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Isolation and characterization of a sodium-dependent phosphate transporter gene in *Dunaliella viridis*

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Abstract

A sodium-dependent phosphate transporter gene, *DvSPT1*, was isolated from a cDNA library using a probe derived from a subtracted cDNA library of *Dunaliella viridis*. Sequencing analyses revealed a cDNA sequence of 2649 bp long and encoded an open-reading frame consisting of 672 amino acids. The deduced amino acid sequence of *DvSPT1* exhibited 31.2% identity to that of TcPHO from *Tetraselmis chui*. Hydrophobicity and secondary structure prediction revealed 11 conserved transmembrane domains similar to those found in PHO89 from *Saccharomyces cerevisiae* and PHO4 from *Neurospora crassa*. Northern blot analysis indicated that the *DvSPT1* expression was induced upon NaCl hyperosmotic stress or phosphate depletion. Functional characterization in yeast Na^+ export pump mutant G19 suggested that *DvSPT1* encoded a Na^+ transporter protein. The gene sequence of *GDvSPT1* (7922 bp) was isolated from a genomic library of *D. viridis*. Southern blot analysis indicated that there exist at least two homologous genes in *D. viridis*.

Keywords: *Dunaliella viridis*; Sodium-dependent phosphate transporter; Induced expression; Yeast functional characterization

Phosphorus, an essential macronutrient for all living organisms, serves various basic biological functions such as a structural element in nucleic acids and phospholipids, a facilitator in energy metabolism and the activation of metabolic intermediates, and a component in signal transduction cascades and the regulation of enzymes [1].

A primary and critical step in the utilization of phosphorus is its uptake into the living cells across the plasma membranes. It has been proposed that, in general, there existed two different types of phosphate transporters. Low-affinity phosphate transporters with a K_m of approximately 1 mM are continuously expressed proteins. On the other hand, high-affinity transporters usually have a much lower K_m (range 1–15 μM), and their expression is regulated and induced when the cells are under phosphate-limiting conditions. In *Saccharomyces cerevisiae*, two derepressive high-affinity phosphate transporters, PHO84 and PHO89, have been identified [2,3]. PHO84 is a H^+-coupled phosphate transporter which also functions as a receptor for ambient phosphate signals. PHO89 is believed to be a sodium-coupled phosphate transporter as its activity is highly correlated with the external Na^+ concentration. The activity of PHO89 is also controlled by the phosphate concentration. Two related enzymes also exist in *Neurospora crassa*, PHO5 and PHO4, respectively [4], PHO5 is a H^+-coupled phosphate transporter and PHO4 is a sodium-coupled phosphate transporter.

Little is known about proteins involved in phosphate uptake in phytoplankton. Homologs of Psts, the phosphate-specific transport system, in *Escherichia coli*, were identified in two marine cyanobacterial genera, *Synechococcus*, and *Prochlorococcus* [5,6]. Polyclonal antiserum raised against PstS has been used to detect the degree of phosphorus depletion in natural picoplankton in a

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mesocosm experiment [6]. In eukaryotic phytoplankton, two membrane-associated proteins with molecular masses of >200 and 50 kDa were identified in *Dunaliella tertiolecta* and *Phaeodacrylum tricornutum* under phosphorus deficiency conditions. The levels of the former compound (>200 kDa) were correlated with various degrees of phosphorus limitation. However, the primary functions of this protein seem to be hydrolysis and mobilization of stored polyphosphates, which means that the protein is not directly involved in phosphate uptake [7,8].

In this study, a full-length cDNA and the corresponding gene *DvSPT1* were isolated from a cDNA library and a genomic library of *Dunaliella viridis*, respectively, using a probe C-2.0-CP1A60 from a hyper-osmotic subtracted cDNA library of the salt tolerant alga *D. viridis*. Sequence analysis indicated that it encoded a sodium-dependent phosphate transporter named as *DvSPT1*. The transcript of *DvSPT1* was investigated by RNA gel blot analysis, and a strong correlation between mRNA abundance and hyperosmotic stress or phosphate starvation was confirmed. Expression of *DvSPT1* in yeast was also primarily reported in this paper. To our knowledge, this is the first time that the sequence and the expression of a sodium-dependent phosphate transporter in *D. viridis* have been reported.

Materials and methods

Cell culture of D. viridis SHU. A SHU strain of *D. viridis* was maintained on a semi-solid growth medium in this laboratory according to [9]. A single colony of *D. viridis* SHU was inoculated to 100 ml of a liquid culture medium containing 0.5 M NaCl [10] and cultured for two weeks to reach the logarithmic growth stage ($\sim 2 \times 10^6$ cells/mL).

Construction of two suppression subtractive libraries. The *Dunaliella* cell culture was divided into two equal parts and pelleted by centrifugation (1000*g* for 5 min at room temperature), one part was cultured in 50 ml of fresh normal liquid culture medium containing 0.5 M NaCl and cultured for 24 h (named as culture C-0.5); one part was cultured in 50 ml of fresh medium containing 1.0 M NaCl for 2 h and then transferred to 50 ml of a fresh medium containing 2.0 M NaCl for 24 h (named as culture C-2.0). About 10^8 cells were harvested by centrifugation from each of the above cultures and processed for total RNA isolation with Trizol (Gibcol-BRL) according to the manufacturer's instructions. Poly(A)$^+$mRNA was purified by using Oligotex mRNA Kit (Qiagen). The RNA concentrations were determined by spectrophotometry (U2000; Hitachi) at 260 and 280 nm. Two sets of double-stranded cDNA fragments were generated from the above Poly(A)$^+$mRNA samples of C-0.5 and C-2.0, while the cDNA fragments were mutually subtracted by using a PCR-selected cDNA subtraction kit (Clontech) according to the manufacturer's instructions [11]. The two sets of subtracted PCR products were cloned into pMD18-T Vector (TaKaRa) to generate two separate subtractive cDNA libraries of C-0.5 and C-2.0.

Screening of the subtracted cDNA library of C-2.0. DNA samples of individual colonies from the C-2.0 library were amplified by PCR using specific primers, about 10 ng of each PCR product was identically blotted onto two membranes, and the blots were subjected to hybridization using the [α-^{32}P]dCTP labeled probes of C-0.5 or C-2.0 which were generated by PCR using the above subtractive hybridization products as templates. The cDNA clones that were hybridized only to the C-2.0 probes but not to the C-0.5 probes were selected for DNA sequencing. The sequences of the cDNA fragments and the deduced coding sequences were analyzed by BLASTX and BLASTP algorithms (http://www.ncbi.nlm.nih.gov).

Screening of cDNA and genomic DNA libraries. The cDNA fragments specifically expressed in the C-2.0 library were used to screen both of the cDNA library (constructed in this laboratory, unpublished) and the genomic library of *D. viridis* SHU [12,13]. A specific full-length cDNA and a specific genomic fragment named *DvSPT1* and *GDvSPT1* were isolated and were further subjected to DNA sequencing analysis followed by possible gene structure, open-reading frame, and sequence alignment assays using the program Vector NTI Suite 8.0. The possible transmembrane regions in the deduced protein were predicted using the program TMpred (http://www.ch.embnet.org/software/TMPRED-form.html) .

Northern blot analysis. Total RNA was extracted from *D. viridis* cells under osmotic shock or phosphate starvation conditions. For osmotic shock, the cells were transferred from the medium containing 1.0 M NaCl to the medium containing 2.0 M NaCl, aliquots of the cells were harvested at 0.5, 1, 2, 3, 6, 9, 12, and 24 h after the hyperosmotic shock and extracted for total RNA. For phosphate starvation treatment, the cells cultured in medium containing 0.2 mM KH_2PO_4 and 1.0 M NaCl were transferred to a medium containing 1.0 M NaCl but no KH_2PO_4 after three thorough washes with the KH_2PO_4-free medium; aliquots of the cells were harvested 0, 1, 3, 6, 18, and 36 h after the treatment and were extracted for total RNA. About 15 μg of the total RNA extracted from these samples was subjected to RNA gel blot analysis, the [α-^{32}P]dCTP labeled probe was generated from the *DvSPT1* cDNA by PCR using two specific primers *DvSPT1*RTS (5′-ccctgttccactcactgc-3′), *DvSPT1*RTA (5′-gaacagggg tggtgcttaca-3′). The rRNA in each sample was used as an internal quantitative reference.

Transformation of a salt sensitive yeast mutant G19. The yeast strain G19 (*MAT*α*leu2-3*, *20112*, *trp1-1*, *ura3-3*, *ade2-1*, *his3-11 can1-100*, *15*(ϕ) *ena1*Δ*::HIS3::ena4*), which is disrupted in the genes encoding Na^+ export pumps and as a result displays salt sensitivity [14], was kindly provided by Professor Alonso Rodriguez-Navarro. Coding regions of *DvSPT1* were amplified by using primers (Y*DvSPT1*FP1: 5′-attgaattcaccatggcggacgtg gacagcttcagc-3′, Y*DvSPT1*RP1: 5′-tccctcgagttaaggcatggtctgctcaga-3′) and then subcloned into the *Eco*RI–*Xho*I site of the yeast shuttle vector pYES2, which formed a vector named as pYSPT1. Yeast cells transformed with pYES2 or pYSPT1 were cultured at 30 °C in minimal medium (0.7% Bacto-yeast nitrogen base without amino acids, pH 6.0, supplemented with 0.002% adenine, 0.002% histidine, 0.003% leucine, 0.003% tryptophan, and 0.002% uracil) plus 2% galactose as carbon source. Yeast transformation was done by the LiAc/ss-DNA/PEG method [15], and transformants were selected in medium without uracil. At least three independent transformants named G19:pYES2 and G19:pYSPT1 were chosen for further growth investigation. Growth (A_{600}) of the cultures was monitored in liquid media supplemented with various concentrations of NaCl (1.71, 50, and 100 mM NaCl) and the mid-log phase ($A_{600} = 0.6$) time differences between G19:pYES2 and G19:pYSPT1 were determined.

Southern blot analysis. Dunaliella viridis cells were grown to stationary phase (about 10^8 cells/ml). Genomic DNA from *D. viridis* was isolated and was digested overnight with *Bam*HI, *Eco*RI, *Hin*dIII, and *Bam*HI/*Eco*RI separately. The digested DNA was separated by 0.8% agarose gel electrophoresis overnight and was then subjected to Southern blot analysis using the same specific probe as in Northern blot analysis but labeled with digoxigenin (DIG) according to manufacturer's instructions (Roche Molecular Biochemicals).

Results

Subtracted cDNA library construction and screening

By the powerful subtractive suppression hybridization (SSH) technique, two SSH cDNA libraries of C-0.5 and C-2.0 representing the cDNA fragments of *D. viridis* SHU cells specifically expressed in the conditions of 0.5 M and 2.0 N NaCl. A total of 384 cDNA clones were selected from the C-2.0 library for further identification

of their specificity by dot-blot hybridization, only those specific to the probes generated from the C-2.0, but not the C-0.5 library, were considered the clones that were expressed in the cells under the condition of 2.0 M NaCl. Ten highly specific clones (CP1A60: DQ285475; CP1F1: DQ285476; CP2F6: DQ285477; CP2F7: DQ285478; CP3B2: DQ285479; CP3D1: DQ286755; CP1E6: DQ286756; CP2B2: DQ286757; CP2A10: DQ286758; and CP2A6: DQ286759) were selected and screened primarily by DNA sequencing for their possible characteristics and most of them were gene fragments exhibiting low levels of similarity to genes in the GenBank database except for CP1E6, which encodes a partial chlorophyll *a-b* binding protein of LHCII type I (data not shown).

cDNA library screening of CP1A60

Then we applied CP1A60 cDNA fragments to screen the cDNA library of *D. viridis* for getting full-length cDNA and further analysis. After two rounds of cDNA library screening (constructed in this laboratory, unpublished), six positive clones were obtained (pBKCMV-A60I∼VI). The inserts of these cDNA clones were further checked by PCR and restriction enzyme mapping (data not shown). Clone pBKCMV-A60I was selected for sequencing analysis. According to sequencing analysis, the cDNA fragment CP1A60 was located between 2064 and 2304 bp in 3′-UTR regions (Fig. 1). The complete length of the cDNA was 2649 bp long, containing an open-reading frame of 672 amino acids (Fig. 1) with a predicted molecular weight of 73.3 kDa. The predicted protein sequence had 40.42% hydrophobic amino acids, with a calculated isoelectric point value of 5.29. A typical green algal polyadenylation signal TGTAA was found 24 bp upstream of the ploy(A) site (Fig. 1).

Computational sequence analysis of cDNA DvSPT1

BLASTP analysis revealed that the deduced amino acid sequence of pBKCMV-A60I had 31.2% identity with a phosphate transporter *TcPHO* from *Tetraselmis chui* [16].

```
                                                                  M  T  D  V    4
   1 CTTGTACATCGGTGCACGCACCGAGCCCCTCCCCACCGCCACTTCGTCTGCGAAAGCAGGAGGGGTAGGCACGCAGCCATGACGGACGTG
     D  S  F  S  G  N  M  P  N  L  S  G  F  E  S  A  A  G  W  Q  G  E  N  F  T  Y  H  D  G  K   34
  91 GACAGCTTCAGCGGCAACATGCCCAACCTCTCGGGCTTTGAGTCCGCAGCGGGCTGGCAAGGCGAGAACTTCACCTACCACGATGGCAAG
     W  S  A  Y  V  W  L  L  V  I  G  S  F  T  S  F  M  A  A  W  G  I  G  A  N  D  V  A  N  S   64
 181 TGGTCTGCCTACGTGTGGCTGCTGGTCATCGGCTCCTTCACCTCATTCATGGCTGCATGGGGCATCGGTGCCAACGATGTGGCCAACTCC
     F  A  T  S  V  G  S  K  A  L  T  M  F  Q  A  C  V  I  A  G  I  F  E  F  V  G  A  V  S  L   94
 271 TTCGCCACAAGTGTGGGCTCCAAGGCTTTGACCATGTTCCAAGCCTGCGTGATTGCGGGCATCTTTGAATTTGTCGGCGCTGTGTCCCTG
     G  G  T  V  V  K  T  V  K  G  S  I  T  D  P  S  M  F  K  H  Q  P  Q  I  F  A  Y  G  M  L  124
 361 GGTGGTACTGTTGTGAAGACCGTGAAGGGCTCCATCACTGACCCCTCAATGTTCAAGCACCAGCCCCAGATTTTTGCGTACGGCATGCTT
     G  A  S  T  S  V  A  I  V  L  F  L  A  T  Y  L  K  Q  A  V  S  T  T  H  T  A  I  G  S  V  154
 451 GGTGCCTCTACTTCTGTAGCCATTGTGCTCTTCCTGGCCACATACTTGAAGCAGGCTGTGTCCACCACACACACTGCAATTGGGTCTGTG
     L  G  F  G  L  V  Y  G  G  A  D  G  V  T  W  N  E  V  S  D  E  F  P  F  R  K  G  F  T  P  184
 541 CTTGGCTTCGGCTTGGTGTATGGAGGCGCTGATGGTGTGACCTGGAACGAGGTGTCCGATGAGTTCCCCTTCCGCAAGGGGTTCACACCT
     V  V  I  S  W  F  V  S  P  V  F  T  A  I  L  S  S  G  F  F  I  I  T  R  I  V  C  L  Q  R  214
 631 GTCGTCATCTCCTGGTTCGTCTCCCCCGTCTTCACTGCCATTCTGTCCTCCGGATTCTTCATCATCACCAGGATTGTCTGCCTGCAGCGC
     Q  Q  S  Y  K  I  A  F  F  M  I  P  V  L  M  M  I  T  I  F  I  V  L  L  A  I  F  L  K  S  244
 721 CAGCAATCCTACAAGATTGCCTTCTTCATGATCCCTGTGCTGATGATGATCACCATCTTCATTGTCCTGCTGGCCATCTTCCTGAAGAGT
     V  D  S  G  H  D  R  E  G  E  L  N  W  S  T  D  K  K  A  W  V  A  I  V  V  G  A  G  A  G  274
 811 GTGGACTCTGGACACGACCGTGAGGGTGAGCTGAACTGGTCCACAGACAAGAAGGCCTGGGTGGCAATTGTTGTCGGTGCTGGTGCCGGT
     L  L  T  I  P  Y  T  I  W  L  R  K  S  M  L  Q  E  D  Q  E  V  H  E  E  N  M  A  R  D  A  304
 901 CTGCTGACCATCCCCTACACCATCTGGCTGAGGAAGTCCATGCTCCAGGAGGACCAGGAAGTGCACGAGGAGAATATGGCTCGTGATGCT
     E  T  A  H  V  A  A  G  G  T  L  K  E  K  N  P  E  D  D  V  H  E  L  T  K  A  E  K  F  M  334
 991 GAGACCGCCCACGTCGCAGCTGGTGGCACACTGAAGGAGAAGAACCCTGAGGATGATGTGCATGAGCTGACCAAGGCTGAGAAGTTTATG
     E  W  F  N  G  T  W  F  G  Q  R  Q  F  V  K  N  F  V  A  A  M  T  Y  D  V  H  A  H  V  A  364
1081 GAGTGGTTCAACGGCACCTGGTTCGGCCAGAGACAGTTTGTGAAGAACTTTGTTGCAGCTATGACATACGATGTGCACGCCCATGTGGCC
     T  M  D  N  P  K  V  A  Q  M  H  A  D  A  E  V  F  D  P  R  T  E  D  V  F  K  R  M  Q  I  394
1171 ACCATGGACAACCCCAAGGTTGCCCAGATGCATGCAGATGCTGAGGTCTTCGACCCCCGCACTGAGGATGTGTTTAAGCGCATGCAGATC
     I  T  A  S  A  V  A  F  V  H  G  A  N  D  V  A  N  G  V  G  P  L  A  G  I  W  D  T  Y  N  424
1261 ATCACTGCCTCTGCTGTGGCCTTCGTGCACGGTGCCAATGATGTCGCCAACGGTGTGGGCCCTCTTGCCGGTATCTGGGACACCTACAAC
     T  Y  Q  S  G  S  G  K  A  S  Q  P  R  W  I  L  V  I  G  A  A  G  I  V  F  G  L  A  M  Y  454
1351 ACCTACCAGTCTGGCAGTGGCAAGGCGTCTCAGCCCCGCTGGATTCTGGTCATCGGTGCTGCTGGCATTGTGTTCGGCCTGGCCATGTAC
     G  Y  R  I  I  A  T  L  G  V  D  L  V  V  M  T  P  N  R  G  Y  S  V  E  L  A  A  A  A  I  484
1441 GGCTACCGTATCATTGCGACCCTGGGTGTGGACTTGGTGGTCATGACCCCCAACCGTGGTTACTCTGTGGAGCTGGCAGCAGCTGCTATC
     I  A  L  A  S  T  Y  G  L  P  V  S  T  T  Q  V  V  T  G  G  K  I  G  V  G  M  C  E  S  W  514
1531 ATTGCCCTGGCCTCCACGTACGGTCTGCCCGTCTCCACTACGCAAGTTGTGACTGGCGGTAAGATAGGGGTGGGCATGTGCGAGAGCTGG
     K  M  T  G  V  N  W  L  L  F  I  R  T  F  W  G  W  V  G  A  L  V  T  G  A  I  L  S  A  L  544
1621 AAGATGACAGGCGTGAACTGGCTCCTGTTTATCCGCACCTTCTGGGGCTGGGTTGGTGCTCTGGTGACTGGCGCCATCCTCTCAGCTCTG
     L  F  S  I  G  V  Y  G  P  S  R  T  D  L  D  Y  V  T  H  Y  Q  R  A  M  K  I  D  I  D  Q  574
1711 CTCTTCTCCATCGGTGTGTACGGCCCCAGCCGCACTGACCTGGACTACGTGACCCACTACCAGAGGGCGATGAAGATCGACATCGACCAG
     S  M  Q  A  L  L  D  R  C  A  A  A  P  A  P  T  N  G  G  G  G  C  G  T  G  D  L  N  C  S  604
1801 TCCATGCAAGCTCTCCTTGACCGATGTGCTGCTGCTCCTGCACCTACCAATGGTGGTGGAGGCTGTGGCACCGGAGACTTGAACTGCTCT
     C  Q  Q  R  I  P  Y  T  M  G  D  L  Q  G  L  K  T  Q  V  D  G  L  F  N  F  E  E  N  G  Q  634
1891 TGCCAGCAGAGGATCCCCTACACCATGGGTGACCTGCAAGGCCTGAAGACGCAGGTTGATGGGCTGTTCAACTTCGAGGAAAATGGCCAG
     I  N  E  Y  N  M  M  Y  M  L  R  Q  I  N  S  L  F  K  Y  T  F  F  D  G  D  F  S  Q  Y  E  664
1981 ATCAACGAGTACAACATGATGTACATGCTGAGGCAGATCAACAGCCTGTTCAAGTACACCTTCTTCGATGGCGACTTCTCTCAGTACGAG
     N  V  S  E  Q  T  M  P  *                                                              672
2071 AACGTGTCTGAGCAGACCATGCCTTAATTCTGAAGTCTTGACAATCTTTTCCCTGTTCCACTCACTGCACTCTTGCTCTTCAGTTACCAA
2161 TTCATCCTTTTTGAAGTCTTGCTTTTGTTCTGAGATTGAACACTAAGCATGACCCGTTCTGTTGTGTTCACATAGCCATATCATTTAGTC
2251 AGGTGTTGTGGCTGGTTCATATCCTGACCAAATTTAGATCTATTGCTGGTATTGTGTACTGGTGGAAGCTTGGGTTCCCAGCATGGTCGT
2341 CTCCCTCAAGTACGCAGGCTGTATTGCATTACCTCTTGTTGACCGAAGACAGTATCTCTTCTACCAATGCTTGTTGCCTGAACCTCAAGC
2431 GTGACTGTTTCGTTGCTTCACTCTTGTGTGTTGGCTCATATTTCATTTTCACCTTCTTCAGCCCACTGAACTGATTGAATAATGCAACTG
2521 CAAGAATAAACACACGTCTATGTATCTGCACTGTTTGTGCAATTGATCCTTCTTGTTGATCATTTTGGAATCCTCCGCTGATTGTGTAAA
2611 TTTTGTAAGCACCACCCCTGTTCAAAAAAAAAAAAAAAA
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Fig. 1. cDNA sequence and deduced amino acids sequence of *DvSPT1*. Conservative sequences "GANDVAN" were double underlined. A poly(A) signal sequence "TGTAA" was underlined. The base number were indicated on the left of the figure and the amino acid number is indicated on the right. Sequence of C-2.0-CP1A60 fragment is marked with character frame.

In addition, the deduced amino acid sequences exhibited high similarities to those of the sodium-coupled phosphate transporters, such as PHO89 in *S. cerevisiae* and PHO4 in *N. crassa* (Fig. 2). The protein encoded by the cDNA shared high similarities to the other sodium-coupled phosphate transporter proteins (Fig. 2) in the N- and C-terminal regions, but poor similarity and large gaps in the central region of the proteins.

A hydropathy plot and secondary structure analysis (http://www.ch.embnet.org) of the deduced peptide indicated that there were 11 transmembrane regions (Fig. 3) with a large hydrophilic loop between TM7 and TM8 as well as the hydrophilic N- and C-termini are intercellular and extracellular, respectively. Furthermore, two short repeats (G-A-N-D-V-A-N), which were highly conservative in the sodium-coupled phosphate transporters, were also identified in the predicated protein located in the positions similar to those of PHO89 and PHO4 (Figs. 1, 2), one in the hydrophilic loop between TM1 and TM2, the other in the large hydrophilic loop TM8. All these features strongly suggested that the cDNA, which was designated as *DvSPT1*, encoded a novel sodium-coupled phosphate transporter protein (*DvSPT1*) in *D. viridis*.

DvSPT1 expression was induced under NaCl osmotic stress and phosphorus depletion

The cells were under hyperosmotic stress by changing the concentration of NaCl in medium from 1.0 to 2.0 M, the amounts of the transcript specific to *DvSPT1* was assayed by Northern blot analysis at various incubation times of 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 h using a 3′-terminal cDNA fragment of *DvSPT1* as probe. The specific transcript was first detected at 2 h after the shock, increased to the maximum in amount at 3 h, and then gradually decreased to the base level at about 24 h after the shock (Fig. 4A).

Previous studies showed the possible presence of a high phosphate-affinity uptake system in *Dunaliella salina* by which the uptake of Pi could be greatly increased by 20-fold in the conditions of phosphate starvation [17]. To observe the possible effect of Pi depletion on the expression of *DvSPT1*, the cells were thoroughly washed with the KH_2PO_4-free medium and incubated in the KH_2PO_4-free medium for different times (0, 1, 3, 6, 18, and 36 h), the RNA samples were extracted from the cells and subjected to Northern blot analysis using 3′-terminal cDNA fragment of *DvSPT1* as probe shown in Fig. 4B. The specific transcript was detected at as early as 1 h after the depletion of KH_2PO_4, increased to the maximal level in amount at 6 h and then decreased gradually to the base level at 18 h after the depletion.

Functional characterization of DvSPT1 in S. cerevisiae Na^+ export pumps mutant G19

The genes encoding plasma membrane Na^+ export pumps have been deleted in the yeast strain G19 and, therefore, it is more sensitive to external Na^+ concentration than wild-type yeast [18]. Any gene product facilitating Na^+ uptake into G19 cells should further increase salt sensitivity. The transformed cells of G19:pYSPT1 were significantly more sensitive to elevated external concentrations of Na^+ than transformed cells of G19:pYES2. Fig. 5 shows growth curve between G19:pYES2 and G19:pYSPT1 at different growth time in liquid minimal medium with various concentrations of NaCl (1.71, 50, and 100 mM). Growth of G19:pYSPT1 cells were more strongly reduced than G19:pYES2 with the elevated external concentrations of Na^+ during the whole exponential phase. For example, the mid-log phase ($A_{600} = 0.6$) between G19:pYES2 and G19:pYSPT1 was deferred more and more long at this point for an average time of 2.20, 4.80, and 7.91 h with the elevated Na^+, respectively. The results were similar to the previous report [14], which indicated that the *DvSPT1* expression in yeast caused the increase of Na^+ uptake in agreement with the functional prediction of *DvSPT1* as a sodium-dependent phosphate transporter.

Isolation and sequence analysis of the DvSPT1 gene

Using the same specific SSH fragment C-2.0-CP1A60 as probe, a clone containing a specific genomic fragment named as G *DvSPT1* (7922 bp) was isolated from the genomic library of *D. viridis* [12]. By the BESTFIT (GCG) analysis, the fragment G *DvSPT1* contained 20 exons and 19 introns (Fig. 6) that covered the full coding region specific to *DvSPT1*. All of the 5′ donor sequences adhered to the 'GT' consensus at the start of the introns; and all of the intron 3′ acceptor sequences at the intron–exon junctions of the gene contained the invariant 'AG' [19] (Table 1).

The core motif of the promoter was predicated to be located in the region of 817–866 bp (CCTATTGGCGAT**TAT AA**GTGCAGGGCCCTGCCGGCATTGC**A**CGAGCG CAT), the high lighted sequences of TATAA and A indicating a possible TATA box and a transcription initiation site, respectively (Fig. 7). The translation initiation and termination sites were located at positions of 1318 and 6860, respectively. In addition, a sequence of 5′-CAC GT-3′ [20] was found to be located at the position of 141–145, which has been known to be the core consensus motif of the binding sites for Pho4p [21,22] during derepression of the genes PHO5 (rAPase) [23], PHO8 (rALPase) [24,25], PHO84 (high-affinity H^+/Pi transporter)[2]; and PHO89 [3]. This suggested that the expression of *DvSPT1* would also be regulated by the PHO regulatory system. In addition, the *cis*-element CACGT is known to be a conservative stress-related regulatory element in plants [26].

There were three $(CA/GT)_n$ repeat sequences different in length in the 5′ promoter region, and one $(TG/AC)_{77}$ long repeat sequence in the sixth intron similar to SSR (simple sequence repeats) found in *Chlamydomonas reinhardtti* [27].

By Southern blot analysis (Fig. 8), two dominant specific bands were detected by the probe of *DvSPT1* cDNA, this

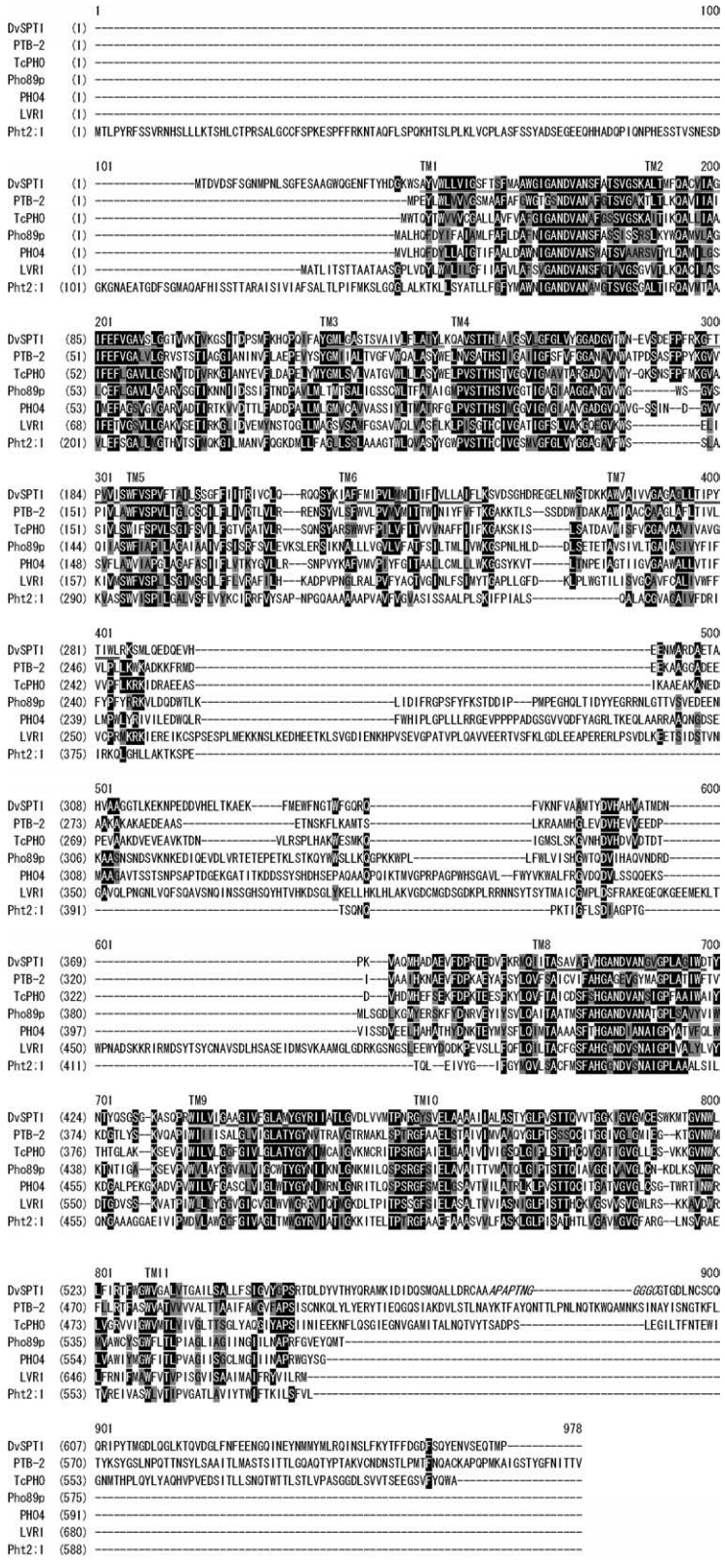

Fig. 2. Alignment of the deduced amino acid sequence of *DvSPT1* and other Na^+/Pi cotransporter. Identical and similar amino acids are shaded black and gray, respectively. The 11 membrane-spanning domains in the two deduced sequences are predicated at website (http://www.ch.embnet.org/) and double-underlined. The double-underlined letters denote sites for two conserved repetitive seven amino acid sequences (GANDVAN). PTB-2: BAB96548, *Chlamydomonas reinhardtii* Pi-transporter homog B2; TcPHO: AA047330, *Tetraselmis chui* high-affinity phosphate transporter; PHO4: P15710, phosphate-repressible phosphate permease of *Neurospora crassa*; Pho89p: NP_009855, *Saccharomyces cerevisiae* Na^+/Pi symporter; LVR1: AAA52572, Leukemia virus receptor 1; Pht2;1: AJ302645.1, a low-affinity phosphate transporter from *Arabidopsis. DvSPT1*: DQ285474, *Dunaliella viridis* sodium-dependent phosphate transporter 1.

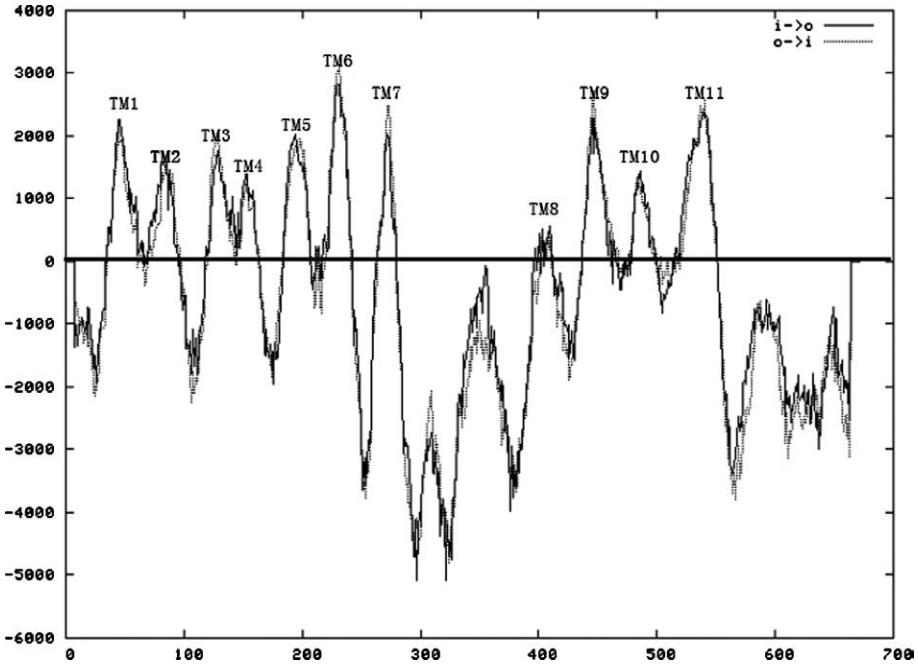

Fig. 3. The transmembrane region prediction was carried out at the website: http://www.ch.embnet.org/. The putative transmembrane regions are indicated with TMn($n = 1,2,3,\ldots11$). Predicated topology of the *DvSPT1* polypeptide is represented with 11 TM domains, a long hydrophilic N-terminus, and a hydrophilic loop between TM7 and TM8.

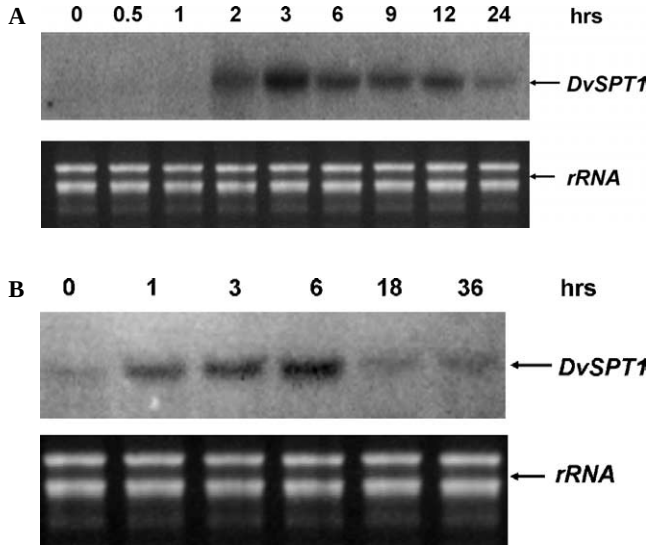

Fig. 4. Northern blot analysis of *DvSPT1* transcripts abundance under NaCl hyperosmotic treatment and phosphate starvation. Northern blot analysis by using [α-^{32}P]dCTP randomly labeled *DvSPT1* 3′ specific cDNA fragment (2121–2138 bp) as probe. (A) Lane 1, *D. viridis* growing in 1.0 M NaCl medium under continuous conditions; lanes 2–9: *D. viridis* RNA shocking from 1.0 to 2.0 M NaCl medium for 0.5, 1, 2, 3, 6, 9, 12, and 24 h, lanes 6–9; (B) *DvSPT1* transcripts abundance during Pi deprivation for 0, 1, 3, 6, 18, and 36 h, 15 μg total RNA was loaded per lane. The lower box was rRNA stained with EB to indicate the consistence of RNA samples in each lane.

suggested that there could exist at least two homologous phosphate transporter genes at different loci in the genome of *D. viridis*.

Discussion

The availability of minerals is recognized as a major limitation for proliferation of phytoplankton in the oceans and in freshwater habitats. Nitrate, phosphate, and iron were identified as the main limiting ions, whose relative contribution varies in different habitats [28]. Phosphate uptake and storage has been studied extensively in freshwater and in marine algae. Phosphate deprivation was found to induce in algae a remarkable enhancement of Pi uptake as well as enhanced extracellular phosphatase activity [29,30]. Uptake of inorganic phosphate was found to depend on Na^+ in marine microalgae indicating a Na^+-coupled symport mechanism [31]. The rapid and large induction of Pi uptake activity, the high-affinity for Pi, combined with the large storage capacity for polyphosphates amounting to 500 mM Pi equivalents in *D. viridis* [32], indicate that *Dunaliella* is well adapted to cope with prolonged phosphate starvation. Calculation shows that within less than 1 h, *D. viridis* may accumulate 500 mM equivalents of Pi from media containing μmol/L Pi concentrations. Recently, Meria Weiss has reported that *Dunaliella* utilized the electrochemical Na^+ gradient also for energization of phosphate and sulfate uptake via Na^+/Pi and Na^+/SO_4 symporters, which suggested that *Dunaliella* recycles Na^+ ion across the plasma membrane as the main ion in transport processes similar to the basic transport mechanisms in animal cells and contrary to most plant and fungi which utilize protons as the main coupling ion in transport processes [17]. This conclusion is not surprising in view of the identification of at least three families

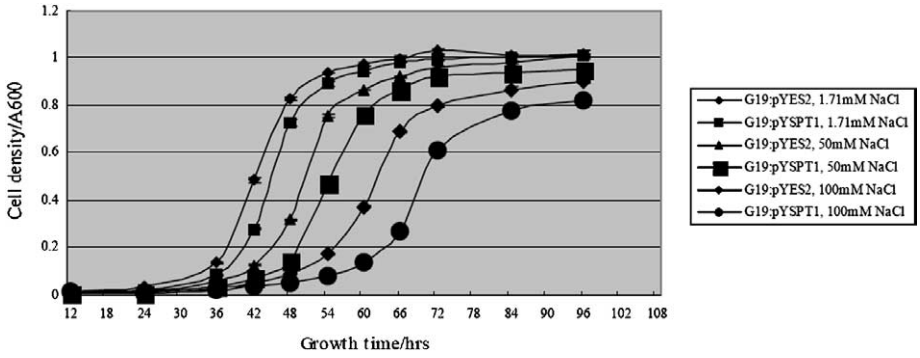

Fig. 5. Growth curve between G19:pY *SPT1* and G19:pYES2 during exponential growth in liquid minimal medium with various concentrations of NaCl (1.71, 50, and 100 mM NaCl). The cell density (A_{600}) was measured at different time for at least three times and the values are means of three experiments ±SD.

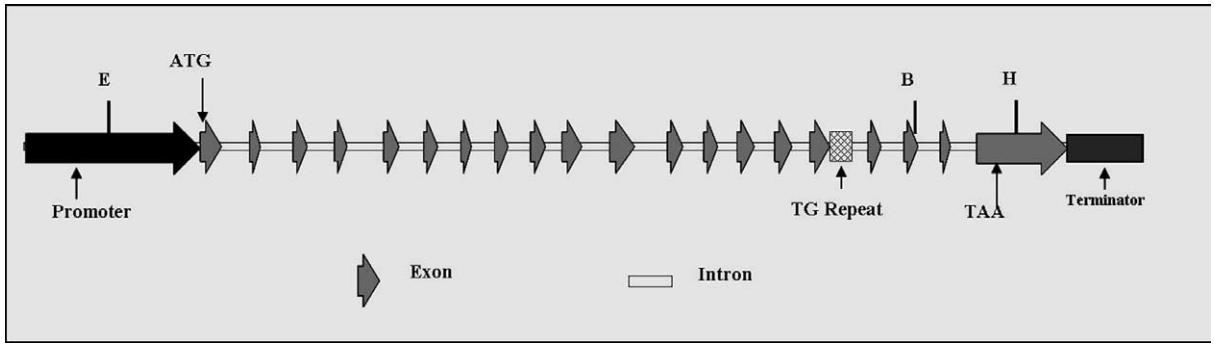

Fig. 6. Gene structure of *GDvSPT1*. The diagram showed that the gene consisted of 20 exons and 19 introns and the 5′, 3′-region of *GDvSPT1*. (B,E, and H) denote restriction sites for *Bam*HIII, *Eco*RI or *Hin*dIII, respectively.

Table 1
Intron and exon organization of *GDsSPT1*

Intron	5′-exon/intron	3′-intron/exon	Intron size (bp)	Preceding exon size (bp)
Intron 1	GGCAAGgtgagcgcg	cacgcacagGCGAGA	221	151
Intron 2	GGCAAGgtgagcgac	gtcctgcagTGGTCT	258	29
Intron 3	CCACAAgtgagcact	gtcctgcagTGGTCT	236	100
Intron 4	TGCGTGgtgggtgcc	gcatcacagATTGCG	267	41
Intron 5	CCCCAGgtatttctt	aacctgcagATTTTT	174	108
Intron 6	CTGCAAgtgagtagc	tttccgcagTTGGGT	166	100
Intron 7	GATGAGgtgagacca	tccctgcagTTCCCC	164	74
Intron 8	CACCAGgtgtgtgcc	ctcctgcagGATTGT	160	98
Intron 9	CTGAAGgtgagcaga	cacgtgcagAGTGTG	119	106
Intron 10	GCTCCAgtgagtctc	ggcctacagGGAGGA	196	140
Intron 11	TGAAGAgtgagtgat	cgcctgcagACTTTG	230	179
Intron 12	ACTGAGgtgggtttc	ctcttgcagGATGTG	149	110
Intron 13	TATCTGgtaagtgaa	ttcctgcagGGACAC	134	101
Intron 14	ATTGCGgtgagtatt	ttcctgcagACCCTG	147	123
Intron 15	GTTGTGgtgagtgat	cccttgcagACTGGC	125	123
Intron 16	CCATCGgtgagtgtg	ttcctgcagGTGTGT	272	142
Intron 17	CTCCTTgtgagtcac	gttctgcagGACCGA	160	95
Intron 18	CCATGGgtaagttct	tccttgcagGTGACC	160	100
Intron 19	AACGAGgtaagctgt	tgtgcgcagTACAAC	186	71
				645

of phosphate transporter genes in plants and at least two in yeast [33]. Unlike in algae, Pi transporters in plants and yeast are predominantly H^+/Pi symporters. Only PHO89 and PHO4 gene products from *S. cerevisiae* and *N. crassa* are high-affinity Na^+/Pi transporters induced under Pi starvation and activated at alkaline pH and high salinity [3].

In this report, a novel Na^+/Pi transporter gene *DvSPT1* was isolated from *D. viridis* by using SSH cDNA fragment

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   1 tcacttagaaagtgaaaaaaactgaatgctaagatggagcaagtaattacttgtttatgaacaaaacaaacaaggagaagcagagggaag
  91 tcctctggcctcacgcattacatcagggcagctaaatgctcacacacacacacgtgcgttaatgcatacgcgcgtgcacacacaagcgcg
 181 cacacacacacacacacacacacacacacacacacacacggtggtggggctcacactagtcacaatgctggtgttgggtctttccttaatgc
 271 ctgctggttaaatccagcgtagacttttacttttttacacacgcatacacgcacgcacgcacgcacgcacgcacgcacgcacgcacgcac
 361 gcacgcacgcacgcgcacacgcacacgcacacacgcacacacacacacacacacacacacacacacacacacacacacacacagaacttgca
 451 atgcacaaaaacagcgaaaaaggaatccttgaattccacaactttcttgcaagtctgaaggagacagaagagtcatggggaggacgtgca
 541 ctcgcgttctgggcatgaaaggcaagaacccagcacgcatacgctaattcacagcgagcaccacacccaaccagaggcgagtggacactc
 631 gccctgagcccacgactgcacctgggcaggcaagccccttaggatgcacattttgccttgacatgcgctgccattccaagcgcacacaag
 721 caggagcaatccttgctgtccacacgcctgcactgccctcacctctcgtgtgcgcgcacatcatgtgtgcgcatgcatgcgtgagacgat
 811 tgcaagcctattggcgattataagtgcagggccctgccggcattgcAcgagcgcatgccttgcaggtgcaccgcgctttgtcccctccat
 901 ctcctggatgtgtgtacaatacctcttgtgataatactaattcaattctcccaaggaggcatgggagctgtgaattttctggcagtgccc
 991 cggcacccatgagacgcgttactcacctgaaatatctgcgctgcatccacatccgatgatacagcaatagcgcgagagcgtctacacact
1081 cttcccccaaagaccaagcagcagagccgcctctctgggcgtgagttcacactgtggtggtgtgaagcggccgtgttcacagtggcatca
1171 tcgagagaagatgccctgcccgaggaaaagtgacccgccgcacccacctgcgccaccaaacaatcatctCTTGTACATCGGTGCACGCAC
1261 CGAGCCCCTCCCCACCGCCACTTCGTCTGCGAAAGCAGGAGGGGTAGGCACGCAGCCATGACGGACGTGGACAGCTTCAGCGGCAACATG
```

Fig. 7. Sequence analysis of the 5′ region of *GDvSPT1* and the following cDNA sequence. The letters of the cDNA sequence are all capitalized. The core motif of the promoter is double underlined with a predicted transcription start site "A" in black font, and the TATA box is framed. The conserved sequence "cacgt" and the two probable initiate codons are in bold font. The three $(ac/gt)_n$ repeat sequences are shaded.

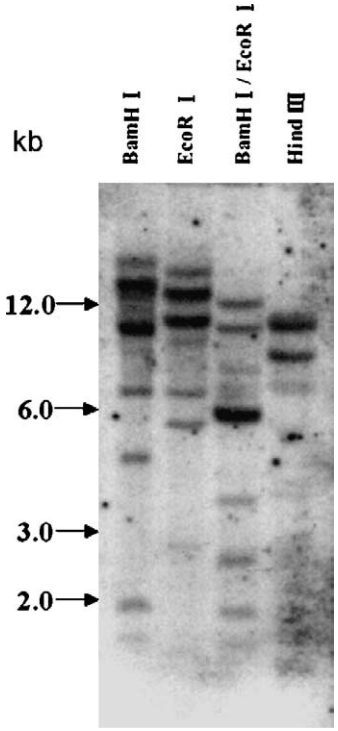

Fig. 8. Southern analysis of *DvSPT1*. Genomic DNA was digested with *Bam*HI, *Eco*RI, *Bam*HI/*Eco*RI, and *Hin*dIII. The *DvSPT1* cDNA was DIG-labeled. The positions of DNA marker fragments and their lengths in kilobases are indicated at left.

C-2.0-CP1A60 as probe. The deduced protein shares high sequence similarity with those Na^+-dependent Pi transport proteins from eubacterial and eukaryotic organisms [3,4]. Two conserved domains exist in both the N- and C-terminal regions of *DvSPT1* and all related sequences (Fig. 3). Conserved amino acids in the sequences of evolutionary distant organisms are likely to represent structurally or functionally important domains and residues. Within these two domains, the two polar amino acids asparagines and aspartate may be particularly relevant for Pi binding and transport activity. Further studies, including site-directed mutagenesis, should give insight into the structure–function relationships within DsSPT1. The largest hydrophilic loop between TM 7 and TM 8 and the hydrophilic N-terminus and C-terminus may also be involved in Pi binding or regulation of transporter activity.

DvSPT1 expression under NaCl hyperosmotic stress and phosphorus depletion starvation showed that the transcripts of *DvSPT1* were enhanced to a high level in a short time for about 1–3 h (Fig. 4), which indicates that *DvSPT1* expression is highly relevant to the NaCl concentration and phosphorus concentration in medium, in agreement with a previous report [17]. They have demonstrated that *Dunaliella* had a high-affinity phosphate uptake system specifically dependent on Na^+ and they have proved that phosphate uptake was dependent on pH and highest rates of uptake were observed at pH 8.

Yeast complementation work further confirmed that *DvSPT1* encoded a Na^+ influx protein.

The previous reports have indicated that intracellular orthophosphate played an important role in triggering osmoregulation in the alga *D. viridis* [34–36]. How should *DvSPT1* function when *D. viridis* is subjected to osmotic stress. We propose that *DvSPT1* may be involved in the process to regulate the intercellular phosphorus concentration under osmotic stress and herein to regulate the intercellular balance of glycerol and starch synthesis or elimination. Under hyperosmotic stress, *DvSPT1* is elevated to a high level to transport more phosphate into the cell and the intracellular phosphate concentration was raised to promote the glycerol synthesis. After the glycerol is enough to balance the osmotic stress, the de novo transcripts of *DvSPT1* will decreased to the normal level.

Acknowledgments

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