REGULATION OF PROLINE METABOLISM UNDER SALT STRESS IN THE PSYCHROPHILIC DIATOM *FRAGILARIOPSIS CYLINDRUS* (BACILLARIOPHYCEAE)¹

Andreas Krell²

Alfred Wegner Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

Dietmar Funck

Department of Plant Physiology and Biochemistry, University of Konstanz, 78457 Konstanz, Germany

Ina Plettner

Department of Marine Botany, University of Bremen, 28334 Bremen, Germany

Research Centre Borstel, Leibniz Center for Medicine and Biosciences, Parkallee 22, 23845 Borstel, Germany

Uwe John and Gerhard Dieckmann

Alfred Wegner Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

Fragilariopsis cylindrus (Grunow) Willi Krieger, a bipolar psychrophilic and highly abundant diatom, experiences strong shifts in salinity during the formation of sea ice. We investigated the effects of osmotic stress due to an increased salt concentration from 34 to 70 practical salinity units (PSU) in conjunction with a temperature decrease from 0°C to -4°C on the anabolic and catabolic pathways of proline metabolism. Gene expression levels of Δ^1 -pyrroline-5-carboxylate synthase (*P5CS*) strongly decreased by a factor of 17.3 in the 70 PSU/0°C treatment, whereas copy numbers of ornithine δ -aminotransferase (*δ -OAT*) increased 7.6-fold. Transcript levels of Δ^1 -pyrroline-5-carboxylate reductase (*P5CR*) and proline dehydrogenase (*ProDH*) were also slightly up-regulated by 2.5 and 2.88, respectively. This contrasts with findings in higher plants where a reverse regulation of *P5CS* and *δ -OAT* was observed and indicates that under elevated external salinities, the ornithine route is preferred to the glutamate pathway in *F. cylindrus*. Photosynthetic quantum yield at PSII instantly dropped from 0.61 to 0.24 after the upshift in salinity, which confirmed a detrimental effect of elevated salt concentrations on the photosynthetic machinery. Reduced photosynthetic energy capture might explain the preference for the ornithine route over the more energy-consuming proline route. Salt proved to be the dominating stressor, while an additional temperature decrease appeared to have an ameliorating effect.

Key index words: *δ -OAT*; *Fragilariopsis cylindrus*; gene expression; osmotic stress; *P5CR*; *P5CS*; photosystem II; *ProDH*; proline metabolism; sea ice

Abbreviations: *δ -OAT*, ornithine δ -aminotransferase; *EST*, expressed sequence tag; *P5CR*, Δ^1 -pyrroline-5-carboxylate reductase; *P5CS*, Δ^1 -pyrroline-5-carboxylate synthase; *ProDH*, proline dehydrogenase

Sea ice is an important structuring feature of polar ecosystems, with strong gradients in temperature, light, space, and salinity through an ice floe (Eicken 1992). Compared to freshwater ice, sea ice is not solid since dissolved constituents of seawater do not enter the ice crystal structure but are expelled as a highly concentrated brine solution into a network of channels and pores within the ice matrix (Weissenberger et al. 1992). The freezing process is very dynamic, and once seawater has reached its freezing point, the rate of ice growth primarily depends on the overlying air temperature. Physicochemical processes within the brine channel system are primarily governed by the relationship between temperature and brine formation, which influences chemical parameters (dissolved inorganic nutrients, dissolved gases, pH), space, and light (Eicken 2003, Papadimitriou et al. 2004). Environmental conditions in the ice are characterized by temperatures between -1.8°C and -20°C, and corresponding salinities ranging from 35 to 212 practical salinity units (PSU; Cox and Weeks 1983). However, despite these harsh conditions, sea ice is densely populated by microorganisms, the most conspicuous of them being pennate diatoms (Günther and Dieckmann 2001). *Fragilariopsis cylindrus* is one of the dominating diatom species in polar realms, thriving equally well in the water column and sea ice (Kang and Fryxell 1992).

Acclimation to changing osmotic conditions is a prerequisite for all cellular life. An altered external

¹Received 10 October 2006. Accepted 8 March 2007.

²Author for correspondence: e-mail andreas.krell@awi.de.

increase or decrease in the concentration of inorganic ions (primarily Na^+ and Cl^-) results in a flow of water across the semipermeable cell membrane and an influx or efflux of ions leading to a disturbance of cellular homeostasis.

To counteract the negative effects of osmotic stress on metabolism—namely, to restore the internal osmotic potential—plants accumulate organic osmolytes, synonymous with the term “compatible solutes” (Brown and Simpson 1972). Compatible solutes are highly soluble, low-molecular-weight organic molecules without net charge at physiological pH. Therefore, they can be accumulated in high concentrations without interfering with the cellular metabolism (Kirst and Wiencke 1995, DasSarma and Arora 2001, Chen and Murata 2002). Among the compatible solutes, proline appears to be the most widely distributed osmolyte accumulated under osmotic stress, not only in higher plants but also in eubacteria, protozoa, marine invertebrates, and algae such as *F. cylindrus* (Kirst 1990, Delauney and Verma 1993, Erdmann and Hagemann 2001). Recently, the ability of osmolytes, especially proline, to scavenge reactive oxygen species has been observed (Hong et al. 2000, Reddy et al. 2004, Rodriguez and Redman 2005).

In eukaryotes, proline is synthesized from glutamate via Δ^1 -pyrroline-5-carboxylate (P5C) in two successive reductions catalyzed by Δ^1 -pyrroline-5-carboxylate synthase (P5CS), a bifunctional enzyme encompassing prokaryotic gamma glutamyl kinase (GK; EC 2.7.2.11) and glutamyl phosphate reductase (GPR; EC 1.2.1.41) activity, and Δ^1 -pyrroline-5-carboxylate reductase (P5CR). The synthesis of proline via ornithine as a precursor is mediated by ornithine δ -aminotransferase (δ -OAT; EC 2.6.1.13). Although an alternative pathway of transamination leading to Δ^1 -pyrroline-2-carboxylate exists, functional complementation of a defective *Escherichia coli* mutant strongly indicated the use of the δ -OAT route (Delauney and Verma 1993). Proline degradation is catalyzed by the subsequent activity of two enzymes located in the mitochondria—proline dehydrogenase (ProDH; EC 1.5.99.8) and P5C dehydrogenase (P5CDH; EC 1.5.1.12).

Proline is the major organic osmolyte in *F. cylindrus* in addition to betaine and dimethylsulfoniopropionate (DMSP; Plettner 2002). The regulation of proline synthesis with respect to time and intracellular concentration, therefore, is of primary interest due to the increase in external salinity when *F. cylindrus* cells are incorporated into growing sea ice. Four cDNAs could be identified from a cold-stress-induced (Mock et al. 2005) and salt-stress-induced (A. Krell, B. Beszteri, T. Mock, G. Glöckner, K. Valentin, unpublished data) expressed sequence tag (EST) library encoding the most relevant enzymes during proline synthesis and degradation: δ -OAT, P5CS, P5CR, and ProDH.

This investigation and the experimental setup were designed to examine the relationship between

intracellular proline concentrations and expression levels of the genes (*P5CS*, *P5CR*, δ -OAT, *ProDH*) expressed under salt-stress conditions, comparable to natural conditions when *F. cylindrus* is enclosed in developing sea ice. The mRNA copy numbers of these genes were determined with Q-PCR techniques concurrent with measurements of intracellular proline concentrations. The aim was to determine whether proline is synthesized primarily via the glutamate or ornithine pathway and which environmental factor leads to the preference of either pathway. Measurements of photosynthetic activity are included to determine the energy availability. This study also differentiates between the impact of salinity as a single stress factor and the impact of increased salinity in combination with decreasing temperature on the regulation of proline metabolism.

MATERIALS AND METHODS

Experimental design and culture conditions. *Fragilariopsis cylindrus* was isolated from Antarctic sea ice during a “Polarstern” expedition (ANT XVI/3) in the eastern Weddell Sea in 1999. Single cells were picked to obtain several clones of *F. cylindrus*. Stock cultures of *F. cylindrus* were grown in 5 L batch cultures using Antarctic seawater having a salinity of 33.6 PSU and enriched with f/2 nutrients after Guillard and Ryther (1962). *Fragilariopsis cylindrus* was maintained in a culture room at 0°C ($\pm 0.2^\circ\text{C}$) under continuous illumination with white fluorescent light (Osram Biolux, Munich, Germany) at a photon flux density of $25 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Bubbling with sterile filtered air and gentle stirring with magnetic stirrers ensured sufficient CO_2 supply and mixing. Cultures of *F. cylindrus* were handled under strict sterile conditions; potential bacterial contamination was strongly reduced as precultures were treated with a combination of penicillin ($100 \mu\text{g} \cdot \text{mL}^{-1}$) and streptomycin ($25 \mu\text{g} \cdot \text{mL}^{-1}$).

Three independently grown stock cultures were used for the following experimental setup: (i) cultures kept at standard salinity and temperature conditions as a control and (ii) two different treatments with cultures exposed to either an increased salinity of 70 PSU at 0°C (70/0) or exposed to increased salinity (70 PSU) combined with a decreased cultivation temperature of -4°C (70/-4). A temperature of -4°C corresponds to the brine salinity of 70 PSU (Assur 1958). Each control/treatment consists of three replicate batch cultures, each of which was inoculated from one of the three independently grown stock cultures.

Shock treatment. The shock treatment started during the early exponential phase by direct addition of sea salt (approx. $44 \text{ g} \cdot \text{L}^{-1}$; Sigma Aldrich Chemie GmbH, Steinheim, Germany) to the cultures up to a final salinity of 70 PSU. The added sea salt was completely dissolved within 15 min. The simultaneously cold treated cultures were transferred to a Light Thermostat (Rumed Model 1301, Rubarth GmbH, Hannover, Germany) at -4°C . Subsamples for the various parameters were taken 1 h before and 4, 24, 48, 288 (12 d), and finally 480 h (20 d) after salt addition.

Determination of cell concentration and growth rate. Cell numbers were determined in triplicate runs using a Multisizer 3 (Beckman Coulter, Krefeld, Germany) particle counter equipped with a $100 \mu\text{m}$ aperture capillary, using a size range from 2.6 to $9.8 \mu\text{m}$. Specific growth rate per day (μ) was calculated according to the following formula:

$$\mu = \frac{\ln(C_1) - \ln(C_0)}{(t_1 - t_0)/24} \quad (1)$$

where C_t denotes cell concentration at time t_1 , and C_0 is the cell number at time t_0 .

Proline analysis. Proline concentration was determined spectrophotometrically with ninhydrine, according to the method described by Bates et al. (1973) and modified by Nothnagel (1995).

Pulse amplitude modulated (PAM) fluorometry measurement.

Variable chl *a* fluorescence, measured with PAM fluorometry, was applied as a proxy to monitor physiological integrity of the photosynthetic apparatus. In vivo quantum yield (Φ_{PSII}) was determined in each culture using a Xenon PAM Fluorometer (WALZ GmbH, Effeltrich, Germany) equipped with a temperature control unit and a magnetic stirrer. In vivo quantum yield was calculated from fluorescence readings of illuminated samples as follows:

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m} \quad (2)$$

where F'_m and F_t denote the maximum and minimum fluorescence in an illuminated sample (Maxwell and Johnson 2000).

RNA extraction and purification. Total RNA extraction was carried out with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cell lysis was improved by shaking for 100 s on a Mini Beatbeater (Biospec Products, Bartlesville, OK, USA). After applying samples to the QIAshredder column (Qiagen), they were centrifuged for 10 min at $\sim 17,900g$ to pellet cell debris and polysaccharides. After elution of RNA, a DNase treatment (Qiagen; 27 Kunitz units $\cdot 100 \mu\text{L}^{-1}$) was performed in liquid for 1 h at 30°C , followed by a second cleanup step including an on column DNase treatment. The RNA was separated on an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) to check for integrity of RNA. Concentrations were determined using NanoDrop (PeqLab, Erlangen, Germany).

Reverse transcription, primer design, and QPCR conditions. Complementary DNA was generated with the Omniscript RT kit (Qiagen) utilizing anchored oligo(dT)₂₀ primer (Invitrogen, Carlsbad, CA, USA) at a final concentration of $25 \text{ ng} \cdot \mu\text{L}^{-1}$. To verify the efficiency of reverse transcription (RT) and to assess the correction of different efficiencies, the reaction mix was spiked with artificial RNA of two genes of $\sim 1.8 \text{ kb}$ size (*MA* and *NSP*) from *Pieris rapae* (cabbage white butterfly, Lepidoptera: Pieridae). As few insects are present in the marine environment, this constitutes an ideal internal

reference. The *MA* was added at a final concentration of $116 \text{ pg} \cdot \mu\text{L}^{-1}$, and *NSP* at $10 \text{ fg} \cdot \mu\text{L}^{-1}$, spanning four orders of magnitude. Reverse transcription of 500 ng of total RNA and the added spike RNA was carried out at 42°C for 1 h, followed by an inactivation cycle at 85°C for 5 min. For each time point and treatment, one RT reaction mix was not supplemented with reverse transcriptase to serve as a control for DNA contamination.

All primers (Table 1) were designed using the Primer Express 2.0.0 (Applied Biosystems, Darmstadt, Germany) software and synthesized from OPERON Biotechnologies, (Köln, Germany). For each primer pair, the reliability of the QPCR was demonstrated by amplification of the purified target sequence in a concentration series spanning six orders of magnitude. Linear regression analysis between the target concentration and the C_t value yielded correlation coefficients close to one for all primer pairs (Table 2), proving the efficiency of the PCR reaction. The control gene *MA* was constantly detected in all samples at a C_t value of $11.4 (\pm 0.28, n = 50)$, and the second control gene *NSP* at a C_t of $33.9 (\pm 1.03, n = 44)$, both values indicative of a consistent efficiency of the RT reaction for high and low copy number genes.

For QPCR, $5 \mu\text{L}$ of a 10 fold diluted RT reaction mix was added to $15 \mu\text{L}$ of a PCR mixture. Instead of using the recommended $2 \times$ SYBR Green PCR Master Mix (Applied Biosystems) dilution, we used a 2.5 fold master mix dilution (i.e., $8 \mu\text{L}$ instead of $10 \mu\text{L}$ per reaction). Each primer was added at a concentration of 50 to 500 nM depending on optimized reaction efficiency. Cycle parameters were as follows: initial denaturation $95^\circ\text{C}/10 \text{ min}$, followed by 40 cycles of $95^\circ\text{C}/15 \text{ s}$ and $59^\circ\text{C}/1 \text{ min}$. Finally, a dissociation step was carried out to check whether a single product was amplified and to check for primer dimers.

Data analysis. Threshold cycle (C_t) values, slope of the standard curve, and correlation were calculated with Sequence Detection Software 1.2.3 (Applied Biosystems). One replicate was removed from the experimental data when the C_t value differed >0.3 from the most similar parallel of the same sampling point. Efficiency of the PCR reaction was calculated from the standard curves according to the following formula:

$$E = 10^{-\text{slope}} - 1 \quad (3)$$

where slope is determined from the linear regression of $\log$ (target concentration) versus C_t .

TABLE 1. Genes investigated during this study and sequences of the primers used to amplify target genes by QPCR.

NCBI accession no.	Function	Primer sequence 5' $\rightarrow$ 3'	Amplicon size (bp)	Best hit	Significance
CF269358	Δ^1 pyrroline 5 carboxylate synthetase (<i>P5CS</i>)	fw: GTGCAATGCAATGGAATCGT rev: TCTGAGCAGCAACTCCGTTACTAA	66	AAB67875	7e 40
DR026040	Δ^1 pyrroline 5 carboxylate reductase (<i>P5CR</i>)	fw: CTCCGCCCTCTTGTGAATGA rev: CCACCCATTTCAAGCGATCT	61	ZP 00357525	5e 47
CF269667	Ornithine δ aminotransferase (δ <i>OAT</i>)	fw: GGTAGGAAATTCGGCCGTAGA rev: GATTGATTCGGACAGCGTTTGA	65	AAH77314	2e 47
DR026487	Proline dehydrogenase (<i>ProDH</i>)	fw: GAAGTTGATGAGGTGATGCCATAC rev: GCACCACCAATAGCACTGTTTTTC	65	A47302	4e 44
DR026674	Actin	fw: ATGAAGATATCGCTGCCCTTGT rev: CCAGCGAAACCGGCTTT	64	CAA42559	8e 125
CF269420	<i>psbA</i>	fw: AGAACCACCAATACACCAGCAA rev: TCCAAGCTGAGCACAACATCTT	71	AAM62069	1e 130
Unpublished	<i>MA</i> control	fw: TCGGTTGACAGATACCTTAAAGGAA rev: TCAAAGGTGACGTTTCGAGTTCAT	100		
AY425622	<i>NSP</i> control	fw: ACGATGCCTTCAGAGCTACCTT rev: TACGCATCAAGCGTTTGAA	100		

TABLE 2. Efficiency of the QPCR reaction derived from standard curves using PCR products of the corresponding plasmid as a template.

Gene	Slope	Efficiency	r^2
Δ^1 pyrroline 5 carboxylate synthetase (<i>P5CS</i>)	-3.472	0.94	0.9979
Δ^1 pyrroline 5 carboxylate reductase (<i>P5CR</i>)	-3.56	0.91	0.9991
Ornithine δ aminotransferase (<i>OAT</i>)	-3.98	0.78	0.9938
Proline dehydrogenase (<i>ProDH</i>)	-3.428	0.96	0.9987
Actin	-3.666	0.87	0.9984
<i>psbA</i>	-3.38	0.98	0.9989
MA control	-3.588	0.90	0.9979
NSP control	-3.634	0.88	0.9981

RESULTS

Salt shock arrests growth for 12 d. All results originate from three different 5 L batch cultures for each setup (control, 70/0, 70/-4). All nine cultures showed an identical growth without lag phase within the 10 d before stress exposure (Fig. 1). At time point 0 (70/0 and 70/-4 exposed to stress conditions), the control cultures were subsequently maintained as semicontinuous batch cultures to prevent them from reaching the stationary growth phase so that undesired effects due to physiological changes would be excluded. Control cultures were kept at a nearly constant cell density of $\sim 1.3 \times 10^6$ cells $\cdot$ mL $^{-1}$, representing a constant growth rate of $\mu = 0.325$ over the course of the experiment. Most of the cells (86% in the 70/0 and 94% in the 70/-4 treatment) survived the salt shock. However, cell growth stopped, and cell numbers remained stationary for about 12 d. Subsequently, both shock treatments regained positive growth, albeit at a low

FIG. 1. Growth kinetics of *Fragilariopsis cylindrus* cultures. Nine cultures were grown in f/2 medium at 0°C, 34 PSU, and 25 μ mol photons $\cdot$ m $^{-2}$ $\cdot$ s $^{-1}$ until day 0. Conditions for three cultures remained unchanged and were kept as semicontinuous cultures (■); in three cultures the salinity was increased to 70 PSU (●); and in the remaining three cultures temperature was additionally decreased to -4°C (▲).

growth rate of $\mu = 0.06$ when compared to the pre-shock phase of the experiment. However, no difference in growth was observed between both treatments (70/0 and 70/-4) after stress exposure.

Photosynthesis is severely inhibited but recovers steadily after salt shock. In the control cultures, in vivo quantum yield (Φ_{PSII}) remained constant at 0.61 ± 0.015 during the course of the experiment (Fig. 2). In the 70/0 shock treatment, average Φ_{PSII} values instantly dropped from 0.59 before treatment to 0.24 within 1 h after the salt addition. During the next 8 h, there was no change in Φ_{PSII} ; but within the first 24 h, Φ_{PSII} increased up to 0.30, and after 48 h, it reached 0.37. Subsequently, photosynthesis recovered slowly but steadily, as shown by increasing Φ_{PSII} , but did not attain values similar to those before the shock treatment or to those of the control cultures. The 70/-4 cultures essentially showed a similar reaction; but the initial drop was even more pronounced (0.18 after 1 h), and the recovery was retarded compared to the 70/0 treatment. Both shock-treated cultures regained Φ_{PSII} values close to the nonstressed cultures toward the end of the experiment.

Proline concentration increases several fold during acclimation to high salinity. Before stress exposure, *F. cylindrus* had intracellular proline concentrations of 3.0 ± 0.48 fmol $\cdot$ cell $^{-1}$. While the concentrations of the control cultures remained constant, the cells of both treatments started to accumulate proline 8 h after stress exposure to both treatments (Fig. 3). After 24 h, the intracellular proline concentration in the treated cultures had already doubled compared to the control. The cultures exposed to lower temperature continued to accumulate proline up to 12.9 ± 1.29 fmol $\cdot$ cell $^{-1}$ at day 12 and 13.6 ± 1.41 fmol $\cdot$ cell $^{-1}$ at day 20. The unchanged intracellular proline concentration within the last two

FIG. 2. Changes of the photosynthetic quantum yield at PSII (Φ_{PSII}) during the course of the experiment. (■), control cultures; (●), 70 PSU/0°C cultures; (▲) 70 PSU/-4°C cultures.

FIG. 3. Temporal development of intracellular proline concentrations. (□), control cultures; (●), 70 PSU/0°C cultures; (▲), 70 PSU/ 4°C cultures.

sampling points might be because of regained growth since day 12 (Fig. 1). However, proline concentrations of *F. cylindrus* 70/0 cultures varied. While increasing simultaneously with the 70/−4 treatment to concentrations of $8.8 \pm 0.09 \text{ fmol} \cdot \text{cell}^{-1}$ on day 4, they decreased to $6.2 \text{ fmol} \cdot \text{cell}^{-1}$ on days 8 and 12 after stress exposure. At the end of the experiment on day 20, a concentration of $15.3 \pm 0.47 \text{ fmol} \cdot \text{cell}^{-1}$ was measured, which slightly exceeds the 70/−4 cultures and is a 4.5-fold increase when compared to the control.

δ-OAT rather than P5CS expression is induced by salt shock. To investigate the genetic mechanisms underlying proline accumulation in salt-stressed cells of *F. cylindrus*, we analyzed the transcript levels of the key enzymes of proline metabolism—P5CS, *δ*-OAT, P5CR and ProDH—by Q-PCR. The *δ* OAT, P5CR, and ProDH genes showed a similar expression pattern during the experiment in both salt-shock

FIG. 4. Quantification of transcript levels of the genes (a) P5CS, (b) *δ* OAT, (c) P5CR, and (d) ProDH during the course of the experiment as revealed by Q-PCR analyses. mRNA copy numbers were calculated from standard curves of DNA templates and normalized to ng of total RNA extracted. (□), control cultures; (●), 70 PSU/0°C cultures; (▲), 70 PSU/ 4°C cultures. *δ* OAT, ornithine *δ* aminotransferase; P5CR, Δ^1 pyrroline 5 carboxylate reductase; P5CS, Δ^1 pyrroline 5 carboxylate synthase; ProDH, proline dehydrogenase.

treatments (Fig. 4, b–d). An increase in mRNA levels of these genes could be detected, reaching its maximum 24 h after the beginning of the stress exposure. After this initial induction, mRNA levels of *P5CR* and δ *OAT* constantly declined in both treated cultures, returning to values close to the control at the end of the experiment on day 20. However, mRNA levels in the cold-treated cultures declined more slowly, reflecting a delay in the recovery. A significant correlation in expression between *P5CR* and δ *OAT* was observed. The *ProDH* expression pattern was different. mRNA levels of the cold-treated cultures remained strongly up-regulated, and the expression level in the 70/0 treatment showed an intermediate decline but increased again at the end of the experiment. This pattern demonstrates a positive correlation between the genetic expression level of *ProDH* and the measured intracellular proline concentration.

In contrast to δ -*OAT* and *P5CR*, transcript levels of *P5CS* and the gene coding for actin strongly decreased during the first hours after stress exposure, reaching their lowest levels at time points of 24 h and 48 h, respectively (Figs. 4a and 5b). However, the expression level of actin in the 70/–4 was less affected when compared with the 70/0 and recovered more rapidly, contrasting with the observations made regarding the up-regulated genes and even *P5CS*. This observation is consistent with the less-affected growth of the additionally cold-treated cultures as described above (Fig. 1). At day 12 after the shock treatment, actin mRNA levels regained control levels.

Surprisingly, *psbA* showed the least variation in its expression level during the course of the experiment (Fig. 5a). Variations in the control and the 70/–4 cultures were within the standard error, and no significant difference between the treatments was observed. Only a slight decrease in the 70/0 treatment at 48 h

was significant. However, this finding strongly contrasts with the inhibition of photosynthetic electron transport revealed by the measurements of Φ_{PSII} (Fig. 2). To summarize, the maximum alteration in the expression level of the observed genes occurred within the first 24 h after stress exposure, while only *P5CS* and *psbA* reached their maximum at 48 h (Table 3). *P5CS* and the gene coding for actin showed the highest magnitude in change of the expression level of all genes analyzed, with a 17.3- and 29.7-fold decrease, respectively. While the adjustment phase in terms of transcript levels of the genes analyzed in this study was completed within 24–48 h, it took 12 d until the adjustment became effective in terms of positive cell growth.

DISCUSSION

It is well known that higher plants and diatoms accumulate the imino acid proline under osmotic stress. The regulation of proline synthesis and degradation upon osmotic stress and relief from it have been extensively studied in higher plants. However, the regulatory mechanisms involved in the proline metabolism in diatoms so far have not been investigated at the molecular level.

To our knowledge, this is one of the first expression analyses involving the use of an absolute quantitative instead of a relative quantification Q-PCR method in diatoms. RNA yield and quality as measured with the Agilent Bioanalyzer lab chip were very similar for all sampling points. The constant detection of the exogenous control genes *MA* and *NSP* verified a consistent efficiency of the RT reaction. Therefore, it can be concluded that expression data gained for the target genes were not biased by the RT step. The strong variability of actin transcript levels demonstrates that methods employing endogenous reference genes for the analyses of expression

Fig. 5. Evaluation of transcript levels of the (a) *psbA* and (b) actin genes during the course of the experiment as revealed by Q-PCR analyses. mRNA copy numbers are normalized to ng of total RNA extracted. (□), control cultures; (●), 70 PSU/0°C cultures; (▲), 70 PSU/–4°C cultures.

TABLE 3. Maximum changes in the expression level compared with the mean of the control and time point when the change occurred.

Gene	70 PSU/0°C		70 PSU/-4°C	
	Fold change	Time (h)	Fold change	Time (h)
Δ^1 pyrroline 5 carboxylate synthetase (<i>P5CS</i>)	-17.26	48	-15.37	48
Δ^1 pyrroline 5 carboxylate reductase (<i>P5CR</i>)	2.5	24	2.7	48
Ornithine δ aminotransferase (δ <i>OAT</i>)	7.55	24	8.93	24
Proline dehydrogenase (<i>ProDH</i>)	2.88	24	3.21	24
Actin	-29.71	24	-20.67	24
<i>psbA</i>	-1.76	48	-1.45	48

changes are very difficult to carry out and that the reference genes must be selected very carefully.

Increasing salinity from 33.6 to 70 PSU proved to be a severe but sublethal stress for *F. cylindrus* as manifested by a strong drop in photosynthesis and growth arrest for 12 d. Both salt-shock treatments—at 0°C as well as at -4°C—induced a strong increase in intracellular proline concentrations. At -4°C, proline increased more rapidly than at 0°C, whereas photosynthesis recovered faster at 0°C. The constantly increasing intracellular proline concentrations (particularly in 70/-4 cultures) as well as the steady recovery of Φ_{PSII} reflected the ongoing acclimation process starting within the first 24 h after the beginning of the stress exposure. The restart of growth after 12 d could be seen as a measure of the success of the acclimation process, as has been shown previously with other species (Plettner 2002). In the 70/0 cultures, the intracellular proline concentration remained constant on days 7 and 12, corresponding to *ProDH* gene expression levels but not reflected by physiological data (cell numbers, recovery of Φ_{PSII}). However, the final intracellular proline concentration of $\sim 14 \text{ fmol} \cdot \text{cell}^{-1}$ was the same as that measured in preliminary experiments (data not shown). Faster down-regulation of *P5CR* expression after the initial peak in the 70/0 cultures might be responsible for a slower rate of proline accumulation during this phase (Fig. 4c). Lower proline concentrations in the 70/0 cultures are correlated to higher and lower transcript levels of *P5CS* and *ProDH*, respectively, which supports the hypothesis that expression of these genes is regulated by proline.

Under salt-stress conditions, *P5CS* mRNA levels in *F. cylindrus* were down-regulated and remained low throughout the experiment (Fig. 6). This finding contrasts with several observations in higher plants, where a strong accumulation, or at least an unchanged level of *P5CS* transcripts, was determined after exposure to osmotic stress (Peng et al. 1996, Igarashi et al. 1997, Hare et al. 1999). The down-regulation of *P5CS* transcript levels indicates possible feedback inhibition of *P5CS* expression by proline, active even after prolonged presence of high salt concentrations (Fig. 4a). Additionally, sequence alignment between diatom and higher

plant *P5CS* proteins revealed conservation of a phenylalanine residue that was shown to mediate feedback-inhibition by proline in plants (Hong et al. 2000). Both observations support the conclusion that *P5CS* is not responsible for proline accumulation under salt stress in diatoms.

In higher plants, proline accumulation during stress was linearly correlated with a decline in *ProDH* transcript levels (Peng et al. 1996, Miller et al. 2005), whereas the results of this study show the opposite tendency (Fig. 4d). We observed a positive correlation between proline levels and *ProDH* gene expression, as was observed in plants treated with proline in the absence of salt or osmotic stress (Kiyosue et al. 1996, Verbruggen et al. 1996). Thus, an autoregulatory induction of *ProDH* expression by proline seems to be present in plants and diatoms, whereas stress-dependent inhibition of proline degradation in diatoms is regulated differently. Unfortunately, *F. cylindrus* does not seem to be able to take up externally applied L-proline under standard conditions and replete nutrients, as no effect on the regulation of either of the investigated genes was observed (data not shown), which might have elucidated the cause and effect.

The inductive effect of proline on *ProDH* expression is supported by the constant elevated copy numbers of *ProDH* transcripts after the shock. In contrast, most other genes investigated in this study showed a transient regulation. It remains to be analyzed whether *ProDH* activity is regulated at the mRNA level in diatoms as observed in higher plants, or whether additional regulatory mechanisms exist. As a high free-proline content might have deleterious effects (Hellmann et al. 2000, Mani et al. 2002, Nanjo et al. 2003), a tight regulation and therefore an increase in *ProDH* transcript levels might be necessary.

The down-regulation of *P5CS* transcript levels opposed by an up-regulation of δ -OAT and *P5CR* strongly argues for proline synthesis via the ornithine pathway in salt-shocked diatoms. This trend would be again in contrast to the results obtained from studies of higher plants, where *P5CS* seems to be the predominant enzyme for proline synthesis, while up-regulation of δ -OAT by salt stress was only observed in young *Arabidopsis* seedlings (Delauney

FIG. 6. Pathways of proline metabolism using either glutamate or ornithine as a substrate, including those enzymes investigated in the present study: P5CS, δ OAT, P5CR, and ProDH. Thick arrows indicate the initial changes in transcript levels after salt shock treatment. δ OAT, ornithine δ aminotransferase; P5CR, Δ^1 pyrroline 5 carboxylate reductase; P5CS, Δ^1 pyrroline 5 carboxylate synthase; ProDH, proline dehydrogenase.

and Verma 1993, Verbruggen et al. 1996, Roosens et al. 1998).

The possibility of the existence of a differentially regulated P5CS isoform in diatoms was also considered, as all known plant genomes contain at least two *P5CS* genes (Strizhov et al. 1997, Ginzberg et al. 1998). However, the search with degenerated primers and cloning of the *P5CS* gene in *F. cylindrus* cDNA libraries and genomic DNA revealed only one copy of this gene (data not shown). This result is supported by a BLAST search (Altschul et al. 1997) of *P5CS* against the complete genome of the centric diatom *Thalassiosira pseudonana* Hasle et Heimdal, also retrieving only one copy. This may imply a potentially different process of regulation in diatoms. Another difference specific to higher plants is the subcellular localization of P5CS, which is cytosolic in higher plants (Kishor et al. 2005). The localization of P5CS in *T. pseudonana*, and probably also in *F. cylindrus*, is very likely to be in the mitochondria (targetp v1.1, mTP 0.797). δ -OAT is predicted to be mitochondrial in both diatoms and higher plants. Copy number also differs for *ProDH*, of which two isoforms were identified in *Medicago*

sativa (Miller et al. 2005), whereas again only one copy was found in *T. pseudonana*. In contrast to a confirmed mitochondrial localization in higher plants (Kavi Kishor et al. 2005), the localization of ProDH in *T. pseudonana* is rather ambiguous (targetp v1.1, cTP 0.620, mTP 0.113 other 0.449). If these findings could be verified in further investigations, it could be hypothesized that the enzymatic equipment and the compartmentalization of proline biosynthesis and degradation are fundamentally different in diatoms and higher plants, potentially reflecting the different evolutionary history of organelles in both taxa.

Energetic aspects of proline synthesis might play a role in the preference of either the glutamate or ornithine route to proline. Although both routes involve the consumption of one molecule of ATP and two molecules of NADPH for the formation of one molecule of proline from glutamate (Fig. 6), ornithine might also be derived via the urea cycle from arginine originating from protein degradation. In the latter case, proline synthesis from ornithine would require only one molecule of NADPH consumed by P5CR (Fig. 6; Hare and Cress 1997). The

presence of a complete urea cycle in diatoms was recently demonstrated at the molecular level (Armburst et al. 2004). Under normal growth conditions, expression of *P5CS* was high and δ *OAT* expression was low, indicating that proline was synthesized via *P5CS* from glutamate, while ornithine was used for the synthesis of arginine. Similar amounts of proline and arginine would be consumed for protein synthesis during growth. Our results show that upon salt shock, the growth of *F. cylindrus* is halted for a considerable period. During this time, arginine utilization is reduced, and hence ornithine might be directed to the synthesis of proline for osmotic adjustment.

The decline in Φ_{PSII} (Fig. 2) suggests a devastating effect of elevated salt concentrations on the photosynthetic apparatus. Measurements of Φ_{PSII} in cold-shocked (+5°C to -1.8°C) cultures of *F. cylindrus* caused only a minor reduction from 0.61 to 0.53 (Mock and Valentin 2004), recovering to 0.60 within 5 d, as opposed to a decrease from 0.59 to 0.24 (70/0) and 0.18 (70/-4) in this study. These results suggest that under the conditions employed in this investigation, linear electron transport and thus the photosynthetic production of reduction equivalents declined and was restored only slowly during acclimation. The unchanged levels of *psbA* transcripts (Fig. 5a) indicate that photodamage to the D1 protein and repair were not the limiting factors for photosynthesis in salt-shocked diatoms. This possibility might be due to a rather stabilizing effect of high salt concentrations on the *psbA* transcript as observed by Allakhverdiev et al. (2002). The drop in Φ_{PSII} may not be an active response but rather induced by water deprivation, as was proposed earlier for salt-stressed green algae and cyanobacteria (Allakhverdiev et al. 2000, Cruz et al. 2001). This interpretation is supported by the correlation between recovery of Φ_{PSII} and the intracellular concentration of proline, which is able to restore the internal water potential.

These results suggest that under conditions such as those employed in this investigation, the contingent of reduction equivalents at least during the first period after the initial salt shock strongly declined and only slowly regained their function. Thus, it might be crucial for *F. cylindrus* to rely on the conversion of arginine and ornithine to proline via δ -OAT rather than inducing de novo synthesis from glutamate via *P5CS*.

In summary, this investigation shows that when *F. cylindrus* is entrapped into newly forming sea ice, experiencing increased external salinities and decreased temperatures, growth is halted and regained only after a considerable acclimation phase, during which proline is accumulated. Analysis of gene expression of those genes involved in proline metabolism suggested that this accumulation is primarily achieved via the ornithine route. Lowering the temperature parallel to the salt shock did not

alter the primary response, although it was initially beneficial for survival, potentially by slowing down deleterious processes. A further dissection of the regulatory mechanisms, including posttranscriptional regulation of the proline metabolism in heterokonts as well as an understanding of the signal transduction pathways mediating salt-stress responses would be of genuine importance for our understanding of the survival strategies of one of the most important primary producers in polar oceans.

We would like to thank Prof. G. O. Kirst for reviewing an earlier version of this manuscript and Sara Beszteri and Heiko Vogel for providing help with the establishment of the spike in control in the Q-PCR assay.

- Allakhverdiev, S. I., Nishiyama, Y., Miyairi, S., Yamamoto, H., Inagaki, N., Kanasaki, Y. & Murata, N. 2002. Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of *psbA* genes in *Synechocystis*. *Plant Physiol.* 130:1443–53.
- Allakhverdiev, S. I., Sakamoto, A., Nishiyama, Y., Inaba, M. & Murata, N. 2000. Ionic and osmotic effects of NaCl induced inactivation of photosystems I and II in *Synechococcus* sp. *Plant Physiol.* 123:1047–56.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–402.
- Armburst, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H., Zhou, S., et al. 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306:79–86.
- Assur, A. 1958. Composition of sea ice and its tensile strength. *Nat. Resour. Council Publ.* 598:106–38.
- Bates, L. S., Waldren, R. P. & Teare, I. D. 1973. Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–7.
- Brown, A. D. & Simpson, J. R. 1972. Water relations of sugar tolerant yeast: the role of intracellular polyols. *J. Gen. Microbiol.* 72:589–91.
- Chen, T. & Murata, N. 2002. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.* 5:250–7.
- Cox, G. F. N. & Weeks, W. F. 1983. Equations for determining the gas and brine volumes in sea ice samples. *J. Glaciol.* 29:306–16.
- Cruz, J. A., Salbilla, B. A., Kanazawa, A. & Kramer, D. M. 2001. Inhibition of plastocyanin to P₇₀₀⁺ electron transfer in *Chlamydomonas reinhardtii* by hyperosmotic stress. *Plant Physiol.* 127:1167–79.
- DasSarma, S. & Arora, P. 2001. *Halophiles. Encyclopedia of Life Sciences*. Macmillan Press, Hampshire, UK, pp.1–9.
- Delauney, A. J. & Verma, D. P. S. 1993. Proline biosynthesis and osmoregulation in plants. *Plant J.* 4:215–23.
- Eicken, H. 1992. The role of sea ice in structuring Antarctic ecosystems. *Polar Biol.* 12:3–13.
- Eicken, H. 2003. From the microscopic, to the macroscopic, to the regional scale: growth, microstructure and properties of sea ice. In Thomas, D. N. & Dieckmann, G. S. [Eds.] *Sea Ice: An Introduction to Its Physics, Chemistry, Biology and Geology*. Blackwell Science, Oxford, UK, pp. 22–81.
- Erdmann, N. & Hagemann, M. 2001. Salt acclimation of algae and cyanobacteria: a comparison. In Rai, L. C. & Gaur, J. P. [Eds.] *Algal Adaptation to Environmental Stresses*. Springer, New York, pp. 323–61.
- Ginzberg, I., Stein, H., Kapulnik, Y., Szabados, L., Strizhov, N., Schell, J., Koncz, C. & Zilberstein, A. 1998. Isolation and characterization of two different cDNAs of Δ^1 pyrroline 5-carboxylate synthase in alfalfa, transcriptionally induced upon salt stress. *Plant Mol. Biol.* 38:755–64.

- Guillard, R. R. L. & Ryther, J. H. 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8:229–39.
- Günther, S. & Dieckmann, G. S. 2001. Vertical zonation and community transition of sea ice diatoms in fast ice and platelet layer, Weddell Sea, Antarctica. *Ann. Glaciol.* 33:287–96.
- Hare, P. D. & Cress, W. A. 1997. Metabolic implications of stress induced accumulation in plants. *Plant Growth Regul.* 21:79–102.
- Hare, P., Cress, W. & van Staden, J. 1999. Proline synthesis and degradation: a model system for elucidating stress related signal transduction. *J. Exp. Biol.* 50:413–34.
- Hellmann, H., Funck, H., Rentsch, D. & Frommer, W. B. 2000. Hypersensitivity of an *Arabidopsis* sugar signaling mutant toward exogenous proline application. *Plant Physiol.* 123:779–89.
- Hong, Z., Lakkineni, K., Zhang, Z. & Verma, D. P. S. 2000. Removal of feedback inhibition of Δ^1 pyrroline 5 carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.* 122:1129–36.
- Igarashi, Y., Yoshida, Y., Sanada, Y., Yamaguchi Shinozaki, K., Wada, K. & Shinozaki, K. 1997. Characterization of the gene for Δ^1 pyrroline 5 carboxylate synthetase and correlation between the expression of the gene and salt tolerance in *Oryza sativa* L. *Plant Mol. Biol.* 33:857–65.
- Kang, S. H. & Fryxell, G. A. 1992. *Fragilariopsis cylindrus* (Grunow) Krieger: the most abundant diatom in water column assemblages of Antarctic marginal ice edge zones. *Polar Biol.* 12:609–27.
- Kirst, G. O. 1990. Salinity tolerance of eukaryotic marine algae. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:21–53.
- Kirst, G. O. & Wiencke, C. 1995. Ecophysiology of polar algae. *J. Phycol.* 31:181–99.
- Kishor, P. B. K., Sangam, S., Amrutha, R. N., Laxmi, P. S., Naidu, K. R., Rao, K. R. S. S., Rao, S., Reddy, K. J., Theriappan, P. & Sreenivasulu, N. 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Curr. Sci.* 88:424–38.
- Kiyosue, T., Yoshida, Y., Yamaguchi Shinozaki, K. & Shinozaki, K. 1996. A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis*. *Plant Cell* 8:1323–35.
- Mani, S., Van de Cotte, B., Van Montagu, M. & Verbruggen, N. 2002. Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in *Arabidopsis*. *Plant Physiol.* 128:73–83.
- Maxwell, K. & Johnson, G. N. 2000. Chlorophyll fluorescence: a practical guide. *J. Exp. Bot.* 51:659–68.
- Miller, G., Stein, H., Honig, A., Kapulnik, Y. & Zilberstein, A. 2005. Responsive modes of *Medicago sativa* proline dehydrogenase genes during salt stress and recovery dictate free proline accumulation. *Planta* 222:70–9.
- Mock, T. & Valentin, K. 2004. Photosynthesis and cold acclimation: molecular evidence from a polar diatom. *J. Phycol.* 40:732–41.
- Mock, T., Krell, A., Glöckner, G., Kolukisaoglu, Ü. & Valentin, K. 2005. Analysis of expressed sequence tags (ESTs) from the polar diatom *Fragilariopsis cylindrus*. *J. Phycol.* 42:78–85.
- Nanjo, T., Fujita, M., Seki, M., Kato, T., Tabata, S. & Shinozaki, K. 2003. Toxicity of free proline revealed in an *Arabidopsis* T DNA tagged mutant deficient in proline dehydrogenase. *Plant Cell Physiol.* 44:541–8.
- Nothnagel, J. 1995. The effects of salinity and light intensity on the osmolyte concentrations, cell volumes and growth rates of the Antarctic sea ice diatoms *Chaetoceros* sp. and *Navicula* sp. with emphasis on the amino acid proline. *Rep. Polar Res.* 161:1–115.
- Papadimitriou, S., Kennedy, H., Kattner, G., Dieckmann, G. S. & Thomas, D. N. 2004. Experimental evidence for carbonate precipitation and CO₂ degassing during sea ice formation. *Geochim. Cosmochim. Acta* 68:1749–61.
- Peng, Z., Lu, Q. & Verma, D. P. S. 1996. Reciprocal regulation of Δ^1 pyrroline 5 carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress. *Mol. Gen. Genet.* 253:334–41.
- Plettner, I. 2002. Stressphysiologie bei antarktischen Diatomeen. Ökophysiologische Untersuchungen zur Bedeutung von Prolin bei der Anpassung an hohe Salinitäten und tiefe Temperaturen. PhD thesis, Universität Bremen, Bremen, Germany, 228 pp.
- Reddy, A. R., Chaitanya, K. V. & Vivekanandan, M. 2004. Drought induced responses of photosynthesis and antioxidant metabolism in higher plants. *J. Plant Physiol.* 161:1189–202.
- Rodriguez, R. & Redman, R. 2005. Balancing the generation and elimination of reactive oxygen species. *Proc. Natl. Acad. Sci. U. S. A.* 102:3175–6.
- Roosen, N. H. C. J., Thu, T. T., Iskandar, H. M. & Jacob, M. 1998. Isolation of the ornithine δ aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. *Plant Physiol.* 117:263–71.
- Strizhov, N., Abraham, E., Okresz, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C. & Szabados, L. 1997. Differential expression of two *P5CS* genes controlling proline accumulation during salt stress requires ABA and is regulated by *ABA1*, *ABI1* and *AXR2* in *Arabidopsis*. *Plant J.* 12:557–69.
- Verbruggen, N., Hua, X. J., May, M. & Van Montagu, M. 1996. Environmental and developmental signals modulate proline homeostasis: evidence for a negative transcriptional regulator. *Proc. Natl. Acad. Sci. U. S. A.* 93:8787–91.
- Weissenberger, J., Dieckmann, G., Gradinger, R. & Spindler, M. 1992. Sea ice: a cast technique to examine and analyze brine pockets and channel structure. *Limnol. Oceanogr.* 37:179–83.