°C) using a refrigerated centrifuge and resuspended in buffer A (50 mM HEPES-NaOH containing 3 mM EDTA, pH 7.5). The cells were then disrupted by sonication (Bioruptor; Cosmo Bio, Tokyo, Japan). After removing insoluble matter by centrifugation (13,000×*g* for 60 min at 4°C), the supernatant was obtained as crude extracts. After desalting by passing through a PD10 column (GE Healthcare Bio-Sciences Inc., Tokyo, Japan) that was equilibrated with buffer B (10 mM sodium phosphate buffer, pH 7.5), the eluate was applied to a DEAE-Toyopearl column (Toso, Tokyo, Japan) equilibrated with buffer B. After washing out unbound compounds five times with the bed volume of buffer B, proteins were eluted out with buffer B containing 0.1 M Na_2SO_4. Each ^{75}Se radioactive fraction was applied to 12.5% SDS-PAGE to detect ^{75}Se protein bands in each fraction.

The EhSEP1-containing fraction was subjected to 100 kDa cut-off size fractionation using Amicon Ultra-15 (100 kDa MWCO; Millipore, Billerica, MA). The filtrate was concentrated by ultra-filtration using a Centriprep YM10 (10 kDa MWCO; Millipore) and then subjected to gel filtration using a Diol-150 HPLC column (Shimadzu, Kyoto, Japan) equilibrated with buffer C (10 mM sodium phosphate buffer containing 0.2 M Na_2SO_4, pH 7.0). Protein elution profiles were monitored by measurement of absorbance at 280 nm in a spectrophotometer (Shimadzu). The radioactivity of the fractions was determined by γ-counting (COBRA II; Packard, Meriden, CT) and ^{75}Se-labeled

proteins were visualized by radioluminography using a BAS2500 system (Fuji Film, Tokyo, Japan).

Purification of non-radioactive EhSEP1 and amino acid sequence analysis

Non-radioactive EhSEP1 was prepared from 50-L cultures using the same protocol as described for ^{75}Se-labeled EhSEP1; this process was necessary to use the amino acid sequencer in a non-radioactive laboratory. To confirm the identity of ^{75}Se-labeled and non-labeled EhSEP1, individual samples and the mixture of the two were loaded separately into adjacent lanes of a 10% SDS-polyacrylamide gel and subjected to electrophoresis. The unlabeled band identical to the ^{75}Se-labeled EhSEP1 was extracted from the gel according to the method of Hanawa *et al.* (2004). For the determination of internal amino acid sequence, the extracted EhSEP1 was treated with lysyl endopeptidase (0.3 μg · mL^{-1}; Wako Pure Chemical, Osaka, Japan) for 2 h. The digested and undigested peptides were resolved in 15% SDS-PAGE slab gels, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The CBB-stained peptide bands were used for N-terminal amino acid sequence analysis by Edman degradation using a Prosice 494HT sequencer (Applied Biosystems, Foster City, CA) at the National Institute for Basic Biology (Okazaki, Japan).

cDNA cloning of E. huxleyi EhSEP1

Total RNA was isolated from *E. huxleyi* cells harvested at the exponential growth phase using the RNAgents Total RNA Isolation System (Promega,

Madison, WI), then mRNA was isolated with the PolyATtract mRNA Isolation System (Promega). A SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, Palo Alto, CA) was used to synthesize first-strand cDNA for 3'- and 5'-RACE PCR. Sense and antisense oligonucleotides were designed from the EST sequence of *E. huxleyi* (GenBank accession number: CV069652), which is identical to the EhSEP1 amino acid sequence. The tblastn program (http://www.ncbi.nlm.nih.gov/BLAST/) was used for searching the EST database of *E. huxleyi*.

First, the cDNA for 3'-RACE PCR was used as the template and amplified with gene-specific sense and antisense primers: 5'-CCCCTGATCAGCTAACACGA-3' and 5'-TCACTTCGGACGACGTCTTC-3', respectively. This PCR product was cloned into the pGEM-T easy vector (Promega) and sequenced with an Applied Biosystems model 310 DNA sequencer using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). The cDNA for 3'-RACE PCR was amplified with two different gene-specific sense primers: 5'-GTAAACGTCGGCTGCATCCCAAAGA-3' (first PCR) and 5'-AAGTCGCTCAACTTCGGGTA-3' (nested PCR). A primer 5'-CTGCATCCCAAAGAAGCTCATGCAC-3' and a nested primer 5'-TCTGCGACTTTGTCAAGCC-3' were used as gene-specific antisense primers for 5'-RACE PCR. The PCR products thus obtained were cloned and sequenced as described above. 3'-RACE and 5'-RACE PCR were performed using LA Taq with GC buffer (Takara Bio, Ohtsu, Japan) for amplification.

Preparation of Se-depleted cells

Cells were pre-grown for one week in the medium without Se to deplete the internal Se concentrations. The cells were then inoculated into fresh medium without Se. The resuspended cells were used as Se-depleted cells.

Northern blotting analysis

Aliquots of 10 μg of total RNA were electrophoresed on 1.17% formaldehyde-denaturing agarose gels. RNAs in the gels were transferred to and then UV-cross-linked with Hybond-N^+ nylon membranes (GE Healthcare Bio-Sciences). These RNAs were hybridized with ^{32}P-labeled *EhSEP1* generated with a BcaBEST labeling kit (Takara Bio). Hybridization was performed as described by Church and Gilbert (1984). After washing off the unhybridized probes, the radioactivity of the bands was determined by BAS2500 with the Image Gauge software (Fuji Film).

TR assay

Cells harvested by centrifugation at 4°C were suspended in phosphate buffer (pH 7.0). The cells were then disrupted in a French press. After removing cell debris by centrifugation (15,000×*g* for 30 min at 4°C), the supernatant was desalted by passing through a PD10 column and concentrated by ultra-filtration using an Amicon Ultra-4 (10 kDa MWCO; Millipore). TR activity was assayed by both the 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) reduction assay (Agorio *et al.* 2003) and the insulin reduction assay (Arner *et al.* 1999). The DTNB reduction assay was performed by a TR assay kit (Sigma-Aldrich, St. Louis, MO) (Agorio *et al.* 2003, Mustacich and

Powis 2000). The reaction was carried out at 25°C and the activity was monitored by the increase in absorbance at 412 nm. The change in A_{412} without NADPH was defined as a base line to calculate the activity. One enzyme unit is defined as 1 micromole of NADPH oxidized per min using the equation $\Delta A_{412}/(13.6\times10^{-3}\times2)$, as 2 mol of 5-thio-2-nitrobenzoic acid ($\varepsilon_{412\ nm}$ = 13.6 mM^{-1} cm^{-1}) is produced when 1 mol of NADPH is used. For the insulin reduction assay (Arner *et al.* 1999), the crude extract was added to a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 80 μM (1.0 mg/ml) insulin, 1.7 μM recombinant human thioredoxin, and 84 μM NADPH. NADPH oxidation was monitored at 340 nm. The change in A_{340} without both human T-cell recombinant thioredoxin and insulin was defined as a base line to calculate the activity. One unit of enzyme was defined as the oxidation of 1 micromole of NADPH ($\varepsilon_{340\ nm}$ = 6.2 mM^{-1} cm^{-1}) per min using the equation $\Delta A_{340}/(6.2\times10^{-3})$. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Results

To identify selenoproteins in *E. huxleyi*, the cells were incubated with ^{75}Se-selenite for one week in axenic culture. After the preparation of crude extracts, ^{75}Se-labeled proteins were separated through a DEAE anion exchange HPLC column, and each fraction was then analyzed by SDS-PAGE. Obata and Shiraiwa (2005) demonstrated the presence of at least six ^{75}Se-labeled proteins, designated EhSEP1 to 6, in *E. huxleyi*. Similarly, I identified five ^{75}Se-labeled protein bands with molecular masses of 59, 27, 29, 23, and 31 kDa by silver staining and radioluminography that corresponded to EhSEP1 to 5, respectively (Fig. 3). In this study, EhSEP6 was not isolated by SDS-PAGE analysis because of its molecular mass that was lower than others. I focused on the largest and second most abundant protein, EhSEP1, of the six ^{75}Se-labeled proteins for the analysis of its molecular properties. Purified EhSEP1, the major fraction containing the greatest amount of EhSEP1, was obtained using a Diol-150 gel filtration HPLC column (Fig. 4*A*). The EhSEP1 band in Fig. 4*B* was extracted, digested with lysyl endopeptidase (Fig. 5*A*), and then the internal and N-terminal amino acid sequences were determined by the Edman degradation (Fig. 5*B*), as described in Experimental procedures. From these analyses, two internal amino acid sequences were obtained (shaded in Fig. 6), but the N-terminal of the whole EhSEP1 was not obtained because of blocking.

For cloning of a full-length EhSEP1 cDNA, the EST sequence and 3' and 5' ends of the sequences were amplified by PCR and RACE PCR, respectively (see Experimental procedures for details). I finally obtained sequences containing the ATG start codon in the 5'-RACE product and a poly-A tail in

the 3'-RACE product. The nucleotides and deduced amino acid sequences of the cloned EhSEP1 cDNA are contained in an open-reading frame of 495 amino acid residues and the 3' UTR consisted of 349 nucleotides (Fig. 6). Analysis of the EhSEP1 amino acid sequence indicated the presence of a characteristic thioredoxin reductase domain, the pyridine nucleotide-disulfide oxidoreductase domain, and a dimerization domain. The strongly conserved sequence GGTCVNVGCIP around the cysteine residues, *i.e.*, Cys54 and Cys59, at the active site, and the C-terminus-located redox active site, GCUG, were also found. Sec was located at codon 494 encoded by an in-frame TGA codon (shown as U with an asterisk in Fig. 6). The SECIS element was found in the 3' UTR of EhSEP1 mRNA by computational search using SECISearch 2.19 (Kryukov *et al.* 2003), and the secondary structure of the SECIS element was drawn using the same program (Fig. 7). The SECIS element in EhSEP1 mRNA contained the conserved functional non-Watson–Crick quartet with the UGAN sequence in the 5'-arm and the NGAN in the 3'-arm, and the unpaired AAN nucleotides in the internal loop. The length of a helix that separated the non-Watson–Crick quartet and the unpaired nucleotides in the internal loop was 15 base pairs as reported in rats (Vendeland *et al.* 1995). Furthermore, the SECIS element of EhSEP1 had a form 2 structure that was similar to animal SECIS (Krol 2002). The amino acid sequences showing homology with EhSEP1 are aligned in Fig. 8. The sequence similarities of EhSEP1 in the public databases were 59.9% to zebrafish (*Danio rerio*) TR1 (NP_898895.2), 57.9% to human (*Homo sapiens*) TR1 (NP_877393.1), 57.4% to the unicellular green alga *Chlamydomonas reinhardtii* TR1 (XP_001696072.1), 56.9% to

mouse (*Mus musculus*) TR1 (NP_001035988.1), 54.0% to the unicellular green alga *Ostreococcus lucimarinus* CCE9901 TR1 (Chr_1 [733779, 735290]), 52.0% to the diatom *Thalassiosira pseudonana* (newV2.0.genewise.318.11.1), and 52.1% to the nematode *Caenorhabditis elegans* TR (AAD41826.1).

The changes in EhSEP1 mRNA levels under Se-sufficient and Se-deficient conditions were analyzed by Northern hybridization (Fig. 9*A*). No significant difference in gene expression of EhSEP1 was observed between the cells into medium with 10 nM selenite and those without Se (Fig. 9*B*).

Cell growth stopped in the Se-deficient culture. However, growth recovered when 10 nM selenite was added to the Se-deficient medium (Fig. 10*A*). The crude extracts of *E. huxleyi* grown in Se-sufficient medium clearly exhibited high TR activity toward both the general substrate DTNB and human thioredoxin (Fig. 10*B* and *C*). When *E. coli* thioredoxin was used as a substrate, the activity was significantly lower than that with human thioredoxin (data not shown).

In both assays, the TR activity was strongly inhibited by the TR inhibitor supplied with the TR assay kit (Sigma-Aldrich). In the insulin reduction assay, the activity determined with the inhibitor was nearly the same level as that determined without thioredoxin, indicating that there was no thioredoxin derived from *E. huxleyi* cells in the extracts (Table 1). TR activity was very low in Se-depleted cells, but the activity increased about four-fold to the same level as that in Se-sufficient cells for about 70 h after the addition of 10 nM selenite (Fig. 10*B* and *C*). The change in TR activity (in ratio to the initial value) was similar in both the DTNB and insulin assays, although the activity

was approximately 5 times higher in the DTNB assay than that in the insulin assay. Such high activity in the DTNB assay may be due to overestimation and/or difference in substrate specificity. The incorporation of Se into EhSEP1 was clearly shown to be essential for exhibiting TR activity.

To predict the other selenoproteins in *E. huxleyi*, homology search was performed in *E. huxleyi* EST database by using selenoproteins which were already known as query. As a result, I found the putative candidates of the selenoproteins in *E. huxleyi* that are homologous to Sel U, T, O, Sep 15, GPX, PHGPX and deiodase (DI) in addition to EhSEP1 (TR) and EhSEP2 (PDI-like protein) (Table 2). The amino acid sequences showing homology with these selenoproteins are aligned in Figs. 11-18, respectively. The secondary structures of the SECIS element of these selenoproteins were drawn using SECISearch 2.19 (Fig. 19). Moreover, all of the SECIS elements of these *Emiliania* EST sequences had the form 2 structure and contained the conserved functional non-Watson–Crick quartet.

Discussion

Two types of TR that reduce thioredoxin in the presence of the electron donor NADPH have been characterized to date: one in animals (mammalian-type TR) (Sun *et al.* 1999) and another in archaea, bacteria, plants, fungi, and some protozoa (NTR) (Russel and Model 1988). Mammalian-type TR is categorized into three TRs: the selenoproteins TR1 (cytosolic TR), TR2 (thioredoxin/glutathione reductase), and TR3 (mitochondrial TR). Although both amino acid sequences and catalytic mechanisms of action differ, the functions of the TRs are almost the same (Mustacich and Powis 2000, Hirt 2002).

In photosynthetic organisms, there are two additional TRs that are not selenoproteins. One is ferredoxin-thioredoxin reductase (FTR), which reduces thioredoxin by using photoreduced ferredoxin as an electron donor and is localized in the chloroplast (Jacquot *et al.* 1997). So, FTR might require NADPH as an electron donor to reduce ferredoxins via ferredoxin-NADPH oxidoreductase. The activity in the insulin reduction assay without addition of human thioredoxin was very low (Table 1) and therefore FTR is not considered to be involved in the TR activity in the crude extracts of *E. huxleyi.*

The other is chloroplast-located NADPH thioredoxin reductase (NTRC), which is non-Se-containing TR that formed by both NTR and a thioredoxin-like domain. NTRC reduces 2-cys peroxiredoxins using NADPH as an electron donor and is functional in the chloroplast (Spinola *et al.* 2008) and its activity can be detected by DTNB reduction, but not by thioredoxin reduction, as reported in *Arabidopsis thaliana* (Serrato *et al.* 2004). As the TR activity in the crude extracts of *E. huxleyi* was detected by thioredoxin

reduction, the activity should not be due to NTRC.

The nucleotide and amino acid sequences of the second most abundant selenoprotein EhSEP1 in *E. huxleyi* (Fig. 6) showed a high degree of identity with TR1 in animals (Fig. 8). Two motifs in the N-terminal region of the protein, namely with the active site disulfide bond (-CVNVGC-) located in the FAD-binding domain and with a pyridine nucleotide-disulfide oxidoreductase class-1 domain, are highly conserved. Furthermore, EhSEP1 possesses a C-terminal dimerization domain and an active site (-GCUG) involving a selenocysteine residue encoded by the TGA codon at the C-terminus. These features of *E. huxleyi* TR are the same as those of TR1.

I also found the SECIS element in the 3' UTR of the cDNA sequences that functions for the insertion of Sec at the translation. Eukaryotic SECIS elements are folded into two slightly different secondary structures, designated form 1 and form 2 (Krol 2002). The green alga *C. reinhardtii*, which does not require Se essentially for its growth, contains TR1 with the form 1 SECIS element (Novoselov *et al.* 2002). Interestingly, the SECIS element of *EhSEP1* differs from the form 1 SECIS in the TR1 of *C. reinhardtii*, and contains the form 2 SECIS like the human TR1, although both *E. huxleyi* and *C. reinhardtii* are photosynthetic organisms. However, both *E. huxleyi* and humans, but not *C. reinhardtii*, are organisms for which Se is essential for life. The haptophyte alga *E. huxleyi* belongs to the kingdom Chromista, possessing the chloroplast that evolved from red algae *via* secondary endosymbiosis, and its phylogenetic position is far distant from the primary symbionts, green algae, and from mammals (Cavalier-Smith 2003). Although

the difference in the SECIS form is thought to lead to no difference in function of the synthesized proteins (Grundner-Culemann *et al.* 1999), it may be related to the Se requirement for transcriptional and/or translational regulation.

The results of the analysis using the available N-terminal transit peptide prediction programs (SOSUI, iPSORT, TargetP 1.1, and SignalP 3.0) (Hirokawa *et al.* 1998, Bannai *et al.* 2002, Emanuelsson *et al.* 2000, Bendtsen *et al.* 2004) showed that EhSEP1 did not include the transit peptide. Therefore, EhSEP1 is thought to be localized in the cytoplasm. The molecular mass of EhSEP1 predicted from the deduced amino acid sequence was 52.2 kDa (Fig. 6). This value is approximately the same as that of EhSEP1, 59 kDa, determined experimentally on SDS-PAGE (Fig. 4*B*). In EhSEP1, the initiating methionine has not yet been precisely identified because the N-terminal residue of EhSEP1 was blocked. However, the nucleotide sequence of EhSEP1 cDNA revealed the presence of an in-frame stop codon upstream of a putative initiation codon. Therefore, EhSEP1 would be translated from the putative initiation codon located at the most upstream region of the sequence.

The molecular masses of other selenoproteins, such as EhSEP3 to 5, determined experimentally on SDS-PAGE (Fig. 3) are 29, 23 and 31 kDa, respectively. The molecular masses of deduced amino acid sequences on the *Emiliania* EST sequences that are homologous to known selenoproteins were calculated (Table 2). The results of comparing molecular masses between EhSEP 3 and 5 calculated and homology search using the EST sequences suggest that EhSEP 3 and 5 may be selenoprotein U (Sel U) and iodotyrosine

deiodase (DI), respectively. In addition, there is a possibility that EhSEP4 may be either Sel T, GPX, PHGPX or PDI-like protein. Sel U is found in some marine organisms, but in mammals, all Sel U homologs are Cys-containing proteins and its function is unknown (Castellano *et al.* 2004). DI is well known in mammals as to activate or inactivate thyroid hormones by reductive deiodination. DI is thought to have evolved in animals, but homologs of this protein were found in bacteria (Zhang *et al.* 2005). Therefore, to search more details about DI in *E. huxleyi* is very interesting for understanding of its evolution and function. GPX (also known as GPX1) and PHGPX (also known as GPX4) are highly efficient antioxidant enzymes that catalyze glutathione dependent hydroperoxide reduction. Mammals contain eight GPX (GPX 1 to 8) homologs, of which five are selenoproteins (GPX 1, 2, 3, 4 and 6). Among them, only PHGPX is known to be essential during embryogenesis in mammals (Yant *et al.* 2003). So the function of PHGPX may be the reason for Se essentiality in *E. huxleyi*. Sel O and Sep 15 are widely distributed in some organisms, but Sec-containing homologs of these proteins are mostly in animals (Kryukov *et al.* 2003, Gladyshev *et al.* 1998). Sel T function is unknown. In Table 2, EST clones marked with both plus at the third column (TGA codon encoded Sec) and minus at the fourth column (SECIS) are thought to be sequences that possess unique SECIS or other mechanisms instead of SECIS for reading TGA as Sec. Interestingly, this type of EST sequences are more homologous to plant than animal despite of its homology to same protein. For example, CX774785.1 possessing a known SECIS is homologous to animal GPX, but CX779257.1 that does not possess a known SECIS is homologous to

plant GPX. If it represents the process of lack of selenoproteins, study in not only *E. huxleyi* but also other algae may give very important information for the evolution of selenoprotein.

I studied the effects of Se on the levels of EhSEP1 mRNA and enzyme activity of TR under Se-deficient and Se-sufficient conditions (Figs. 9 and 10). According to genomic information of photosynthetic aquatic phototrophs, a cyanobacterium (*Synechocystis* sp. PCC 6803: Hishiya *et al.* 2008) and a red alga (*C. merolae*) possess FTR (NP_442409.1, BAC76288.1) and NTR (NP_443054.1, c09f0014). The green alga *C. reinhardtii* possesses FTR (XP_001693262.1), NTR (XP_001699827.1), NTRC (XP_001689807.1), and TR1, and *O. lucimarinus* possesses FTR (XP_001421554.1), NTRC (XP_001422184.1), and TR1. The diatom *T. pseudonana* possesses FTR (estExt_fgenesh1_pm.C_chr_130010), NTR (estExt_gwp_gw1.C_chr_40101), and TR1. Therefore, I cannot completely exclude the possibility that the TR activity determined experimentally in this study was due to TRs other than EhSEP1.

However, I suggest that the TR activity in the extracts of *E. huxleyi* was due to EhSEP1 because: 1) when I searched homologues of various TRs of human (TR1: NP_877393.1) and *Arabidopsis thaliana* (NTRC: NP_565954.1, FTR: NP_568195.1, NP_197735.1 and NP_178547.1, NTR: Q39243.2 and Q39242.2) as queries in the *E. huxleyi* EST database, only EhSEP1 was found. Actually, 5 EST clones were found to be homologous to EhSEP1 while no EST clones were to *Arabidopsis thaliana* TRs; 2) there are no other selenoproteins of which molecular mass is similar to the mammalian-type TRs on

2D-SDS-PAGE in the extracts of *E. huxleyi* (Obata and Shiraiwa 2005); 3) TR activity detected in Se-depleted cells was quickly recovered when selenite was added to the medium. Altogether, our findings strongly suggest that TR activity in the crude extracts of *E. huxleyi* is due to EhSEP1 that is induced in the presence of Se in the medium during growth.

The mRNA levels of EhSEP1 remained nearly constant, except for a transient slight increase within 2 h when Se-deficient cells were transferred to Se-sufficient conditions (Fig. 9). In rat liver, TR activity was markedly down-regulated under Se-deficient conditions, but mRNA level decreased only slightly (Hadley and Sunde 2001). My observations are similar to results in rat liver. The results of transcript and enzyme activity suggest that the translation of EhSEP1 might be in response to the levels of available Se. In contrast, Obata and Shiraiwa (2005) reported that the mRNA levels of EhSEP2 were strongly stimulated by the addition of selenite to Se-deficient cells. This suggests that the regulation of selenoprotein synthesis by Se differs depending on proteins.

Higher plants have no mammalian-type TRs. On the other hand, in marine photosynthetic organisms, mammalian-type TRs have been found with the accumulation of genomic information over the last several years. Lobanov *et al.* (2007) reported that marine organisms contain more selenoproteins than terrestrial organisms, suggesting that Se is used more efficiently in marine organisms (Lobanov *et al.* 2007).

TR is a very important enzyme because it reduces cytosolic thioredoxins, which regulate many oxidoreductases that play key roles in cellular

metabolism. For example, cytosolic thioredoxins are thought to provide electrons for the reduction of ribonucleotide, hydrogen peroxide, peroxiredoxins, etc (Meyer *et al.* 2008). In contrast, chloroplastic thioredoxins, which are reduced by FTR, mainly regulate the activity of enzymes implicated in photosynthetic carbon assimilation. However, there are some thioredoxins for which functions have not yet been identified. Therefore, further analyses of mammalian-type TRs (EhSEP1, etc.) in marine photosynthetic organisms would be useful to elucidate how the thioredoxin system operates in these organisms.

In conclusion, I identified *EhSEP1*, a gene encoding the selenoprotein TR1, from the haptophyte *E. huxleyi.* The secondary structure of SECIS is same as form 2 in *E. huxleyi*, which is similar to that of humans, but different from that of the green alga *C. reinhardtii*. The changes in transcription of EhSEP1 and TR activity with Se availability suggest that EhSEP1 is regulated by Se upon translation. I also found another seven selenoproteins which are homologous to selenoproteins known in other organisms in *E. huxleyi* EST sequences. The analysis by comparing molecular masses, suggested that EhSEP 3 and 5 may be Sel U and DI, respectively, and EhSEP4 may be either Sel T, GPX, PHGPX or PDI-like protein.

In Fig. 20, I summarized the uptake, transport and metabolism of Se in *E. huxleyi* including results obtained in this study. Further studies on selenoproteins in various marine organisms will be necessary to elucidate how marine organisms have developed the strategy for Se utilization to be an essential element and how it related to survive in marine environments.

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Tables and Figures

Table 1. Comparison of TR activity determined by the DTNB reduction assay and the insulin reduction assay in crude extracts of *E. huxleyi*.

Assay	Components	inhibitor	NADPH oxidation (μmol min^{-1} mg $protein^{-1}$)	
Insulin reduction[a]				
	Complete[c]	-	36.4	(100%)
		+	6.2	(17%)
	Insulin -	-	14.1	(39%)
	Thioredoxin -	-	3.4	(9%)
DTNB reduction[b]				
	Complete[d]	-	150.6	(100%)
		+	38.6	(26%)

[a and b] Cells used for the insulin and DTNB reduction assays were 7- and 3-d cultures, respectively.

[c and d] Human T-cell recombinant thioredoxin and DTNB were added as substrates, respectively.

Table 2. Homology search of selenoproteins known in other organisms in *E. huxleyi* ESTsequences.

Selenoproteins (molecular weight) [the accession number of query]	The accession number of EST clone	TGA codon encoded Sec	SECIS
MsrA (17.8kDa) [AAN32904.1]	-	-	-
MsrB, Sel R, Sel X (12.6kDa) [NP_057416.1]	-	-	-
Sel H (13.3kDa) [Q8IZQ5.1]	-	-	-
Sel I (45.1kDa) [NP_277040.1]	-	-	-
Sel K (10.5kDa) [AAH13162.]	-	-	-
selenophosphate synthetase 2 (47.2kDa) [NP_036380.2]	-	-	-
Sel N (65.5kDa) [NP_065184.2]	-	-	-
Sel P (41.7kDa) [AAH15875.1]	-	-	-
Sel W (9.3kDa) [NP_003000.1]	-	-	-
Sel S (21kDa) [NP_060915.2]	-	-	-
Sel V (36.7kDa) [NP_874363.1]	-	-	-
Sel M (16.1kDa) [AAH13421.1]	-	-	-
Sel U (27.5kDa) [EDO99700.1]	CX774313.1	+	+
	CX779243.1	-	+
Sel T (22.2kDa) [AAH26350.2]	GE159669.1	+	+
Sel O (73.3kDa) [AAP85540.1]	GE171520.1	+	+
	GE171521.1	+	?
Sep 15 (18kDa) [NP_004252.2]	CX773474.1	+	+

GPX (22.2kDa) [AAH00742.3]	CX774785.1	+	+
	CX779257.1	+	-
PHGPX, GPX4 (19.2kDa) [AAM18080.2]	GE168244.1	+	+
DI (31.3kDa) [AAH17717.2]	GE198993.1	+	+
	CX775453.1	+	-
PDI like protein (24.4kDa) [Q50KB1.2]	GE147892.1(EhSEP2)	+	+
	GE148906.1	+	?
	GE156283.1	+	?
	GE144352.1	Cysteine homolog	
	EG030949.1	+	-
TR1 (54.6kDa) [NP_877419.1]	EhSEP1	+	+
TR2 (56.4kDa) [NP_006431.2]	-	-	-
TR3 (82.3kDa) [XP_001130163.1]	-	-	-

+ and - indicate detected and undetected by computational search using SECISearch 2.19, respectively. The question mark indicates that the EST sequence does not contain 3'-UTR.

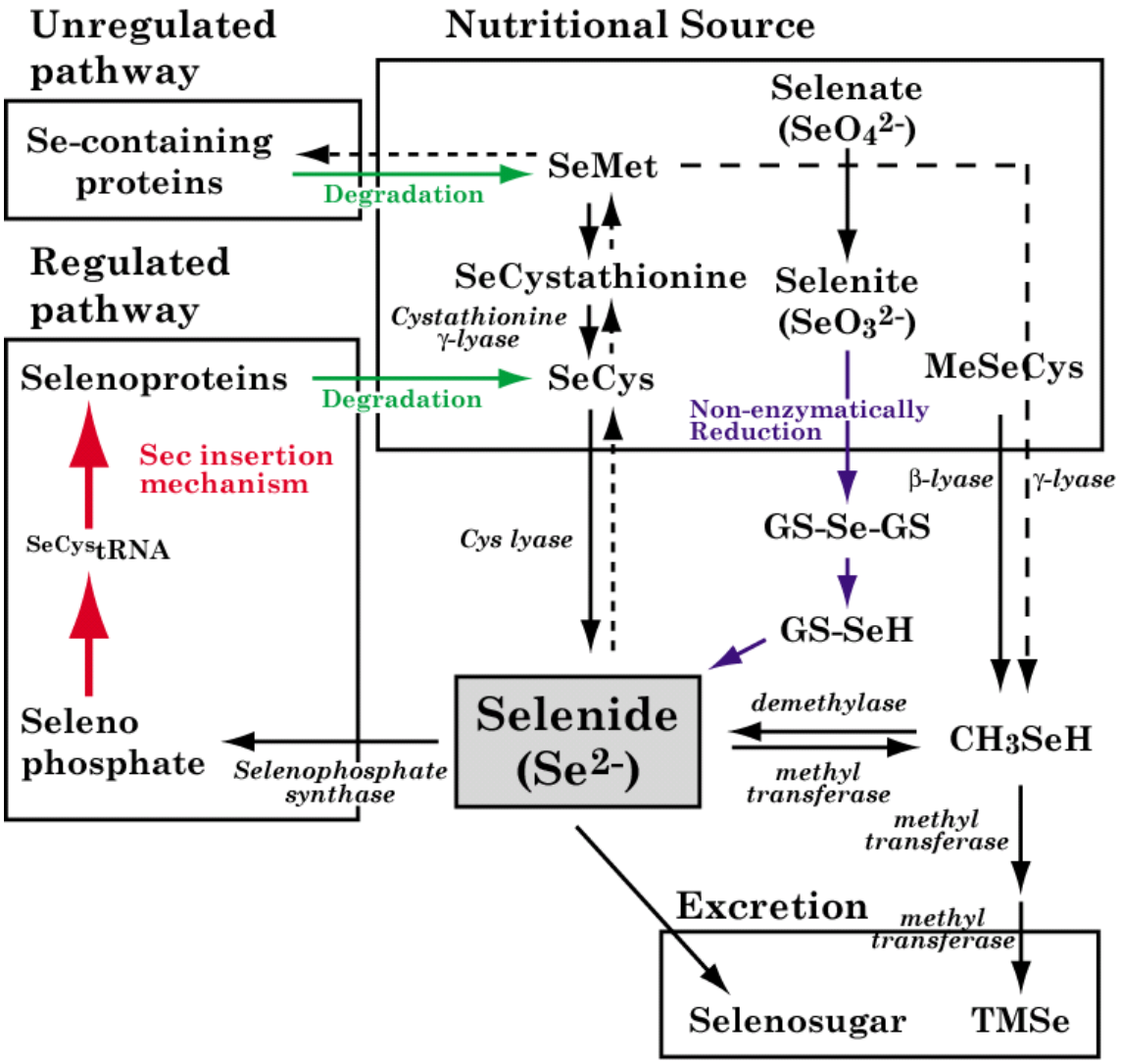


Fig. 1. Proposed pathways for the metabolism of Se in animals compiled from Ganther and Lawrence (1997), Suzuki (2005) and Suzuki *et al.* (2006). Abbreviations: Cys, cysteine; SeMet, selenomethionine; MeSeCys, Se-methylselenocysteine; GS-SeH, selenoglutathione; GS-Se-SG, selenodiglutathione; TMSe, trimethylselenonium.

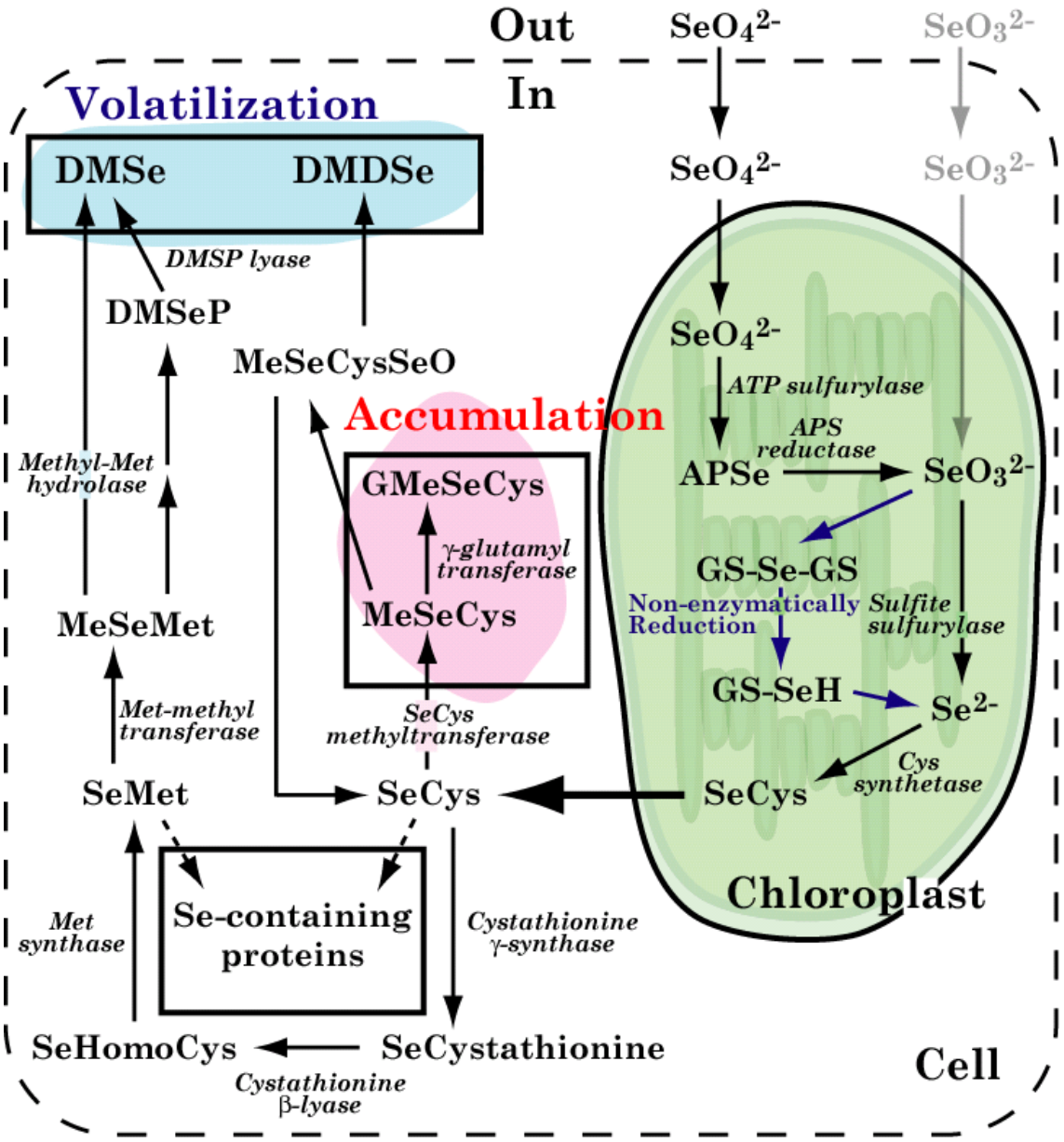


Fig. 2. Proposed pathways for the metabolism of Se in land plants compiled from Terry *et al.* (2000) and Ellis and Salt (2003). Abbreviations: APS, adenosine 5'-phosphosulfate; APSe, adenosine 5'-phosphoselenate; DMDSe, dimethyl-diselenide; DMSe, dimethylselenide; DMSeP, dimethyl-selenoniopropionate; MeSeCys, Se-methylselenocysteine; SeHomoCys, selenohomocysteine; MeSeMet, Se-methyl-selenomethionine; GMeSeCys, g-gultamyl-MeSeCys; MeSeCysSeo, MeSeCys Se-oxide.

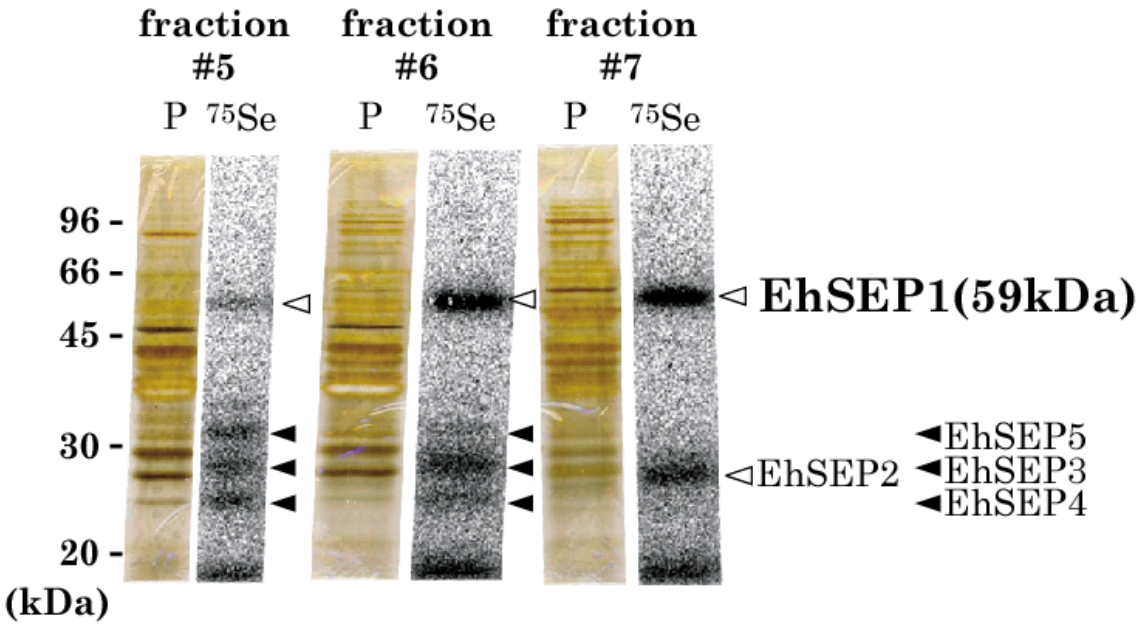


Fig. 3. Selenoproteins in a coccolithophorid, *E. huxleyi*. SDS-PAGE pattern of ^{75}Se radio-labeled proteins in each fraction obtained by DEAE anion exchange HPLC column chromatography. P, Protein bands visualized by the silver staining method. ^{75}Se, ^{75}Se radioactive bands visualized by BAS5000. Arrowheads indicate a 59, 27, 29, 23, and 31 kDa ^{75}Se radioactive bands, which were identified as EhSEP1 to 5, respectively.

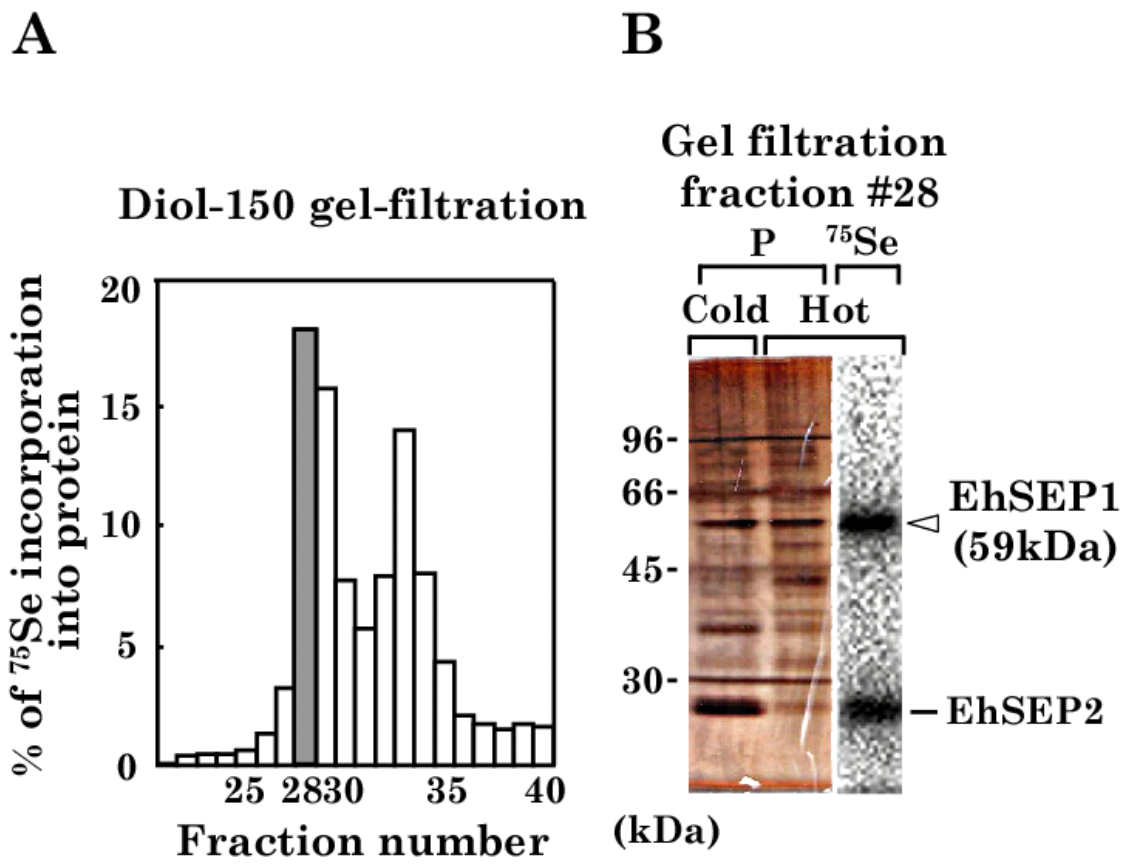
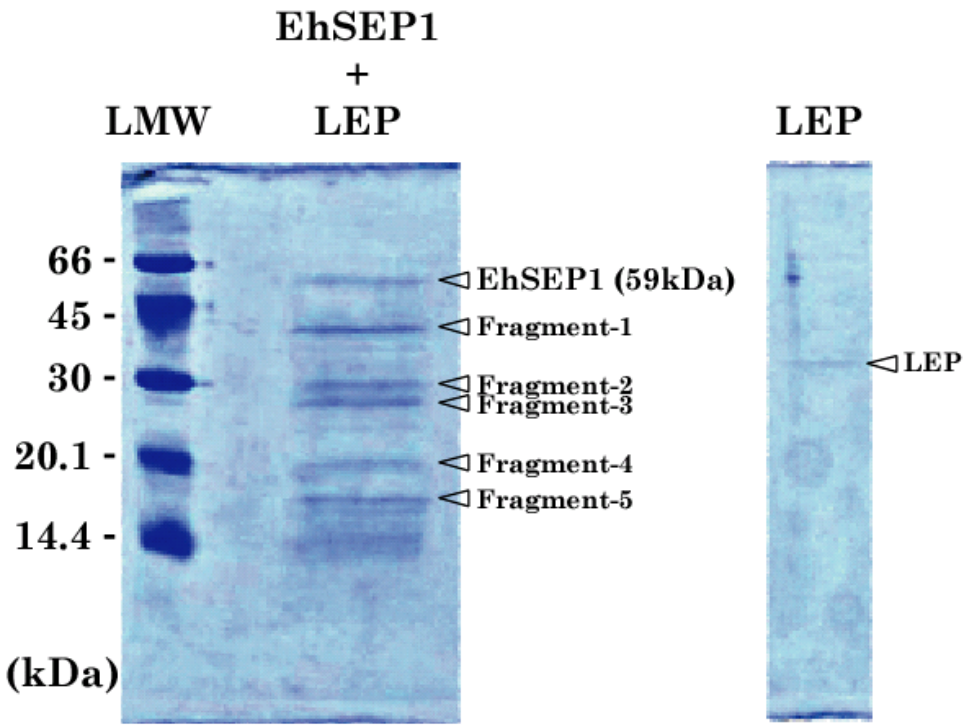


Fig. 4. Purification of EhSEP1 from a coccolithophorid, *E. huxleyi*. A, Fractionation of EhSEP1 containing fraction by Diol-150 gel filtration HPLC column chromatography. EhSEP1 containing fraction was obtained by DEAE anion exchange HPLC column chromatography. The bars represent the percentage of total ^{75}Se radioactivity in the eluted fractions. B, SDS-PAGE analysis of fraction #28 obtained by Diol-150 column chromatography. P, Protein bands visualized by the silver staining method. ^{75}Se, ^{75}Se radioactive bands visualized by BAS5000. Cold and Hot, samples prepared as non-radiolabeled and ^{75}Se-labeled proteins, respectively. The 59-kDa band in the non-radiolabeled sample was used in the subsequent experiments, such as amino acid sequence analysis. Arrowheads indicate a 59-kDa-^{75}Se-radioactive band, which was identified as EhSEP1.

A



B

Protein	Amino acid sequences
EhSEP1	Blocked
Fragment-1	ELGITSDDVFAL
Fragment-2	ELGITSDDVFAL
Fragment-3	Blocked
Fragment-4	X-(A/L)-(L/N)-(A/G)-(G/D)-X
Fragment-5	RAASHGKKVAV

Fig. 5. Purification of EhSEP1 from a coccolithophorid, *E. huxleyi*.

A, Lysyl endopeptidase (LEP, 20 ng) was added to the EhSEP1 (10 μg) and incubated at 37°C for about 2 h. Those digested and undigested peptides (Fragment-1 to -5 and EhSEP1, respectively) were resolved on 15% SDS-PAGE gel and transferred to PVDF membranes. The CBB stained peptide bands were applied to the analysis of N-terminal amino acid sequence. B, The resulted N-terminal amino acid sequences of Fragment-1 to -5 and EhSEP1, respectively.

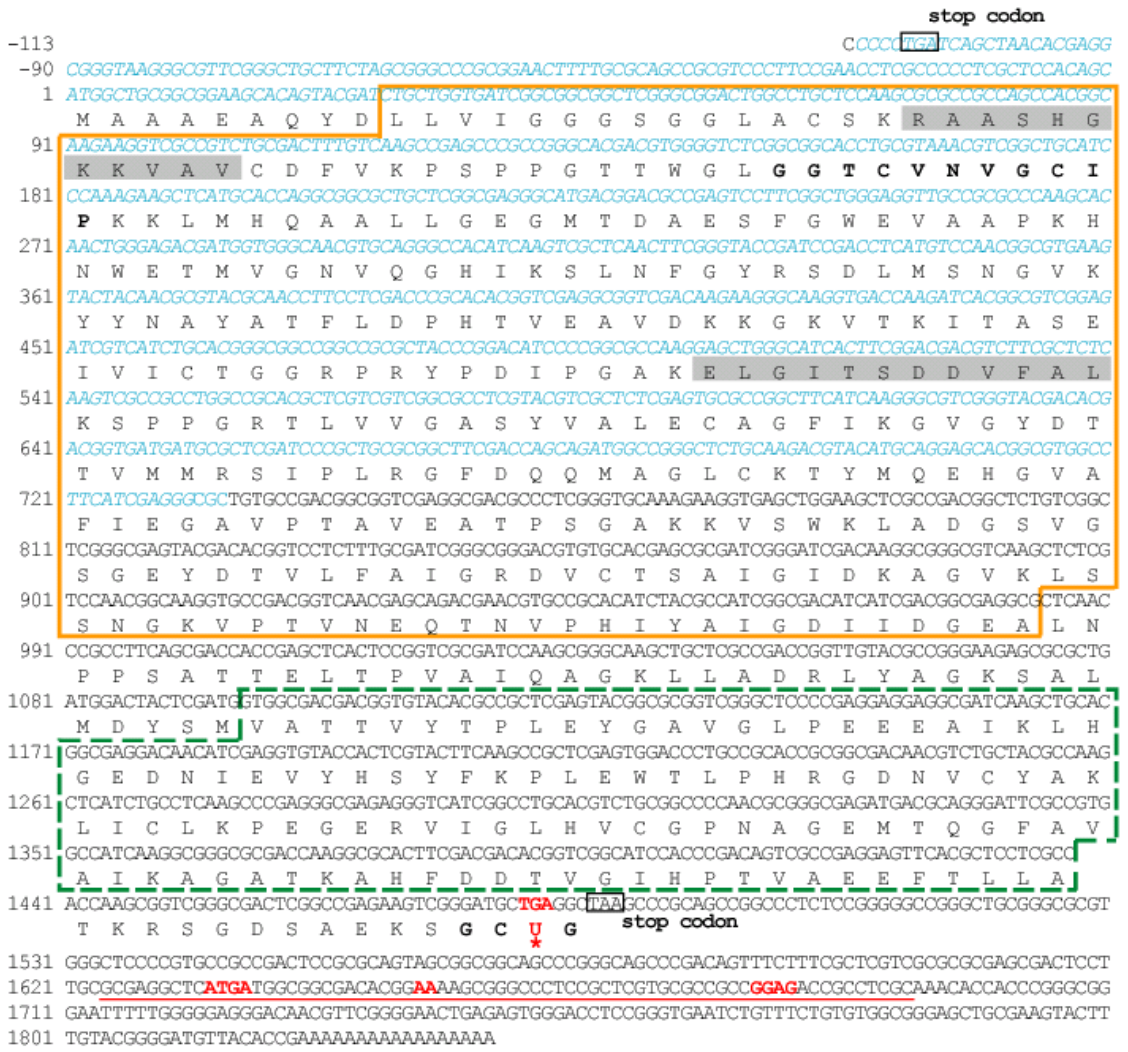


Fig. 6. The nucleotide sequence and deduced amino acid sequence of EhSEP1. Shaded residues correspond to sequences that were confirmed by peptide sequencing. The nucleotide sequence in blue (-112 to 731) was obtained from the EST sequence of *E. huxleyi* (NCBI accession number: CV069652). A pyridine nucleotide-disulfide oxidoreductase domain (28-984) and a dimerization domain (1096-1437) are shown in a box and with a dotted line, respectively. The pyridine nucleotide-disulfide oxidoreductases class-I and its C-terminal GCUG active site are shown in bold. The deduced stop codons were shown in box. The putative SECIS element sequence is underlined and the non-Watson-Crick quartet and AA pair at an apical loop are highlighted with a red color.

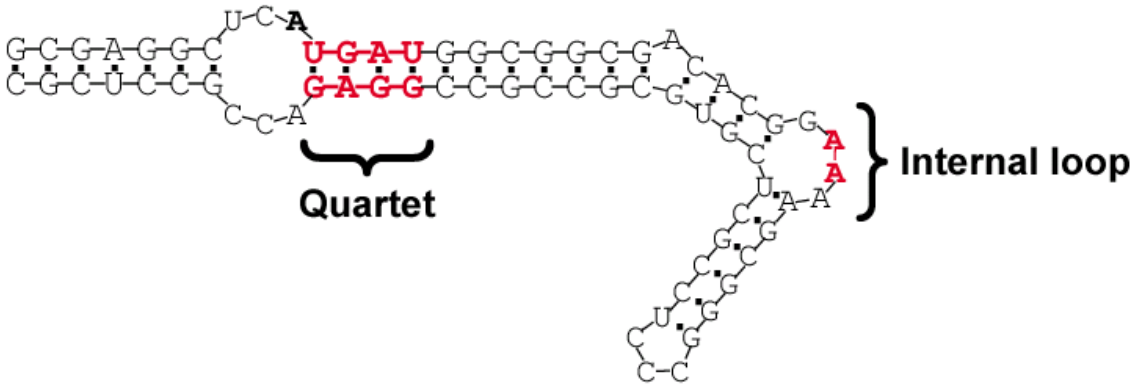


Fig. 7. Structure of the SECIS element in the 3'-UTR region of EhSEP1. The structure of the SECIS element was analyzed and drawn by SECISearch 2.19. Conserved nucleotides in the apical loop and the quartet are shown in bold with a red color.

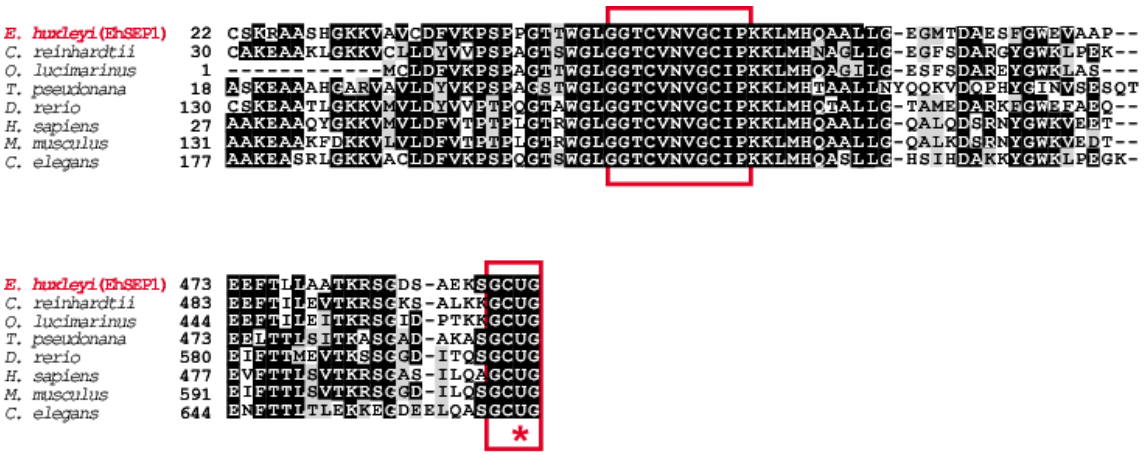


Fig. 8. Multiple sequence alignment of amino acid sequences in various organisms that show homology with EhSEP1. The TRs and GenBank accession numbers of each sequence are XP_001696072.1 (*Chlamydomonas reinhardtii* TR1), Chr_1 [733779, 735290] (*Ostreococcus lucimarinus* CCE9901 TR1), newV2.0.genewise.318.11.1 (*Thalassiosira pseudonana*), NP_898895.2 (*Danio rerio* TR1), NP_877393.1 (*Homo sapiens* TR1), NP_001035988.1 (*Mus musculus* TR1), and AAD41826.1 (*Caenorhabditis elegans* TR). Residues conserved in all of the sequences are highlighted. The highly conserved active sites are boxed. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

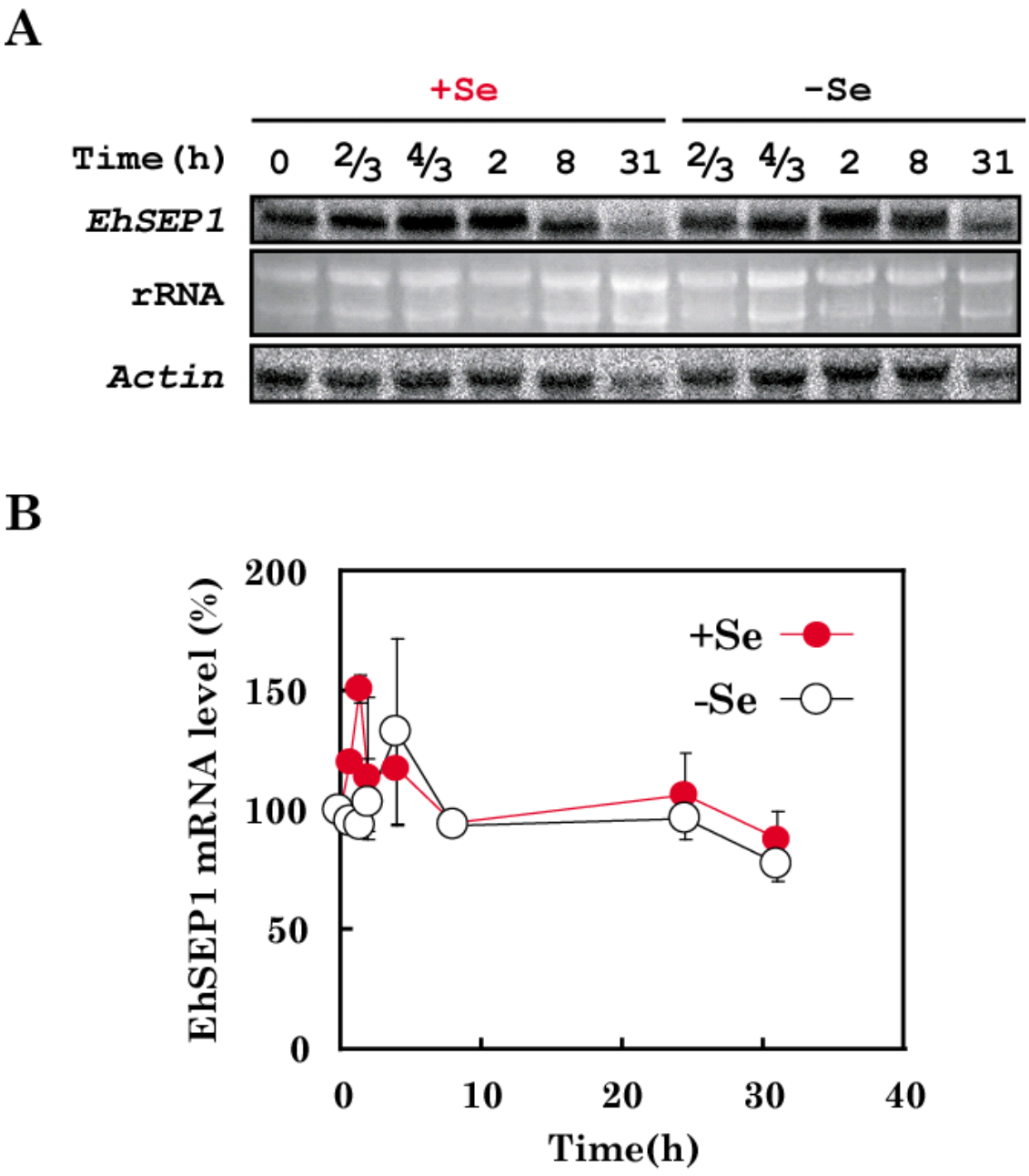


Fig. 9. Effects of selenium on the expression of EhSEP1. A, Representative mRNA levels of EhSEP1. B, After 3 days incubation of cells in Se-depleted medium, no (-Se) and 10 nM selenite (+Se) was added at 0 h (open and closed circles, respectively). Values are average of 2-6 experiments. Error bars represent standard error of means (n = 2-6).

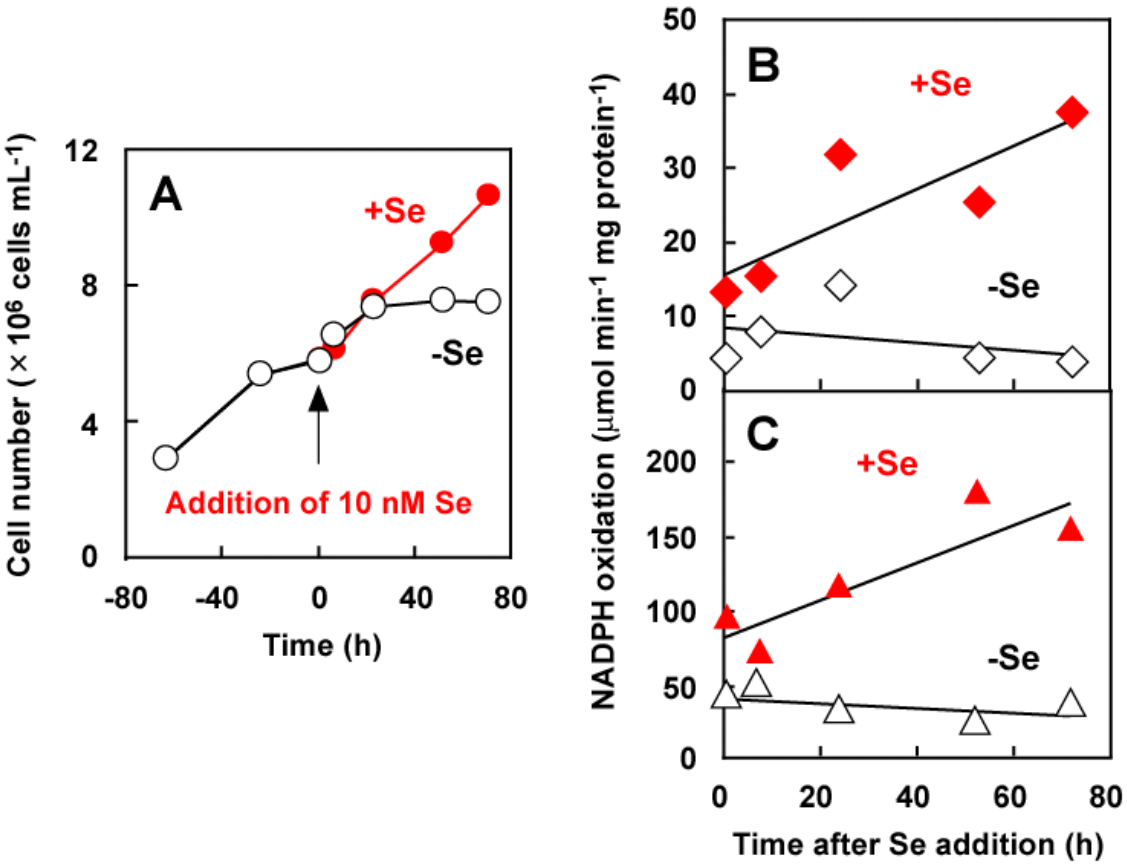


Fig. 10. Effects of selenium on the induction of TR activity in a coccolithophorid, *E. huxleyi*. A, Effects of Se limitation and repletion on cell growth. Changes in cell growth of the resuspended cells for Se-depletion. Se (final concentration, 10 nM) was added at 0 h (closed circles), whereas the other culture was maintained without Se (open circles). Preincubated cells were incubated without Se for 7 days. B, Changes in TR activity after the addition of Se (final concentration, 10 nM), measured by the insulin reduction assay. C, Changes in TR activity after the addition of Se (final concentration, 10 nM), measured by the DTNB reduction assay. The lines in the figures were drawn according to approximate curve fitting of the Excel (Microsoft Corp., Redmond, USA).

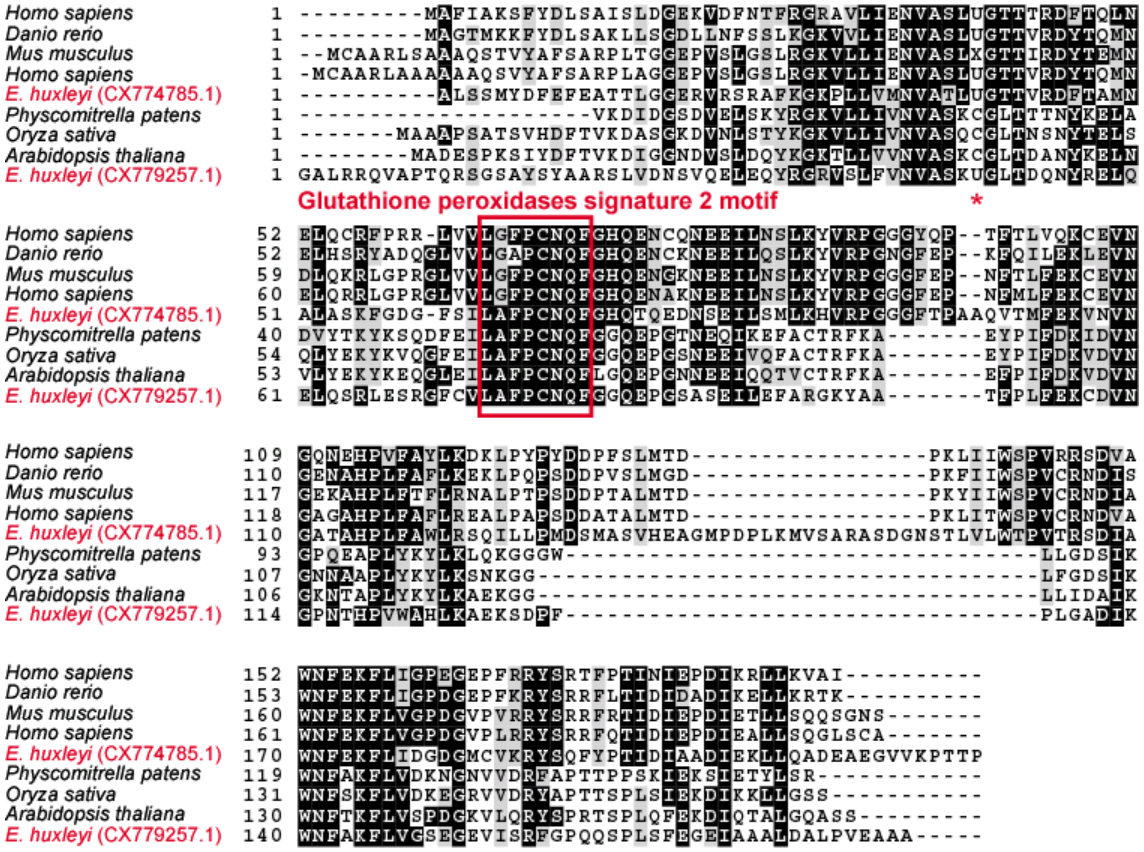


Fig. 11. Multiple sequence alignment of GPX in various organisms and the EST sequences of *E. huxleyi* that were homologous with GPX. The GenBank accession numbers of each sequence are AAA67540.2, NP_002074.2 (*Homo sapiens*), NP_001007282.2 (*Danio rerio*), CAA27558.1 (*Mus musculus*), ABG37901.1 (*Physcomitrella patens*), NP_001053524.1 (*Oryza sativa*), NP_180715.1 (*Arabidopsis thaliana*), CX774785.1 and CX779257.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The highly conserved motif is boxed. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

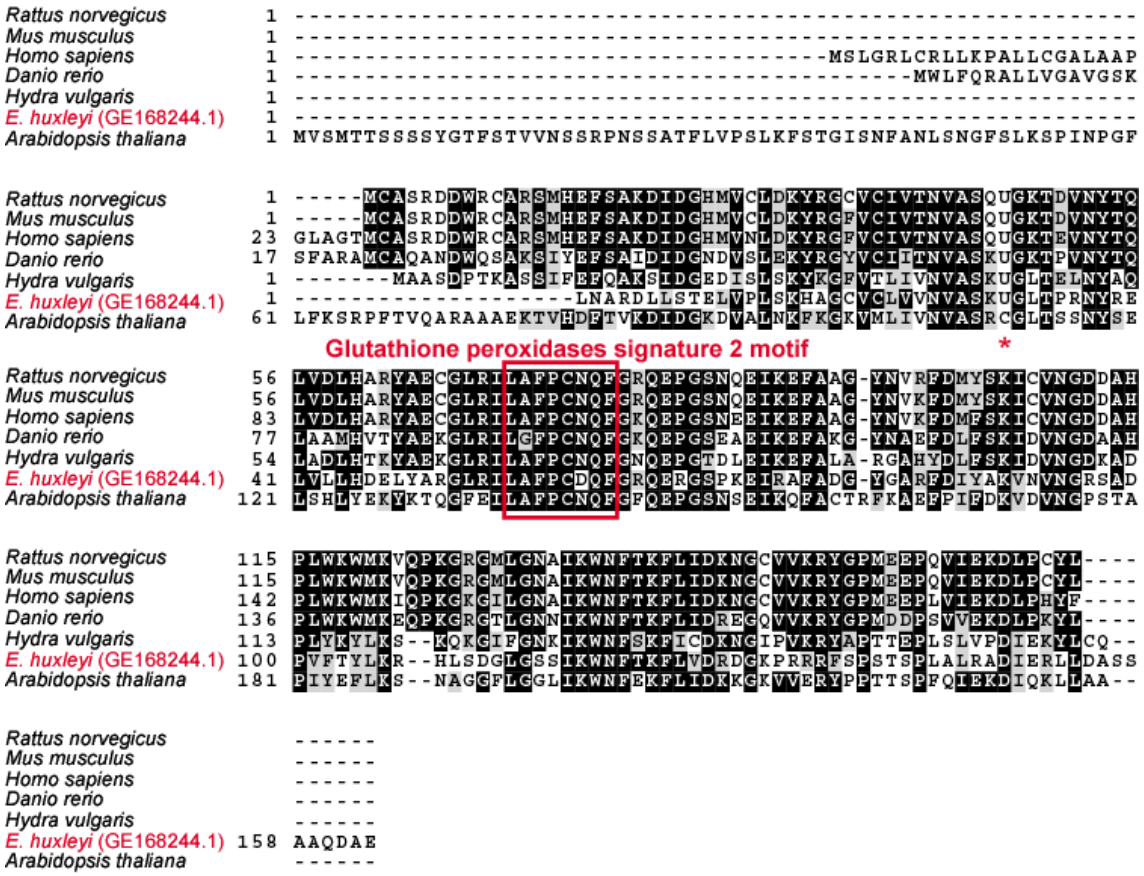


Fig. 12. Multiple sequence alignment of PHGPX in various organisms and the EST sequences of *E. huxleyi* that were homologous with PHGPX. The GenBank accession numbers of each sequence are NP_002076.2 (*Homo sapiens*), NP_001025241.2 (*Danio rerio*), AAC15833.1 (*Mus musculus*), CAD61277.1 (*Rattus norvegicus*), NP_180080.1 (*Arabidopsis thaliana*), ABC25027.1 (*Hydra vulgaris*) and GE168244.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The highly conserved motif is boxed. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

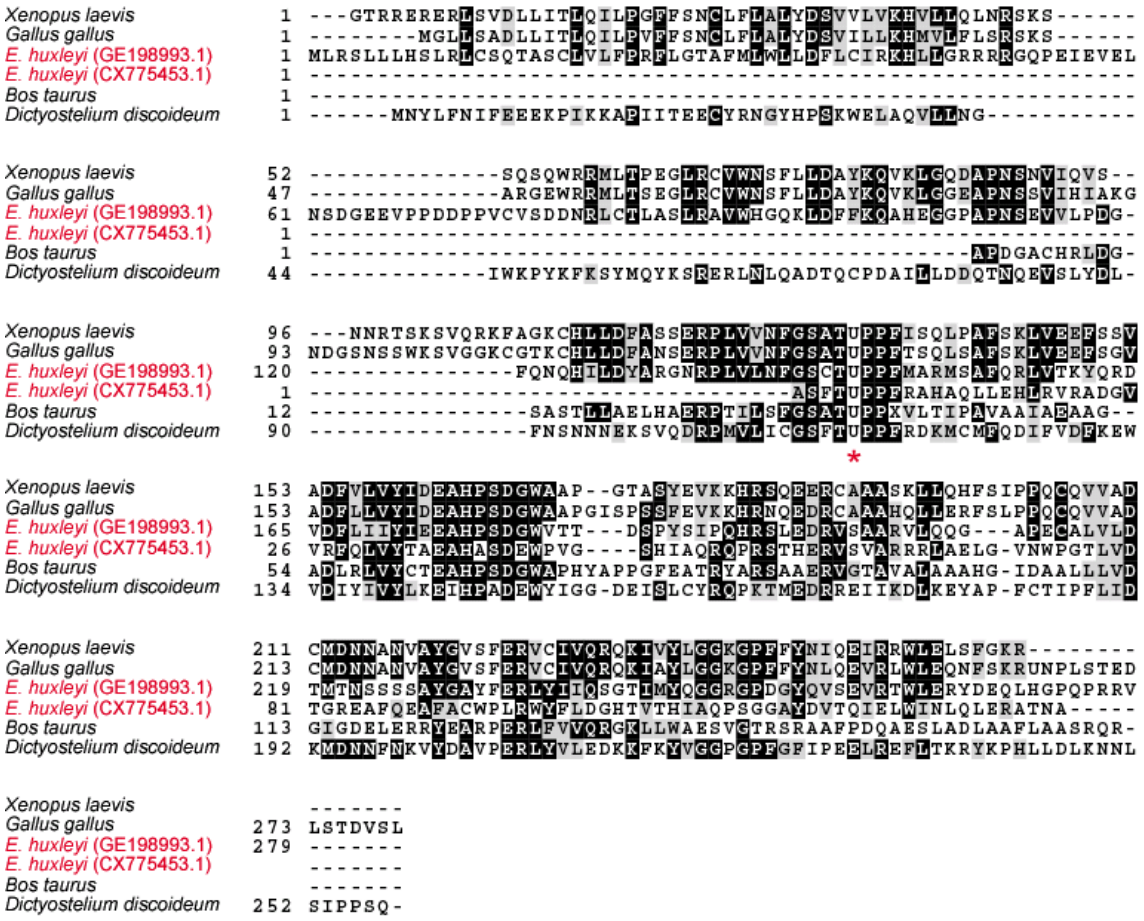


Fig. 13. Multiple sequence alignment of DI in various organisms and the EST sequences of *E. huxleyi* that were homologous with DI. The GenBank accession numbers of each sequence are AAK40121.1 (*Xenopus laevis*), Q9IAX2.3 (*Gallus gallus*), NP_001010993.1 (*Bos taurus*), XP_643546.2 (*Dictyostelium discoideum* AX4), GE198993.1 and CX775453.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

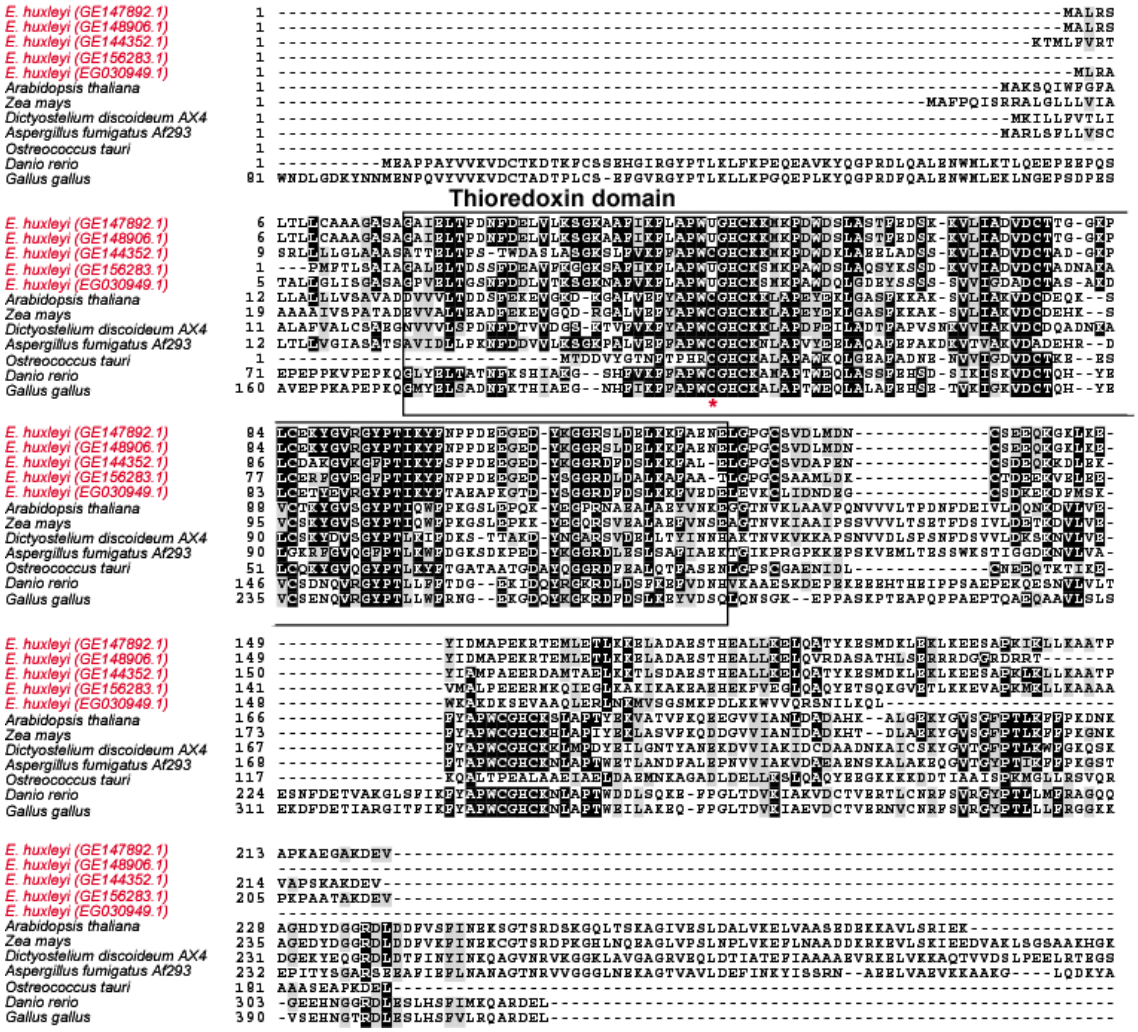


Fig. 14. Multiple sequence alignment of PDI-like protein in various organisms and the EST sequences of *E. huxleyi* that were homologous with PDI-like protein. The GenBank accession numbers of each sequence are NP_998181.1 (*Danio rerio*), CAL51723.1 (*Ostreococcus tauri*), XP_643357.1 (*Dictyostelium discoideum* AX4), XP_753425.1 (*Aspergillus fumigatus* Af293), NP_001006374.1 (*Gallus gallus*), NP_001078074.1 (*Arabidopsis thaliana*), NP_001105759.1 (*Zea mays*), GE147892.1, GE148906.1, EG030949.1, GE144352.1 and GE156283.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The highly conserved domain is boxed. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

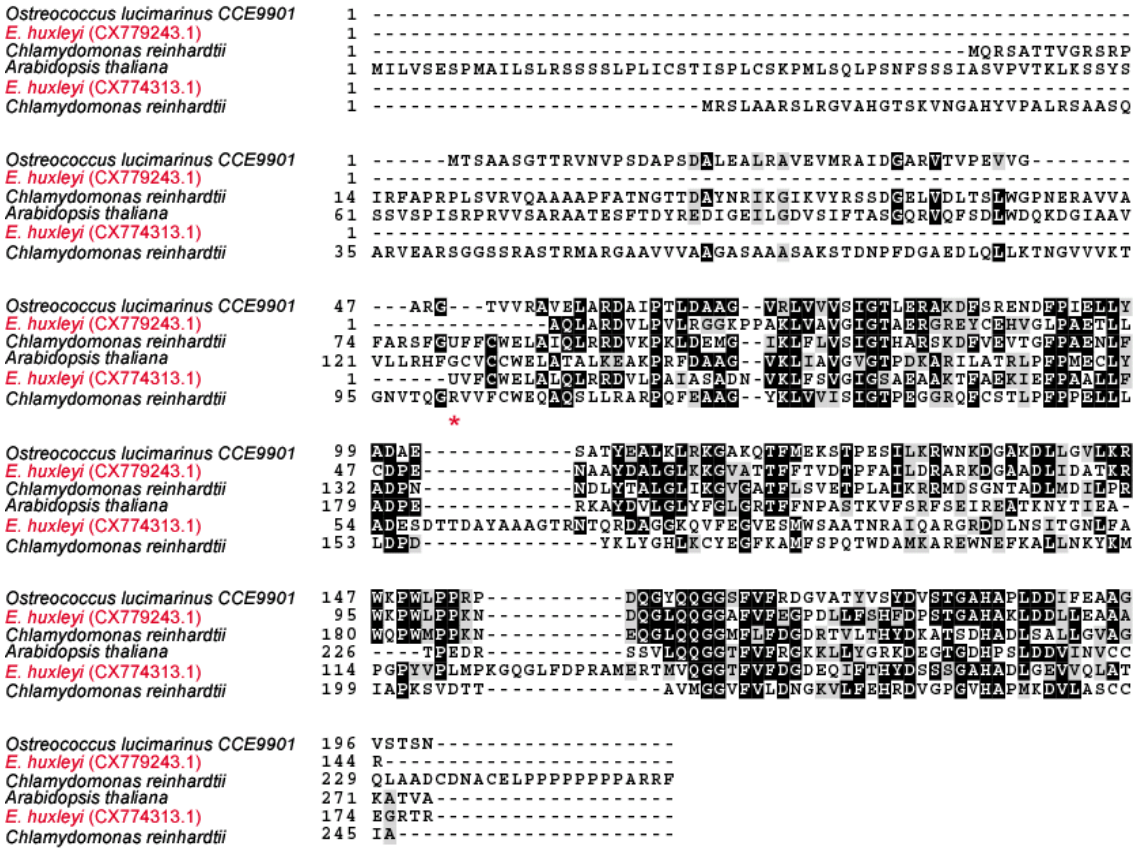


Fig. 15. Multiple sequence alignment of Sel U in various organisms and the EST sequences of *E. huxleyi* that were homologous with Sel U. The GenBank accession numbers of each sequence are XP_001698115.1, XP_001699290.1 (*Chlamydomonas reinhardtii*), XP_001415644.1 (*Ostreococcus lucimarinus* CCE9901), NP_201385.2 (*Arabidopsis thaliana*), CX774313.1 and CX779243.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

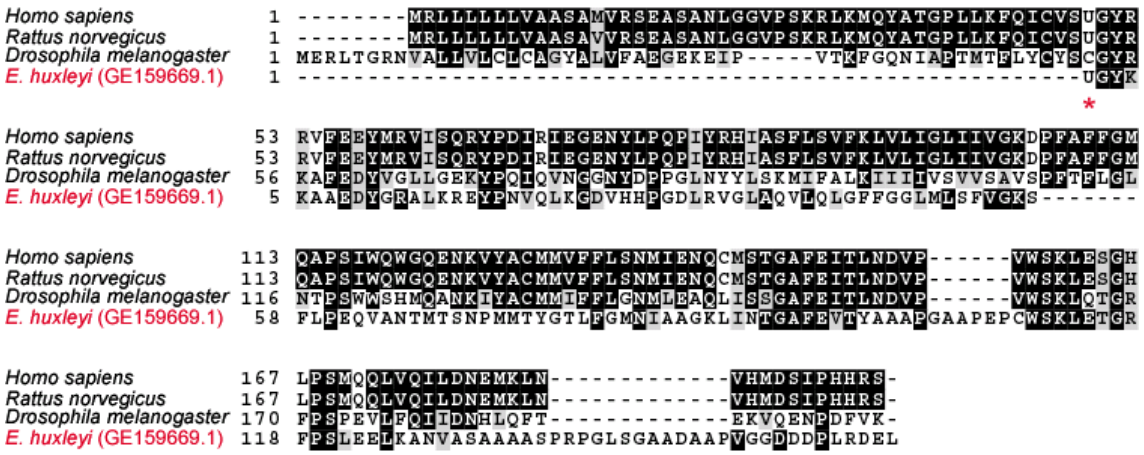


Fig. 16. Multiple sequence alignment of Sel T in various organisms and the EST sequences of *E. huxleyi* that were homologous with Sel T. The GenBank accession numbers of each sequence are NP_057359.2 (*Homo sapiens*), NP_001014275.2 (*Rattus norvegicus*), NP_608897.1 (*Drosophila melanogaster*) and GE159669.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

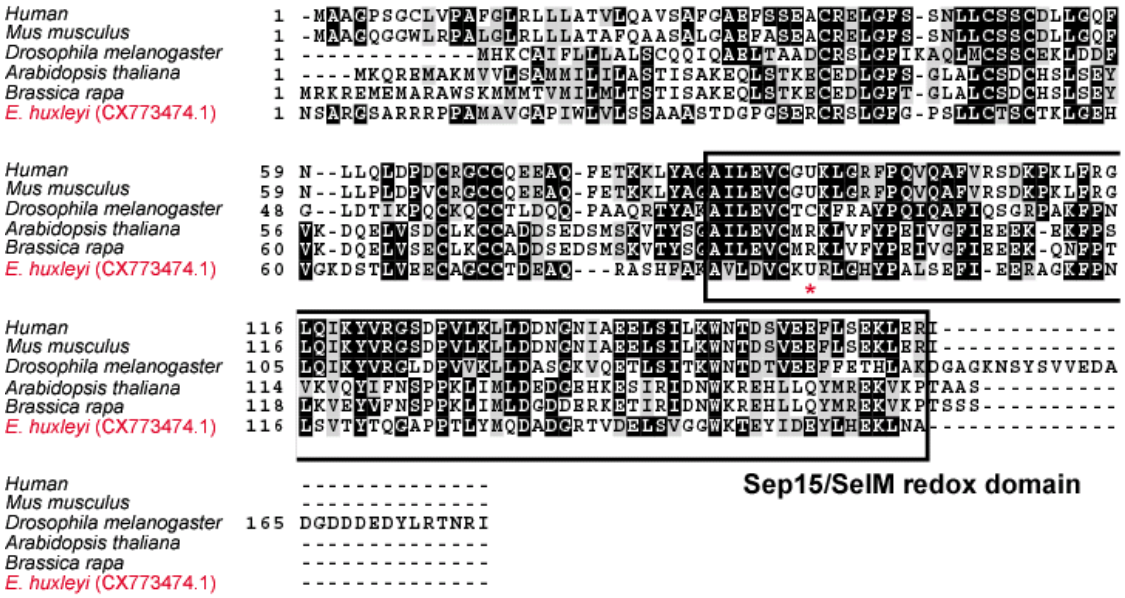


Fig. 17. Multiple sequence alignment of Sep 15 in various organisms and the EST sequences of *E. huxleyi* that were homologous with Sep 15. The GenBank accession numbers of each sequence are O60613.3 (Homo sapiens), NP_444332.1 (Mus musculus), NP_563747.1 (Arabidopsis thaliana), BAF81524.1 (Brassica rapa), CAL26599.1 (Drosophila melanogaster) and CX773474.1 (Emiliania huxleyi). Residues conserved in all of the sequences are highlighted. The highly conserved domain is boxed. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

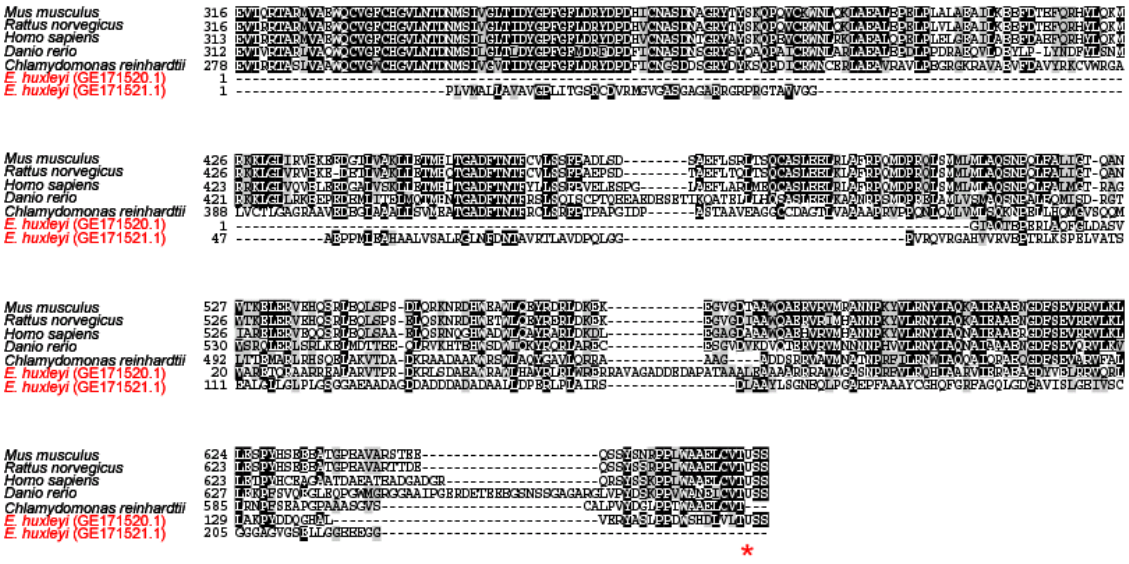


Fig. 18. Multiple sequence alignment of Sel O in various organisms and the EST sequences of *E. huxleyi* that were homologous with Sel O. The GenBank accession numbers of each sequence are NP_113642.1 (*Homo sapiens*), NP_001078954.1 (*Rattus norvegicus*), NP_082181.2 (*Mus musculus*), Q1LVN8.2 (*Danio rerio*), XP_001699727.1 (*Chlamydomonas reinhardtii*), GE171520.1 and GE171521.1 (*Emiliania huxleyi*). Residues conserved in all of the sequences are highlighted. The alignment was constructed with ClustalW. "U*" indicates the position of Sec.

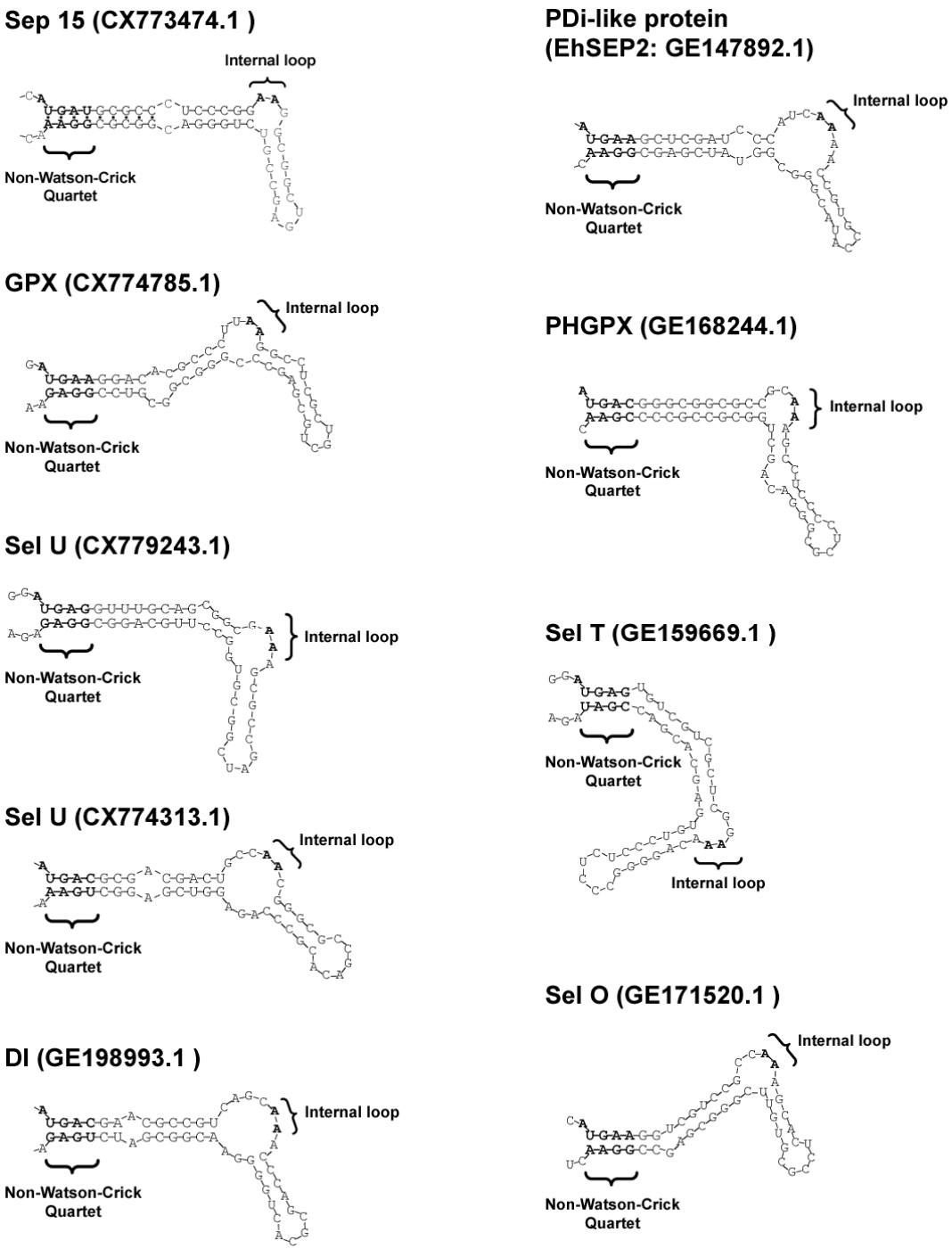


Fig. 19. Structure of the SECIS element in the 3'-UTR region of *Emiliania* EST sequences that are homologous with Sel U, T, O, Sep 15, GPX, PHGPX, DI and PDI-like protein. The structure of the SECIS element was analyzed and drawn by SECISearch 2.19. Conserved nucleotides in the apical loop and the quartet are shown in bold.

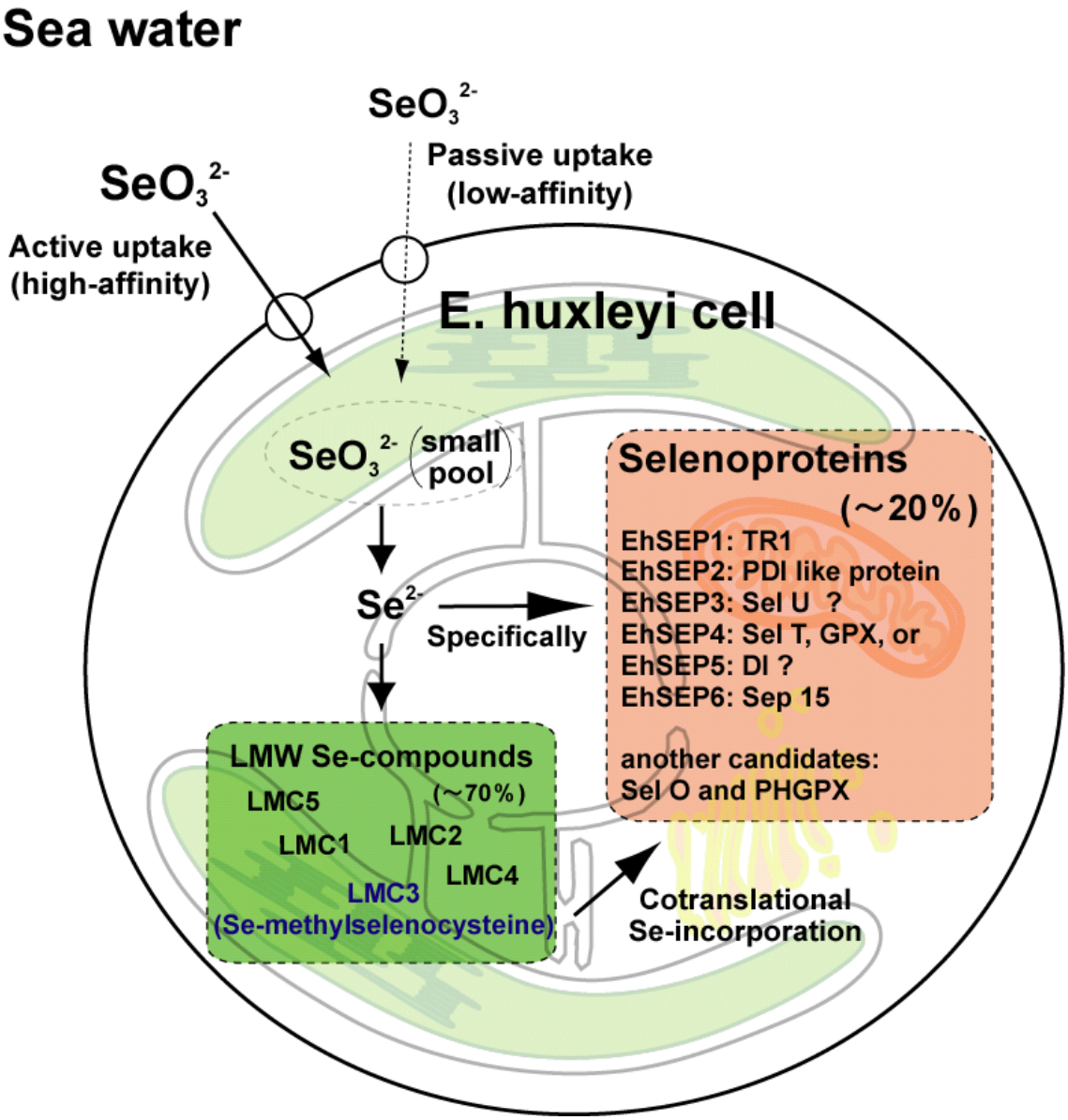


Fig. 20. The summary of proposed pathway for the metabolism of Se in *E. huxleyi* compiled from Obata *et al.* (2004), Obata and Shiraiwa (2005) and Araie *et al.* (the present thesis).

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