

Identification and Isolation of Differentially Expressed Gene in Response to Cold Stress in a Green Alga, *Spirogyra varians* (Zygnematales)

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The expression of genes responding to cold stress in a freshwater alga, *Spirogyra varians*, was studied by using differential expression gene (DEG) method. A gene strongly up-regulated in 4°C was isolated and designated as SVCR2 (*Spirogyra varians* cold regulated) gene. The cDNA encoding SVCR2 was cloned using λ ZAP cDNA library of *Spirogyra varians*. The deduced amino acid had a sequence similarity with trans-membrane protein in *Arabidopsis thaliana* (Q9M2D2, 52.7%). Northern blot analysis demonstrated that transcript level of SVCR2 increased about 10 fold under low temperature (4°C), compared with that cultured at warm (20°C) conditions. The expression of SVCR2 was also affected by light conditions. When the plants were exposed to high light (HL) (1200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$), the expression of SVCR2 began within 2 hrs. This gene expression lasted for 4 hrs and decreased afterwards. Under the blue light (470 nm) condition, the expression of this gene was induced in same way as HL treatment, even under less than 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. But red light (650 nm) and UV-A irradiation did not affect the expression of SVCR2.

Key Words: cold stress, differentially expressed gene (DEG), *Spirogyra varians*

INTRODUCTION

In plants, cold adaptation is characterized by various morphological and physiological transitions. Plant survives cold stress using various methods. Some plants produce antifreeze protein (Guy *et al.* 1985) or change the composition of lipid and carbohydrate in the cell (Guy 1990) to increase the ability to endure cold stress; but most plants regulate expression of certain gene group to adjust to cold stress (Thomashow 1990).

There are many reports on the cold stress regulated genes of higher plants as wheat, barley and *Arabidopsis* spp. (Graham and Paterson 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), but little is known about the cold stress genes and cold acclimation mechanism in algae. Cold shock can damage a freshwater alga much more seriously than higher plants because most of over-wintering freshwater algal cells are directly exposed to freezing waters during winter time. In order to maintain their functions under cold stress, freshwater alga must have been developing some unique cold accli-

mation mechanisms.

Spirogyra varians grows in shallow ponds in Korea from late January to April. The water repeats freezing and thawing during this period. This species is often found growing under the ice. When blue light is given, plants move toward to the light source and this phototropic movement is affected by temperature (Kim *et al.* 2005).

Differential displayed (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 small amount of RNA since this technique is PCR-based. However, relatively high chance to have false-positive acts is a major handicap in this technique. Many efforts have been attempted to improve the specificity of DD-PCR (Liang *et al.* 1993, 1994). Recently modification to eliminate false-positive acts has been developed by increasing the annealing specificity with specially designed annealing control primers (ACP) system (Hwang *et al.* 2003; Kim *et al.* 2004). In this study, we analyzed the cold stress associated genes in *Spirogyra varians* by differential expression gene (DEG) technique using ACP system. A cold-

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Table 1. Primers for PCR

Primer name	Sequence (5'-3')	Base
SVCR2-F-1	AGC TCC ATT AAT ACC AGA GCA C	22
SVCR2-R-1	GCA ATC ACG CCT AAG GGA AGC AG	23
SVCR2-R-2	TAG TCA TGG ATG AGC GCA C	19
SK	CGC TCT AGA ACT AGT GGA TC	20
dT-ACP1	CTG TGA ATG CTG CGA CTA CGA TXX XXX (T) ₁₈	45
dT-ACP2	CTG TGA ATG CTG CGA CTA CGA TXX XXX (T) ₁₅	42

regulated gene was identified with this method. The expression of this gene was observed under combinations of various monochromatic lights and temperatures.

MATERIALS AND METHODS

Plant materials and growth conditions

Spirogyra varians was collected from several shallow ponds in Kongju (36°20'34" 127°12'28"). Collected sample was cleaned and kept in Bold's basal medium (Bischoff and Bold 1963) at 20°C, 50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ under 12/12 hrs light and dark cycle. For cold treatments, *Spirogyra varians* was transferred to 4°C growth chamber with the same light intensity and photoperiod. For high light treatment, *Spirogyra varians* was exposed to 1200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 2-12 hrs. Monochromatic light conditions (Red and Blue light, 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) were obtained using LED panels (Goodfeelings Co, Seoul, Korea) and UV-A lamp.

RNA extraction

Total RNA was isolated from plants grown under two (4°C and 20°C) temperature conditions and various light conditions using the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). Isolated total RNA was cleaned with RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The RNA concentration was determined spectrophotometrically using U-3300 (HITACHI Ltd, Tokyo, Japan) and its integrity was assessed by electrophoresis in 1.2% formaldehyde gels (Sambrook and Russell 2001).

Differential expressed gene screening

GeneFishing kits (ACP 51-60, Seegene, Seoul, Korea) were used for differential display following the manufacturer's instructions and displayed on 2% agarose gel electrophoresis. The PCR products were cloned into pGEM-T Easy cloning vector (Promega). The full length cDNA sequence of cold up-regulated gene was obtained

using a PCR with λ ZAP cDNA library.

cDNA library construction

Total RNA from cold treated *Spirogyra varians* was provided to OligotexTM (Qiagen) for Poly A⁺ RNA purification and cDNA library construction using Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA). The synthesized double strand cDNAs (with EcoRI restriction site on the 5'-end and XhoI on the 3'-end) were cloned into EcoRI/XhoI 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.

Obtaining full-length cDNA

To obtain full-length cDNA, PCR primers were designed based on the partial DNA sequence obtained from DEG (Table 1). 1 ng/ μL of the λ ZAP 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 (SVCR2-R-1) and 0.5 μM of universal primer (SK), 1X Taq buffer, 2.5 mM MgCl₂, and 1 unit Taq DNA polymerase (Takara, Tokyo, Japan). PCR was performed for 35 cycles at 95°C for 20 sec, 55°C for 30 sec (fragment I) and 72°C for 1min 30 sec, followed by 72°C for 10 min. The resultant PCR products were resolved on 1% agarose gel.

DNA sequence analysis

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

Northern blot analysis

Total RNA was extracted from plants exposed to various combinations of light and temperature conditions. Equal amounts of total RNA (3 μg) from each treatment were loaded and separated on 1.2% (w/v) formalde-

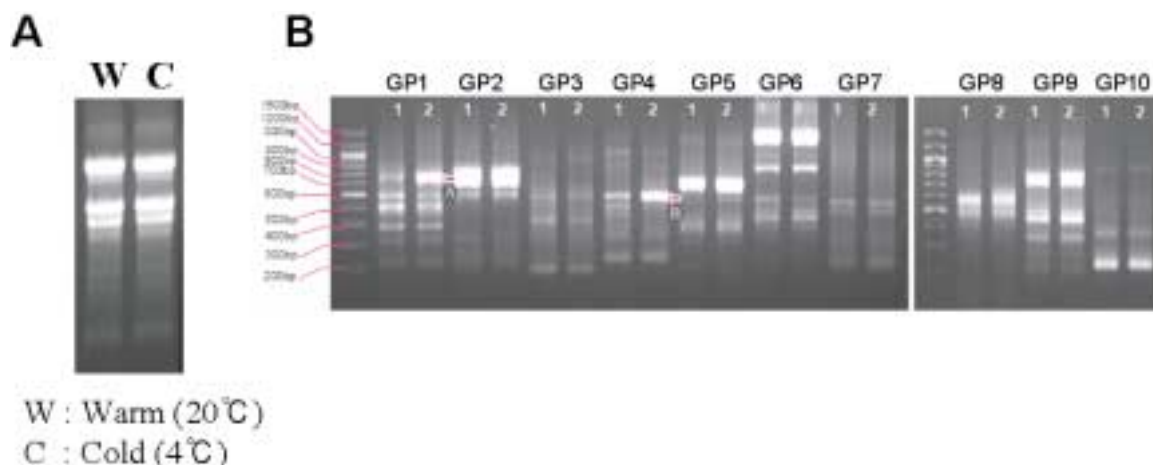


Fig. 1. RT-PCR display using DEG method. (A) Total RNA from control (20°C) and cold (4°C) treated *Spirogyra varians*. (B) Amplified RT-PCR products using ACP51-60 primers. Arrows show differentially expressed genes. GP4 Ⓡ indicates SVCR2.

hyde-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, USA) by capillary transfer and was immobilized to the membranes by UV cross-linking.

The DNA probe was directly amplified and labeled with DIG-dUTP by PCR of SVCR2 gene from *Spirogyra varians* first strand cDNA using DIG probe Synthesis Kit (Roche, USA). For reverse transcriptase (RT) reactions, 10 μ g of total RNA was used as template to synthesize cDNA. Total RNA was mixed with 300 ng of oligo-dT primer, incubated at 65°C for 5 min and cooled slowly at room temperature. The RT reaction was carried out in a 50 μ L volume with 2 unit of M-MLV reverse transcriptase (Stratagene, La Jolla, CA, USA) at 42°C for 1 hr, followed by heating at 95°C for 5 min to template reaction. RT reaction product was diluted to 50 μ L with autoclaved DDW, and 5 μ L was used as a template DNA for PCR. SVCR2-F-1 and SVCR2-R-1 primers were used for PCR. PCR product was separated on 1.2% (w/v) agarose gel with low DNA mass ladder (Intron Biotechnology, Seoul, Korea). The blots were then exposed to X-ray films (CP-BU, Agfa).

RESULTS

Spirogyra varians can survive in shallow ponds which repeat freezing and thawing during winter. In the culture, plants could grow at low temperature (4°C) and survived freezing followed by thawing. To identify differentially expressed genes at cold condition, we compared the mRNA expression profile by differentially expressed gene method (DEG). Total RNA from plants

Table 2. Amino acid composition of SVCR2

Amino acid	Number	Percent (%)
Ala	32	7.4
Arg	28	6.5
Asn	22	5.1
Asp	15	3.5
Cys	4	0.9
Gln	12	2.8
Glu	18	4.1
Gly	18	4.1
His	13	3.0
Ile	26	6.0
Leu	52	12.0
Lys	21	4.8
Met	15	3.5
Phe	21	4.8
Pro	23	5.3
Ser	46	10.6
Thr	20	4.6
Trp	7	1.6
Tyr	10	2.3
Val	31	7.1
Total	434	100

grown under two different conditions (4°C and 20°C) were extracted and subjected to ACP-based RT-PCR, using a combination of 10 arbitrary primers and anchored oligo dT primer of GeneFishing PCR kit (Seegene, Seoul, Korea). Two cold-specific RT-PCR products were found using ACP51-60 primers (Fig. 1). The 600 bp DNA fragment of GP4 was extracted from the gel and cloned into pGEM-T Easy vector. To obtain full-length cDNA, we amplified 5' end using internal specific primers (SVCR-R-1, SVCR-R-2) and SK universal primer

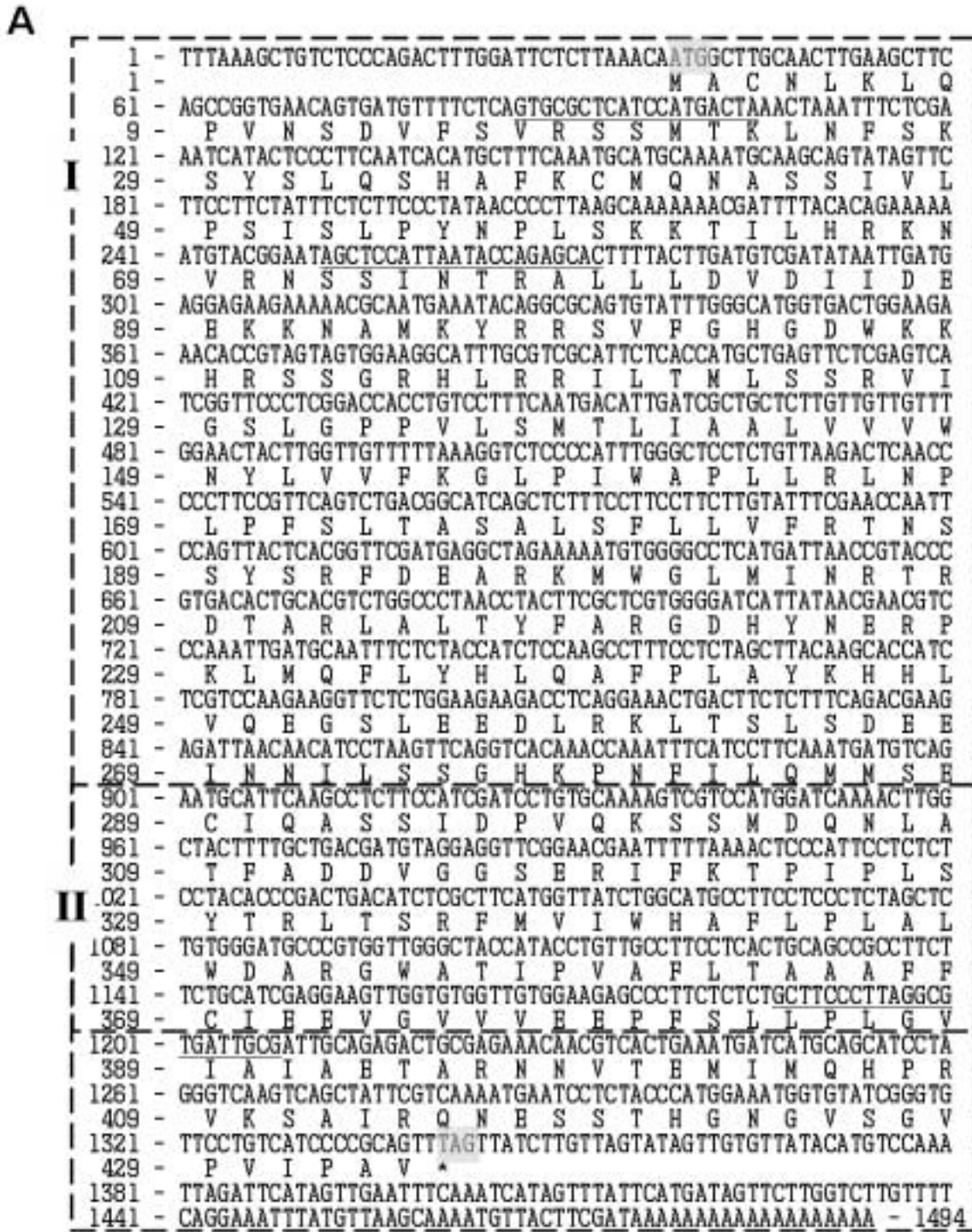


Fig. 2. (A) Nucleotide sequence of the cDNA encoding SVCR2 and deduced amino acid sequence. Box I indicates 5' RACE region. Box II indicate DEG amplified region. Underline shows specific primer site. Gray boxes indicate start and stop codon. (B) Scheme of PCR procedure. Arrows indicate primers and lines indicate amplified regions.

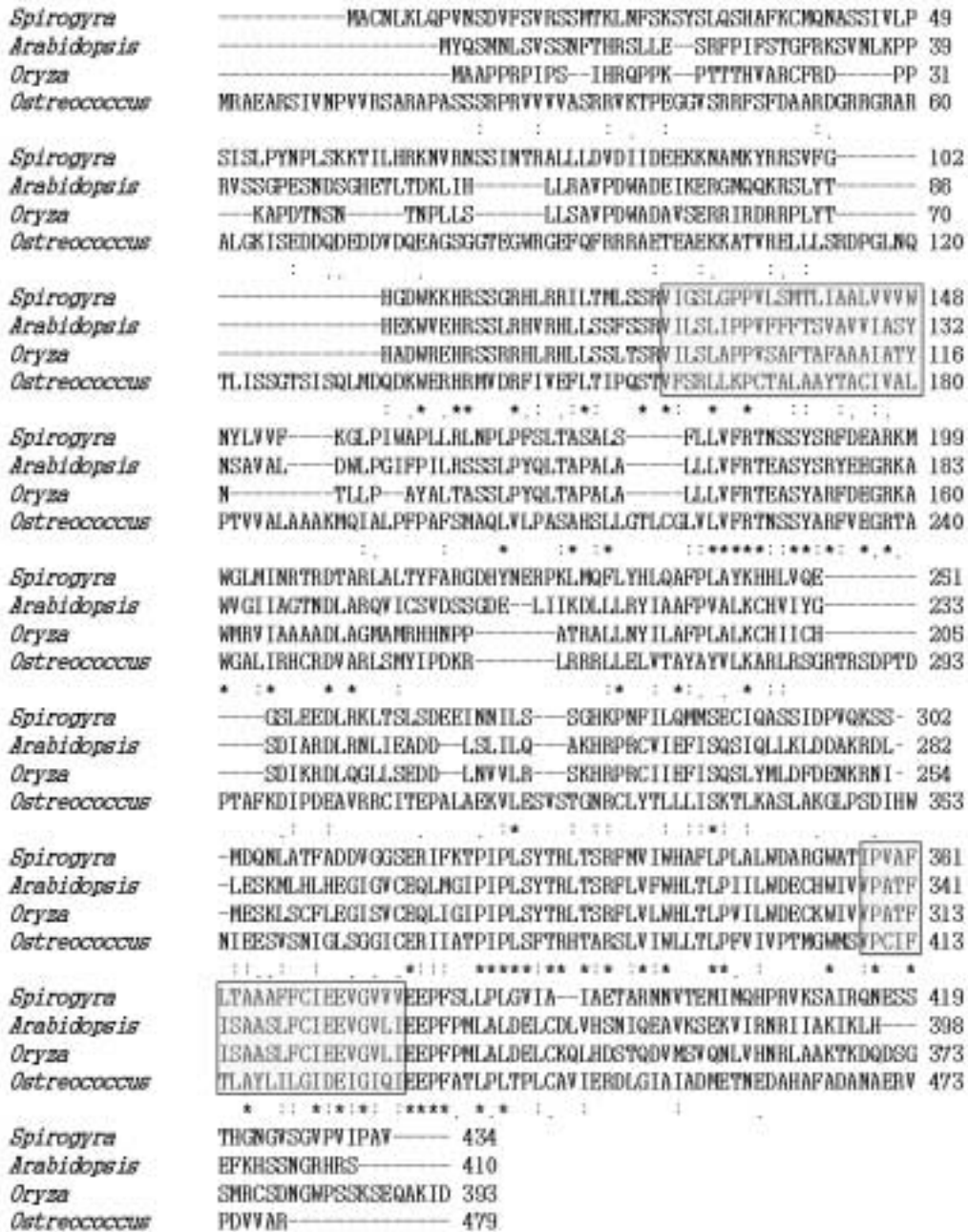


Fig. 3. Alignment of the deduced amino acid sequences of SVCR2 with other members of plant UPF0187 using Clustal W. *Arabidopsis thaliana* (Q9M2D2), *Oryza sativa* (japonica cultivar-group, NP_001048682) and *Ostreococcus tauri* (CAL54763). [*] indicates that all amino acid residues are identical, and [:] means that all but one are identical. The two predicted transmembrane regions are boxed.

with cDNA library as described in figure 2B. The full sequence of the cDNA encoding one of these genes was successfully obtained, which consists of 1,494 bp with an open reading frame (ORF) of 1,302 bp encoding 434 amino acid residues (Fig. 2A). The estimated molecular weight of the protein product was about 48.8 kDa. Amino acid composition of this gene is shown in Table 2.

The deduced amino acid sequence of this gene was searched for homology on BLAST. As the gene was

novel, it was named as SVCR2 (*Spirogyra varians* cold regulated). Alignment of the deduced amino acid sequences of SVCR2 with UPF0187 gene from other members of plant and algae showed that SVCR2 contained two transmembrane domains (Fig. 3, marked area).

SVCR2 showed a significant similarity with transmembrane protein from a higher plant, *Arabidopsis thaliana* (Table 3). In green algae, we could find only one homo-

Table 3. Comparison of amino acid sequence for UPF0187 for other organisms

Species	Accession Number	Identity(%)	Similarity(%)
<i>Arabidopsis thaliana</i>	Q9M2D2	33.1	52.7
<i>Oryza sativa</i>	NP_001048682	31.0	48.9
<i>Ostreococcus tauri</i>	CAL54763	22.2	33.6
<i>Rhodospseudomonas palustris</i>	YP_570078	19.0	31.5
<i>Nitrococcus mobilis</i>	ZP_01127385	20.5	30.9

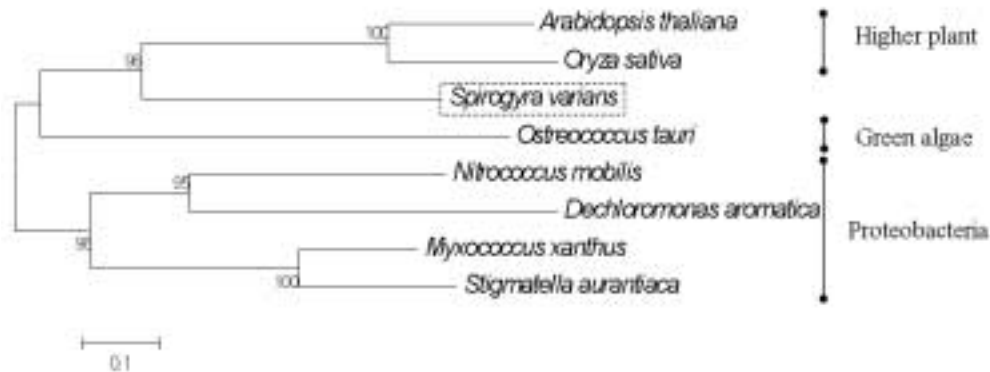


Fig. 4. Phylogenetic tree based on deduced amino acid sequence of UPF0187 constructed by neighbor joining method. The numbers at the nodes are the bootstrap values. The SVCR2 are delineated in box.

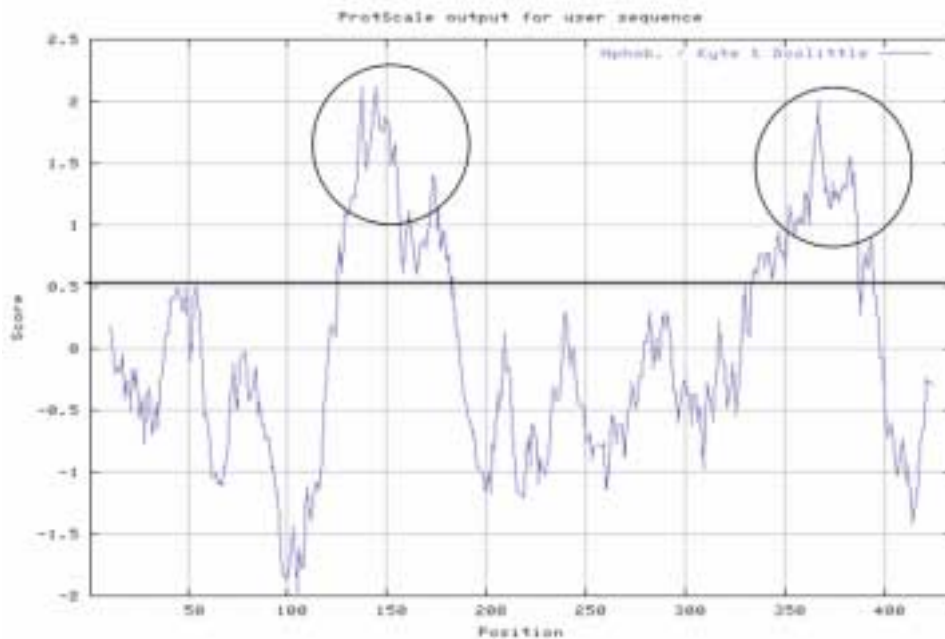


Fig. 5. Hydropathy plots of the translated cDNA sequence of SVCR2. The circles indicate transmembrane regions.

logue of this gene in a unicellular alga *Ostreococcus tauri*, but it showed 33.6% of similarity which was much lower than that of *Arabidopsis thaliana* (57.7%) and *Oryza sativa* (48.9%). A phylogenetic tree based on deduced amino acid sequence of SVCR2 and UPF0187 is shown in figure 5. The hydropathy plot result showed that SVCR2 had two transmembrane areas, which were the same with

UPF0187 from *Arabidopsis thaliana*. These results suggested that SVCR2 might be a chloroplast targeted gene.

Northern blot analysis was performed to see the effect of cold and light condition to the expression of SVCR2 (Fig. 6). The transcript level of SVCR2 gene increased more than 10 fold at low temperature (4°C) than at 20°C under 50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ of light condition (Fig. 6B).

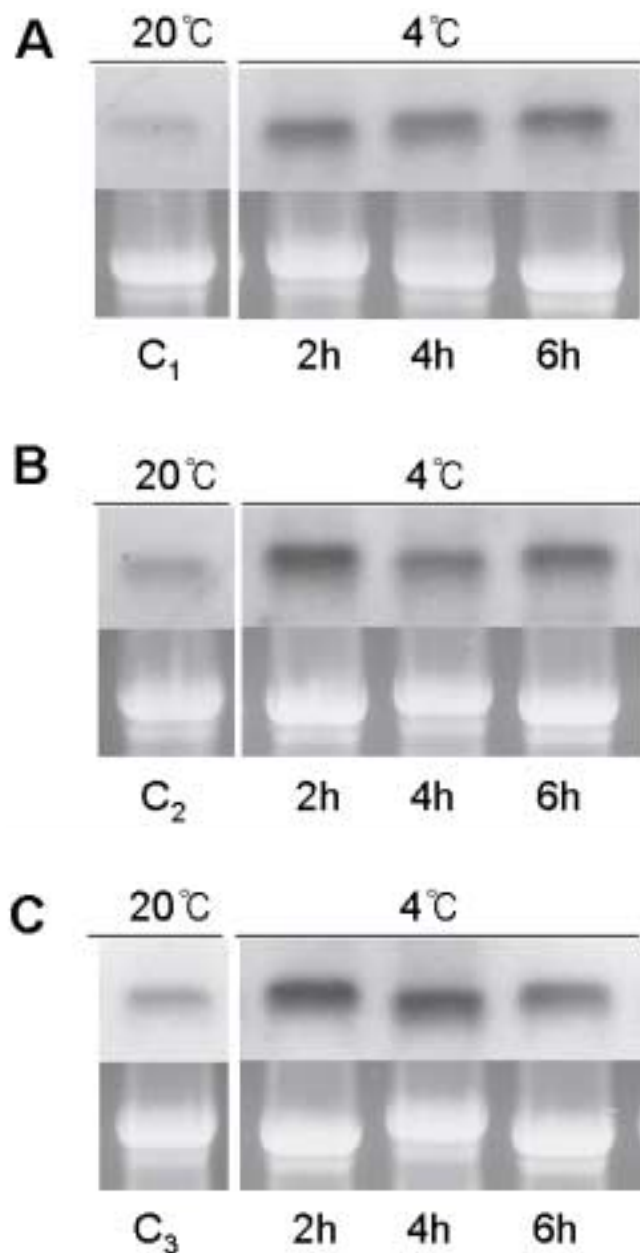


Fig. 6. Northern blot analysis of SVCR2 gene using total RNA in response to cold treatment (4°C) under different light intensities. Ethidium bromide stained rRNA bands are shown at the bottom of each figure. Lanes $C_{1,2,3}$ are control (20°C). (A) Induction of SVCR2 under cold condition (4°C) for 2, 4, 6 hrs, dark. (B) 4°C and $50 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. (C) 4°C, $200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$.

SVCR2 mRNA began to accumulate and reached to maximum at 2 hr after transfer to 4°C culture chamber from 20°C (Fig. 6). Under dark condition with 4°C, SVCR2 increased 5 to 10 fold of control (20°C, dark) within 2 hr (Fig. 6A).

High-light condition ($1200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) induced strong accumulation of SVCR2 mRNA in 2 hrs

even at control (20°C, $50 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, Fig. 7A, 7B), but it decreased rapidly at this temperature (Fig. 7B). The effect of monochromatic light on the expression of SVCR2 gene was observed (Fig. 7C). Blue light (470 nm) could induce the accumulation of SVCR2 mRNA in 2 hrs at 20°C, but red light (650 nm) and UV-A irradiation did not show any effect.

DISCUSSION

We isolated a gene, SVCR2, from a freshwater green alga *Spirogyra varians* using DD-PCR together with annealing control primers (DEG method). The expression of SVCR2 was induced by low temperature (4°C). Although the gene expression could be induced by low temperature alone in dark condition, a stronger expression was observed when light was given at the same time. A high light condition ($1200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$), monochromatic blue (470 nm) and UV-A light could induce the gene expression in 20°C.

The highest homology of SVCR2 was found in UPF0187 gene of *Arabidopsis thaliana* (57.7%). As the name indicates, UPF0187 gene is a family of unidentified protein identified through *Arabidopsis* genome project. Although the presences of similar genes were reported in other higher plant, *Oryza sativa* (48.9%), we could not find any functional studies on this gene group. The conserved transmembrane region common in SVCR2 and UPF0187 gene suggested that SVCR2 is a transmembrane protein targeted to chloroplast. Cold stress was reported to induce the production of transmembrane proteins and the proteins are targeted to various organelles in the cell to protect membrane structure.

The photosynthetic potential of plants exposed to low temperature is set to high excitation pressure similar to high light condition (Gray *et al.* 1996, 1997). Low temperature (0-12°C) treatment of plants affects the chloroplast ultrastructure, resulting in swelling and distorting of thylakoids (Gemel *et al.* 1986; Kratsch *et al.* 2000). It is known that chilling treatment in both the light and the dark may affect different sites within the chloroplast, and thus may account for the differences in the effect between chilling in the light and in the dark. Northern blot analysis of SVCR2 in various combinations of light and temperature suggested that this gene belongs to the superfamily of the chlorophyll-binding proteins in eukaryota which are synthesized in response to environmental stresses, which do not appear to play a role in light harvesting.

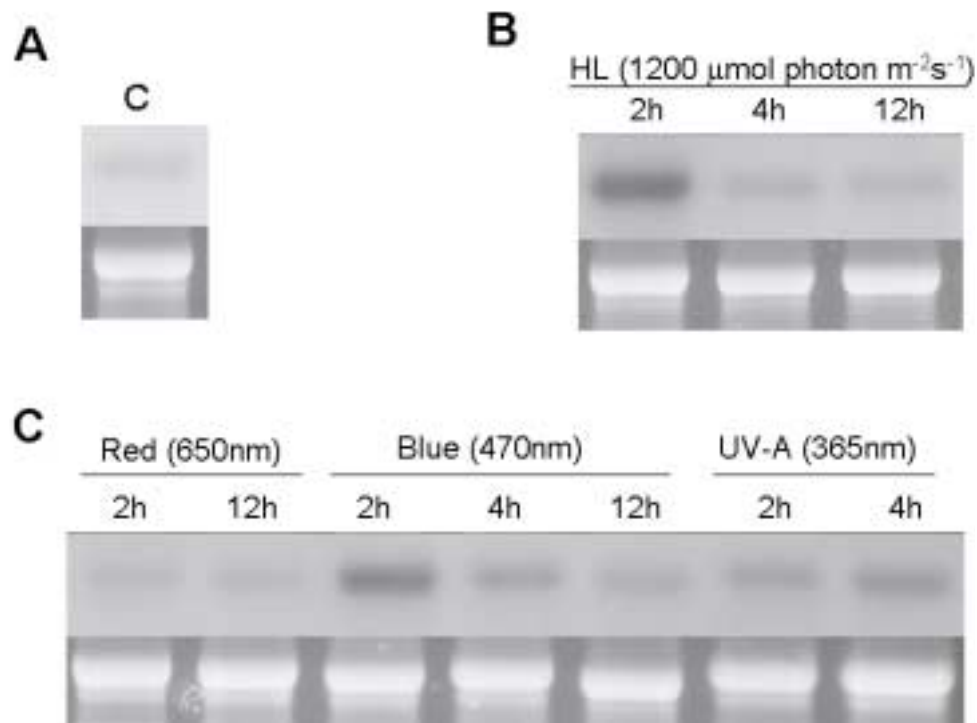


Fig. 7. Northern blot analysis of SVCR2 gene using total RNA in response to different light conditions. Ethidium bromide stained rRNA bands are shown at the bottom of each figure. (A) C: control, 20°C, 50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. (B) 20°C, highlight (1200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$). (C) Effect of monochromatic light at 20°C for 2-12 hrs.

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