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Cold stress regulation of a bi-functional 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH)-like gene in the freshwater green alga *Spirogyra varians*

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Abstract

A low temperature up-regulated gene was isolated from *Spirogyra varians* using the differentially expressed gene method. The full cDNA sequence consisted of 1798 bp with an open reading frame of 1560 bp encoding 520 amino acid residues, which had neither a cleavage site nor a signal peptide. The deduced amino acid sequence was highly similar to the bi-functional 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH) gene of higher plants. As the gene was cold regulated, we named it SVCR3 (*Spirogyra varians* cold regulated gene). SVCR3 had two conserved domains of DHQase I and SDH (AroE), as well as a shikimate-binding site as found in other DHQ/SDH genes. Northern blot analysis showed that SVCR3 was up-regulated at 4°C. When combined with light (>50 μmol photon m^{-2} s^{-1}) the expression of SVCR3 increased more at 4°C, but the expression was not affected by light intensity alone up to 200 μmol photon m^{-2} s^{-1} at temperatures higher than 10°C. Using the zymogram method (which is a visualization method for gel areas containing specific enzymes based on chromogenic reactions), we showed that there were three isozymes of DHQ/SDH in *Spirogyra varians*. This is the first report of DHQ/SDH-like gene regulation by cold stress.

Keywords: bi-functional 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH) gene; cold stress; green algae; Shikimate pathway; *Spirogyra varians*.

Introduction

In plants, cold adaptation is characterized by various morphological and physiological transitions. Some plants produce antifreeze proteins (Guy et al. 1985) or change the composition of lipids and carbohydrates in the cell (Guy 1990), but most plants regulate expression of certain gene groups to adjust to cold stress (Thomashow 1990). There are many reports on cold stress regulated genes of higher plants, such as wheat, barley, and *Arabidopsis* spp. (Graham and Patterson 1982, Cattivelli and Bartels 1990, Hajela et al. 1990, Gilmour et al. 1992, Danyluk et al. 1994, Houde et al. 1995, Pearce et al. 1998). As freshwater algae are directly exposed to freezing waters during wintertime, cold stress can damage their cells much more seriously than higher plants in air. In order to maintain their functions under cold stress, freshwater algae may have developed unique cold acclimation mechanisms (Han et al. 2007), but little is known about cold stress genes in algae.

The shikimate pathway (aromatic biosynthesis) plays a pivotal role in the production of precursors for aromatic compounds in microorganisms and plants, but is absent in humans and other higher animals (Herrmann 1995a,b, Ding et al. 2007). It has been estimated that, under normal growth conditions, 20% of the carbon fixed by plants may be directed towards the shikimate pathway (Haslam 1993). The shikimate pathway consists of seven metabolic steps beginning with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate to form 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), and ending with the synthesis of chorismate from 5-enolpyruvylshikimate 3-phosphate (EpSP). The organization of the seven enzymes is markedly different between three kingdoms. The pathway provides the precursors of phenylalanine, tyrosine, and tryptophan, but also precursors for a very diverse range of other aromatic compounds derived, such as vitamins, flavonoids, and alkaloids from chorismate, the end product of the shikimate pathway (Weaver and Herrmann 1997).

In plants, the bi-functional enzyme 3-dehydroquinate dehydratase (DHQ, EC 4.2.1.10)/ shikimate dehydrogenase (SDH, EC 1.1.1.25) (DHQ/SDH) catalyzes the dehydration of dehydroquinate to dehydroshikimate and the reversible reduction of dehydroshikimate to shikimate, which are third and fourth steps of the shikimate pathway (Herrmann and Weaver 1999, Ding et al. 2007). Its enzymatic activities have been reported from several plant species, and the respective proteins from pea and tobacco have been purified to homogeneity (Mousdale et al. 1987, Bonner and Jensen 1994, Deka et al. 1994). The molecular characterization of DHQ/SDH was reported for the first time in tomato (Bischoff et al. 2001).

In some plants, the shikimate pathway works to respond to environmental stresses, such as light, pathogens, and wounding (Weaver and Herrmann 1997). The increment of DHQ/SDH activities is caused by mechanical wounding, microbial attacks, and copper stress in

pepper (Díaz and Merino 1998, Díaz et al. 2001). Stress-induced expression of the genes encoding enzymes of the prechorismate pathway has so far been mainly analyzed in the context of plant-pathogen interactions. A comprehensive analysis of the elicitor inducibility of these genes has been carried out with cultured tomato cells (Görlach et al. 1995, Bischoff et al. 1996), but there is no report on the induction of these genes by cold stress.

Spirogyra varians (Hass.) Kuetzing is a cosmopolitan freshwater alga, which grows in a wide range of temperatures. This species is often found growing under ice during wintertime in Korea (Kim et al. 2005, Han et al. 2007). In our studies on cold stress-related genes in *Spirogyra varians*, we isolated a DHQ/SDH-like gene using a differential expression gene (DEG) method. We analyzed the full cDNA sequence of the gene and considered its cold stress regulation in relation to high light and oxidative stresses.

Materials and methods

Plant material and growth conditions

Spirogyra varians was collected from several shallow ponds in Kongju, Korea (36°20′34″ N, 127°12′28″ E). Each collected sample was cleaned and kept in modified Bold's basal medium (Bischoff and Bold 1963, Kim et al. 2007) at 20°C, 50 μmol photon m^{-2} s^{-1} under 12:12 h light:dark cycle.

Stress conditions

The plants were transferred from 20°C chamber to three different temperature conditions (4°C, 10°C, and 35°C) and incubated for 24 h. To observe the effect of light stress together with temperature shock, three light conditions (0, 50 and 200 μmol photon m^{-2} s^{-1}) were supplied simultaneously and samples were collected over a time course (0 h, 3 h, 6 h, 12 h, and 24 h). Samples were oxidatively stressed using different concentrations of hydrogen peroxide (0.001%, 0.003%, 0.01%, 0.025%, 0.05%, and 0.1%) for 12 h. After the treatments, the expression level of SVCR3 was analyzed by Northern blot.

RNA extraction

Total RNA was isolated from plants grown under each of the above conditions using TRI reagent (MRC, Cincinnati, USA) based on the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). Isolated total RNA was cleaned with an RNeasy Plant Mini Kit (Qiagen, Valencia, USA) following the manufacturer's instructions. The RNA concentration was determined spectrophotometrically using a U-3300 (Hitachi Ltd, Tokyo, Japan) and its integrity was assessed by electrophoresis in 1.2% formaldehyde gels (Sambrook and Russell 2001).

Differentially expressed gene screening

GeneFishing kits (ACP41–50, Seegene, Seoul, Korea) were used for differential display following the manufacturer's instructions. A total of 3 μg of total RNA isolated from the plants grown under each treatment condition was used to synthesize first strand cDNA using 200 U of M-MLV reverse transcriptase, 2 μl dT-ACP1 (10 μM), 0.5 mM dNTP and 20 U of RNase inhibitor in a total volume of 20 μl. Reaction mixtures were incubated at 42°C for 90 min and then heat inactivated at 94°C for 2 min. Synthesized cDNA was diluted to 10 vol with ddH_2O. Gene fishing PCR was performed with ACP primers (2 μl of 50 μM arbitrary ACP and 1 μl of 10 μM dT-ACP2), 5 μl of diluted first strand cDNA and 10 μl of SeeAmp™ Master Mix (Seegene, Seoul, Korea), in a total volume of 20 μl. The PCR was performed as follows: 1 cycle for step 1 (5 min denaturation at 94°C, 3 min annealing at 50°C and extension at 72°C for 1 min), 40 cycles for step 2 (40 s at 94°C, 40 s at 65°C, and 1 min at 72°C) and 1 cycle of final step at 72°C for 5 min. The products were displayed by 2% agarose gel electrophoresis. Differentially expressed genes were purified using a gel extraction kit (Intron, Seoul, Korea). The isolated products were cloned into pGEM-T Easy cloning vector (Promega, Madison, WI, USA). The full length cDNA sequence of cold regulated gene was obtained using a PCR with λZAP cDNA library and 5′-RACE kit (Roche, Mannheim, Germany).

cDNA library construction

A mRNA was isolated using total RNA from cold treated *Spirogyra varians* by oligo dT cellulose (Oligotex™, Qiagen). The cDNA library was constructed using Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA). The synthesized double strand cDNAs (with *Eco*RI restriction site on the 5′-end and *Xho*I on the 3′-end) were cloned into *Eco*RI/*Xho*I predigested Uni-Zap XR vector to make the phagemid cDNA library. The estimated titer for the primary cDNA library was 2×10^6 pfu ml^{-1}.

5′-RACE

To obtain full-length cDNA, PCR primers were designed based on the partial cDNA sequence obtained from DEG (Table 1). A total of 1 ng μl^{-1} of the λZAP cDNA library was used as template. PCR was carried out in a 20-μl reaction mixture containing DNA template, 0.5 μM of specific primer (SVCR3-SR1) and 0.5 μM of universal primer (SK), 1× Taq buffer, 2.5 mM of $MgCl_2$, and 1 U Taq DNA polymerase (Takara, Tokyo, Japan). PCR was performed for 35 cycles at 95°C for 20 s, 55°C for 30 s and 72°C for 1.5 min, followed by 72°C for 10 min. To confirm the 5′-region, a 5′-RACE kit (Roche) was used following the manufacturer's instructions. The 3′-end of first strand cDNA was A-tailed using 80 U of terminal transferase and 0.2 mM dATP at 37°C for 20 min. The dA-tailed cDNA was amplified with oligo-dT anchor primer (0.75 μM) and SVCR3-SR2 (0.25 μM). Nested PCR was performed with 1 μl of PCR product, anchor primer (0.25 μM) and SVCR3-R3 (0.25 μM). The PCR products were displayed by 1.2% agarose gel electrophoresis and the band was cloned into pGEM T-easy vector (Promega) for sequencing.

Table 1 Primers used for PCR.

Primer name	Sequence (5′-3′)
SVCR3-F1	TGC CGA ACT TAC GGG GAA G
SVCR3-R1	ACG GCT TTG TGA AGT GCG G
SVCR3-R2	AGC GAA GGA TAT GGT GGG TGT C
SVCR3-R3	TCG ACA GCA TAG ATG GCA GCT CC
SK	CGC TCT AGA ACT AGT GGA TC
Oligo dT anchor primer (Roche)	GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV
PCR anchor primer (Roche)	GAC CAC GCG TAT CGA TGT CGA C

DNA sequence analysis

Nucleotide sequences were determined according to the protocol of a sequencing kit using the ABI 373S or 310 sequencer (Applied Biosystems, Forster, CA, USA). Nucleotide sequence analyses were performed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan). Sequence identity was confirmed by BLAST searches (http://www.ncbi.nlm.nih.gov/).

Northern blot analysis

The DNA probe was directly amplified and labeled with DIG-dUTP by PCR of SVCR3 gene from *Spirogyra varians* cDNA library using a DIG probe Synthesis Kit (Roche). PCR product was separated on 1.2% (w/v) agarose gel with a low DNA mass ladder (Intron Biotechnology, Seoul, Korea). Total RNA was extracted from plants exposed to various combinations of light and temperature. Equal amounts of total RNA (5 μg) from each treatment were loaded and separated on 1.2% (w/v) formaldehyde-agarose gels and photographed to confirm RNA quality and to verify equal sample loading. RNA was transferred onto Biodyne Nylon B membranes (Pall Life Science, East Hills, NY, USA) by capillary transfer with 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and immobilized to the membranes by UV cross-linking at 120 mJ for 1 min. The membrane was incubated with prehybridization solution (DIG Easy Hyb, Roche) for 2 h and then in hybridization solution (DIG Easy Hyb with 25 ng ml^{-1} DIG probe) overnight at 50°C. The blots were washed in 2× SSC, 0.1% sodium dodecyl sulfate (SDS) and then 0.5× SSC, 0.1% SDS. The mRNA was immunologically detected by anti-digoxigenin-AP. The blots were then exposed to X-ray films (CP-BU, Agfa, Mortsel, Belgium) for 2 h.

Enzyme extraction and determination of protein concentration

All steps were performed at 4°C. Samples grown at 20°C and 4°C were separately homogenized in the extraction buffer (Tris-HCl, pH 7.5, containing 1 mM dithiothreitol [DTT], 2 mM ethylenediaminetetraacetic acid [EDTA], 1 mM phenylmethanesulphonylfluoride [PMSF]). The extract was centrifuged at 30,000×*g* for 30 min, and the supernatant (crude extract) was collected. This crude extract was dialyzed using dialysis tubing (MWCO 3500, Spectra, Hills, CA, USA) with extraction buffer. Samples were stored at -70°C. The protein concentration was determined using the Bradford method (Bradford 1976) using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as a standard.

Detection of SDH on electrophoretic gels

Extracted total proteins (25 μg) from plants grown in two different temperatures were loaded on 8% polyacrylamide gels (0.375 M of Tris-HCl, pH 8.8) to perform native PAGEs as previously described (Díaz and Merino 1998). The electrode buffer consisted of 0.2 M Tris, 2 mM EDTA and 0.15 mM boric acid, pH 8.5. Gels were stained for SDH activity using a solution containing 25 mg shikimic acid, 5 mg NADP, 6 mg MTT (tetrazolium salt) and 1 mg phenazine methosulphate (PMS) in 30 ml of 0.1 M Tris-HCl pH 7.6 for 30 min in the dark (Manchenko 1994). Isozymes were numbered by increasing mobility toward the anode. Relative amounts of DHQ/SDH between 4°C and 20°C were measured using an image analysis program (Olysia ver 3.2, Olympus, Tokyo, Japan).

Results

Spirogyra varians is a freshwater green alga commonly occurring in shallow waters from late January to April in Korea. The water repeats freezing and thawing during January to February and the temperature in the ponds rises to 22°C during the day in late April.

Total RNA (Figure 1A) from plants grown under two different conditions (4°C and 20°C) was extracted and subjected to ACP-based reverse transcriptase polymerase chain reaction (RT-PCR). Three cold-specific RT-PCR products were found using ACP1-10 primers (Figure 1B). We isolated a distinctively up-regulated DNA fragment (A6), approximately 1.5 kb in size, from cold-acclimated plants. The full length cDNA sequence was obtained using the strategy shown schematically in Figure 2. The cDNA sequence obtained and deduced amino acid sequence was submitted to the NCBI Genbank as accession no. EU769117, ACF05190. The gene consisted of 1798 bp with an open reading frame of 1560 bp and encoded 520 amino acid residues (Figure 3). The deduced amino acid sequence had neither a cleavage site nor a signal peptide. The calculated molecular weight from deduced amino acids was approximately 56 kDa and p*I* was 5.6. As it was a cold regulated gene, we named it SVCR3 (*Spirogyra varians* cold regulated gene).

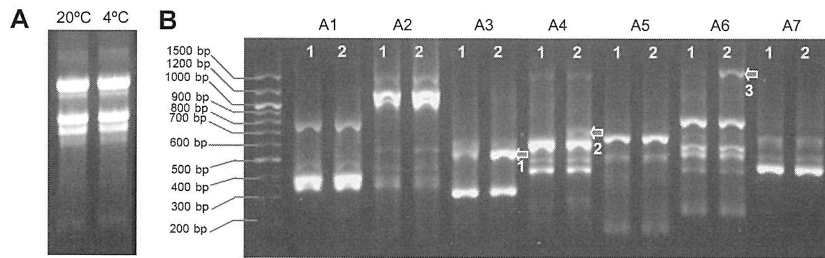

Figure 1 *Spirogyra varians*: RT-PCR display using the DEG method.
(A) Total RNA from control (20°C) and cold (4°C) treated filaments. (B) Amplified RT-PCR products from warm (lane 1, 20°C) and cold (lane 2, 4°C) treated filaments using ACP1-10 primers. Arrows show differentially expressed genes. A6, number 3 indicates SVCR3.

Figure 2 *Spirogyra varians*: schematic of PCR procedure.
Arrows indicate primers and bars indicate amplified regions.

The homology search using the deduced amino acid sequence on NCBI Blast databank showed that SVCR3 was an algal homolog of Type I 3-dehydroquinase/shikimate 5-dehydrogenase (DHQ/SDH or AroDE in abbreviation), which is a bi-functional enzyme involved in the third and fourth steps of the shikimate pathway in higher plants. SVCR3 contained two conserved domain regions like other bi-functional genes, and the catalytic site was conserved as in other DHQ/SDH enzymes (Figure 3).

Comparison of the deduced amino acid sequence with other known DHQ/SDH genes showed that SVCR3 had significant identity values to those genes of higher plants (40.4% to *Juglans regia* and 38.5% to *Arabidopsis thaliana*). The genomic database of *Chlamydomonas reinhardtii* and *Ostreococcus lucimarinus* also contained a gene similar to DHQ/SDH, but the identity value (33.0% and 36.4%, respectively) to SVCR3 was lower than that of higher plants (Table 2).

Northern blot analysis showed that expression of the SVCR3 gene increased at low temperature (4°C) and high light condition, confirming results from the DEG method. When the light intensity was maintained at 50 μmol photon m^{-2} s^{-1}, the expression of SVCR3 was observed at 4°C (Figure 4). Very little SVCR3 was expressed at 20°C regardless of the light intensity, but was expressed at 4°C even in the dark (Figure 5). SVCR3 accumulated more

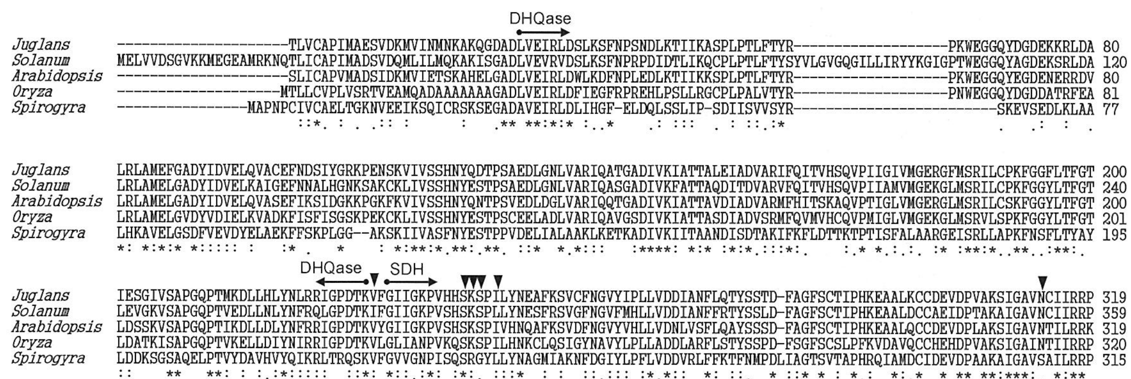

Figure 3 *Spirogyra varians*: alignment of the deduced amino acid sequences of SVCR3 with other DHQ/SDH genes: *Arabidopsis thaliana* (AAW63134), *Juglans regia* (AAW65140), *Solanum lycopersicum* (AAC17991), and *Oryza sativa* (*japonica* cultivar-group, BAD61388).
Black arrowheads indicate shikimate binding site; box enclosed by dashed lines indicates NADPH binding site; [*] indicates that all amino acid residues are identical; [:] indicates that all amino acid residues are conserved; [.] indicates that amino acid residues are semi-conserved; DHQase and SDH domains are marked above the aligned sequence.

Table 2 Comparison of amino acid sequence of SVCR3 with DHQ/SDH sequence of other organisms.

Species	GenBank accession no.	Similarity (%)	Identity (%)
Arabidopsis thaliana (L.) Heynh	AAW63134	60.0	38.5
Chlamydomonas reinhardtii P.A. Dangeard	EDP02779	52.6	33.0
Juglans regia Linn.	AAW65140	60.7	40.4
Lycopersicon esculentum Mill.	AAC17991	56.4	37.3
Nicotiana tabacum L.	AAS90325	53.1	34.5
Oryza sativa L.	BAD61388	58.7	38.8
Ostreococcus lucimarinus	ABO98979	54.8	36.4

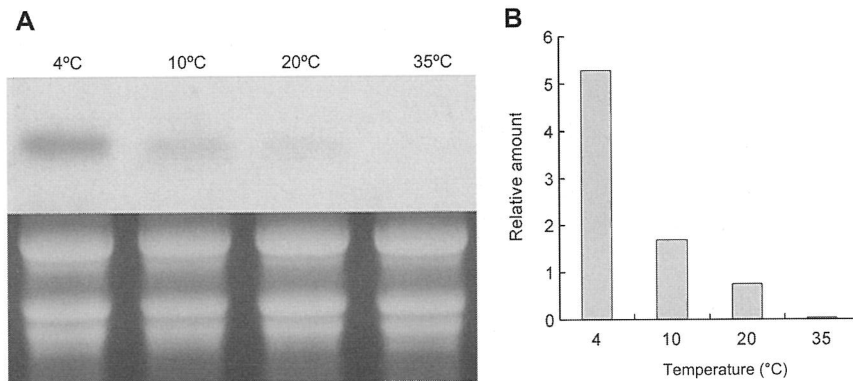

Figure 4 *Spirogyra varians*: Northern blot analysis of SVCR3 gene using total RNA in response to different temperatures.
(A) Lane 1, 4°C; lane 2, 10°C; lane 3, 20°C; lane 4, 35°C. Light intensity was maintained at 50 μmol photons m^{-2} s^{-1} for 12 h. Ethidium bromide stained rRNA bands are shown at the bottom of the Figure. (B) Relative amount of SVCR3 mRNA obtained using image analysis.

rapidly and strongly when high light (50, 200 μmol photon m^{-2} s^{-1}) was provided at low temperatures (Figure 5). SVCR3 was up-regulated in 3 h after transfer to low temperature and the expression level reached a maximum at 12 h (Figure 5). Oxidative stress was given to *Spirogyra varians* by incubation with different concentration of hydrogen peroxide. No up-regulation of SVCR3 was observed at any concentration of hydrogen peroxide (Figure 6).

The SDH display using native PAGEs and SDH staining method showed three isozymes of DHQ/SDH in *Spirogyra varians*. The relative amounts of the enzymes were

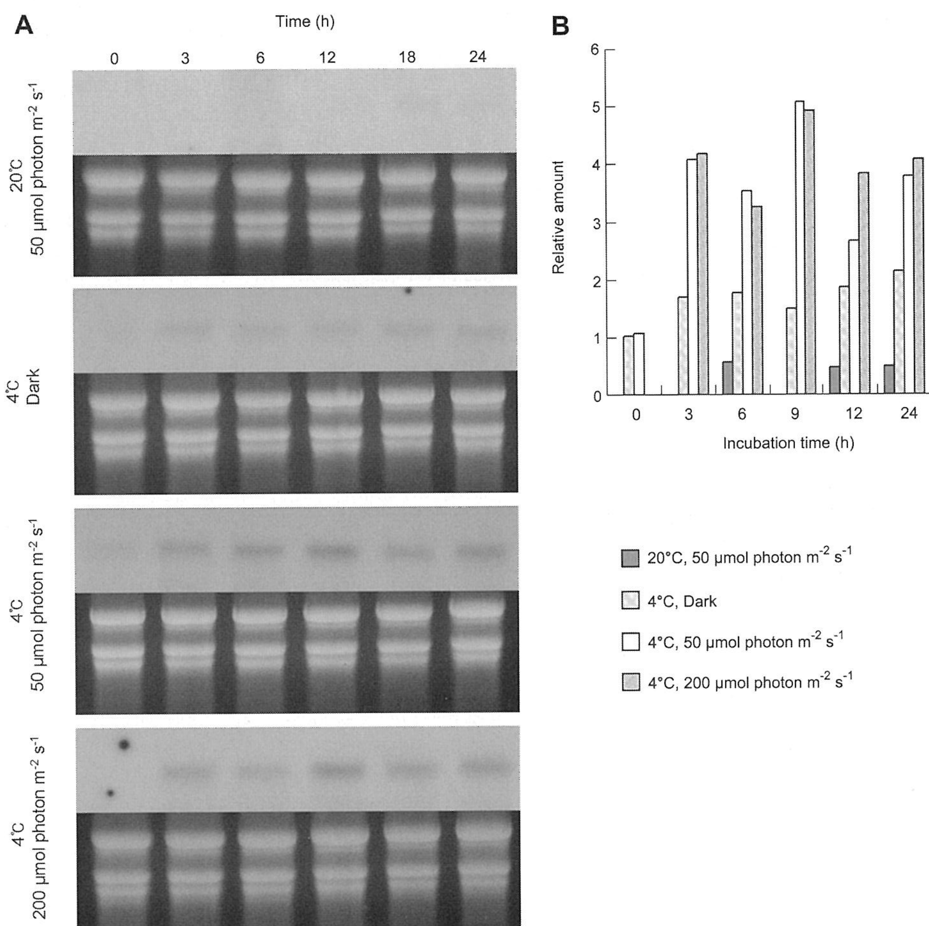

Figure 5 *Spirogyra varians*: Northern blot analysis of SVCR3 gene using total RNA in response to different light intensities and temperatures (20°C, 50 μmol photons m^{-2} s^{-1}; 4°C, dark; 4°C, 50 μmol photons m^{-2} s^{-1}; 4°C, 200 μmol photons m^{-2} s^{-1}) over time (0, 3, 6, 12, 18, 24 h).
(A) Ethidium bromide stained rRNA bands are shown at the bottom of each Figure. (B) Relative amount of SVCR3 mRNA obtained using image analysis.

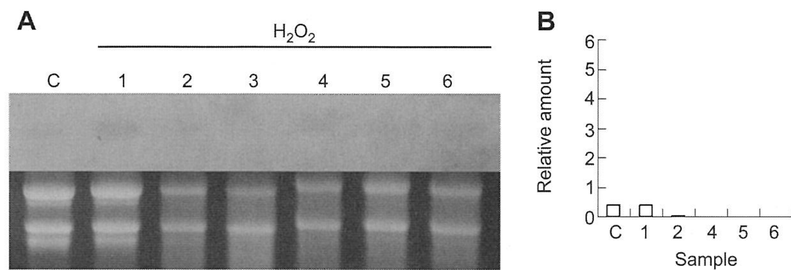

Figure 6 *Spirogyra varians*: Northern blot analysis of SVCR3 gene using total RNA in response to oxidative stress.
(A) Plants incubated in different concentrations of hydrogen peroxide for 1 h (20°C, 50 μmol photons m^{-2} s^{-1}). C, control; lanes 1–6, superoxide treated samples (lane 1, 0.001%; lane 2, 0.003%; lane 3, 0.01%; lane 4, 0.025%; lane 5, 0.05%; lane 6, 0.1%). Ethidium bromide stained rRNA bands are shown at the bottom of each Figure. (B) Relative amount of SVCR3 mRNA obtained using image analysis.

measured using image analysis. Isozymes 1 and 2 were up-regulated (38% and 24%, respectively) and isozyme 3 was down-regulated (28%) at 4°C (Figure 7).

Discussion

Plants must adapt to changes in their environment. The list of environmental conditions to which plants respond includes stimuli, such as light, pathogens, and wounding, but temperature always overlaps with these environmental factors and may change their influence. In plants, cold adaptation is characterized by various morphological and physiological transitions regulated by cold stress related genes (e.g., Graham and Patterson 1982, Cattivelli and Bartels 1990, Hajela et al. 1990, Gilmour et al. 1992, Danyluk et al. 1994, Houde et al. 1995, Pearce et al. 1998). Considering that there are many algae growing in very harsh environments, such as the polar regions, where no higher plant could survive, it is surprising that so little is known about the cold stress genes in algae. We chose *Spirogyra varians* as a model species to study the cold adaptation mechanism of algae, because it is a cosmopolitan freshwater alga that grows in a wide range of temperature regimes (Kim et al. 2005, Han et al. 2007). Previous studies on cold stress related genes have reported that cold acclimation processes are closely connected to light and other oxidative stresses in *Spirogyra varians* (Han et al. 2007). SVCR3 was strongly up-regulated when cold stress was given together with high light condition. However, the induction of SVCR3 was possible only when the plants were exposed to temperatures lower than 4°C. Although high light stress intensified the transcription of SVCR3 at 4°C, it did not up-regulate the gene when temperatures were above 10°C. Hydrogen peroxide treatment alone did not induce the expression of SVCR3, suggesting that this gene might not be directly related to oxidative stress in this species. These results showed that the expression of SVCR3 could be induced specifically by cold stress.

Plants are exposed to multiple environmental stressors which cause oxidative stress in the cell, and for long-term survival they have several responsive mechanisms. One of these appears to be an enhancement of phenolic biosynthesis, including the shikimate pathway (Dixon and

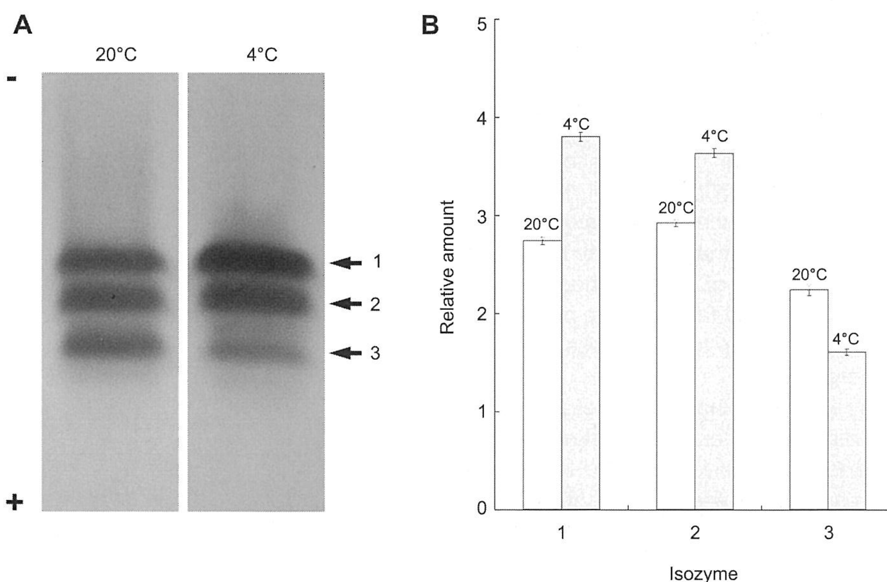

Figure 7 *Spirogyra varians*: SDH display and isozyme pattern using the zymogram method.
(A) Native PAGE gel showing three isozymes of SDH. (B) Relative activity of SDH obtained using image analysis. Values in bar diagram are means±SD. Numbers on the right hand side of (A) and at the bottom of (B) indicate the isozyme number of the enzyme.

Paiva 1995, Weaver and Herrmann 1997). The antioxidative response of the cell is mediated by two mechanisms. One uses antioxidative protein to scavenge the free radicals enzymatically. Another response is accumulating antioxidative low molecular weight chemicals which bind free radicals in the cell and reduce the damage. Most antioxidant chemicals (including polyphenols) known so far are aromatic compounds. The shikimate pathway is one of the main processes producing aromatic compounds in plant cells. It has been estimated that under normal growth conditions 20% of the carbon fixed by plants can be directed towards the shikimate pathway (Haslam 1993). Under cold stress, the enzymatic scavenging of free radicals may be less efficient, because most enzymatic activity drops significantly at temperatures close to 0°C. Therefore, enhanced expression of DHQ/SDH under low temperature might be necessary to produce aromatic antioxidant chemicals.

The shikimate pathway is also involved in the production of aromatic amino acids in microorganisms and plants (Weaver and Herrmann 1997, Herrmann and Weaver 1999, Knaggs 1999). The cytoplasmic shikimate pathway in plants uses these amino acids not only as protein building blocks but also as precursors for secondary metabolitic compounds used in UV light protectant, lignin biosynthesis (Bentley 1990, Schmid and Amrhein 1995, Cabané et al. 2004), etc. All higher plants have five isoforms of DHQ/SDH (Díaz and Merino 1998), but our results from the SDH display showed only three isoforms in *Spirogyra varians*. The isoforms of SDH in this species were differentially regulated under the same conditions; isozymes 1 and 2 were up-regulated but isozyme 3 was down-regulated at 4°C. This result implies that these isozymes may be present in different organelles of the cell. Further study is necessary to confirm the subcellular location of each isozyme.

The shikimate pathway is activated by nitrogen and amino acid starvation, UV irradiation, heavy metals, ozone, and high light (Henstrand et al. 1992, Umeda et al. 1994, Díaz et al. 2001, Cabané et al. 2004, Ahsan et al. 2007). An increase of DHQ/SDH activities caused by mechanical wounding, microbial attacks, and copper stress has been reported in higher plants (Díaz and Merino 1998, Díaz et al. 2001). A comprehensive analysis of the elicitor inducibility of these genes has been performed on cultured tomato cells (Görlach et al. 1995, Bischoff et al. 1996). Stress-induced expression of the genes involved in the prechorismate pathway has been analyzed mainly in the context of plant-pathogen interactions (Bischoff et al. 2001). So far, there are no reports on DHQ/SDH activities in relation to cold stress in higher plants or any other organisms.

Differential display (DD)-PCR has been developed to identify and isolate differentially expressed genes (Liang and Pardee 1992) and is extensively applied to various ranges of gene expression analyses because of its effectiveness and convenience. One of the advantages of this technique is that it requires only a small amount of RNA since it is a PCR-based process. However, there is a relatively high probability of obtaining false-positives, which is a major shortcoming of this technique. Many efforts have attempted to improve the specificity of DD-PCR (Liang et al. 1993, 1994). Recently, modification to eliminate false-positives has been developed by increasing the annealing specificity of primers with a specially designed annealing control primer (ACP) system known as the differential expressed gene (DEG) method (Hwang et al. 2003, Han et al. 2007). Our Northern blotting results confirmed that the DEG method is sensitive enough and useful in detecting environmentally regulated genes.

At this point, it is not easy to determine whether accumulation of SVCR3 is directly related to shikimate accumulation in *Spirogyra varians*. Ding et al. (2007) reported that DHQ/SDH-deficient plants may also accumulate shikimate. When expression of DHQ/SDH is suppressed by RNAi in transgenic tobacco plants, the end product, shikimate, unexpectedly increases, suggesting that the shikimate pathway is not operating in a straightforward manner. It is more likely that the organization of the enzymes involved in the shikimate pathway works differently among organisms. Further isozyme studies are necessary to compare the organization of the shikimate pathway between algae and higher plants.

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