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Physiological and molecular responses to environmental stresses in a sterile mutant of *Ulva pertusa* (Ulvales, Chlorophyta)

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Abstract A sterile mutant of *Ulva pertusa* is tolerant to growth in a wide range of temperature, salinity, and nutrient conditions, suggesting that this is an appropriate species for examining physiological and molecular responses to environmental stresses in intertidal macroalgae. We have investigated thermal and salinity stress responses in the sterile *U. pertusa*. When the alga was cultivated at 20℃ and 30℃, remarkable changes in chemical components due to thermal stress were found in the amounts of the photosynthetic pigments and the major free amino acids. In response to thermal stress, these components increased 1.4-2.4 and 1.9-10.5 fold, respectively. Isozyme assays for metabolic enzymes showed that the 20℃- and 30℃-cultivated algae expressed a NAD^+- and $NADP^+$-specific glutamate dehydrogenase (NAD/NADP-GDH), whereas the 30℃-cultivated alga expressed an additional $NADP^+$-specific GDH (NADP-GDH). Two types of cDNA clones encoding GDH, designated pGDH-L and pGDH-S, were isolated from the 30℃-cultivated alga. mRNA encoding GDH-L was constitutively expressed in the alga, whereas the expression of that encoding GDH-S was induced by thermal stress. Differential gene expression in the alga grown under thermal stress conditions was investigated by using differential display reverse transcriptase-PCR (DDRT-PCR). Some DDRT-PCR fragments encoded proteins which were closely connected with carbon and nitrogen metabolism, and photosynthetic and molecular chaperone systems. The salinity stress responses of the alga were investigated at 20, 100, and 180% seawater (low, normal, and high salinity conditions, respectively). Analysis of free amino acid content showed that proline significantly increased in response to high salinity stress. Some DDRT-PCR fragments corresponding to mRNAs whose expressions were altered by low and high salinity conditions, were similar to membrane, transport, and transcription factor proteins.

Key words: Chlorophyta, gene expression, salinity stress, stress response, thermal stress, *Ulva*

Introduction

Land plants are often exposed to severe environmental conditions that dramatically change on a daily and yearly basis. Plants have developed a number of mechanisms in order to respond and adapt to such fluctuating environmental conditions. In recent years, some of the adaptation mechanisms in response to various environmental changes in the plants have been identified at the molecular level. Intertidal algae are also often exposed to a range of environmental factors such as extreme temperature and sunlight, desiccation, rapid salinity changes, and various forms of pollution. Responses to these environmental stresses in algae are well investigated at physiological and biochemical levels. For example, upper intertidal algae often show enhanced rates of photosynthesis and nutrient uptake in response to desiccation (Quadir *et al.*, 1979; Thomas and Turpin, 1980). In addition, as part of an adaptation mechanism to desiccation, remarkable increases in enzymatic activity are found in nitrate reducatse, amylase, invertase, and peroxidase (Murthy *et al.*, 1986, 1988; Murthy and Sharma, 1989). However, molecular mechanisms underlying responses and adaptation to fluctuating environmental conditions in intertidal algae have received little attention, since most of the intertidal algal species grown in the laboratory are maintained with difficult under axenic condi-

tions.

Ulva pertusa (Chlorophyta) grows in the intertidal zone along the entire coast of Japan, and is tolerant to fluctuating environmental conditions. However, the 'wild' strain of *U. pertusa* undergoes sexual reproduction in laboratory culture which involves the release of zoospores and the subsequent deterioration of tissue. In contrast, a sterile mutant of *Ulva pertusa* found at Omura Bay, Kyusyu province in Japan in 1973 is suitable for laboratory experiments, since the alga only develops vegetatively and can be maintained with ease under laboratory conditions (Migita, 1985). In addition, the alga has high tolerance to extensive ranges of temperature, nutrient salts, and salinity, suggesting that this alga is an appropriate species for investigations designed to elucidate molecular responses and adaptation mechanisms to various environmental stresses.

We have recently investigated some of the physiological and molecular responses to thermal and salinity stresses in intertidal algae using the sterile mutant of *U. pertusa* as a model species. This paper deals with the results and problems in studies on physiological and molecular responses and adaptation mechanisms to the thermal and salinity stresses in the algae.

1. Thermal stress responses

Effect of thermal stress on growth

Previous experiments have shown that growth is optimal when the sterile mutant of *U. pertusa* is continuously cultured in our laboratory in one-fifth strength Provasoli's enriched seawater (1/5 PES) medium (Provasoli, 1968) at a light intensity of 100-200 μ E/m^2/s with a 12 : 12 h (light : dark) cycle at temperatures between 20 and 25℃ (Murase *et al.*, 1993; Amano *et al.*, 1997). Thalli cultivated at 20℃ grow rapidly and have a normal flattened appearance (Fig. 1A). When the sterile mutant is cultivated at 30℃ for 7 days, the thalli also have the same normal flattened appearance as the 20℃-cultivated thalli. However, the cells of the thallus become dark green in color and rich in cytoplasmic content, and the cell wall thickens (Fig. 1B)

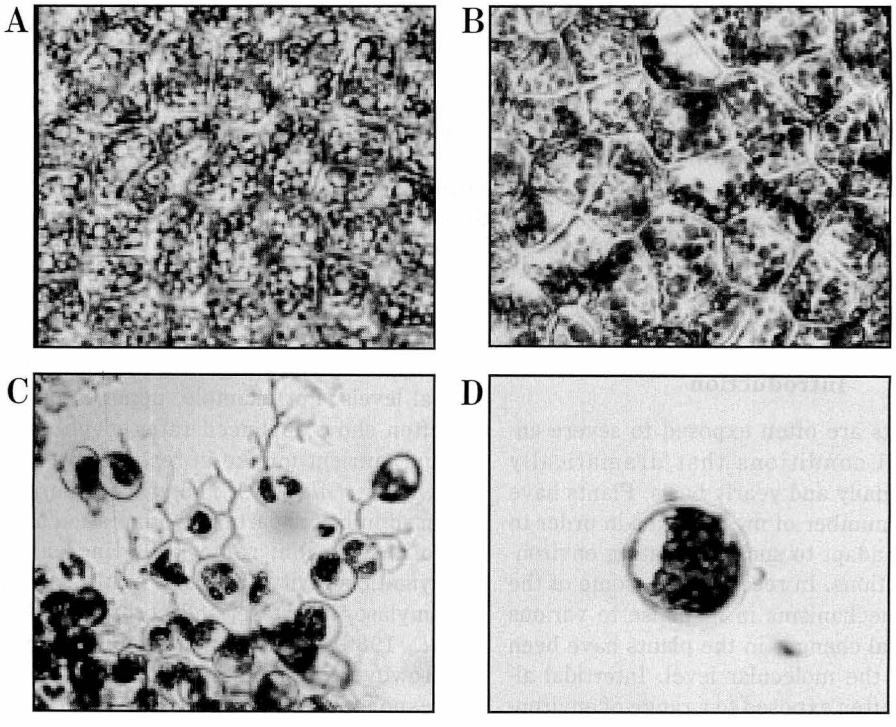

Fig. 1. Morphological changes of sterile *Ulva pertusa* grown under different thermal conditions. Thallus tissue cultivated at 20℃. (A) and 30℃. (B) for 7 days, respectively. When the alga is cultivated at 33℃ for additional 7 days, the thallus cuticle layer breaks down. (C) with the release of marginal cells from thallus periphery. The released single cell (D) regenerates to a thallus by cultivation at 20℃.

(Amano *et al.*, 1997). A further 7 days of growth at 33℃ results in the breakdown of the cuticle layer with the release of marginal cells from its periphery (Fig. 1C and D). When the released cells are inoculated to fresh medium and cultivated at 20℃, the cells regenerate into normal thalli. These morphological changes, resulting from a change in culture temperature (20 to 30 ℃ and 30 to 33℃), may reflect one of the thermal stress responses of the alga. However, the large amount of single cells released at 33℃ are of little use in biochemical and molecular biological studies on thermal stress responses, since about 50% of the cells are dead (Amano *et al.*, 1997). Therefore, we investigated algal thermal stress responses using the thalli maintained either at 20℃ (controls) and 30℃ (experimental growth conditions).

Changes in chemical components due to thermal stress

Total pigment contents for the 20℃- and 30 ℃-cultivated algae were approximately 580 and 990 mg per 100 g dry weight, respectively (Table 1) (Kakinuma *et al.*, 2001a). Individual pigment contents in the 30℃-cultivated alga were about 1.4-2.4 times higher than those in the 20 ℃-cultivated alga. The darkening of the chloroplasts at high temperature is consistent with the high photosynthetic pigment contents and accounts for the higher photosynthetic activity (Murase *et al.*, 1993). In addition, total carbon and nitrogen contents of the 30℃-cultivated alga were significantly higher than the levels of the 20℃-cultivated (Table 2) (Kakinuma *et al.*, 2001a). The increase of the total carbon content may correspond to the thickened cell walls of the stressed cells.

Total free amino acid content also significantly increased in the 30℃- cultivated alga. Amounts of major components in the 20℃- and 30℃-cultivated algae, which accounted for approximately 80-90% of the total free amino acids, are shown in Table 3 (Kakinuma *et al.*, 2001a). The amounts of asparagine, aspartic acid, glutamine, glutamic acid, glycine, and serine in the 30℃-cultivated alga were 1.9-10.5 times higher than levels in the 20℃-cultivated alga. A remarkable increase was found in the asparagine content. In higher plants, excess

Table 1. Photosynthetic pigment contents for 20℃- and 30℃-cultivated sterile *Ulva pertusa* (Kakinuma *et al.*, 2001)

	Photosynthetic pigment content (mg/100 g dry wt)[1]	
	20℃	30℃
β-Carotene*	19.7 ± 1.09	46.5 ± 10.30
Chlorophyll a***	427.3 ± 31.86	677.5 ± 9.16
Chlorophyll b***	91.4 ± 7.13	208.3 ± 12.08
Lutein**	27.8 ± 1.01	37.6 ± 2.90
Neoxanthin**	6.9 ± 0.74	9.5 ± 0.42
Violaxanthin**	8.5 ± 0.36	14.0 ± 1.72
Total***	581.5 ± 24.24	993.3 ± 40.79

[1]Data represent mean ± SD ($n=3$) with significant differences at $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

Table 2. Total carbon and nitrogen contents for 20℃- and 30℃-cultivated sterile *Ulva pertusa* (Kakinuma *et al.*, 2001)

	Content (% of dry wy)[1]	
	20℃	30℃
Carbon*	25.8 ± 0.17	29.1 ± 1.59
Nitrogen**	2.1 ± 0.07	2.6 ± 0.15

[1]Data represent mean ± SD ($n=3$) with significant differences at $^{*}P<0.05$ and $^{**}P<0.01$.

Table 3. Free amino acid contents for 20℃- and 30℃-cultivated *sterile Ulva pertusa* (Kakinuma *et al.*, 2001)

	Free amino acid content (mg/100 g dry wt)[1]	
	20 ℃	30 ℃
Asparagine**	96.6 ± 79.40	1010.3 ± 214.89
Aspartic acid**	20.7 ± 4.94	68.0 ± 14.26
Glutamine*	7.7 ± 1.93	31.9 ± 9.06
Glutamic acid*	101.4 ± 17.32	192.0 ± 31.74
Glycine*	7.0 ± 1.27	33.7 ± 15.22
Serine***	9.0 ± 1.58	21.4 ± 1.37
D-Cysteinolic acid	460.8 ± 67.86	312.3 ± 84.25
Other free amino acids[2]	134.1 ± 13.35	144.2 ± 21.41
Total***	837.4 ± 13.09	1813.7 ± 176.17

[1]Data represent mean ± SD ($n=3$) with significant differences at $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.
[2]Total amount of free amino acids exept for asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, and d-cysteinolic acid.

nitrogen from protein degradation is converted into asparagine and glutamine, and consequently these amino acids are pooled in tissue (Givan, 1979; Sieciechowicz *et al.*, 1988; Dembinski and Bany, 1991; Oask, 1994; Lam *et al.*, 1996). Thermal stress may have influenced protein and amino acid metabolism in the alga. On the other hand, glutamine and glutamic acid accumulated in the 30℃-cultivated alga play important roles in ammonia assimilation and in carbon and nitrogen metabolism in higher plants and algae (Cullimore and Smis, 1981; Haxen and Lewis, 1981; Srivastava and Singh, 1987; Oask, 1994; Lam *et al.*, 1996; Sakakibara *et al.*, 1995). Nevertheless, total nitrogen content in the 20℃- and 30℃-cultivated algae differed only slightly (Table 2), suggesting that the increases of these amino acids in the 30℃-cultivated alga reflect thermal stress responses involved in regulating some of the different pathways of nitrogen metabolism.

Glutamate dehydrogenase isozymes

Comparison of isozyme patterns for 20 metabolic enzymes from the 20℃- and 30℃-cultivated algae showed obviously different banding patterns in the case of glutamate dehydrogenase (GDH) (Fig. 2) (Kakinuma *et al.*, 2001a). Generally, GDH is classified into three types by coenzyme requirement: NAD^+-specific GDH (NAD-GDH, EC 1.4.1.2), NAD^+- and $NADP^+$-specific GDH (NAD/NADP-GDH, EC 1.4.1.3), and $NADP^+$-specific (NADP-GDH, EC 1.4.1.4). The result of the GDH isozyme assay indicates that NAD/NADP-GDH is constitutively expressed in algal tissue, whereas NADP-GDH is expressed in response to thermal stress. In higher plants, GDH plays an important role for regulation of carbon and nitrogen metabolism and oxidative deamination of glutamate to 2-oxoglutalate for the TCA cycle (Lam *et al.*, 1996; Robinson *et al.*, 1992; Moyano *et al.*, 1995). In contrast, macroalgal GDH may be essential for amination reactions and is thought to be a key enzyme in the primary assimilation of ammonia (Sato *et al.*, 1984; Inokuchi *et al.*, 1997, 1999). The NADP-GDH expressed in response to thermal stress seems to compensate for the drop in NAD/NADP-GDH activity to maintain ammonia assimilation and regulate nitrogen metabolism under thermal stress conditions.

The primary structure and expression of GDH

Different types of GDH were expressed in the algal tissue in response to changes of culture temperature. We isolated two types of cDNA clones encoding GDH from the sterile *U. pertusa* from a cDNA library constructed from 30℃-cultivated thalli (Kakinuma *et al.*, 2001b). The clone with the longest insert, designated pGDH-L, was 1610 bp and contained an open reading frame (ORF) encoding 447 amino acids (Fig. 3). Compared with pGDH-L, the other

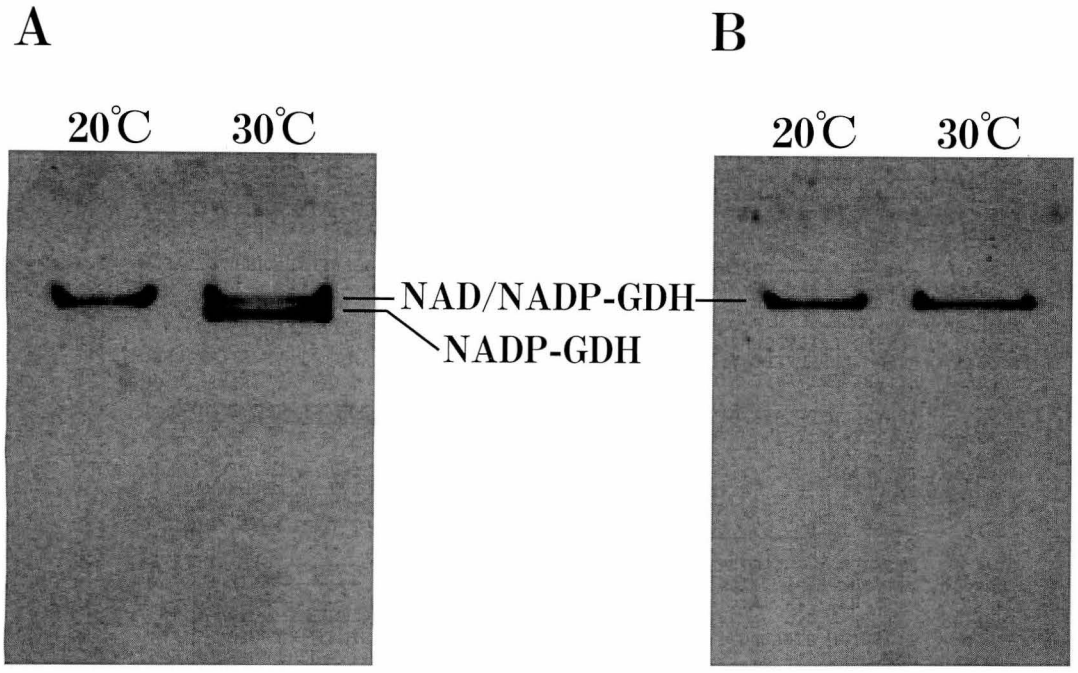

Fig. 2. Distribution of banding patterns of glutamate dehydrogenase (GDH) from sterile *Ulva pertusa*. The soluble protein fractions for the 20℃- and 30℃-cultivated algae were resolved on an acrylamide-gel and stained with two types of GDH staining solutions containing NADP+ (A) or NAD+ (B) as the coenzyme.

type of clone, designated pGDH-S, lacked 78 bp corresponding to the region from the 161st to 186th amino acid (Fig. 3). The alignment of amino acid sequences deduced from sterile *U. pertusa* GDH cDNAs revealed approximately 50% identity with those from higher plants (Sakakibara *et al.*, 1995; Melo-Oliveira *et al.*, 1996; Syntichakis *et al.*, 1996; Tsurano *et al.*, 1997). Two regions that were presumed to be 2-oxoglutarate-binding site (Fig. 3, Box I) containing Lys_{136} for catalytic function and pyridine nucleotide-binding site (Fig. 3, Box II) containing the nucleotide-binding motif GXGXXG(A) were highly conserved in GDH-L and GDH-S (Britton *et al.*, 1992; Lilly and Engel, 1992). The missing region of GDH-S is located near these binding sites, suggesting that the region influences the binding characteristics of GDH.

As shown in Fig. 4, expression analysis by RT-PCR indicated that mRNA encoding GDH-S was only expressed in thallus tissue cultivated at 30℃ for more than 24 h, whereas mRNA encoding GDH-L was constitutively expressed at both temperatures. Genomic DNA from sterile *U. pertusa* probed with the GDH-L cDNA gave several bands, suggesting that GDH is a multigene family in the alga (data not shown). Although it is not clear whether GDH-L and GDH-S transcribed from the same or different genes, transcription of these mRNAs seems to be regulated by thermal stress.

The GDH in higher plants and algae is a hexameric enzyme and composed of two types of subunits, suggesting that the two subunits make up all the possible hexameric combinations (Sakakibara *et al.*, 1995; Syntichakis *et al.*, 1996; Inokuchi *et al.*, 1999). mRNA levels for these subunits change under conditions of plant stress and during development and ripening (Loulakakis and Roubelakis-Angelakis, 1991; Loulakakis *et al.*, 1994; Melo-Oliveira *et al.*, 1996; Syntichakis *et al.*, 1996). Therefore two possibilities are suggested for the GDH isozymes found in sterile *U. pertusa*. First, the algal NAD/NADP-GDH and NADP-GDH may correspond to GDH-L and GDH-S, respectively. Second, GDH-L and GDH-S may encode subunits of the hexameric GDH, and the different biochemical functions of GDH regulated by changes in subunit composition. Expression and purification of GDH-L and GDH-S using a

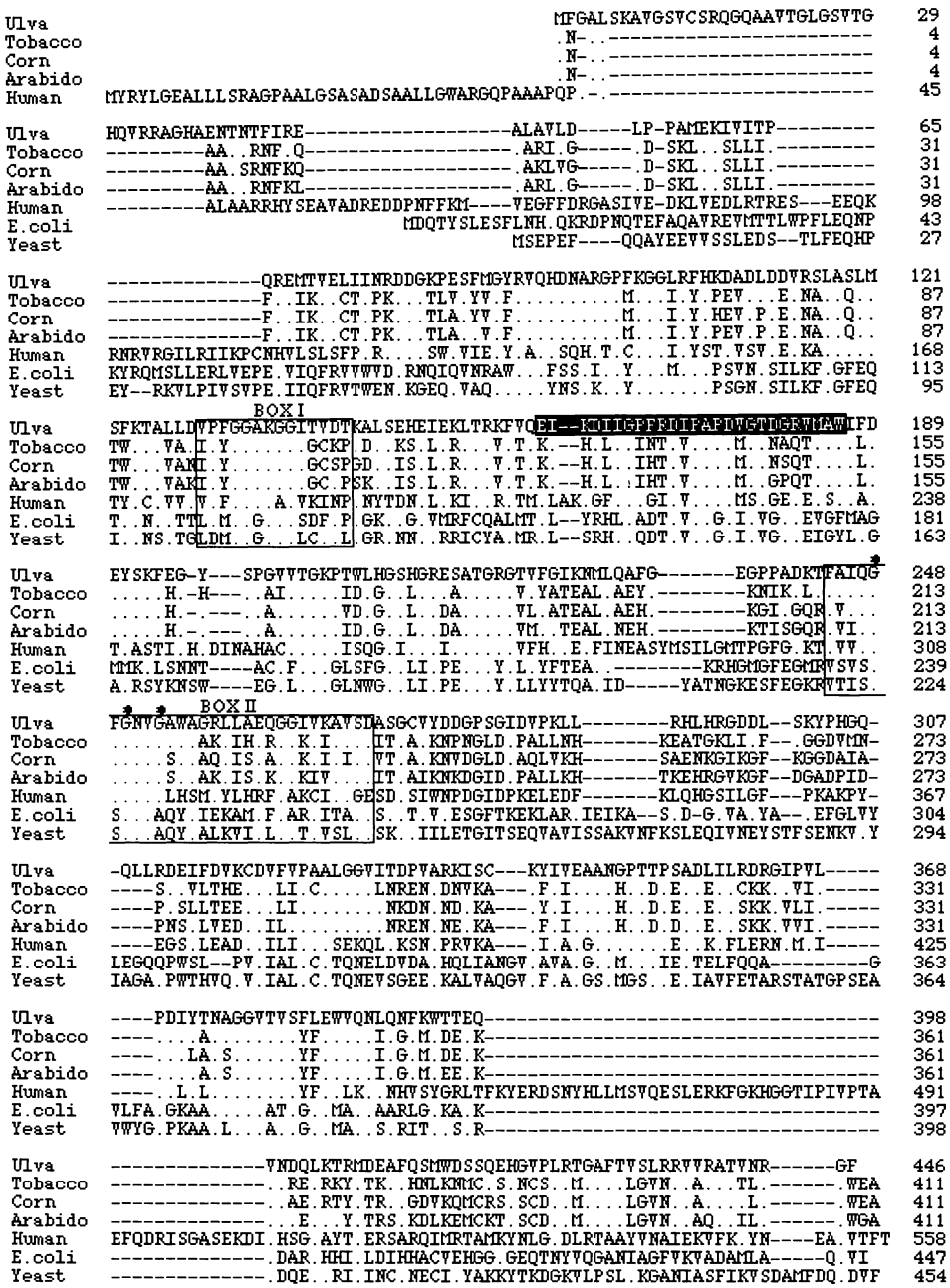

Fig. 3. The amino acid sequence of sterile *Ulva pertusa* glutamate dehydrogenase (GDH) in comparison with those of other species. The deduced amino acid sequence of GDH-L for sterile *U. pertusa* (*Ulva*) (Kakinuma *et al.*, 2001b) is shown on the upper line in single letter code. The amino acid sequences of *N. plumbaginifolia* GDH A (Tobacco) (Ficarelli *et al.*, 1999), *Zea mays* GDH1 (Corn) (Sakakibara *et al.*, 1995), *A. thaliana* GDH1 (Arabido) (Melo-Oliveira *et al.*, 1996), *H. sapiens* GDH (Human) (Amuro *et al.*, 1988), *E. coli* K-12 GDH A (*E. coli*) (Valle *et al.*, 1984), and *S. cerevisiae* GDH1 (Yeast) (Moye *et al.*, 1985) are shown below in optimal alignment. Shaded box indicates sequence gap between GDH-L and GDH-S. Boxed sequences (represented as Box I and Box II) are functionally important regions. The asterisks in Box II denote residues of a consensus sequence (GXGXXG) which forms a pyridine nucleotide-binding site.

bacterial expression system are currently being carried out to fully characterize enzymatic

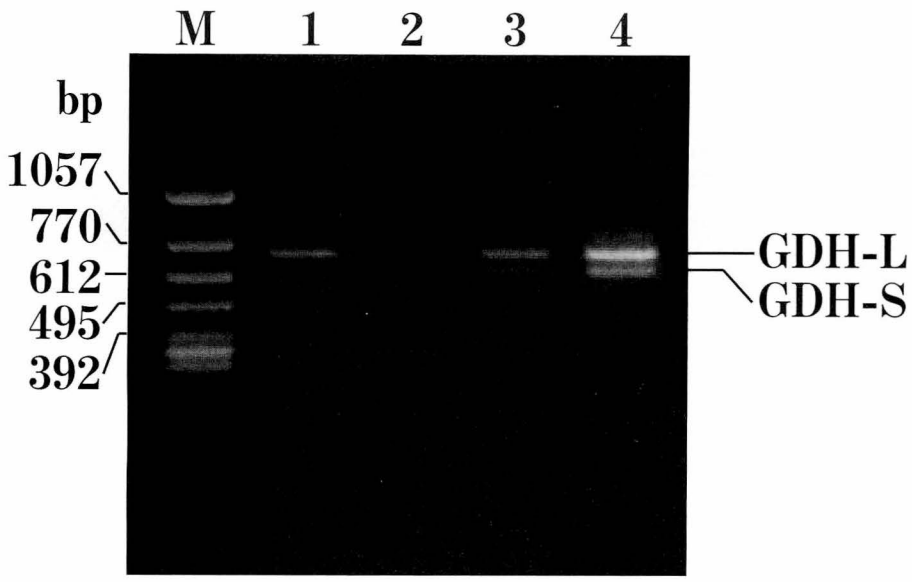

Fig. 4. Detection of mRNAs encoding GDH-L and GDH-S of sterile *Ulva pertusa* by RT-PCR. First-strand cDNA was synthesized from 5 μg of total RNA isolated from the alga subjected to 20℃ (lane 1) and 30℃ for 6 h (lane 2), 24 h (lane 3), and 1 week (lane 4). The PCR amplification was carried out using 0.5 μg of first-strand cDNA as template. Sense and antisense primers used for detection of GDH cDNA were designed to produce DNA fragments of approximately 700 and 620 bp for GDH-L and GDH-S, respectively. Lane M is the DNA size marker.

properties and functions of sterile *U. pertusa* GDH isozymes.

Other proteins related to thermal stress responses

Higher plants exposed to various environmental stresses accumulate a variety of small heat shock proteins (HSPs) ranging from 16 to 30 kDa (Boston *et al.*, 1996). Two-dimensional electrophoretic analysis of total protein for the 20℃- and 30℃-cultivated algae showed that some low molecular proteins (15-30 kDa) accumulated in response to thermal stress, suggesting that these proteins may be algal small HSPs. Other HSPs such as HSP60, 70, and 90, which are major components of molecular chaperonin system, are well characterized in various species. These HSPs are expressed during heat stress and are related to acquisition of thermotolerance in higher plants (Boston *et al.*, 1996). We isolated cDNA clones encoding sterile *U. pertusa* HSP60, 70, and 90 from the 30℃-cultivated algal cDNA library. These clones, designated pHSP60, pHSP70, and pHSP90, cover an entire ORF encoding 573, 663, and 705 amino acids, respectively. The deduced amino acid sequence of each HSP for the alga exhibited between 60 and 80% similarity with that of higher plant HSPs (Prasad and Stewart, 1992; Wu *et al.*, 1994; Milioni and Hatzopoulos, 1997; Li *et al.*, 1999). mRNA encoding HSP60, 70, and 90 were detected by RT-PCR using sense and antisense primers specific to each HSP. The mRNA levels of these sterile *U. pertusa* HSPs increased in the thallus tissue treated with high temperature (Fig. 5), suggesting that these HSPs were also involved in adaptation to thermal stress.

Recently, we used differential display reverse transcriptase-PCR (DDRT-PCR) to identify sterile *U. pertusa* genes whose expression are specifically induced and repressed by thermal stress. We have isolated 33 and 14 DDRT-PCR fragments from two experimental conditions, 20℃ → 35℃ and 20℃ → 5℃, respectively. BLAST analysis of these cDNA nucleotide sequences showed that 12 of them encoded proteins which were closely connected with carbon and nitrogen metabolism, and photosynthetic and molecular chaperone systems (Table 4). On the other hand, remaining 35 DDRT-PCR fragments were not homologous to genes deposited in DNA databases.

2. Salinity stress responses

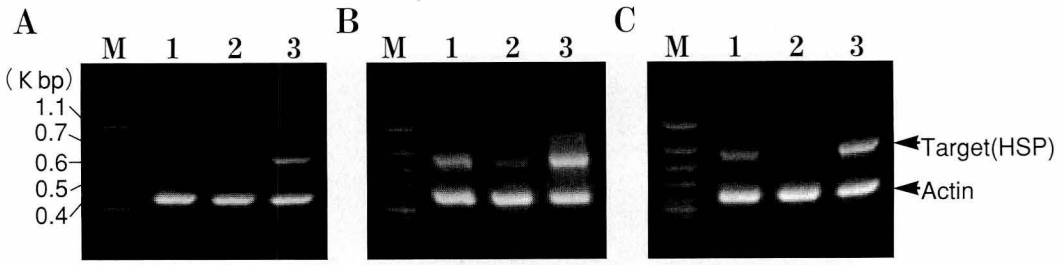

Fig. 5. Detection of mRNAs encoding heat shock protein (HSP) 60 (A), 70 (B), and 90 (C) of sterile *Ulva pertusa* by RT-PCR. First-strand cDNA was synthesized from 5 μg of total RNA isolated from the alga subjected to 20℃ (lane 1), 5℃ for 6 h (lane 2), or 35℃ for 6 h (lane 3). The PCR amplification was carried out using 0.5 μg of first-strand cDNA as the template and primers specific to each HSP cDNA. Relative expression changes of each HSP was estimated by using cDNA encoding actin as an internal standard. Lane M is the DNA size marker.

Changes in growth and chemical components due to salinity stress

Sterile *U. pertusa* can withstand extreme changes in salinity. The algal strain maintained in our laboratory grows in salinity ranging from 20 to 180%. After five days in culture, the thalli grown under normal salinity conditions (100% seawater) were approximately 31-fold larger in surface area than initial thalli, whereas the thalli treated with high and low salinity conditions (180 and 20% seawater, respectively) increased only about 7- and 8-fold, respectively. However, thalli cultivated in these different conditions have the same flattened appearance. Comparison of growth rates of thalli cultivated at normal, high, and low salinity conditions for 24 h showed that growth was significantly repressed by salinity stress.

No significant difference was found in total pigment, carbon, and nitrogen contents for the algae cultivated under normal, high, and low salinity conditions for 24 h. In contrast, free amino acid analysis showed obvious differences in proline content in the algae grown under different salinity conditions. The proline content of the algae cultivated at normal, high, and low salinity conditions were 33.1 ± 4.64, 475.2 ± 18.84, and 15.8 ± 7.32 mg per 100 g dry weight, respectively. The amount of proline in the alga cultivated under hypersaline condition was approximately 14-30 times higher than levels in alga treated with normal and low salinity conditions. It has been reported that the adjustment of osmotic potential can be achieved by changes in the concentrations of internal inorganic ions and organic osmolytes, such as proline, betaines, and mannitol, in higher plants and algae (Edwards *et al.*, 1987; Ahmad and Hellebust, 1988; Kirst, 1990; Kalinkina and Naumova, 1992; Delauney and Verma, 1993; Singh *et al.*, 1996; Liu *et al.*, 2000). In particular, proline, one of the nontoxic osmotica, functions as a protectant of macromolecules such as proteins and membranes, as a nitrogen-storage compound and energy source after the release of stress, and as a regulator of cellular redox status (Delauney and Verma, 1993; Hare and Cress, 1997; Hare *et al.*, 1999; Liu *et al.*, 2000). Therefore, we are interested in expression and regulation of genes related to proline biosynthesis, since the result of free amino acid analysis shows that the sterile *U. pertusa* may adapt to hypersaline condition by accumulating proline in tissues.

Other proteins related to salinity stress responses

We have employed DDRT-PCR for isolation and identification of genes which are specifically expressed in salinity-stressed algae and may associate with acquisition of osmotolerance. Nine and twenty-eight DDRT-PCR fragments were isolated from two experimental conditions, normal → high salinity and normal → low salinity, respectively. cDNA nucleotide and deduced amino acid sequences of nine of the DDRT-PCR fragments are homologous to variety of plant proteins as listed in Table 5. These membrane-, transporter-, and transcription-associated proteins may play important roles for adaptation to salinity-stress conditions in sterile *U. pertusa*. In contrast, the genes and associated proteins corresponding to the other 28 DDRT-PCR fragments have not been identified.

Table 4. DDRT-PCR fragments isolated from sterile *U. pertusa* treated with high and low temperatures.

cDNA fragment (bp)[1]	Expression pattern[2]	Homologous protein (species)	similarit (%)[3]
DDF-4-4 (763)	induced by 5℃*	Ferredoxin (*Dunaliella salina*)	61
DDF-6-3 (224)	induced by 5℃	Elongation factor-Tu (*Mesostiguma viride*)	80
DDF-11-4 (807)	induced by 35℃	Frabin-binding monooxygenase (*Arabidopsis thaliana*)	33
DDF-13-5 (577)	induced by 35℃*	Methylenetetrahydrofolate dehydrogenase (*Mesorhizobium loti*)	43
DDF-17-2 (811)	induced by 35℃	Peptidylprolyl cis-trans isomerase (*Eschelichia coli*)	38
DDF-22-1 (393)	induced by 35℃*	Oxygen-evolving enhancer protein 2 (*Chlamydomonas reinhardtii*)	75
DDF-22-5 (1019)	induced by 35℃*	Heat shock protein 70 (*Pisum sativum*)	70
DDF-24-8 (751)	induced by 5℃	TCP-1 chaperonin-like protein (*Arabidopsis thaliana*)	65
DDF-27-39 (441)	repressed by 5℃*	Sedoheptulose-1,7-bisphosphatase (*Chlamydomonas reinhardtii*)	72
DDF-31-57 (484)	induced by 35℃*	RIESKE iron-sulfer protein (*Solanum tuberosum*)	69
DDF-33-60 (341)	induced by 35℃*	20S proteasome β subunit (*Oryza sativa*)	40
DDF-36-9 (588)	induced by 35℃	Ca^{2+}-ATPase (*Arabidopsis thaliana*)	55

[1]Length of fragment including arbitrary and anchor primers.
[2]Expression patterns estimated from fingerprint patterns of DDRT-PCR products.
Asterisks (*) indicate that the differential expression was confirmed by Northern blot analysis.
[3]Values represent amino acid sequence similarity.

Conclusions and problems for future studies

Sterile *U. pertusa* is one of the most useful model species to elucidate the mechanism of adaptation to environmental stress conditions in the intertidal algae, since the sterile mutant develops vegetatively and can be maintained easily in laboratory conditions. In this study, we showed that morphological changes in the sterile *U. pertusa* grown under thermal and salinity stress conditions correlated with changes in chemical components. In addition, we showed that the alga expressed GDH isozymes and various HSPs in response to thermal stress. GDH and HSPs are thought to play important roles in carbon/nitorogen metabolism and in the molecular chaperone system, respectively. We are currently carrying out experiments to fully characterize enzymatic properties and functions of these proteins in stressed tissues.

On the other hand, we have attempted to isolate and identify other proteins related to thermal and salinity stress responses by DDRT-PCR. Some DDRT-PCR fragments corresponding to mRNAs whose expressions were altered by changes in temperature and salinity conditions have been isolated. BLAST analysis of nucleotide and amino acid sequences showed possibilities that some of the DDRT-PCR fragments were homologous to genes encoding proteins closely connected with carbon/nitrogen metabolism, photosynthesis, the molecular chaperone system, gene transcription, and

Table 5. DDRT-PCR fragments isolated from sterile *U. pertusa* treated with high and low salinities.

cDNA fragment (bp)[1]	Expression pattern[2]	Homologous protein (species)	similarit (%)[3]
DDFS-1-1 (379)	induced by low salinity	20S proteasome β subunit (*Spinacia oleracea*)	80
DDFS -3-1 (534)	induced by low salinity	Membrane-associated prostaglandin E synthase 2 (*Macaca fascicularis*)	36
DDFS -3-2 (523)	induced by high salinity	Translation initiation factor eIF-2, γ subunit (*Arabidopsis thaliana*)	71
DDFS -3-3 (1120)	induced by high salinity	Translational endoplasmic reticulum ATPase (*Arabidopsis thaliana*)	76
DDFS -59-1 (828)	induced by low salinity	Syntaxin-related protein Nt-syr1 (*Nicotiana tabacum*)	32
DDFS -59-2 (911)	induced by low salinity	CHP-rich zinc finger protein (*Aridopsis thaliana*)	27
DDFS -61-1 (459)	induced by high salinity	Geranyl-geranyl hydrogenase (*Glycine max*)	79
DDFS -61-2 (448)	induced by high salinity	GTP-binding protein type A (*Arabidopsis thaliana*)	57
DDFS -71-1 (632)	induced by low salinity	Plastidic ATP/ADP transporter (*Solanum tuberosum*)	58

[1]Length of fragment including arbitrary and anchor primers.
[2]Expression patterns estimated from fingerprint patterns of DDRT-PCR products.
[3]Values represent amino acid sequence similarity.

membrane functions. However, it has been impossible to estimate functions of genes and/or proteins corresponded to the other cDNAs isolated by DDRT-PCR, since these isolates consist of a 3'-terminus encoding a partial ORF and untranslated regions. In addition, expression sequence tag (EST) database resources for macroalgae are not advanced enough compared with that of higher plants. In order to elucidate the mechanism of adaptation to both stress conditions in the alga, we are planning to isolate and identify mRNAs corresponding to these DDRT-PCR fragments from a sterile *U. pertusa* cDNA library.

In land plants, various methods for stable nuclear transformation have been established, which resulted in production of transgenic plants (Harms and Potrykus, 1978; Hayashimoto, *et al.*, 1990; Hiei *et al.*, 1994; Shimada *et al.*, 1995). Transgenic plants are an effective system for functional analysis of unidentified genes and for production of stress-tolerant mutants. The same systems are necessary for transgenic algae and for analyses of the properties of translated products encoding unknown genes isolated from sterile *U. pertusa*. In recent years, studies on development of algal transgenic systems have been attempted, but no transformation vectors for marine macroalgae are available.

Construction and enrichment of algal genomic, protein, and EST databases, and the establishment of algal transgenic systems are essential to further development of studies on molecular responses to various environmental stresses in sterile *U. pertusa*.

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緑藻不稔性アオサにおける ストレス応答と分子情報

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緑藻不稔性アオサは，自然条件下，湾内や河口域で浮遊あるいは付着生育している。このような環境条件下で生育する本藻種は，幅広い温度，海水濃度，栄養塩濃度などに適応することが知られている。さらに，本藻種は室内培養が容易なため，種々の環境ストレスに対する海藻の応答・適応機構を調べる格好のモデル生物と考えられる。我々はこのような特徴を有する不稔性アオサを対象とし，生化学および分子生物学的手法を利用して，温度および塩ストレス応答・適応機構の解明に取り組んでいる。

ここでは，以下に挙げた不稔性アオサの温度および塩ストレスに対する応答反応について紹介すると共に，海藻の分子生物学研究の現状と今後の課題について述べる。20℃培養藻体と比較して，30℃培養藻体の光合成色素および特定の遊離アミノ酸含量は，それぞれ1.4～2.4倍および1.9～10.5倍に増加していた。代謝系酵素のアイソザイム分析により，20℃および30℃培養藻体にはNAD / NADP依存性グルタミン酸脱水素酵素（GDH）が存在するのに対し，高温培養藻体では加えてNADP依存性GDHが存在することが明らかとなった。cDNAクローニングにより，GDHをコードする2種類のクローン（pGDH-LおよびpGDH-S）が単離された。発現解析により，GDH-Lは藻体内で恒常的に発現しているのに対し，GDH-Sは30℃で24時間以上培養した藻体でのみ発現することが明らかとなった。Differential display reverse transcriptase-PCR（DDRT-PCR）を利用した遺伝子発現解析により，温度ストレスにより少なくとも47種類のmRNAで発現変化が認められた。そのうち12種類は，炭素・窒素代謝系，光合成系，分子シャペロン系に関連するタンパク質をコードしていた。塩ストレス処理により，藻体内のプロリン含量が顕著に変化した。不稔性アオサは細胞内プロリン含量を調節し，塩ストレス環境下に適応していることが示唆された。DDRT-PCRにより，塩ストレスにより少なくとも37種類のmRNAで発現変化が認められた。そのうち9種類は膜タンパク質，輸送タンパク質，転写調節因子をコードしていた。