European Journal of Phycology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713725516

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Online Publication Date: 01 November 2008

To cite this Article Krell, Andreas, Beszteri, Bánk, Dieckmann, Gerhard, Glöckner, Gernot, Valentin, Klaus and Mock, Thomas(2008)'A new class of ice-binding proteins discovered in a salt-stress-induced cDNA library of the psychrophilic diatom Fragilariopsis cylindrus (Bacillariophyceae)',European Journal of Phycology,43:4,423 — 433

To link to this Article: DOI: 10.1080/09670260802348615

URL: http://dx.doi.org/10.1080/09670260802348615

Eur. J. Phycol., (2008), **43(4)**: 423–433

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A new class of ice-binding proteins discovered in a salt-stress-induced cDNA library of the psychrophilic diatom *Fragilariopsis cylindrus* (Bacillariophyceae)

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(Received 22 May 2007; accepted 16 June 2008)

The psychrophilic pennate diatom *Fragilariopsis cylindrus* is a dominant polar diatom adapted to grow at extremely low temperatures and high salinities prevailing in the brine channels of sea ice. As a basis for deeper investigations of this physiological trait, we used an EST approach to find candidate genes involved in acclimation to salt stress in this diatom. From 2880 cDNA clones sequenced from the 5′ end, 1691 high-quality tentative unique sequences were assembled and analysed. Only 62% of these sequences have homologues in the genomes of two mesophilic diatoms, *Thalassiosira pseudonana* (centric) and *Phaeodactylum tricornutum* (pennate), which share 73–78% of their genes. Of the 1691 sequences, 44.2% could be functionally characterized by comparison with the Swiss-Prot and RefSeq databases. These include sequences encoding different ionic transporters and antiporters, reflecting the requirement to re-establish the ion homeostasis disturbed by exogenous salt stress. Furthermore, numerous genes encoding heat shock proteins (hsps), genes related to oxidative stress, and three key genes involved in the proline synthesis pathway, the most important organic osmolyte synthesized in *F. cylindrus* were identified. A major outcome of this analysis is the finding of four full-length ORFs showing significant similarities to ice-binding proteins (IBPs), which have been shown in a parallel study to be specific to sea ice diatoms, giving evidence of their ability to shape their habitat.

Key words: Antarctic, Arctic, *Fragilariopsis cylindrus*, EST, salt stress, gene expression, ice-binding protein

Introduction

Adaptation to salinity changes is of significance for many marine organisms and this holds even more for the sea ice habitat, which is characterized by extremes in salinity. Thus, it is of major interest to understand how diatoms as major primary producers in sea ice cope with short- and long-term acclimation to disturbances in salinity within the sea ice brine channel habitat. As in higher plants, salt stress in algae severely disturbs the cellular homeostasis brought about by differences between the internal and exogenous concentration of inorganic ions (predominantly Na^+ and Cl^-), which causes water efflux, i.e. a decrease in cell volume and ion influx (Bisson & Kirst, 1995; Kirst & Wiencke, 1995; Kirst, 1996; Erdmann & Hagemann, 2001). As a consequence, salt stress has a severe impact on a variety of metabolic pathways ranging from photosynthesis (Allakhverdiev *et al*., 2002), membrane lipid biosynthesis (Sakamoto & Murata, 2002; Singh *et al*., 2002) to protein folding and turnover (Madern *et al*., 2000; Thomsen *et al*., 2002). Some of the impacts are mediated by the liberation of reactive oxygen species (ROS), causing additional oxidative stress (Mittler, 2002; Jahnke & White, 2003).

The ubiquitous bi-polar diatom *Fragilariopsis cylindrus* was selected as a model species to understand how diatoms are adapted to polar conditions, particularly those within sea ice. This species has been investigated thoroughly in the past, an EST library is available and the genome is currently under sequencing. However, little information is available on the molecular adaptation of *F. cylindrus* to elevated external salinities (Plettner, 2002; Mock *et al*., 2006).

While high external salt concentrations in terrestrial habitats and in rock pools occur in conjunction with elevated temperatures, the reverse is

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ISSN 0967-0262 print/ISSN 1469-4433 online/08/040423–433 © 2008 British Phycological Society
DOI: 10.1080/09670260802348615

true for the polar sea ice habitat, where a combination of salt stress and freezing temperatures prevails. Since very little is known about the molecular underpinnings of this adaptation, we addressed this topic by investigating the acclimation to enhanced salinity using an EST approach.

Recent EST-based as well as whole-genome sequence analyses of diatoms have lead to the finding of genes and pathways novel in photosynthetic eukaryotes (Armbrust *et al.*, 2004; Allen *et al.*, 2006). These investigations have mainly focussed on species from temperate marine environments and the genetic repertoire of the polar diatom *F. cylindrus* as an eukaryotic extremophile will thus contribute valuable new genetic data on stress tolerance and adaptation.

The EST project reported here was conducted to find candidate genes involved in the acclimation response of *F. cylindrus* to the high external salinity that occurs in the brine channel system of sea ice. Furthermore, this study was intended to complement a previously established cold-stress-induced EST library of *F. cylindrus* (Mock *et al.*, 2006) with regard to habitat constraints.

Materials and methods

Culture conditions and experimental setup

Fragilariopsis cylindrus was grown non-axenically in 4.5-l batch cultures at 0°C, using double f/2 medium (Guillard & Ryther, 1962) prepared from Antarctic seawater having a salinity of 33.6. Cultures were continuously illuminated with white fluorescent light (Osram Biolux, Germany) at an irradiance of 15 µmol photons $m^{-2} s^{-1}$. Bubbling with sterile filtered air and gentle stirring with magnetic stirrers ensured sufficient CO_2 supply and mixing. To induce salt stress, a concentrated brine solution was prepared by adding sea salt (Instant Ocean, Aquarium Systems, France) to the original medium. A salinity of 60 was chosen, since previous experiments (Bartsch, 1989) showed that *F. cylindrus* could survive and grow at this salinity, although at only half the rate that it grows at 34. The salt was added during the early exponential growth phase (ca. 1.7×10^6 cells l^{-1}) to prevent subsequent nutrient exhaustion.

In the first experimental set-up, the brine was gradually added to the batch culture with a peristaltic pump to a final salinity of 60 within 3 hours. In the second experimental set-up, to increase the stress conditions, the brine was added at one time. Samples for RNA isolation were taken at various times up to 96 hours after salt addition, thus ensuring the acquisition of transiently regulated genes and genes up-regulated during the later course of the acclimation process.

PAM measurements

In vivo quantum yield of photosystem II (Φ_{PSII}) of the *F. cylindrus* cultures was measured with a Xenon Pulse-Amplitude-Modulated (PAM) Fluorometer (Walz GmbH, Germany), to serve as an indicator of the physiological integrity of the photosynthetic apparatus and, more generally the fitness of the cells. *In vivo* quantum yield was calculated from fluorescence readings of illuminated samples as:

$$\Phi_{PSII} = F'_m - F_t/F'_m$$

where F'_m and F_t denote the maximum and minimum fluorescence in an illuminated sample (Maxwell & Johnson, 2000). Therefore, the measured quantum yield does not reflect the potential maximum yield but reveals the *in situ* conditions.

Isolation of mRNA

For RNA extraction, subsamples of 30 ml of the culture were filtered onto 25-mm, 1.2-µm pore size polycarbonate membrane filters (Millipore) and washed with 20 ml of 0.2 µm filtered seawater. The filters were shock frozen in liquid nitrogen and stored at −80°C. Total RNA from all sampling points of the two experimental set-ups was extracted using an *RNAqueous* kit (Ambion Inc, USA) and subsequent poly A^+ mRNA purification was achieved with a *Poly(A) Purist MAG* kit (Ambion Inc, USA). A total of 2 µg poly A^+ mRNA containing equal fractions of poly A^+ mRNA from both experimental set-ups was employed for first strand synthesis.

cDNA synthesis and library construction

cDNA library construction was carried out using the CloneMiner™ cDNA library construction kit (Invitrogen, Germany). In contrast to other library construction methods, the CloneMiner™ kit does not use restriction enzymes in order to yield longer cDNA fragments and eventually full length clones. During first and second strand synthesis, attB1/2 adaptors were ligated to the 3′and 5′ ends of double stranded cDNA. First strand cDNA was synthesized using the following program: 70°C for 5 min, 37°C for 5 min, allowed to cool for 2 min, before the buffer mix was added (leave tube in cycler). After a further 3 min, Superscript II RT was added, 37°C for 1 : 10 hours, 70°C for 5 min. After second-strand synthesis, a second cleaning step with 160 µl chloroform:isoamylalcohol was carried out. Size fractionation of flanked cDNA was carried out in two steps: first, cDNA was purified with a *SizeSep 400 Spin Column* (Amersham Biosciences, UK). After checking cDNA quality of a sub-sample, cDNA was loaded on a 1% agarose mini-gel having a ladder stained with Sybr-green running parallel to the cDNA, which was not stained at all. Three size fractions, 0.5–1.5 kbp, 1.5–4 kbp and >4 kbp were cut out using a dark reader and extracted with a *Mini Elute Gel extraction* kit (Qiagen, Hilden, Germany), finally yielding 10 µl cDNA per fraction.

The different cDNA size fractions were directionally cloned into the attP- containing vector pDONR™ 222 through site specific recombination. Afterwards the vector was transformed into competent *E. coli* (ElectroMax™ DH10B™) phage-resistant cells

through electroporation. Insert screening to determine the percentage of recombinants was performed by *BsrG I* digestion and revealed a low value of 54% for the 4+ kbp fraction. Therefore, this fraction was omitted during later sequencing.

Sequencing and annotation

Plasmid DNA extracted from overnight cultures of cDNA clones from the library were sequenced starting at the 5′ end using the M13 forward sequencing primer and BigDye terminator chemistry (Applied Biosystems, Germany). Sequencing reaction products were separated on ABI3700 96 capillary machines. Base calling, vector masking, rRNA masking using known ribosomal sequences deposited in GenBank and sequence quality assessment was performed using Phred (Ewing & Green, 1998; Ewing *et al*., 1998). Sequences with a Phred score of less than 20 were rejected from the data set.

EST clustering and assembly analysis. The Phrep algorithm with standard parameters was employed for clustering of sequences. Sequence clusters were inspected manually with the help of the Staden package (Staden *et al*., 1998).

Sequence comparison and functional classification. To yield a possible function of sequences in the non-redundant set (NRS), matches to existing sequences of different non-redundant datasets were performed querying a six-frame translation of the sequences using tBLASTX (Altschul *et al*., 1997) against the Swiss-Prot and RefSeq databases on a local Sun system. Furthermore, individual databases of all available genome sequences from *T. pseudonana*, (version3; genome.jgi-psf.org/Thaps3/Thaps3.home.html), *P. tricornutum* (version2; genome.jgi-psf.org/Phatr2/Phatr2.home.html), *Chlamydomonas sp*. (chlamy.org/chlamydb.html), *Arabidopsis thaliana* (arabidopsis.org) and *Oryza sativa*, (tigr.org/tdb/e2k1/osa1/), as well as EST information from *Physcomitrella patens* (moss.leeds.ac.uk/), *Cyanidioschyzon merolae* (merolae.biol.s.u-tokyo.ac.jp/) and *Porphyra yezoensis* (kazusa.or.jp/en/plant/porphyra/EST/) were queried in the same way. Functional domains were searched against the InterPro (ebi.ac.uk/interpro/) database and genes were classified according to GeneOntology (geneontology.org/).

Sequence analysis of candidate IBPs. After the identification of ORFs yielding known ice-binding proteins from *Typhula ishikariensis* and *Navicula glaciei* as BLAST hits, different bioinformatics approaches were used to further test whether these sequences indeed belong to a family of ice-binding proteins. Amino acid sequences of potential homologues were collected using PSI-BLAST (Altschul *et al*., 1997) from the non-redundant protein sequence database (nr) of NCBI and a translated EST from *Lentinula edodes* was added to the data set. Multiple alignments were calculated using the F-INS-i algorithm of MAFFT (Katoh *et al*., 2005). The aligned sequences were classified into putative protein subfamilies using SciPhy (phylogenomics.berkeley.edu/cgi-bin/SCI-PHY/input_SCI-PHY.py) and by phylogenetic analyses. Maximum likelihood trees were calculated and bootstrapped in 100 replicates using PhyML (Guindon & Gascuel, 2003). Neighbour-joining trees were calculated and bootstrapped using PHYLIP (Felsenstein, 2005), using the JTT substitution model (200 bootstrap replicates), with QuickTree (Howe *et al*., 2002), using uncorrected distances and with a modified version of this program using Scoredist distances (Sonnhammer & Hollich, 2005; Beszteri & Frickenhaus, pers. comm.) with 1000 bootstrap replicates. Maximum parsimony analyses were performed and bootstrapped in 1000 replicates using PAUP* (Swofford, 1998) with heuristic search.

Results and discussion

Description of the cDNA library

After base calling, assessment of sequence quality, vector trimming and trimming for ribosomal sequences, an initial EST collection of 2099 high-quality 5′ sequences larger than 100 bp, having an average edited length of 612 bp were obtained from the cDNA library. This initial collection of ESTs was clustered and assembled to yield a set of 1691 tentative unique sequences (TUs). This set contained a total of 209 tentative consensus sequences (TCs) assembled from 608 sequences and an additional 1482 sequences, uniquely represented in the EST collection, which are referred to as singletons (Table 1). All high quality sequences were deposited at the dbEST section of NCBI (www.ncbi.nlm.nih.gov/dbEST/) under accession numbers DR025674 to DR027056 and EL737249 to EL737964. A comprehensive online database of *F. cylindrus* EST sequences, so far containing sequences from this cDNA library and a previously described cold-shock library (Mock *et al*., 2006) is accessible at www.genome.fli-leibniz.de/ALGAE/cgi-bin/Index.pl. Assuming that the 11 242 predicted genes from the genome of *T. pseudonana*

Table 1. Overview of the *F. cylindrus* salt-stress cDNA library.

Sequence type	No. of sequences	Average length (bp)	Total characters (bp)
Clones sequenced	2880		
High quality single reads (Phred20, no rRNA, min 100 bp)	2099	612	1 283 603
Tentative unique sequences (TUs)	1691	630	1 065 577
Singletons	1482	598	886 599
Tentative consensus sequences (TCs)	209	856	178 978
G/C content	0.41		

Table 2. Number of sequences from the *F. cylindrus* NRS (1691 sequences) finding significant hits (BLAST *e*-values $\leq 1e^{-04}$) in pairs of other protein sequence datasets.

	Total number of hits in each database	Swiss-Prot/ RefSeq	*Chlamydomonas reinhardtii*	*C. merolae*/ *P. EST*	*T. pseudonana*/ *P. tricornutum*	*Physcomitrella patens*	*A. thaliana*/ *O. sativa*
Swiss-Prot/RefSeq	748	**41**	363	454	595	351	520
Chlamydomonas reinhardtii	385		**3**	325	281	263	352
C. merolae/P. EST	482			**5**	378	300	407
T. pseudonana/*P. tricornutum*	1014				**396**	293	436
Physcomitrella patens	359					**1**	336
A. thaliana/*O. sativa*	549						**3**

Bold values in the diagonal give the number of sequences producing hits exclusively within the respective dataset.

(Armbrust *et al.*, 2004) also represent the total number of genes present in *F. cylindrus* (the genome size is comparable; Mock & Glöckner, pers. comm.); we have identified roughly 15% of the expressed genome within this EST collection. Together with the 996 sequences from the cold-shock library this equals approx. 20%. Compared to the 10 025 genes predicted in the present version v.2 (genome.jgi-psf.org/Phatr2/Phatr2.home.html) from *P. tricornutum*, 27% of the transcriptome has been identified. A total of 1147 sequences (68%) retrieved a significant hit ($e < 10–4$) in at least one of the queried databases. A putative function via significant similarities to Swiss-Prot and RefSeq could be assigned to 747 (44.2%) of the TUs. Five hundred and forty-four TCs or singletons (32% of the TUs) remained unique, i.e. produced no significant hit against any sequence database. Furthermore, functional protein domains could be assigned to 70 of the 544 unique sequences via an InterPro search. A similar proportion of ESTs could be identified in other cDNA libraries of chromophytic algae, e.g. *Laminaria digitata* 39–48%, (Crépineau *et al.*, 2000); *P. tricornutum* < 40%, (Scala *et al.*, 2002; Montsant *et al.*, 2005) and green alga *Ulva linza* 48% (Stanley *et al.*, 2005). The number of sequences producing significant hits might have been higher in a library established under unstressed conditions, since it has been shown that strong abiotic stress may lead to an increase in 'non-meaningful' ESTs, i.e. no hit in GenBank (Bohnert *et al.*, 2001).

Of the 1691 *F. cylindrus* TUs, 1014 sequences shared similarities with genes in the genomes of *T. pseudonana* or *P. tricornutum* and 796 sequences were common in all three datasets. The remaining 677 (40%) TUs were specific to *F. cylindrus*, i.e. have no homologues in the two mesophilic species. *F. cylindrus* shared more sequences exclusively with *P. tricornutum* (184) than with *T. pseudonana* (34), which is indicative of the closer phylogenetic relationship between the pennate diatoms. Furthermore, of the sequences common to all three diatoms, the *F. cylindrus* sequences showed a significantly higher similarity to their counterparts from *P. tricornutum* than to those from *T. pseudonana* (paired *t*-test based on bit score values: mean difference: 8.9; $n = 796$; $t = -3.89$; $p < 0.0001$). The fact that 396 TUs (Table 2) were exclusively present in diatoms but in no other database is consistent with the fact that a large fraction of diatom genes is still functionally uncharacterized and might be specific to them. To compare the gene content of *F. cylindrus* with that of the two mesophilic diatoms with a genome sequence available (*P. tricornutum* and *T. pseudonana*) taking into account the limitations inherent to the EST approach, we compared our EST data set to EST sets from these diatoms using BLASTX (with an *e*-value cut-off of 10-4). From this comparison it can be concluded that the two mesophilic species *P. tricornutum* and *T. pseudonana* share a larger proportion of genes with each other (71.8% of 1243 *P. tricornutum* ESTs found in *T. pseudonana*, and 73.4% of 1631 *vice versa* – these numbers are similar when comparing the complete sets of predicted proteins), than *F. cylindrus* shares with *P. tricornutum* or *T. pseudonana*, respectively (52% of 1691 *F. cylindrus* ESTs found in *T. pseudonana* and 55% of them found in *P. tricornutum*). This higher proportion of unknown sequences in *F. cylindrus* might be attributed to the diverse habitats they occur in and the requirement of *F. cylindrus* to adapt to the extreme environment of the polar realm.

Twenty-seven TCs consisted of four or more single reads, representing the most abundant TCs. Most (89%) of the abundant TCs in our library could be assigned a putative function (Table 3), in contrast to other EST libraries from *F. cylindrus* (Mock *et al.*, 2006) and from *P. tricornutum* (Montsant *et al.*, 2005), where only 40% and 60%, of the most abundant ESTs were identified, respectively. However, the most abundant TC (Fcyl0044d08) comprising 2.8% of all high quality sequences is a yet unknown sequence. Two other unidentified TCs (Fcyl0043a06 and Fcyl0043h05)

Table 3. Most abundant (≥4 single reads) tentative consensus sequences (TCs) and best hit to the Swiss-Prot database.

Internal name	No. of ESTs	TC length	Function	*e*-value
Fcyl0044d08	59	1313	(no hit)	
Fcyl0051h06	42	817	Fucoxanthin–chlorophyll *a*–*c*-binding protein	8e-67
Fcyl0044a07	10	1532	S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1)	1e-179
Fcyl0047c10	7	2127	Heat shock 70 kDa protein.	6e-250
Fcyl0036b05	7	1070	NADP-dependent L-serine/L-allo-threonine DH	3e-6
Fcyl0047h05	6	702	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	2e-61
Fcyl0054g05	6	1265	Hypothetical protein sll1483 precursor.	2e-28
Fcyl0027b06	6	585	Clathrin heavy chain.	2e-50
Fcyl0051d11	5	1410	Hypothetical protein yqjG.	1e-48
Fcyl0042g12	5	892	NAD(P)H:quinone oxidoreductase (EC 1.6.5.2)	6e-28
Fcyl0041b08	4	934	Chlorophyll *a*–*c*-binding protein	2e-33
Fcyl0041g02	4	1065	Fructose-bisphosphate aldolase (EC 4.1.2.13)	1e-81
Fcyl0044h09	4	972	60S ribosomal protein L5-1.	2e-77
Fcyl0045b02	4	1124	Hypothetical 57.5 kDa protein	1e-9
Fcyl0042d03	4	903	Chloroplastic quinone-oxidoreductase homolog	1e-23
Fcyl0043a06	4	808	(no hit)	
Fcyl0045e07	4	1384	Eukaryotic initiation factor 4A (EC 3.6.1.-)	1e-143
Fcyl0048b07	4	765	Putative protein disulfide-isomerase (EC 5.3.4.1)	3e-14
Fcyl0039d09	4	1534	Probable serine hydrolase	1e-10
Fcyl0051b05	4	807	Fucoxanthin–chlorophyll *a*–*c*-binding protein	1e-32
Fcyl0047d12	4	926	Fucoxanthin–chlorophyll *a*–*c*-binding protein	7e-5
Fcyl0030h03	4	734	Hypothetical protein	4e-18
Fcyl0049b05	4	1176	Pyrroline-5-carboxylate reductase (EC 1.5.1.2)	1e-34
Fcyl0043h05	4	1303	(no hit)	
Fcyl0039b06	4	1359	Enolase (EC 4.2.1.11)	2e-135
Fcyl0038g05	4	979	DNA translocase ftsK 1.	6e-6
Fcyl0050f10	4	346	Protein ccdA (Protein letA) (Protein H) (LynA)	1e-29

seemed to be specific for diatoms, since similar sequences were found only in the diatom genomes. The occurrence of three fucoxanthin–chlorophyll *a*–*c*-binding proteins and one chlorophyll *a*/*b*-binding protein among the 27 most abundant TCs is comparable to their occurrence in the *P. tricornutum* EST set (Montsant *et al.*, 2005) and the cold-shock library. This reflects the importance of the light harvesting complex (LHC) protein family in stress acclimation. Severe photoinhibition, manifested as a strong decline in photosynthetic quantum yield has been shown to occur upon cold stress (Mock & Valentin, 2004) and even more upon salt stress (Fig. 1). Thus, a restructuring of the LHC in response to these stressors is a major part of stress acclimation in *F. cylindrus*. The occurrence of four ESTs encoding enolase (Fcyl0039b06), which is one of the enzymes involved in the energy yielding phase of glycolysis, emphasizes the importance of catabolic energy gain under conditions where photosynthetic energy production is limited.

Sequences encoding proteins belonging to the class of peptidyl-prolyl cis-trans isomerases (PPIases) and a disulphide isomerase are also represented within the most abundant TCs (Fcyl0047h05, Fcyl0048b07). They catalyse the isomerization of peptide bonds of proline residues, a rate-limiting step in the folding of newly synthesized proteins, activated by different stressors, including cold and drought stress (Miernyk, 1999). A total of six different TCs and singletons

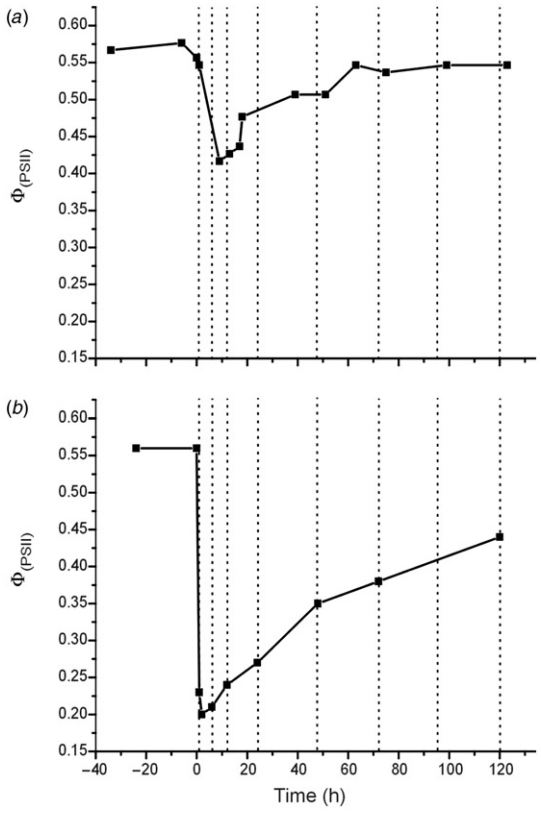

Fig. 1. Temporal development of the photosynthetic quantum yield at photosystem II (Φ_{PSII}) during the course of the two salt shock experiments ($n = 2$). (*a*) Gradual increase of salinity to 60 within 3 hours; (*b*) instantaneous increase of salinity to 60. The addition of the concentrated brine solution in both experiments started at time point 0. The dashed lines denote sampling points for RNA extraction.

encoding cyclophilins (CYP20, CYP40, CYP-A) and two FK506-binding proteins (FKBPs) could be identified. Cyclophilins also protect cells against oxidative stress (Doyle *et al*., 1999) and are potentially regulated by thioredoxin (Motohashi *et al*., 2003). Reactive oxygen species (ROS) generated by salt stress is another source of damage in plant cells (Jahnke & White, 2003). Our cDNA library contained a number of genes coding proteins involved in the detoxification and scavenging of ROS, e.g. glutathione synthetase (Fcyl0045c07), peroxiredoxin, and thioredoxin (Table 4). Glutathione content has been shown to be increased in higher plants subjected to salt stress (Ruiz & Blumwald, 2002) and in algae under high light intensities, since it acts as an intermediate in

Table 4. Selected genes potentially related to osmotic stress tolerance found in this cDNA library.

Internal name	Function	No. of ESTs	*e*-value
Organic osmolytes			
Fcyl0049b05	Pyrroline-5-carboxylate reductase	4	1e-34
Fcyl0039a09	Prolin dehydrogenase	1	3e-37
Fcyl0044g07	Ornithine aminotransferase, mit. precursor (EC 2.6.1.13)	1	3e-70
Fcyl0045a03	Argininosuccinate synthase (EC 6.3.4.5)	1	2e-70
Fcyl0033f11	Spermidine synthase 1 (EC 2.5.1.16)	1	2e-06
Fcyl0053g06	S-adenosylmethionine synthetase (EC 2.5.1.6)	1	4e-63
Transporter/ion homoeostasis			
Fcyl0032b02	Na+/H+ antiporter	1	4e-15
Fcyl0052d12	Glutathione-regulated K^+/H^+ antiporter	1	1e-34
Fcyl0048a11	$Na^+/K^+/Ca^{2+}$-exchange protein 1	1	1e-41
Fcyl0049f08	Sodium-dependent phosphate transporter 1	1	2e-31
Fcyl0049f03	Calcium-transporting ATPase 1 (EC 3.6.3.8)	1	8e-14
Fcyl0047b10	V-ATPase A subunit (EC 3.6.3.14)	1	7e-104
Fcyl0053g12	V-ATPase 16 kDa proteolipid subunit (EC 3.6.3.14)	1	6e-51
Fcyl0044g02	V-ATPase 21 kDa proteolipid subunit (EC 3.6.3.14)	1	2e-34
Fcyl0029c03	Putative cation-transporting ATPase (EC 3.6.3.-)	1	3e-34
Fcyl0046e06	Probable cation-transporting ATPase F(EC 3.6.3.-)	2	6e-32
Fcyl0048g08	Probable calcium-transporting ATPase (EC 3.6.3.8)	1	7e-32
Fcyl0049a11	PP-energized vacuolar membrane proton pump (EC 3.6.1.1)	1	4e-28
Fcyl0041f03	PP-energized vacuolar membrane proton pump (EC 3.6.1.1)	2	3e-95
Chaperones			
Fcyl0041b10	Heat shock protein STI (Stress-inducible protein)	1	4e-27
Fcyl0030f10	GroEL (60 kDa chaperonin 1)	1	8e-39
Fcyl0034b11	GroES (10 kDa chaperonin)	2	2e-13
Fcyl0047c10	Heat shock 70 kDa protein	7	6e-250
Fcyl0038e01	Heat shock 70 kDa protein	1	7e-50
Fcy0052e08	DNAJ protein homolog 2	2	1e-66
Fcyl0031g04	Heat shock protein 83	1	3e-24
Fcyl0048f04	Heat shock protein 83	1	1e-78
Fcyl0041f05	Probable chaperone HSP31	1	2e-15
Oxidative stress defense			
Fcyl0045c07	Glutathione synthetase (EC 6.3.2.3)	1	2e-21
Fcyl0047f09	Peroxiredoxin (EC 1.11.1.15)	1	4e-16
Fcyl0045b09	putative thioredoxin peroxidase	1	1e-10
Fcyl0046d05	Tocopherol O-methyltransferase, chloroplast precursor	1	3e-8
Fcyl0042b10	Pyridoxin biosynthesis protein PDX1	1	4e-96
Signalling/stress perception			
Fcyl0037h12	Cell wall integrity and stress response component 3 prec.	3	4e-15
Fcyl0023a08	ATPase-like:Histidine kinase A	1	2e-08
Fcyl0036b01	Sensory box sensor histidine kinase/response regulator	1	6e-10
Fcyl0052e03	Two-component system sensor histidine kinase	1	3e-11
Fcyl0046a06	14-3-3-like protein	2	1e-87
Fcyl0043e09	Putative two-component sensor	1	3e-18
Fcyl0052a07	Phosphatidylinositol 4-kinase	1	5e-19
Fcyl0023a09	Protein SIS2 (Halotolerance protein HAL3)	1	9e-9
Protein degradation			
Fcyl0032g01	Ubiquitin-conjugating enzyme E2 (EC 6.3.2.19)	1	1e-18
Fcyl0054f05	Ubiquitin	1	3e-32
Fcyl0049d01	Ubiquitin carboxyl-terminal hydrolase 19 (EC 3.1.2.15)	1	1e-16
Fcyl0043e02	Ubiquitin carboxyl-terminal hydrolase 3 (EC 3.1.2.15)	1	7e-21
Fcyl0029f11	Ubiquitin-activating enzyme E1	1	3e-17

ROS removal during light saturation of photosynthesis (Dupont *et al.*, 2004). Taken together these results imply that ROS scavenging pathways might play a role in the salt tolerance of *F. cylindrus* as is the case in higher plants, where oxidative stress-related genes were highly abundant in EST libraries established under salt stress (Wang *et al.*, 2006).

Physiological response on salt stress and a new class of ice-binding proteins

To assess the impact of changes in external salinity on the physiological integrity of the photosynthetic machinery, measurements of the *in vivo* quantum yield of PS II (Φ_{PSII}) were carried out, serving as a proxy for overall cellular fitness. This data demonstrated that the cultures were stressed by the applied salinity shock. In the first experiment, Φ_{PSII} dropped from 0.57 to 0.42 within 9 hours after salt addition (Fig. 1*a*). During the course of the experiment, Φ_{PSII} gradually recovered, reaching values of ~0.55, which were close to pre-shock levels. During the second shock treatment (Fig. 1*b*), average Φ_{PSII} values instantly dropped from 0.56 before treatment to 0.20 within 2 hours after the salt addition. Thereafter photosynthesis recovered steadily as shown by increasing Φ_{PSII}, yet during the sampling period did not attain values as high as before the salt shock. In both experiments, the decrease of the photosynthetic quantum yield showed that increased salinity led to a disturbance of cellular homoeostasis. Adding the brine at once had a much stronger inhibitory effect on Φ_{PSII} than adding it gradually. This reflects an increased influence of the osmotic component of the salt shock. Hence, metabolic pathways to counteract the negative effects of this salt stress need to be activated. Our data shows, that a large number of tentative unique sequences could be assigned to genes involved into various metabolic processes crucial in salt stress acclimation, e.g. osmolyte production, ion homeostasis, ROS scavenging and protein degradation (Table 4). They include several genes required for the synthesis and degradation of proline, the main organic osmolyte in *F. cylindrus* (Plettner, 2002). One of the most abundant TCs (Table 3) is a gene coding pyrroline-5-carboxylate reductase (P5CR), which catalyses the final step in the synthesis of proline, thus stressing the importance and need for the synthesis of organic osmolytes under salt stress. In addition to P5CR, we also found a sequence encoding proline dehydrogenase, the first enzyme in the catabolic pathway (Verma, 1999) of proline. Furthermore, a singleton encoding ornithine aminotransferase was found, suggesting that L-ornithine is used in addition to L-glutamate as a substrate for the synthesis of proline (Fig. 3). The importance of the ornithine pathway was verified by the up-regulation of ornithine aminotransferase during salt stress (Krell *et al.*, 2007). This also established a link to the urea cycle; which was recently found to be present in diatoms (Armbrust *et al.*, 2004). No sequence encoding the rate-limiting step in proline synthesis from glutamate (Δ-1-pyrroline-5-carboxylate synthase, P5CS) was found among the salt stress-induced ESTs sequenced. Although P5CS is known from the cold-shock library, its absence in the salt stress library was not surprising in view of the recent finding that it was strongly down-regulated upon salt stress (Krell *et al.*, 2007). One singleton and one TC (Fcyl0044a07) are homologues to genes involved in Glycine-betaine (Gly-betaine) synthesis, another important osmolyte in *F. cylindrus* (Plettner, 2002). The synthesis of Gly-betaine involves several methylations of phosphoethanolamine. The gene encoding *S*-adenosyl-homocysteine (SAH),-hydrolase (Fcyl0044a07), responsible for the degradation of SAH a potent inhibitor of *S*-adenosyl-Methionin(SAM)-dependent methyltransferases, belongs to the most abundant TCs (Table 3) in this library, stressing the importance of this protein in maintaining methylation activity during salt stress (Weretilnyk *et al.*, 2001). The destruction of cellular ion homoeostasis is one of the most immediate and most severe damages caused by salt stress (Erdmann & Hagemann, 2001). The need for the cells to re-establish ion homoeostasis is reflected by the high number of different ionic transporter and antiporter genes found in this library. The finding of several sequences encoding different types and subunits of V-type H^+ ATPase, some even represented by more than one clone, as well as antiporters for various ions (Na^+, K^+, Ca^{2+}) (Table 4) stresses the significance of these proteins in salt stress response (Allakhverdiev *et al.*, 2000; Shi *et al.*, 2003). Two other TUs encode a high-affinity fructose transporter (Fcyl0039g06) and a monosaccharide transporter (Fcyl0036h07) both membrane proteins are involved in the synthesis of exopolysaccharides. The exudation of exopolymeric substances has been recognized as a common trait of eukaryotic and prokaryotic organisms dwelling in sea ice to help maintain a liquid environment important for the survival of organisms in sea ice (Krembs *et al.*, 2002; Mancuso Nichols *et al.*, 2004).

Unexpected in the context of salt stress acclimation was the finding of sequences similar to sequences encoding antifreeze proteins. Antifreeze proteins, originally discovered in polar fish (DeVries, 1969) have been isolated from a number of higher plants (Griffith *et al.*, 2005), fungi (Hoshino *et al.*, 2003), bacteria (Duman &

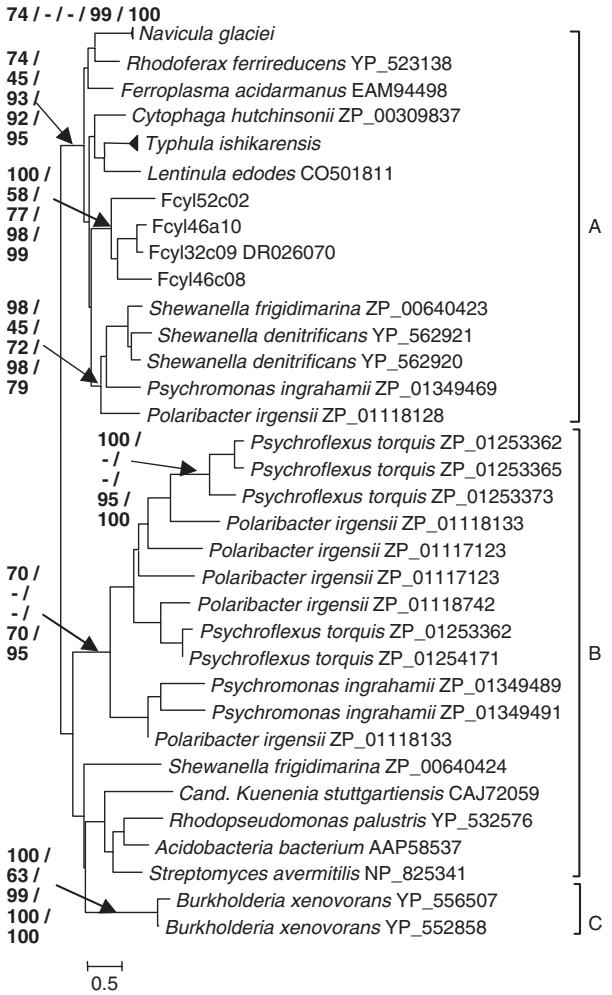

Fig. 2. Maximum likelihood phylogenetic tree of the candidate ice-binding protein sequences from our EST library and homologues (incl. Accession number) found in the Genbank database. The group marked *Typhula ishikarensis* comprises seven homologues (BAD02891–BAD02897) and four homologues from the diatom *Navicula glaciei* (AAZ76250–AAZ76254), (Janech *et al*., 2006) are also grouped together. The latter sequences were found with primers based on the *F. cylindrus* 32c09 sequence (DR026070). Numbers on the nodes indicate bootstrap values from neighbour joining analyses with uncorrected, JTT and ScoreDist distances, maximum parsimony and maximum likelihood analysis, respectively (only bootstrap values above 60% are shown). The three groups marked A, B and C are the 'subfamilies' identified by SciPhy.

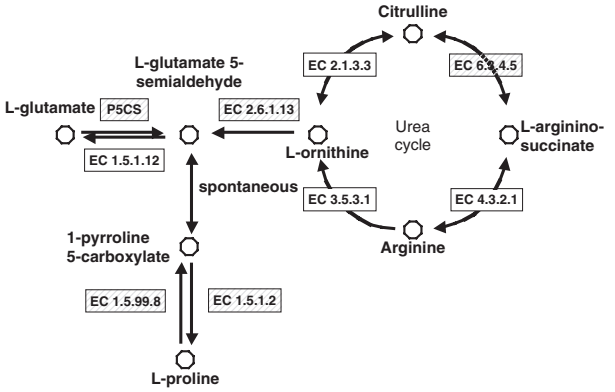

Fig. 3. Substrates and enzymes involved in proline metabolism and linked to the urea cycle. Genes encoding the shaded enzymes (ornithine δ-aminotransferase (δ-OAT, EC 2.6.1.13), Δ^1-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2), proline dehydrogenase (ProDH, EC 1.5.99.8) and argininosuccinate synthase (EC 6.3.4.5) were found in this library, while the gene for Δ^1-pyrroline-5-carboxylate synthase (P5CS, EC not assigned) was identified in the cold-shock library.

Olsen, 1993) and insects (Duman, 2001). Whereas proteins isolated from animals typically have the ability to depress the freezing point to actually avoid the formation of ice crystals, those proteins from plants and bacteria mainly inhibit ice re-crystallization, i.e. the growth of large ice crystals at the expense of smaller ones with smaller ones having less damaging effects on biological tissues (Griffith *et al*., 2005; Raymond *et al*., 2007). Four TUs from our library were similar to sequences encoding antifreeze proteins isolated from the snow mould fungi *Typhula ishikariensis* (Fig. 2) (Hoshino *et al*., 2003). One of these sequences (Fcyl0032c09) has already been described in detail and degenerated primers based on this sequence have lead to the identification of similar sequences in *Navicula glaciei* (Janech *et al*., 2006). All four sequences contained complete ORFs. Different sequence clustering methods (Flower Power, SciPhy and different phylogenetic analysis methods) all classified the *F. cylindrus* IBP sequences with the *Typhula* and *Navicula* IBPs in a single subfamily (Fig. 2). Notably, this group of sequences shows no significant similarity to any other antifreeze proteins from either the animal or plant kingdoms, suggesting that these sequences represent a new class of antifreeze proteins formerly unknown in photosynthetic eukaryotes. The group contains a high proportion of non-polar (alanine, glycine, leucine and isoleucine: around 40% together) and polar, neutral amino acids (threonine and serine: 20%). Secondary structure prediction results from the SAM-T2k pipeline reveal the presence of two alpha helices and several beta sheets, the latter separated in part by short coiled regions, which might provide the basis for a beta-helical tertiary structure as observed in insect and budworm antifreeze proteins (Graether *et al*., 2000; Leinala *et al*., 2002). The length of the open reading frame (ORF) between three of the isoforms varied only by 12 (266–278) amino acids, while one isoform (Fcyl0052c02) was considerably longer (359 amino acids, Table 5). In contrast to the former, this sequence contained no signal peptide according to the neural network prediction in SignalP (Bendtsen *et al*., 2004), while the others belonged to the secretory pathway with high probability

Table 5. Sequence characteristics of ice-binding proteins found in *F. cylindrus* and BLAST alignment score against the most similar *T. ishikariensis* antifreeze proteins.

Internal name	No. of ESTs	Total length	5′UTR	ORF	3′UTR	Score
Fcyl0032c09	3	963	64	834	49	177
Fcyl0046a10	2	948	65	822	47	182
Fcyl0046c08	1	886	48	798	25	155
Fcyl0052c02	1	1280	66	1077	119	161

(0.795–0.930), and may thus be released into the extracellular space. The AFPs found in *Typhula ishikariensis* were secreted into the extracellular space of the fungi and observations of Hoshino *et al*. (2003) indicated that these AFPs can bind to surfaces of ice crystals to inhibit their growth. This physiological ability would be of fundamental importance in the sea ice diatom *F. cylindrus*, in order to grow in brine channels between sea ice crystals without being damaged by freezing. Furthermore, the release of such substances by diatoms that bind to and affect the growth of ice was already proposed by Raymond *et al*. (1994) and Raymond and Knight (2003). None of the new IBP sequences could be found in the genomes of *T. pseudonana* and *P. tricornutum*, supporting evidence that these proteins are essential for the adaptation to polar environments. In addition, none of these sequences could be found in the previously established cold-shock library (Mock *et al*., 2006). Thus, the principal stimulus triggering the release of these proteins might be salt stress. Meijer *et al*. (1996) showed that salt stress was able to enhance the expression of a winter flounder AFP in *E. coli*. Thus, these proteins might ameliorate negative effects of salt stress, in addition to exhibiting antifreeze activity. Further expression studies aimed at the involvement of these proteins in the responses to freezing and salt stress will be carried out.

Conclusions

A non-redundant set of 1691 genes was produced from a salt stress-induced *F. cylindrus* cDNA library. Of the ESTs whose putative functions were identified, many were associated with abiotic stress responses in general and some were specifically associated with salt stress acclimation. Many of the ESTs from the *F. cylindrus* library were not found in the genomes of two mesophilic Bacillariophyceae, possibly reflecting the adaptation of *F. cylindrus* to its cold and salty habitat in its genetic repertoire. The finding of numerous sequences related to the synthesis of osmolytes and transporters to re-establish ion homoeostasis proved this EST approach to be a valuable tool for mining for salt-stress-related genes. The finding of a new class of ice-binding proteins formerly unknown in animals and plants and obviously also not present in mesophilic species of diatoms is a major result of this study and sheds new molecular light on the manipulation of the environment by sea ice diatoms. These results should pave the way for large-scale expression studies in *F. cylindrus* and may even provide new target genes for the engineering of improved drought and salt resistance in crop plants.

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