Salt stress tolerance in the psychrophilic diatom *Fragilariopsis cylindrus*

Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften
- Dr. rer. nat. –

vorgelegt von

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Bremen April 2006

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Part I

Abstract

This thesis was conducted to find mechanisms responsible for the adaptation success of *Fragilariopsis cylindrus* to the extreme polar environment, especially sea ice, manifested in the genetic repertoire. The generated molecular information was afterwards utilized in expression studies focused on the regulation of the proline metabolism during acclimatisation to elevated external salt concentrations.

An expressed sequence tag (EST) approach was used to establish two complementaryDNA (cDNA) datasets, based on cultures subjected to temperature and salt stress conditions, the major abiotic constraints in sea ice. The genetic information (ca. 2600 tentative unique sequences) gathered with these two cDNA libraries covered about 20 % of all genes present in *F. cylindrus,* taken the genome of the centric diatom *Thalassiosira pseudonana* as a reference. A comparison of the salt stress cDNA library to the genomes of the mesophilic species *T. pseudonana* and *Phaeodactylum tricornutum* revealed about one third of the sequences to be unique to *F. cylindrus*, indicating substantial genomic variation between the mesophilic and psychrophilic lifestyle. This indicates the potential of yet unknown adaptation mechanisms. A putative function could be assigned to 44 % of the sequences and a large number of genes involved in transport processes, oxidative stress defence, osmolyte synthesis and protein turnover as well as chaperones could be identified, stressing the importance of these mechanisms in salt stress acclimatisation.

Furthermore, four different full length sequences encoding a new class of ice-binding proteins yet unknown in animals and plants were found and further studies proved its occurrence in a number of polar diatom species, but not in mesophilic ones. These proteins are most probably exuded into the extracellular space and hence might be of fundamental importance in enabling survival in the brine channel system.

The finding of all relevant proteins involved in the ana- and catabolic pathways of proline metabolism enabled a detailed expression study of these genes in a physiological salt shock (elevation from 34 to 70 PSU) experiment. Expression levels of Δ^1-pyrroline-5-carboxylate synthase (P5CS), the rate limiting enzyme in synthesis from glutamate, strongly decreased,

whereas copy numbers of ornithine δ-aminotransferase (δ-OAT) increased, indicating a shift from the glutamate to the ornithine route under elevated external salinities. This contrasts with findings in higher plants, where the opposite regulation of P5CS and δ-OAT was observed. A shortage in reduction equivalents caused by a severe inhibition of linear electron transport revealed by the measurement of the photosynthetic quantum yield might force *F. cylindrus* to use energy saving pathways of proline synthesis.

Interestingly, a further temperature decrease in addition to the elevated salt concentration exhibited no more negative effects in a number of physiological parameters, thus leading to the conclusion that salt is the dominating abiotic stressor in sea ice.

Introduction

This thesis deals with the physiological acclimatisation processes and their molecular basis in the sea ice diatom *Fragilariopsis cylindrus*, as they occur during the formation and development of sea ice. To elucidate these mechanisms investigations were carried out on separately applied cold and salt stress, or a combination of both. Emphasis was placed on the regulation of proline metabolism, since this is the main organic osmolyte in *F. cylindrus* which is synthesized under hyperosmotic conditions.

The sea ice habitat

Polar perennial sea ice at its maximum extent constitutes one of the largest ecosystems in the world covering an area of up to $20x10^6$ km^2 in the Antarctic and $16x10^6$ km^2 in the Arctic (Thomas & Dieckmann, 2002a). Total annual primary production of Antarctic sea ice assemblages has been estimated to be in the range between 63 and 70 Tg C yr-1, which is about 5% of estimated total primary production in the Antarctic sea ice zone (Lizotte, 2001). Especially in the Antarctic, where the majority of sea ice lasts only one season it constitutes a highly dynamic system. It provides a unique range of ephemeral habitats for planktonic organisms which during their life time in open water are buffered against dramatic changes in their physicochemical environment, with the exception of solar irradiance and at times changes in the availability of inorganic nutrients. When incorporated into sea ice, these organisms are subjected to very different chemical and physical constraints which vary greatly during the annual cycle of ice formation, consolidation and melt (Eicken, 2003).

Salt does not enter the ice crystal structure, and so during the process of ice formation, salt and other dissolved constituents of seawater are expelled and collect as a highly concentrated brine solution within a labyrinth of brine channels and pores in the ice matrix, ranging in size from a few µm to several mm (Eicken, 1992, Weissenberger *et al.*, 1992, Eicken, 2003). This brine channel system provides a habitat that is controlled by the confines of the channel / pore diameters and the salinity of the brine. The internal surface area of brine channels ranges from

0.6 to 4.0 m^2 kg^{-1} of ice at -2.5°C, constituting a large surface area for organisms like algae and bacteria to colonise (Krembs *et al.*, 2000).

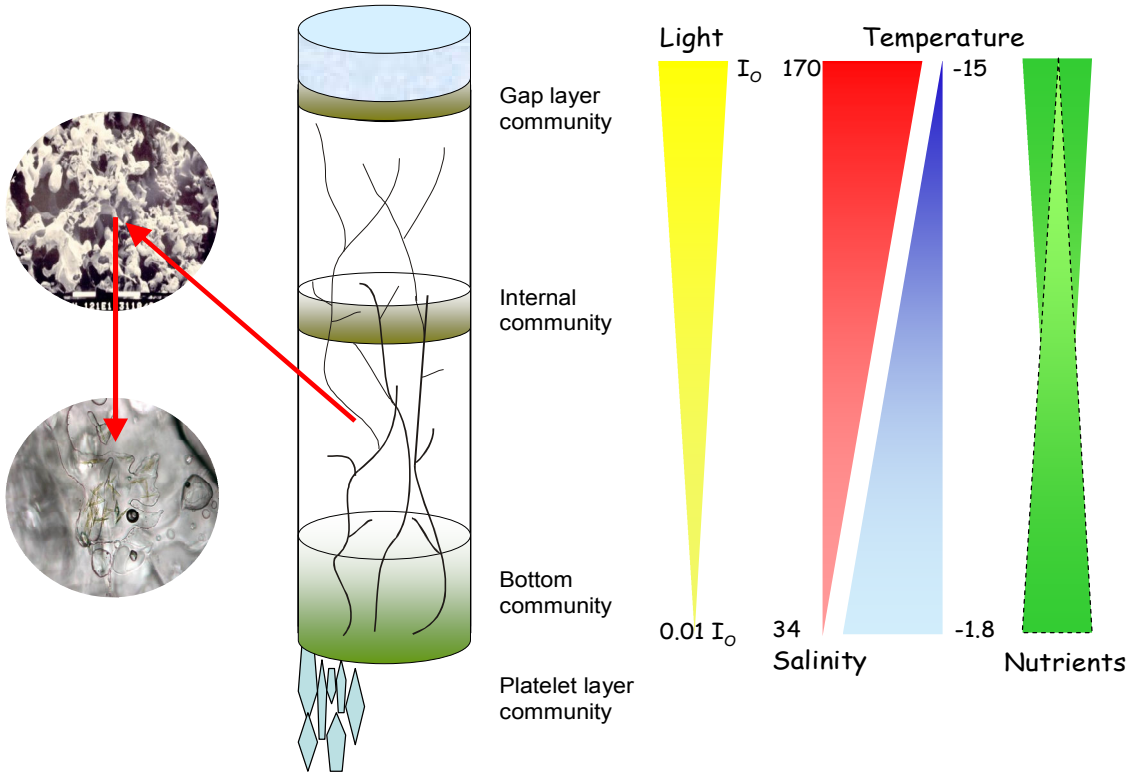


Figure 1:
Schematic drawing of a sea ice column, with the different communities that can be encountered. The two close ups depict the brine channel system and a single brine pocket with diatoms dwelling in it (courtesy C. Krembs). To the right gradients of abiotic factors are shown, in cases of flooding events nutrient supply might be also from the top of the ice sheet.

Abiotic parameters in the ice exhibit strong gradients between the top of the ice and the ice-water interface (Figure 1). Brine channel size and brine concentration within the channels is directly proportional to the temperature of the ice (Eicken *et al.*, 2000). Temperatures are lowest at the top of the ice, ranging from -4 to -20°C and are strongly influenced by the ambient air temperature. At the ice water interface it is almost constant at -1.89°C, depending on sea water salinity. Brine salinities range from 35 to 212g/l (Cox & Weeks, 1983) and in sea ice is subjected to gravity drainage resulting in a gradual desalination of sea ice as it ages. Light availability in sea ice depends on backscatter, ice thickness and snow cover, and may be strongly reduced. The amount of incident light at the bottom of the ice is only about 1% of surface scalar irradiance (Eicken, 1992). The continuous supply of inorganic nutrients is limited to the ice water interface

(Thomas & Dieckmann, 2002b) and is severely restricted within the ice. High photosynthetic activity leads to a much altered chemistry within the ice matrix. It reduces the availability of major inorganic nutrients, dissolved inorganic carbon and as a consequence a shift of pH to high values and a strong oxygen supersaturation (Gleitz *et al.*, 1995, Günther *et al.*, 1999). Despite these harsh conditions sea ice provides a habitat for a diverse, well adapted community comprising mainly heterotrophic bacteria, autotrophic flagellates, ciliates and unicellular algae, especially pennate diatoms (Kirst & Wiencke, 1995, Thomas & Dieckmann, 2002a).

Biomass concentration in sea ice is mostly much higher than in the underlying sea water and often even exceeds that in open polar waters. In Antarctic sea ice standing stocks of <400 µg chl*a* l^{-1} have been observed, while concentrations in surface waters of the southern ocean typically range from 0 to 5 µg chl*a* l^{-1} (Lizotte, 2001). Ice algae generally possess photosynthetic characteristics well adapted to low light conditions, with a high photosynthetic efficiency, fast saturation and photoinhibition at low irradiances (Cota, 1985, MacIntyre *et al.*, 2002). They are capable of maintaining a positive net photosynthetic rate down to photon flux densities as low as 0.2 – 2.9 µmol photons m^{-2} s^{-1} (Mock, 2002). Tilzer & Dubinsky (1987) found that light was the growth limiting factor rather than low temperatures. Although high light intensities cause damage by photoinhibition, a threshold light availability is necessary to withstand abiotic stress, especially osmotic stress, because of the energy dependency of repair mechanisms, e.g. the production of osmolytes.

The dominant ice organisms need to be equally well adapted to a dynamic salinity regime coping with both hypersaline stress during sea ice formation and hyposaline stress during melt of the ice cover (Bates & Cota, 1986, Kirst, 1990, Kirst & Wiencke, 1995, Thomas & Dieckmann, 2002b).

Fragilariopsis cylindrus

One of the most abundant diatoms, especially in the southern polar oceans, is *Fragilariopsis cylindrus* (Grunow) Krieger (Bacillariophyceae), thriving equally well in the open water column as well as being dominant in sea ice assemblages (Kang & Fryxell, 1992)(Figure 2). Thus this species contributes significantly to the formation of blooms. The distribution of *F. cylindrus* in the Antarctic ranges from high latitudinal fast ice communities (Thomas *et al.*, 2001) to the polar front and its occurrence is also been reported from the Arctic regions (Medlin & Priddle, 1990, von Quillfeldt, 1997). The optimum growth temperature of *F. cylindrus* is +5°C (Fiala & Oriol, 1990) and a lower survival limit has not been reported so far, but is expected to be limited by high salinities. Growth experiments in which salinity was consecutively increased to 150 PSU showed that growth halted at 110 PSU - equalling a temperature of -6.7°C – and could be regained at lower salinities (Bartsch, 1989). Altogether, this makes *F. cylindrus* an ideal representative model organism for physiological studies related to polar conditions.

The cultures used in this thesis derive from samples isolated during the Polarstern expedition ANT XVI/3 to the Weddell Sea in 1999 by Thomas Mock.

a) b)

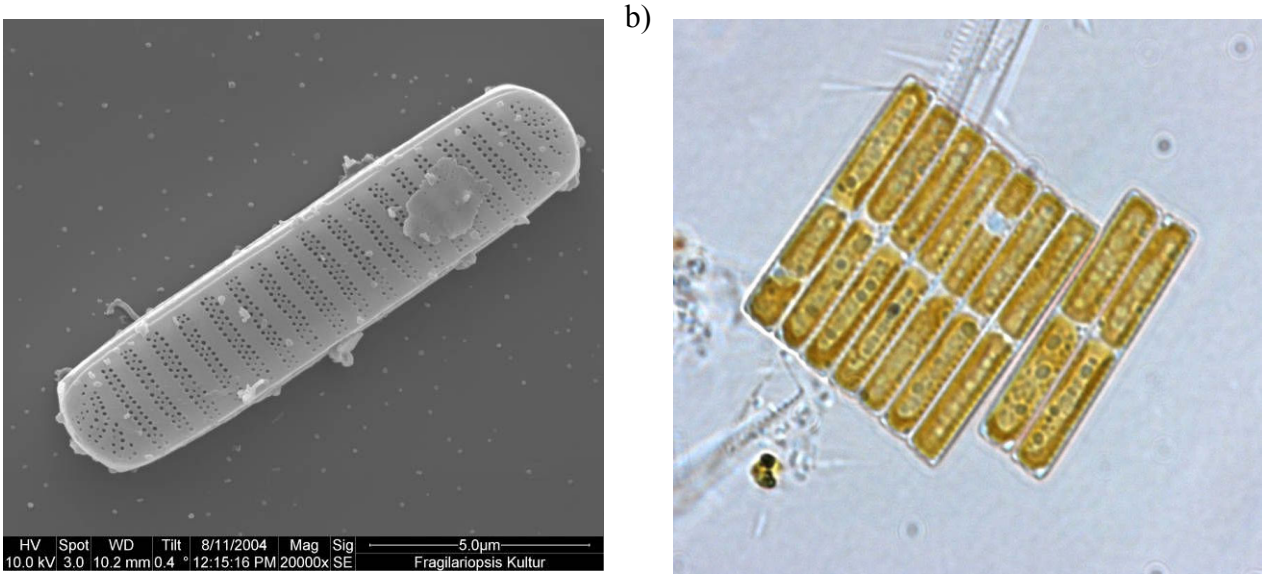


Figure 2: a) SEM picture of a single cleaned *F. cylindrus* frustrule in valve view b) small chain of *F. cylindrus* cells having two chloroplasts. The small droplets visible might be lipid inclusions

Salt stress and organic osmolytes

The ability to adapt to changing osmotic conditions is a prerequisite for all cellular life. Upon osmotic stress, higher plants and algae exhibit a wide range of adaptations at the molecular, cellular and organism level (Hare & Cress, 1997, Bohnert *et al.*, 2001). In this thesis the term osmotic acclimation describes the immediate reaction to osmotic and ionic stress involving the re-establishment of cellular homeostasis through transport processes and the production of osmolytes. In contrast to this, osmotic adaptation implies the evolutionary adaptation manifested on the genetic level.

The alteration of external salinities, either as an increase or decrease mainly influences the internal homeostasis of the cells in three different ways (Kirst, 1990, Erdmann & Hagemann, 2001):

(1) Osmotic stress caused by a flux of water across the semi permeable cell membrane, which leads to a change of the cellular water potential. This osmotically forced water flux directly affects the cell within seconds to a few minutes. Hyperosmotic conditions lead to a shrinkage of the plasmalema, (Bisson & Kirst, 1995). In contrast to this, hypoosmotic conditions cause a water influx resulting in an increased turgor pressure, which is better tolerated by algae possessing a rigid cell wall.

(2) Ionic stress caused by the passive loss and uptake of inorganic ions (mainly Na^+, Ca^{2+}, K^+ and Cl^-). Salinity stress, in addition to osmotic stress, has this ionic component, i.e. the electrochemical charge ions possess, causes the disturbance of the hydration sphere around proteins and other macromolecules, affecting their confirmation or charge interaction, thus rendering their proper function impossible (Xiong & Zhu, 2002). Hence this effect is more deleterious to cells than osmotic stress alone and therefore ion homeostasis plays a critical role in the response to osmotic stress.

(3) Changes in the cellular ionic composition due to the selective ion permeability of membranes.

To counteract the negative effects on cell homeostasis brought about by osmotic and ionic stress, plants and algae pursue a combination of two different mechanisms:

(1) salt extrusion from the cell across the plasma membrane involving ion transporters. This response includes various ATPases, water channel proteins and ion transporters which are regulated by salt stress. Na^+ ions are extruded from the cells or compartmentalized in their vacuole mainly by Na^+/H^+ antiporters (Apse *et al.*, 1999, Shi *et al.*, 2003), which are driven by a pH gradient generated by P-type (plasma membrane localized) or V-type (tonoplast) H^+-ATPases. A number of genes encoding these transporters could be identified in the salt stress induced cDNA library described in this thesis.

(2) osmotic adjustment of the cytoplasm due to the accumulation of organic osmolytes to restore their cellular water potential. Simultaneously, the term "compatible solute" is used, stressing the characteristic feature of these compounds, i.e. their ability to be accumulated in high concentrations of more than 2 M without being toxic to the cell (Brown & Simpson, 1972). Although the uptake of inorganic ions would be the energetically most favourable way to alleviate osmotic stress; this mode of action is restricted to some halophilic archea of the order Halobacteriales and bacteria of the order Haloanaerobiales (Oren, 1999), which possess enzymes especially adapted to high ion concentrations (Madern *et al.*, 2000).

There are about 20 different compatible solutes known in algae (Erdmann & Hagemann, 2001). They mainly belong to three different classes: (1) highest osmotic tolerance is conferred by the accumulation of quaternary ammonium compounds - e.g. glycine betaine and homarine – and tertiary sulfonium compounds like 3-dimethylsulfonium propionate (DMSP) (Kirst, 1996). It has been shown recently that DMSP and its breakdown product DMS is also able to scavenge reactive oxygen species (ROS), thus serving as an antioxidant system (Sunda *et al.*, 2002). (2) moderate osmotic tolerance is achieved by synthesis of amino acids, (3) least osmotic tolerance is exhibited by organisms utilizing polyols and disaccharides. The osmoprotective quality of these compounds corresponds for the majority, with their energetic demands for synthesis (Erdmann & Hagemann, 2001).

In addition to their function in alleviation of osmotic stress, some organic osmolytes have shown cryoprotective properties during freezing. These include the protection of membranes and proteins by sugars (Uemura & Steponkus, 2003), the promotion of recovery processes in freeze

damaged cells by glycine betaine (Chen & Murata, 2002) and the scavenging of reactive oxygen species by proline, as described below.

In recent years the engineering - with emphasis on crop plants - of more osmotolerant varieties has been attempted by the overexpression of certain genes involved in the synthesis of organic osmolyte and transporter genes (Zhang *et al.*, 2001, Chen & Murata, 2002, Wang *et al.*, 2003). However, this has proved to be a difficult task, due to the fact that stress tolerance is a complex multigenic trait and a lot of cross-talk between different regulatory pathways exists. Developing salt-tolerant crop plants remains a challenging task (Yamaguchi & Blumwald, 2005). As a side effect of this work, the genes encoding a new class of antifreeze proteins found in *F. cylindrus* are of potential interest.

Salt stress perception and signalling pathways

Drought and cold stress also cause osmotic stress, which is why salt; drought (water deficit through freezing) and cold stress induce some common sets of plant genes. Different sensors for the direct perception of osmotic stress in algae are under discussion. The water deficit caused either by freezing or high salinity has ionic, osmotic and even mechanical impact on the cell, and it is likely that all these different signals have their own cognate receptor. The only true Na^+ sensor has so far been identified in *Escherichia coli* and controls the expression of a Na^+/H^+ antiporter (Wood, 1999).

In algae, to date no signal transduction chain is known in detail, whereas in higher plants and yeast different signalling pathways are known. In Arabidopsis the salt overly sensitive (SOS) pathway has been discovered, coping specifically with the ionic aspect of salt stress. Three genes are involved in this pathway (Zhu, 2001): SOS1 encodes a plasma membrane localized Na^+/H^+ antiporter, which is regulated by SOS2 and SOS3, two protein kinases. SOS3 senses cytosolic calcium changes that are elicited by salt stress. This is in general one of the early responses to salinity and osmotic stress; a rapid increase in cytosolic free Ca^{2+} concentration, triggering a host of downstream biochemical reactions. Furthermore, plant cells contain a variety of phospholipid-based signalling pathways, including phospholipase C (PLC). PLC upon activation, hydrolyses phosphatidylinositol 3,5 bisphosphate into two second messengers, one of which in turn releases

Ca^{2+} from intracellular stores (Munnik & Meijer, 2001). These authors have also proposed osmotic stress to be a graded phenomenon that activates different receptors dependent on the stress level.

The involvement of mitogen-activated protein kinase (MAPK) cascades as a common mechanism to translate external stimuli into cellular responses in osmotic stress signalling has been demonstrated to be highly conserved in higher plants as well as algae (Jimenez *et al.*, 2004, Teige *et al.*, 2004).

While four different kinds of posttranscriptional regulation have been identified in the biosynthesis of compatible solutes – (1) enzyme activation by ions, (2) covalent modification through protein phosphorylation/dephosphorylation, (3) activity control by a regulatory protein, (4) protein processing through partial proteolysis – transcriptional regulation was thought to be of minor importance for osmolyte sensing enzymes (Erdmann & Hagemann, 2001). However, with the increasing number of investigations on a genetic level, this picture may change (Vinnemeier & Hagemann, 1999). The clear exception to this mode of regulation is the accumulation of proline, the synthesis and degradation of which has been shown to be under tight transcriptional control in higher plants (Strizhov *et al.*, 1997, Hong *et al.*, 2000, Kavi Kishor *et al.*, 2005). This was one reason why the metabolic pathways of proline synthesis and degradation were chosen for molecular genetic investigations in this thesis.

Proline

Although commonly referred to as an amino acid, proline is biochemically an imino acid. The structure of proline differs from the structure of other amino acids in that the aliphatic side chain is bonded to the nitrogen of the amino group as well as to the α carbon atom (Figure 3). This makes the amino group a secondary amine, and because of this, proline is also described as an imino acid. This ring structure is responsible for the hydrophilic characteristics of proline strongly influencing the secondary structure of proteins.

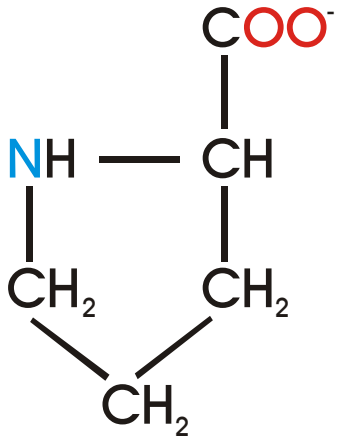


Figure 3: Chemical structure of proline

Proline appears to be the most widely distributed osmolyt 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 & Verma, 1993, Erdmann & Hagemann, 2001). In addition to its function as an organic osmolyte, there are a number of other functions which are associated with proline, e.g. an improved tolerance to freezing (e.g. (Helliot & MortainBertrand, 1999, Takagi *et al.*, 2000) and maintenance of the cellular redox potential after stress events (Hare & Cress, 1997). It is also believed that proline synthesis, through the consumption of NADPH and ATP, may serve as an energy sink, thus continually restoring the terminal electron acceptor of the electron transport chain and preventing photoinhibition (Hare *et al.*, 1999) in stressed cells. Furthermore, increased intracellular proline concentrations lead to an enhanced tolerance against heavy metal induced toxicity (Siripornadulsil *et al.*, 2002). Recently the ability of proline to scavenge reactive oxygen species (ROS) has gained increasing attention (Schriek, 2000, Reddy

et al., 2004, Rodriguez & Redman, 2005), since much of the damage caused by various abiotic stress conditions, including salt stress, is thought to be mediated by ROS. In this context it has also been shown that proline is able to alleviate the negative effects of various abiotic stresses such as UV light, heat, (Liu & Zhu, 1997) and hydrogen peroxide, thus preventing cellular stress and eventually apoptosis (Chen & Dickman, 2005).

However, there is some controversy about the protective function of proline. For instance, it has been shown that in some cases the accumulation of high proline levels made organisms even more susceptible to salt stress (Liu & Zhu, 1997, Rout & Shaw, 1998). Nanjo *et al.* (2003) even attributed toxic effects to elevated proline concentrations.

Proline has been identified as the main organic osmolyte in *F. cylindrus*, besides homarine, betaine and DMSP. Also the concentration of other free amino acids increased with increasing salinity (Plettner, 2002). Thus the identification and the regulation of the proline metabolism is of outstanding interest regarding its central function in the acclimation to elevated external salinities, as well as possessing cryoprotective characteristics.

Proline synthesis

The molecular and genetic basis for the biochemical pathways involved in proline synthesis and degradation have essentially been elucidated in higher plants in the 1990s and has been reviewed by Delauney & Verma (1993), Hare *et al.* (1999) and Verma (1999).

In eukaryotes, proline can either be synthesized starting from glutamate via Δ^1-pyrroline-5-carboxylate (P5C) in two successive reductions catalysed 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, EC 1.5.1.2), whereby P5CS is generally regarded as the rate limiting step in the glutamate route (Figure 4). P5CS has been shown to be feedback regulated via the proline concentration, thus favouring alternative synthesis routes.

An alternative pathway exists in which ornithine is used as the substrate. In this case P5C is again synthesized as the intermediate through the transamination of ornithine mediated by the

ornithine δ-aminotransferase (δ-OAT, EC 2.6.1.13). The subcellular localization of P5CS is thought to be cytosolic in higher plants (Kavi Kishor *et al.*, 2005), whereas findings in this thesis suggest a mitochondrial localization in diatoms. A thorough characterization of the relative contributions of the two P5C- synthesizing routes and the mechanisms whereby they are coordinated is still warranted in higher plants and diatoms. A shift between both pathways in

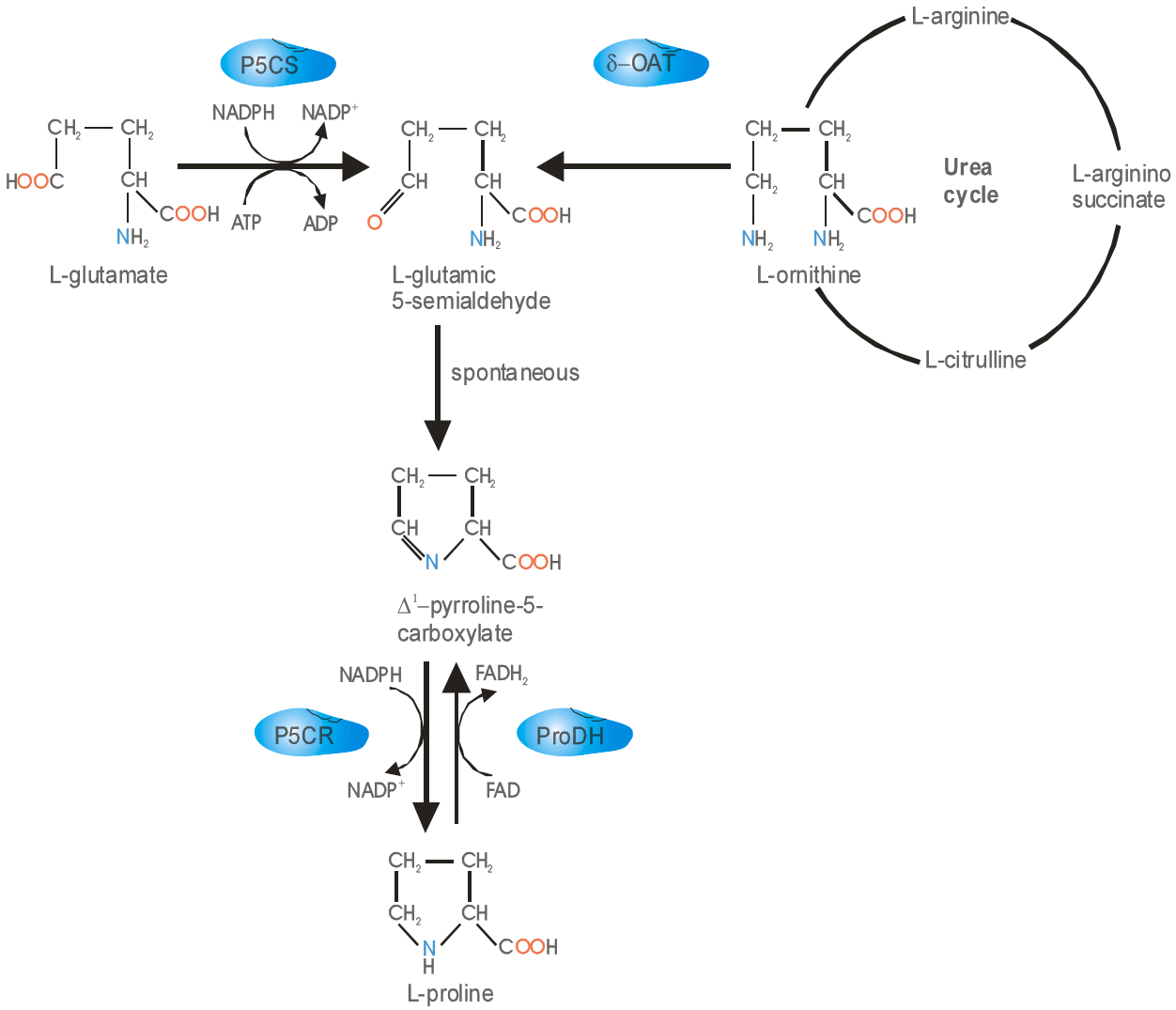


Figure 4: Pathways of proline metabolism using either glutamate or ornithine as a substrate, including the enzymes involved in synthesis and degradation: P5CS, δ-OAT, P5CR and ProDH.. Due to the ambiguous localization of various enzymes no cell compartmentation is depicted.

response to salt stress seems to be likely as data in this thesis and from (Plettner, 2002) suggest. An important aspect in this context may be the nutrient status, especially the availability of nitrogen, since most of the organic osmolytes produced in algae are nitrogenous compounds. This is supported by the fact that in the salt stress cDNA library, three different genes involved

in the fixation of externally supplied nitrogen - a nitrate reductase (NADH consuming), the key enzyme in nitrate assimilation; a nitrite as well as an ammonium transporter - could be found. The recent finding of a full functional urea cycle in diatoms (Armbrust *et al.*, 2004) enhances the role of the urea cycle in protein degradation thus increasing the supply of nitrogen via ornithine. This might be at least in parts determine, whether the glutamate or ornithine synthesis route is preferred under salt stress conditions.

Quite unusual in the degradation of proline is the occurrence of the intermediate P5C. The first degradation step leading to P5C is catalyzed by proline dehydrogenase (ProDH, EC 1.5.99.8), followed by P5C dehydrogenase (P5CDH, EC 1.5.1.12) finally leading to glutamate. Both of these enzymes are mitochondrially located in higher plants, whereas the localisation of ProDH in *Thalassiosira pseudonana* is rather ambiguous.

Antifreeze proteins

Antifreeze proteins, originally discovered in polar fish have been the object of investigations since the 1960s (DeVries, 1969, Scholander & Maggert, 1971). They have also been found in mussels and insects as well as bacteria, fungi and higher plants (Duman & Olsen, 1993, Hoshino *et al.*, 1999). A few years ago the release of macromolecules that bind to and affect the growth of ice was observed in sea ice diatoms (Raymond *et al.*, 1994).

Freezing describes the process of ice crystallization from supercooled water. This process is initiated by the stage of ice nucleation followed by the growth of ice (Du *et al.*, 2003). Subsequently, recrystallization, i.e. the growth of large ice crystal grains at the expense of smaller grains, takes place and larger grains arise which may have deleterious effects on biological tissues. Thus the inhibition of ice nucleation, ice growth and ice recrystallization are equally important functions of ice binding proteins.

Damaging effects of low temperatures and freezing conditions on plant material are characterized by mechanical injury (cell and tissue disruption), which is caused by ice formation, and dehydration injury caused by water loss associated with ice formation. In the case of sea ice diatoms, damage is also associated with dehydration caused by water efflux elicited by rising

external salinities. Under freezing conditions, intracellular bulk water and water oriented on the surface of macromolecules and on the polar heads of lipids in cellular membranes are effectively removed, causing severe dehydration and structural and functional damage to plasma membranes (Webb *et al.*, 1996, Uemura *et al.*, 2006).

With the onset of freezing conditions, some plants produce colligative cryoprotectants such as sucrose and proline; in others, changes in membrane lipids and proteins that render membranes more stable against cold have been reported (Webb *et al.*, 1996). Some plants are able to produce cold-regulated cryoprotective proteins referred to as antifreeze proteins (AFPs), ice recrystallization inhibitors, ice nucleators, or ice active substances (IAS). In the context of this thesis the term ice binding protein (IBP) will be mainly used.

The structure of animal IBPs is well known. They are classified into six types according to the homology of amino acid sequences; antifreeze glycopeptides (AFGPs) and antifreeze types I to IV in fish and hyper-antifreeze proteins present in the body fluid of insects. The molecular mass of animal AFPs ranges from 3 to 33 kD. AFPs similar to those found within the animal kingdom have been identified in plants (Griffith *et al.*, 1992, Griffith & Ewart, 1995). However, the molecular masses of AFPs isolated from plants are considerably larger (11 to 81 kD), than those isolated from animals (Hoshino *et al.*, 1999). It has been shown by Griffith *et al.* (2005) that plant AFPs appear to behave similarly at freezing temperatures, i.e. by inhibiting ice crystal growth and ice recrystallization. The exact functional mechanism underlying the capacity to inhibit ice formation, growth and recrystallization is still a matter of ongoing research.

Ice nucleation is inhibited by adsorption of IBPs to the surfaces of ice nuclei and dust particles leading to an increase in the ice nucleation barrier (Du *et al.*, 2003). Similarly to surfactant molecules, IBPs accumulate and self-assemble on the surface of ice, due to the fact that each IBP molecule possesses a hydrophobic and hydrophilic portion. The adsorbed IBP repels other approaching water molecules, causing a direct impact on ice crystallization.

Further growth of ice is inhibited by adsorption of IBPs to surfaces of growing ice crystals causing a curvature of the ice front (Figure 5).

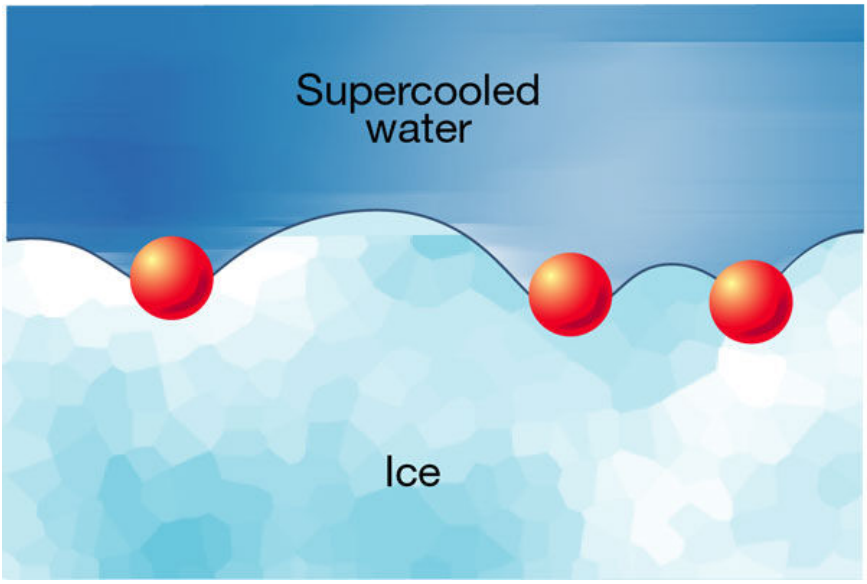


Figure 5: IBPs (red dots) causing a curvature of the ice front and thereby reducing the local freezing point (Knight, 2000)

Since the energetic costs of adding a water molecule to a convex surface is high, this results in a local decrease of the freezing point, while the melting point remains constant (Knight, 2000). This is known as the Kelvin effect.

Recrystallization is thermodynamically favourable because it minimizes the ice interfacial surface area between ice crystals. However, the functioning of IBPs on a molecular level is not yet well understood.

At least in animal AFGPs it has been shown that proline is an important compound of these proteins (Nguyen *et al.*, 2002). It is also hypothesized that IBPs produced by plants not only possess properties to ameliorate the damaging effects of ice formation, but also aids in the tolerance to other abiotic stresses, e.g. drought.

Aims

Resulting from the habitat constraints with which *F. cylindrus* is confronted, a major challenge is to gain physiological information on how *F. cylindrus* is able to cope with these adverse conditions. Emphasis will be placed on investigating differences between the acclimation to low temperatures and acclimations associated with elevated salinities. Since salinity and temperature are physically coupled in the sea ice habitat it is important to investigate each of these stressors separately in order to discriminate between the singular effects of low temperature and high salinity, as well as the combined consequences.

The major aim of this thesis is to obtain an insight into the genetic repertoire of *F. cylindrus* as a starting point for further investigations on a molecular level.

The organic osmolyte proline is synthesized by *F. cylindrus* so as to become acclimated to elevated salinities. The regulation of proline metabolism in diatoms has so far been studied on the enzymatic level. However, data concerning the regulation on the genetic level is still scarce but of prime interest, since it has been shown that the proline metabolism in higher plants is tightly regulated on the transcript level. Therefore, one focus in this thesis is the transcriptional regulation of proline metabolism.

Results and Discussion

Expressed sequence tag (EST) analysis has proven to be an effective method in discovering novel genes and investigating gene expression in different organs and tissues, as well as different environmental conditions. This method was employed in this thesis to establish the genetic basis for further expression analysis and to find genes relevant for the acclimation to environmental stress events.

The first cDNA library of a psychrophilic diatom

The complementaryDNA (cDNA) library analysed in publication I provides first insights into the genome of the psychrophilic diatom *F. cylindrus* and was aimed at elucidating possible adaptations to freezing temperatures. Therefore, the construction of this library was carried out as a cold induced library, i.e. cultures for RNA isolation were subjected to a temperature shock from +5 to -1.8 °C for 5 days in order to enhance the expression of genes related to cold stress.

Of the 2372 clones sequenced from the 5´ end, a set of 996 high quality tentative unique sequences was retrieved after cluster analysis and assembly of sequences. This set comprised 196 tentative unique consensus sequences (TCs) and 800 singletons. The set was subjected to a sequence comparison based on 11 different non-redundant datasets using tBLASTX (Altschul *et al.*, 1997) with a cut off expectancy (e-value) of e 10^{-4}, in order to functionally characterize sequences and to find homologues in other organisms.

A total of 340 sequences gave a significant hit to any database. The *F. cylindrus* sequences showed highest similarity to the *Thalassiosira pseudonana* database (271 significant matches) and 84 of these sequences were found in no other database, indicating their specificity to diatoms. The origin of these sequences showed the highest degree of similarity to eukaryotic algae/plants (30 %), animals (27 %), bacteria (16 %) and fungi (4 %), the remaining twenty-three percent were of unknown affiliation but had EST support. Thus, about one third of the sequences belonged to heterotrophic eukaryotic organisms which could possibly originate from the heterotrophic secondary host, although gene loss in the plant/red algal lineage could not be ruled out (Armbrust *et al.*, 2004).

The most highly expressed sequences (Table 1) encoded two fucoxanthin, chlorophyll a,c-binding proteins (fcps) (31 and 9 clones, respectively), the major protein components of the light-harvesting antenna complexes of photosystem I and II and a calmodulin like protein with no specific function assigned. Genes coding fcps were also highly abundant in other EST libraries stressing their general importance (Scala *et al.*, 2002), being enhanced under stress conditions. Calmodulin is a major calcium sensor and possesses regulatory functions, interacting with a series of cellular proteins like protein kinases, GTPase-activating enzymes, sodium channel proteins and multidrug resistance proteins (Rhoads & Friedberg, 1997).

Table 1: Most abundant TCs (tentative unique consensi)

Internal name of TC	Gene definition	No. of ESTs
F.cyla04h04.s1	Fucoxanthin, chlorophyll a,c-binding protein	31
F.cyla16se09.s1	Calmodulin like protein	20
F.cyla19g12.s1	Unknown function; signal peptide predicted	9
F.cyla01c06.s1	Fucoxanthin, chlorophyll a,c-binding protein	9
F.cyla19e03.s1	Unknown function	8
F.cyla19h06.s1	Sm-like protein	7
F.cyla10g01.s1	Unknown function; signal peptide predicted	7
AVIEST.0.231	Unknown function	7
F.cyla08d09.s1	Unknown function; signal peptide predicted	7
F.cyla12e12.s1	Unknown function	6

Six out of the 10 most abundant TCs belong to the group of genes with unknown function. This differs from results in other eukaryotic EST libraries, where the most highly expressed genes had defined functions. The sequences of unknown function, together with the sequences yielding no significant match to any database, formed 77.1% of all sequences. This was again more than reported so far. However, this might be in part due to the fact that in cDNA libraries established under stress conditions, the number of genes with unknown function generally increases (Bohnert *et al.*, 2001).

Interestingly, a high number of 10 different ABC transporters were found in this cDNA library. These transporters appear to be involved in a number of processes such as fungal resistance,

stomatal conductance or signal transduction (Martinoia *et al.*, 2002). Common to all these transporters is at least one membrane-spanning domain coupled to an ATP-binding cassette. Six of the ABC transporters could not be functionally characterized due to their diverse substrate specificities. However, four clones were of particular interest, since they all encoded proteins without a membrane-spanning domain, but two ATP-cassettes. They belong to two protein classes involved in translational control in yeast (Decottignies & Goffeau, 1997), but their function in other organisms remains to be elucidated. The finding of these genes in this cDNA library might suggest a possible function of the group of ABC transporters in cold acclimation of *F. cylindrus*.

In summary most of the genes functionally characterized and found in this library could be attributed to processes of translation, ribosomal structure, amino acid transport, metabolism and post-translational modification indicating an increased importance of protein metabolism under cold stress. The large number of highly expressed but functionally uncharacterized sequences even hold the potential of yet unknown proteins relevant in cold stress acclimation.

While the aim of publication I was to provide a first insight into the genome of *F. cylindrus*, the aim of publication II was to broaden the basis of available sequence information, with the emphasis on a second major constraint in the sea ice habitat, namely salinity.

Many genes related to salt stress acclimatisation discovered

A total of 2099 high quality sequences (phred 20, min. 100bp length, no ribosomal RNA) was produced from a salt stress induced – elevation of salinity from 34 PSU to 60 PSU, while keeping temperature and light constant – cDNA library of *F. cylindrus*. After the assembly of sequences a non-redundant set (NRS) of 1691 sequences was obtained, reflecting approximately 15% of the expressed *F. cylindrus* genome. This NRS was further subjected to sequence comparison with 10 databases and genomes as described in publication I, in order to assign a putative functional annotation to sequences and to find overlaps to other genomes, especially to those of the pennate *Phaeodactylum tricornutum* and the centric *T. pseudonana.*

Of the NRS, a potential function could be assigned to 44.2% of the sequences, which were considerably more identifiable sequences compared to the first library (27%). The intention of this cDNA library was to identify many genes involved in salt stress response. Among those were a number of genes related to the synthesis and degradation of proline (Figure 6), the major organic osmolyte in *F. cylindrus* accumulated after subjection to elevated external salinities (Plettner, 2002). One of these sequences, encoding Δ^1-pyrroline-5-carboxylate reductase (P5CR) was highly abundant (4 copies), stressing the importance of this pathway. Together with the gene coding for Δ^1-pyrroline-5-carboxylate synthase (P5CS) reported on in publication I, this established the basis for later expression analysis carried out in publication IV.

Salt stress severely disturbs cellular ion homeostasis, which needs to be re-established during stress acclimatisation. Essential to this stress response are different ion transporters and antiporters. A number of antiporters for various ions (Na^+, K^+, Ca^{2+}), as well as different subunits of a V-type H^+-ATPase, responsible for the generation of a proton gradient across the tonoplast to drive sodium sequestration into the vacuole (Shi *et al.*, 2003), were found.

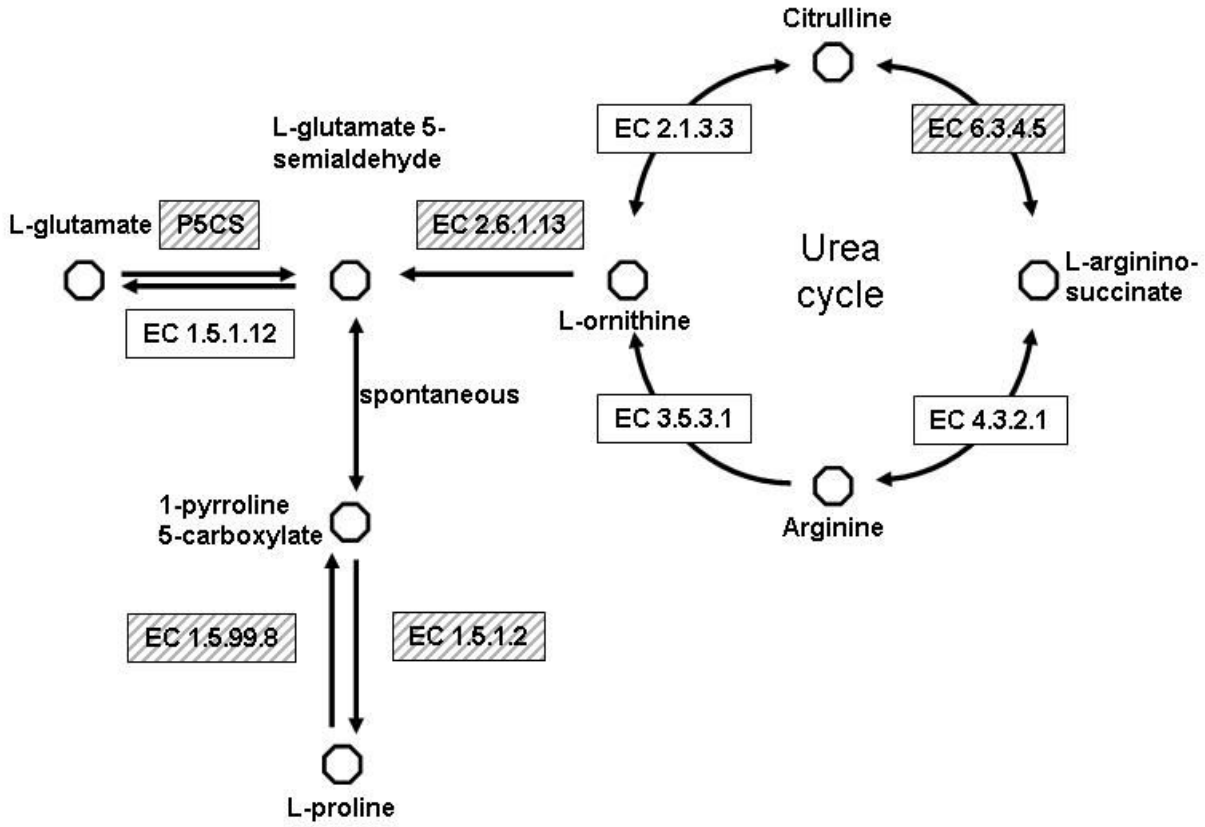


Figure 6: Substrates and enzymes involved in proline metabolism and linked to the urea cycle. Shaded enzymes (ornithine δ-aminotransferase (δ-OAT, EC 2.6.1.13), Δ^1-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2), proline dehydrogenase (ProDH, EC 1.5.99.8) and argininosuccinate synthase (EC 6.3.4.5) were found in this library, while Δ^1-pyrroline-5-carboxylate synthase (P5CS, EC not assigned) was identified in the cold shock library.

The liberation of reactive oxygen species (ROS) following stress events is a major cause of damage in plant cells. Therefore the detoxification of such ROS is of great importance, also in salt stress response as indicated by the finding of genes for glutathione synthase, peroxiredoxin, thioredoxin and a pyridoxine biosynthesis protein (essential for vitamine B6 synthesis), all involved in the scavenging of ROS (Ehrenshaft *et al.*, 1999, Wood *et al.*, 2003, Dupont *et al.*, 2004).

Many sequences coding heat shock proteins (hsps) of different classes were identified in this cDNA library. A member of the hsp70/Dna K family even belonged to the most abundant sequences found; represented by 7 ESTs. Hsps are molecular chaperones, which are responsible for protein and membrane stabilization and assist in protein re-folding during stress acclimatisation (Wang *et al.*, 2004). Hsp70 members have been shown to be fundamental in

conferring salt stress tolerance by overexpression in higher plants (Sugino *et al.*, 1999), thus stressing its importance certainly also in this case.

These examples illustrate the importance and success of a stress induced library in identifying genes related to stress response and acclimatisation. A larger set of sequences, in the range of 10,000 sequences and from libraries established under different conditions would even allow to draw also quantitative conclusions in addition to the more qualitative ones stated her.

A comparison of the *F. cylindrus* NRS with the genomes of the two mesophilic diatoms *P. tricornutum* and *T. pseudonana* showed that 38.3% of the sequences had no homologue in either of the genomes. This variation might be attributed in parts to phylogenetic diversity, but also to the different habitat these diatoms live in. Thus, this fraction of sequences specific to *F. cylindrus* might harbour genes necessary for adaptation to its extreme environment of the polar oceans and sea ice.

Taken together, the cold and salt shock library, which showed only a marginal overlap of 95 non-redundant sequences, we have identified approximately 20 % of all open reading frames in *F. cylindrus*. This forms a critical mass for further microarray studies, especially related to abiotic stress conditions and furthermore, the EST support gained with these two libraries might prove to be very helpful in a future annotation of the *F. cylindrus* genome.

A new class of ice-binding proteins was discovered

In the cDNA library described in publication II, 7 EST sequences were found forming 4 different consensus sequences after assembling. They exhibit highest homology to antifreeze proteins described from the snow mold *Typhula ishikariensis* (Hoshino *et al.*, 2003). This is the first time that homologues from this gene were discovered in a photosynthetic eukaryote (Figure 7). According to the prediction in SignalP (Bendtsen *et al.*, 2004), three of these sequences possess a signal peptide targeting the secretory pathway, thus they might be released into the extracellular space. Observations by Hoshino *et al*. (2003) showed that the AFPs released by *T. ishikariensis* were able to lower the freezing point of water by 0.2 degree, probably by binding to ice crystals and thus inhibiting their growth. The release of such substances by diatoms was already

proposed by Raymond *et al.* (1994) and Raymond & Knight (2003), but no molecular evidence existed so far.

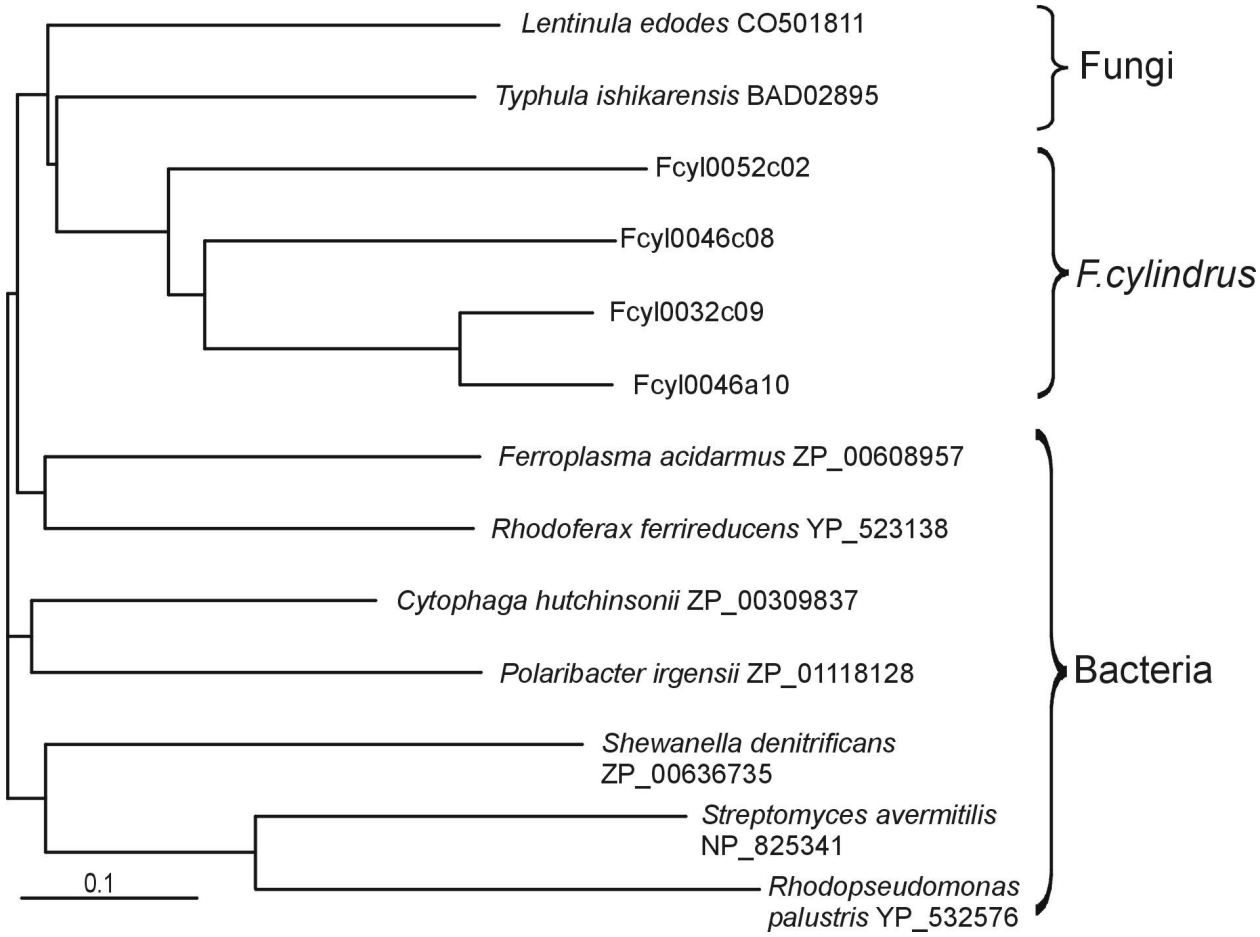


Figure 7: Phylogenetic tree of *F. cylindrus* IBP isoforms and homologues (incl. Accession number) found in Genbank

The ability to produce and exude such proteins would be of fundamental importance for surviving at freezing temperatures in brine channels, with the constant threat of damage by growing ice crystals. Similar genes encoding the IBPs in *F. cylindrus*, could not be detected in the genomes of the mesophilic diatoms *T. pseudonana* and *P. tricornutum,* which implies that these genes might be necessary in adaptation to the polar environment and thus exclusively occur in psychrophilic organisms.

Since none of these sequences were found in the previously established cold shock library (Mock *et al.*, 2006), the possible stimuli for the production of these proteins might rather be salt than temperature. Thus, they might also play a role in ameliorating negative effects of salt stress.

Many polar diatom species possess ice-binding proteins

The study of ice active substance released by sea ice diatoms has been the focus of research carried out by James Raymond (Raymond *et al.*, 1994, Raymond & Knight, 2003). The approach that was employed so far was to purify culture media in which sea ice diatoms have been grown. This supernatant was then subjected to freezing tests and has been shown to possess ice-pitting activities. The aim of further studies was to obtain the purified protein exhibiting ice-binding properties and find out its primary structure. In publication III this was tried with tandem mass spectrometry of a ~25 kDa protein spot separated by 2-D electrophoresis of *Navicula glaciei*. However, the four-peptide sequences which could be identified were too short for detailed characterization. With the already known full length nucleotide sequence retrieved from clones in the salt stress library described in publication II, we were able to design primers to amplify the IBP gene in *N. glaciei* using 5′/3′ rapid amplification of complementary DNA ends (RACE) techniques. Resulting from this work, a full-length *N. glaciei* cDNA could be identified containing a 75 bp 5′untranslated region (UTR), a 726 bp open reading frame (ORF) encoding 242 amino acids and a ~ 121 bp 3′UTR.

The predicted molecular mass of 24.461 kDa agreed well with the results of the 2-D electrophoresis and with the value of 30 kDa estimated from a *Nitzschia stellata* IBP (Raymond *et al.*, 1994). The *F. cylindrus* cDNA contained a slightly larger ORF of 831 bp, encoding 277 amino acids and possessed a molecular mass of 27.961 kDa. The *F. cylindrus* and *N. glaciei* sequences showed a 48 % identity. Both sequences showed considerable similarity to the two fungi *Lentinula enodes* and *T. ishikariensis* and to a number of bacteria, but not to any other organism from the plant or animal kingdom as described in publication II.

The present results obtained from axenic cultures of sea ice diatoms confirm the production of proteins with ice-binding activity. The possible function is still ambiguous, but it is likely that

they appear to act as cellular cryoprotectants rather than antifreeze proteins, since they have little effect on the freezing point. Results of Kang & Raymond (2004) indicated that IBPs protect the cell membrane.

Different mechanisms of preventing freeze induced damage are described in the introduction. The inhibition of recrystallization has been implicated in plant freezing tolerance and proteins being potent re-crystallization inhibitors have been found in several cold-hardy plants (Griffith *et al.*, 2005). Diatom IBPs have also been proposed to act as re-crystallization inhibitors (Raymond & Knight, 2003). Since IBPs produced by diatoms have been shown to be exuded they may protect the cells by preventing the re-crystallization of external ice, thus shaping their habitat. This is supported by the fact that preservation of brine pockets in sea ice appears to be essential for the survival of diatoms at low temperatures (Krembs *et al.*, 2002).

However, a detailed study verifying the proposed function of the IBPs is still warrant. Therefore, next steps are directed in the expression of these genes in *Escherichia coli* to yield the encoded protein. After purification of the protein, X-ray crystallographic analysis and Nuclear Magnetic Resonance (NMR)-spectroscopy studies will be carried out to determine the three-dimensional structure of the protein. Furthermore, after purification of the protein, antibodies matching the protein will be synthesized. This will enable us to carry out studies revealing the localisation of the protein. Centering around the question: does it occur both intra- and extracellularly? And if exuded, can the amount be determined in environmental sea ice samples?

The analysis of the cold and salt shock libraries in publications I and II led to the discovery of all relevant genes coding enzymes involved in the proline metabolism in *F. cylindrus*. The only gene missing is that for Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) coding the enzyme catalysing the second step in proline degradation to glutamate.

Under salt stress proline is synthesized with ornithine as a substrate rather than glutamate

To obtain an insight into the regulation of proline metabolism under hyperosmotic conditions as they occur during incorporation of *F. cylindrus* into a growing ice sheet, a physiological culture experiment was carried out. The results of this experiment are described in publication IV.

The experimental set up consisted of three biological replicates of the following treatments: a) control cultures kept at standard salinity (33.6 PSU) and a temperature of 0 °C; b) an increased salinity (70 PSU) at constant temperature (70/0) and c) increased salinity and decreased temperature (70/-4).

The salt shock applied proved to be a severe but sub-lethal stress for *F. cylindrus* as manifested by a strong drop in photosynthetic quantum yield (Φ_{PSII} dropped from 0.61 to 0.25) within the first 4 h after salt addition and growth arrest for 12 days. Φ_{PSI} constantly recovered in both treatments, but did not attain values as before the shock application. In both salt shock treatments the intracellular concentration of proline increased. After one day it had already doubled and at the end of the experiment it had roughly increased 4.5-fold compared to pre-stress conditions and the control. The constantly increasing intracellular proline concentrations as well as the steady recovery of Φ_{PSII} reflected the ongoing acclimatisation process starting within the first 24 h after the beginning of stress exposition. The accumulation of proline upon salt and hyperosmotic stress is a common stress response in higher plants as well as in algae (Kirst, 1990, Delauney & Verma, 1993, Plettner, 2002). However, the mode of regulation on the transcriptional level of proline synthesis seems to vary between higher plants and algae.

To investigate the regulatory mechanisms underlying proline accumulation in salt stressed cells of *F. cylindrus*, the transcript levels of the key enzymes of proline metabolism, P5CS, δ-OAT, P5CR and ProDH were analysed, employing quantitative real-time PCR techniques (Q-PCR). This analysis revealed a strong (17.3-fold) down-regulation of P5CS (Figure 8), contrasting 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). Together with the finding of a conserved phenylalanine residue in

the amino acid sequence of P5CS, responsible for the feedback inhibition of this enzyme by proline (Hong *et al.*, 2000), this supports the conclusion that P5CS is not responsible for proline accumulation under salt stress in diatoms.

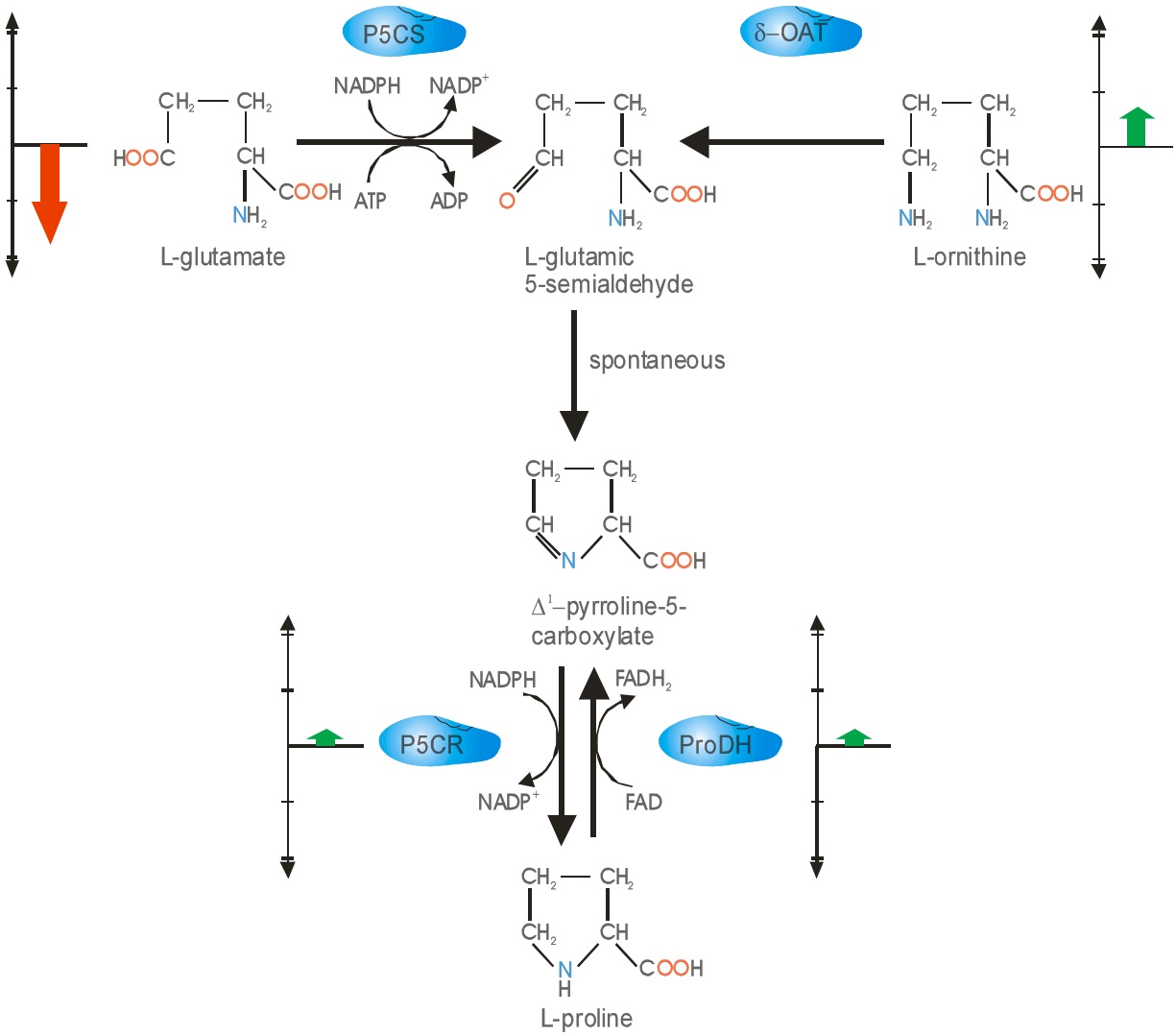


Figure 8: Pathways of proline metabolism using either glutamate or ornithine as a substrate, including those enzymes investigated in the present study: P5CS, δ-OAT, P5CR and ProDH. Thick arrows indicate the initial changes in transcript levels after salt shock treatment.

In contrast to the decline of P5CS, the δ-OAT expression level increased by a factor of 7.6 and 8.9 (70/0; 70/-4), respectively. This obvious up-regulation of δ-OAT and an increase, although to a lesser extent, of P5CR expression and the simultaneous down-regulation of P5CS transcript levels strongly argues for proline synthesis via the ornithine pathway in salt shocked diatoms. This is again in contrast to higher plants where the glutamate route is clearly enhanced following

osmotic stress. The presence of a complete urea cycle in diatoms has recently been demonstrated on the molecular level (Armbrust *et al.*, 2004), supporting the preference of the ornithine route, since the synthesis from arginine originating from protein degradation would only need half the energy necessary for the synthesis from glutamate. In contrast to a number of other polar diatom species, the concentration of free amino acids in *F. cylindrus* increases after a hyper-osmotic shock (Plettner, 2002). The fact that many genes related to proteolysis, especially ubiquitin mediated, were found in the salt stress induced cDNA library supports this hypothesis. The strong inhibition of photosynthesis and hence decline in reduction equivalents by salt shock, might force diatoms to employ energy saving routes of acclimatisation.

In higher plants, proline accumulation during stress was linearly correlated with a strong decline in ProDH transcript levels (Peng *et al.*, 1996, Miller *et al.*, 2005), whereas the results of this study showed the opposite tendency. ProDH levels were positively correlated with the proline concentration and increased three-fold after 24 h, suggesting a turnover of proline and the absence of a stress dependent inhibition of proline degradation in diatoms. However, it still needs to be elucidated, if ProDH activity is regulated at the mRNA level in diatoms as was observed in higher plants, or if additional regulatory mechanisms at the posttranscriptional level exist.

The sub-cellular localization of P5CS and ProDH, as well as the number of isoforms of both enzymes differed between diatoms and higher plants, potentially reflecting the different evolutionary history of organelles in both taxa.

To summarize, in diatoms high external salinities lead to an increase in intracellular proline concentration, as was observed in many organisms and higher plants. However, the regulation of the proline synthesis pathway, as well as the catabolic route is obviously different. In *F. cylindrus*, proline is primarily synthesized via the ornithine route. The combination of increased external salinities and lowered temperature produced no marked differences in the stress response of *F. cylindrus* employing a variety of physiological parameters. This leads to the conclusion that salt is the dominant abiotic stressor.

However, since there are fundamental differences on the mRNA level, it will be essential to measure enzyme activities in order to obtain more definite results. Although this has been shown to be a difficult task, especially related to the measurement of P5CS (Plettner, 2002), this is planned in the near future.

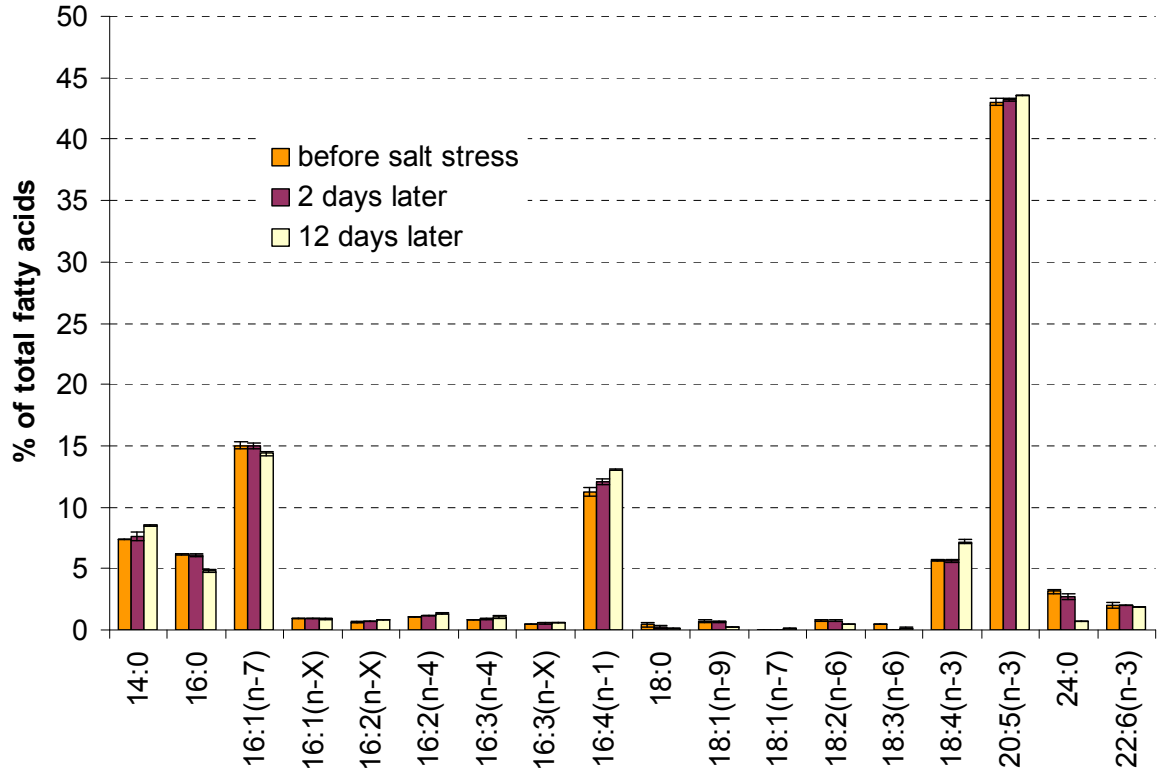
In addition to this, the use of RNAinterference (RNAi) techniques would allow the selective inhibition of the translation of genes involved in proline metabolism, thus clearly dissecting between the contributions of the glutamate or ornithine pathway.

In addition to the parameters already described in publication IV (cell numbers, photosynthetic quantum yield, proline concentration and expression data), further samples for the determination of fatty acid composition were taken. The results of these analyses are depicted here; for further details concerning the experimental set-up refer to publication IV.

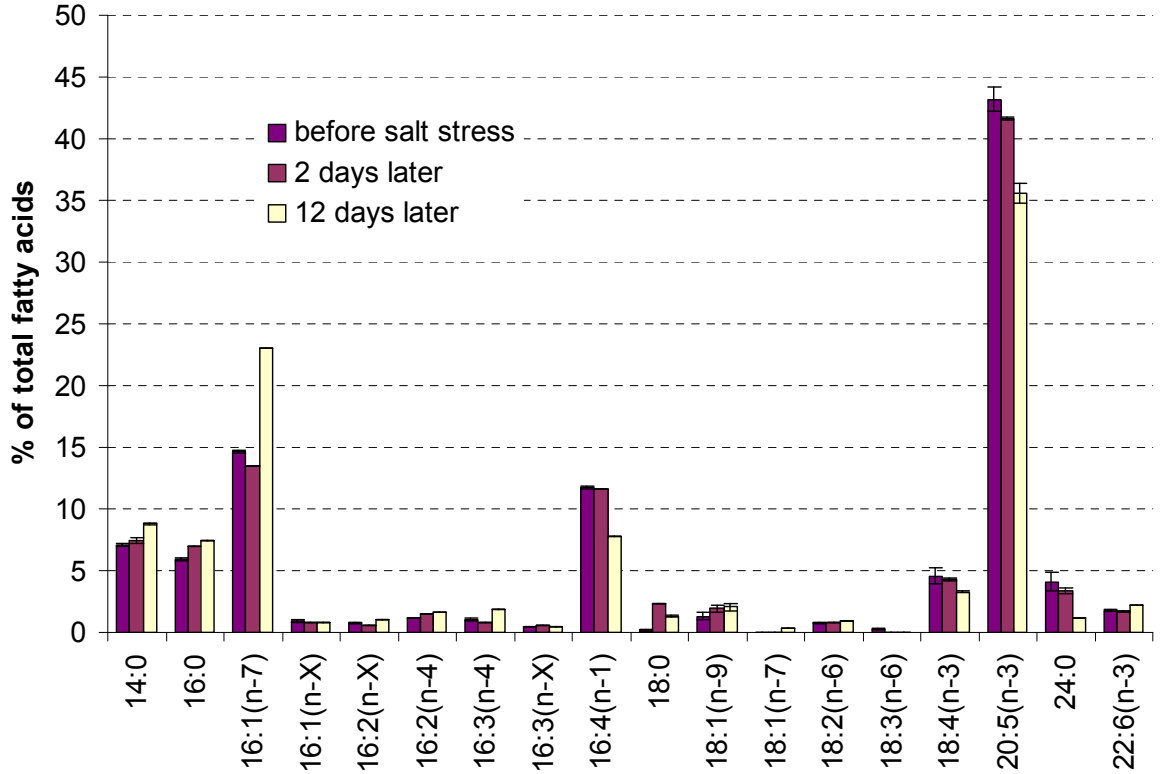
The proportion of poly unsaturated fatty acids decreases upon salt stress

Polyunsaturated fatty acids (PUFAs) are essential membrane components in higher eukaryotes and are the precursors of many lipid-derived signalling molecules. Changes in the fatty acid composition and lipid classes in response to nutrient status (Mock & Kroon, 2002a), light regime (Mock & Kroon, 2002b) and cold stress have been intensively studied (Nishida & Murata, 1996, Mikami & Murata, 2003, Uemura *et al.*, 2006). In general, decreasing temperature is associated with a reduction in membrane fluidity, thus negatively affecting passive and active transport processes. In the case of the sea ice habitat temperature and salinity are inevitably coupled, therefore the investigation of the fatty acid composition under salinity stress might elucidate the dominant stressor.

a)


% of total fatty acids
50
45
40
35
30
25
20
15
10
5
0
before salt stress
2 days later
12 days later
14:0
16:0
16:1(n-7)
16:1(n-X)
16:2(n-X)
16:2(n-4)
16:3(n-4)
16:3(n-X)
16:4(n-1)
18:0
18:1(n-9)
18:1(n-7)
18:2(n-6)
18:3(n-6)
18:4(n-3)
20:5(n-3)
24:0
22:6(n-3)

b)


% of total fatty acids
50
45
40
35
30
25
20
15
10
5
0
before salt stress
2 days later
12 days later
14:0
16:0
16:1(n-7)
16:1(n-X)
16:2(n-X)
16:2(n-4)
16:3(n-4)
16:3(n-X)
16:4(n-1)
18:0
18:1(n-9)
18:1(n-7)
18:2(n-6)
18:3(n-6)
18:4(n-3)
20:5(n-3)
24:0
22:6(n-3)

c)

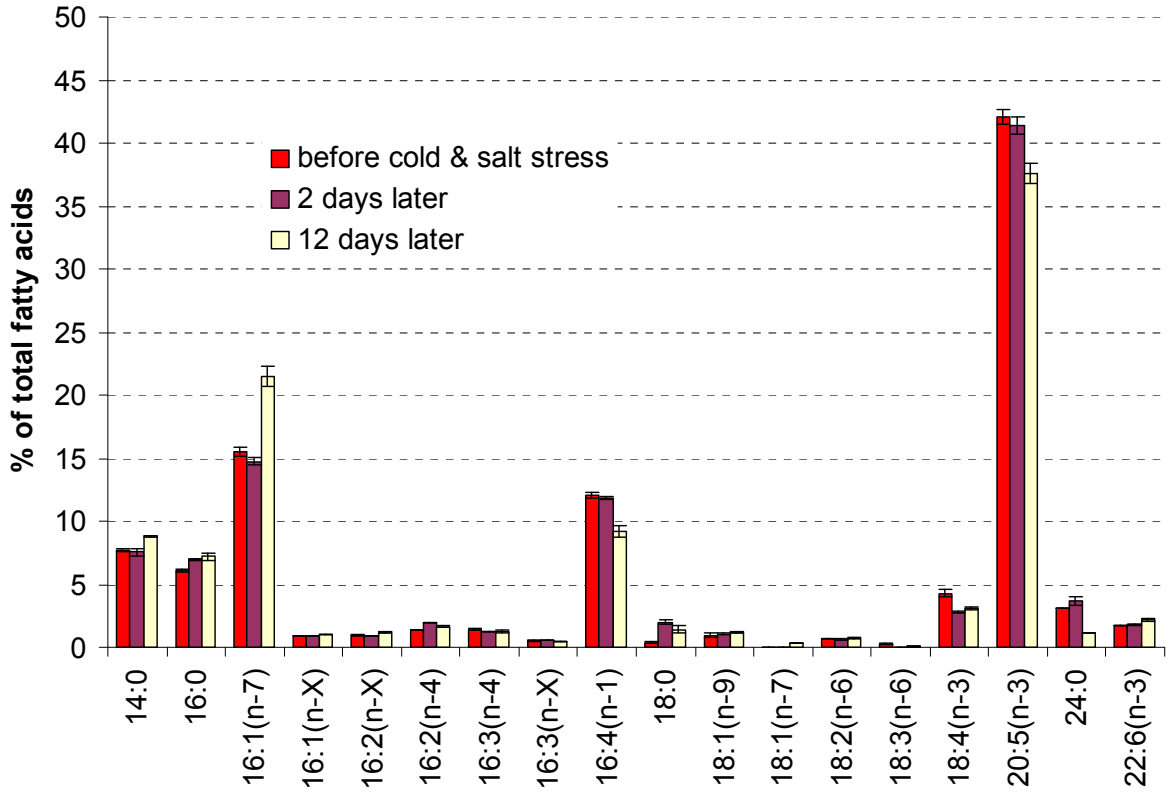


Figure 9: Fatty acid profiles of *F. cylindrus* and its temporal changes in a) control, b) salt stress (70/0) and c) salt and cold stress (70/-4). Fatty acids with unknown position of double bonds are denoted with an 'X'. Error bars denote standard deviation; n = 3, except 70/0 2 and 12 days n = 2

The main fatty acids found in *F. cylindrus* were the unsaturated 16:1, 16:4 and 20:5 with 14:0 and 16:0 being the dominant saturated fatty acids (Figure 9). This general composition pattern neither changed in the 70/0 nor 70/-4 treatment. During the course of the experiment, only a marginal increase of PUFAs could be observed in the control cultures. However, after 12 days of stress exposure the amount of saturated and monounsaturated fatty acids (MUFAs) increased at the expense of the PUFAs (Table 2) in both treatments. The decline of PUFAs concerned mainly the 16:4 and 20:5, whereas the amount of 16:1 increases similarly in the 70/0 and 70/-4 treatment (Figure 9b, c).

The fact that no significant changes could be observed within the first 2 days may be either attributed to the generally low temperatures causing a slow down of metabolism (Q_{10} rule), or to an energy deficit resulting from impaired photosynthesis, hindering the modification of fatty acids. The psychrophilic diatom *F. cylindrus* already contains a very high degree of PUFAs

compared to the mesophilic species *P. tricornutum*. When *F. cylindrus* was subjected to a cold shock from +7 to -1.8 °C changes in FA profile were minimal compared to changes in *P. tricornutum* which was cold stressed from +15 to +5°C (Lange, 2004). These results indicated that *F. cylindrus* in terms of FA composition is strongly pre-adapted to low temperatures, which might be one reason why changes in fatty acid composition were mainly due to the increase in salinity and only marginal after subjection to additional cold stress. However, the decrease of PUFAs and the increase of saturated FAs were still quite remarkable. This decrease in the FA saturation level upon salt stress has been observed in other organisms.

Table 2: Composition of fatty acids in % according to their degree of saturation, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFAs)

	before salt stress			after 2 days			after 12 days		
	control	70/0	70/-4	control	70/0	70/-4	control	70/0	70/-4
Saturated FA	17.10	17.36	17.26	16.65	20.12	20.20	14.26	18.78	18.63
MUFA	16.66	16.88	17.38	16.55	16.25	16.66	15.63	26.31	24.00
PUFA	66.24	65.76	65.36	66.80	63.63	63.14	70.11	54.91	57.36

Outlook

To summarize, about 2500 different genes have been characterized in the first two publications of this thesis. One additional cDNA library under CO_2 limiting conditions is currently established, with the planned sequencing of 5000 clones. Afterwards the genetic basis will be comprehensive enough (ca. 5000 genes) to carry out expression analysis using microarrays. The required technique is currently established at the Alfred-Wegner-Institute and will allow us to investigate the effects and response mechanisms following stress events on a much broader scale, deducing coherence and cross-talk between different sets of genes.

Furthermore, this analysis might provide hints of potential functions of genes, since a major challenge will be to further functionally characterize the newly discovered genes. First of all this is planned for the newly identified ice-binding proteins as described above, since up to now the attributed function is based only on sequence homologies.

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Part II
Publications

List of publications:

Publication I

Thomas Mock, **Andreas Krell,** Gernot Glöckner, Üner Kolukisaoglu, Klaus Valentin (2005) Analysis of expressed sequence tags (ESTs) from the polar diatom Fragilariopsis cylindrus, *Journal of Phycology*, **42**:78-85

Publication II

Andreas Krell, Gernot Glöckner, Thomas Mock, Klaus Valentin (2006) Generation and analysis of a salt stress induced cDNA library of the psychrophilic diatom *Fragilariopsis cylindrus,* and the finding of a new class of ice-binding proteins (to be submitted)

Publication III

Michael Janech, **Andreas Krell,** Thomas Mock, Jae–Shin Kang, James Raymond (2006) Ice-binding proteins from sea-ice diatoms, *Journal of Phycology*, **42**: 410-416

Publication IV

Andreas Krell, Dietmar Funck, Ina Plettner, Uwe John, Gerhard Dieckmann (2006) Regulation of proline metabolism under salt stress in the sea ice diatom *Fragilariopsis cylindrus*, *Plant, Cell and Environment* (submitted)

Publication I

Analysis of expressed sequence tags (ESTs) from the polar diatom *Fragilariopsis cylindrus*

Thomas Mock, Andreas Krell, Gernot Glöckner, Üner Kolukisaoglu and Klaus Valentin

J. Phycol. **42,** 78–85 (2005)

DOI: 10.1111/j.1529-8817.2005.00164.x

ANALYSIS OF EXPRESSED SEQUENCE TAGS (ESTS) FROM THE POLAR DIATOM *FRAGILARIOPSIS CYLINDRUS*[1]

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Analysis of expressed sequence tags (ESTs) was performed to gain insights into cold adaptation in the polar diatom *Fragilariopsis cylindrus* Grunow. The EST library was generated from RNA isolated 5 days after *F. cylindrus* cells were shifted from approximately $+5^\circ$ C to -1.8° C. A total of 1376 ESTs were sequenced from a non-normalized cDNA library and assembled into 996 tentative unique sequences. About 27% of the ESTs displayed similarity (tBLASTX, *e*-value of $\leq 10^{-4}$) to predicted proteins in the centric diatom *Thalassiosira pseudonana* Hasle & Heindal. Eleven additional algae and plant data bases were used for annotation of sequences not covered by *Thalassiosira* sequences (7%). Most of the ESTs were similar to genes encoding proteins responsible for translation, ribosomal structure, and biogenesis (3%), followed by genes encoding proteins for amino acid transport and metabolism and post-translational modifications. Interestingly, 66% of all the EST sequences from *F. cylindrus* displayed no similarity (*e*-value $\leq 10^{-4}$) to sequences from the 12 non-redundant databases. Even 6 of the 10 strong to moderately expressed sequences in this EST library could not be identified. Adaptation of *F. cylindrus* to freezing temperatures of seawater may require a complex protein metabolism and possibly also genes, which were highly expressed but still unknown. However, it could also mean that due to low temperatures, there might have been a stronger pressure to adapt amino acid sequences, making it more difficult to identify these unknown sequences and/or that there are still few protist sequences available for comparison.

Key index words: **EST; cold acclimation; diatom; gene expression; polar; genome**

Abbreviations: **cDNA, complementary DNA; *e*, expectancy value; EST, expressed sequence tag; fcp, fucoxanthin/chl binding protein; TC, tentative consensus**

The polar seas are one of the least studied and least understood ecosystems on the planet. Photosynthesis and oxygen production in polar regions are mainly accomplished by single-celled phytoplankton that live in the top portion of the ocean and within sea ice (Legendre et al. 1992, Lizotte 2001, Boyd 2002, Thomas and Dieckmann 2002). Relatively few classes of phytoplankton and a small number of genera and species appear to play key roles in trophic interactions and biogeochemical fluxes such as that of carbon (Falkowski et al. 2004). The most important class of phytoplankton in the polar marine food-web are diatoms (Bacillariophyceae, Smetacek 1999), with many species that are highly stenothermal with upper lethal temperature limits of only approximately $+10^\circ$ C (Fiala and Oriol 1990). Polar diatoms are well adapted to the main stressors in polar seas: constant low and freezing temperatures, physical disturbances from sea ice, and extreme seasonality (Cota 1985). However, they probably benefit from the relatively high concentrations of dissolved silicate ($Si(OH)_4$) in polar seawater. Species prominent in sediments also serve as proxies in the reconstruction of paleoclimate (Leventer 1998). In the past, because of the

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important global role of diatoms in polar marine ecosysterms, many investigations were carried out using *in situ* perturbation experiments (e.g. EISENEX, EIFEX, SOFeX, ISPOL), meso- and microcosm studies, and physiological and biochemical experiments to assess their adaptation and to understand their role in the biogeochemical cycles of polar oceans (Boyd 2002, Coale et al. 2004, Falkowski and Davis 2004). Despite these efforts, it is still unclear what features allow polar diatoms to survive under polar conditions.

Genome sequencing has become a powerful tool and many genomes from key organisms have been sequenced to provide the basis for understanding biochemical and physiological activities in these organisms. Early reports of genome sequences from autotrophic prokaryotes by Rocap et al. 2003, Palenik et al. 2003, along with a paper by Dufresne et al. 2003, demonstrate how genomic studies can lead to a new understanding of biodiversity, ecology, biological efficiency, and biogeochemistry in marine systems. Recently, the first complete genome of an ecologically important marine diatom (*Thalassiosira pseudonana*) was sequenced (Armbrust et al. 2004). New metabolic pathways were discovered such as the urea cycle in *T. pseudonana* and the uptake of cyanate as a nitrogen source in *Prochlorococcus* MED4. Up to now, biological oceanographers had not considered cyanate as a nitrogen source of any significance. Genome research may therefore influence paradigms on biogeochemical cycling of elements.

About 50% of sequences from newly sequenced genomes and expressed sequence tags (ESTs) bear no similarity to genes identified previously (Armbrust et al. 2003, Ronning et al. 2003). This proportion may be even larger for organisms from polar environments (Clark et al. 2004). These genes could point to novel physiological and ecological phenomena and may also have the potential for biotechnological applications. The first whole genome sequences from polar microorganisms (e.g. *Colwellia psychrerythraea*, Psychrobacter sp. 273–4) and BAC libraries from ice fishes demonstrate the presence of specific metabolical pathways (http://www.genome.gov/10001852). Recently, the U.S. National Academy of Sciences has mapped a strategy for advancing our understanding of polar regions (Hoag, 2003, National Research Council USA 2003). Genomic sequencing and expression analysis will be at the center of this endeavor.

We selected the pennate diatom *Fragilariopsis cylindrus* for molecular studies on mechanisms of polar adaptation. *Fragilariopsis cylindrus* is regarded as the most important cold water diatom of the polar oceans (von Quillfeldt 2004). This diatom has been reported as a dominant species in sea ice, but can also dominate in open water blooms. The goal of this work was to provide a functionally annotated preliminary set of ESTs from *F. cylindrus* expressed under an important polar environmental condition: freezing temperature. Our results provide the first insights into the genome of a polar diatom and provide evidence for specific sequences that may underlie adaptation to freezing conditions in polar waters.

MATERIALS AND METHODS

Fragilariopsis cylindrus *cDNA library and EST generation*. *Fragilariopsis cylindrus* was isolated from Antarctic sea ice during a "Polarstern" expedition (ANT XVI/3) in the eastern Weddell Sea. Axenic cultures were grown at $+5^{\circ}$C in a 10 L batch culture under 35 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (16:8 L:D) in double f/2 medium (Guillard and Ryther 1962). Bubbling with air (150 mL/min) ensured sufficient CO_2 supply and continuous mixing. Samples for the cDNA library were taken 5 days after chilling the cells to the freezing point of seawater (approximately -1.8°C). *Fragilariopsis cylindrus* is acclimated to freezing conditions after five days at this irradiance, as shown in an expression study (Mock and Valentin 2004).

Total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The mRNA was isolated from approximately 100 µg total RNA with an Oligotex mRNA Midi-Kit (Qiagen). Approximately 800 ng poly A^+ mRNA was used for first-strand cDNA synthesis. The cDNA library was synthesized with a SMART™ cDNA Library Construction Kit (Clontech, Mountain View, CA, USA). Total poly A^+ mRNA was used for first-strand synthesis with SMART IV™ oligonucleotides and CDS III/3′PCR primer. Double-stranded cDNA synthesis was performed by LD PCR with an Eppendorf Thermocycler (Hamburg, Germany) using the following program: 95° C for 5 min denaturation, and subsequent 20 cycles at 95° C (2 min) and 68° C (6 min). The cDNA was digested with *SfiI* and fractionated with CHROMA Spin™-400 columns (Amersham, Braun-Schweigen, Germany). The resulting cDNAs were ligated at 16° C overnight into pTriplEX2 vectors. A separate λ-phage packaging reaction (Promega, Madison, WI, USA) was used to obtain an amplified library with a titer of 2.7×10^9 pfu/mL. Blue/white screening with IPTG and X-gal revealed a recombination efficiency of approximately 70%. We recovered DNA from the clones with the magnetic bead kit from Qiagen. Sequencing of cDNA clones from 5′ end were performed using BigDye terminator chemistry from Applied Biosystems, Foster City, CA, USA. The sequencing reaction products were separated on ABI3700 96 capillary machines. Base calling, vector masking, and sequence quality assessment were performed using Phred (Ewing and Green 1998, Ewing et al. 1998). Sequences with a Phred score less than 20 were rejected from the data set.

EST clustering and assembly analysis. The Phrap algorithm with a minimum quality score of 20 was used 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. Individual tentative unique sequences were compared (tBLASTX) to swissprot and genpept data bases on a local Sun system. Furthermore, individual data bases of all available sequences from *Thalassiosira pseudonana* (http://genome.jgi-psf.org/thaps1/thaps1.home.html), *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp/), *Porphyra yezoensis* (http://www.kazusa.or.jp/en/plant/porphyra/EST/), *Oryza sativae* (http://www.tigr.org/tdb/e2k1/osa1/), *Arabidopsis thaliana* (http://www.arabidopsis.org/), *Physcomitrella paten* (http://www.moss.leeds.ac.uk/)s, and *Chlamydomonas reinhardtii* (http://www.chlamy.org/chlamydb.html) were built and queried the same way. Functional domains were searched against COG (http://www.ncbi.nlm.nih.gov/COG/old/xognitor.html) and Interpro (http://www.ebi.ac.uk/interpro/) data bases using default query parameters.

RESULTS

Clustering and assembly analysis. The 5′ ends of 2372 randomly chosen clone inserts were sequenced from a non-normalized cDNA library. Phred analysis (Ew-

TABLE 1. Clustering and redundancy within the cDNA library.

	No. sequences
Total number of ESTs	1376
Singletons	804
ESTs in tentative unique consensi (TCs)	572
TCs	192
Tentative unique sequences	996

EST, expressed sequence tag.

ing et al. 1998) identified 1376 high-quality sequences. To determine the redundancy in the present EST data set and to identify tentative unique sequences, all 1376 ESTs were subjected to sequence clustering and assembly analysis using the Phrap algorithm with standard parameters. Sequence clusters were inspected manually with the help of the Staden package (Staden et al. 1998). The tentative unique sequences consisted of tentative unique singletons and tentative consensus (TC; Ronning et al. 2003) sequences. The ESTs with less than 95% sequence identity over the matching range to other EST sequences from the library were defined as singletons, and TCs were derived from aligned groups of ESTs sharing significant sequence similarity. Approximately 58% of the 1376 *F. cylindrus* ESTs were identified as singletons, whereas approximately 42% of the ESTs were aligned into 192 tentative unique consensus sequences. Finally, a set comprising 996 tentative unique sequences was derived (Table 1). The length of these sequences ranged from 18 to 1580 bp with an average of 415 bp. The number of ESTs in the TCs ranged from two to 31, with an average of 3 ESTs per consensus sequence. The number of ESTs corresponding to the tentative unique sequences (singletons and TCs) ranged from one to 31, with an average of 1.4 ESTs per tentative unique sequence (Fig. 1).

Functional analysis of tentative unique sequences. The 996 tentative unique sequences were compared with 11 non-redundant data bases, of which two (Interpro and COG) were used to identify functional protein domains (supplementary Table 1). Because the *Chlamydomonas* database was composed of a genomic database and an EST database, the total number of databases increased to 12. The comparison was conducted using BLASTX (Altschul et al. 1997) with a cut-off expectancy (e) value of 10^{-4}. Using the distribution of *e*-values for the best matching sequence identified in these 10 data bases, the *F. cylindrus* sequences were most similar to those of the *Thalassiosira* database. Conversely, *Chlamydomonas* sequences showed the lowest similarities to sequences from the *F. cylindrus* data base (Fig. 2). Most of the significant (*e*-value $\leq 10^{-4}$) matches were also retrieved from the *Thalassiosira* data base (total matches: 271). Sixty-nine sequences displayed no similarity to sequences from *Thalassiosira* but were similar to sequences from the

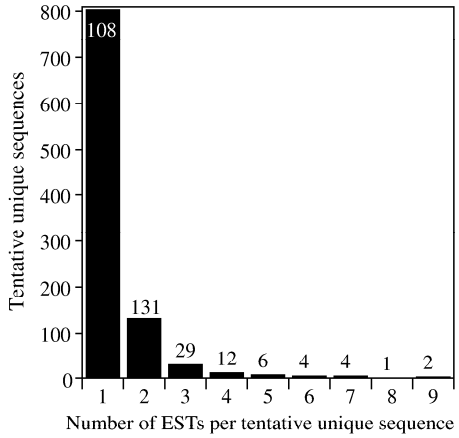

FIG. 1. Distribution of the number of Expressed sequence tags (ESTs) per assembled tentative unique sequence. In total, 1376 EST sequences were analyzed.

other data-bases. A comparison of all matches from all data-bases identified 340 sequences in *F. cylindrus* that were similar to a data-base sequence (Table 2). Interestingly, 84 sequences from *F. cylindrus* had sim-

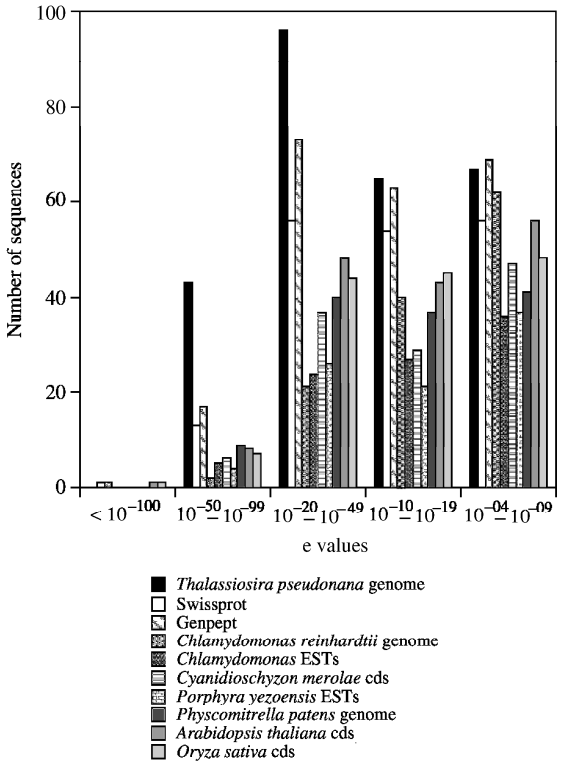

FIG. 2. Distribution of *e*-values from 10 non-redundant databases. Functionally defined database entries were used as resource for assignment of potential functions to the tentative unique sequences. Matches with *e*-values higher than 10^{-10} are most likely insignificant and can may due to e.g. biased nucleotide usage as in *Chlamydomonas reinhardtii*.

TABLE 2. Similarity of the annotated *Fragilariopsis cylindrus* EST database to other databases (cutoff *e*-value $\leq 10^{-4}$).

		996 unique EST sequences from *Fragilariopsis cylindrus*						
Data banks	Total number of similar sequences in data banks	Similar sequences in SwissProt/ GenPep	Similar sequences in *T. pseudonana*	Similar sequences in *C. reinhardtii*	Similar sequences in *C. merolae*	Similar sequences in *P. yezoensis*	Similar sequences in *P. patens*	Similar sequences in higher plants	Similar sequences in *F.cylindrus*
SwissProt/GenPep	229	**17**	179	127	115	77	122	160	229
Thalassiosira pseudonana	271	179	**84**	115	109	70	110	143	271
Chlamydomonas reinhardtii	145	129	115	**3**	93	73	96	115	145
Cyanidioschyzon merolae	119	115	109	93	**0**	64	88	105	119
Porphyra yezoensis	86	77	70	73	64	**2**	67	71	86
Physcomitrella patens	125	122	110	94	88	67	**1**	111	125
Higher plants	173	160	143	115	105	71	111	**3**	173
Fragilariopsis cylindrus	*340*	229	271	147	119	88	127	173	*656*

The table reads as follows: the left and right columns give the total number of similar/homologous sequences found in a data base when compared with the *F. cylindrus* data set, e.g. out of the 996 unique *F. cylindrus* sequences 229 showed similarity to sequences from SwissProt or GenPep, 271 were similar to sequences from *Thalassiosira pseudonana* etc., *340* of the 996 ESTs could be identified by comparison with all databases; *656* could not be identified. The diagonal (in bold) gives the number of unique sequences shared by *F. cylindrus* and a given data base, e.g. 84 of the 996 EST matched only sequences from *T. pseudonana*, only 2 sequences from *Porphyra yezoensis*, etc. The remaining values give the number of sequences shared by three databases, e.g. out of the 996 ESTs, 179 were found in SwissProt/GenPep and in *T. pseudonana*, 93 were found in *C. reinhardtii* plus *C. merolae*.

EST, expressed sequence tag.

FIG. 3. Distribution of matches either in the genome of *Thalassiosira pseudonana* (a) or non-redundant protein data bases (Swissprot and genpept) (b). These sequences from *T. pseudonana*, swissprot, and genpept shared similarity with sequences from either bacteria, fungi, other algae, plants, or animals.

ilar sequences only in *Thalassiosira*, three only in *Chlamydomonas*, two only in *Porphyra*, one only in *Physcomitrella*, three only in higher plants, and 17 only in protein data bases (Swissprot and Genpept) (Table 2, supplementary Table 2). Most of the 84 sequences that occurred exclusively in the genome of *Thalassiosira* showed the highest degree of similarity to eukaryotic counterparts from other algae/plants (30% of sequences), animals (27% of sequences), and fungi (4% of sequences) (Fig. 3a). Twenty-three percent of these sequences were predicted open reading frames (ORFs) with EST support but unknown function (not related to conserved hypothetical proteins), and 16% showed similarity to bacterial sequences. In contrast, matches only found in protein data bases (17 matches) showed the highest degree of similarity to prokaryotic counterparts from bacteria (65% of 17 matches, Fig. 3b). Eighteen percent of these sequences were similar to fungi and 17% to animals. Although bacterial contaminations of the algal culture cannot be ruled out entirely, the relatively high *e*-values (close to 10^{-4}) point to either fast-evolving, horizontally transferred sequences, or to non-significant matches. Sequences found only in *Chlamydomonas*, *Porphyra*, and *Physcomitrella* are related to conserved hypothetical proteins with unknown function, except for one sequence from *Porphyra*, which was identified as an unspecific monooxygenase (*e*-value 4×10^{-23}).

A total of 656 sequences seem to be specific to *F. cylindrus*, a number that may change as more sequences become available from new genome projects (e.g. *Phaeodactylum tricornutum*). In addition, these results from sequence comparison (Table 2 and Fig. 2) are based on heterogenous data sets. For instance, there are many more sequences available from the genome of *Chlamydomonas* than from the genome of *Porphyra*

TABLE 3. Most abundant TCs (tentative unique consensi).

Internal name of TC	Gene definition	No. of clones in sequence
F.cyla04h04.s1	Fucoxanthin chl *a,c*-binding protein	31
F.cyla16se09.s1	Calmodulin-like protein	20
F.cyla19g12.s1	Unknown function; signal peptide predicted	9
F.cyla01c06.s1	Fucoxanthin chl *a,c*-binding protein	9
F.cyla19e03.s1	Unknown function	8
F.cyla19h06.s1	Sm-like protein	7
F.cyla10g01.s1	Unknown function; signal peptide predicted	7
AVIEST.0.231	Unknown function	7
F.cyla08d09.s1	Unknown function; signal peptide predicted	7
F.cyla12e12.s1	Unknown function	6

because the genome of *Chlamydomonas* is about 90% complete, and over 150,000 ESTs (dbEST NCBI) are available. In contrast, genome sequence from *Phorphyra* is not yet available, but approximately 21,000 ESTs (dbEST NCBI) have been sequenced. A comprehensive data bank for *F. cylindrus* ESTs was constructed and can be found at http://genome.imb-jena.de/ALGAE/index.html. Additionally, all ESTs were submitted to http://www.ncbi.nlm.nih.gov/dbEST/.

Most abundant sequences in TCs. Several TCs were composed of mulitple EST sequences (Table 3). The most abundant EST detected encoded a fucoxanthin/chl *a,c* binding protein (fcp) (31 ESTs), followed by a calmodulin-like protein (20 ESTs) and a protein of unknown function with a predicted signal peptide sequence (Interpro data base match). Another abundant fcp (9 ESTs) as well as an Sm-like protein (7 ESTs) could be identified among the 10 most abundant TCs. The remaining five most abundant TCs encoded proteins with unknown function. Two of these TCs had a putative signal peptide sequence followed by an open reading frame (Table 3).

Most abundant protein domains and assigned functional categories. All tentative unique sequences were compared with the Interpro (EBI) and COG (clusters of orthologous groups of proteins, NCBI) data base to identify protein domains. From all tentative unique sequences (996 sequences), 245 sequences (approximately 25%) were found to contain conserved protein domains (supplementary Table 1). The COG database and its functional categories were used to cluster all domains (Table 4). Biological processes such as translation, ribosomal structure, and biogenesis (category J) were represented by 26 protein domains, followed by amino acid transport and metabolism (category E) (17 protein domains), post-translational modifications (category O) (15 protein domains), energy production and conversion (category C) (11 protein domains), and carbohydrate metabolism (category G) (10 protein domains) and general metabolism (category R) (10 protein domains) (Table 4). The most abundant protein domains from both data bases (Interpro, COG) are shown in Table 5.

DISCUSSION

Abundance and function of tentative unique sequences. Only 0.2% (two sequences) of all sequences were highly expressed, i.e. they are represented by TCs comprising more than 10 ESTs (Zhang et al. 2004). A total of 31 ESTs encode a fucoxanthin, chl *a,c*-binding protein (fcp), the major protein components of the light-harvesting antenna complexes of PSI and PSII within diatom plastids (Grossman et al. 1990). In addition, a second gene encoding an fcp was supported with nine ESTs. Some fucoxanthin, chl *a,c* binding proteins are known to be highly expressed in *F. cylindrus*, as indicated by an expression study using macroarrays (Mock and Valentin 2004). A gene encoding an fcp was also supported by the most ESTs in a *P. tricornutum* EST library (Scala et al. 2002), suggesting that members of this gene family (fcps) may be highly expressed under certain stresses or changing environmental conditions. The second TC in this study is composed of 20 ESTs and encodes a calmodulin-like protein whose specific function cannot be

TABLE 4. Most abundant functional categories identified by the COG (cluster of orthologous groups of proteins) data base (http://www.ncbi.nlm.nih.gov/COG/old/xognitor.html).

Rank	Functional category (symbol of category)	No. of protein domains
1	Translation, ribosomal structure, and biogenesis (J)	26
2	Amino acid transport and metabolism (E)	17
3	Post-translational modification, protein turnover, chaperones (O)	15
4	Energy production and conversion (C)	11
5	Carbohydrate transport and metabolism (G)	10
5	General function prediction only (R)	10

TABLE 5. Most abundant protein domains identified by the COG (Cluster of Orthologous Groups of Proteins) (http://www.ncbi.nlm.nih.gov/COG/old/xognitor.html) and Interpro (http://www.edi.ac.uk/interpro/) data bases.

Rank	Description of protein domain	No. of unique sequences
1	Ribosomal	19
2	ABC transporter	10
3	Peptidases	9
4	Chl *a/b* binding	8
5	DNA/RNA helicases	6
5	GTP binding	6
6	Chaperons	5
6	TonB box, N-terminal	5
7	Elongation factor	4
7	Zn-finger	4

defined by sequence comparison. However, of the 996 tentative unique sequences, two IQ calmodulin binding regions (IPR00048) could be identified: one interpro domain matched the annotated calmodulin-like protein and the other a conserved protein with unknown function. Calmodulin acts as a major calcium sensor and orchestrator of regulatory events through its interaction with a diverse group of cellular proteins (Rhoads and Friedberg 1997). Three classes of recognition motifs exist for many of the known calmodulin binding proteins. The IQ motif is a consensus for Ca^{2+}-independent binding, and two related motifs termed 18–14 and 1–5–10 based on the position of conserved hydrophobic residues indicate Ca^{2+}-dependent binding. The calmodulin binding IQ motif occurs in a variety of proteins such as protein kinases, GTPase-activating enzymes, sodium channel proteins and multidrug resistance proteins (Rhoads and Friedberg 1997). The question remains regarding which specific function this protein carries out in *F. cylindrus*.

A moderately expressed sequence (six to nine ESTs per TC) encoded an Sm-like protein. Proteins from the Sm family are known to interact with small mRNAs for mRNA processing (e.g. splicing). The Sm proteins are essential for pre-mRNA splicing and are implicated in the formation of stable, biologically active spliceosomal small nuclear ribonucleoproteins (snRNP) structures that are involved in Sm protein–protein interactions (Hermann et al. 1995).

The remaining six TCs out of the 10 most abundant TCs could not be functionally defined based on analysis of all 12 data bases (tBLASTX and BLASTN), which is unusual based on EST libraries from other eukarytoes (chl Scala et al. 2002, Ronning et al. 2003, Shrager et al. 2003, Habermann et al. 2004, Ida et al. 2004) in which the most highly expressed genes had defined functions. The TCs from *F. cylindrus* were not related to conserved genes with unknown function, nor to genes from other diatoms (*T. pseudonana* and *P. tricornutum*). However, all six TCs had an open reading frame either at the 5′ end or in the middle of the sequence, and five had a polyA tail and a length between 671 and 1263 bp. For 129 ESTs with a polyA tail, wc could localize the gene end relative to the polyA tail and were therefore able to calculate the average length of the 3′ UTR, which was 138 bp (5–500 bp). The smallest TC of the six most abundant TCs with unknown function is longer than the longest 3′ UTR sequence. Consequently, it is unlikely that the six abundant unknown TCs are UTRs. They could therefore be specific sequences at least moderately expressed under freezing conditions.

Sequence comparison with data bases. Sixty-six percent of sequences in the EST library from *F. cylindrus* are related to genes of unknown function. However, it is possible that some of these sequences that were shorter (less than 100 bp) and without a polyA tail may have corresponded to UTRs. The remaining 34% of the ESTs showed similarities, expressed as low *e*-values (*e*-value $\leq 10^{-04}$), to sequences from *T. pseudonana* and ESTs from *P. tricornutum*, which were integrated into the annotation of the genome from *T. pseudonana*. This high similarity of sequences between *F. cylindrus* and *T. pseudonana* is related to the fact that they both belong to the class Bacillariophyceae.

Eighty-four EST sequences were similar to sequences found only in the *T. pseudonana* data base but not in the other plant/algae databases examined here. Thirty-one percent of these 84 genes had similarities to genes from heterotrophic eukaryotic organisms (fungi and animals), which were possibly derived from the heterotrophic secondary host, although gene loss in the plant/red algal lineage cannot be ruled out (Armbrust et al. 2003). Twenty-three percent were novel diatom genes with unknown function. Interestingly, all similarities to sequences exclusively found in protein databases (genpept and swissprot) were related to bacteria and other heterotrophs, and none of them were found in the plant databases or the *T. pseudonana* data base examined here. For instance, there are 3 "animal-like" *F. cylindrus* sequences that are absent from *T. pseudonana*: one is most similar to a human calpain protease (PalBH/CAPN7, Acc. Nr. Q9Y6W3), and the other two to the *Dictyostelium discoideum* kinase (SNF1/AMP, Acc. Nr. AAD30963) and MkpA protein (Acc. Nr. AAO51390). Interestingly, all three sequences were related to protein metabolism.

Abundant protein domains and functional distribution. The three most common functional categories, comprised of 58 protein domains (23% of all identified domains), were related to translation, including post-translational modifications and transport of amino acids/peptides. Six different DNA/RNA helicases in the EST library revealed that DNA and RNA coiling/uncoiling are important for this organism. Minimizing secondary structures and duplexes of mRNAs, which could easily form under low temperatures, is necessary to initiate translation. Up-regulation of a DEAD/DEAH box RNA helicase under freezing temperatures already demonstrated the importance of this process in *F. cylindrus* (Mock and

Valentin 2004). However, protein domains of DNA/RNA helicases are the 8th most abundant protein domain in the genome of *T. pseudonana* (Armbrust et al. 2003), and therefore more evidence is necessary to conclude that these enzymes are essential to cope with freezing temperatures.

Nine genes encoding peptidases were identified in the EST library of *F. cylindrus*: two sequences with signal peptidase domains were identified and two with domains for cystein peptidases. Peptidases are enzymes responsible for either the complete digestion of proteins or cleavage of localization peptides required for protein targeting and activation. Three of the 10 most highly expressed sequences with unknown function in *F. cylindrus* possess signal peptides (predicted by Interpro). In addition, peptidases may also be required to repair photodamaged proteins (e.g. D1 of PSII) under freezing temperatures (Mock and Valentin 2004).

Membrane transport of substances other than proteins also seems to play a pivotal role in cold adaptation. The high number of ABC transporters in plants (Henikoff et al. 1997, Sánchez-Fernández et al. 2001) has been hypothesized to reflect the fact that sessile organisms rely upon detoxification as an important means of resisting different stresses. Interestingly, few plant ABC transporters have actually been shown to play a role in detoxification. Instead, these transporters appear to be required for a variety of other processes such as fungal resistance, stomatal conductance, or signal transduction (Martinoia et al. 2002).

Two out of the ten ABC transporter EST sequences, both represented by a single clone, displayed homologies to bacterial permeases. Four of the ABC transporters are homologous to ABC transporters exclusively found in eukaryotes. There are two sequences with homologies to WBC proteins: one PGP-like EST and the other one with similarities to ABC1 proteins. All these proteins are found in animals and plants, and they consist of at least one membrane-spanning domain coupled to an ATP-binding cassette in one polypeptide, like the WBC proteins, or they harbor both domains in tandem. Functional characterizations of these transporters according to their structure were not possible, because of their diverse substrate specificities and functions. Four ABC transporter ESTs isolated in this study are of particular interest. They are all homologous to genes encoding proteins with two ATP-binding cassettes without membrane spanning domains. One of them belongs to the GCN20 class, first characterized in yeast and then also found in other higher eukaryotes (Vazquez de Aldana et al. 1995, Dean and Allikmets 2001, Sánchez-Fernández et al. 2003). Another two ESTs encode two different YEF3 homologs. These proteins are structurally and functionally related to GCN20 proteins in yeast. Both protein classes are involved in translational control in yeast (Decottignies and Goffeau 1997), but their function in other organisms remains to be elucidated. The fourth EST clone shows homology to bacterial *uup* genes and also to a protein with two ABC domains but without a membrane-spanning domain. These proteins have been reported to control transpositional processes in *E. coli* (Reddy and Gowrishankar 1997). Although all these structurally related proteins are relatively well characterized in bacteria and yeast, almost nothing is known about them in higher eukaryotes. The appearance of these four different clones in this EST collection suggests a functional role for this particular group of ABC transporters in cold acclimation of *F. cylindrus*.

To date, polar diatoms are not a major subject in the field of cold acclimation/adaptation, despite their important role as the basis of the entire polar food web. Therefore, this EST study was conducted to provide the basis for further molecular studies with polar diatoms and in particular with *F. cylindrus*. Most of the annotated sequences are related to translation, ribosomal structure, biogenesis, and post-translational modifications of proteins. New enzymes/proteins are required to acclimate to freezing temperatures. The occurrence of signal peptides in some of the most highly expressed sequences indicates secretion of these proteins.

We are grateful for the constructive advice from two anonymous referees, Chris Bowler and particularly, Ginger Armbrust, who helped us with the final editing of this paper.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. COG and IPR IDs.
Table S2. Similarity of ESTs to other data bases.

Publication II

Generation and analysis of a salt stress induced cDNA library of the psychrophilic diatom *Fragilariopsis cylindrus* and the finding of a new class of ice-binding proteins

Andreas Krell, Thomas Mock, Gernot Glöckner, Bank Beszteri and Klaus Valentin

Generation and analysis of a salt stress induced cDNA library of the psychrophilic diatom *Fragilariopsis cylindrus* and the finding of a new class of ice-binding proteins

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Keywords:

Antarctic, Arctic, *Fragilariopsis cylindrus,* EST, osmotic stress, salt stress, gene expression, ice-binding protein

Abstract

We have used an EST approach to find genes related to salt stress in the polar diatom *Fragilariopsis cylindrus*. From 2880 clones sequenced from the 5 primed end 1691 high quality tentative unique sequences were established and analysed. 38% of these sequences have no homologues in the genomes of two mesophilic diatoms, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* indicating substantial genomic variation between mesophilic and psychrophilic diatoms. Of the 1691 sequences 55.8 % could not unequivocally identified by comparison to the SwissProt and refseq data base. Among the 44.2 % tentative unique sequences with homologues in databases a high proportion could be assigned to stress related genes. Most of these encode for various different heat shock proteins (hsps) and proteins involved into transport processes, especially different ionic transporter and antiporter genes reflecting the requirement to re-establish the disturbed ion homeostasis caused by salt stress. But also genes related to oxidative stress and apoptosis were found. Furthermore, many genes involved in the proline synthesis pathway, one of the most important organic osmolytes in *F. cylindrus* were identified. A major outcome of this analysis was the unexpected finding of a new class of ice-binding proteins (IBPs) identified in *F. cylindrus*, providing the first molecular evidence for the already proposed existence of such proteins related to the ability of diatoms in shaping their habitat. A comparison to a recently established cold-shock library from the same species (Mock *et al.*, 2006) revealed several stress related genes common in both libraries, suggesting some general physiological response mechanisms to both cold and salt stress. Thus, this *F. cylindrus* cDNA library may serve as a rich genetic resource for the identification of novel genes unknown in Bacillariophycea associated with environmental stress and salt stress tolerance.

Introduction

Salt stress tolerance and salt stress adaptation of algae have been a point of focus during the past decades (Kirst, 1990, Erdmann & Hagemann, 2001). These studies, however mostly focussed on macroalgae of the intertidal and rock pools. Salt stress has also been studied extensively in the microalgae *Clamydomonas sp*., *Synechocystis sp.* (Kanesaki *et al.*, 2002) and *Dunaliella sp*. (Liska *et al.*, 2004) but they are not representative for the marine environment. Salt stress in algae as well as in other plants severely disturbs the cellular homeostasis brought about by differences between the internal and exogenous concentration of inorganic ions (predominantly Na^+ and Cl^-), causing water efflux, i.e a decrease in cell volume and ion influx.

Thus 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/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).

Fragilariopsis cylindrus (Grunow) Krieger (Bacillariophyceae) is a psychrophilic, bipolar distributed pennate diatom and a major primary producer in open polar waters, especially in the marginal ice-edge zone (Kang & Fryxell, 1992), as well as in sea ice. Its occurrence in this extreme habitat is well documented (Gleitz & Thomas, 1993, Günther & Dieckmann, 2001) *F. cylindrus* is obligatory adapted to the polar environment, i.e. it is not able to survive temperatures above +8°C (Fiala & Oriol, 1990).

The most prominent feature of sea ice is its porous structure, since during the freezing process only water molecules form a solid matrix, whereas other ions are rejected into a system of interconnected channels and isolated pockets (Weissenberger *et al.*, 1992), varying in size from <5µm to >1 cm depending on ice type (granular/columnar) and temperature. Salinity in the brine channel system thus increases due to salt rejection during ice crystal formation and can attain values of 70 to 144 at -4 to -10°C respectively (Cox & Weeks, 1983). Incoming irradiance is strongly attenuated so that values ranging between 0.3 and 100 µmol photons m^{-2} s^{-1} reflect typical habitat conditions (McMinn *et al.*, 2000). *F. cylindrus* was chosen as a model organism to

study osmotic stress adaptation since this diatom is capable of tolerating salinities up to 150 PSU and still grows at a salinity of 100 PSU (Grant & Horner, 1976, Bartsch, 1989). It can be expected that this rather extreme habitat stipulates metabolic adaptation with respect to temperature, and with regard to the survival of *F. cylindrus* in sea ice, also to elevated osmotic and hence oxidative stress. 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 conditions, to combine the impact of multiple stressors.

To understand molecular processes underlying osmotic stress response it is essential to study the adaptation mechanisms inherent in *F. cylindru*. Whereas *P. tricornutum* and *T. pseudonana*, whose genome has recently been published (Armbrust *et al.*, 2004), may serve as good model organisms for temperate regions, they are inadequate representatives of polar realms. It is ecpected that the genetic repertoire of *F. cylindrus* differs considerably from both. Hence, the EST sequence information of *F. cylindrus* as an eukaryotic extremophile may add valuable genetic data on stress tolerance and adaptation.

Expressed sequence tag (EST) approaches, although entitled as the “poor man’s genome” (Rudd, 2003) have been shown to be an effective mean of rapidly gaining information about an organism at fundamental levels and to gather data to investigate particular subsets of genes related to different tissues, growth, developmental and stress conditions. Furthermore a cDNA library established under determined stress conditions is thought to amplify genes responsible for compensating this stress. This method has already demonstrated its suitability in resolving different questions in the context of osmotic stress response in higher plants as well as in algae (Redkar *et al.*, 1996, Zhang *et al.*, 2001, Oztur *et al.*, 2002, Wang *et al.*, 2006).

Information gathered from cDNA libraries has provided significant aid in later annotation of whole genomes (Haas *et al.*, 2002, Shrager *et al.*, 2003). Furthermore a stress induced cDNA library can be regarded as an excellent method to find relevant genes of stress response and establish a valuable basis for later expression analysis, equally in the form of hybridisation techniques (northern blotting and microarray analysis), real time quantitative PCR (Q-PCR) and to a lesser extent for siRNA and transformation experiments.

Here we present a cDNA library generated from *F. cylindrus* subjected to elevated salinity, which allows the identification of genes putatively critical for survival at high salinities.

Material and Methods

Culture conditions

Fragilariopsis cylindrus was grown in 4.5 l batch cultures at 0 °C under an irradiance of 15 µmol photons m^{-2} s^{-1} (continuous illumination), using double f/2 medium (Guillard & Ryther, 1962) prepared from Antarctic seawater with a salinity of 34. Two different salt shock experiments were carried out during the early exponential growth phase (ca. 1.7 mio. cells l^{-1}) to prevent subsequent nutrient exhaustion.

To excite osmotic stress, a concentrated brine solution was prepared from the original medium and added sea salt (Instant Ocean). During the first experimental set-up, the brine solution was added to the batch culture with the help of a peristaltic pump, whereby salinity was increased to a final salinity of 60 within 3 h.

During the second essay more artificial stress conditions were employed, i.e. all the concentrated brine solution was added instantaneously. Samples for RNA isolation from both experimtal set-ups were taken every 6h following the start of the salt addition on the first day and subsequently once per day for a period of 4 days, thus ensuring the acquisition of transiently regulated genes.

In vivo quantum yield of photosystem II (F_v/F_m) of the *F. cylindrus* cultures was monitored once per day with the aid of a Xenon Puls-Amplitude-Modulated (PAM) Fluorometer (Walz GmbH, Germany). F_v measurements were done on not dark adapted samples but at an illumination of 15 µmol photons m^{-2} s^{-1}, which means that the measured quantum yield reflects *in situ* conditions rather then the potential maximum yield.

Isolation of mRNA

Subsamples of 30 ml for RNA extraction of the culture were filtered onto 25 mm, 1.2 µm pore size polycarbonate membrane filters (Millipore), washed with 20 ml 0.2 µm filtered seawater, shock frozen in liquid nitrogen and stored at –80 °C. Total RNA was extracted using the

RNAquous kit (Ambion Inc, USA) and subsequent poly A^+ mRNA purification with the *Poly(A) Purist MAG* kit (Ambion Inc, USA). A total amount 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 of Invitrogen. In contrast to other library construction methods the use of restriction enzymes is omitted 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. FS synthesis was performed using the following program: 70°C/ 5min, 37°C/ 5min, allowed to cool for two minutes, before buffer mix was added (leave cup in cycler). After three more minutes Superscript II RT was added, 37 °C/ 1:10 h, 70°C/ 5min. 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 Spun Column* from Amersham. After checking cDNA quality on a subsample 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 more than 4 kbp were cut out using a dark reader and extracted with the *Mini Elute Gel extraction* kit from Qiagen, 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 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 over night cultures of cDNA clones from the library were sequenced starting at the 5 primed end using the M13 forward sequencing primer and BigDye terminator chemistry from Applied Biosystems. 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 less than 20 were rejected from the data set.

EST clustering and assembly analysis. The Phrap 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 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 swissprot and refseq databases on a local Sun system. Furthermore, individual databases of all available genome sequences from *T. pseudonana,* (genome.jgi-psf.org/thaps1/ thaps1.home.html), *P. tricornutum* (unpublished), *Chlamydomonas sp.* (www.chlamy.org /chlamydb.html), *Arabidopsis thaliana.* (www.arabidopsis.org) and *Oryza sativa,* (www.tigr.org/tdb/e2k1/osa1/), as well as EST information from *Physcomitrella patens* (www.moss.leeds.ac.uk/), *Cyanidioschyzon merolae* (merolae.biol.s.u-tokyo.ac.jp/) and *Porphyra yezoensis* (www.kazusa.or.jp /en/plant/porphyra/EST/) were build and queried the same way. Functional domains were searched against the InterPro (www.ebi.ac.uk/interpro/) and GeneOntology (www.geneontology.org/) databases.

Results and Discussion

Assembling and functional analysis

After base calling, assessment of sequence quality, vector trimming and trimming for ribosomal sequences an initial EST collection of 2099 5' high quality sequences larger than 100bp, having an average edited length of 612bp were obtained from the cDNA library. This initial collection of ESTs was further clustered and assembled to yield a non-redundant set (NRS) of tentative unique sequences (TUs). This set contained a total number of 209 tentative consensus sequences (TCs) assembled from 608 sequences. 1482 sequences remained being uniquely represented in the EST collection and 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. A further 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 genome.imb-jena.de/ALGAE/index.html. Assuming that the 11,242 predicted genes from the genome of *T. pseudonana* (Armbrust *et al.*, 2004) represent the total number of genes present, also in *F. cylindrus;* we have identified roughly a 15 % proportion of the expressed genome within this EST collection.

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

	No. of sequences	**average length bp**	**Total characters bp**
Clones sequenced	2880		
High quality single reads (phred20, no rRNA, min 100bp)	2099	612	1283603
Singletons	1482	598	886599
Tentative consensus sequences (TCs)	209	856	178978
Non-redundant set (NRS)	1691	630	1065577
G/C content	0.41		

Comparison of the F. cylindrus *NRS to other databases*

A total of 1147 sequences (68%) retrieved a significant hit in at least one of the queried databases and a potential function via significant similarities to SwissProt and refseq could be assigned to 747 (44.2%) sequences of the NRS (Table 2). Of the non-redundant set, 544 TCs or singletons remained unique, i.e. produced no significant hit against any sequence database equalling 32 % of the NRS. Although these sequences may in part represent 3` untranslated regions (UTRs), this fraction is believed to be rather small, since sequencing was performed from the 5´end and it has been shown by (Mock *et al.*, 2006) that the average length of the 3´UTR identified was 138 bp and in this library it was only 116 bp (identified 24 times). Furthermore, functional protein domains could be assigned to 70 of the 544 unique sequences via InterPro.

Table 2: Significant similarities (≤ 1e-04) of the *F. cylindrus* NRS (1691 sequences) to other datasets. To enhance the meaning some datasets have been combined. Bold values in the diagonal give the number of hits unique to this dataset

	Total number of hits in each database	Swissprot / refseq	*C.reinhardtii*	*C.merolae/ P.yezoensis EST*	*T.pseudonana/ P.tricornutum*	*P. patens*	*A. thaliana/ O. sativa*
Swissprot/refseq	747	**41**	362	453	659	351	519
Clamydomonas reinhardtii	384		**3**	324	354	263	351
C. merolae/ P.yezoensis EST	481			**5**	451	300	406
T. pseudonana/ P. tricornutum	1043				**349**	344	513
P. patens	359					**1**	336
A. thaliana/ O. sativa	548						**3**

Compared to EST collections from streptophytes, where in general roughly 60% of genes could be assigned a potential function, the number of genes producing no hit in the Swissprot and refseq database was larger. However, an equal proportion of EST sequences 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); *Ulva linza* 48%

(Stanley *et al.*, 2005). The number of significant hits might have been even 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). Therefore, it is assumed that a significant proportion of the unique sequences might represent genes which are involved in adaptation of *F. cylindrus* to its extreme environment and in this case especially to potential adaptations to increased external salinities.

Comparison of the F. cylindrus *NRS with the* T. pseudonana *and* P. tricornutum *genome reveal one third to be unique to* F. cylindrus

Polar centric diatoms are believed to be the origin of pennate diatoms, which first emerged about 70 mill years ago. Pennate and centric diatoms fundamentally differ in their cell symmetry, but also in their mode of sexual reproduction and mechanisms for cell motility, leading to a variation in their gene content. In addition to these phylogenetic traits, differences in the physiology might also play a role, since *T. pseudonana* and *P. tricornutum* both represent mesophilic diatoms, whereas the occurrence of *F. cylindrus* is restricted to polar regions, obligatory adapted to temperatures below +8°C. Such essential differences in their habitat and consequently adaptations to it, would be expected to be reflected in their genetic repertoire.

A total of 1043 sequences in the *F. cylindrus* NRS shared similarities with either of the two genomes (Figure 1) and 744 sequences were common in all three datasets. The fact that 349 TUs (Table 2) were exclusively present in diatoms, is indicative for the close phylogenetic relationship. However, *F. cylindrus* shared slightly more sequences with *T. pseudonana* than with *P. tricornutum,* which is very astonishing given the fact that *P. tricornutum* is also a pennate diatom. However, a 158 versus 141 sequences overlap is not a large difference and given the size of the dataset this finding may change if more sequences become available. Furthermore, of those sequences common to all three diatoms, *F. cylindrus* shared a significant higher similarity with *P. tricornutum* according to a t-test based on mean bit score values (453.6 versus 395.7; Mean difference: 57.9; $n = 744$; $t = 10.2$; $p < 0{,}001$).

A total of 648 (38.3%) TUs are specific to *F. cylindrus*, i.e. there is no homologue in the two mesophilic species.

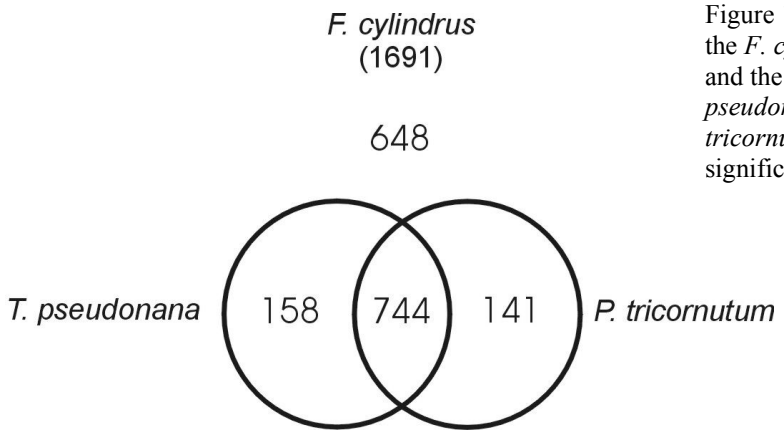

Figure 1: Overlap between the *F. cylindrus* NRS set and the genomes of *T. pseudonana* and *P. tricornutum* based on a significance level of 1e-04.

One hundred and four non-redundant sequences (= 6.2%) produced no significant hit against either of the diatom genomes, but to other databases. Some of the TUs in this fraction even belonged to the group of most abundant TCs (Table 2).

Most abundant ESTs can be functionally characterized

Twenty-seven TCs consisted of 4 or more single reads, representing the most abundant TCs. In contrast to the previously described most abundant TCs in (Mock *et al.*, 2006) and in (Montsant *et al.*, 2005) where 60% and 40%, respectively remained unidentified, a potential function could be assigned to almost all (89%) of the most abundant TCs in this library (Table 3). In this respect, this EST set is comparable to those of higher plants (Bräutigam *et al.*, 2005, Wang *et al.*, 2006).

However, the most abundant TC comprising 2.8% of all high quality sequences was a unique sequence showing no homology to any database. Two other unidentified TCs Fcyl0043a06 and Fcyl0043h05 seemed to be specific for diatoms, since they only showed a homology to both diatom genomes but not to any other database. Fcyl0043a06 yielded an InterPro hit spanning a 185 amino acid long domain of a twin_arginine translocation pathway signal (IPR006311). Further four TCs encoded conserved hypothetical proteins with unknown function. The occurrence of three fcps and one cab protein among the 26 most abundant TCs is comparable to

the *P. tricornutum* EST set (Montsant *et al.*, 2005) and the cold shock library and represents the importance of the LHC protein family in stress acclimation. Severe photoinhibition, manifested as a strong decline in photosynthetic quantum yield has been shown to occur upon cold (Mock & Valentin, 2004) and even more upon salt stress (data not shown), thus a restructuring of the LHC in response to these stresses seems to be necessary. The frequent occurrence of ESTs encoding enolase, which is one of the enzymes involved in the energy yielding phase of glycolysis, might hint at the increasing importance of catabolic energy gain under conditions where photosynthetic energy production is limited.

Table 3: Most abundant (≥ 4 single reads) tentative consensus sequences (TCs) and best hit to the swissprot database

Internal name	No. of ESTs	TC length	Function	e-value
Fcyl0044d08	59	1313		
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-b 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		
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		
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

TC Fcyl0043h05 encodes a full length heat-shock protein (hsp) 70, which together with a high number of others ESTs encodes a whole suit of different heat shock proteins that have been identified in this library.

The production of organic osmolytes in response to salt stress seems to be of major importance; since a TC coding pyrroline-5-carboxylate reductase (P5CR) could be identified within the most abundant TCs. P5CR catalyzes the final step in the synthesis of proline, which has been shown to be the major organic osmolyte in *F. cylindrus* (Plettner, 2002).

The class of peptidyl-prolyl cis-trans isomerases (PPIases) is also represented within the most abundant TCs, they catalyze the isomerisation of peptide bonds of proline residues, a rate limiting step in the folding of newly synthesized proteins. They are also considered as molecular chaperons, activated by different stressors, including cold and drought stress. A total of six different representatives of this class could be identified. This class of proteins encompasses cyclophilins (CYP20, CYP40, CYP-A) and two FK506 binding proteins (FKBPs). In higher plants most of the immunophilins are targeted to the thylakoid lumen. Isoformes of CYP40 and CYP20 in the thylakoid lumen serve as protein folding catalysts, but also regulate the acitivity of the PSII specific protein phosphatase (Edvardsson *et al.*, 2003). In addition to this, evidence exists that cyclophilins protect cells against oxidative stress (Doyle *et al.*, 1999), potentially regulated by thioredoxin (Motohashi *et al.*, 2003). One singleton exclusively found in the diatom genomes contained a 176 aa long cyclophilin typ PPIase domain, suggesting that it might be a new representative of this class.

Many potentially salt stress related genes could be identified

The usefulness of information which can be gained through cDNA libraries established under determined stress conditions was supported by the fact, that a large number of tentative unique sequences could be assigned to genes potentially involved in salt stress response (Table 4). This comprises several genes required for the synthesis and degradation of proline, the major organic osmolyte in *F. cylindrus*, namely Δ^1-pyrroline-5-carboxylate reductase, which catalyzes the final step of proline synthesis and prolin dehydrogenase the first enzyme in the catabolic pathway (Verma, 1999). A singleton encoding ornithine aminotransferase was found, representing an

alternative route of proline synthesis, compared to the glutamate pathway (Figure 2). In a parallel study (Krell *et al.*, 2006) it could be shown that this gene is strongly up-regulated following salt stress, verifying the importance of this pathway and at the same time establishing a potential link to the urea cycle. The unexpected finding of a full urea cycle in diatoms (Armbrust *et al.*, 2004) and one of its components in this library (argininosuccinate synthase) allows one to speculate on its importance in stress induced protein turnover, as well as organic osmolyte synthesis. The rate limiting step in proline synthesis from glutamate – Δ-1-pyrroline-5-carboxylate synthase - could not be found, but this was not surprising considering the fact that it was strong down-regulated upon salt stress (Krell *et al.*, 2006).

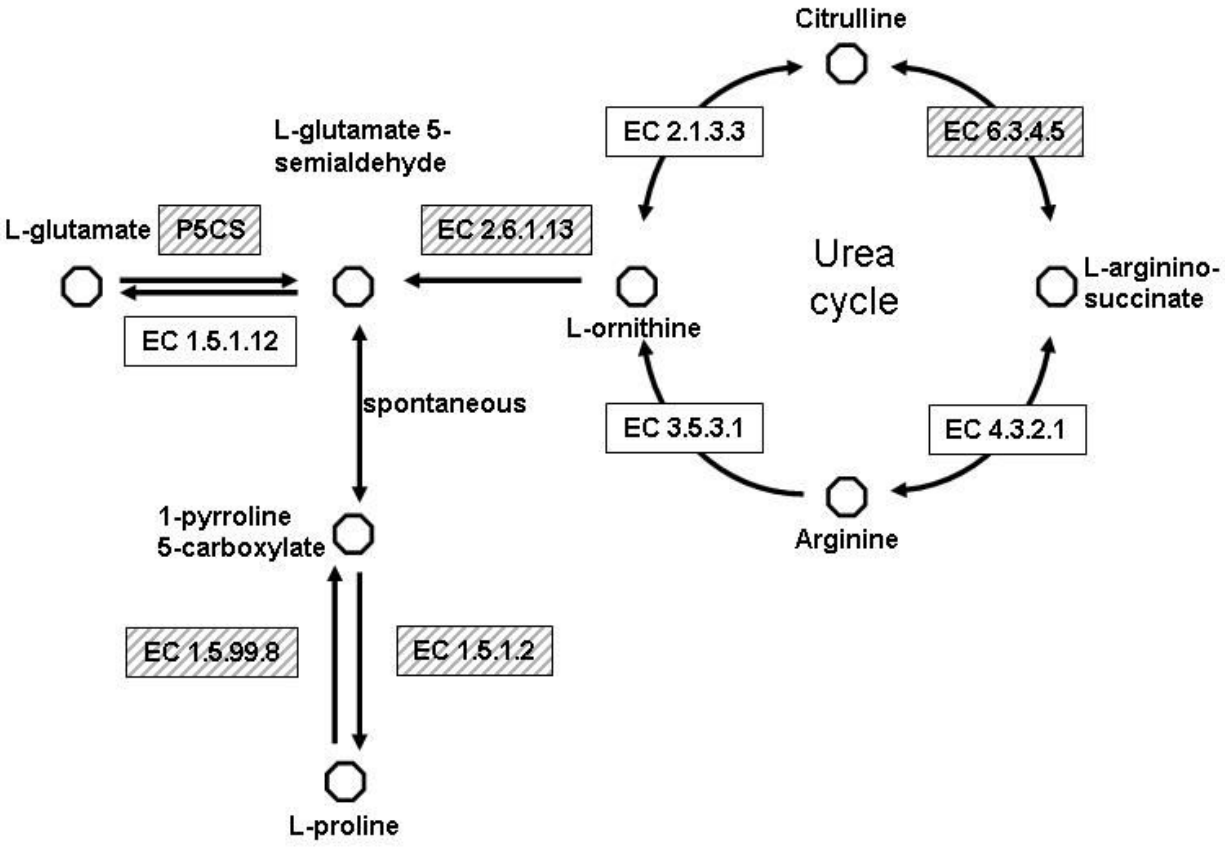

Figure 2: Substrates and enzymes involved in proline metabolism and linked to the urea cycle. Shaded enzymes (ornithine δ-aminotransferase (δ-OAT, EC 2.6.1.13), Δ^1-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2), proline dehydrogenase (ProDH, EC 1.5.99.8) and argininosuccinate synthase (EC 6.3.4.5) were found in this library, while Δ^1-pyrroline-5-carboxylate synthase (P5CS, EC not assigned) was identified in the cold shock library.

One singleton (Fcyl0033f11) encoded a spermidine synthase. Spermidine as well as spermine are important osmolytes in higher plants, where they function in preventing chlorophyll loss under osmotic stress (Capell *et al.*, 2004).

Another important osmolyte in *F. cylindrus* is Glycine-betaine (Gly-betaine). The synthesis of its precursor phosphocholine involves three successive *S*-adenosyl-Methionine (SAM)-dependent *N*-methylations of phosphoethanolamine. A singleton encoding SAM-synthase could be found. With each transmethylation, a molecule of *S*-adenosylhomo-Cys (SAH) is produced, a potent inhibitor of *S*-adenosyl-Met-dependent methyltransferases. Therefore *S*-adenosylhomo-Cys (SAH) needs to be catabolized to prevent feedback inhibition of SAM-dependent methyltransferases. The gene encoding SAH-hydrolase, responsible for the degradation of SAH to adenosine plus homo-Cys belonged to the most abundant TCs in this library, stressing the cruxial role of this protein in maintaining methylation activity during salt stress (Weretilnyk *et al.*, 2001).

The destruction of cellular ion homeostasis is one of the prompt and most severe damages caused by salt stress. The need for the cells to re-establish this disturbed ion homeostasis is reflected by the high number of different ionic transporter and antiporter genes found in this library. Several copies coding different types and subunits of V-type H^+ ATPase, some even present by more than one clone, as well as antiporters for various ions (Na^+, K^+, Ca^{2+}) (Table 4) stress the significance of this genes (Allakhverdiev *et al.*, 2000). For salinity tolerance the V-type H^+-ATPase is of prime importance in establishing an electrochemical H^+ gradient across the tonoplast to drive sodium sequestration into the vacuole (Shi *et al.*, 2003).

Reactive oxygen species (ROS) generated by salt stress might be an important source of damage in plant cells. In this cDNA library a number of genes involved in the detoxification and scavenging of ROS, e.g. glutathione synthetase, peroxiredoxin, thioredoxin (Wood *et al.*, 2003) could be found. Two singletons unique to *F. cylindrus* contained an InterPro catalase domain (65aa, Fcyl0029c11) and peroxidase domain (79aa, Fcyl0035a10), respectively. 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 ROS removal during light saturation of photosynthesis (Dupont *et al.*, 2004).

Two genes involved in the synthesis of vitamins could also be identified, a tocopherol O-methyltransferase and a pyridoxin biosynthesis protein, essential for vitamine B6 synthesis. This protein is able to quench singlet oxygen at a rate comparative to vitamin C and E,

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 homeostasis			
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

Table 4 continued

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

suggesting a previously unknown role for pyridoxine in active oxygen resistance (Ehrenshaft *et al.*, 1999). Taken together this might imply that ROS scavenging pathways may play an exceptional 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). However, it should also be mentioned that ROS might act as secondary

messengers in the activation of stress-response signal transduction pathways and defence mechanisms (Mittler, 2002).

TUs involved in signalling pathways potentially related to salt stress, such as the 14-3-3 protein and histidin kinases could also be detected. Histidin kinases act as sensors in the perception of various stressors like changes of the redox status and salt stress (Marin *et al.*, 2003). The 14-3-3 proteins bind to a range of transcription factors and other signalling proteins, regulating stress response, including the osmotic regulation of H^+-ATPases in plasma membranes (Roberts, 2003).

Molecular chaperons play a crucial role in protecting plants against a variety of abiotic stressors and in re-establishing cellular homeostasis. They are responsible for protein folding, assembly, translocation and degradation. Under stress they stabilize proteins and membranes and assist in protein refolding (Fulda *et al.*, 1999) (Wang *et al.*, 2004). A number of sequences coding hsps belonging to different classes were found in this library. Hsp 70/DnaK, and its co-chaperon (DnaJ/hsp40) were present by many different variations, as well as in high abundance in terms of copy numbers. The overexpression of hsp70 members in higher plants and yeast lead to an enhanced tolerance to salt stress (Sugino *et al.*, 1999, Sugimoto *et al.*, 2003), thus stressing its importance potentially also in this case. They are also believed to play a regulatory role in stress associated gene expression. (Thomsen *et al.*, 2002, Diamant *et al.*, 2003) reported that protein-disaggregation by a chaperone network composed of ClpB and DnaK + DnaJ + GrpE was essential for the recovery after salt stress. Interestingly, ClpB a member of the hsp100 class was strongly and specifically activated by the osmolyte Gly-betaine, thus linking osmolyte and chaperone metabolism.

Under stress conditions, a number of proteins are damaged, or need to be degraded in order to readjust to the new situation. The major proteolytic system in eukaryotes is the ubiquitin mediated degradation in the proteasome. The analysis of this cDNA library revealed a number of TUs encoding proteins involved in this degradation process, e.g. Fcyl0032g01, Fcyl0043e02 and Fcyl0029f11. This is consistent with previous findings where the expression of ubiquitin related proteins and various proteases was found to be enhanced under drought and salt stress and with

the requirement of protein degradation under stress conditions. Furthermore, at least three different genes (Fcyl0043h12, Fcyl0045d02, Fcyl0053d05) encoding FtsH proteases could be identified in *F. cylindrus*. A number of FtsH proteases is targeted to chloroplasts, where they are thought to be involved in the degradation of several photosystem-II (PS II) proteins, especially the reaction-center D1 protein (Adam & Clarke, 2002). Measurements of photosynthetic quantum yield revealed a detrimental effect of elevated salt concentrations on the photosynthetic machinery in *F. cylindrus* (Krell *et al.*, 2006), thus showing the need for such repair mechanisms.

A new class of antifreeze proteins could be identified

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 & Olsen, 1993) and insects (Duman, 2001). While proteins isolated from animals typically have substantial thermal hysteresis activity (TH), i.e. are able to depress the freezing point to actually avoid the formation of ice crystals, those proteins from plants and bacteria are able to 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.

Table 5: Sequence characteristics of ice-binding proteins found in *F. cylindrus* and best match to *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

In this library surprisingly four TUs could be identified, which showed a high similarity to sequences encoding antifreeze homologues originally isolated from the snow mold fungi *Typhula ishikariensis* (Hoshino *et al.*, 2003), all of them being full length sequences. These sequences were subjected to a further detailed phylogenetic analysis (Figure 3). No significant

similarity to any other antifreeze protein, neither from the animal nor plant kingdom could be found, suggesting that these sequences might represent a new class of antifreeze proteins formerly unknown in plants.

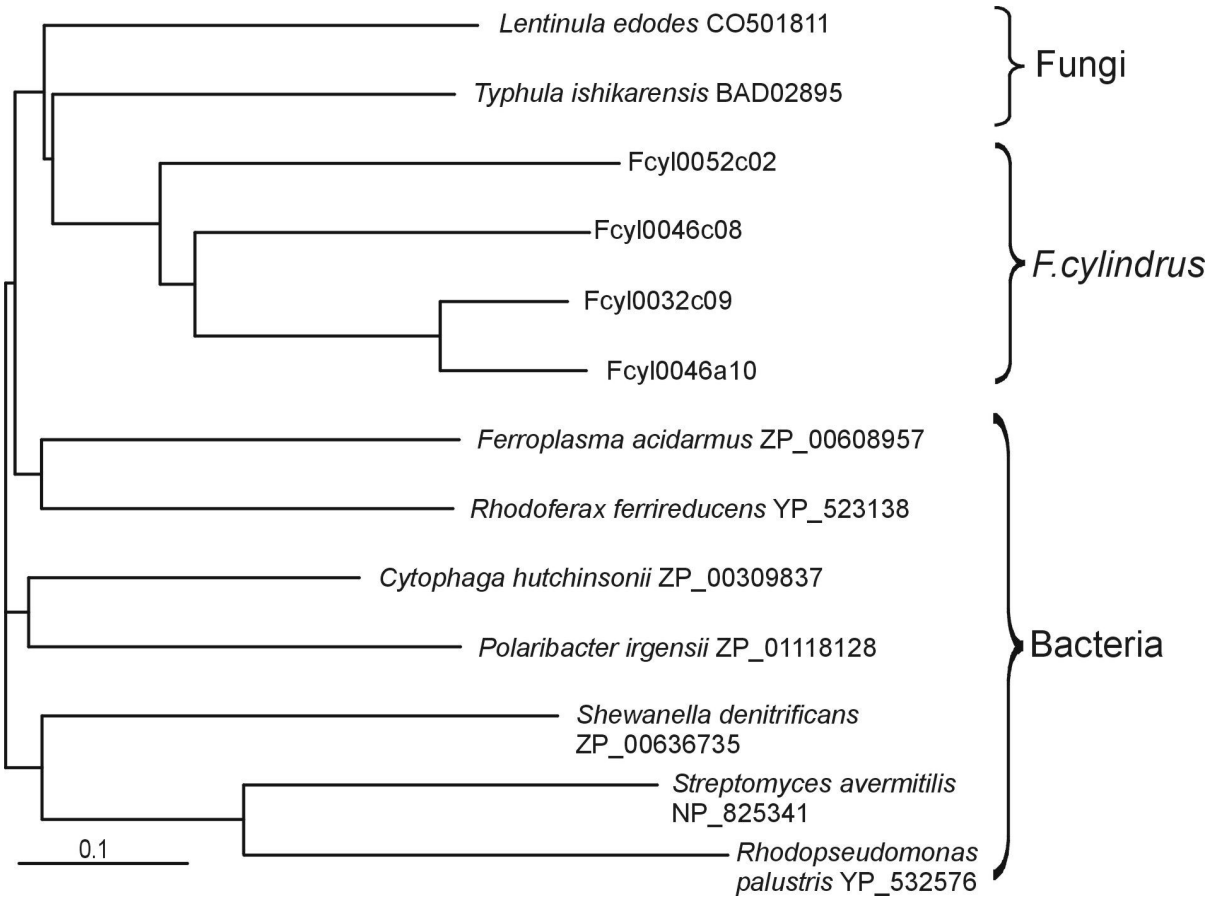

Figure 3: Phylogenetic tree of *F. cylindrus* full length IBP isoforms and homologues (incl. Accession number) found in the Genbank database

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 aa)(Table 6). 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 with high probability (0.795 – 0.930), to the secretory pathway and may thus be released into the extracellular space. The AFPs found in *Typhula ishikariensis* were also secreted into the extracellular space of the fungi and observations of Hoshino et al. (2003) indicated that these AFPs can probably 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 damage 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 & Knight (2003). This would be the first molecular evidence for the existence of such proteins. Neither of these sequences could be found in the genomes of *T. pseudonana* and *P. tricornutum*, respectively, supporting evidence that these proteins are essential for the adaptation to polar environments. The fact that neither of these sequences could be found in the previously established cold shock library (Mock *et al.*, 2006), allows one to speculate on the possible stimuli triggering the release of these proteins. It has been shown that salt stress was able to enhance the expression of an antifreeze protein in *E.coli* (Meijer *et al.*, 1996). Thus one might also speculate on the potential role of these proteins in ameliorating negative effects of salt stress, in addition to exhibiting antifreeze activity. Further expression studies aiming at the involvement into freezing and salt stress response will be carried out.

Many stress related genes common in the salt and cold induced cDNA library

In order to detect similarities potentially related to a common stress response upon cold and salt stress, the previously described cold shock library was merged with the salt stress library. This produced only a marginal overlap of 95 TUs, which might be due to the limited number of sequences in both libraries, but also owing to different physiological responses following cold and salt stress.

However, of the 95 matching TUs more than 10 could be significantly attributed to stress related proteins like, e.g. chaperones (hsp 70, hsp 31), enhanced disease susceptibility 5 (Fcyl0042d07), cell wall integrity and stress response component 3 precursor (Fcyl0037h12), light repressed protein A homolog (Fcyl0036a05), peroxiredoxin HYR1 (Fcyl0047f09) and SAM synthetase (Fcyl0053g06). Some of these proteins even belonged to the most abundant TUs in this library, e.g the peptidyl-prolyl cis-trans isomerase (Fcyl0047h05), some fcps and one hsp70. Among the other matching sequences identified, many belonged to general metabolism. Transporters and proteins related to the protein metabolism were only represented in a limited number. One TU

coding a UDP-sulfoquinovose synthase (Fcyl0046h07) and one coding a delta-5/delta-6 fatty acid desaturase (Fcyl0051c03) were found in both libraries, suggesting an important role of changes in lipid composition, as a common trait in stress response.

A high-affinity fructose transporter (Fcyl0039g06) was also found in both libraries and together with the identification of a monosaccharide transporter (Fcyl0036h07) both integral to membranes this might hint at the formation of exopolysaccharides. The exudation of exopolysaccharide proteins have been suggested to be a common trait of eukaryotic and prokaryotic organism dwelling in sea ice (Krembs *et al.*, 2002, Mancuso Nichols *et al.*, 2004), thus being able to shape their habitat.

Twenty-one sequences of the merged libraries were specific to diatoms and 22 were exclusively present in *F. cylindrus*. These "unknown" genes are very likely the source of candidate cold- and salt-tolerant genes associated with the necessary adaptation of F. *cylindrus* to its extreme habitat. Further functional analysis will help elucidate their specific role in stress tolerance.

Conclusion

A non-redundant set of 1691 genes was produced from a salt stress induced *F. cylindrus* cDNA library. Further analysis of ESTs with putative functional annotation identified a large number of genes involved in abiotic stress response in general and especially to salt stress acclimation. The comparison to two genomes of mesophilic Bacillariophyceae revealed a large proportion of genes unique to the psychrophilic *F. cylindrus*, thus reflecting the adaptation to its extreme habitat in its genetic repertoire. The finding of numerous sequences related to the synthesis of osmolytes and transporters to re-establish ion homeostasis proved this EST approach to be a valuable tool for mining 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 provide the basis to facilitate 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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Publication III

Ice-binding proteins from sea ice diatoms (Bacillariophyceae)

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J. Phycol. **42,** 410–416 (2006)

DOI: 10.1111/j.1529-8817.2006.00208.x

ICE-BINDING PROTEINS FROM SEA ICE DIATOMS (BACILLARIOPHYCEAE)[1]

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Sea ice diatoms thrive under conditions of low temperature and high salinity, and as a result are responsible for a significant fraction of polar photosynthesis. Their success may be owing in part to secretion of macromolecules that have previously been shown to interfere with the growth of ice and to have the ability to act as cryoprotectants. Here we show that one of these molecules, produced by the sea ice diatom *Navicula glaciei* Vanheurk, is a ~ 25 kDa ice-binding protein (IBP). A cDNA obtained from another sea ice diatom, *Fragilariopsis cylindrus* Grunow, was found to encode a protein that closely matched the partially sequenced *N. glaciei* IBP, and enabled the amplification and sequencing of an *N. glaciei* IBP cDNA. Similar proteins are not present in the genome of the mesophilic diatom *Thalassiosira pseudonana*. Both proteins closely resemble antifreeze proteins from psychrophilic snow molds, and as a group represent a new class of IBPs that is distinct from other IBPs found in fish, insects and plants, and bacteria. The diatom IBPs also have striking similarities to three prokaryotic hypothetical proteins. Relatives of both snow molds and two of the prokaryotes have been found in sea ice, raising the possibility of a fungal or bacterial origin of diatom IBPs.

Key index words: **cryoprotection; diatoms; *Fragilariopsis cylindrus*; ice-binding proteins; *Navicula glaciei*; sea ice**

***Abbreviations:* AB, ammonium bicarbonate; CFB, Cytophaga–Flavibacterium–Bacterioides; DTT, dithiothreitol; EST, expressed sequence tag; IBP, ice-binding protein; UNLV, University of Nevada, Las Vegas**

Sea ice is a habitat of extremophiles, organisms that not only survive but thrive under extremes of temperature, salinity, and light (Thomas and Dieckmann 2002). Prominent among these organisms are diatoms, which during periods of bloom form a dense brown layer at the bottom of sea ice that is responsible for a significant fraction of polar photosynthesis (Thomas and Dieckmann 2002). The diatoms appear to grow best during periods of ice growth (Garrison et al. 1983, Smetacek et al. 1992), and grow well even in brine pockets at temperatures as low as -6° C (Aletsee and Jahnke 1992). One factor that may contribute to survival is the release by the diatoms of exopolymeric substances that can help to preserve a liquid environment as sea ice freezes (Krembs et al. 2002). We have been investigating another potential survival mechanism that involves the production of proteins that have an affinity for ice crystals. Associated with the diatom community are extracellular proteins (formerly called ice-active substances) that strongly interact with ice (Raymond et al. 1994, Raymond 2000). The most conspicuous effect of these proteins is to cause pitting and other deformities on the surface of growing ice crystals, which is a sign of adsorption of an impurity to a crystal surface (Buckley 1951). Accordingly, we call these proteins ice-binding proteins (IBPs). Ice-binding activity has been found in all unialgal populations of Antarctic and Arctic ice diatoms that have been examined so far, and it has not been found in mesophilic diatoms. Thus, it appears that the molecules have a function related to cold or icy environments.

IBPs have been found in many organisms that are exposed to cold, including fish, insects, plants, fungi, and bacteria, in which they act as antifreezes, ice recrystallization inhibitors, and ice nucleators (for reviews of plant antifreezes, see Smallwood and Bowles 2002, Griffith and Yaish 2004). Diatom IBPs resemble plant antifreezes in that they do not appreciably lower the freezing point, which suggests that they mainly

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function as cryoprotectants. Many IBPs, including over a dozen from plants, have been sequenced, and it is now evident that they have little in common beyond an ability to bind to ice. Little is known of the nature of diatom IBPs other than their having the characteristics of proteins. Here we report the cDNA sequences of two diatom IBPs and show that they are more closely related to fungal antifreezes than to any known plant antifreezes.

MATERIALS AND METHODS

Diatoms. Cells of the sea ice diatom *Navicula glaciei* (identified by J. S. K.) were obtained from a dense colony in a crack in the sea ice at Cape Evans, Antarctica. The cells were almost exclusively *N. glaciei*. Additional cells of *N. glaciei*, *Fragilariopsis pseudonana*, *Chaetoceros neogracile*, and *Stellarima microtrias* were obtained near Maxwell Bay, King George Island, Antarctica and were axenically cultured at approximately 3° C under 25 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (24 h light) in modified f/2 medium (Guillard and Ryther 1962) at the Korea Polar Research Institute or at UNLV. Cells of the Arctic sea ice diatoms *Synedra* sp., *Amphora* sp. and *Attheya* sp. were cultured at approximately 2° C at the Provasoli–Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine. Cells of *F. cylindrus* were isolated from Antarctic sea ice during a "Polarstern" expedition (ANTXVI/3) in the eastern Weddell Sea. Nonaxenic cultures were grown at 0° C under 20 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (24 h light) in double f/2 medium at the Alfred Wegener Institute. Bubbling with air (approximately 150 mL/min) ensured sufficient CO_2 supply and continuous mixing.

Sequencing of F. cylindrus *IBP.* A cDNA library from *F. cylindrus* was constructed from nonaxenic cultures subjected to salt stress conditions. The salinity of double ANT f/2 medium cells was increased by a concentrated brine solution (Instant Ocean, Aquarium Systems, Mentor, OH, USA) from approximately 34 to 60 psu in the middle of the exponential growth phase to induce expression of genes related to osmotic stress. Samples for RNA isolation were taken at increasing intervals within 4 days after brine addition. All samples were pooled for total RNA extraction and mRNA isolation (Ambion Inc., Austin, TX, USA). A cDNA library was synthesized with the CloneMiner cDNA Library Construction Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The 5′ ends of over 1000 clones were sequenced (unpublished data). The IBP sequence from *F. cylindrus* was obtained by annotation of this expressed sequence tag (EST) library using the Genpept data bank (translated NCBI) and the tBlastX algorithm with default options.

Purification of N. glaciei *IBP.* IBP was purified from the Cape Evans ice as described previously (Raymond 2000). In this method, cell-free meltwater was adjusted to an osmolality of approximately 300 mOsm/kg and frozen in centrifuge bottles at −15° C overnight. The bottles were centrifuged upside down at approximately −4° C to expel brine and impurity proteins, leaving IBPs relatively enriched in the ice. The ice fraction was melted and the freeze–thaw cycle was repeated another six times. The final solution was dialyzed, freeze-dried, and subjected to two-dimensional (2-D) (SDS-PAGE/*p*I) electrophoresis at Kendrick Laboratories (Madison, WI, USA) using a 10% acrylamide gel and *p*I range of 3.5–10. Other electrophoresis conditions were as described previously (Raymond and Fritsen 2001). The ice-binding activity of a solution was qualitatively assayed by observing the growth of ice single crystals submerged in the solution at a temperature of approximately 0.1° C below the freezing point. A rough, pitted surface is an indication of the presence of IBPs.

De novo *sequencing of peptides by tandem mass spectrometry (MS/MS).* Part of a protein spot of interest was cut from the gel and subjected to mass spectroscopy analysis at the Molecular Structure Facility, University of California, Davis, CA, USA. The spot was washed with 100 mM ammonium bicarbonate (AB), diced, dried in a SpeedVac (Savant, Instruments, Farmingdale, NY, USA), reduced with 10 mM dithiothreitol (DTT) in AB, pH 8 for 1 h at 55° C, washed with AB to removed excess DTT, alkylated with 55 mM iodoacetamide in 100 mM AB for 45 min in the dark at room temperature, separated from excess reagent, washed with AB, partially dehydrated with acetonitrile, completely dehydrated in a SpeedVac, and digested in 50% AB containing sequence grade, modified trypsin (Promega, Madison, WI, USA) at a final concentration in the range of 10–25 ng/μL at 37° C for 17 h. Peptides were extracted once each with 0.1% TFA in water and 5% formic acid in 50% acetonitrile. The extraction volume was carefully controlled, not exceeding 50 μL, and reduced to 15 μL as the final volume for mass spectrometry analysis. Aliquots of tryptic peptides were cleaned and concentrated using POROS R2 resin (Perceptive Biosystems, Framingham, MA, USA) in a microcolumn following the method described in the Protana manual (Protana, Odense, Denmark). In brief, the peptide mixture (dissolved in 5% formic acid) was loaded onto the microcolumn. The column was then washed with 5% formic acid and a gold-coated nanospray ES capillary obtained from Protana was aligned in continuation of the microcolumn. Peptides were eluted into the nanospray ES capillary using 50% methanol–5% formic acid. Tryptic peptides were analyzed by a hybrid nanospray/ESI-Quadrupole-TOF-MS and MS/MS in a QSTAR mass spectrometer (Applied Biosystems Inc, Foster City, CA, USA). Peptides in 50% methanol–5% formic acid were sprayed from the gold-coated capillary. The QSTAR instrument was calibrated with a standard peptide mixture that gives mass accuracies of 5 ppm or better. Argon gas was used as the collision gas. *De novo* sequencing of peptides was carried out using the QSTAR software (Analyst QS) and double-checked via a manual interpretation of MS/MS spectra.

Initial PCR amplification of N. glaciei *IBP cDNA.* Total RNA was isolated from axenically grown cells using TRIZOL reagent (Invitrogen) and reverse transcribed with a SuperScript first-strand synthesis kit (Invitrogen) using the polydT oligo included in the kit. PCR was performed using a forward primer 5′-CTG CTG TCA ACC TTG GAA CTG C-3′, based on sequences of the IBPs of *F. cylindrus* and *T. ishikariensis*, and a degenerate reverse primer 5′-TAT GCT GCN GAC TAC ACN GC-3′, based on one of the peptide fragments of *N. glaciei* IBP and the corresponding nucleotide sequence of *F. cylindrus* IBP. First-strand-synthesized cDNA was amplified by Taq polymerase (1 u; Promega) in a 50 μL reaction volume containing the following reagents (expressed as final concentration): 5 μL of 10× Taq buffer B, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 μM forward primer, and 0.2 μM reverse primer. Products were amplified using a Eppendorf Mastercycler with initial denaturing for 3 min at 95° C and then 33 cycles as follows: 30 s at 95° C, 30 s at 53° C, and 40 s at 72° C, followed by a final extension for 7 min at 72° C. The PCR products were separated on a 2% TAE agarose gel and a single spot of 210 bp was excised, gel purified (Wizard gel prep, Promega) and cloned into a sequencing vector (pCR2.1, Invitrogen) and transformed into TOP10 competent *E. coli* (Invitrogen) for sequencing. Plasmids were isolated using a Wizard Plus Miniprep kit (Promega) and a subset was subjected to restriction enzyme digestion (*Eco*RI) for positive identification of the insert. The PCR inserts were sequenced with the M13F primer at the Nevada Genomics Center, University of Nevada, Reno, NV, USA.

5′/3′ Rapid amplification of cDNA ends (5′/3′ RACE). SMART RACE (Clontech, Mountain View, CA, USA)

technology was utilized to identify the entire open-reading frame of the *N. glaciei* IBP cDNA as well as identify the full 5′ and 3′ untranslated regions (UTRs). Total RNA was isolated from axenically grown *N. glaciei* cells and was reverse transcribed with Powerscript reverse transcriptase (Clontech) using either the 5′ RACE cDNA synthesis primer or 3′ RACE cDNA synthesis primer. 5′ RACE was performed using a gene-specific reverse primer 5′-GAC GAT GTT GAG AAT GTG TTG CTG GAA TCC-3′ and 3′ RACE was performed using a gene-specific forward primer 5′-CTC ACT ACT GGT CCT ACT GAA GTG ACC GG-3′). The PCR was performed in a 50 µL reaction volume using the 10× universal primer mix (5 µL), the gene-specific primer (0.2 µM final concentration), and reagents, and protocols in the AdvanTAge PCR cloning kit (Clontech). The 5′ RACE amplification was carried out as follows: 2 min at 95° C followed by 30 cycles of 30 s at 95° C, 30 s at 64° C, 2 min at 72° C. The 3′ RACE amplification was carried out as follows: 2 min at 95° C followed by 30 cycles of 30 s at 95° C, 30 s at 68° C, 2 min at 72° C. All amplification reactions were allowed to extend at 72° C for 10 min. The PCR products were gel purified and cloned into the pGEM-T sequencing vector (Promega) and transformed into TOP10 competent *E. coli* (Invitrogen) for sequencing. Plasmids were isolated using a Wizard Plus Miniprep kit (Promega) and a subset was subjected to restriction enzyme digestion (EcoRI) for positive identification of the insert. Twelve plasmids, six containing the 5′ RACE and six containing the 3′ RACE inserts, were sequenced in both directions as described above. Overlapping 5′ and 3′ RACE products allowed the reconstruction of the *N. glaciei* IBP cDNA.

To confirm the continuous open-reading frame of *N. glaciei* IBP cDNA as predicted by RACE and to investigate possible polymorphic variations in *N. glaciei* IBPs, we amplified first strand *N. glaciei* cDNA (described above) with primers designed within the 5′ UTR and 3′ UTR: 5′ UTR: 5′-CCCAA CAATTCAATCAACTCAATTTCC-3′; 3′ UTR: 5′-GGAGATC AACCCGGAACGATGAG-3′. First-strand synthesized cDNA was amplified with Pfx polymerase (1 u, Invitrogen) in a 50 µL reaction volume containing the following reagents (expressed as final concentration): 5 µL of 10×Pfx buffer, 1 mM MgSO4, 0.6 mM dNTP mix, 0.4 µM 5′ UTR primer, and 0.4 µM 3′ UTR primer. Products were amplified using a DNA thermal cycler (9700, Perkin Elmer, Norwalk, CT, USA) with initial denaturing for 2 min at 95° C and then 30 cycles as follows: 30 s at 95° C, 30 s at 55° C, and 2 min at 72° C, followed by a final extension for 10 min at 72° C. The PCR product was separated on a 1% TAE agarose gel and a single band of approximately 850 bp was excised, gel purified and cloned into a Blunt-end sequencing vector (pCR4, Invitrogen) for sequencing. Six positive clones were isolated by miniprep and sent for sequencing in both directions (described above). Sequences were aligned with ClustalW.

Other. Predicted molecular mass and predicted *p*I of the open reading frame (ORF) were obtained with Protein Calculator (http://www.scripps.edu/cgi-bin/cdputnam/protcalc). The N-terminal sequences were examined for signal peptides with the neural network algorithm of SignalP (http://www.cbs.dtu.dk/services/SignalP/) and for mitochondrial and chloroplast targeting signals with TargetP (http://www.cbs.dtu.dk/services/TargetP). Full sequences were examined for *N*-glycosylation sites using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Putative homologs of the diatom IBPs were found using the BLAST search algorithm against the protein data base hosted by the National Center for Biotechnology Information (NCBI).

RESULTS

Origin of ice-binding activity. Sea ice diatoms have been suspected as being the source of ice-pitting activity in diatom-rich sea ice, but other members of the sea ice community such as bacteria and fungi could not be ruled out. Axenic cultures of seven sea ice diatoms, *N. glaciei*, *C. neogracile*, *F. pseudonana*, and *S. microtrias* from the Antarctic and *Synedra* sp., *Amphora* sp. and *Attheya* sp. from the Arctic, were tested and all were found to release ice-pitting activity into the culture medium. These results demonstrate that sea ice diatoms can produce IBPs and suggest that production of such proteins is a common trait of sea ice diatoms.

N. glaciei *IBP.* An example of the ice-pitting activity of supernatant from melted sea ice in which *N. glaciei* accounted for >99% of the cells is shown in Fig. 1A. The activity was retained through the purification process. 2-D polyacrylamide gel electrophoresis of nearly pure material yielded a spot of approximately 25 kDa with a *p*I of about 5.0 (Fig. 1B). Tandem mass spectrometry sequencing of the band yielded four peptide sequences, NTFSTSSLVTGK, LYAADYTAPTPSK, SDFSTAFTDAAGR, and VGDQ QFYLTGTAK. These sequences were found to be similar to several antifreeze isoforms of the snow mold *Typhula ishikariensis* (NCBI GenBank Acc. Nos. AB109742-AB109748) submitted by T. Hoshino et al. The *T. ishikariensis* protein was identified as an antifreeze because of its ability to depress the freezing point of water by approximately 0.1° C (Hoshino et al. 2003a). A second IBP cDNA from the sea ice diatom *F. cylindrus* Grunow (Acc. No. DR026070) was discovered from an *F. cylindrus* salt stress EST library. Primers based on the nucleotide sequences of the

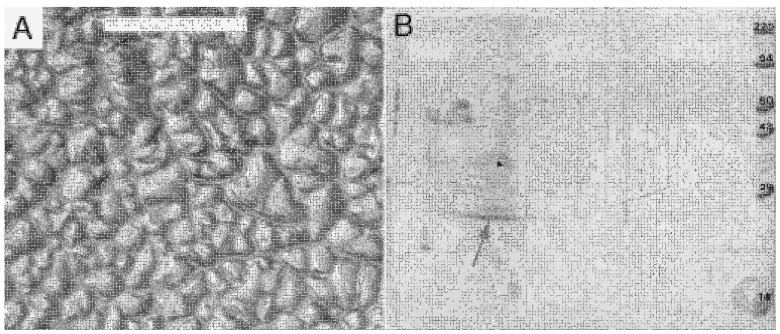

FIG. 1. Ice-binding proteins of *Navicula glaciei*. (A) Surface (basal plane) of an ice crystal grown in the presence of melted sea ice rich in *N. glaciei* cells. Adsorbed proteins inhibit growth on the pit faces, allowing vertical growth on the basal plane surrounding the pits. Scale bar, 500 µm. (B) Polyacrylamide gel of nearly pure ice-binding protein (IBP) of *N. glaciei*. Arrow indicates IBP. The *p*I range of the gel is from 3.5 to 10. Black triangle indicates a protein standard (tropomyosin) with *p*I of 5.2.

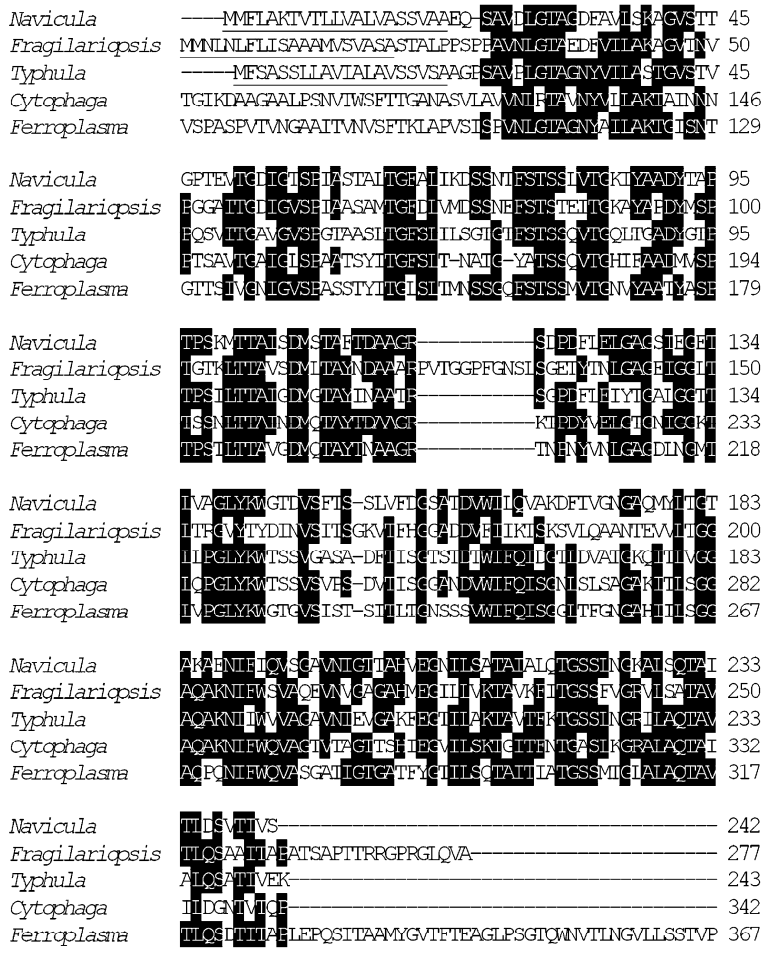

FIG. 2. ClustalW alignment of ice-binding proteins from *Navicula glaciei* (Acc. No. DQ062566), *Fragilariopsis cylindrus* (CN212299) and *Typhula ishikariensis* (AB109745), and hypothetical proteins from *Cytophaga hutchinsonii* (ZP_00309837) and *Ferroplasma acidarmanus* (ZP_00608957). Predicted signal peptides are underlined. Gaps have been inserted to improve alignment. Conserved residues are shaded. The N-terminal sequence of the *Cytophaga* protein and the N-and C-terminal sequences of the *Ferroplasma* protein are truncated. Residue numbers are shown at right.

T. ishikariensis and *F. cylindrus* antifreeze genes and the *N. glaciei* peptide sequences resulted in the amplification and cloning of a 210 bp *N. glaciei* cDNA whose predicted amino-acid sequence closely matched the MS-derived *N. glaciei* peptides. Using gene-specific primers designed from the *N. glaciei* 210 bp product and 5′/3′ RACE, nine 5′ and seven 3′ RACE products were sequenced and found to be similar to both the *T. ishikariensis* and *F. cylindrus* IBP sequences. The RACE products overlapped for 63 nts between nucleotides 220 and 282 of the reconstructed IBP shown in Fig. 2. Although 5′ RACE products were not equal in length, all sequences at a common position within the 5′ UTR did not show any sequence variation and were considered identical. Similar to the 5′ UTR, the 3′ RACE products did not show sequence variation in the 3′ UTR until nucleotide 914, which is just proximal to the polyadenylation site where the sequencing results were less reliable. A consensus polyadenylation site (ATAAA) was found at nucleotides 901–905, which is 14 nucleotides upstream of a polyadenylated region. Six PCR products obtained with flanking UTR primers were cloned and sequenced, and none were redundant. They confirmed the 5′/3′ RACE results and revealed a fairly wide range of single nucleotide polymorphisms within the open-reading frame. One of the clones was identical to a corresponding set of 5′ and 3′ RACE products and so it and the 5′ and 3′ RACE products were used to reconstruct a full-length *N. glaciei* IBP (Acc. No. DQ062566, Fig. 2) (Accession numbers of the other five clones are DQ062561–DQ062565). The confirmed full-length *N. glaciei* cDNA contains a 75 nt 5′ UTR, a 726 nt ORF encoding 242 amino acids, and an approximately 121 nt 3′ UTR. The predicted molecular mass and predicted *p*I of the ORF 24,461 Da and 4.45, agree well with the values estimated by 2-D electrophoresis. The closeness of the two molecular mass estimates suggests that the protein has little or no carbohydrate moiety. The molecular mass is consistent with a value of 30 kDa estimated for an IBP associated with the sea ice diatom *Nitzschia stellata* (Raymond et al. 1994). Together, these data cast doubt on estimates of larger sizes of IBPs based on retention by dialysis membranes from two other species of ice diatom (Raymond 2000).

F. cylindrus *IBP*. The above-mentioned cDNA from the *F. cylindrus* salt stress library was found to have high similarity (e-value 5e-43) to isoforms of the *T. ishikariensis* antifreeze, which identified its putative function. The full-length *F. cylindrus* cDNA contains a 63 nt 5′ UTR, an 831 nt ORF encoding 277 amino-acids, and an approximately 49 nt 3′ UTR. The predicted molecular mass and predicted *p*I of the ORF are 27,961 Da and 5.55. A putative polyadenylation signal is located at nucleotides 921–926 (ATTAAA), which is approximately 20 nucleotides upstream of the poly(A+) tail. The predicted amino-acid sequence (Fig. 2) shows 48% identity to that of *N. glaciei* IBP. Although *F. cylindrus* was not tested to see whether it expresses an IBP, axenically grown *F. pseudonana* was confirmed to release ice binding activity into the culture medium and to express an IBP mRNA, whose partial sequence (~530 bp, unpublished data) is very similar to the sequence of *F. cylindrus* IBP.

IBP properties. The N-terminal sequences of the *N. glaciei* and *F. cylindrus* IBPs and each of the *T. ishikariensis* antifreeze isoforms have high probabilities of being signal peptides (with lengths of 21, 20, and 21 residues, respectively; Fig. 2) and low probabilities of being mitochondrial or chloroplast targeting peptides. *F. cylindrus* IBP has one potential *N*-glycosylation site, whereas *N. glaciei* IBP has none, in agreement with its predicted low degree of glycosylation.

Other IBP-like proteins. Other proteins similar to the diatom IBPs were found in the NCBI data base. H. S. Kwan et al. (The Chinese University of Hong Kong) submitted 18 ESTs from the commercially grown shiitake mushroom, *Lentinula enodes* (e.g. Acc. No. CO501811) that closely resembled (~70% amino-acid identity) the *T. ishikariensis* antifreeze sequence (43% amino-acid identity to *N. glaciei* IBP). Shiitake mushrooms are typically exposed to freezing temperatures during their growing period. The supernatant of a cell homogenate of this species was found to have modest ice-pitting activity (our unpublished data), presumably owing to expression of the IBP-like genes. However, the function of these genes remains unclear.

The IBPs also show considerable similarity to hypothetical proteins from a Gram-negative soil bacterium, *Cytophaga hutchinsonii*, (Acc. no. ZP_00309837; 48% amino-acid identity to the *N. glaciei* IBP), an acidophilic archaeon, *Ferroplasma acidarmanus* (Acc. no. ZP_00608957; 51% identity), a denitrifying estuarine bacteria, *Shewanella denitrificans* (Acc. no. ZP_00636736; 43% identity), and a psychrotolerant sediment bacteria, *Rhodoferax ferrireducens* (Acc. no. ZP_00693230; 58% identity) in the regions of overlap. Portions of the *C. hutchinsonii* and *F. acidarmanus* proteins are aligned with the diatom and *T. ishikariensis* IBPs in Fig. 2. Several bacterial strains related to *Cytophaga* and several species of *Shewanella* have been isolated from polar ice. A few other bacterial proteins that are similar to the *Cytophaga* and *Ferroplasma* proteins show lower sequence identities (~30%) to the IBPs. It is interesting that the *Cytophaga* and *Ferroplasma* proteins are also weakly similar (21%–25% identities) to some bacterial ice-nucleating proteins (e.g. Acc. Nos. P09815, P20469, and P06620), although the resemblance may be owing to chance. The phylogenetic relationships of these proteins will be described separately.

Sequence information from the nearly full genome of *Thalassiosira pseudonana* (Armbrust et al. 2004) and from EST data for *Phaeodactylum tricornutum* (Scala et al. 2002), both mesophilic diatoms, have recently become available. No predicted translation products similar to IBPs were found in either species, in agreement with a previous finding that ice-pitting activity in diatoms is limited to polar regions (Raymond et al. 1994).

DISCUSSION

Previous studies have described "ice-active substances" that are "associated" with sea ice diatoms (Raymond et al. 1994, Raymond 2000) because the nature of the substances and their origin were not definitely known. The present results obtained from axenic cultures of diatoms confirm that diatoms produce proteins with ice-binding activity. It seems likely that the activity that we observe in natural communities largely originates from diatoms, although we cannot rule out the possibility that some of the natural activity originates from bacteria. Our results identify the source of the ice-binding activity in *N. glaciei* as a 25 kDa protein and strongly suggest that the IBP gene of *F. cylindrus* expresses a similar protein. Over 200 species of sea ice diatom are found in both the Arctic and Antarctic, representing a total of 58 genera (Horner 1985). Because each of the axenic species of sea ice diatom that we of examined so far have exhibited ice-pitting activity, we expect that each of these genera will have one or more IBP homologs.

IBP homologs. Our results show that the diatom IBPs are likely homologs of "antifreeze" proteins from the snow mold *T. ishikariensis*. Other snow molds appear to have similar proteins (Hoshino et al. 2003a, b). The diatom and snow mold IBPs do not resemble any of the numerous antifreeze proteins that have been identified so far in fish, insects, plants, and bacteria, and thus represent a new class of IBP.

The finding of IBP-like hypothetical proteins in *Cytophaga hutchinsonii* and *Shewanella denitrificans* is interesting because both species are related to well-known sea ice bacteria. One of the most prominent groups of sea ice bacteria are the so-called Cytophaga–Flavibacterium–Bacterioides (CFB) phylum group (Brown and Bowman 2001). Bacteria from this group have been isolated from sea ice in the Arctic (Junge et al. 2002, Groudieva et al. 2004) and Antarctic (Brown and Bowman 2001), and in ice overlaying Lake Vostok in Antarctica (Christner et al. 2001). Furthermore, several lines of evidence suggest that CFB

or CFB-like bacteria contribute strongly to bacterial populations in well-established sea-ice algal assemblages (Brown and Bowman 2001). Several psychrophilic species of *Shewanella* have been isolated from sea ice, often in association with algal assemblages (Bowman et al. 1997a, b). It will thus be interesting to see whether these polar bacteria express IBPs.

Function and mechanism of IBPs. Because the diatom IBPs appear to be limited to icy environments, it is reasonable to believe that they have a role in cold hardiness. It seems most likely that their role is to protect cells in the frozen state because they have little effect on the freezing point. In fact, IBPs have been shown to increase the survival of cells subjected to a freeze–thaw cycle: semi-pure *Fragilariopsis kerguelensis* IBP increased the survival of both polar and nonpolar diatoms (Raymond and Knight 2003, Raymond and Janech 2003) and semipure *N. glaciei* IBP was found to reduce the hemolysis of frozen human red blood cells (Kang and Raymond 2004). The latter results indicate that the IBP protected the cell membrane.

A possible mechanism by which IBPs could prevent freezing injury to membranes is inhibition of the recrystallization of ice, a phenomenon that has been implicated in plant freezing tolerance (Knight et al. 1995, Thomashow 1998, Griffith and Yaish 2004). Recrystallization is a process in which large grains of ice grow at the expense of small grains, which are thermodynamically unstable. The growth of the larger grains is thought to be physically disrupting to cell membranes. Protein recrystallization inhibitors have been found in several cold-hardy plants, including ryegrass (Pudney et al. 2003), Antarctic hair grass (John and Spangenberg 2005), carrot (Worrall et al. 1998, Smallwood et al. 1999), lichens and mosses (Doucet et al. 2000), and winter rye (Griffith et al. 2005). Diatom IBPs are also strong recrystallization inhibitors (Raymond and Janech 2003, Raymond and Knight 2003, Kang and Raymond 2004), as are fish antifreezes (Knight et al. 1988). In hair grass (John and Spangenberg 2005) and winter rye (Griffith et al. 2005), recrystallization inhibitors have been found to be expressed or to accumulate in the extracellular region where ice forms, which supports the idea that they serve to prevent freezing injury. Because diatoms secrete IBPs, the IBPs may also act extracellularly, where they may protect the cells by preventing the recrystallization of external ice. However, other functions cannot be ruled out. For example, diatom IBPs may have a role in preserving brine pockets in sea ice, which appear to be essential for the survival of diatoms in sea ice at low temperatures (Krembs et al. 2002).

Origin of IBPs. Although the diatom IBPs and snow mold antifreezes almost certainly have a role in cold hardiness, their original function may have been different. In other organisms, antifreezes appear to have arisen from a variety of proteins with other functions (Cheng 1998, Griffith and Yaish 2004), although some may retain the original functions (Griffith and Yaish 2004). These proteins fortuitously shared an ability to bind to ice that appears to have been exploited to allow survival in freezing environments. The function of the IBP-like transcripts of the mushroom *Lentinula enodes* is unclear. This species is typically grown over a period greater than a year, during which time temperatures can fall below freezing, so they may act as cryoprotectants, but other roles cannot be ruled out. The route by which the diatoms acquired IBP genes is unknown. Horizontal transfer from fungi is a possibility, as basidiomycotic fungi (which include *Lentinula* and *Typhula*), are known to inhabit sea ice (Melnikov 1997, Gunde-Cimerman et al. 2003), and are believed to have arisen hundreds of millions of years before the diatoms (Heckman et al. 2001, Falkowski et al. 2004). Horizontal transfer from a *Cytophaga*-like bacteria or a species of *Shewanella* is another possibility because of their association with algal assemblages in sea ice and relation to species with IBP-like genes. However, it is too early to rule out the possibility of convergent evolution from different ancestral genes. We are presently attempting to isolate IBPs from other genera of sea ice diatoms, which we hope will shed light on the evolution of these proteins in polar diatoms and possibly on the evolution and radiation of sea ice diatoms themselves.

This study was supported by grants from the National Science Foundation (OPP0088000) and Korean Research and Development Institute (PE87200) to J. R., a post-doctoral Fellowship to J. K. from the Korea Science and Engineering Foundation (KOSEF), and an NSF International Research Fellowship Program Grant to M. J. We thank Y.-M. Lee for MS/MS analyses, R. Anderson and J. Sexton for growing the Arctic sea ice diatoms, M. Kuiper for assistance with the electrophoresis experiments, and E. Bachère and F. Van Breukelen for their help and use of their laboratories.

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Publication IV

Regulation of proline metabolism under salt stress in the sea ice diatom *Fragilariopsis cylindrus*

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Regulation of proline metabolism under salt stress in the sea ice diatom *Fragilariopsis cylindrus*

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Abstract

Fragilariopsis cylindrus, a bipolar psychrophilic and highly abundant diatom, experiences strong shifts of external salinity in its environment during the formation of sea ice. The effects of osmotic stress due to an increased salt concentration from 34 to 70 PSU alone and in combination with a temperature decrease from 0°C to -4°C, on the ana- and catabolic pathways of proline metabolism were investigated during a 20 day period. Expression levels of Δ^1-pyrroline-5-carboxylate synthase (P5CS) strongly decreased by a factor of 17.3, 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 2.9, respectively. This contrasts with findings in higher plants where an opposite regulation of P5CS and δ-OAT was observed and leads to the conclusion that under elevated external salinities the ornithine route is preferred to the glutamate pathway in *F. cylindrus*. Potentially due to a shortage in reduction equivalents, since photosynthetic quantum yield at photosystem II instantly dropped from 0.61 to 0.24, confirming a detrimental effect of elevated salt concentrations on the photosynthetic machinery. Salt stress proved to be the dominating stressor, an additional temperature decrease rather having ameliorating effects.

Keywords:

Sea ice, *Fragilariopsis cylindrus*, osmotic stress, proline metabolism, photosystem II, gene expression, P5CS, δ-OAT, P5CR, ProDH

Introduction

Sea ice is one of the most structuring features of polar ecosystems, with strong gradients of temperature, light, space and salinity through an ice floe (Eicken, 1992). Compared to freshwater ice, sea ice is not solid since dissolved constituents of sea water 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). Physicochemical processes within the brine channel system are primarily governed by the relationship between temperature and brine formation influencing chemical parameters (dissolved inorganic nutrients, dissolved gases, pH), space and light (Eicken, 2003, Papadimitriou *et al.*, 2004). Environmental conditions in the ice are characterised by temperatures between –1.8 and –20°C and corresponding salinities ranging from 35 to 212 PSU (practical saliniy units) (Cox & Weeks, 1983). However, despite these harsh conditions sea ice is densely populated by micro-organisms, the most conspicuous of them being pennate diatoms (Günther & Dieckmann, 2001, and references therein). *Fragilariopsis cylindrus* is one of the dominating diatom species in polar realms thriving equally well in the water column and sea ice (Kang & Fryxell, 1992).

Acclimation to changing osmotic conditions is a prerequisite for all cellular life. An altered external increase or decrease in the concentration of inorganic ions (primarily Na^+ and Cl^-) results in a flow of water across the semi permeable 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 & 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 & Wiencke, 1995, DasSarma & Arora, 2001, Chen & Murata, 2002). Among the compatible solutes proline appears to be the most widely distributed osmolyt 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 & Verma, 1993, Erdmann & Hagemann, 2001). Recently, the ability of

osmolytes, especially proline to scavenge reactive oxygen species has been indicated (Hong *et al.*, 2000, Reddy, Chaitanya & Vivekanandan, 2004, Rodriguez & Redman, 2005)

In eukaryotes proline is synthesised from glutamate via Δ^1-pyrroline-5-carboxylate (P5C) in two successive reductions catalysed 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 *E. coli* mutant strongly indicated the use of the δ-OAT route (Delauney & Verma, 1993). Proline degradation is catalysed by the subsequent activity of two mitochondrial located enzymes 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* besides betaine and dimethylsulfoniopropionate (DMSP) (Plettner, 2002). The regulation of proline synthesis with respect to time and intracellular concentration is of primary interest regarding an increase in external salinity during the incorporation of *F. cylindrus* cells into growing sea ice. Four cDNAs could be identified from a cold stress (Mock *et al.*, 2005) and salt stress (Krell et al. unpublished) induced Expressed Sequence Tag (EST) library encoding the most relevant enzymes during proline synthesis and degradation: δ-OAT, P5CS, P5CR and ProDH.

This investigation and experimental set up was 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 into developing sea ice. The mRNA copy numbers of these genes were determined with Q-PCR techniques along with intracellular proline concentrations. The aim was to determine if proline is synthesised primarily via the glutamate or ornithine pathway, or which environmental factor leads to the preference of either one. Measurements of photosynthetic activity are included to monitor the energy availability. This study also differentiates between the

impact of salinity as a single stress factor as well as a combination of salinity and decreased 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 Antarctic seawater enriched with f/2 nutrients after (Guillard & Ryther, 1962) at a salinity of 33.6 PSU in 5l batch cultures. *F. cylindrus* was kept in a culture room at 0°C (± 0.2°C) under continuous illumination with white fluorescent light (Osram Biolux, Germany) at a photon flux density of 25 µmol photons m^{-2} 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 since pre-cultures were treated with a combination of penicillin (100 µg ml^{-1}) and streptomycin (25 µg ml^{-1}).

Three independently grown stock-cultures were used for the following experimental set-up: a) cultures kept at standard salinity and temperature conditions as a control; and b) 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 3 independently grown stock cultures.

Shock treatment

The shock treatment started during the early exponential phase by direct addition of sea salt (Sigma) (approx. 44 g l^{-1}) 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.

Sub-samples for the various parameters were taken one hour before and 4h, 24h, 48h, 288h (12d) and finally 480h (20d) after salt addition.

Determination of cell concentration and growth rate

Cell numbers were determined in triplicate runs using a Multisizer 3 (Beckman Coulter, Germany) particle counter equipped with a 100 µm aperture capillary; using a size range from 2.6 to 9.8 µm. Specific growth rate (µ) was calculated according to the formula:

$$\mu = [\ln (C_1) - \ln (C_0)]/ [(t_1 - t_0)/24]$$

where C_1 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, Waldren & Teare, 1973) modified by (Nothnagel, 1995).

PAM measurements

Variable chlorophyll a fluorescence, measured with Pulse Amplitude Modulated (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 Germany) equipped with a temperature control unit and a magnetic stirrer. *In vivo* quantum yield was calculated from fluorescence readings of illuminated samples as:

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

where $F\mathrm{m}'$ and F_t denote the maximum and minimum fluorescence in an illuminated sample (Maxwell & Johnson, 2000).

RNA extraction and purification

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

Reverse transcription, primer design and Q-PCR conditions

Complementary DNA was generated with the Omniscript RT kit (Qiagen Hilden Germany) utilising anchored oligo$(dT)_{20}$ primer (Invitrogen) at a final concentration of 25ng μl^{-1}. To verify the efficiency of reverse transcription and to get a handle for the correction of different efficiencies the reaction mix was spiked with artificial RNA of two genes of approx. 1.8kb in size (MA and NSP) from *Pieris rapae* (cabbage white butterfly, Lepidoptera: Pieridae). Since hardly any insects are present in the marine environment this constitutes an ideal internal reference. MA was added at a final concentration of 116 pg μl^{-1} and NSP at 10 fg μl^{-1} spanning 4 orders of magnitude. Reverse transcription of 500ng total RNA and the added spike RNA was carried out at 42°C for 1h followed by an inactivation cycle at 85°C for 5 min. For each time point and treatment one reverse transcription 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 Germany) software and synthesised from OPERON Biotechnologies Germany. For each primer pair, the reliability of the Q-PCR 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 1 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 (±0.28 n=50), the second control gene NSP at a C_t of 33.9 (±1.03 n=44); both values indicative of a consistent efficiency of the reverse transcription reaction for high and low copy number genes.

For Q-PCR, 5µl of a 10-fold diluted RT reaction mix was added to 15µl of a PCR mixture. Instead of using the recommended 2x Sybr Green PCR Master Mix (Applied Biosystems) dilution, we used a 2.5-fold master mix dilution, i.e. 8µl instead of 10µl per reaction. Each primer was added at a concentration of 50 to 500nM depending on optimised reaction efficiency. Cycle parameters were as follows: initial denaturation 95°C/10min, followed by 40 cycles of 95°C/15sec and 59°C/1min. Finally a dissociation step was carried out to check if a single product was amplified and for primer dimers.

Data analysis

Threshold cycle (C_t) values, slope of the standard curve and correlation were calculated with the Sequence Detection Software 1.2.3 (Applied Biosystems Germany). One replicate was removed from the experimental data when the C_t value differed more than 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 formula: $E = 10^{-slope} - 1$ where slope is determined from the linear regression of Log (target concentration) versus C_t.

Results

Salt shock arrests growth for 12 days

All results originate from 3 different 5l batch cultures for each set-up (control, 70/0, 70/-4). All nine cultures showed an identical growth without lag phase within the 10 days before stress exposition (Figure 1).

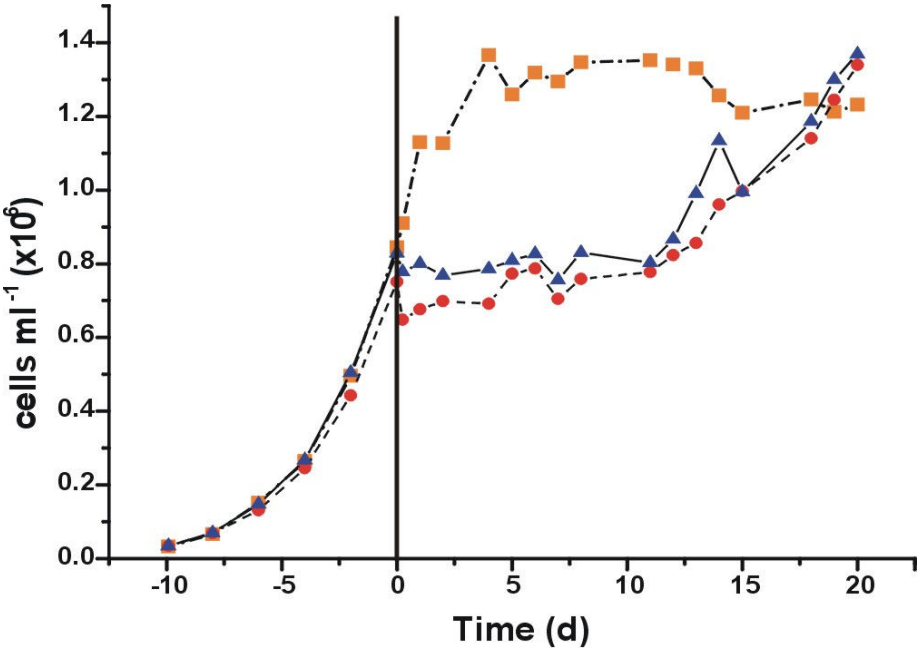

Figure 1: Growth kinetics of *F.* cylindrus cultures. Nine cultures were grown in f/2 medium at 0°C, 34 PSU and 25 µmol photons m^{-2} s^{-1} until day 0. Conditions for three cultures remained unchanged and were further on kept as semi-continuous cultures (■), in three cultures the salinity was increased to 70 PSU (●), in the remaining three cultures temperature was additionally decreased to -4°C (▲).

At time point 0 (70/0 and 70/-4 exposed to stress conditions) the control cultures were subsequently maintained as semi-continuous batch cultures to prevent them from reaching the stationary growth phase to exclude undesired effects due to physiological changes. Control cultures were kept at a nearly constant cell density around 1.3 x 10^6 cells ml^{-1} representing a constant growth rate of µ=0.325 over the course of the experiment. Due to cell death caused by the salt shock, cell density of both treatments decreased by approx. 10^5 cells ml^{-1} and remained stationary for about 12 days. Subsequently both shock treatments regained positive growth albeit

at a lowered growth rate of μ=0.06 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 exposition.

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 (Figure 2). In the 70/0 shock treatment, average Φ_{PSII} values instantly dropped from 0.59 before treatment to 0.24 within one hour after the salt addition. During the next 8 hours there was no change in Φ_{PSII}, but within the first 24 hours Φ_{PSII} increased up to 0.30 and after 48 hours it reached 0.37.

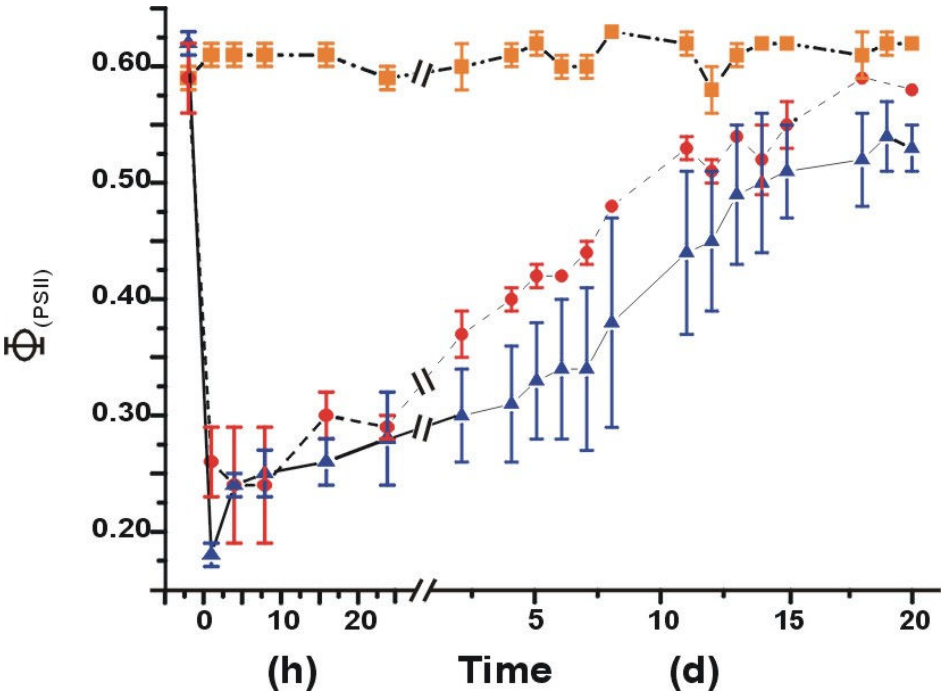

Figure 2: Changes of the photosynthetic quantum yield at PS II during the course of the experiment; (■) control cultures, (●) 70PSU/0°C cultures, (▲) 70PSU/-4°C cultures

Afterwards photosynthesis recovered slowly but steadily as shown by increasing Φ_{PSII}, yet did not attain values as before the shock treatment and as the control cultures. The 70/-4 cultures essentially showed a similar reaction, but the initial drop was even more pronounced (0.18 after one hour) and the recovery was retarded compared to the 70/0 treatment. Both shock treated cultures regained quantum yield values close to the non-stressed cultures towards the end of the experiment.

Proline concentration increases several fold during acclimation to high salinity

Before stress exposition *F. cylindrus* had intracellular proline concentrations of 3.0±0.48 fmol cell^{-1}. Whereas the concentrations of the control cultures remained constant, the cells of both treatments started to accumulate proline eight hours after stress exposition in both treatments (Figure 3). After 24 hours the intracellular proline concentration in the treated cultures had already doubled compared to the control.

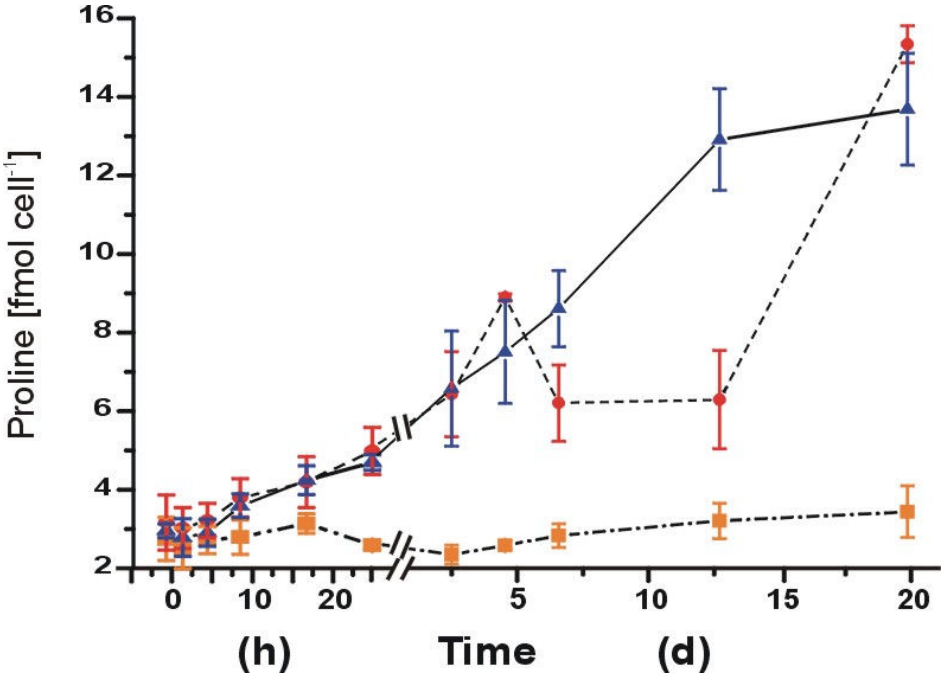

Figure 3: Temporal development of intracellular proline concentrations (■) control cultures, (●) 70PSU/0°C cultures, (▲) 70PSU/-4°C cultures

Furtheron, the cultures exposed to lower temperature kept constantly accumulating proline up to 12.9±1.29 fmol cell^{-1} at day 12 and 13.6±1.41 fmol cell^{-1} at day 20. The unchanged intracellular proline concentration within the last two sampling points might be due to regained growth since day 12 (Figure 1). However, proline concentrations of *F. cylindrus* 70/0 cultures varied: While increasing simultaneously with the 70/-4 treatment to concentrations of 8.8±0.09 fmol cell^{-1} on day four, they decreased to 6.2 fmol cell^{-1} on days 8 and 12 after stress exposition. At the end of the experiment on day 20 a concentration slightly exceeding the 70/-4 cultures of 15.3±0.47 fmol cell^{-1} was measured; which is a 4.5 fold increase 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 analysed the transcript levels of the key enzymes of proline metabolism, P5CS, δ-OAT, P5CR and ProDH, by Q-PCR. δ-OAT, P5CR and ProDH showed a similar expression pattern during the experiment in both salt shock treatments (Figure 4b-d). An increase in mRNA levels of theses genes could be detected reaching its maximum 24h after the beginning of the stress exposition. After this initial induction, mRNA levels of P5CR and δ-OAT constantly declined in both treated cultures returning almost to the values of 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. 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. Showing 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 actin strongly decreased during the first hours after stress exposition, reaching their lowest level at timepoints 24h and 48h, respectively (Figures 4a, 5b). However, the expression level of actin in the 70/-4 was less affected compared to the 70/0 and recovered more rapidly, contrasting the observations made regarding the up-regulated genes and even P5CS. This is consistent with the less affected growth of the additionally cold treated cultures as described above (Figure 1). At day 12 after the shock treatment actin mRNA levels regained control levels.

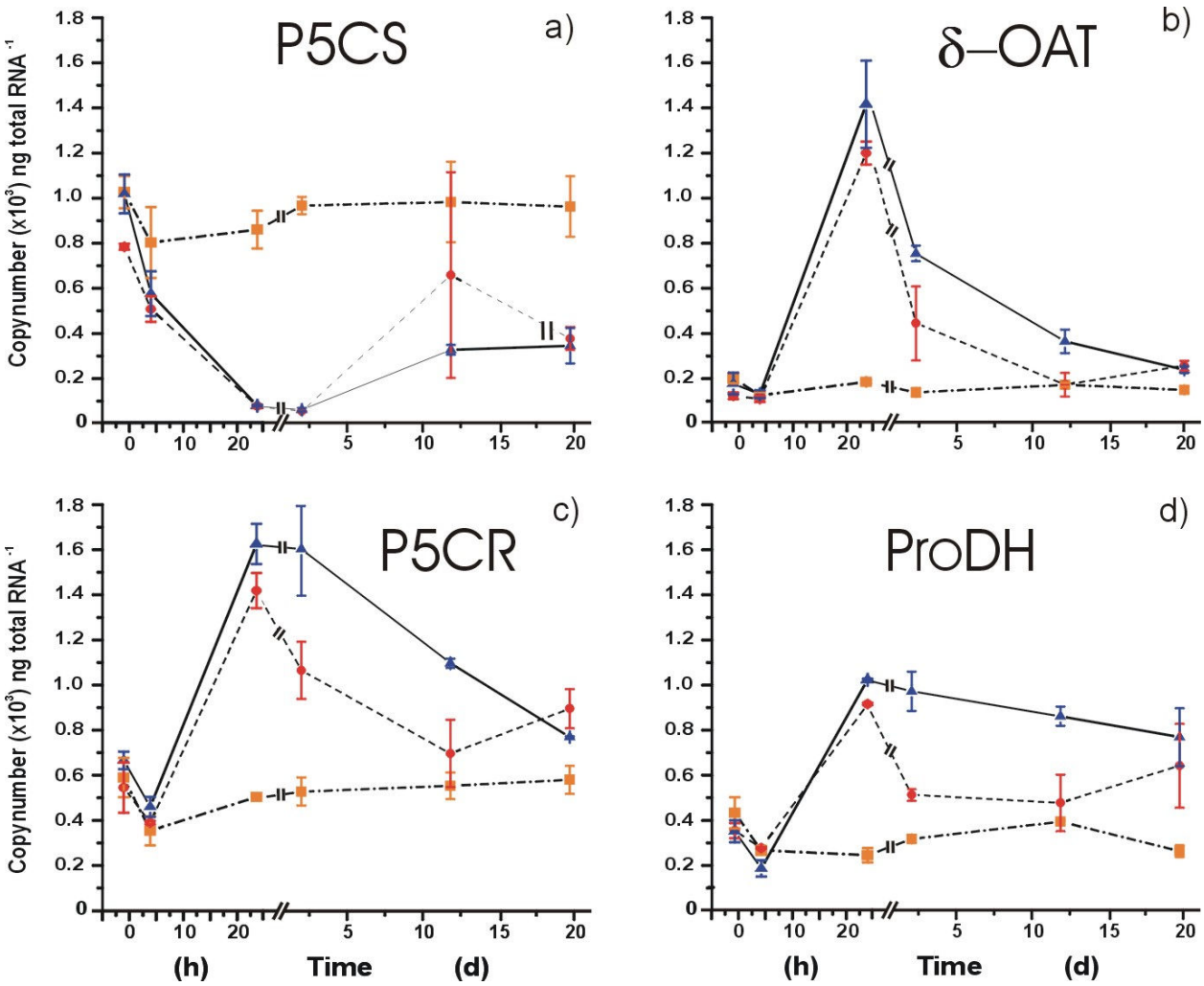

Figure 4: Quantification of transcript levels of the genes a) P5CS, b) δ-OAT, c) P5CR and d) ProDH during the course of the experiment as revealed by Q-PCR analyses. mRNA copy-numbers were calculated from standard curves of DNA templates and normalised to ng of total RNA extracted. (■) control cultures, (●) 70PSU/0°C cultures, (▲) 70PSU/-4°C cultures

Surprisingly, psbA showed the least variation in its expression level over the course of the experiment (Figure 5a). Variations of the control and the 70/-4 cultures were within the standard error and also no significant difference between the treatments was observed. Only a slight decrease in the 70/0 treatment at 48h was significant. However, this strongly contrasts to the inhibition of photosynthetic electron transport revealed by the measurements of Φ_{PSII} (Figure 2). To summarise: the maximum alteration in the expression level of the observed genes occurred within the first 24h after stress exposition, only P5CS and psbA reached their maximum at 48h (Table 3). P5CS and actine showed the highest magnitude in changes of the expression level of all genes analysed with a 17.3 and 29.7 fold decrease, respectively. While the adjustment phase

in terms of transcript levels of the genes analysed in this study was completed within 24 to 48 h, it took 12 days until the adjustment became effective in terms of positive cell growth.

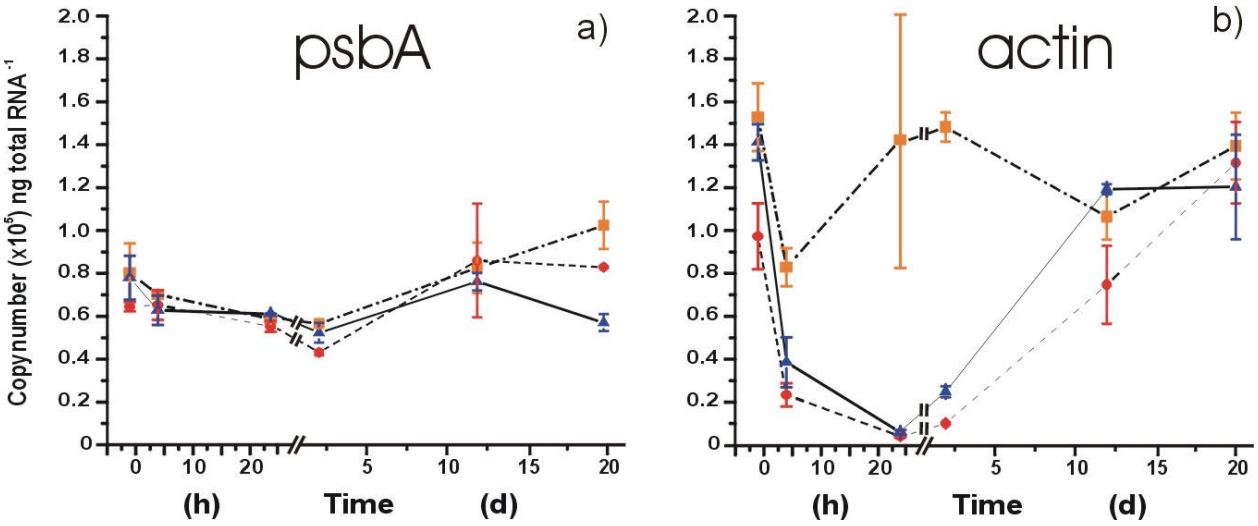

Figure 5: Evaluation of transcript levels of the genes a) psbA and b) actin during the course of the experiment as revealed by Q-PCR analyses. mRNA copy-numbers are normalised to ng of total RNA extracted (■) control cultures, (●) 70PSU/0°C cultures, (▲) 70PSU/-4°C cultures

Discussion

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

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 was very similar for all sampling points. The constant detection of the exogenous control genes MA and NSP verified a consistent efficiency of the reverse transcription reaction, therefore it can be concluded that expression data gained for the target genes were not biased by the reverse transcription step. The strong variability of actin transcript levels demonstrates that methods employing endogenous reference genes for the analyses of expression 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 sub-lethal stress for *F. cylindrus* as manifested by a strong drop in photosynthesis and growth arrest for12 days. 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 (esp. in 70/-4 cultures) as well as the steady recovery of Φ_{PSII} reflected the ongoing acclimatisation process starting within the first 24 h after the beginning of the stress exposition. The restart of growth after 12 days could be seen as a measure for 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 day 7 and 12 corresponding with ProDH gene expression levels, but not reflected by physiological data (cell numbers, recovery of Φ_{PSII}). However, the final intracellular proline concentration of ~14 fmol cell^{-1} was the same as 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 (Figure 4c). Lower proline concentrations in the 70/0 cultures are correlated to higher and lower transcript levels of P5CS and ProDH, respectively, supporting the hypothesis that expression of these genes is regulated by proline.

Under salt stress conditions P5CS mRNA levels in *F. cylindrus* were clearly down-regulated and remained low throughout the experiment (Figure 6). This 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, Lu & Verma, 1996, Igarashi *et al.*, 1997; Hare, Cress & van Staden 1999). The strong down-regulation of P5CS transcript levels indicates feedback inhibition of P5CS expression by proline, active even after prolonged presence of high salt concentrations (Figure 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 the plant enzymes (Hong *et al.*, 2000). Both observations support the conclusion that P5CS is not responsible for proline accumulation under salt stress in diatoms.

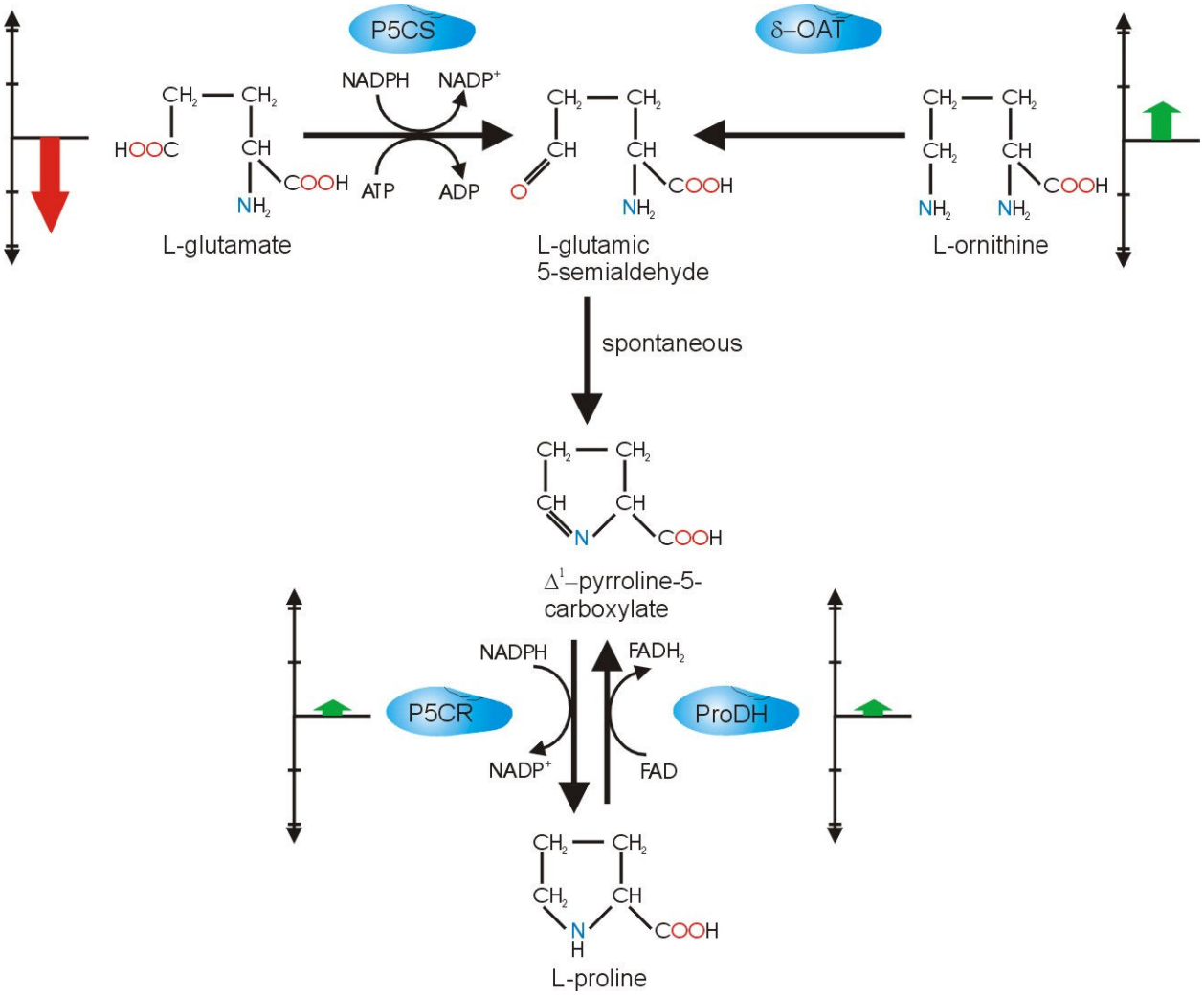

Figure 6: Pathways of proline metabolism using either glutamate or ornithine as a substrate, including those enzymes investigated in the present study: P5CS, δ-OAT, P5CR and ProDH. Thick arrows indicate the initial changes in transcript levels after salt shock treatment.

In higher plants proline accumulation during stress was linearly correlated with a strong decline in ProDH transcript levels (Peng *et al.*, 1996, Miller *et al.*, 2005), whereas the results of this study show the opposite tendency (Figure 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 is absent in diatoms or acts at the posttranscriptional level. Obviously, *F. cylindrus* does not seem to be able to take up externally applied L-proline under standard conditions and replete nutrients, since 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 is supported by the constant elevated copy-numbers of ProDH after the shock treatment positively correlated to proline concentration, while most other genes investigated in this study showed a transient regulation. It remains to be analysed, if ProDH activity is regulated at the mRNA level in diatoms as was observed in higher plants, or if additional regulatory mechanisms exist. Since an overshot in 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 is again in contrast to 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 (Delauny, 1993; Verbruggen, 1995; Roosens et al. 1998)

The possibility of the existence of a differentially regulated P5CS isoform in diatoms was also considered, since 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 of P5CS against the complete genome of the centric diatom *Thalassiosira pseudonana*, equally retrieving only one copy. This may imply a potentially different process of regulation in diatoms. Another difference to higher plants is the subcellular localization of P5CS, which is cytosolic in higher plants (Kavi Kishor *et al.*, 2005). The localisation 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 is also differing for ProDH, of which 2 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 localisation in

higher plants (Kavi Kishor *et al.*, 2005) the localisation of ProDH in *T. pseudonana* is rather ambiguous (targetp v1.1, cTP 0.620, mTP 0.113 other 0.449). These findings suggest that the enzymatic equipment and the compartmentalization of proline biosynthesis and degradation is fundamentally different in diatoms and higher plants, potentially reflecting the different evolutionary history of organelles in both taxa. Additionally, the extensive search for putative isoforms supports the conception, that the genes analysed in this investigation enclose all steps relevant for proline accumulation in *F. cylindrus*.

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 ATP and two molecules of NADPH for the formation of one molecule of proline from glutamate (Figure 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 (Figure 6) (Hare & Cress, 1997). The presence of a complete urea cycle in diatoms was recently demonstrated at the molecular level (Armbrust *et al.*, 2004). Under normal growth conditions, expression of P5CS was high and δ-OAT expression was low, indicating that proline was synthesised 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 utilisation is reduced, and hence ornithine is directed to the synthesis of proline for osmotic adjustment. The strong inhibition of photosynthesis and hence decline in reduction equivalents by salt shock might force diatoms to employ energy saving routes of acclimation.

The strong decline in Φ_{PSII} (Figure 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 & Valentin, 2004) recovering to 0.60 within 5 days, opposed to a decrease from 0.59 to 0.24 (70/0) and 0.18 (70/-4) in this study, respectively. These results suggest that under the conditions employed in this investigation, linear electron transport and thus the photosynthetic production of reduction

equivalents strongly declined and was restored only slowly during acclimation. The unchanged levels of psbA transcripts (Figure 5a) indicate, that photodamage to the D1 protein and repair were not the limiting factors for photosynthesis in salt shocked diatoms. This might be due to a rather stabilising effect of high salt concentrations on the psbA transcript as observed by (Allakhverdiev *et al.*, 2002). A similar complete inhibition of linear electron transport was observed by (Cruz *et al.*, 2001) in *Clamydomonas reinhardtii* after salt shock. They attributed the inhibition to the shrinkage of luminal space hindering the docking of plastocyanin to PS I or cytochrome c_6. An observation which is supported by the findings of (Allakhverdiev *et al.*, 2000) who equally asserted the inactivation of PS I and II in salt stressed cyanobacteria to a water deficit in the cytoplasm. This could explain the slow recovery of Φ_{PSII} since in our study the magnitude of recovery was correlated to the concentration of proline, which is able to restore the internal water potential and hence luminal space.

These results suggest that under such conditions as 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 its function. Thus, the ornithine pathway might be preferred under such circumstances of energy deficiency and in turn lead to an elevated expression of δ-OAT.

Conclusion

The accumulation of proline as a mean to counteract the negative effects of osmotic stress has been maintained throughout evolution in bacteria, higher plants, and in diatoms. However, there seem to be differences regarding the number of isoforms of the genes and subcellular localisation of the proteins involved in the proline metabolism. Additionally, the mechanisms regulating proline accumulation in response to osmotic stress at the transcriptional level seem to differ between higher plants and diatoms. High external salt concentrations lead to an accumulation of proline in *F. cylindrus*, primarily synthesised via the ornithine route. Proline accumulation caused a feedback inhibition of P5CS and induction of ProDH, possibly to overcome a shortage in reduction equivalents caused by a severe inhibition of linear electron transport. 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. At later stages, low temperature induced a more steady increase in proline concentration and acclimation was equally successful as measured by regained growth. This investigation furthermore shows that if *F. cylindrus* is entrapped into newly forming sea ice, experiencing increased external salinities and decreased temperatures, growth is halted and only regained after a considerable adaptation phase during which proline is accumulated. A further dissection of the regulatory mechanisms, including post-transcriptional 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.

Acknowledgements

We would like to thank Prof. G. O. Kirst for reviewing an earlier version of this manuscript.

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Table 1: Genes investigated during this study and sequences of the primers used to amplify target genes by Q-PCR

NCBI Accession no.	Function	Primer sequence 5′ → 3′	Amplicon size (bp)	Best hit	Significance
CF269358	Δ^1-pyrroline-5-carboxylate synthetase	fw: GTGCAATGCAATGGAATCGT rev: TCTGAGCAGCAACTCCGTTACTAA	66	AAB67875	7e-40
DR026040	Δ^1-pyrroline-5-carboxylate reductase	fw: CTCCGCCCTCTTGTGAATGA rev: CCACCCATTTCAAGCGATCT	61	ZP_00357525	5e-47
CF269667	δ-Ornithin aminotransferase	fw: GGTAGGAAATTCGGCCGTAGA rev: GATTGATTCGGACAGCGTTTAGA	65	AAH77314	2e-47
DR026487	Proline dehydrogenase	fw: GAAGTTGATGAGGTGATGCCATAC rev: GCACCACCAATAGCACTGTTTTC	65	A47302	4e-44
DR026674	Actin	fw: ATGAAGATATCGCTGCCCTTGT rev: CCAGCGAAACCGGCTTT	64	CAA42559	8e-125
CF269420	psbA	fw: AGAACCACCAAATACACCAGCAA rev: TCCAAGCTGAGCACAACATCTT	71	AAM62069	1e-130
unpublished	MA control	fw: TCGGTTGACAGATACCTTAAAGGAA rev: TCAAAGGTGACGTTCGAGTTCAT	100		
AY425622	NSP control	fw: ACGATGCCTTCAGAGCTACCTT rev: TACGCATCAAGCGTTTGGAA	100		

Table 2: Efficiency of the Q-PCR reaction derived from standard curves using PCR products of the corresponding plasmid as a template.

Gen	slope	Efficiency	r^2
Δ^1-pyrroline-5-carboxylate synthetase	-3.472	0.94	0.9979
Δ^1-pyrroline-5-carboxylate reductase	-3.56	0.91	0.9991
δ-Ornithin aminotransferase	-3.98	0.78	0.9938
Proline dehydrogenase	-3.428	0.96	0.9987
Actin	-3.666	0.87	0.9984
psbA	-3.38	0.98	0.9989
MA control	-3.588	0.90	0.9979
NSP control	-3.634	0.88	0.9981

Table 3: Maximum changes in the expression level compared to the mean of the control and time point when it occurred.

Gen	70PSU/0°C		70PSU/-4°C	
	fold change	time (h)	fold change	time (h)
Δ^1-pyrroline-5-carboxylate synthetase	-17.26	48	-15.37	48
Δ^1-pyrroline-5-carboxylate reductase	2.5	24	2.7	48
δ-Ornithin aminotransferase	7.55	24	8.93	24
Proline dehydrogenase	2.88	24	3.21	24
Actin	-29.71	24	-20.67	24
psbA	-1.76	48	-1.45	48

Danksagung

Mein herzlicher Dank gilt Herrn Professor Gunter Otto Kirst, der mich seit der Einführungsvorlesung zur Meereskunde im ersten Semester, bei der Vordiplomsprüfung und auf Exkursionen durch mein gesamtes Studium begleitet hat, und schließlich die Betreuung meiner Promotion übernommen hat.

Vielen Dank an Herrn Professor Christian Wiencke für die Übernahme des Zweitgutachtens.

Mein ganz besonderer Dank gilt Dr. Gerhard Dieckmann der mich auf den Pfad der Meereisforschung gebracht hat, der mich stets tatkräftig bei der Anfertigung dieser Arbeit unterstützt hat und für alle Probleme ein offenes Ohr hatte. Gemeinsame Abenteuer in den Weiten Russlands (der Zoll ist allein ein Phänomen für sich) und zwei Monate auf einer vier Quadratmeter Kammer schweißen schon zusammen. Das event cooking mit mongolischen Feuertöpfen wird auch in lebhafter Erinnerung bleiben.

Ein großes Dankeschön gilt Dr. Klaus Valentin, dem Ratgeber in allen Lebenslagen. Er hat nicht nur wesentlich zu meinem tieferen Verständnis der molekularbiolgischen Arbeitsweisen und Methoden beigetragen, sondern mir auch gezeigt, wie die vorderen Bremsscheiben bei meinem Peugeot 205 gewechselt werden. Dank seiner Initiative und Idee wird mir zudem ein weiterer bezahlter Aufenthalt in der Wissenschaft für die nächste Zeit ermöglicht. Den nächsten Cappuccino bezahl ich, versprochen!

Dr. Thomas Mock danke ich besonders für die Hinführung von der Ökologie zur Molekularbiologie, sowie für spannende fachliche Diskussionen.

Bei Henrik Lange und Nikolai Hoch möchte ich mich ganz herzlich für die Unterstützung und einige nächtliche Probennahmen während meines großen Salzschock Experimentes bedanken. Niko insbesondere für die Erhebung der PAM Daten und Henrik für die aufwendige Analyse der Fettsäuren. Es hat viel Spaß gemacht mit Euch beiden.

Birte Gerdes danke ich für eine Vielzahl von hilfreichen Hinweisen, netten Gesprächen und Plaudereien über den Schreibtisch und den Tresen im Zillertal hinweg.

Erika Allhusen, ohne deinen unermüdlichen Einsatz im Kulturlabor, in der Bestellung großer wie kleiner Dinge, die Probennahme bei diversen Experimenten und nicht zu Letzt durch die Versorgung mit Kuchen bei einer Reihe von Geburtstagen hast du meine Doktorandenzeit sehr erleichtert und verschönert. Ein ganz großes Dankeschön an dich.

Bei Dr. Dietmar Funck bedanke ich mich herzlich für die intensive und hilfreiche Diskussion an einem schwierigen Punkt meiner Arbeit. Ich hoffe, ich kann per Gelegenheit mal in Konstanz vorbei schauen und mich dafür revanchieren.

Bei Dr. Uwe John bedanke ich mich ganz besonders für die Möglichkeit tiefer in die Materie der quantitativen Real-time PCR einzusteigen und die vielen technischen Tipps und Tricks, ohne die ich wohl doppelt so lang im Labor gestanden hätte. Des Weiteren an den Rest der AG Cembella, die mich immer freudig in ihren Laboren begrüßt hat, wenn ich mal wieder auf der Suche nach Dingen war, die ich mal eben kurz brauchte.

Bei Jessica Kegel und Christiane Uhlig bedanke ich mich für die Durchführung eines Salz- und Kälteschockexperiments im Rahmen eines Praktikums. Hoffentlich werden euch die Nachwirkungen, die dieser erste Kontakt zu den kleinen grünen Zellen scheinbar mit sich brachte, weiterhin viel Spaß bereiten.

Meiner Frau Zuzana danke ich von ganzem Herzen für die liebevolle Unterstützung aus dem sozialwissenschaftlichen Blickwinkel, ihre Geduld und das entgegengebrachte Verständnis bei der Entstehung dieser Arbeit. Meiner Tochter Annika danke ich für die vielen tollen Momente, die ich im letzten Jahr erleben durfte.

Meine Eltern haben mich während meines gesamten Studiums auf vielfältigste Weise unterstützt, sie haben mich stets ermuntert mein Ziel zu verfolgen und dabei auch mal links und rechts des Weges zu schauen. Vielen Dank!