Abiotic stress response in Ectocarpus siliculosis : a global approach

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Abiotic Stress Response in *Ectocarpus siliculosus*: a global approach

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I would like to thank…

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Introduction

1. Introduction

1.1 The intertidal zone, a unique changing habitat with many stressors

All organisms living in the intertidal zone are subject to recurring, harsh changes in the environment associated to life in the interface of terrestrial and marine habitats. These changes include, but are not limited to, mechanical strain, biotic attacks, pollution, temperature, light (UV), desiccation, changes in salinity (Figure 1), and control the distribution of organisms in the intertidal zone (Davison & Pearson 1996). They can therefore be considered stressors, according to the definition of stress as environmental conditions that decrease growth and reproduction (Davison & Pearson 1996). The following section will give a brief overview of different abiotic stressors in the intertidal zone.

1.1.1 Abiotic stresses in the intertidal zone

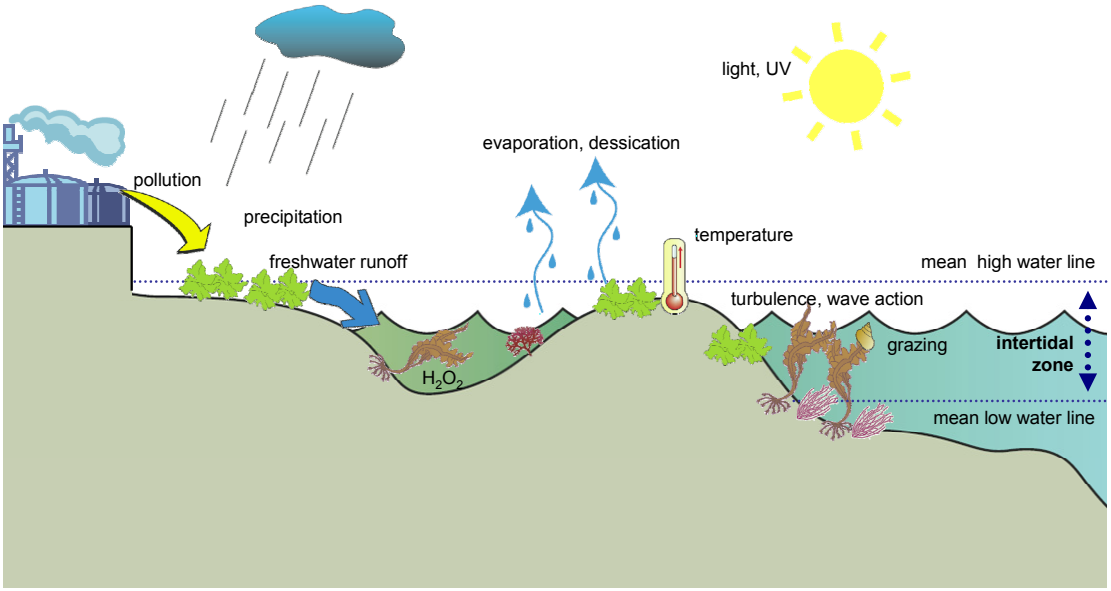

Figure 1: Graphical representation of different abiotic (and biotic) constraints in the intertidal zone

One of the most obvious stressors is **wave action**. Close to the water-land interface, waves exert mechanical stress on algae, so that only very well adapted species, or even no algae colonize very exposed areas of the shoreline at all.

In more sheltered areas, waves remain a stress factor, but other stressors gain in relative importance, most of them related to the changing tides. **Light**, and in

particular ultraviolet (UV) light, is quite well absorbed by water so that changing water levels as well as emersion and immersion will greatly change intensity and composition of light perceived by organisms. Light is necessary for photosynthesis, but at the same time, can cause damage to cells if too intense by inducing the production of oxygen radicals and also, in the case of UV light, directly damaging DNA. Finally, the availability and intensity of sunlight has a direct influence on temperature.

While water **temperature** in the open ocean remains relatively constant throughout the day, water temperature in tidal pools or the temperature in the absence of water during low tide is subject to great variability. Depending on the latitude, water temperature in tidal pools might be close to the freezing point at low tide in winter. The opposite is true in summer, when elevated temperatures might become a problem. During practical courses at the Helgoland Biological Station (http://www.awi.de/en/institute/sites/helgoland/), for instance, we have measured the temperature in some rock pools to be 10°C over that of the open water on a sunny summer afternoon. These changes in temperature affect algae and other organisms in several ways. An increase in temperature will generally cause an increase in respiration and at the same time a decrease in $\mathbf{O_2}$ solubility, thus creating an environment deficient in O_2. Parallel changes in the **pH** might also influence algae directly and via the availability of HCO_3 and $\mathbf{CO_2}$.

Similar effects also occur in the course of emersion. In addition, algae will start to **desiccate**. Some algae can loose up to 90 % of their intracellular water and entirely recover when re-immersed into water (Dring and Brown 1982). The effects of desiccation are generally very similar to those of **hypersaline stress,** as in both cases a loss of intracellular water will cause a parallel increase in intracellular ion concentration. Salinity is generally highly variable in the coastal region and particularly in the intertidal zone, e.g. in tidal pools (Davison & Pearson 1996), where evaporation as well as precipitation and freshwater runoff have the highest impact (Kirst 1989). Collén et al. (2007) suggested that saline stress (both hypo- and hypersaline) is one of the most important stresses in the intertidal zone.

Over the last decades, **human activities** have also started to generate additional stressors through various forms of pollution. Oil spills, heavy metal contaminated waste water from ships or from industries, anti-fouling paint, or un-treated sewage

water have drastically changed the costal areas around the more heavily populated regions. As, from an evolutionary point of view, these stressors are novel, not many organisms possess mechanisms to tolerate them. Many of these stressors, however, have some similarities to natural stresses. Heavy metal and in particular copper stress, for example, is known to cause an intracellular accumulation of **reactive oxygen species** (ROSs) just as many other biotic and abiotic stresses do (Mithöfer et al. 2004).

1.1.2 Photosynthetic organisms in the intertidal zone: a focus on brown seaweeds

Not very many photosynthetic organisms have adapted to life in the intertidal zone. While the majority of organisms that inhabit the intertidal zone can follow the tides and avoid the stresses related to immersion and emersion, seaweeds are usually immobile and, in order to survive in the intertidal zone, have to rely mainly on cellular mechanisms to tolerate stress (Ocon 2007).

Probably the most abundant photosynthetic inhabitants of the intertidal zone are brown algae. Brown algae belong to the chromalveolate kingdom and have evolved to multicellularity independently from red and green algae, as well as the most commonly studied eukaryotic lineages - animals, fungi, or green plants (Baldauf 2003, Figure 2).

The phylogenetic distance of brown algae from other multicellular eukaryotes can be illustrated by a number of unique metabolic pathways and features. Brown algal plastids, for example, like those of all heterokonts, derive from a secondary endosymbiosis event with a red alga (Keeling 2004), and are surrounded by four membranes. Recent genomic analyses furthermore indicate that plastids derived from secondary endosymbiosis might have replaced an earlier green plastid in heterokonts (Moustafa et al. 2009), traces of which can still be found in heterokont genomes. Other unique features include the ability of brown algae to synthesize both C18 (plant-like) and C20 (animal-like) oxylipins (Ritter et al. 2008), their original cell wall composition and the associated pathways of cell wall synthesis (Kloareg & Quadrano 1988, Nyvall et al. 2003, Tonon et al. 2008), and the ability to accumulate high concentrations of iodine in their cell walls, a trait recently discovered in the kelp *Laminaria digitata* (Leblanc et al. 2006).

Brown algae are also of high ecological importance. One eminent example for this are kelp forests, which are formed mainly by brown algae of the order of *Laminariales* (Bartsch et al. 2008) and provide habitats for thousands of species. Most kelp forests are found in shallow coastal areas of temperate regions (Mann, 1973), but they have recently also been discovered in deep-water areas (>30 m) in tropical regions (Graham et al. 2007).

The economic interest of brown algae is related mainly to their use in the industrial production of polysaccharides (McHugh 2003). For example, alginate, a brown algal cell wall polysaccharide, is used both for pharmaceutical purposes (Tønnesen & Karlsen 2002) and in food industry (Jensen 1993). Another example is the production of the beta-1,3-glucan laminarin, which can act as an efficient stimulator of plant defense (Klarzynski et al. 2000).

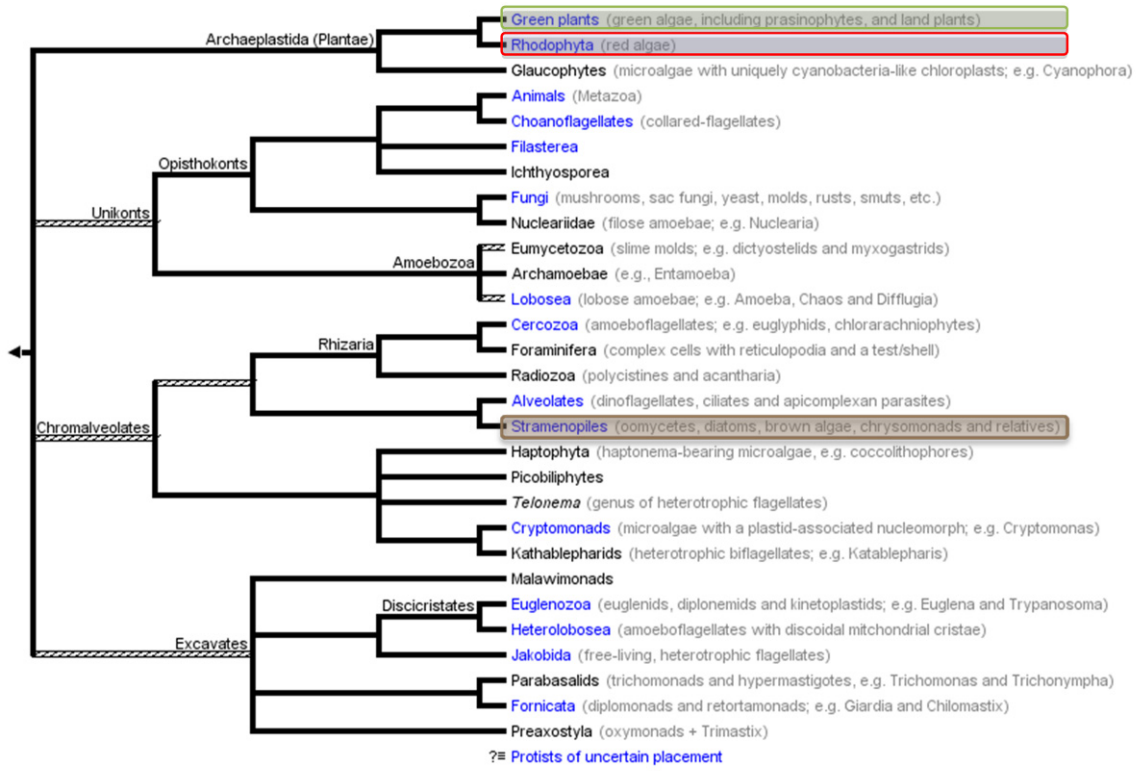

Figure 2: The tree of eukaryotes (taken from the tree of life project http://tolweb.org/, Maddison et al. 2007)

Considering the economic and ecological importance of brown algae, it is essential to understand the mechanisms underlying the adaptation of these seaweeds to the intertidal zone. Understanding how an organism interacts with its natural environment is the first step towards understanding how (anthropogenic) changes will impact an

organism, and the basis of deciphering complex interactions between an organism and its biotic surroundings. This is particularly true for brown algae, as, due to their phylogenetic distance from the green lineage including terrestrial plants, they might have developed very distinct mechanisms of stress tolerance.

Before summarizing what is known about the stress response in seaweed in section 1.3, and in spite of the phylogenetic distance of brown algae from terrestrial plants, the next section will give a brief overview of the stress response in land plants. There are several similarities between terrestrial plants and algae including brown algae (e.g. the fact that they are both sessile photosynthetic organisms with cell walls), and the approaches that were taken to study terrestrial plants have much inspired my work on *Ectocarpus*.

1.2 Abiotic stress tolerance in terrestrial plants

1.2.1 Approaches to studying stress tolerance in terrestrial plants

In terrestrial plants, numerous studies have already addressed the question of the mechanisms underlying the abiotic stress response, and research in this field was greatly accelerated by the availability of the *Arabidopsis* genome sequence (The Arabidopsis Genome Initiative 2000). Soon after the publication of the genome, a range of transcriptomic studies examined gene expression profiles in response to several stresses, starting with mechanical and biotic stress (Reymond et al. 2000), drought and cold stress (Seki et al. 2001), salt stress (Kawasaki et al. 2001), oxidative stress (Desikan et al. 2001), iron deficiency (Thimm et al. 2001), and high light stress (Rossel et al. 2002).

Most of these data are deposited in public repositories in a standardized format according to the MIAME standards (minimum information about microarray experiments, Brazma et al. 2001). As of July 2009, Gene Expression Omnibus (Edgar et al. 2002), one of the largest public repositories available, already contained standardized information from over 300,000 hybridizations performed with different organisms, many of which were plants. This ever increasing amount of data now forms the basis for meta-analyses such as that of Benedict et al. (2006), who managed to identify a network of genes involved in cold signaling by re-analyzing a large number of publicly available datasets.

Gene expression is still a major element in studies of plant stress response (as can be seen from Table 1) although, after an initial "boom" of microarray studies, the scientific community became increasingly aware of the fact that transcriptomic regulation was only one several levels of regulation. Recent comparative studies have examined gene expression profiles along with protein abundance (Branco-Price et al. 2008), enzyme activity (Gibon et al. 2004), and metabolite concentration (Kaplan et al. 2007; Kempa et al. 2008). Although there was a general correlation between these measures and transcript abundance, several exceptions to this general trend were found. Especially in complex metabolic networks, it is very difficult to predict what a plant will do (metabolite changes, changes in enzyme activity) from what a plant "thinks" (transcription, Sweetlove 2008). Studies such as those of Branco-Price et al. (2008), Kaplan et al. (2008) and Kempa et al. (2008) have greatly contributed to our understanding of the stress response in terrestrial plants by highlighting the importance of additional levels of regulation.

Table 1: One hundred most frequently used words in the titles and abstracts of publication referenced in PubMed and containing the keywords "plant" and "stress" (7046 publications in total) as of July 2009. The most commonly used words are located in the left column. Word counts were performed using Rankwords 2.0.4 (www.mechanicwords.com). Numbers, conjunctions, articles, etc. were removed manually.

plant(s)	conditions	support	specific	used
stress(es)	different	soil	significant	function(s)
expression	showed	signaling	seedlings	metabolism
gene(s)	proteins	production	rice	key
growth	analysis	concentrations	enzyme(s)	identified
response(s)	role	leaf/leaves	compared	decreased
salt	level(s)	concentration	cellular	biomass
acid	high	treatment	abiotic	peroxidase
induced	Arabidopsis	reduced	related	laboratory
water	effect(s)	using	important	suggest
activity/activities	resistance	higher	NaCl	signal
protein	content	antioxidant	membrane	wheat
increased	regulation	observed	including	present
drought	development	accumulation	ROS	exposure
tolerance	type(s)	molecular	revealed	UV
cell(s)	increase	low	physiological	seed
process	environmental	changes	functional	model
species	control	transgenic	mechanisms	wild
ABA	root(s)	tissues	glutathione	superoxide
oxidative	involved	significantly	extract	salinity

Recently, transcriptomic and metabolomic studies have been extended to comparisons of the stress response between different ecotypes or similar species. The most

prominent object of this type of study is *Thellungiella halophila*, a particularly (salt) stress resistant species, closely related to *Arabidopsis thaliana* (the average sequence identity is 92 %, Inan et al. 2004). The study of *T. halophila* has already yielded valuable information on the mechanisms underlying its stress tolerance, e.g. with respect to ion uptake and exclusion (reviewed in Amtmann 2009), and the development of further tools including the *Thellungiella* genome project (www.jgi.doe.gov/sequencing/why/50029.html) is currently underway.

The probably most important contribution to understanding the abiotic stress response in plants, however, comes from more targeted studies, as the analysis of changes in gene expression will only yield relevant information if complemented by functional studies of the genes and proteins in question. In *Arabidopsis* (and other terrestrial plants) a number of tools have facilitated a seemingly endless number of studies elucidating the function of specific genes or pathways. These tools include a large number of mutants (see e.g. Ito et al. 2005), as well as resources for gene silencing using RNAi (e.g. Hilton et al. 2004).

All together, these studies lead to a relatively complete (from a phycological point of view) picture of how plants perceive and respond to stress. In the following section, I will give a few examples illustrating some of the aspects of stress signaling and response in terrestrial plants, focusing on drought and saline stress.

1.2.2 Stress perception and signaling

Stress, and in particular saline and drought stress, is known to have several effects on plant cells that lead to the activation of stress signaling pathways, which include changes in cell turgor, to a certain extent changes in cell volume (Xiong & Zhu 2002), and in the worst case membrane damage and protein denaturation (Schluze et al. 2005). Membrane proteins such as receptor-like kinases, stretch-dependent ion (calcium) channels and redox-mediated systems (Urao et al. 1999, Kacperska 2004) have been identified as osmosensors which activate downstream signaling cascades (Figure 3).

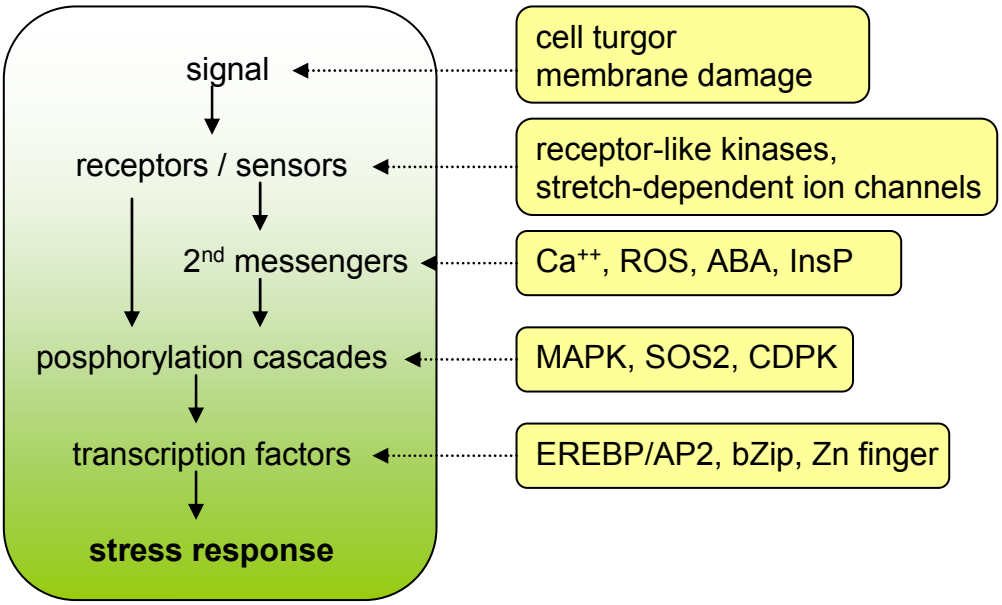

Figure 3: Stress signaling cascade after Xiong et al. (2002) with some examples for each step given on the right. ROS = reactive oxygen species, ABA = abscisic acid, InsP = inositol-5-phosphate, MAPK = mitogen-activated protein kinase, SOS = salt overly sensitive, CDPK = cyclin-dependent protein kinase, EREBP = ethylene-responsive element binding proteins, AP2 = apetala2, bZIP = basic leucine zipper, Zn = zinc.

With respect to (drought) stress signaling in terrestrial plants, a distinction is frequently made between abscisic acid (ABA)-dependent and ABA-independent pathways (Tuteja 2007). ABA is a C15 isoprenoid derived from the cleavage of C40 carotenoids (Nambara & Marion-Poll 2005). As can be seen from Table 1, ABA is a common subject in studies examining plant stress. ABA levels in plants increase in response to most abiotic stresses, but in particular in response to osmotic stress, and activate a number of other second messengers such as inositol triphosphate, reactive oxygen species, or phospholipid-based signals (Zhu 2002). ABA has been shown to be involved in processes such as stomatal closure, but also lipid synthesis and plant development. Although most commonly studied in plants, ABA has also been shown to be present in several metazoans, where it appears to play a protective role against injuries (reviewed in Wasilewsk et al. 2008), and in algae including brown algae, where it might be an inhibitor of growth (Schaffelke 1995).

One of the first elements of the ABA-independent signaling described in detail was the dehydration responsive element (DRE) in the cis-terminal region of RD29A, a gene strongly induced in response to drought and salt stress (Yamaguchi-Shinozaki & Shinozaki 1994). Other genes are the *Arabidopsis thaliana* SHAGGY-related protein kinase (AtSK) and the calcium-dependent protein kinase (CDPK), which are thought

to be activated directly by osmotic stress or by Ca^{2+} respectively, or the CBF4 transcription factor (Boudsocq & Lauriere 2005).

1.2.3 General mechanisms of response

These ABA-dependent and ABA-independent pathways, and several other mechanisms, coordinate the actual abiotic stress response, which, according to Vinocur and Altman (2005), consists of four essential parts: synthesis of compatible osmolytes (in the case of osmotic stress), synthesis of chaperones, activation of detoxification mechanisms, and activation of water and ion transporters. In addition, a 5^{th} element, the regulation of growth, has been attributed increasing importance (Kovtun et al. 2000, Achard et al. 2008, Figure 4). In the following section, I will give a brief overview of a few of the mechanisms known for each of these five parts in terrestrial plants.

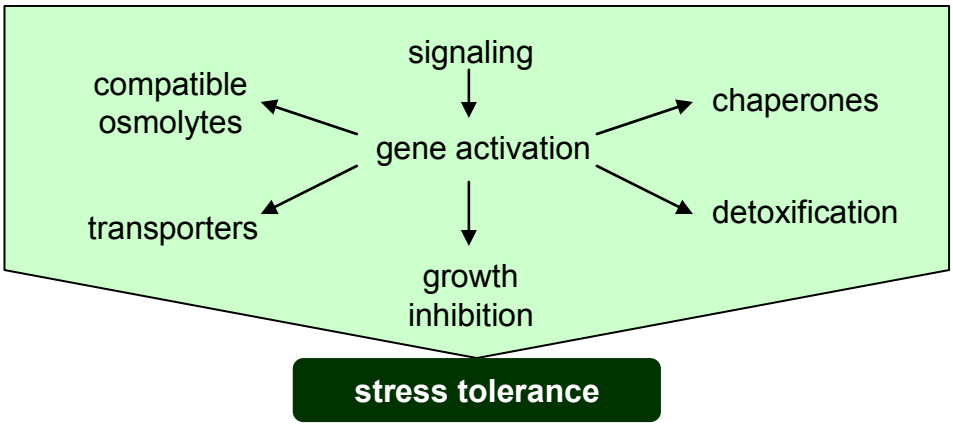

Figure 4: Elements of stress response in terrestrial plants. Based on Vinocur and Altman (2005), with modifications.

1.2.4 Synthesis of compatible osmolytes

Compatible osmolytes, i.e. non-perturbing and osmotically active substances, are commonly employed in all domains of life to control water balance, in particular in response to different salinities or to drought (Yancey et al. 1982). Two prominent examples of compatible osmolytes in terrestrial plants are glycine-betaine and proline, both of which have been shown to be very abundant in halophytes and to correlate with salt tolerance (reviewed in Hellebust 1976). Mannitol seems to be less important in terrestrial plants, although artificially increased mannitol levels have been shown to enhance the stress tolerance in tobacco (Tarczynski et al. 1993). Many substances,

deemed compatible osmolytes, might also play a more direct role in the stress response as osmoprotectors, making it difficult to distinguish between the osmotic and the protective effect of a compound. Glycine-betaine concentrations, for example, are highest in chloroplasts, where glycine-betaine plays an important role in maintaining photosynthetic activity by stabilizing the thylakoid membrane (Robinson & Jones 1986). Proline also was shown to be an efficient hydroxyl radical scavenger (Smirnoff & Cumbes 1989). In many ways, these functions resemble those of chaperone proteins, some of which will be mentioned in section 1.2.6.

1.2.5 Activation of water and ion transporters

In the case of salt stress, one mechanism that is of particular importance for restoring homeostasis and growth are transporters (reviewed in Blumwald 2000, Munns & Tester 2008). As high intracellular Na^+ concentrations are toxic for most plants, salt tolerance is often achieved by actively extruding Na^+ from the cell. Two enzymes seem to be vital for this process: H^+-ATPases, which create an H^+ gradient over a membrane, and Na^+/H^+ antiporters, which use this gradient to transport Na^+. This transport can either be directed out of the cell or into specific cell compartments such as the vacuole, where salts will have fewer or no toxic effects.

1.2.6 Synthesis of chaperones

Molecular chaperones (reviewed in Boston et al. 1996, Wang et al. 2004) are classically considered proteins that are able to bind to many other proteins and assist correct protein folding and maturation. Chaperones consequently also play a role in stabilizing the correct protein conformation, in particular under stress conditions, where their synthesis is frequently increased. Families of common (plant) chaperones include the calmodulin-like protein (CLP) family, the heat shock protein (HSP) 90 family, the HSP70 family, calnexins, and small HSPs, many of which are in turn associated to co-chaperones such as DNAJ/HSP40, glucose regulated protein (GPR) E, or chaperonin (CPN) 10.

1.2.7 Activation of detoxification mechanisms

Abiotic stress is often related to the production of oxygen radicals that play an important role in cell signaling, but which at the same time are cytotoxic and need to be detoxified (Mittler et al. 2004). Plant cells contain a repertoire of ROS scavenging

enzymes such as superoxide dismutases (Monk et al. 1989), catalases, glutathione peroxidases (Halliwell 1974), ascorbate peroxidases (Asada 1992), or peroxiredoxins (Wood et al. 2003), which, in most cases, catalyze the reaction of reactive oxygen species with antioxidants such as ascorbic acid and glutathione. These antioxidants are regenerated, mainly using NAD(P)H, by enzymes such as glutathione reductases or monodehydroascorbate reductases. Furthermore, several other compounds have been shown to exhibit antioxidant properties independently of the ROS scavenging enzymes. These include lipid-related substances like carotenoids (Burton & Ingold 1984, Liebler & McClure 1996), but also the putative compatible osmolytes sorbitol, mannitol, myo-inositol and proline (Smirnoff & Cumbes 1989).

A comprehensive description of the role of only ROS scavenging enzymes and antioxidants alone would be enough information to fill an entire thesis; however they only represent a part of the detoxification mechanisms known in plants. Depending on the type of stress, other detoxification mechanisms are activated. Some examples are ferritins (detoxification of heavy metals), gluthathione S-transfereases (removal of peroxidised lipids and xenobiotics), ubiquitination and protein degradation (removal of damaged proteins), or DNA repair.

1.2.8 Inhibition of growth

The last aspect of plant stress tolerance I would like to mention has received comparatively little attention in the literature: the regulation of growth. Growth is the fifth most common word in abstracts related to plant stress (Table 1) and, by definition, is negatively affected by stress (see figure 5 for an example). However, it is oftentimes forgotten that the inhibition of growth is based on complex regulatory mechanisms, which are essential for survival. One example for this regulation was given by Kovtun et al. (2000), who demonstrated that the *Arabidopsis* mitogen-activated protein kinase ANP1, which is activated by H_2O_2 and initiates several phosphorylation cascades related to the stress response, also inhibited the effect of auxin, an important growth hormone in plants. More recently, more complex connections between growth and stress signaling have been discovered. Again in *Arabidopsis*, the changes in expression of a transcriptional regulator controlling genes involved in the response to cold stress, the CBF1/DREB1b gene, were shown to have a positive effect on the accumulation of DELLA protein, an important repressor of plant growth (Achard et al. 2008). Under normal conditions the DELLA protein is

rapidly targeted for degradation by the activity of a specific SCF ubiquitin ligase. However, this targeting requires binding of the gibberelin receptor GID1 and biologically active gibberelin. CBF1 regulates the concentration of biologically active gibberellin via the activation of gibberellin oxidase genes, thus decreasing the degradation of the DELLA protein and inhibiting growth.

These two examples are only a small part of the molecular bases underlying the crosstalk between stress signaling and regulation of growth, and much research will still be required before this part of plant life will be fully understood.

Figure 5: Effects of abiotic stress on plant growth (photo courtesy of René Bürgi)

1.3 Stress tolerance in seaweeds

The first section of this introduction (1.1) demonstrated that seaweeds live in a unique habitat with high levels of abiotic stress, and the previous section (1.2) illustrated that terrestrial plants possess several mechanisms for tolerating abiotic stresses, and that these mechanisms have been relatively well studied. In this third section, I will give an overview of what is known about specific mechanisms of abiotic stress tolerance in seaweeds. Although most of the work regarding stress in intertidal seaweeds aims towards understanding the distribution of different seaweeds in the intertidal zone (reviewed in Davison and Pearson 1996), a few studies relate the tolerance towards a particular stress to an underlying mechanism such as the activation of certain detoxification pathways. As the focus of my thesis is on brown algae, I will

concentrate on this lineage, but also cite some results obtained for the closely related diatoms as well as other seaweeds.

1.3.1 Stress perception and signaling

Very little is known about stress perception and signaling, although several hypotheses exist. In response to osmotic stress, for example, it is generally believed that these changes are perceived by the cell via sensors probably located in the cell membrane (Kirst 1989). Observations in the brown alga *Fucus serratus* also demonstrate that hyperosmotic stress induces ROS production at the plasma membrane and cytosolic Ca^{2+} release, which in turn is responsible for a second oxidative burst in the mitochondrion (Coelho et al. 2002). These early events may form the starting point for subsequent signaling cascades that coordinate the stress response.

In the red alga *C. crispus*, exposure to methyl jasmonate, an important hormone involved in the abiotic and biotic stress response in terrestrial plants (Creelman & Mullet 1997) enhanced both the synthesis of other oxylipins (Bouarab et al. 2004) and the expression of genes with putative functions in stress response (Collén et al. 2006a). The same is true for brown algae (*Laminariales*), where oxylipin synthesis of both plant-like (C18) and animal-like (C20) oxylipins was stimulated in response to methyl jasmonate treatment (Küpper et al. 2009) and in response to copper stress (Ritter et al. 2008). Even though no experimental evidence of methyl jasmonate production exists in either red or brown seaweeds, these results suggest a possible stress signaling role of both methyl jasmonate and other oxylipins, as reported for terrestrial plants (Blechert et al. 1995) and animals (Funk 2001).

In diatoms, a calcium- and nitric oxide based signaling mechanism has been revealed in response to treatments with polyunsaturated aldehydes (Vardi et al. 2006). More recently, Vardi et al. (2008) identified a chloroplastic nitric oxide-associated protein (PtNOA) and demonstrated that the over-expression of this gene increases nitric oxide production in several organisms as well as the sensitivity of diatoms to polyunsaturated aldehydes, thus further supporting the role of nitric oxide in stress

signaling in diatoms. To my knowledge, no studies so far have demonstrated a signaling role of nitric oxide in brown algae[1].

Similarly to polyunsaturated aldehydes in diatoms, which are produced upon cell death, in brown algae, oligoguluronates, i.e. oligomeric degradation products of alginate, a brown algal cell wall polysaccharide, are produced as a direct result of grazing. In *L. digitata*, treatment with oligoguluronates was shown to elicit a strong oxidative burst with a parallel efflux of potassium (Küpper et al. 2001). ROS formation is likely to occur via the activity of an oxidase with a flavoprotein subunit, and signaling events involved in this process probably include phospholipase A_2 as well as calcium and possibly chloride channels. Although several studies have examined theses events, the precise signaling pathways involved still remain unknown (Cosse et al. 2007).

1.3.2 Osmoadaptation

In response to saline stress, signaling events such as the ones described above need to coordinate the adaptation to rapid changes in cell turgor and / or size (Figure 6), changed ion concentration, and in ion- and water exchange. Very high salinities have been shown to cause plasmolysis, i.e. the detachment of cell membrane from the cell wall due to a loss of water within the cell. Very low salinities, on the other hand, could cause an influx of water that could eventually damage the cell or even cause it to burst (even if the latter is rather unlikely, considering the solid cell walls). Although the sensory mechanisms underlying the saline stress response are yet unknown, several reactions to saline stress can be observed within the cell.

As maintaining a strong osmotic difference between intracellular and extracellular medium is costly, algae generally adapt their intracellular concentration of osmotically active compounds. These might either be inorganic (i.e. ions) or organic compatible osmolytes. The cost of producing compatible osmolytes, along with the effect of increased ion concentrations, were often thought to be the reasons for a reduction of algal growth in response to stress (Kirst 1989 and references therein). Mannitol is considered the main osmolyte in brown (Reed et al. 1985, Thomas &

[1] Outlook: a highly homologous gene to PtNOA also exists in *Ectocarpus siliculosus* (Esi0060_0044), but the two associated ESTs, LQ0AAA11YM21FM1.SCF and LQ0AAB14YO11FM1.SCF, had too low expression levels to be detected in the microarray experiments described in chapter 2.2.2.

Kirst 1991) and in several species of red algae (Mostaert et al. 1995, Eggert et al. 2007), some of which also actively exclude sodium in response to hypersaline stress (Mostaert et al. 1995).

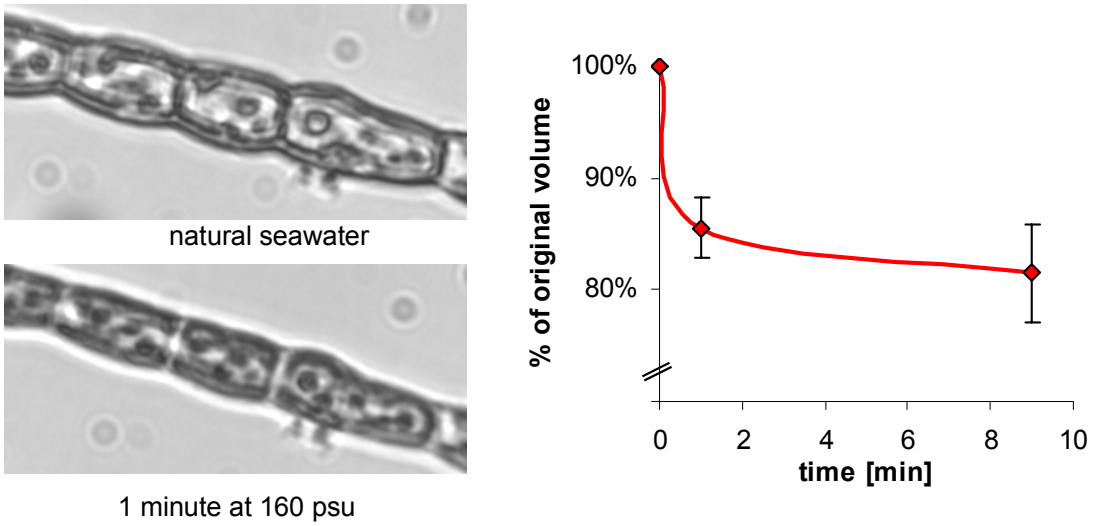

Figure 6: *Ectocarpus siliculosus* exhibits slight (15 to 20 %) changes in cell volume after exposure to very high salinities (5x concentrated seawater, i.e. 160 practical salinity units). These results were obtained during my thesis.

Indications for the role of mannitol as an osmolyte (besides its accumulation) are provided for brown algae by observations of Davidson and Reed (1985). Under non-stress conditions, mannitol concentrations were inversely correlated to intracellular nitrate, and when nitrate was high, mannitol was converted to the osmotically less active polysaccharide laminarin. Furthermore, mannitol at concentration of up to 0.85 M has no or only minor effects on enzyme activities in extracts of *Fucus vesiculosus*, *Laminaria saccharina*, and *L. digitata*, while corresponding concentrations of sodium or potassium salts inhibited certain enzymes (Davison & Reed 1985). This latter observation would exclude ions as major osmotically active substances in the examined species. In *Ectocarpus*, however, intracellular potassium concentrations exceed 200 mM, making potassium a possible candidate for osmoregulation in this species (Thomas & Kirst 1991).

Other potential osmolytes have received relatively little attention within the brown algae, although Wright and Reed (1985) suggested that altriol might play a role in osmoregulation in *Himanthalia elongata* in addition to mannitol. Likewise, several macro- and microalgae have been shown to produce varying concentrations of dimethylsulfoniopropionate in response to different salinities (Dickson & Kirst

1987ab). The amino acid proline has also been suggested to function as an osmolyte in the green alga *Ulva pertusa* (Kakinuma et al. 2006), as well as in the Antarctic ice diatom *Fragilariopsis cylindrus* (Krell et al. 2007). Finally, also in diatoms, one of the possible functions of the urea cycle might be the accumulation of urea as compatible osmolyte (Armbrust et al. 2004), although no experimental evidence for this hypothesis exists so far.

In conclusion, we can say that, among the potentially osmotically active substances in seaweeds, only mannitol has been well studied and accumulates under hypersaline stress conditions in most or all brown algae. Ions may play a role as osmolytes only in some species, but their concentrations vary a lot between species and their repartition between cytoplasm and vacuole has not been sufficiently examined.

1.3.3 Chaperones

Just as in terrestrial plants, HSPs also constitute a significant element in the response to most abiotic stresses in algae. In the red alga *Chondrus chrispus*, HSPs were observed to be transcriptionally activated under osmotic-, light-, temperature-, and natural stress (Collén et al. 2007), as well as in regenerating protoplasts (Collén et al. 2006b). In brown algae Roeder et al. (2005) also observed the overrepresentation of ESTs coding for a HSP70 proteins in a protoplast library compared to the control library, and, in addition, Henkel and Hofmann (2007) demonstrated that the rate of transcriptional up-regulation of HSP70 under heat stress correlated with the competitiveness of two different species of seaweed. Finally, in the Antarctic diatom *Chaetoceros neogracile*, the protein concentration of two chaperone proteins (DnaK and ClpB) increased in response to cold stress (Park et al. 2008). This demonstrates that molecular chaperones are important actors in seaweeds.

1.3.4 UV- and light related proteins

Light stress and the effects of increased UV radiation, as well as protective mechanisms have recently been the focus of several studies. This is due to the degradation of the ozone layer by human influences and the consequential increase in the proportion of UV light that reaches the (water) surface. UV radiation was shown to potentially inhibit algal growth under natural conditions (Huovinen et al 2006), with young individuals being more affected than older ones (Altamirano et al. 2004).

Even though algae are generally able to adapt well to current levels of UV radiation, this stress might act synergistically with other stressors such as temperature or salinity (Fredersdorf et al. 2009). Many marine algae, especially those that occur higher up in the intertidal zone, possess UV-absorbing mycosporine-like amino acids, which provide a possible protection (Shick & Dunlap 2002). Brown algal tissues, however, contain only very low concentration of these substances and probably rely on other UV absorbers such as phlorotannins (Pavia et al. 1997). In red algae, luteine and beta-carotene might also be involved in the recovery of the photochemical capacity after UV-stress (Krabs & Wiencke 2005). Furthermore, in *C. crispus*, genes coding for early light induced proteins (ELIPs) were strongly induced in response to light and other abiotic stresses (Collén et al. 2007). In an Antarctic diatom, a group of genes coding for fucoxanthin a/c binding proteins (related to ELIPs) was up-regulated in response to heat (Hwang et al. 2008). These proteins might be involved in non-photochemical quenching under different stress conditions, when photosynthesis is less efficient. Finally, many (if not all) seaweeds possess the pigments of the xanthophyll cycle carotenoids: violaxanthin, antheraxanthin and zeaxanthin (Vershinin & Kamnev 1996). During high light stress, violaxanthin can be converted to zeaxanthin (de-epoxidation reaction), to assist non-photochemical quenching.

1.3.5. ROS-scavenging enzymes

Light stress, as well as all other forms of abiotic stress, is closely related to the production of ROSs, which, in algae just as in terrestrial plants, can be cytotoxic if accumulated in the cell. In brown algae, the cellular activity of ROS scavenging enzymes (catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase) appeared to correlate with vertical zonation of different species of *Fucales* in the intertidal zone (Collén & Davison 1999). In addition, Aguilera et al. (2002) observed an increase in superoxide dismutase and catalase activity in green and red but not brown algae, with increasing radiation (photosynthetically active radiation + UV). Genes coding for these and related enzymes were also found to be transcriptionally up-regulated in the red alga *Chondrus chrispus* during natural stress, but not in response to saline or temperature stress (Collén et al. 2007). Finally, an activation of ROS scavenging enzymes is most frequently observed in the short-term response to heavy metal stress (reviewed in Pinto et al. 2003). Overall, ROS scavenging enzymes and antioxidants seem to play an important role for abiotic stress

tolerance in seaweeds, and, at least in some cases, their production and activity might be regulated transcriptionally.

In addition to ROS scavenging enzymes, a recent study of Küppers et al. (2008) demonstrated that the brown alga *Laminaria digitata* can use iodine, which it can store at high concentrations in the apoplast, as ROS scavenger. To my knowledge, this study presents the first description of the active use of an inorganic antioxidant in a living organism.

1.3.6 Cell wall composition

The cell wall plays an important role in the algal stress response as it constitutes an additional barrier between a cell and its surroundings. Marine algae, unlike freshwater algae or terrestrial plants, contain sulphated cell wall polysaccharides (Kloareg & Quatrano 1988) such as carrageenans (red algae), fucans (brown algae, McCandless & Craigie 1979), or ulvans (green algae, Ray & Lahaye 1995), which have been suggested to be involved in ionic regulation. Although the exact role of sulphated polysaccharides in marine organisms is still unknown, they do exert antioxidant activities in laboratory experiments (Ray & Lahaye 1995) and might therefore not only play a role in the adaptation to salt, but in the stress response in general. Some enzymes involved in cell-wall biosynthesis or modification such as the brown algal c5-epimerases (Nyvall et al. 2003, Tonon et al. 2008) or vanadium-dependent bromoperoxidases, enzymes involved in cell-wall cross-linking (Berglin et al. 2004) and in the generation of halogenated metabolites (Colin et al. 2003), are thought to be involved in the response to biotic stress and have been shown to be up-regulated in protoplasts, but they might also play a role in the abiotic stress response.

1.3.7 Stress response in seaweeds – a conclusion

These examples underline that, even though several medium-throughput studies have addressed different aspects of stress response in seaweeds, our knowledge about this topic is still rather limited and scattered. Furthermore, data is available usually only for one species, making it difficult to develop a complete picture of possible interactions. In particular with respect to stress perception and stress signaling, very little is known – a fact, which is partially due to the lack of genomic resources for

marine macroalgae. Our hope is to gradually fill these gaps, with the help of the new brown algal model *Ectocarpus siliculosus* presented in the next section.

1.4 The new model *Ectocarpus siliculosus*

Ectocarpus siliculosus represents a species or possibly a complex of species (Starche-Crain et al. 1997) closely related to *Laminariales* (Kawai et al. 2007; Figure 7A). It grows epiphytically or on solid substrates in temperate regions all over the world [www.algaebase.org]. Although it is mainly a marine and brackish water species, there are a few reports in freshwater. Geissler (1983), for example, reported finding a strain of *E. siliculosus* in a heavily salt-contaminated section of the Werra river in Germany, and West and Kraft (1996) isolated a strain, which was recently confirmed as *E. siliculosus* (McCauley & Wehr 2007), from a true freshwater habitat in Australia.

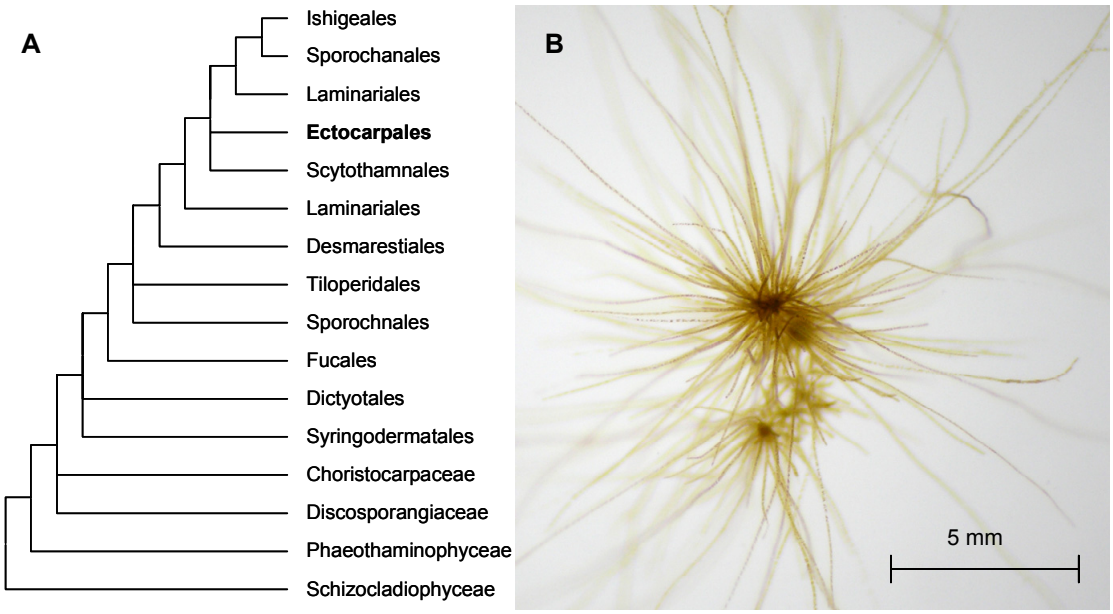

Figure 7: A. Phylogeny of brown algae based on 18S rDNA sequences (modified from Kawai et al., 2007) **B**. *E. siliculosus* strain CCAP 1310/196 (West & Kraft 1996), cultured in natural seawater in Petri dishes.

Research on *E. siliculosus* has a long history, dating back to the 19th century, as recently reviewed by Charrier et al. (2008). Many of the very early studies (e.g. Berthold 1881) focused on reproduction and the complex life cycle of *Ectocarpus*, which includes three different life cycle stages (a diploid sporophyte as well as haploid parthenosporophytes and gametophytes), and which was finally unraveled by Dieter Müller in the 1960s (Müller 1964, 1966, 1967). More recently, extensive work

has been carried out on the salt stress response of different *Ectocarpales* (e.g. Thomas & Kirst 1991), different aspects of carbon uptake (reviewed in Schmid & Dring 1996) and photosynthesis, and on the integration and ecology of the *E. siliculosus* virus 1 (Bräutigam et al. 1995; Delaroque et al. 1999, 2001).

The sum of research already carried out on *E. siliculosus*, its relatively short life cycle, its small genome of ca. 200 Mbp, and its close relation to ecologically relevant species, contributed to the decision to develop this species as a new model for brown algae (Peters et al. 2004). In the end of 2007, the final 10.5X genome sequence became available to members of the *Ectocarpus* consortium, and a public release of the sequences is planned for the end of 2009. The *E. siliculosus* genome is thus the first multicellular heterokont sequenced, and at the same time the first genome of seaweed to become available to researchers (Table 2).

Table 2: Genome projects on aquatic photosynthetic eukaryotes as of July 2009. All genome projects are marked "in progress" according to the public release of the sequence date

Kingdom	spcecies	phylum	habitat	status
chromalveolata	Guillardia theta	cryptophyte	brackish water	Douglas et al. 2001
	Thalassiosira pseudonana	diatoms	marine	Armbrust et al. 2004
	Phaeodactylum tricornutum	diatoms	marine	Bowler et al. 2008
	Ectocarpus siliculous*	brown algae	marine	awaiting submission (Genoscope)
	Fragilariopsis cylindrus	diatoms	marine	in progress (JGI)
	Cyclotella meneghiniana	diatoms	freshwater	in progress (Forensic Science Laboratory Madhuban)
	Thalassiosira rotula	diatoms	marine	planed (JGI)
plantae	Cyanidioschyzon merolae	red algae	hot springs	Matsuzaki et al. 2004
	Chondrus crispus*	red algae	marine	in progress (Genoscope)
	Porphyra purpurea*	red algae	marine	in progress (JGI)
	Ostreococcus tauri	green algae	marine	Derelle et al. 2006
	Chlamydomonas reinhardtii	green algae	freshwater	Merchant et al. 2007
	Micromonas pusilla	green algae	marine	Worden et al. 2009
	Chlorella sp. NC64A	green algae	freshwater	compled (JGI)
	Chlorella vulgaris	green algae	freshwater	compled (JGI)
	Dunaliella salina	green algae	salt fields	in progress? (JGI)
	Ostreococcus lucimarinus	green algae	marine	Palenik et al. 2007
	Volvox carteri*	green algae	marine	in progress (JGI)
	Zostera marina*	Angiosperms	marine	in progress (JGI)

* multicellular organism

In parallel to the actual sequencing of the genome, a number of tools have been and are still being developed, including improved methods for culturing and cryostorage (F. Küpper, pers. comm.), a collection of approximately 60 morphological mutants as well as two life-cycle mutants (B. Charrier, pers. comm.), the sequences of about

90,000 ESTs obtained from different culture conditions and life-cycle stages, a protocol for 2D differential proteomics (Contreras et al. 2008), a genetic map, and recently also a TILLING approach (Targeting Induced Local Lesions in Genomes, O. Godfroy, pers. comm.). Even though some important tools such as a reliable transformation protocol are still in the very early stages of development, the resources accumulated for *E. siliculosus*, once made available to the public, certainly have the potential to greatly increase our understanding of the evolution and the specific adaptations of brown algae. As an example, the first genome-wide analysis of a multigenic family conducted in our team has shown that cytosolic Glutathion S-transferases (GSTs), enzymes involved in detoxification mechanisms, are members of the Sigma class GSTs, identified so far mainly in mammals and invertebrates (de Franco et al. 2009).

One of the most interesting novel studies made possible by the availability of the *Ectocarpus* genomic resources is the evolution of multicellularity (Coelho et al. 2007), as brown algae have evolved to become multicellular organism independently from terrestrial plants or animals. However, in the context of global warming and increasing human impacts on coastal ecosystems (Broecker 1978, Florides & Christodoulides 2009, Brierley & Kinsford 2009), auto-ecological studies, i.e. studies of the interactions of an individual with its biotic and abiotic environment, are increasing in importance.

1.5 Objectives and approach

The objective of this thesis is to address the question of how *Ectocarpus* adapts to its unique abiotic environment, the intertidal zone, by developing an integrated approach. My work focused in particular on three topics that are related to this question (Figure 8).

1. Study of primary metabolism

Understanding primary metabolism forms the basis for understanding the stress response, however, very little is known so far about primary metabolism in brown algae. Although primary storage compounds have been quite thoroughly investigated (Yamaguchi et al. 1966, Reed & Barron 1983, Reed et al. 1985, Thomas & Kirst 1991), and basic amino acids profiling was carried out for some species of brown algae (Smith & Young 1955, Nasr et al. 1967, Nagahisa et al. 1995), it is not known how the concentrations of these compounds change in the course of the day, and what

role transcriptional regulation plays in modulating these changes. This question was addressed by extensive metabolite profiling during diurnal cycle (in collaboration with Antoine Gravot and Alain Bouchereau, UMR 118, Université Rennes 1), combined with an additional experiment on carbon starvation / enrichment, targeted transcript analysis, and genome annotation. In my opinion, knowledge of the basic metabolic processes dominating the life of *E. siliculosus* without stress is essential to the understanding of the changes that are induced in response to stress.

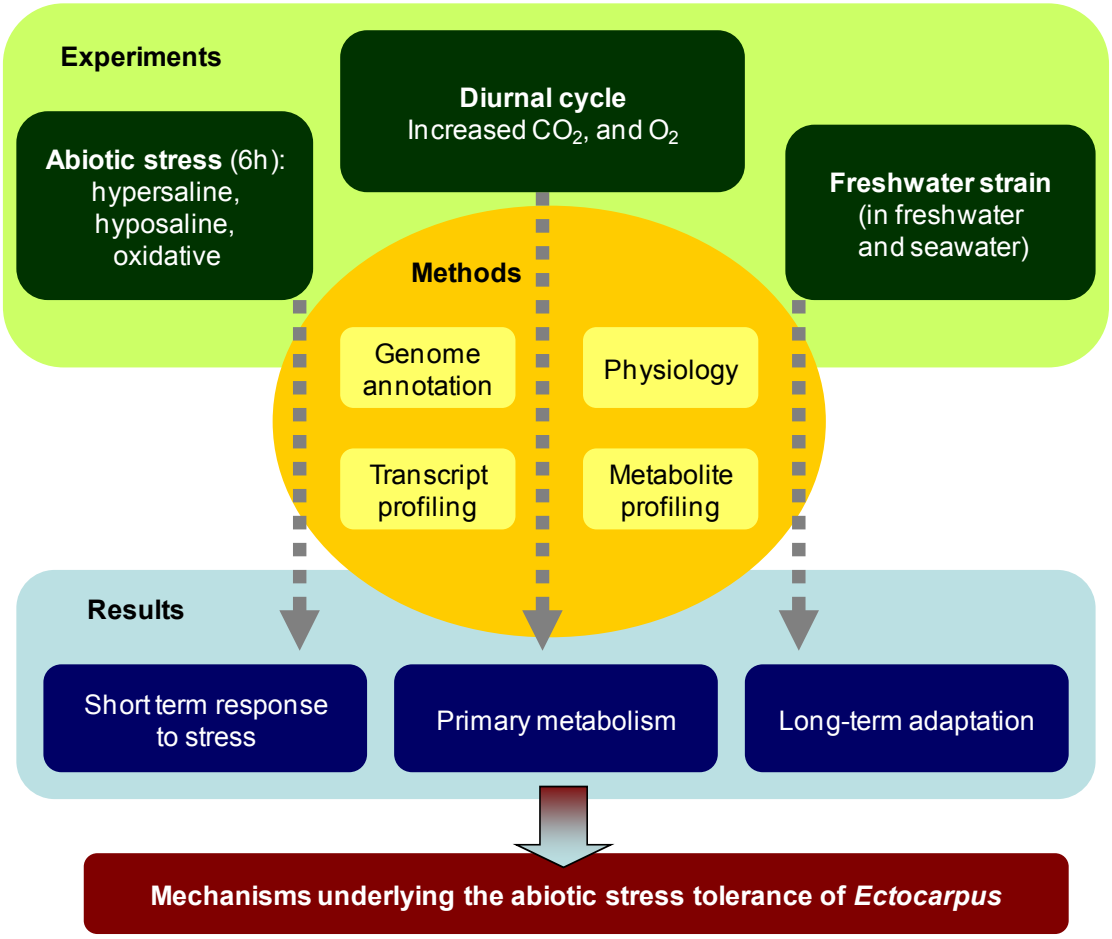

Figure 8: Overview of the approaches taken during my thesis.

2. Short-term stress response

In parallel we sought to examine short term-responses to changes in the environment. For this approach we chose to focus on very few conditions (six hours of sublethal hypersaline, hyposaline, and oxidative stress), but to try to examine these conditions as thoroughly as possible. This was done by combining transcriptomic analyses using a microarray developed as a part of this thesis and metabolic profiling performed by

collaborators (Antoine Gravot and Alain Bouchereau from the Université Rennes 1, and Sophie Goulitquer from the Université de Bretagne Occidentale). These experiments shed light onto some the adaptations of *E. siliculosus* to its highly variable natural environment, the intertidal zone.

3. Long-term adaptation to saline stress (i.e. freshwater)

The third topic dealt with the long-term changes in a strain of *E. siliculosus* that has adapted to life in freshwater. Again, using an integrated approach combining metabolite and transcriptomic profiling, genome annotation, and this time also comparative genome hybridization and morphological observations, we attempted to point out differences between freshwater and seawater strains in different salinities and to compare these differences with what was observed during the short-term response to saline stress. This section highlights the role of plasticity (transcriptomic, metabolic and morphological) in the adaptation to different environments and underlines the value of the freshwater strain of *E. siliculosus* as a model to study the mechanisms of early speciation, as well as evolutionary changes underlying the transition from marine to freshwater habitats.

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Chapter 2

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Primary metabolism

2. Primary metabolism

2.1 Preface

Even though *Ectocarpus siliculosus* has been established as a genetic model for brown algae, very little is known about its primary metabolism or about changes in metabolite concentrations in the course of the diurnal cycle. However, such data is essential for the correct interpretation of changes in response to stress. The aim of this first chapter was therefore to provide a basis for the analyses that followed, by combining four complementary approaches:

1. Manual annotation of 238 loci (corresponding to 147 genes) related to primary metabolism (and in particular metabolism of amino acids) in the *Ectocarpus* genome.
2. Metabolite profiling of amino acids and non-structural carbohydrates in the course of the light-dark cycle during 48 hours.
3. Metabolite profiling after 6 and 12 hours of incubation in seawater repleted or depleted in CO_2.
4. Expression profiling of genes involved in key metabolic processes, during the diurnal cycle and after alteration of growh conditions, by real-time quantitative PCR.

The presented data show that mannitol and several amino acids exhibited strong diurnal oscillations and allowed us to formulate several hypotheses on primary metabolic processes in *Ectocarpus*. As will become clear in chapters 3 and 4, the results presented in this chapter contributed to the understanding of several of the metabolic changes observed in the short-term and the long-term response to stress. This chapter is the result of a collaborative effort of numerous scientists, of which several, and in particular Antoine Gravot (University of Rennes 1), had a major contribution.

2.2 Primary metabolism in *E. siliculosus*

Diurnal oscillations of metabolite abundances and genome analysis provide new insights into central metabolic processes of the brown alga *Ectocarpus siliculosus*

- preliminary manuscript -

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Abstract

In this study we examined the primary metabolism of the model brown alga *Ectocarpus siliculosus* by extensive metabolite profiling across two light-dark cycles and under different CO_2/O_2 concentrations, as well as by targeted gene expression analysis and genome annotation. Apart from anticipated results such as changes in the mannitol concentration, several interesting features emerged from this exploratory approach. I) We detected very low levels of γ-aminobutyric acid (GABA), a stress-related compound usually present in high concentrations in plants, and linked this to the absence of a glutamate decarboxylase gene from the *Ectocarpus*- and also diatom genomes. II) We discuss diurnal changes in the concentration of aspartate and the absence of malate in the context of a possible carbon concentrating mechanism (CCM) in *Ectocarpus*. In combination with genome annotations, our results point to the conclusion that *Ectocarpus* does probably not possess a classical C4 or CAM like organic CCM, although indications for an inorganic, carbonic anhydrase-based CCM were found. III) In agreement with this, we observed an accumulation of glycine and serine, as well as an increase in the ratio of glycine to serine during the light phase and in response to carbon starvation or oxygen enrichment. Glycine, serine, and their ratio are considered markers of photorespiration. IV) We detected a strong inverse correlation between the intracellular concentrations of glutamate and alanine, which might be related to a possible role of these compounds in the temporary storage of pyruvate during photosynthesis.

Keywords: Brown algae, diurnal cycle, carbon concentrating mechanisms, photorespiration, targeted metabolite profiling, quantitative real-time PCR

Introduction

Brown algae, the dominant photosynthetic organisms in the intertidal zone, are part of the chromalveolate kingdom and are phylogenetically distant from red/green algae and land plants. Their primary metabolism has been investigated in several species, with special emphasis on storage compounds and biochemical mechanisms of carbon assimilation. Unlike green plants, brown algae do not accumulate starch or sucrose, but store assimilated carbon essentially as laminarin and mannitol. In particular, mannitol has been intensively studied and is considered a storage compound and osmolyte (Reed & Barron 1983, Reed et al. 1985, Thomas & Kirst 1991).

Similarly, the organic acid citrate is known to be present in high concentrations in brown algae including *Ectocarpus spp.* (Hillrichs & Schmid 2001), and many of the primary amino acids directly associated to nitrogen assimilation also reach substantial levels. However, the concentrations of amino acids have, however, only been examined in a few studies mainly using field samples (Smith & Young 1955, Nasr et al. 1967, Nagahisa et al. 1995). Moreover, these studies neither examined amino acid concentrations in the course of the diurnal cycle, nor the metabolic pathways involved in their synthesis and their regulation under different physiological conditions.

A number of studies (e.g. Yamaguchi et al. 1966, Kawamitsu & Boyer 1999, Moulin et al. 1999, Axelsson et al. 2000, Schmid & Dring 2001, Hillrichs & Schmid 2001) have been conducted to examine the possible presence of inorganic and organic carbon concentrating mechanisms (CCMs) in different brown algal species. Various components of inorganic CCMs, such as external and internal carbonic anhydrases (Schmid & Dring 1996a) and direct bicarbonate uptake (Schmid 1998) were identified, yet the situation is not very clear with respect to the presence or absence of an organic CCM. In a classical organic CCM, the enzymatic decarboxylation of a C4 compound by malic enzymes or phosphoenolpyruvate carboxykinases (PEP-CK) generates CO_2 for the fixation by the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) in the first step of the Calvin cycle. Both enzymatic activities were detected in brown algae, although the activity of malic enzymes is usually very low (Akagawa 1972a), and PEP-CK activity in brown algae is likely to be involved in the light-independent fixation of CO_2 rather than decarboxylation (Kremer & Küppers 1977, Axelsson et al. 1989). Moreover, even though efficient CCMs repress the oxygenase activity of RuBisCO, the immediate product of this oxygenase function, 2-

phosphoglycolate, and its metabolism (photorespiratory glycolate cycle), were detected in *Spatoglossum pacificum* (Iwamoto & Ikawa 1997). On the other hand, several species of brown algae display a weak Warburg effect (i.e. inhibition of photosynthesis by high oxygen concentrations, Kremer 1980).

Basic knowledge about these primary metabolic processes is essential for the understanding of the physiology and ecology of seaweeds, and constitutes a basis for the design and interpretation of future experiments. In this study, we therefore attempt to fill some gaps in our knowledge about primary metabolism in the model brown alga *Ectocarpus siliculosus* (Charrier et al. 2008). This small and filamentous species can easily be cultivated under controlled conditions in the laboratory, therefore alleviating possible influences of seasonal and environmental variations. Moreover, *E. siliculosus* has been selected as a genetic model (Peters et al. 2004) and its genome has recently been sequenced (Cock et al. in prep.). The availability of genomic data allowed us to combine metabolite profiling with manual genome annotation and targeted transcriptomic profiling, thus improving our knowledge of primary metabolism in *E. siliculosus* at the molecular level. Here we present metabolite profiling of the cellular content of free amino acids (20 protein coding amino acids as well as γ-aminobutyric acid (GABA), *β*-alanine, and ornithine), and 24 sugar alcohols (7 of which were detectable), in 3 h intervals during 48 h as well as under different CO_2 and O_2 concentrations. In addition, annotation and expression profiling was carried out for a number of genes of interest. This approach revealed several interesting features about primary metabolism in *Ectocarpus*, including the presence of traces of GABA, the probable absence of a C4 or CAM like organic CCM, the importance of photorespiration, and a striking opposition between the diurnal changes of alanine and glutamate.

Materials and Methods

Experimental setup

For the diurnal cycle experiment, three replicate cultures of *Ectocarpus siliculosus* (Dilwyn) Lyngbye (Ectocarpales, Phaeophyceae) unialgal strain 32 (accession CCAP 1310/4, origin San Juan de Marcona, Peru) were cultivated in 10 L plastic flasks in a culture room at 14 °C using artificial seawater (ASW) (450 mM Na^+, 532 mM Cl^-, 10 mM K^+, 6 mM Ca^{2+}, 46 mM Mg^{2+}, 16 mM $SO4^{2-}$) enriched in Provasoli nutrients

(Starr & Zeikus 1993). This culture medium provides NO_3^- at a final concentration of 0.88 mM (75 mg L^{-1}) in the growth medium. Photosynthetically active radiation (PAR) was provided by Philips daylight fluorescence tubes at a photon flux density of 40 µmol m^{-2} s^{-1} for 10 hours per day. Cultures were aerated with filtered (0.22 µm) compressed air to avoid CO_2 depletion. The diurnal cycle experiment was started about 15 min after the beginning of the light phase. Every three hours, about 200 ml of each of the replicate cultures were harvested by filtration, quickly dried with a paper towel, divided into two sub-samples and frozen in liquid nitrogen. The first sub-sample was used to extract total RNA, while the second sub-sample was freeze-dried and used for GC- and UPLC-analysis.

The carbon starvation / enrichment experiment was performed under the same conditions, with a few exceptions: natural seawater (NSW) adjusted with HCl and NaOH to a pH of 8.3-8.5 was used instead of ASW, PAR was provided 12 hours a day at an intensity of 30 µmol m^{-2} s^{-1}, and cultures were kept in 250 ml glass flasks. In the carbon starvation / enrichment experiment, four different CO_2 / O_2 conditions were tested: a control condition, aerated with compressed air; a carbon depletion condition in which the culture medium was acidified with HCl (pH 2.0), stirred for 4 hours before restoring the pH to 8.4, and then aerated with a mixture of pure N_2 and O_2 (80 % and 20%, respectively); a carbon saturation condition in which 10 g L^{-1} $NaHCO_3$ was added to seawater and the pH was again adjusted to 8.4 (here the air used for bubbling was also enriched with CO_2 by aeration through a saturated solution of $NaHCO_3$); and finally, an O_2-enriched condition, in which control medium was aerated with 50 % O_2, 50 % air starting 12 hours prior to the experiment. This experiment was started in the beginning of the light phase by adding about 2 g FW or *E. siliculosus* to the prepared culture media. Three biological replicates were made on three subsequent days. Each culture was harvested as described above after 6 hours and 12 hours.

Metabolite profiling

For metabolite profiling, 10 mg of the freeze-dried sample were ground using a ball mill. A methanol-chloroform-water-based extraction was performed according to the following procedure: ground samples were suspended in 400 µL of methanol containing two internal standards: 200 µM 3-aminobutyric acid (for amino acid quantification) and 400 µM ribitol (GC-analysis). Suspensions were agitated for 15

min at room temperature. 200 µL of chloroform were then added, followed by a 5-min agitation step. Finally, 400 µL of water were added, and samples were vortexed vigorously and centrifuged at 13,000 g for 5 min to induce phase separation. The upper phase, which contained non-structural carbohydrates, organic acids and amino acids, was transferred to a clean microtube and used for subsequent analysis.

For amino acid profiling, 50 µL of each methanol/water extract were dried under vacuum. Dry residues were resuspended in 50 µL of ultra-pure water and 10 µL were used for the derivatization using the AccQ-Tag Ultra derivatization kit (Waters, Milford, MA, USA). Derivatized amino acids were analyzed using an Acquity UPLC-DAD system (Waters) according to Jubault et al. (2008). 3-aminobutyric acid was used as internal standard. As this protocol provides only poor separation of glutamate and citrulline, an alternative gradient was also run to verify that no citrulline was present in our samples: initial, 99.9% A; 2 min, 99.9% A, curve 6; 6 min, 98% A, curve 7; 10 min, 96% A, curve 7; 12 min, 80% A, curve 6; 15 min, 40.4% A, curve 6; 16 min, 40,4% A, curve 6; 17 min, 99.9% A, curve 6; and 18 min, 99.9% A, curve 6.

Non-structural carbohydrates and organic acids were analyzed by GC-FID according to Adams et al. (1999) and Lugan et al. (2009). Fifty µL of polar extract were dried under vacuum. The dry residue was dissolved in 50 µL of 20 mg mL^{-1} methoxyaminehydrochloride in pyridine at 30°C for 90 min under orbital shaking. Then 50 µL of N,O-bis(trimethylsisyl)trifluoroacetamide (BSTFA) were added and samples were incubated at 37°C for 30 min, then at room temperature overnight before injection. One µL of the mixture was injected in a Trace 2000 GC-FID (Thermo-Fisher Scientific, Waltham, CA, USA) equipped with an AS2000 autosampler (Thermo-Fisher Scientific), a split/splitless injector (split mode set to 1:25) at 230°C, a J&W DB5 30 m x 0.32 mm x 0.25 mm column and an FID detector at 250°C. The temperature gradient was: 5 min at 70°C, 5°C min^{-1} until 220°C, 2°C min^{-1} until 260°C, 20 °C min^{-1}until 300°C and finally 5 min at 300°C. Ribitol was used as internal standard.

GC-MS profiling was used with several samples to check the identity and purity of peaks used for GC-FID quantification.

Genome annotation and gene expression analysis

All genes were identified by sequence homology in the version 2 of the *Ectocarpus* genome (Cock et al., in prep.). The subcellular protein localization of genes was

predicted using HECTAR, a prediction method developed specifically for heterokonts (Gschlössl et al. 2008), and PTS1 predictor, a method used to predict the c-terminal peroxisomal targeting signal 1 (PTS1, Neuberger et al. 2003). Real time quantitative polymerase chain reaction (qPCR) analyses were performed for the time points 0, 6, 12, 18, and 24 hours of the diurnal cycle experiment and for the 6 hours samples of the carbon starvation / enrichment experiment (3 replicates each). Primer design, RNA extraction, reverse transcription, and qPCR analyses were performed as previously described (Le Bail et al. 2008).

Expression profiles were examined for the following genes (Table 1): two alanine aminotransferases (ALT1 and ALT2, where ALT2 has a peroxisomal target peptide PTS1 and is likely to function as glutamate:glyoxylate aminotransferase (GGT) in photorespiration), three additional genes involved in the photorespiratory pathway (one phosphoglycolate phosphatase (PGP), one glycolate oxidase (GOX1), and one protein of the glycine cleavage system (GCS-L)), two pyruvate dehydrogenases (acetyl transferase E1 subunit, PDH1 and PDH2), three genes potentially involved in known C4 metabolic pathways, one phosphoenolpyruvate carboxykinase (PEP-CK), one phosphoenolpyruvate carboxylase (PEP-C), one cytosolic NADP-Malic Enzyme (MDH1), and three carbonic anhydrases (CA2, CA3, CA4). Genes coding for an elongation factor 1 alpha (EF1alpha) and an ubiquitin conjugating enzyme (UBCE) were used as reference genes for normalization (Le Bail et al. 2008).

Table 1: Genes used for qPCR experiments. The table includes gene names, accession numbers and primer sequences, as well as the number of expressed sequence tags (ESTs) associated to each gene.

Short name	Name	ESTs	Genome	Forward primer	Reverse primer
ALT1	alanine aminotransferase	4	Esi0298_0027	GTGTTGTCAGCAGCCTCAAGTAG	AGCATTTCTCGTCAGCCTTTCC
ALT2/GGT	alanine aminotransferase (peroxisomal) glutamate:glyoxylate aminotransferase	10	Esi0008_0209	ACCGCCTCAGAAAATCCACAAC	ACGGACGACAAGCAGTAGACC
PGP	phosphoglycolate phosphatase	3	Esi0130_0079	CACGGGATTATAGAAGGCGAAGTAG	GCTCAGCGGCGGATAAGTTTC
GOX1	glycolate oxidase	19	Esi0012_0104	GCAGCAGCAGTCTAATGGTCTAC	CTACACTTGTTAGCCGCCTTCC
GCS-L	glycine-cleavage system L-protein	4	Esi0322_0005	CATAGTTACATCCATCCTAAACAGTGC	CTGCCCATTAGACCATTTACATTTACG
PDH2	pyruvate dehydrogenase E1 component alpha subunit	7	Esi0122_0080	CCAGCCAAGACCTAAGCACAAG	CCATCACACGACATATTCCCTCTC
PEP-CK	phosphoenolpyruvate carboxykinase (ATP)	2	Esi0035_0144	TGGCTGGAGTGTGCTGGAC	TCGTCATGGCGGCTTTCAATC
PEP-C	phosphoenolpyruvate carboxylase	14	Esi0009_0060	GGTCACCAGCAATCCGTGTAAG	TCAATCATCGCCTCACCTCTCC
MDH1	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+)	2	Esi0003_0295	GCAATCGGTTCCTCGTGAGAAG	CGGGCGTTGTTTAGGCTTGG
CA2	gamma carbonic anhydrase (mitochondrial)	4	Esi0011_0163	TTCGCCTGATTCAGCATGGTTG	AGGAACGCAGAGAGGGAAAGG
CA3	carbonic anhydrase (chloroplast)	1	Esi0062_0116	CTCTCACTTCTTCATTCGCTTCTAAAC	AACTCCGTGCCAGGTGTAGG
CA4	carbonic anhydrase (no signalp.)	70	Esi0368_0022	AGTTGGTACACGGTGCTTTGC	CGCTACCTGGATTTGACCTTCTG
LHCA	chlorophyll binding protein	9	Esi0278_0013	GCGTACTCCTCCTCGGTGTAG	ATCGTGGTGACTATCCTCATCCTC
Ef1alpha	elongation factor 1 alpha	230	Esi0387_0021	GCAAGGGCCTCAGCTCTG	ACAAGCCGTCTGGGTATATGTTAGC
UBCE	ubiquitin conjugating enzyme	6	Esi0054_0059	AACAATGGCCTTTGCGAAAA	GCGTACGTCTTGAAGCCCAG

Statistical analysis

Statistical analysis was carried out using R 2.9.0 [www.r-project.org] together with R Commander (Fox 2005). For the diurnal cycle experiment, both metabolites and log2-transformed gene expression data were analysed by means of a one-way analysis of variance (ANOVA) with time as predictor. In the carbon starvation / enrichment

experiment, metabolite data were analysed using a two-factor ANOVA with time and treatment as predictors, while log2-transformed gene expression data was tested using a Student's t-test. In the carbon starvation / enrichment experiment, every treatment was compared separately to the control.

Results

Metabolic profiling during the diurnal cycle

GC-profiling and quantification of polar organic solutes in *Ectocarpus* was performed using GC-FID and the nature of compounds was confirmed using GC-MS. As expected, mannitol was found to be the major solute in *Ectocarpus* tissues, reaching between 200 and 350 µmol g^{-1} DW (3.6 – 6.4 % DW). Mannitol content was subject to strong variations in the course of the light-dark cycle, with a clear increase during light periods, and a subsequent decrease in the dark ($p < 0.001$, Figure 1). Citrate was the major organic acid found in *Ectocarpus* (Table 2), but exhibited only minor changes in concentration during the diurnal cycle ($p = 0.013$). Detailed diurnal profiles of all examined metabolites are presented in Supplementary file 1.

Table 2: Organic polar solute profiling in *Ectocarpus siliculosus* (6h time point) in comparison with 3 weeks old rosette leaves of *Arabidopsis thaliana*. All values are given in µmol g^{-1} DW and represent means of three replicates ± standard error. ND = not detected, ? = not examined.

	Ectocarpus	***Arabidopsis***
total amino acids	**88.1**	**82.4**
aspartate	14.5 ± 2.0	10.9 ± 0.9
asparagine	0.7 ± 0.1	6.1 ± 0.2
methionine	0.3 ± 0.1	0.2 ± 0.0
isoleucine	0.2 ± 0.1	0.2 ± 0.0
lysine	0.3 ± 0.1	0.3 ± 0.0
threonine	0.7 ± 0.1	4.5 ± 0.3
glutamate	22.9 ± 4.6	23.5 ± 1.1
glutamine	10.8 ± 2.1	16.0 ± 1.4
arginine	0.1 ± 0.0	0.2 ± 0.0
GABA	< 0.05	1.3 ± 0.4
proline	1.3 ± 0.2	3.2 ± 0.5
ornithine	0.1 ± 0.0	0.2 ± 0.1
α-alanine	29.9 ± 5.1	5.8 ± 0.3
valine	0.7 ± 0.4	0.9 ± 0.0
leucine	0.3 ± 0.1	0.3 ± 0.0
tyrosine	< 0.05	0.2 ± 0.0
phenylalanine	0.2 ± 0.1	0.3 ± 0.1
tryptophane	0.0 ± 0.0	0.1 ± 0.0
serine	3.1 ± 1.0	6.8 ± 0.6
glycine	1.7 ± 0.7	1.4 ± 0.2
glycine/serine	0.5 ± 0.0	0.2
organic acids	**18.4**	**178.2**
fumarate	ND	86.8 ± 31.9
malate	ND	34.7 ± 5.4
citrate	16.7 ± 2.1	56.7 ± 7.1
succinate	1.7 ± 0.2	?
sugars and polyols	**334.7**	**50.6**
Fructose	< 0.05	11.4 ± 0.7
glucose	3.3 ± 0.7	21.7 ± 0.7
myo -Inositol	< 0.05	5.5 ± 0.7
sucrose	< 0.05	12.0 ± 0.1
mannitol	331.4 ± 68.7	ND
total organic solutes	**441.1**	**311.3**

The total free amino acid content was found to be roughly stable, with a mean of 86.1 ± 10.8 µmol g^{-1} DW (Figure 1). Glutamate and alanine were the major free amino acids in *Ectocarpus* tissues, with global means of 28.1 ± 5.4 µmol g^{-1} DW and 24.5 ± 4.4 µmol g^{-1} DW, respectively. Concentrations of these compounds were subject to large fluctuations ($p < 0.001$) during the light-dark cycle (Figure 1): the

light phase was characterized by a decrease in glutamate and a slight increase in the alanine content. In the first part of the dark phase, we observed a strong decrease in alanine and a parallel increase in glutamate, followed by a slow decrease in glutamate and an increase in alanine in the second half of the dark phase. Aspartate and glutamine were the next most concentrated amino acids in *Ectocarpus*, with mean concentrations of 13.9 ± 2.2 and 10.7 ± 1.7 µmol g^{-1} DW, respectively. A slight oscillation of aspartate levels was detected ($p = 0.032$), reaching maximum concentrations towards the end of the light phase and minimal concentrations at the end of the dark phase. Changes in glutamine concentrations were even smaller but also statistically significant (Figure 1, $p = 0.005$).

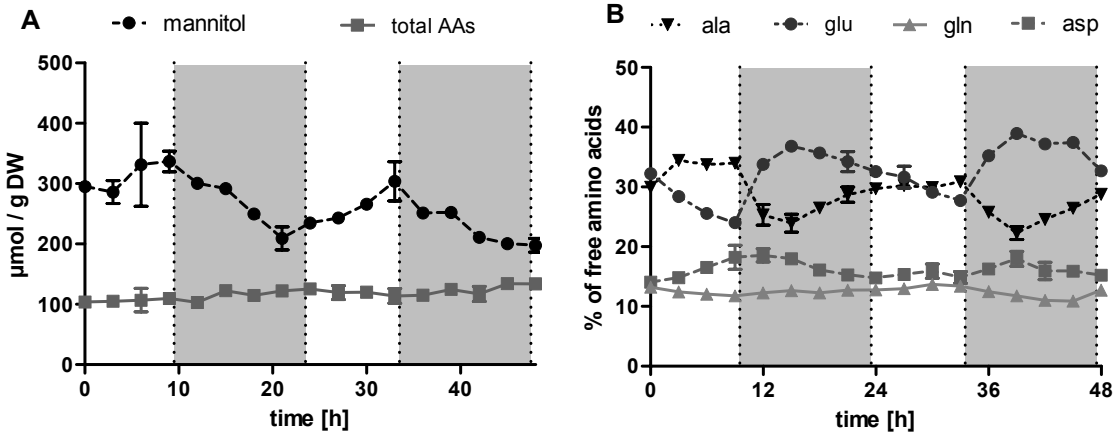

Figure 1: Changes of mannitol and total free amino acid concentrations (panel A) as well as the relative concentrations of the predominant amino acids (asp = aspartate, glu = glutamate, gln = glutamine, and ala = α-alanine; panel B) in the course of two light-dark cycles. Grey areas indicate the dark phase. The graph shows means of three replicates ± standard errors.

The content of glycine and serine was highly variable compared to other amino acids. Both amino acids generally accumulated during the light phase and declined during the dark phase (Figure 2, $p = 0.017$ for glycine and $p < 0.001$ for serine). This resulted in an overall increase in the ratio (mol/mol) of glycine to serine during the light phase (Figure 2), although this increase was not statistically significant ($p = 0.077$).

Finally, we observed significant oscillations ($p < 0.050$) in the content of asparagine, arginine, threonine, proline, lysine, methionine, valine, leucine, isoleucine, and phenylalanine (supplementary file 1). These oscillations were minor compared to those of alanine, glutamate and mannitol.

Many compounds classically found in vascular plants, were not detectable in *Ectocarpus* tissues, even after concentrating the samples, as for example sucrose, fructose, malate, fumarate, trehalose and oligosaccharides of the raffinose family

(Table 2). We also did not detect any altrose, a polyol previously described as typical of Fucales (Raven et al. 2001), and a sample without ribitol as internal standard was analyzed to check the absence of endogenous ribitol. Furthermore, β-alanine and GABA were present only in traces (<0.01 µmol g^{-1} DW, Table 2), but 34 peaks potentially corresponding to yet unidentified biogenic amines were detected through the AccQ-Tag derivation in *Ectocarpus* samples (data not shown).

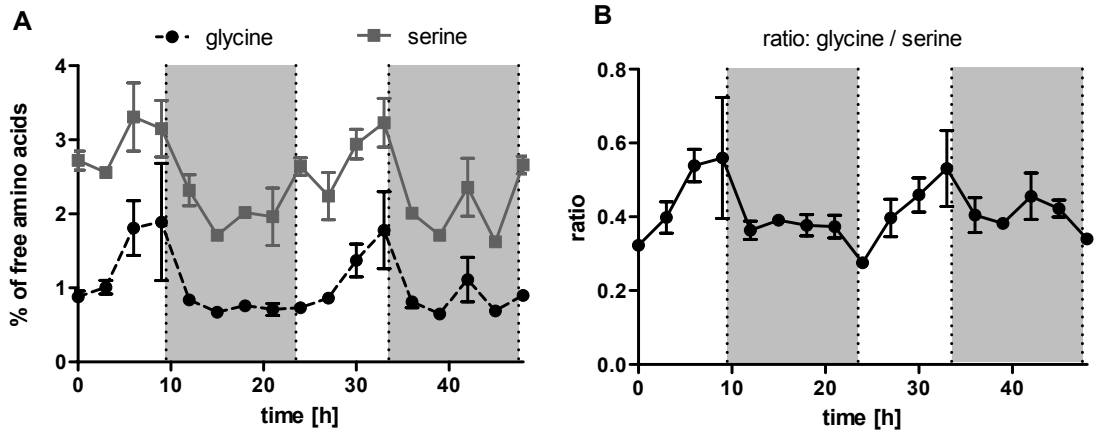

Figure 2: Changes in the concentrations of glycine, serine (panel A) as well as in the ratio (mol/mol) of glycine to serine (panel B) in the course of two light-dark cycles. Grey areas indicate the dark phase. The graph shows means of three replicates ± standard errors.

Metabolic profiling under altered growth conditions

In order to complete our observations on the changes of primary metabolites throughout the light-dark cycle, we conducted an experiment during which the availability of CO_2 and O_2 was modified in order to potentially affect photorespiration.

No change in the quantity of total free amino acids was observed in this experiment, but O_2 enrichment, a treatment that was expected to enhance photorespiration, triggered significant accumulation of glycine ($p = 0.022$), serine ($p = 0.023$) and an increase of the glycine/serine ratio ($p = 0.027$, Figure 3). In contrast, CO_2 enrichment, which was expected to lower photorespiration, clearly led to a decrease of all three biochemical markers of this process (serine: $p < 0.001$; glycine: $p = 0.004$; glycine/serine: $p = 0.006$). CO_2 starvation also led to a slight increase in all three markers, although this increase was not statistically significant ($p > 0.130$). Other significant changes in this experiment comprised a decrease in alanine in response to O_2-enrichment (16 %, $p = 0.008$), a decrease in glutamate (19 %, $p = 0.047$) and

aspartate (45 %, p = 0.003) in response to CO_2 starvation, a decrease in methionine under CO_2-enriched conditions (46 %, p = 0.001), and a parallel increase in tyrosine (45 %, p = 0.008) and leucine (73 %, p = 0.002). Finally, we observed a strong decrease in the NH_4^+ concentration in the CO_2 enriched condition (p < 0.001, Figure 4). A complete table of metabolite concentrations measured in this experiment is available in supplementary file 2.

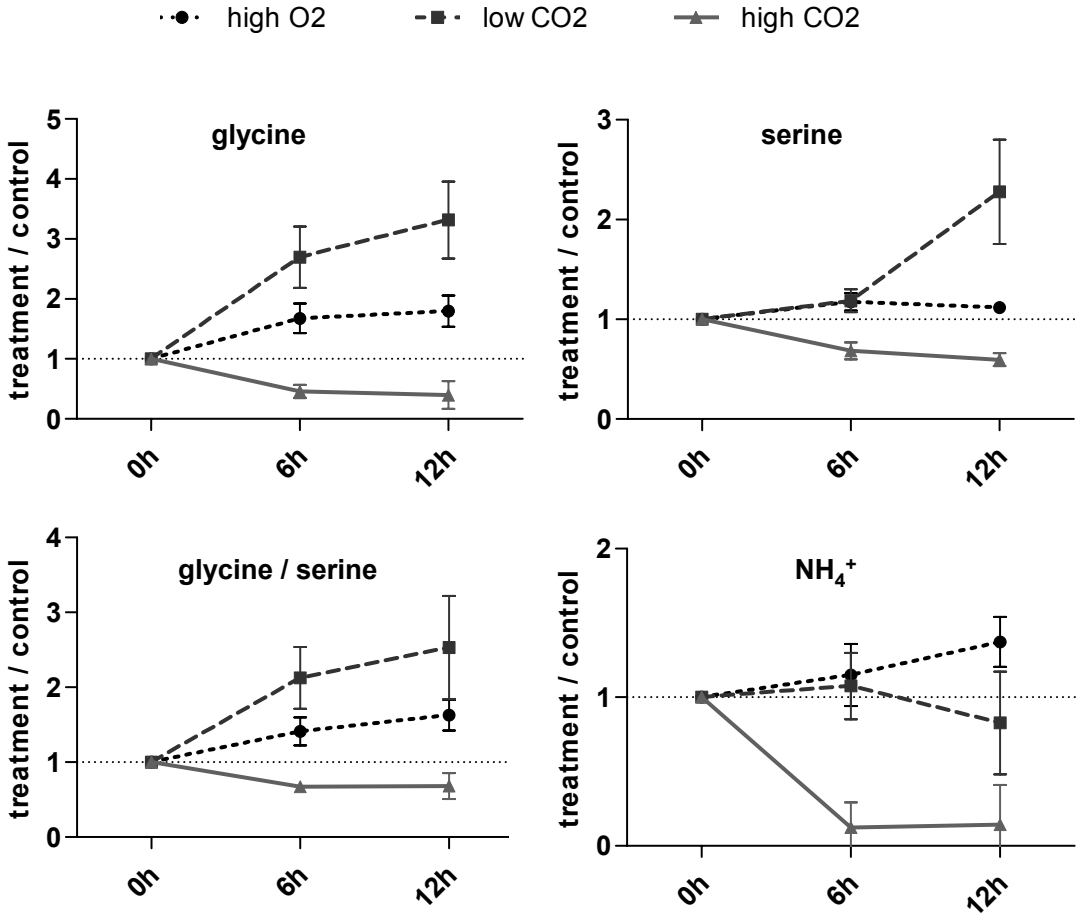

Figure 3: Changes in relative metabolite concentrations in response to different growth conditions. All values are displayed as ratios (mol/mol) of treatment (high O_2, low CO_2, and high CO_2) and control (mean of three replicates ± standard errors).

Genome annotation and changes in expression of key genes involved in basic metabolic processes

Changes in expression of selected genes corresponding to enzymes involved in primary metabolism (Table 1) were monitored throughout the light-dark cycle and after modification of the growth conditions (Figure 4).

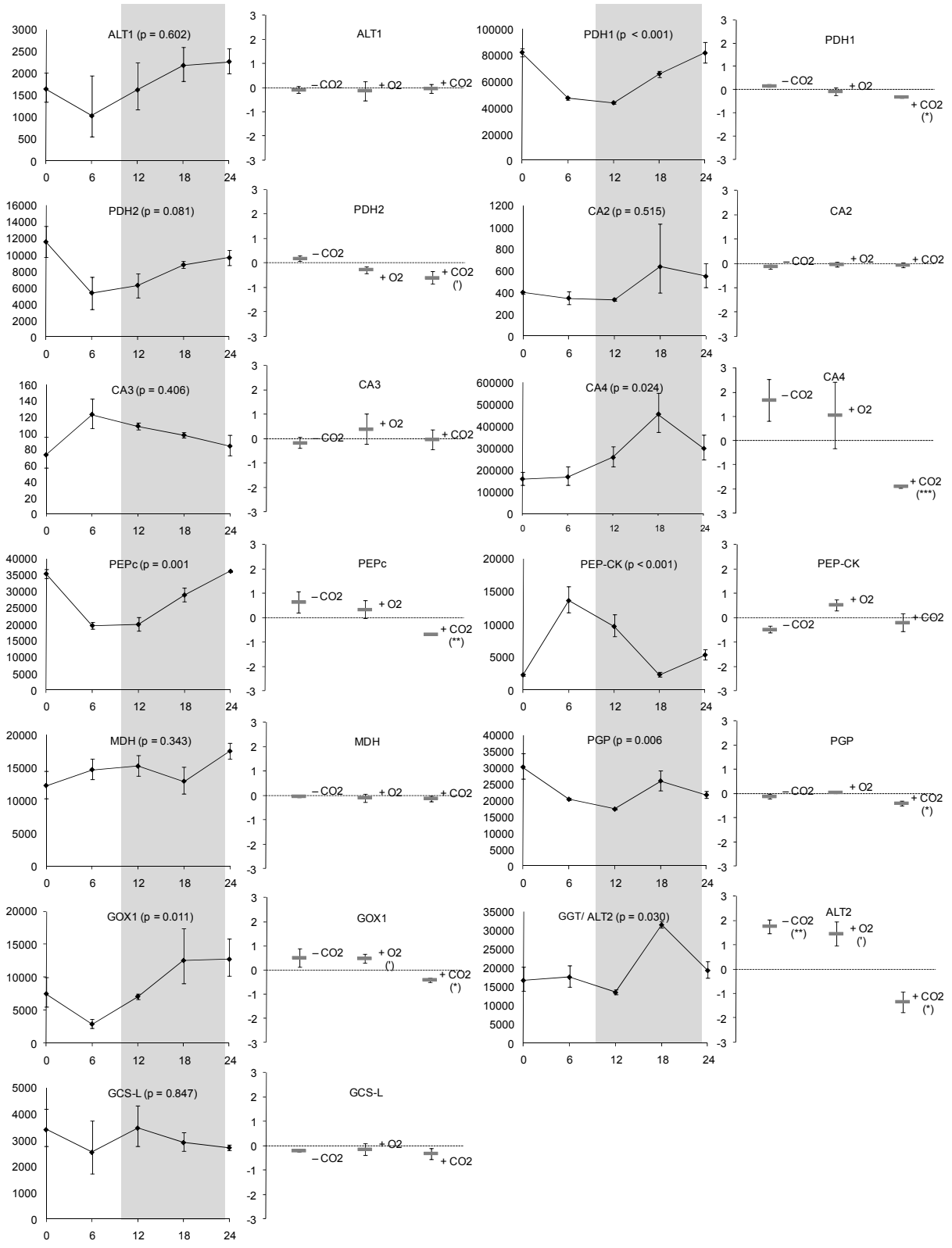

Figure 4: Gene expression profiles during the light-dark cycle and in different growth conditions (means of three replicates ± standard error). Profiles of each gene during the diurnal cycle show the copy number, while changes in expression (log2-ratios with the control condition) in response to the different growth conditions are shown in the adjacent graph to the right. In the latter graph, positive values indicate that a gene was up-regulated, negative values that it was down-regulated; p-values are indicated as follows: ' $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The shaded area indicates the dark phase (from 9.5 to 23.5 h). See Table 1 for a definition of the examined genes.

The non-peroxisomal alanine transaminase ALT1 is likely to catalyze the transfer of an amino group from glutamate to pyruvate, thereby producing alanine and α-ketoglutarate, and was therefore possibly involved in the strong antagonism between alanine and glutamate. ALT1 was found to be constitutively expressed. We then monitored the changes in expression of two genes coding for pyruvate dehydrogenases, enzymes using pyruvate as a substrate to furnish acetyl-CoA for the tricarboxylic acid (TCA) cycles. The TCA cycle represents a metabolic crossroad between carbon and nitrogen metabolism, furnishing carbon skeletons (pyruvate, oxaloacetate and α-ketoglutarate) as precursors for alanine, aspartate and glutamate biosynthesis. Only PDH1 was significantly regulated on a transcriptional level. Its expression decreased during the light phase and increased during the dark phase – a feature that could suggest an increase in TCA activity during the dark phase. PDH2 followed the same trends, but the changes in expression were not statistically significant ($p=0.080$) and the overall expression level was ten-fold lower than that of PDH1. Both PDH1 and PDH2 were also down-regulated (1.2 and 1.5-fold, respectively) in CO_2 enriched conditions, although, again, changes for PDH2 were not statistically significant (PDH1: $p = 0.013$; PDH2: $p = 0.093$).

Carbonic anhydrases (CAs) are involved in inorganic carbon concentration. The *Ectocarpus* genome contains five CAs with EST support (Table 3), three of which (CA2, CA3, and CA4) were tested in this study. CA4 was clearly the most strongly expressed carbonic anhydrase (70 associated ESTs, Table 1) and was also the only one to be differentially expressed in our experiments. This gene was up-regulated during the dark phase ($p = 0.024$) and strongly down-regulated in CO_2 enriched conditions (3.75-fold, $p < 0.001$). Corresponding to the presence of carbonic anhydrases, we found two genes coding for putative bicarbonate transporters, one sodium bicarbonate cotransporter predicted to be located in the chloroplast, and one family 2 anion exchanger with no detected signal peptide (Table 3).

Several genes with a putative role in a C4-like organic CCM are shown in Table 3. In this study, the C4-related genes examined by qPCR were mainly regulated during the diurnal cycle experiment and not in response to different carbon concentrations, although the phosphoenolpyruvate carboxylase (PEP-C) was also slightly down-regulated under CO_2 enriched conditions (1.60-fold, $p = 0.003$). PEP-Cs are involved in carbon fixation in C4 and CAM plants by producing oxaloacetate from

phosphoenolpyruvate and CO_2. The *Ectocarpus* PEP-C was down-regulated during the light period and expression levels increased in the course of the dark phase. In C4 plants, stored CO_2 is released either by the activity of malic enzymes (e.g. MDH) or phosphoenolpyruvate carboxykinases (PEP-CK). In *Ectocarpus*, data on the CO_2 affinity of PEP-CK suggest that this enzyme functions rather in the carboxylation sense (Akagawa et al. 1972a). In our experiments, PEP-CK showed the exactly opposite profile as PEP-C: it was strongly up-regulated during the light phase and expression levels decreased during the dark phase. This is in agreement with data on the enzymatic activity of PEP-CK observed in *E. siliculosus* during the circadian cycle under permanent red light (Busch & Schmid 2001). No significant regulation of the tested cytosolic NADP-malic enzyme (MDH1) was observed in any of the conditions tested, but high basal transcription levels were observed for this gene.

Table 3: Carbonic anhydrases, bicarbonate transporters, and genes possibly involved in a C4-like carbon concentrating mechanism as well as their predicted subcellular localization in *Ectocarpus*. "*" marks genes that were tested by qPCR (Table 1).

Protein	genome	ESTs	predicted localisation
carbonic anhydrase (EC 4.2.1.1)	Esi0000_0144	7	signal peptide
	Esi0011_0163*	4	mitochondiron
	Esi0062_0116*	1	chloroplast
	Esi0368_0022*	70	no target peptide
	Esi0125_0083	1	no target peptide
putative sodium bicarbonate cotransporter	Esi0172_0020	1	chloroplast
putative chloride bicarbonate anion exchanger	Esi0423_0017	2	no target peptide
phosphoenolpyruvate carboxylase (EC 4.1.1.31)	Esi0009_0060*	14	mitochondrion
phosphoenolpyruvate carboxykinase (EC 4.1.1.32)	Esi0035_0144*	2	mitochondrion
pyruvate carboxylase (EC 6.4.1.1)	Esi0011_0185	2	mitochondrion
malate dehydrogenase (EC 1.1.1.37)	Esi0209_0031	0	signal peptide
	Esi0344_0024	0	no target peptide
	Esi0006_0195	9	mitochondiron
NAD-malic enzyme (EC 1.1.1.39)	Esi0061_0055	0	no target peptide
	Esi0061_0051	0	mitochondiron
NADP-malic enzyme (EC 1.1.1.40)	Esi0003_0295*	2	no target peptide

We also investigated the expression profiles of four genes directly involved in the photorespiratory pathway: phosphoglycolate phosphatase (PGP), glycolate oxidase (GOX1), peroxysomal glutamate/alanine:glyoxylate amino-transferase (ALT2/GGT), and glycine cleavage system subunit L (GCS-L). Surprisingly, three of them (PGP, GOX1 and ALT2/GGT) were expressed at a slightly but significantly higher level during the dark phase. ALT2/GGT was up-regulated during CO_2 starvation (3.37-fold,

p = 0.009) and in the O_2-enriched (2.73-fold, p = 0.055) condition, and down-regulated in the CO_2 enriched condition (2.54-fold, p = 0.035), corroborating the potential role of this predicted peroxysomal amino-transferase in the photorespiratory process. We also observed an approximately 1.3-fold down-regulation of GOX1 and PGP in CO_2 enriched conditions (p = 0.021 and 0.044 respectively), while expression levels of GCS-L did not change significantly in either of the experiments (p > 0.6). A second glyoxylate oxidase present in the genome (GOX2) was not examined in this study, due to problems in designing specific primers. However, this gene model is supported by only 1 EST vs. 16 for GOX1. Manual annotations of genes related to photorespiration are listed in Table 4.

Table 4: Genes possibly involved in the photorespiratory pathway and their predicted subcellular localization in *E. siliculosus* compared to *A. thaliana*. "*" marks enzymes with at least one gene tested by qPCR (Table 1). A serine-glyoxylate aminotransferase, a glycerate dehydrogenase, and a hydroxypyruvate reductase could not be clearly identified in either *E. siliculosus* or diatom (not shown) genomes.

	Arabidopsis		*Ectocarpus*	
Name	**genes**	**localiszation**	**genes**	**localiszation**
phosphoglycolate phosphatase (EC 3.1.3.18)*	At5g36700	chloroplast	Esi0130_0079	chloroplast
glycolate oxidase (EC 1.1.3.15)*	At5g06580	peroxysome	Esi0012_0104, Esi0017_0040	peroxysome
alanine aminotransferase = glutamate-glyoxylate aminotransferase (EC 2.6.1.4)*	At1g23310, At1g70580	peroxysome	Esi0008_0209	peroxysome
glycine decarboxylase complex (GCS)*	At1g32470, At2g35120, At2g35370, At2g26080, At4g33010	mitochondrion	Esi0046_0032, Esi0046_0033, Esi0221_0023, Esi0322_0005	mitochondrion
serine hydroxymethyltransferase (EC 2.1.2.1)	At4g37930	mitochondrion	Esi0148_0037, Esi0340_0018	no signal peptide / mitochondium
serine-glyoxylate aminotransferase (EC 2.6.1.45)	At2g13360	peroxysome	-	
glycerate dehydrogenase (EC 1.1.1.29)	At1g68010	peroxysome	-	
hydroxypyruvate reductase (EC 1.1.1.81)	At1g79870	cytosolic	-	
putative glycerate kinase (2.7.1.31)	At1g80380	chloroplast	Esi0157_0054	no signal peptide
catalase (1.11.1.6)	At1g07890, At1g20630, At4g35090	peroxysome	Esi0045_0115	possibly peroxysomal

Finally, we searched for genes classically involved in GABA synthesis via the GABA shunt, and found that *Ectocarpus*, as well as *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, lack genes coding for a glutamate decarboxylase and a GABA-transaminase, key enzymes of GABA biosynthesis and GABA catabolism respectively (Table 5). The traces of GABA detected in our samples could have been synthesized from putrescine by the activity of a diamine oxidase (two copies in the *Ectocarpus*: Esi0076_0063 and Esi0076_0061) and an aminobutyraldehyde dehydrogenase (candidate genes with this function could be Esi0010_0069, Esi0451_0004, and Esi0003_0206).

Table 5: Enzymes of the GABA shunt in *E. siliculosus*, *Phaeodactylum tricornutum*, *Thalassiosira peudonana, Phytophthora sojae,* and *A. thaliana*. Neither glutamate decarboxylase nor GABA transaminase were found in *E. siliculosus* or diatom genomes.

Enzyme	*Arabidopsis*	*Ectocarpus*	diatoms	*Phytophtora*
glutamate decarboxylase (GAD, EC 4.1.1.15)	AT5G17330, AT2G02010, AT1G65960, AT2G02000, AT3G17760	-	-	jgi|sojae1|109436, jgi|sojae1|108138, jgi|sojae1|109780
gaba transaminase (GABA-T, EC 2.6.1.19)	AT3G22200	-	-	jgi|sojae1|157659
succinic semialdehyde dehydrogenase (EC 1.2.1.24)	AT1G79440	Esi0003_0206	jgi|Thaps3|268262, jgi|Phatr2|28191	jgi|sojae1|108179, jgi|sojae1|109030

Discussion

This exploratory study presents the most extensive profiling of primary metabolites carried out in brown algae so far, and revealed the prevalence of only a small number of organic acids, polyols, and soluble non structural carbohydrates in *E. siliculosus* tissues. The predominant soluble carbohydrates were mannitol and citrate, representing 90% and 6% of the total polyols/organic acids measured respectively. These results are in accordance with early biochemical reports on brown algae (Jones 1956, Yamaguchi et al. 1966). As previously shown for the brown algae *Macrocystis integrifolia* and *Nereocystis luetkeana* (Rosell & Srivastava 1985), the predominant amino acids in our samples were alanine, glutamate, aspartate, and glutamine, together accounting for about 90 % of the total amino acids.

Absence of the GABA shunt in sequenced photosynthetic heterokonts

Compared to previous studies in brown algae, we used more sensitive methods, facilitating the separation and detection of several compounds present only at very low concentrations in our samples. For example, we detected traces of β-alanine and GABA, both of which had not been detected in a previous study of the brown alga *Himanthalia elongata* (Jones 1956). In particular, the quasi-absence of GABA is an unusual feature, as this molecule is generally considered ubiquitous (reviewed in Clark et al. 2009), even if, in terrestrial plants, GABA contents are highly regulated by environmental conditions such as heat. In animals, GABA is considered an important neurotransmitter (Li & Xu 2008). The GABA shunt is believed to be the main pathway of GABA synthesis in both plants and animals (Petroff 2002). In plants, this pathway is also thought to furnish intermediates for the TCA cycle (Allan & Shelp 2006). The quasi-absence of GABA in *Ectocarpus* in our culture conditions is likely to be related to the absence of a gene encoding a glutamate decarboxylase in the

Ectocarpus genome (Table 5). It is interesting that the absence of this gene is common to *Ectocarpus* and both sequenced diatoms, but not to the non-photosynthetic heterokont *Phytophthora sojae.* This suggests that the GABA shunt was either already present in the last common ancestor of chromists, ophistokonts, and plants, and then lost in *Ectocarpus* and the two known diatoms or one of their common ancestors, or that it was acquired independently by *Phytophthora*.

Indications of inorganic but not organic carbon concentrating mechanisms in Ectocarpus

The presence of an inorganic CCM was suggested for several algae (Reiskind et al. 1989, Badger & Price 1994) including brown algae (Axelsson et al. 2000, Klenell et al. 2004, Mercado et al. 2005), although species-specific differences in the efficiency of such a mechanism can be expected. In *Ectocarpus* the existence of such a mechanism, is supported by the presence of two putative bicarbonate transporters and several carbonic anhydrases in the *Ectocarpus* genome (Table 3), as well as the strong regulation of the carbonic anhydrase CA4 in response to changes in carbon availability (Figure 4).

However, our data does not provide support for the presence of an organic CCM. Many reports have been published on the possibility of organic CCMs in brown algae (e.g. Schmid & Hillrichs 2001). Unlike many terrestrial C4 plants, which rely on two different cell types (mesophyll cells for fixating carbon and bundle sheath cells for photosynthesis), *Ectocarpus*, due to its simple morphology, would need to rely on a CAM-like mechanism (Ranson & Thomas 1960) or on intracellular compartmentation in order to effectively concentrate CO_2. The latter was described in the unicellular marine diatom *Thalassiosira weissflogii* (Reinfelder et al. 2000). Although the decarboxylating enzymes employed by terrestrial plants, i.e. malate dehydrogenases or PEP-CKs (Hatch 1987), are present in the *Ectocarpus* genome, unlike in *T. weissflogii*, none of the enzymes are predicted to be targeted to the chloroplast in *Ectocarpus*. Moreover, previous reports in other brown algae (Akagawa et al. 1972a, Kremer & Küppers 1977) have suggested that brown algal PEP-CKs may function as a carboxylating rather than a decarboxylating enzyme. Overall, the situation in *E. siliculosus* much resembles that in *Phaeodactylum tricornutum* (Kroth et al. 2008), as decarboxylation of C4-compounds would need to take place in the mitochondrion. Although in *Ectocarpus*, as in *P. tricornutum*, mitochondria are preferentially located

in close proximity of the chloroplast (A. Le Bail, personal communication), transport of CO_2 between the two organelles would involve crossing six membranes and would probably be inefficient.

Secondly, classical C4 or CAM mechanisms, present for example in maize or pineapple, employ malate as a C4 compound to store and efficiently deliver CO_2 to the RuBisCo. In our samples, the absence of detectable malate at any time point is inconsistent with such a malate-based CCM. As suggested by Schmid & Dring (1996b), an alternative C4 compound could be aspartate. Aspartate can be decarboxylated by three reactions (*via* oxaloacetate and phosphoenolpyruvate) producing alanine as a final product and providing one CO_2 molecule for photosynthesis. Indeed, we observed cyclic changes in the concentrations of aspartate and alanine in our diurnal experiments (Figure 1). However, aspartate concentrations clearly increased during the light phase, which does not agree with the increased demand for CO_2, when photosynthesis is active. In addition, no other amino acid, organic acid, polyol, or sugar was found to behave as expected for a carbon storage compound. These findings make the presence of an active CAM-like organic CCM in *Ectocarpus* unlikely.

Altogether, our observations therefore point to the conclusion that *Ectocarpus* does not posses a classical C4 / CAM-like organic CCM, although we cannot eliminate the possibility of a yet unknown organic CCM.

Photorespiration

Photorespiration is a process that occurs in plants, mainly in response to high light, high temperature, or CO_2 deficiency. Under these stress conditions, an increased proportion of ribulose-1,5-bisphosphate (RuBP) may be oxygenized rather than carboxylated. This marks the starting point for the photorespiratory glycolate pathway (C2-cycle), which finally leads to the regeneration of RuBP (Foyer et al. 2009). The presence of a highly efficient CCM would be expected to minimize photorespiration, even under the aforementioned conditions.

In our experiments, the fluctuations of glycine, serine and the ratio of glycine to serine during the diurnal cycle could be explained by the activation of a photorespiration pathway in *E. siliculosus.* In terrestrial plants, the accumulation of glycine and serine, and an increase in their ratio are considered reliable markers of photorespiration (Foyer et al. 2003, Igarashi et al. 2006). The hypothesis that this is also valid for

Ectocarpus is supported by the observations made under different growth conditions: all three of these markers increased not only during the light phase but also in response to carbon starvation and increased O_2 concentrations, and decreased under carbon-enriched conditions. Moreover, changes in expression of the peroxysomal alanine aminotransferase (ALT2/GGT) were highly correlated with the expected level of photorespiration in CO_2 / O_2 altered growth conditions. In contrast, alanine, aspartate, and glutamate contents, as well as the expression of genes potentially involved in a C4 cycle, were not clearly altered in CO_2 / O_2 enriched or deficient growth conditions. Moreover, photorespiration might also explain the rather unusual changes in ammonium concentrations in our samples. In CO_2 enriched conditions, NH_4^+ levels were very low, suggesting that little NH_4^+ was produced from photorespiration and/or that NH_4^+ assimilation is higher when carbon is not limiting. Together, these findings provide a strong indication for the induction of the photorespiratory pathway in *Ectocarpus*. However, while a putative glycerate kinase, an essential gene for a classical photorespiratory pathway, which seems to be absent in *P. tricornutum* (Kroth et al. 2008), was found in *Ectocarpus*, we did not identify genes coding for serine-glyoxylate aminotransferases, glycerate dehydrogenases, or hydroxypyruvate reductases. One possible explanation could be that these genes were not indentified merely due to the lack of well-characterized heterokont sequences. Indeed, homologous genes (identified e.g. as phosphoglycerate dehydrogenases) can be found in the *Ectocarpus* genome. Moreover, hydroxypyruvate reductase activity has been demonstrated experimentally in the brown alga *Egregia menziesii* (Gross 1990), and all enzymatic activities necessary to complete the glycolate cycle were previously detected in the brown alga *Spatoglossum pacificum* (Iwamoto & Ikawa 1997). This underlines the importance of more targeted enzymatic studies to identify the missing links in the photorespiratory pathway of *Ectocarpus*.

Pyruvate storage as alanine

One of the most striking results of this study was the observed antagonism between alanine and glutamate (Figure 1). Alanine can be synthesized by the reversible transfer of an amino group from glutamate to pyruvate also producing one molecule of α-ketoglutarate. The latter can then be recycled for further glutamate / glutamine synthesis via the GS/GOGAT system (Figure 5). Although the mitochondrial alanine transaminase thought to catalyze the synthesis of alanine (*ALT1*) was not

transcriptionally regulated in our experiments, a direct conversion of glutamate to alanine would provide the most plausible explanation for the observed inverse correlation between the concentrations of these two compounds. One hypothesis could be, that under laboratory conditions, when nitrate is not limiting, this accumulation constitutes a way of temporarily storing pyruvate produced from photosynthesis, which might be complementary to the role of mannitol as storage compound.

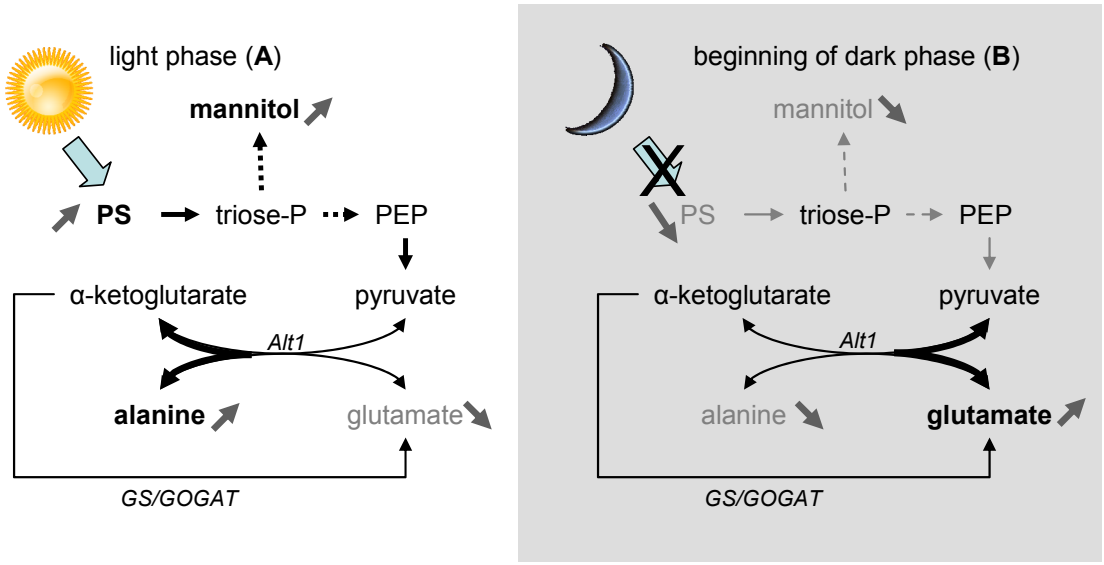

Figure 5: Schematic representation of the proposed mechanism underlying the changes in alanine and glutamate concentrations. During the light phase (A), pyruvate produced from photosynthesis can be stored as alanine and released in the beginning of the dark phase (B). Arrows pointing up indicate an increase or activation, arrows pointing down a decrease. PS = photosynthesis, Alt = alanine aminotransferase, GS/GOGAT = glutamine synthetase / glutamine:2-oxoglutarate aminotransferase.

In the beginning of the dark phase, the supply of pyruvate from photosynthesis will cease. Since the alanine transaminase works in both directions, this would shift the alanine / glutamate balance towards glutamate and therefore liberate pyruvate, explaining both the rapid decrease in alanine concentrations at the transition from the light to the dark phase and the parallel increase in glutamate. Finally, after a latency period of a few hours, the rapid changes observed in the beginning of the dark phase came to a halt. This could be due to an activation of another pyruvate source, e.g. glycolysis. Such fluxes between pyruvate and alanine were previously reported in terrestrial plants, for instance in maize phloem (Valle & Heldt, 1991), and would moreover be consistent with the work reported by Akagawa et al. (1972b), who illustrated that alanine was one of the first amino acids to be synthesized from radiolabeled carbon in several brown algae. Although this hypothesis provides a

possible explanation for the observed relationship between alanine and glutamate, further experimental validation, e.g. using pulse-chase experiments, will be required to test this hypothesis in *Ectocarpus*.

Conclusion

This study represents the most extensive metabolite analysis of a brown algal species so far, and highlights several new features related to primary metabolism in the model brown alga *E. siliculosus*. First, *Ectocarpus* tissues contained dramatically low levels of GABA, a quality most likely related to the apparent absence of a glutamate decarboxylase. This important enzyme involved in GABA synthesis might have been lost after the separation of marine and terrestrial heterokonts. Secondly, several observations such as the absence of malate, the increase of aspartate during the light phase, and the predicted subcellular localizations of several enzymes, are not compatible with the presence of a classical C4- or CAM-like organic CCM in *Ectocarpus*, although the high degree of regulation of the carbonic anhydrase CA4 in response to changes in CO_2 highlights the possible activity of an inorganic CCM. Thirdly, changes in the concentrations of glycine and serine, as well as expression profiles of ALT2/GGT, provided a strong indication for the increase of photorespiration depending on the availability of O_2 and CO_2. Finally, we observed an inverse relationship between glutamate and alanine concentrations, which could, for instance, be explained by a role of the glutamate-alanine system in the storage of photosynthetic pyruvate.

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Supplementary files

Supplementary file 1: Complete profiles during the light-dark cycle. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

Supplementary file 2: Complete profiles under different growth conditions. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis

Chapter 3

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Short-term response to abiotic stress

3. Short-term response to abiotic stress

3.1 Preface

After having discussed metabolite and transcriptomic changes during the diurnal cycle, this section deals with changes observed during the short-term response to abiotic stress. My approach was focused exclusively on three stress conditions (6h of hyposaline, hypersaline, and oxidative stress), for which I tried to obtain as much information as possible. This chapter contains three sub-sections, each represented by a separate manuscript or draft.

The first section presents a global transcriptomic analysis of the response to the selected stress conditions. This article describes the choice of the three sub-lethal stress conditions based on physiological parameters, the establishment of protocols and resources for performing and analyzing microarray experiments with *Ectocarpus siliculosus* (details on our *Ectocarpus transcriptomics* web site: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/), and the profound transcriptomic changes observed in *E. siliculosus* in response to the chosen stress conditions.

In the second section, these transcriptomic changes were correlated to metabolite changes of amino acids, sugars/polyols (measured by Antoine Gravot, University of Rennes 1), and fatty acids (measured by Sophie Goulitquer, University of Brest). This part of my thesis, which is presented as a manscript to be submitted for publication, shows that complex interactions, which would be hard to foresee based on the transcriptomic changes only, were at the root of many of the observed changes in metabolite concentration. This section also further addresses the topic of compatible osmolytes which is raised in the first section.

Finally, the third section of this chapter provides an example of more targeted studies possible, based on the results of the microarray experiments. It describes the phylogenetic analysis of a group of fucoxanthin chlorophyll a/c binding proteins (FCPs) shown to be induced under abiotic stress conditions. The data presented indicate that stress responsive FCPs are likely to form a subfamily of FCPs, which is conserved in several macro- and microalgal species. Part of the work in this last section was carried out by Gurvan Michel, and more extensive analyses (notably the inclusion of red-algal genes) are still in progress.

3.2

Transcriptomic response to short-term saline and oxidative stress

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Global expression analysis of the brown alga *Ectocarpus siliculosus* (Phaeophyceae) reveals large-scale reprogramming of the transcriptome in response to abiotic stress

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Abstract

Background: Brown algae (Phaeophyceae) are phylogenetically distant from red and green algae and an important component of the coastal ecosystem. They have developed unique mechanisms that allow them to inhabit the intertidal zone, an environment with high levels of abiotic stress. *Ectocarpus siliculosus* is being established as a genetic and genomic model for the brown algal lineage, but little is known about its response to abiotic stress.

Results: Here we examine the transcriptomic changes that occur during the short-term acclimation of *E. siliculosus* to three different abiotic stress conditions (hyposaline, hypersaline and oxidative stress). Our results show that almost 70% of the expressed genes are regulated in response to at least one of these stressors. Although there are several common elements with terrestrial plants, such as repression of growth-related genes, switching from primary production to protein and nutrient recycling processes, and induction of genes involved in vesicular trafficking, many of the stress-regulated genes are either not known to respond to stress in other organisms or are have been found exclusively in *E. siliculosus*.

Conclusions: This first large-scale transcriptomic study of a brown alga demonstrates that, unlike terrestrial plants, *E. siliculosus* undergoes extensive reprogramming of its transcriptome during the acclimation to mild abiotic stress. We identify several new genes and pathways with a putative function in the stress response and thus pave the way for more detailed investigations of the mechanisms underlying the stress tolerance ofbrown algae.

Background

The brown algae (Phaeophyceae) are photosynthetic organisms, derived from a secondary endosymbiosis [1], that have evolved complex multicellularity independently of other major groups such as animals, green plants, fungi, and red algae. They belong to the heterokont lineage, together with diatoms and oomycetes, and are hence very distant phylogenetically, not only from land plants, animals, and fungi, but also from red and green algae [2]. Many brown algae inhabit the intertidal zone, an environment of rapidly changing physical conditions due to the turning tides. Others form kelp forests in cold and temperate waters as well as in deep-waters of tropical regions [3,4]. Brown algae, in terms of biomass, are the primary organisms in such ecosystems and, as such, represent important habitats for a wide variety of other organisms. As sessile organisms, brown algae require high levels of tolerance to various abiotic stressors such as osmotic pressure, temperature, and light. They differ from most terrestrial plants in many aspects of their biology, such as their ability to accumulate iodine [5], the fact that they are capable of synthesizing both C18 and C20 oxylipins [6], their use of laminarin as a storage polysaccharide [7], the original composition of their cell walls, and the associated cell wall synthesis pathways [8-10]. Many aspects of brown algal biology, however, remain poorly explored, presenting a high potential for new discoveries.

In order to fill this knowledge gap, *Ectocarpus siliculosus*, a small, cosmopolitan, filamentous brown alga (see [11] for a recent review) has been chosen as a model [12], mainly because it can complete its life cycle rapidly under laboratory conditions, is sexual and highly fertile, and possesses a relatively small genome (200 Mbp). Several genomic resources have been developed for this organism, such as the complete sequence of its genome and a large collection of expressed sequence tags (ESTs). Although *Ectocarpus* is used as a model for developmental studies [13,14], no molecular studies have been undertaken so far to study how this alga deals with the high levels of abiotic stress that are a part of its natural environment. This is also true for intertidal seaweeds in general, where very few studies have addressed this question.

In the 1960s and 1970s several studies (reviewed in [15]) examined the effects of abiotic stressors such as light, temperature, pH, osmolarity and mechanical stress on algal growth and photosynthesis. However, only a few of the mechanisms underlying the response to these stressors - for example, the role of mannitol as an osmolyte in brown algae [16,17] - have been investigated so far. Developing and applying molecular and biochemical tools will help us to further our knowledge about these mechanisms - an approach that was suggested 12 years ago by Davison and Pearson [18]. Nevertheless, it was only recently that the first transcriptomic approaches were undertaken to investigate stress tolerance in intertidal seaweeds. Using a cDNA microarray representing 1,295 genes, Collén *et al.* [19,20] obtained data demonstrating the up-regulation of stress-response genes in the red alga *Chondrus crispus* after treatment with methyl jasmonate [19] and suggesting that hypersaline and hyposaline stress are similar to important stressors in natural environments [20]. Furthermore, in the brown alga *Laminaria digitata*, Roeder *et al.* [21] performed a comparison of two EST libraries (sporophyte and protoplasts) and identified several genes that are potentially involved in the stress response, including the brown alga-specific vanadium-dependent bromoperoxidases and mannuronan-C5-epimerases, which are thought to play a role in cell wall modification and assembly. These studies have provided valuable information about the mechanisms and pathways involved in algal stress responses, but they were nevertheless limited by the availability of sequence information for the studied organisms at the time.

With the tools and sequences available for the emerging brown algal model *E. siliculosus*, we are now in a position to study the stress response of this alga on the level of the whole transcriptome. For this, we have developed an EST-based microarray along with several tools and annotations (available on our *Ectocarpus* transcriptomics homepage [22]), and used this array to study the transcriptomic response of *E. siliculosus* to three forms of abiotic stress: hyposaline, hypersaline, and oxidative stress. Hypersaline stress is a stress experienced by intertidal seaweeds - for example, in rockpools at low tide (due to evaporation) or due to anthropogenic influences - and is comparable to desiccation stress. Hyposaline stress is also common in the intertidal zone, and can arise, for example, due to rain. Furthermore, organisms with a high tolerance to saline stress can inhabit a wide range of habitats. *E. siliculosus* strains have been isolated from locations covering a wide range of salinity. A specimen was found in a highly salt-polluted area of the Werra river in Germany, where chloride concentrations at times reached 52.5 grams per liter [23]. At the same time, *E. siliculosus* can be found in estuaries, in the Baltic sea, and one strain of *E. siliculosus* was isolated from freshwater [24]. Oxidative stress is commonly experienced by living organisms. Reactive oxygen species (ROSs) are produced intracellularly in response to various stressors due to malfunctioning of cellular components, and have been implicated in many different signaling cascades in plants [25]. In algae, several studies have demonstrated the production of ROSs in response to biotic stress (reviewed in [26]). Therefore, protection against these molecules is at the basis of every stress response and has been well studied in many organisms. We simulated this stress by the addition of hydrogen peroxide to the culture medium.

Results

Determination of sub-lethal stress conditions

The aim of this study was to determine the mechanisms that allow short-term acclimation to abiotic stress. To be sure to monitor the short-term response to stress rather than just cell death, the intensity of the different stresses needed to be cho-

sen carefully. Using a pulse amplitude modulation fluorometer (see Materials and methods), we measured the effects of different stress intensities on photosynthesis. Figure 1 shows the change in quantum yield of photosynthesis in response to different intensities of the different stresses, where values of over 0.5 indicate low stress. The quantum yield can vary during the course of the day even under controlled conditions, as changes in light have a strong impact on this parameter. Stress conditions were chosen to have a clear effect on the photosynthesis rate, but to be sub-lethal, allowing the alga to acclimate and recover. The conditions that corresponded best to these criteria were 1.47 M NaCl (hypersaline condition, approximately three times the salinity of normal seawater), 12.5% seawater, and 1 mM H_2O_2 (oxidative stress condition), although, for this last stressor, we can assume that the H_2O_2 concentration in the medium decreases over the course of the experiment. Each stress was applied for 6 hours because this corresponds to the time span between high and low tide. In addition, experiments carried out on land plants [27] and red algae [19] have indicated that the application of stress for 6 hours induces the most marked changes in transcription.

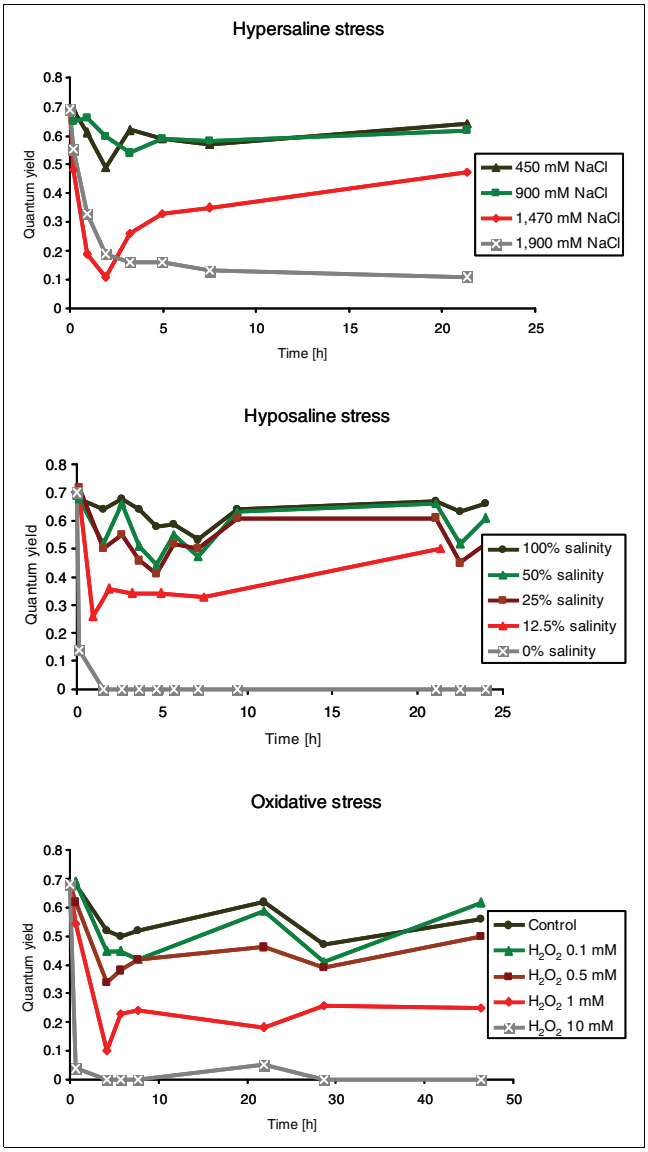

Figure 1
Effects of saline and oxidative stress of different intensities on the photosynthetic efficiency (quantum yield) of *E. siliculosus*. The conditions in red (1,470 mM NaCl, 12.5% seawater, and 1 mM H_2O_2) were the conditions chosen for the microarray analysis.

Initially, we had considered a fourth stress condition, 2 M sorbitol in artificial sea water (ASW), to imitate the osmotic pressure of the hypersaline treatment without the possible effects of the salts. However, this treatment was not included in the final experiment because cultures did not survive this treatment for 6 hours. For the other stresses, we observed 100% recovery of photosynthesis after about 6 days, even after 24 hours of stress (Additional data file 1).

Intracellular osmolarity and Na^+ concentration

Apart from the photosynthetic activity, we also measured intracellular osmolarity and Na^+ concentrations (Figure 2). After 6 hours of exposure to different salinities, the intracellular osmolarity was always about 500 mOsm higher than that of the extracellular medium. The intracellular Na^+ concentration was about 500 mM lower than in the extracellular medium under hypersaline stress, 60 mM lower under control conditions, and the same under hyposaline stress. Oxidative stress had no detectable effect on the intracellular ion composition or osmolarity (data not shown).

The *E. siliculosus* microarray represents 17,119 sequences

We designed a microarray based on 90,637 ESTs obtained by sequencing clones from 6 different cDNA libraries: immature sporophyte (normalized and non-normalized), mature sporophyte, immature gametophyte, mature gametophyte, and stress (sporophyte). Cleaning and assembly resulted in the generation of 8,165 contigs and 8,874 singletons. In addition, 21 genomic sequences and 231 *E. siliculosus* Virus 1 (EsV-1) genes were included. The array design file has been deposited under the accession number [ArrayExpress:A-MEXP-1445] and is also available on our *Ectocarpus* transcriptomics homepage [22].

Of the 17,119 genes represented on the array, 12,250 gave a significant signal over background in our experiments and were considered to be expressed under the conditions tested. The analysis focused on these 12,250 genes (see Materials and methods). A first comparison with the data obtained from a tiling experiment with *E. siliculosus* (MP Samanta and JM Cock, personal communication), where 12,600 genes were considered strongly expressed, demonstrates that our array offers a rather complete coverage of at least the highly transcribed parts of the *E. siliculosus* genome, suggesting that we are working at the whole genome scale.

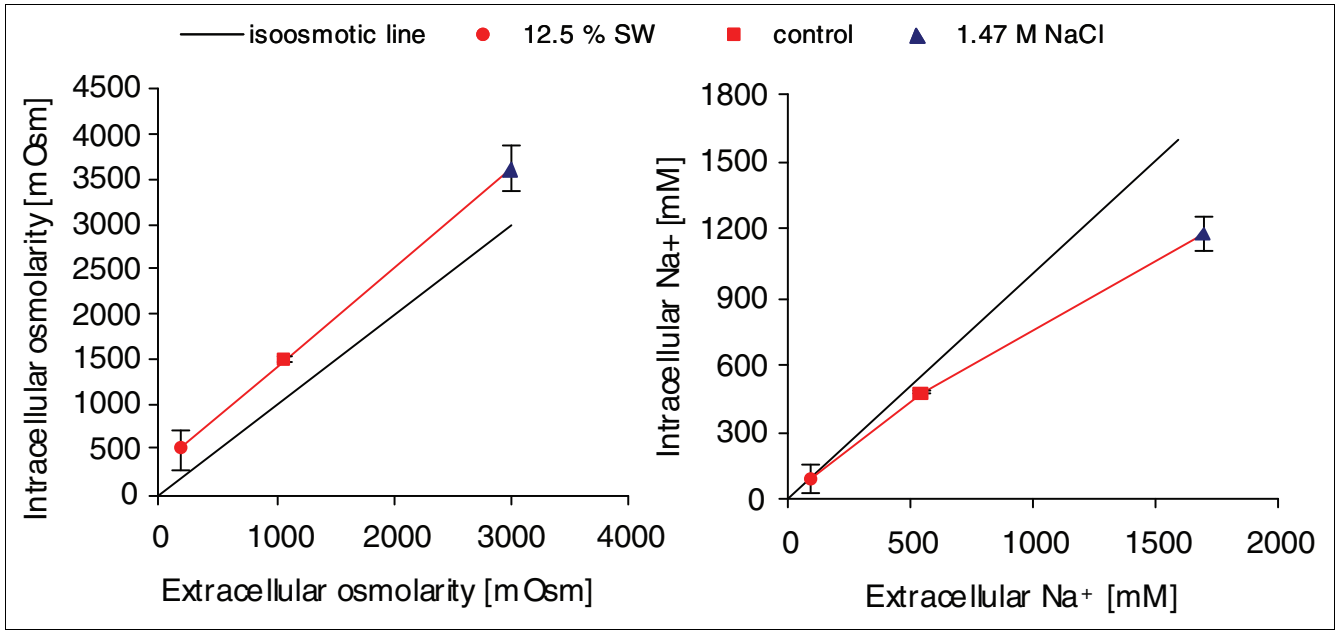

Figure 2
Intracellular versus extracellular osmolarity and Na^+ concentration under saline stress. Oxidative stress samples are not shown as they did not differ significantly from the control sample. Every point represents the mean of five biological replicates ± standard deviation.

Table 1

Comparison of microarray and RT-qPCR results for genes changing expression

ID	Genome ID	Name	r	Function
CL4038Contig1	[Esi0355_0025]	*HSP70*	0.87	HSP70
LQ0AAB7YD09FM1.SCF	[Esi0155_0065]	*NADH*	0.94	NADH dehydrogenase
CL7513Contig1	[Esi0269_0011]	*ProDH*	0.79	Proline dehydrogenase
CL3741Contig1	[Esi0024_0066]	*TF*	0.90	Putative transcription factor
LQ0AAB12YN05FM1.SCF	[Esi0399_0008]	*WD_rep*	0.66	WD repeat gene
CL1Contig3	[Esi0085_0055]	*CLB1*	0.95	Chlorophyll binding protein
CL43Contig1	[Esi0199_0054]	*CLB2*	0.98	Fucoxanthin binding protein
CL7742Contig1	[Esi0026_0055]	*TagS*	0.69	TAG synthase
CL2765Contig1	[Esi0526_0006]	*NH4-Tr*	0.96	Ammonium transporter
CL3832Contig1	[Esi0437_0012]	*FOR*	0.67	Phycoerythrobilin:ferredoxin oxidoreductase
LQ0AAA16YN10FM1.SCF	[Esi0153_0004]	*Arg-MetTr*	0.71	Arginine N-methyltransferase
CL7099Contig1	[Esi0018_0111]	*HSD*	0.83	Homoserine dehydrogenase
CL6576Contig1	[Esi0107_0059]	*IGPS*	0.97	Indole-3-glycerol-phosphate synthase
CL7231Contig1	[Esi0686_0001]	*CDPK*	0.85	cAMP-dependent protein kinase
CL4027Contig1	[Esi0122_0054]	*mGST*	-0.48	Microsomal glutathione S-transferase
CL4274Contig1	[Esi0023_0183]	*SNR*	0.57	SNR (vesicular transport)
CL5850Contig1	[Esi0109_0088]	*mG*	0.99	Glycin-rich protein
CL455Contig1	[Esi0159_0021]	*G6PD*	0.91	Glucose-6-phosphate 1-dehydrogenase
CL6746Contig1	[Esi0116_0065]	*IF4E*	0.91	Eukaryotic initiation factor 4E

R is the Pearson correlation coefficient between the microarray and the RT-qPCR expression profile. ID corresponds to the name of the sequence on the array.

cDNA synthesis and amplification provided consistent results with both mRNA and total RNA samples

For reasons as yet unknown, cDNA synthesis reactions with *E. siliculosus* are inhibited at high concentrations of RNA. Therefore, we decided to synthesize cDNAs from a small quantity of total RNA or mRNA, and to include a PCR amplification step in the protocol to obtain sufficient double-stranded cDNA (4 μg) for each hybridization. A comparison of the four four-fold replicates synthesized from 30 ng of mRNA and the single four-fold replicate synthesized from 100 ng total RNA showed that these two protocols yielded similar results. All total RNA replicates clustered with the mRNA replicates of the same stress (data not shown). Nevertheless, at a false discovery rate (FDR) of 5%, 163 transcripts gave significantly different results with the two types of sample. These transcripts represented mainly constituents of the ribosome, as revealed by a Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology Based Annotation System (KOBAS) analysis and by an analysis of overrepresented GO terms (Additional data file 2).

Validation of microarray results using quantitative PCR

Nineteen genes that exhibited significant changes in their expression patterns in the microarray analysis were analyzed by real time quantitative PCR (RT-qPCR). Eighteen of these had similar expression profiles in both the microarray experiment and the RT-qPCR experiment (correlation coefficient r of between 0.57 and 0.99; Table 1). Only one gene, which codes for a microsomal glutathione S-transferase, displayed a different pattern in the two experiments (r = -0.48). Furthermore, the seven most stable 'housekeeping genes' as identified by qPCR in a previous report [28] showed only statistically non-significant relative changes of <1.5-fold (log2-ratio <0.58) in the microarray experiment (Table 2). This demonstrated that the protocol for cDNA amplification provided reliable measures of the relative transcript abundances. Although this method has been successfully applied in several small-scale expression studies [29-35], to our knowledge, the use of this technique has not been reported with commercial photolithographically synthesized arrays.

Ribosomal protein genes are among those whose transcript abundances are least affected by stress

The 100 most stably expressed genes in these microarray experiments included 51 genes with unknown functions. Nineteen genes code for ribosomal proteins, and 21 genes are known housekeeping genes with functions related to protein turnover (transcription, 4 genes; translation, 3 genes; degradation, 3 genes), energy production (6 genes), and the cytoskeleton (5 genes). For a detailed list of these most stably expressed genes, please see Additional data file 3.

Classification of stress response genes using automatic annotations

Overall, 8,474 genes were identified as being differentially expressed in at least one of the conditions compared to the control, allowing a FDR of 10% (5,812 were labeled significant at an FDR of 5%). As can be seen in Figure 3, the relative change for these genes ranged from 1.2-fold (log2-ratio ≈0.3) to more than 32-fold (log2-ratio >5). Of these 8,474 genes, 2,569 (30%) could be automatically annotated with GO terms using the GO-term Prediction and Evaluation Tool (GOPET) [36] and 1,602 (19%) with KEGG orthology annotations using the KOBAS software [37]. These automatic annotations were analyzed for each stress condition individually, to identify GO categories and KEGG pathways that were significantly overrepresented.

The KOBAS results (Figure 4; Additional data file 4) indicated that under hyposaline and hypersaline stresses most of the changes involved down-regulation of the synthesis and metabolism of amino acids. More precisely, genes involved in the synthesis of valine, leucine, and isoleucine, as well as that of the aromatic amino acids (phenylalanine, tyrosine, tryptophan), and arginine and proline metabolism were affected. This effect on amino acid synthesis was less marked for oxidative stress, where glutamate metabolism was the only

Table 2

Comparison of microarray and RT-qPCR results for housekeeping or stable genes

ID	Genome ID	Name	Maximum change ARRAY	Maximum change QPCR	Function
LQ0AAB30YA12FM1.SCF	[Esi0298_0008]	Dyn	0.23	0.77	Dynein
CL1914Contig1	[Esi0021_0024]	ARP2.1	0.22	0.44	Actin related protein
CL3Contig2	[Esi0387_0021]	EF1A	0.08	0.46	Elongation factor 1 alpha
CL8Contig12	[Esi0053_0059]	TUA	0.57	0.91	Alpha tubulin
CL1073Contig1	[Esi0054_0059]	UBCE	0.22	0.38	Ubiquitin-conjugating enzyme
CL29Contig4	[Esi0302_0019]	UBQ	0.18	0.82	Ubiquitin
CL461Contig1	[Esi0072_0068]	R26S	0.22	n/a	Ribosomal protein S26

The table displays the maximum log2-ratio between any stress and the control condition for both the microarray and the RT-qPCR analysis. No RT-qPCR value is available for R26S, as this gene was used for normalization of the RT-qPCR samples. ID corresponds to the name of the sequence on the array.

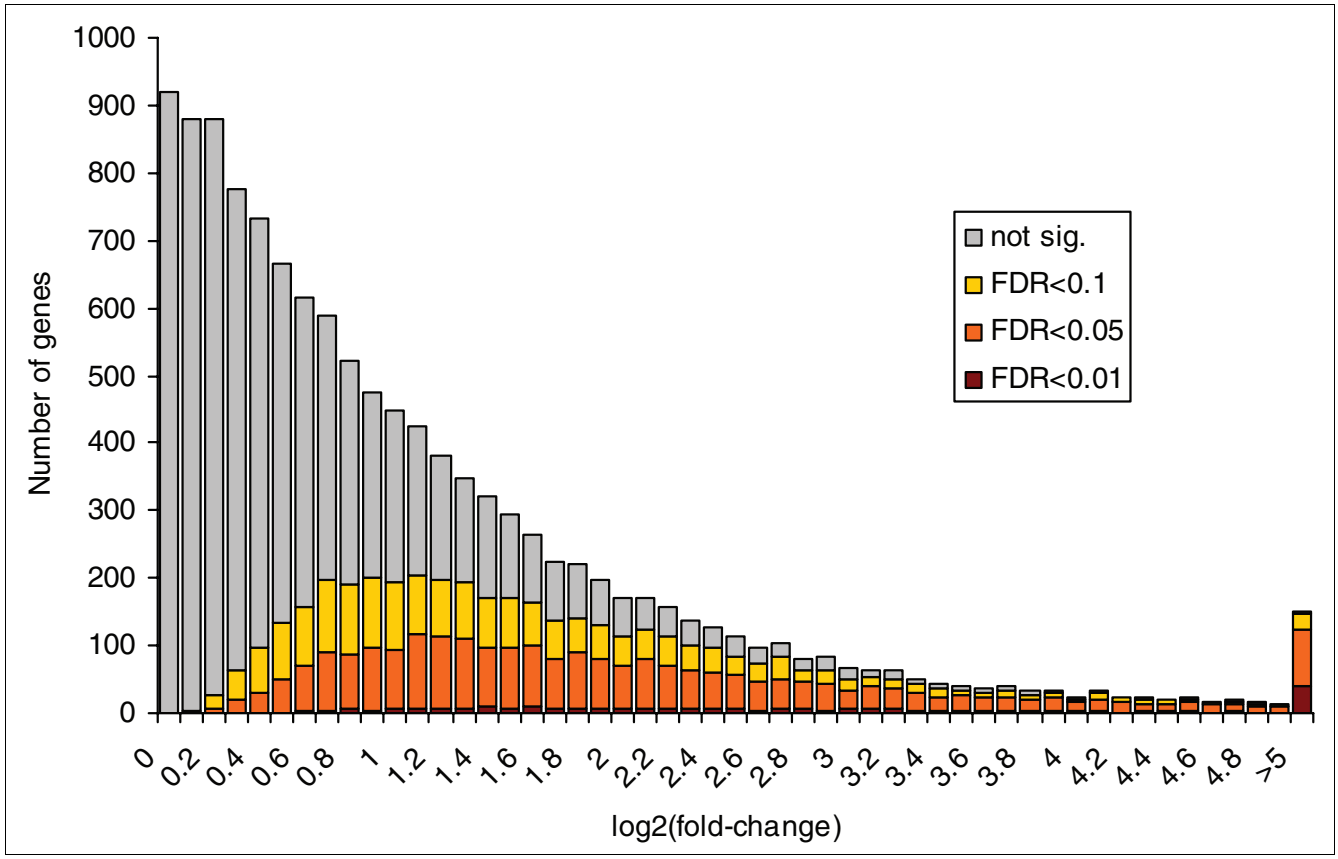

Figure 3
Distribution of observed fold-changes (log2-ratios of stress and control samples). All three comparisons between stress and control treatments were considered and the observed frequencies averaged. The color coding shows how many transcripts were labeled as differentially expressed at different FDRs. Not sig., not significant.

amino acid metabolism affected. Under hypersaline conditions, there was also an increase in transcripts coding for enzymes that metabolize valine, leucine, and isoleucine. In addition, photosynthesis and vesicular transport seemed to be altered by both hyposaline and oxidative stress. Pathways that appeared to be specifically affected by one stress included the up-regulation of fatty acid metabolism and down-regulation of translation factors under hypersaline stress, the up-regulation of the proteasome and down-regulation of nitrogen metabolism under hyposaline stress, and an increase in glycerophospholipid metabolism under oxidative stress (Figure 4). A complete list of the pathways identified is available in Additional data file 4, with possible artifacts arising from the automatic annotation marked in grey.

The GOPET analysis (Table 3; Additional data file 5) was focused on the molecular function of the individual genes rather than their role in a specific pathway. Only three GO terms were identified as being over-represented among the up-regulated genes: arginase and agmatinase activity under hypersaline conditions, and microtubule motor activity under oxidative stress. Most GO terms were found to be significantly over-represented among the down-regulated genes. In agreement with the down-regulation of amino acid metabolism identified by the KOBAS analysis, we observed a decrease in the abundance of transcripts encoding aminoacyl-tRNA ligases in hypersaline and hyposaline conditions using the GOPET annotations. Also, under hypersaline stress, we observed down-regulation of genes associated with the GO terms RNA binding and translation factor activity, which corresponds to the KEGG category translation factors, and down-regulation of transcripts coding for proteins with a CTP synthase activity, which are involved in purine and pyrimidine metabolism. Under hyposaline stress, we observed that $NAD(P)^+$ transhydrogenases, a number of transferases and oxidoreductases involved in amino acid metabolism, as well as genes with functions in nucleic acid and chlorophyll binding, were most affected, the latter matching well with the pathways 'photosynthesis-antenna proteins' identified by KOBAS. Under oxidative stress, using the GOPET annotations, we detected down-regulation of several different categories of transferases, nitrate transporters, oxidoreductases involved in steroid metabolism, and 3-isopropylmalate dehydratase-like enzymes that are involved in amino acid metab-

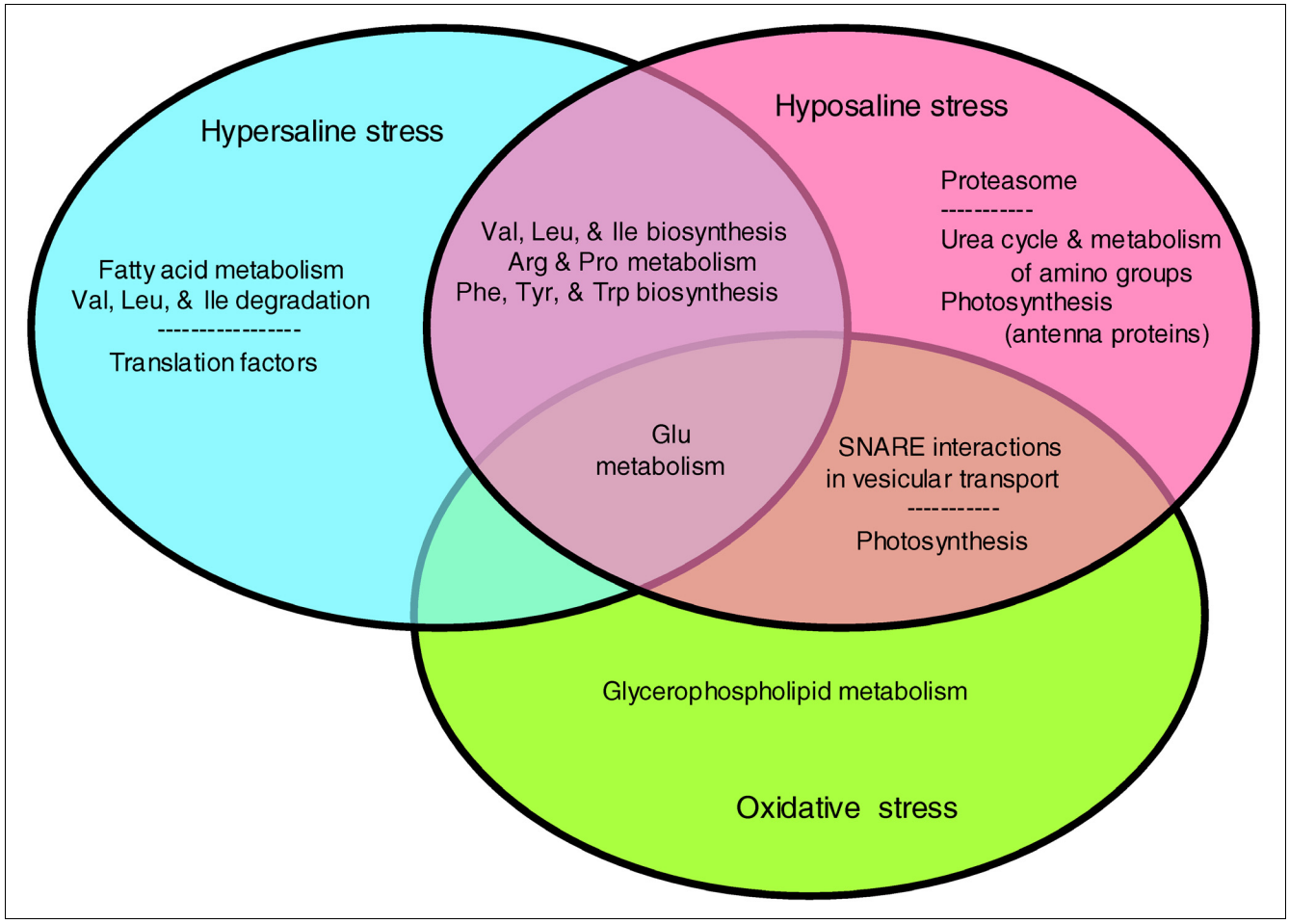

Figure 4
Venn diagram of KEGG pathways identified as over-represented among the transcripts significantly up- or down-regulated (FDR <0.1) in the different stress conditions. Only KEGG pathways with q-values < 0.1 in at least two conditions or for both datasets (FDR of 0.05 and FDR of 0.1) were considered. The general category 'other enzymes' was not included. Further 'SNARE interactions in vesicular transport' includes the category 'SNARE', and 'photosynthesis' includes 'photosynthesis proteins' and 'porphyrin and chlorophyll metabolism'. No pathways were found to be common only to hyposaline and hypersaline stress. SNARE, soluble N-ethylmaleimide-sensitive factor attachment receptor.

olism. Here, the KOBAS analysis did not identify any significantly up- or down-regulated pathways. Also in contrast to the KOBAS results, no GO terms were significantly over-represented among the genes identified as being up- or down-regulated in both oxidative and hypersaline stresses, or in all three stresses at the same time.

Manual classification of stress response genes with the most significant changes in expression

To identify the most important mechanisms involved in the stress response, we manually classified and examined in detail 966 genes that exhibited the most significant changes in one of the stress conditions compared to the control (that is, genes that meet both criteria: significance at an FDR <1% and a relative change in expression of more than two-fold). A complete list of these genes, including their putative function, assigned manually based on sequence homology of the corresponding genome sequence to public protein databases, can be found in Additional data file 3.

We identified 519 genes (53.7%) with no homologues in either the National Center for Biotechnology Information (NCBI) databases or other heterokont genomes (e-value > 1e-10). An additional 122 genes (12.6%) code for conserved genes with unknown function. Of these 122 conserved genes, 23 (18.9%) are conserved only within the heterokont lineage. The remaining 325 genes (33.6%) were divided into 12 groups according to their putative functions in amino acid metabolism, DNA replication and protein synthesis, protein turnover, carbohydrate metabolism, photosynthesis-related processes, fatty acid metabolism, transporters, vesicular trafficking and cytoskeleton, classical stress response pathways, autophagy, signaling, and other processes. The following sec-

Table 3

GO terms identified to be over-represented among the transcripts of significantly up- or down-regulated in the different stress conditions

Condition	Change in expression	Category	Function	GO ID
Hyper	Down	Nucleic acid binding	RNA binding (mRNA, rRNA, snoRNA)	[GO:0003723]; [GO:0003729]; [GO:0019843]; [GO:0030515]
		Nucleic acid binding	Translation factor activity (elongation and initiation)	[GO:0008135]; [GO:0003746]; [GO:0003743]
		Lyase	UDP-glucuronate decarboxylase activity	[GO:0048040]
		Ligase	CTP synthase activity	[GO:0003883]
		Isomerase activity	Intramolecular oxidoreductase activity	[GO:0016860]
	Up	Hydrolase	Agmatinase activity	[GO:0008783]
		Hydrolase	Arginase activity	[GO:0004053]
Hyper Hypo	Down	Ligase	Aminoacyl-tRNA ligase activity (inlcuding Pro, Ser, Ile, Glu)	[GO:0004812]; [GO:0016876]; [GO:0004828]; [GO:0004829]; [GO:0004822]
Hypo	Down	Oxidoreductase (S, peroxide)	Antioxidant activity (glutathione-disulfide reductase and catalase, cytochrome-c peroxidase)	[GO:0016209]; [GO:0004362]; [GO:0004096]; [GO:0004130]
		Nucleic acid binding	Structure-specific DNA binding	[GO:0000404]; [GO:0032134]; [GO:0000403]; [GO:0032137]; [GO:0032138]; [GO:0032139]
		Tetrapyrrole binding	Chlorophyll binding	[GO:0016168]
		Lyase	Carbon-oxygen lyase activity	[GO:0016835]
		Transferase (N)	Transaminase activity (including TYR, ASP, histidinol-P, aromatic amino acids)	[GO:0008483]; [GO:0004838]; [GO:0004400]; [GO:0008793]; [GO:0004069]
		Transferase (C1)	Aspartate carbamoyltransferase activity	[GO:0004070]
		Transferase (glycosyl)	Transferase activity, transferring pentosyl groups	[GO:0016763]
		Oxidoreductase CH-CH	Biliverdin reductase activity	[GO:0004074]
		Oxidoreductase (CH-NH2)	Glutamate synthase activity	[GO:0015930]
		Isomerase	Isomerase activity	[GO:0016853]
		Transporter	NAD(P)+ transhydrogenase (B-specific) activity	[GO:0003957]
Hypo	Down	Oxidoreductase	Oxidoreductase activity	[GO:0016491]
Oxi			Oxidoreductase activity, acting on NADH or NADPH	[GO:0016651]; [GO:0016652]
			Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (including L-iditol 2-dehydrogenase activity)	[GO:0016616]; [GO:0016614]; [GO:0003939]
Oxi	Down	Lyase	3-Isopropylmalate dehydratase activity	[GO:0003861]

Table 3 *(Continued)*

GO terms identified to be over-represented among the transcripts of significantly up- or down-regulated in the different stress conditions

	Transferase (P)	Amino acid kinase activity	[GO:0019202]
	Transporter	Nitrate transmembrane transporter activity	[GO:0015112]
	Transferase (C1)	S-adenosylmethionine-dependent methyltransferase activity (including nicotinate phosphoribosyltransferase)	[GO:0008757]
	Oxidoreductase (steroids)	Steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	[GO:0033764]
	Transferase (glycosyl)	Transferase activity, transferring pentosyl groups	[GO:0016763]; [GO:0004853]
	Transferase (glycosyl)	Uracil phosphoribosyltransferase activity	[GO:0004845]
	Transferase (P)	Phosphoribulokinase activity	[GO:0008974]
Up	Motor activity	Microtubule motor activity	[GO:0003777]

The table shows only pathways that were labeled significant at an FDR <10% in both sets of significant genes (5% FDR and 10% FDR).

tion gives a brief overview of the different groups of genes identified among the most significantly regulated genes.

Among genes involved in amino acid metabolism, we found a total of 32 down-regulated genes related to the metabolism of all 20 standard amino acids except aspartic acid. In contrast, nine genes were induced in at least one abiotic stress condition. These were involved in the metabolism of proline, arginine, cysteine, alanine, phenylalanine, tyrosine, tryptophan, leucine, isoleucine, and valine. Highly regulated genes involved in the different steps of DNA replication and protein synthesis coded for proteins, including helicases, DNA polymerases and related enzymes, proteins involved in purine and pyrimidine synthesis, DNA repair proteins, transcription factors, RNA processing enzymes, proteins involved in translation, ribosomal proteins, and proteins for tRNA synthesis and ligation. Most of these genes were down-regulated in all stress conditions, but some genes were up-regulated in response to abiotic stress. These genes include some helicases, transcription factors, and DNA repair proteins. We also found seven genes related to protein turnover to be down-regulated and six to be up-regulated in one or more of the stress conditions. Among the up-regulated genes, there were two ubiquitin conjugating enzymes, which play a potential role in targeting damaged proteins to the proteasome, or control the stability, function, or subcellular localization of proteins.

The situation was similar for genes involved in carbohydrate metabolism, where we found both glycolysis- and citric acid cycle-related genes to be strongly down-regulated under all the stresses tested (six and seven genes down-regulated, respectively). However, four genes, encoding a gluconolactonase, a xylulokinase, a phosphoglycerate kinase, and an isocitrate lyase, were up-regulated. In particular, an isocitrate lyase gene was 19- to 212-fold up-regulated under the different stress conditions. Photosynthesis-related genes that were regulated in response to abiotic stress included eight chlorophyll a/c binding proteins as well as genes responsible for the assembly of photosystem 2, electron transport, light sensing, and carotenoid synthesis. Many of these genes were strongly affected in the hypersaline condition, with the majority being down-regulated (17 versus 11 that were up-regulated). There was at least one gene that was up-regulated under one or more stress condition in every group. Genes with roles in fatty acid metabolism altered their expression patterns in a similar way under all stress conditions. We were able to distinguish between two groups: three genes involved in the synthesis of fatty acids, which were down-regulated; and genes functioning in the degradation of fatty acids, among which five of six genes were up-regulated. We further observed that three genes involved in lipid synthesis were up-regulated, and genes involved in inositol metabolism were also affected.

With respect to transporters, we identified five genes encoding nitrogen transporters (all down-regulated) as well as three genes encoding sugar transporters (all up-regulated). Genes coding for ion transporters were also mainly down-regulated under hypersaline and hyposaline conditions, although two potassium and magnesium transporter genes were up-regulated under hypersaline stress. Among genes responsible for the transport of solutes and proteins to the mitochondrion, we observed an up-regulation mainly in the hyposaline stress condition. Regarding genes related to vesicular trafficking and the cytoskeleton, we identified 13 up- and 6 down-regulated genes, many of these genes containing an ankyrin repeat domain and showing strongest changes in transcription under hyposaline and oxidative stress conditions.

We further found several classical stress response genes to be up-regulated. Four genes coding for heat shock proteins (HSPs) were up-regulated mainly under hyposaline and oxi-

dative stress, but there were also two genes coding for a chaperonin cpn60 and a prefoldin, each of which was down-regulated. In addition, we found genes involved in protection against oxidative stress to be induced. These include a glutaredoxin (oxidative stress), a methionine sulfoxide reductase (hyposaline stress), and three glutathione peroxidases (mainly hypersaline stress). At the same time, however, a catalase-coding gene was down-regulated in all stress conditions, most strongly under hyposaline stress.

Two genes involved in autophagy, one of which is represented by two sequences on the microarray, were up-regulated in all stress conditions and several genes with putative signaling functions were affected. Six protein kinases were among the most significantly up-regulated genes: three equally under all stress conditions, and one each specifically under hyposaline, hypersaline and oxidative stress. Furthermore, one protein kinase and one WD-40 domain containing gene were down-regulated under hyper- and hyposaline stress, respectively.

Several other genes are not mentioned here, either because only a very vague prediction of their function was possible, or because they are difficult to put into categories with other genes. More detailed information about these genes can be found in Additional data file 3.

Stress response genes with unknown functions

All unknown and conserved unknown genes present among the most significantly regulated genes were sorted into groups according to their sequence similarity (Additional data file 6). Among the groups with three or more members, there were three (I to III) that had no known homologs in species other than *E. siliculosus*, and three (IV to VI) for which we were able to find homologs in other lineages for most of the sequences. A more detailed description of all of the unknown and unknown conserved stress response genes, including an analysis of conserved protein and transmembrane domains, is available in Additional data file 6.

Known brown algal stress genes

Many of the brown alga-specific stress response genes identified in *L. digitata* by Roeder *et al.* [21] were not among the most regulated genes identified in this study. Nevertheless, we decided to examine their expression patterns in more detail. The array used in this study contained probes for one vanadium-dependent bromoperoxidase (CL83Contig2), but this gene was not strongly regulated under the different stress conditions (1.06-fold to 1.4-fold induced, $P = 0.75$). Twenty-four C5-epimerases were represented, but none of these genes were among the most significantly regulated loci, although several of them were either induced or repressed under the different stress conditions. A detailed list of these genes, including their expression profiles, can be found in Additional data file 7. Finally, we decided to consider genes involved in the synthesis of mannitol, a well-known osmolyte in brown algae [16,17]. Only one enzyme specific to the synthesis of this polyol could be clearly identified based on sequence homology: mannitol 1-phosphate dehydrogenase (see [38] for a description of the mannitol synthesis pathway in brown algae). Our array contains probes for two genes identified as potential mannitol 1-phosphate dehydrogenases: one (CL200Contig2 corresponding to Esi0017_0062 in the *Ectocarpus* genome), which was among the most significantly regulated genes and six-fold down-regulated in hyposaline condition, and one (CL2843Contig corresponding to Esi0020_0181), which was generally expressed at a very low level but was up-regulated approximately five-fold under hypersaline stress ($P = 0.066$).

Clusters of genes with similar expression patterns

Based on a figure of merit (FOM) graph, we decided to divide the set of expressed genes into seven different clusters (A to G). These clusters, along with the GO terms and KEGG pathways that are over-represented among each of them, are shown in Figure 5. We identified one cluster (A) representing the stably expressed genes, three clusters included mainly up-regulated genes (B-D), and the remaining three clusters included mainly down-regulated genes (E-G). Among both the up- and down-regulated clusters, we found one cluster each that was equally affected by all stress conditions (B and E), one each where gene expression was affected only by hyposaline and oxidative stress conditions (C and G), and one cluster each where gene expression was affected mainly by hypersaline stress (D and F). Most of the principal functions identified for each cluster by GOPET and KOBAS fit well with the results from our earlier analysis of the up- and down-regulated genes.

Discussion

This study presents the first global gene expression analysis of a brown alga. Our goal was to determine the transcriptomic changes in response to short-term hypersaline, hyposaline and oxidative stress - three stresses that play an important role in the natural habitat of many brown algae, the intertidal zone [20,26]. Our results show that almost 70% of the expressed genes had a modified expression pattern in at least one of the examined stress conditions. This is in contrast to what has been observed in flowering plants, where the proportion of significantly regulated genes generally ranges from 1% to 30%, depending on types of abiotic stress examined, their number, and the statistical treatment applied (see [27,39,40] for some examples). Our findings demonstrate that, rather than relying on a few specific stress response proteins, *E. siliculosus* responds to abiotic stress by extensive reprogramming of its transcriptome.

A more detailed analysis of the manual annotation of the 966 most significantly regulated genes and the results for the GOPET and KOBAS analysis for all three stress conditions, reveals two major themes concerning the short-term stress response of *E. siliculosus*: down-regulation of primary

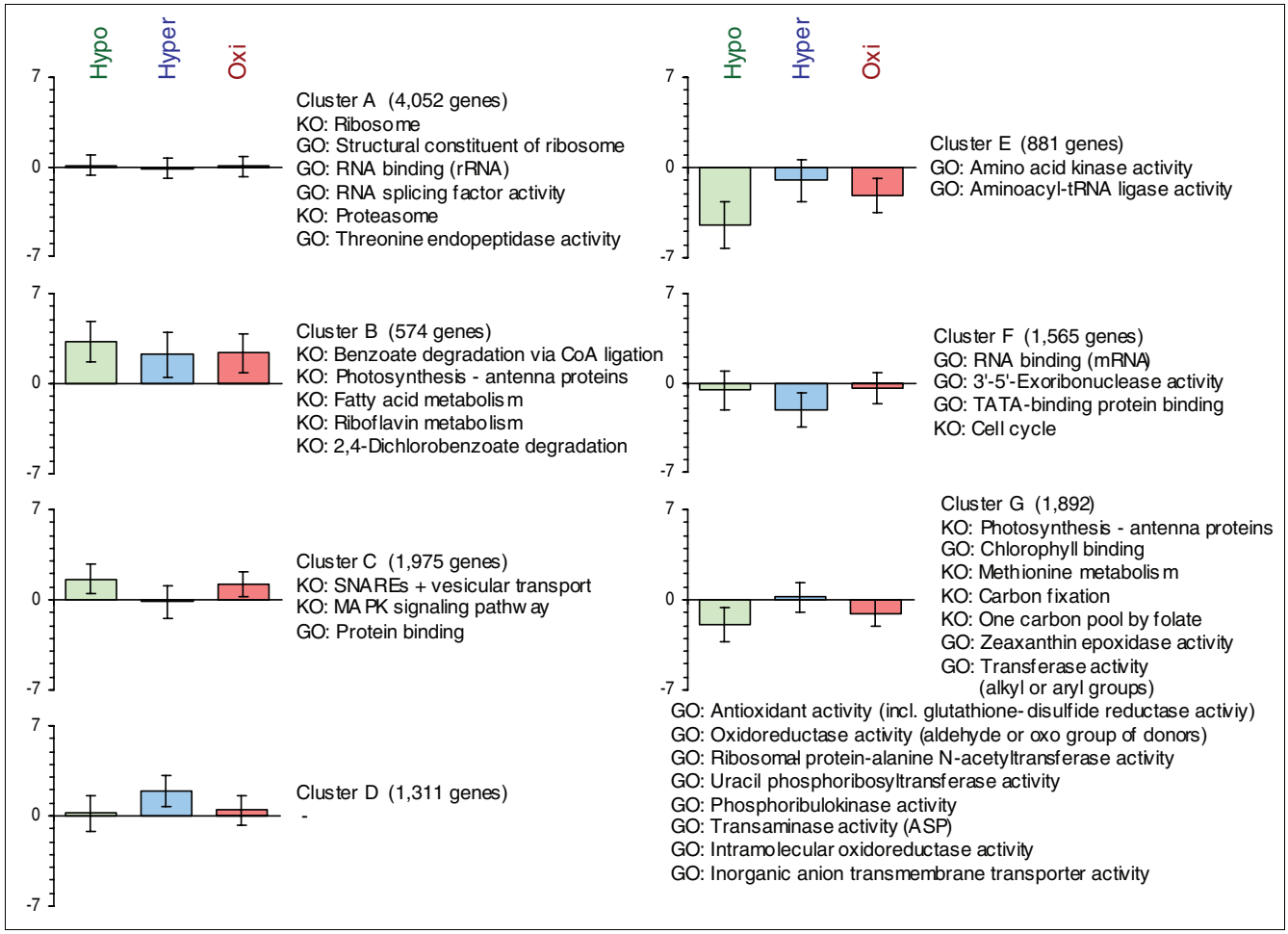

Figure 5
Expression graphs of clusters identified by the k-means algorithm. The graphs display the log2-ratio of all stress conditions (hypo = green, hyper = red, oxi = blue) with the control condition. GO terms and KEGG pathways (KO) identified as over-represented in these clusters (FDR = 10%) are shown next to the graph.

metabolism and growth processes; and activation of energy stores and of genes and pathways involved in 'stress management'. These findings are summarized in Figure 6. In the following sections we will first discuss the differences observed between the different stress conditions that were tested, and then highlight some of the general trends that emerged from our data.

Comparison of stress conditions

We have compared each stress condition to the control condition, and analyzed these results using KOBAS and GOPET. Due to the necessity to control the FDR with multiple testing, the chance of beta-errors (that is, the chance of falsely labeling a gene or a pathway as not significantly regulated) greatly increased, making a direct comparison of the genes and groups of genes identified as being up- or down-regulated under the different stress conditions prone to false conclusions. Therefore, we based our comparison of the different stresses on the cluster analysis and the results from the manually analyzed 966 most significantly changing genes.

The first and most apparent observation from the clustering was that the changes in gene expression induced by hyposaline and oxidative stress were more similar to each other than to those observed under hypersaline stress. One explanation for this might be that hypersaline stress, although it is a common stressor in the natural habitat of brown algae, is not likely to occur at the same intensity in the field as that used for our laboratory experiments (about three times the concentration of normal seawater). Even though we did not observe a strong difference in the efficiency of photosynthesis under the different stress conditions, it is possible that hypersaline stress, at the intensities applied in our experiments, represents a condition the alga is less able to adjust to. This hypothesis is supported by the fact that in cluster F (down-regulated in hypersaline conditions) cell cycle-related genes were over-represented, indicating that growth was most strongly

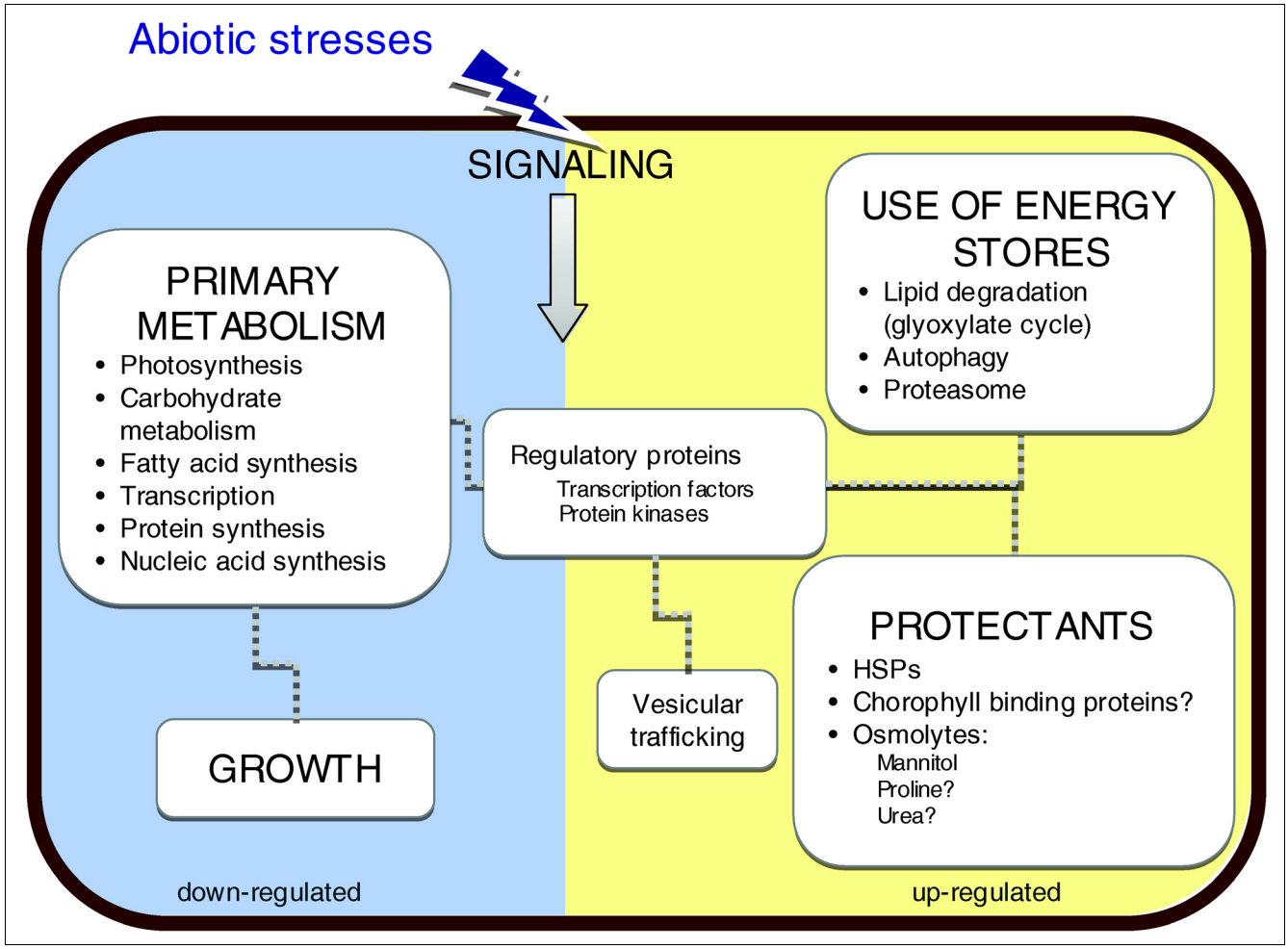

Figure 6
Major transcriptomic changes in *E. siliculosus* under short-term oxidative and saline stress. This schema summarizes the most important transcriptomic changes discussed in the text. Processes on the left (blue) were repressed, while processes on the right (yellow) were activated. Please note that this graph displays only the general trends; some of the pathways are not regulated in all stress conditions and not all genes of one pathway are always regulated in the same way (see text for details).

affected under hypersaline stress conditions. Furthermore, other classical stress responses, such as the up-regulation of SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor)-related genes, which are important for cellular transport of vesicles and their fusion with membranes and play a role in plant development and abiotic stress response [41], were observed mainly under oxidative and hyposaline stress.

There are also several smaller differences between oxidative and hyposaline stress, such as the induction of a glutaredoxin gene (Additional data file 2) under oxidative stress conditions. Glutaredoxins are known to play a central role in the protection against oxidative damage [42], as they can be oxidized by diverse substrates, including ROSs, and are reduced by glutathione. Other genes did not change expression under oxidative stress, but were specifically regulated under saline stress. Such examples are given in the following paragraphs.

Down-regulation of growth and primary metabolism

Many genes involved in several pathways related to growth and primary metabolism were identified to be down-regulated by more than one of our analyses (GOPET, KOBAS, k-means clustering, and manual analysis). We observed a decrease in the abundance of transcripts of genes that are important in the synthesis of purine and pyrimidine nucleotides and, correspondingly, of several genes responsible for the replication of DNA. This function is essential for cell division, a process affected in all of the stress conditions examined.

A reduction in growth implies a reduction in the requirements for primary metabolites necessary to fuel this growth. Within our dataset, we found widespread evidence for down-regulation of processes involved in primary metabolism. This was most pronounced in the case of protein synthesis. Several genes encoding enzymes involved in this and related proc-

esses (the synthesis of amino acids, their ligation with the appropriate tRNAs, the production of mRNA (that is, transcription), and the actual assembly of polypeptide chains (that is, translation)) were down-regulated under stress conditions. Furthermore, genes responsible for the uptake of nitrogen, which is used mainly for the synthesis of amino acids, were also down-regulated. Together, this provides a strong indication that the overall rate of protein synthesis was reduced under the stress conditions examined.

However, there were additional primary metabolic processes that were at least partially affected by the stress treatments. These included the synthesis of fatty acids, photosynthesis and pigment synthesis, and carbohydrate metabolism. Again, these changes probably reflected a decreased need for metabolites for growth.

One possible explanation for the observed down-regulation of genes involved in growth and primary metabolism can be found in the results of our pulse amplitude modulation fluorometer experiment: we observed that the efficiency of photosynthesis decreased almost immediately after the application of the stress treatments. Photosynthesis is strongly affected by environmental stress [43]. A decrease in photosynthetic efficiency is synonymous with a decrease in energy production, and we can assume that reducing all of these aspects of primary metabolism might represent a means of conserving energy. This phenomenon is known in higher plants, where Kovtun *et al.* [44] have reported cross-talk between oxidative stress and auxin signaling, auxin being a major growth hormone in higher plants. Most likely, this cross-talk allows plants to shift their energy from growth-related processes to stress protection and survival. This might also be true for *E. siliculosus*, where the observed down-regulation of growth- and primary metabolism-related genes might represent a way of compensating for reduced energy production under stress conditions and redirecting energy to specific stress response processes.

Activation of protein degradation, energy stores, and nutrient recycling

We observed an up-regulation of two genes involved in autophagy in all three of the stresses examined. Under nutrient-limited and under stress conditions, this process has been shown to play a role in the re-allocation of sugar and nutrients to essential biological processes in several organisms [45]. The activation of autophagy-related genes might, therefore, just like the down-regulation of growth-related processes, represent a mechanism of compensating for reduced energy production under stress conditions and provide sugars and nutrients for both core biological processes and synthesis of stress proteins. This coincides with the strong up-regulation of an isocitrate lyase gene under all stress conditions. Isocitrate lyases are enzymes located in the glyoxysome and catalyze a rate-controlling step in the glyoxylate cycle (reviewed in [46]), one function of which is the conversion of lipids to carbohydrates when non-lipid-derived storage reserves are depleted. Corresponding to this and the up-regulation of autophagy-related genes, we observed an up-regulation of three genes coding for sugar transporters in all stress conditions and of genes coding for mitochondrial exchange proteins under the hyposaline and oxidative stress conditions. These transporters may direct recycled sugars and nutrients to the mitochondrion, where they can be used for energy production.

Recently, additional roles of autophagy have been discovered in higher plants, including the degradation and removal of oxidized or damaged proteins during stress [47]. To a certain degree, these roles overlap with the role of the proteasome. Although we found some genes involved in protein turnover to be down-regulated, the KEGG pathway 'proteasome' was identified as up-regulated in the hyposaline stress condition and we identified two genes involved in ubiquitination among the most significantly up-regulated genes under all stress conditions. Ubiquitination is a process in which proteins are labeled with a small polypeptide (ubiquitin) [48], thereby modifying their stability, function, or subcellular localization. This could serve regulatory purposes (for example, by targeting transcription factors or other regulatory proteins for degradation by the proteasome), accelerate translation of the large-scale transcriptomic changes into changes in protein abundance, and/or, just like autophagy, facilitate nitrogen recycling [49].

Activation of signaling pathways

Large-scale transcriptomic reprogramming as observed in our dataset most certainly requires a large number of signals for coordination. We have already discussed a possible regulatory role of ubiquitination in the previous paragraph. This, however, is not the only regulatory mechanism highlighted by the transcriptomic changes in our dataset. We found several other genes that might play roles in orchestrating the abiotic stress response of *E. siliculosus*. For example, three protein kinases with a potential role in cell signaling were strongly up-regulated under all stress conditions, while three other members of this large family appeared each to be specific to one particular stress. Furthermore, several potential transcription factors were strongly regulated under different stress conditions. Since there is still very little known about the molecules and proteins involved in the stress sensing signaling cascades of brown algae, these genes provide particularly interesting candidates for more targeted experiments such as targeted mutagenesis and chromatin immunoprecipitation in the case of the putative transcription factors.

In addition to transcription factors and protein kinases, we detected an up-regulation of genes involved in fatty acid metabolism, and more specifically fatty acid catabolism. Fatty acid derivates such as oxylipins have been shown to function in signaling in both terrestrial plants [50] and marine algae [51]. Thus, we can conclude that our data show an up-regula-

tion of genes putatively involved in several different signaling pathways.

Synthesis of 'classical' stress response proteins

The only medium throughput transcriptomic analysis of the abiotic stress response in brown algae so far [21], conducted with protoplasts of *L. digitata*, reported the transcriptional activation of vanadium-dependent bromoperoxidases and C5-epimerases. Neither of these genes was regulated in our study. While in *L. digitata* vanadium-dependent bromoperoxidases (enzymes implicated in the synthesis of halogenated organic compounds associated with defense of seaweeds against biotic stressors [52]) comprise a multigenic family [21], the *E. siliculosus* genome contains only a single copy of a vanadium-dependent bromoperoxidase, indicating a possibly different or subordinate role in this organism. Regarding C5-epimerases, which are enzymes responsible for the modification of brown algal cell walls [10] highly represented in the *Ectocarpus* genome, we observed differences in regulation between the study of Roeder *et al.* [21] and our study. This can be explained by the nature of the stress (generation of protoplasts (that is, removal of the cell wall) in [21] versus milder saline or oxidative stress in our study).

Generally, only a few 'classical' stress response genes changed expression in our experiments. In most organisms, up-regulation of genes coding for HSPs and other chaperones has been observed under abiotic stress conditions. These molecules stabilize proteins and membranes, and have been shown to play a vital role in protecting against stress by re-establishing normal protein conformation and, thus, cellular homeostasis [53]. In *E. siliculosus*, we observed four HSPs or chaperones to be among the most significantly up-regulated genes in hyposaline and oxidative stress conditions. However, this was not the case under hypersaline stress conditions. Moreover, two chaperone-like proteins were down-regulated under all stress conditions. The situation was similar for genes coding for proteins with antioxidant activity. Three genes coding for glutathione peroxidases were up-regulated under hypersaline and oxidative stress, and one glutaredoxin under hyposaline stress. At the same time, two genes encoding a glutathione S-transferase and a catalase were among the most significantly down-regulated in all stress conditions. Consequently, the GO term 'antioxidant activity' was significantly over-represented among the most down-regulated genes in the hyposaline condition. In flowering plants all of these proteins are known to carry out important functions in the protection against reactive oxygen species [25]. Our finding that these genes were not induced was, at first, surprising, but is in accordance with a transcriptomic analysis of the abiotic stress response of the intertidal red alga *C. crispus*. Collén *et al.* [20] have reported that the average expression of HSP-coding genes was only moderately elevated (approximately 1.3-fold) under hypersaline and hyposaline conditions. Furthermore, in *E. siliculosus*, the average expression of genes coding for proteins with antioxidant activity was slightly repressed (1.17-fold) under hyposaline stress conditions and slightly induced under hypersaline stress conditions (1.15-fold). One possible explanation for this might lie in the fact that transcriptional regulation is not the most important mechanism regulating the activity of these enzymes. This hypothesis would be compatible with an earlier study by Collén and Davison [54], who found that the cellular activity of ROS scavenging enzymes correlated with vertical zonation of different species of the brown algal order Fucales in the intertidal zone. To our knowledge, it is currently not known whether the activity of the ROS scavengers that were examined also changes upon exposure to abiotic stress. Such studies could greatly aid our understanding of the role of these enzymes in the brown algal stress response.

An alternative or additional explanation to that of non-transcriptional mechanisms regulating the activity of ROS scavenging enzymes could be that other, yet unknown proteins and mechanisms play more important roles in the defense against ROS in brown algae. One candidate for this kind of protein could be the chlorophyll a/c binding proteins. Thirty chlorophyll a/c binding proteins were represented on our microarray, most of them being down-regulated mainly under hyposaline and oxidative stress conditions. As chlorophyll a/c binding proteins serve as light-harvesting antennae, this down-regulation is likely to represent a response to the reduced photosynthesis efficiency (quantum yield) under stress conditions. Reducing the amount of energy that reaches the photosynthetic reaction center would also reduce the need for non-photochemical quenching and decrease the risk of the formation of ROS. However, there were also three genes coding for chlorophyll a/c binding proteins among the most significantly up-regulated genes under hyposaline and hypersaline stresses. A similar observation was made by Hwang *et al.* [55] in the Antarctic diatom *Chaetoceros neogracile*, where heat stress induced the up-regulation of five and the down-regulation of ten genes coding for chlorophyll a/c binding proteins. It is possible that these up-regulated chlorophyll a/c binding proteins, in spite of their high sequence similarity with the other transitionally down-regulated ones, have evolved or are evolving to serve different functions within the heterokont lineage.

Ions and potential osmolytes

In parallel to the induction of 'classical' stress response genes, we observed the transcriptional regulation of several genes involved in the synthesis or degradation of potential organic osmolytes and the transport of ions under saline stress. Under hypersaline stress, changes in the extracellular salt concentration as well as cell volume are likely to cause imbalances in ion concentrations, explaining the need for transporters to maintain homeostasis. Two genes coding for a magnesium and a potassium transporter were up-regulated specifically under hypersaline stress. Interestingly, we did not observe transcriptional activation of sodium transporter genes under salt stress, although many glycophytes (non- or

moderately salt tolerant terrestrial plants) use these transporters to exclude NaCl from their cytosol, allowing a certain degree of salt tolerance [56]. The latter observation suggests that the large quantities of NaCl accumulated upon exposure to saline stress (Figure 2) are stored within the cytoplasm rather than in the vacuole. A similar observation was made by Miyama and Tada [57] in the Burma mangrove. In this tree, exposure to sub-lethal NaCl stress did not cause an activation of Na transporters but led to a slow increase of the NaCl concentration in the leaves. One possible explanation for this, as proposed by Miyama and Tada [57], is that NaCl itself could serve as an osmolyte within the cells of the Burma mangrove. This may also be true for brown algae - a hypothesis that is strengthened by the observation that, in *Ectocarpus* as well as in *Lamninaria* [58] and the Burma mangrove [57], sorbitol, added at the same osmolarity as NaCl, had irreversible toxic effects.

While there is no evidence of the synthesis of additional compatible osmolytes in the Burma mangrove, we observed an increasing difference between intracellular osmolarity and intracellular Na^+ concentration with rising salinity in *E. siliculosus*, demonstrating the accumulation of other osmotically active substances in the cell. Mannitol has frequently been suggested to be a compatible osmolyte in brown alga [16,17], and indeed, one of the two mannitol 1-phosphate-dehydogenases in *E. siliculosus* was down-regulated in hyposaline stress, and the other up-regulated in hypersaline stress (though with a weak *P*-value of 0.066). In addition, under hyposaline conditions, we observed a strong up-regulation of a proline dehydrogenase gene (Additional data file 3), an enzyme responsible for the degradation of proline, which is known as a compatible osmolyte in higher plants [59] and diatoms [60]. Degrading proline under conditions of low salinity might help *E. siliculosus* to reduce the osmotic pressure between the intracellular and extracellular medium. Finally, a possible role of urea as a compatible osmolyte was suggested in diatoms [61]. *E. siliculosus* possesses the complete urea cycle and genes encoding arginases were up-regulated in hypersaline conditions. As arginases catalyze the last reaction of this cycle - that is, the degradation of arginine to ornithine and urea - their up-regulation supports the hypothesis of urea as a compatible osmolyte in heterokonts. There are, however, other or additional possible roles of arginases: in higher plants, for example, arginases have been shown to play a regulatory role in nitric oxide metabolism, increasing both the synthesis of proline and polyamines [62], both of which, in turn, are part of their osmotic stress response [59]. Additional experiments addressing the question of the possible compatible osmolytes in brown algae - for example, metabolite profiling - are now required to further test these hypotheses.

Stress response genes with unknown functions

Although our study has revealed several major themes underlying the abiotic stress response of *E. siliculosus*, we should not forget that this analysis was based on only a subset of the genes that actually changed expression. Our manual analysis of the 966 of the most significantly regulated genes has shown that 53.7% of these genes, to date, have no known homologs in current databases, including diatoms, and for another 12.6% there is no indication of their function, even though homologs exist in other organisms. This demonstrates both the discovery potential working with *E. siliculosus* and the amount of work that lies ahead for the phycological community.

Conclusions

In this study, which presents the first large-scale transcriptomic study within the brown algal lineage, we have developed and compiled the tools and protocols necessary to perform microarray experiments in the emerging model brown alga *E. siliculosus*, and used these tools to study the transcriptional response to three different stresses. Our results show that *E. siliculosus* undergoes large-scale transcriptomic reprogramming during the short-term acclimation to abiotic stress. The observed changes include several modifications to transcription, translation, amino acid metabolism, protein turnover, and photosynthesis, and indicate a shift from primary production to protein and nutrient recycling.

Although *E. siliculosus* shares many stress responses with flowering plants, for example, the induction of genes involved in vesicular trafficking, some classical stress responses, such as the induction of several ROS scavengers, could not be observed. On the other hand, our data highlighted many novel reactions such as the up-regulation of several genes coding for chlorophyll a/c binding proteins or the regulation of a large percentage of unknown genes, many of which are unique to *E. siliculosus*. In particular, the latter result, that is, that the functions of two-thirds of the regulated genes are unknown, underlines the fact that many of the molecular mechanisms underlying the acclimation to environmental stresses in brown algae are still entirely unknown. Understanding these mechanisms is a challenge that will still require much research, and our study provides a valuable starting point to approach this task.

Materials and methods

Growth conditions, stress treatments, and measurements of osmolarity and Na^+ concentration

E. siliculosus (Dillwyn) Lyngbye (Ectocarpales, Phaeophyceae) unialgal strain 32 (accession CCAP 1310/4, origin San Juan de Marcona, Peru) was cultivated in 10-liter plastic flasks in a culture room at 14°C using filtered and autoclaved natural seawater enriched in Provasoli nutrients [63]. Light was provided by Philips daylight fluorescence tubes at a photon flux density of 40 μmol m^{-2} s^{-1} for 14 h per day. Cultures were bubbled with filtered (0.22 μm) compressed air to avoid

CO_2 depletion. Ten days before the stress experiments, tissues were transferred to ASW with the following ion composition: 450 mM Na^+, 532 mM Cl^-, 10 mM K^+, 6 mM Ca^{2+}, 46 mM Mg^{2+}, 16 mM SO_4^{2-}.

Three different stress media were prepared based on ASW. For hyposaline stress, ASW was diluted to 12.5% of its original concentration with distilled water, resulting in a final NaCl concentration of 56 mM. For hypersaline stress, ASW with a final concentration of 1.47 M NaCl was used. For oxidative stress, H_2O_2 (30% w/w; Sigma-Aldrich, St. Louis, MO, USA) was added to the ASW immediately before beginning the stress experiment at a final concentration of 1 mM. Identical quantities of Provasoli nutrients were added in each of these media.

In order to monitor the intensity of a stress, we measured the quantum yield, a fluorometric marker for the photosynthetic efficiency, using a Walz Phyto-pulse amplitude modulation fluorometer (Waltz, Effeltrich, Germany) and default parameters (actinic light intensity 3, approximately 90 $\mu E\ m^{-2}\ s^{-1}$; saturation pulse intensity 10, approximately 2,000 $\mu E\ m^{-2}\ s^{-1}$, 200 ms) before harvesting the cultures.

The stress experiment was started by filtering 20 liters of ASW-acclimated *E. siliculosus* and transferring approximately 4 g of tissue each to 20 flasks (5 replicates per condition) containing 1 liter of one of the three stress media or the control medium (ASW with Provasoli nutrients). After 6 h the content of each flask was harvested by filtration, dried with a paper towel, and immediately frozen in liquid nitrogen.

Immediately after harvesting, about 300 mg (wet weight) of sample were thoroughly ground at room temperature (RT) and centrifuged for 1 minute at 16,000 g. Both the supernatant and a sample of the culture medium were then used to measure the concentration of osmolytes employing a freezing point depression osmometer (Osmometer Type 15, Löser Messtechnik, Berlin, Germany), and to determine the intracellular concentration of Na^+ with a FLM3 flame photometer (Radiometer, Copenhagen, Denmark).

Sequence preparation and array design

The 90,637 EST sequences used for the microarray design were cleaned using Phred [64] (trim-cutoff 0.05) and SeqClean and assembled using TGICL [65] and default parameters. Forty-one sequences that had been removed by Phred were re-included in the dataset, because they had significant BLAST hits with known eukaryotic proteins. In addition, 231 *E. siliculosus* virus 1 (Es-V1) sequences and 21 genomic intron sequences were included in the design. All assembled sequences are available directly from our homepage [22] and the ESTs have been deposited in public databases [EMBL: FP245546-FP312611]. Relevant accession numbers are listed in Additional data file 8.

Four 60-mere probes were designed for 17,119 of the 17,332 sequences (132 genes are not represented on the array) by Roche NimbleGen (Madison, WI, USA) and synthesized on 4-plex arrays with 72,000 features per hybridization zone. Roche NimbleGen also carried out cDNA labeling and hybridization as part of their gene expression array service.

Automatic annotation and correspondence table

All sequences were automatically annotated with KEGG orthology (KO) numbers using KOBAS [37] and with GO terms [66] using GOPET [36]. Protein sequences corresponding to the assembled EST sequences were then predicted using ORF predictor [67]. The automatic annotation of these sequences yielded 2,383 and 3,148 annotated sequences, respectively. As 37.5% of the cDNA sequences that were represented on the array contained mainly, or exclusively, 3' untranslated region sequence, their function could not be assessed directly. In these cases the corresponding genome sequence was annotated, yielding an additional 1,047 KO and 2,743 GO annotations. The correspondence table used to relate the assembled ESTs to supercontigs was generated by blasting all of the assembled EST sequences against the full *E. siliculosus* genome (coding and non-coding sequences) and selecting the best hit (best identity, longest alignments). Wherever this best hit was part of a predicted coding sequence (CDS), the corresponding CDS was chosen. In cases where the hit region was upstream of only one CDS, this CDS was chosen. In some cases the best hit was located upstream of two CDSs on opposite strands. Here the closest CDS was selected, if the distance to the closer CDS was half as long as or shorter than that to the other CDS. Otherwise no corresponding genome sequence was selected. In total, 3,430 (20%) of all represented genes were annotated with KEGG and 5,891 (34%) were annotated with GO terms.

Sample preparation, hybridization and verification

RNA was extracted from approximately 100 mg (wet weight) of tissue following Apt *et al.* [68] with modifications as described by Le Bail *et al.* [28], using a cetyltrimethylammonium bromide (CTAB)-based buffer and subsequent phenol-chloroform purification, LiCl-precipitation, and DNAse (Turbo DNAse, Ambion, Austin, TX, USA) steps. RNA quality and quantity was then verified on 1.5% agarose gel stained with ethidium bromide and a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). For four of the five flasks, mRNA was isolated from the total RNA using the PolyATtract® mRNA Isolation System III (Promega, Madison, WI, USA). These samples were concentrated in a SpeedVac concentrator (Savant, Ramsy, MN, USA) and again quantified using the NanoDrop. One replicate was used to verify if our procedure would directly work from total RNA.

Double-stranded cDNA was synthesized and amplified with the SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA) and the M-MuLV reverse transcriptase (Finnzymes,

Espoo, Finland) starting from 30 ng of mRNA or 100 ng of total RNA. In this kit, the first strand of cDNA is synthesized using an oligo(dT) primer with an attached SMART priming site. The terminal C-transferase activity of the reverse transcriptase will create an oligo(dC) tail at the 5' end of each mRNA, which is used to add a second SMART priming site. The two priming sites are then used to produce the second strand of the cDNA and to amplify it by PCR according to the Clontech protocol, using the Advantage2 polymerase (Clontech). The optimal number of amplification cycles was determined by semi-quantitative PCR, and ranged between 20 and 25 for our samples.

The PCR reactions were purified by first vortexing with one volume of phenol:chloroform:isoamyl alcohol (25:24:1), then by precipitating the aqueous phase with 0.5 volume of 7.5 M NH_4OH, 6 μg of nuclease-free Glycogen (Ambion), and 2.4 volumes of ethanol. After centrifugation at RT (20 minutes at 14,000 g), the pellet was washed with 70% EtOH, centrifuged (10 minutes at 14,000 g and RT) and resuspended in 14 μl of H_2O. To finish, cDNAs were once more quantified and checked on an agarose gel to ensure that they met the requirements of Roche NimbleGen for hybridization (concentration >250 μg l^{-1}, $A_{260/280} \geq 1.7$, $A_{260/230} \geq 1.5$, median size ≥ 400 bp).

RT-qPCR validation of the microarray was performed as described by Le Bail *et al.* [28].

Statistical analysis

Expression values were generated by Roche NimbleGen using quantile normalization [69], and the Robust Multichip Average algorithm [70,71].

To increase the power of subsequent statistical tests, non-expressed genes were removed from the dataset. This was done by comparing the raw expression values of each gene with those of the random probes included in the array design by Roche NimbleGen. Most of the random probes (>99.66%) had raw expression values inferior to 450, so all genes that had raw expression values over 450 in one of the replicates of the different experimental conditions were considered expressed. The probability p' of labeling a non-expressed gene as being expressed can be calculated according to the laws of a binomial distribution. Since 16 hybridizations were considered, for our dataset p' equals 5.3%, meaning that we have theoretically removed 94.7% of all genes without detectable expression.

The most stable genes were defined by ranking the genes according to the sum of squares of the log2-ratios of all stress conditions with the control. Differentially expressed genes were identified in the reduced dataset by *t*-test using TigrMEV 4.1 [72] with subsequent calculation of the FDR according to Benjamini and Hochberg [73]. Clustering was performed on the log2-ratios of all expressed genes with the control, considering all mRNA replicates using TigrMEV 4.1. A k-means algorithm [74], 'Euclidian distance', and 'average linkage' were selected. The ideal number of clusters for our dataset (k = 7) was determined using a figure of merit graph [75]. After clustering, the different replicates were averaged to generate the expression graphs. Both the clusters and the over-expressed and repressed genes identified by the *t*-test (FDR 10%) were analyzed to identify over-represented groups of genes. Over-expressed KEGG categories were identified using the KOBAS web-site [37] and a binomial test. Over-represented GO terms were identified using the GO Local Exploration Map (GOLEM) software (version 2.1) [76] and the Benjamini and Yekutieli algorithm to determine the FDR [77]. Additionally, the 966 genes that showed the most significant changes in expression (that is, genes that meet both criteria: significance at an FDR <1% and a relative change in expression compared to the control of more than twofold) were annotated and grouped manually.

Stress response genes with unknown functions

Genes were considered unknown stress response genes if they significantly changed expression in at least one stress treatment compared to the control (FDR <1%, more than twofold change compared to the control) and when, for both the assembled EST sequence itself and for the corresponding genome sequence, no BLAST hits were found in either the NCBI databases or among the known heterokont genomes (*Phytophthora sojae*, *P. ramosum*, *Thalassiosira pseudonana*, and *Phaeodactylum tricornutum*). Where homologs (e-value < 1e-10) were found, but these homologs had no functional annotations, genes were considered conserved unknown stress response genes. To group these genes, all unknown stress response genes and conserved unknown stress response genes were blasted against themselves using the NCBI BLAST program (version 2.2.18) [78] and a cut-off e-value of 1e-10. If there were several alignments between two genes, only the alignment with the lowest e-value was considered. Self-hits were removed. All groups of genes with homologs among the (conserved) unknown stress response genes in *E. siliculosus* were then visualized using Cytoscape 2.6.0 [79]. Their subcellular localizations were identified using HECTAR [80] and transmembrane domains were searched for using TMAP [81].

Data deposition

Microarray data have been deposited in a public database [ArrayExpress:E-TABM-578].

Abbreviations

ASW: artificial sea water; CDS: coding sequence; EST: expressed sequence tag; FDR: false discovery rate; GO: Gene Ontology; GOPET: GO-term Prediction and Evaluation Tool; HSP: heat shock protein; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOBAS: KEGG Orthology-Based Annotation System; NCBI: National Center for Biotechnology

Information; ROS: reactive oxygen species; RT: room temperature; RT-qPCR: real time quantitative PCR; SNARE: soluble N-ethylmaleimide-sensitive factor attachment receptor.

Authors' contributions

SMD, together with and under supervision of TT, performed the experiments, analyzed the data and wrote the manuscript, with help from CB and JMC; DS, BS and JLP have generated cDNA libraries (normalized and non-normalized); CDS was involved in EST data treatment and data formatting for sequence submissions; EC helped assemble the ESTs and set up the internet site; RK and KHG provided the GOPET annotations; MD modified ArrayLIMS and EMMA to work with the Roche NimbleGen arrays; JMC, PR, YVDP, and LS were involved in genome-wide gene annotation; all authors read and approved the manuscript.

Additional data files

The following additional data are available with the online version of this paper: a figure showing the recovery of the photosynthetic efficiency (quantum yield) in *E. siliculosus* cultures after 24 hours of stress treatment (Additional data file 1); a table listing the KEGG pathways and GO terms identified to be over-represented among the transcripts significantly different in the mRNA and total-RNA-derived samples (Additional data file 2); a table listing the 966 most regulated (FDR <0.01) and the 100 most stable genes on the microarray in all stress conditions (Additional data file 3); a table listing the KEGG pathways identified to be over-represented among the transcripts significantly up- or down-regulated in the different stress conditions (Additional data file 4); a table listing the GO categories identified to be over-represented in the different conditions at a FDR of 10% (Additional data file 5); description of the unknown stress response genes identified in this study (Additional data file 6); a table listing the C5-epimerase-genes represented on the microarray and their expression profiles (Additional data file 7); accession numbers of all sequences used in the design of the *E. siliculosus* microarray (Additional data file 8).

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3.3

Metabolite changes in response to short-term saline and oxidative stress

Integrative analysis of metabolite and transcript abundance during the short-term response to abiotic stress in the brown alga *Ectocarpus siliculosus.*

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Abstract

Brown algae constitute an important functional group of the benthic vegetation in the intertidal zone. A recent study has shown that the brown alga *Ectocarpus siliculosus* undergoes extensive transcriptomic changes in response to abiotic stress, many of them related to primary metabolism and in particular amino acid metabolism.

In this study we seek to combine transcriptomic data with selected metabolite analysis to improve our knowledge of the mechanisms underlying the stress tolerance of brown algae, in particular with regard to compatible osmolytes. To this end, we performed extensive metabolic profiling (urea, amino acids, sugars, polyols, organic acids and fatty acids) of *Ectocarpus* samples subjected to hyposaline, hypersaline, and oxidative stress.

Our results show that the most pronounced changes in terms of metabolite concentration occur under hypersaline stress. Both mannitol and proline were accumulated in response to salt stress, and their possible role in osmoprotection or osmoregulation is discussed. Urea was not detected in any of our samples. Furthermore, we provide evidence for a shift in fatty acid composition from n-3 to n-6 fatty acids and, for the first time in brown algae, demonstrate the salt stress-induced accumulation of small amounts of γ-aminobutyric acid (GABA). We propose that GABA could be synthesized in *Ectocarpus* through a salt-stress induced putrescin-degradation pathway.

Key words: abiotic stress response, brown algae, amino acid metabolism, gamma-aminobutyric acid (GABA), polyunsaturated fatty acids, osmolytes, proline, urea.

Introduction

Brown algae are an important component of coastal ecosystems as they are the dominant vegetation in the intertidal and sub-tidal zone. They can be found from tropical to Polar regions, and some species form large kelp forests providing habitats for thousands of other species. Being sessile, intertidal seaweeds must be able to withstand many stresses such as desiccation, sunlight, UV-radiation, changes in salinity (rain, evaporation), mechanical stress (waves), as well as anthropogenic stresses such as heavy metal pollution. The capacity of algae to tolerate different types of abiotic stress has been related to their distribution within the intertidal zone (reviewed in Davison & Pearson 1996). Despite the broad scientific interest in physiological responses of algae to environmental stresses, the biochemical pathways involved and the underlying genetics were largely unexplored. The sequencing of algal genomes including the *Ectocarpus siliculosus* genome has enabled a new suite of approaches in the search for pathways involved in stress tolerance.

Recently, we have started to gather increasing amounts of transcriptomic data on the adaptation of seaweeds to their environments (Roeder et al. 2005, Collén et al. 2007, Dittami et al. 2009), but only few studies have additionally examined changes in metabolites in response to stress. Within the heterokont lineage, Krell et al. (2007) examined gene expression levels and the accumulation of proline in the salinity stressed Antarctic diatom *Fragilariopsis cylindris*, and Ritter et al. (2008) evaluated the expression of genes and the production of oxylipins in copper-stressed individuals of the brown alga *Laminaria digitata*. However, very little is known about how transcriptomic changes translate into changes in metabolites in algae. This is especially noteworthy as several recent comparative studies in terrestrial plants have demonstrated that gene expression profiles do not always correlate directly with protein translation (Branco-Price et al. 2008), enzyme activity (Gibon et al. 2004, 2006), and metabolite concentrations (Kaplan et al. 2007, Kempa et al. 2008). As metabolites play essential roles in the stress response both for signaling and for protection, comparative metabolite and gene expression studies are now necessary to further improve our understanding of the abiotic stress response in algae.

Here, we examined changes in metabolite profiles in the cosmopolitan brown alga *Ectocarpus siliculosu*s (Charrier et al. 2008) upon exposure to mild hyposaline, hypersaline and oxidative stress. We took advantage of our previous transcriptomic

study (Dittami et al. 2009) to link metabolic with transcriptomic changes, and to examine how large-scale transcriptomic reprogramming, which was to a large extent related to pathways associated to primary metabolism, translated into changes in metabolite concentrations. Moreover, we tested several hypotheses about accumulation of possible compatible osmolytes such as urea and proline. Finally, this study permitted the discovery of the accumulation of new stress-related metabolites, such as γ-aminobutyric acid (GABA), which was not predicted by genomic / transcriptomic analyses, and detected only in traces under normal growth conditions in *E. siliculosus* (chapter 2.2).

Materials and Methods

Culture conditions

Ectocarpus siliculosus (Dilwyn) Lyngbye (Ectocarpales, Phaeophyceae) unialgal strain 32 (accession CCAP 1310/4, origin San Juan de Marcona, Peru) was cultivated in 10 L plastic flasks in a culture room at 14 °C using filtered and autoclaved seawater (SW) with a salinity of 33 psu enriched in Provasoli nutrients (Starr & Zeikus 1993). Light was provided by Philips daylight fluorescence tubes at a photon flux density of 40 μmol m^{-2} s^{-1} for 14 hours per day. Cultures were aerated with filtered (0.22 μm) compressed air to avoid CO_2 depletion. One week before the experiment, about 0.5 g (fresh weight) of algae per condition and replicate (three replicates per condition) were transferred to individual Petri dishes containing 100 ml of fresh culture medium.

Experimental setup

Stress experiments were started about 30 minutes after the beginning of the light phase by replacing the culture media with pre-prepared stress media. In order to be able to compare this study with a previous transcriptomic study (Dittami et al. 2009), identical stress conditions were chosen. For hyposaline stress, 33 psu SW was diluted to 12.5 % of its original concentration with distilled water (i.e. final salinity: 4 psu). For hypersaline stress, 60 g NaCl were added per liter of 33 psu SW (i.e. final salinity: 93 psu). For oxidative stress, H_2O_2 (30 % w/w, Sigma-Aldrich, St. Louis, MO, USA) was added immediately before beginning the stress experiment at a final concentration of 1 mM. For the control condition, 33 psu SW was used. Identical quantities of Provasoli nutrients were added to each of these media.

Transcriptomic changes were monitored after 6 h in our previous study (Dittami et al. 2009). In this report, two time points (6 h and 24 h) were examined as transcriptomic changes might also translate into variation of metabolites later. Approximately 50 % of the biomass of each setup were harvested by filtration after 6 h, and the remaining biomass was harvested after 24 h incubation time in the conditions described above. All samples were quickly dried on a paper towel and immediately frozen in liquid nitrogen.

Metabolite analysis

For amino acid, sugar, polyol, organic acid, and urea analysis, samples were ground in liquid nitrogen and freeze-dried. Amino acids, non-structural carbohydrates, and organic acids were extracted and quantified as follows: between 10.0 and 12.0 mg of freeze-dried sample, corresponding to approximately 50 mg fresh weight, were weighted and ground using a ball mill. The powder was suspended in 400 µL of a methanolic solution containing 100 µM of DL-3-aminobutyric acid and 200 µM ribitol, followed by 15 min of agitation at room temperature. 200 µL of chloroform were added, followed by a 5-min agitation step. Finally, 400 µL of water were added, and samples were vortexed vigorously before centrifugation at 13,000 g for 5 min. 50 µL and 200 µL aliquots of the upper phase, which contained polar metabolites including amino acids, polyols and sugars, were transferred to clean vials and vacuum-dried for subsequent chromatographic analysis.

For amino acid profiling, the vacuum-dried polar phase aliquots were resuspended in 50 µL of ultra-pure water, and 10 µL was used for the derivatization using the AccQ-Tag Ultra derivatization kit (Waters). Derivatized amino acids were analyzed using an Acquity UPLC system (Waters) according to Jubault et al. (2008), using DL-3-aminobutyric acid as internal standard.

For gas chromatography / mass spectrometry (GC-MS) profiling of non-structural carbohydrates and organic acids, the 200 µL vacuum-dried polar phase aliquots were resuspended in 40 µL of 20 g L^{-1} methoxyamine-hydrochloride (Sigma-Aldrich) in pyridine before incubation under orbital shaking at 30°C for 1h30. After addition of one volume of N,O-bis(trimethylsisyl)trifluoroacetamide (Sigma-Aldrich), samples were incubated 37 min at 30°C, transferred to glass vials, and incubated at room temperature overnight before injection. GC-MS analysis was performed according to Roessner et al. (2001). The GC-MS system consisted of a TriPlus autosampler, a

Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). Chromatograms were deconvoluted using the AMDIS software v2.65 (http://chemdata.nist.gov/mass-spc/amdis/) associated with NIST libraries. Metabolite levels were quantified using ribitol as internal standard and by comparison with individual external standards.

In addition, **mannitol** concentrations were confirmed by an alternative method, based on an extraction in 1 ml of 70% aqueous ethanol (v/v) at 70°C for 4 h according to Karsten et al. (1991). After centrifugation for 5 min at 5,000 g, 700 µL of the supernatant were evaporated to dryness under vacuum, re-dissolved in the same volume of distilled water, and analyzed with an isocratic Agilent HPLC system equipped with a differential refractive index detector, a BioRad resin-based column (Aminex Fast Carbohydrate Analysis, 100 x 7.8 mm) and a Phenomenex Carbo-Pb2+ (4 x 3 mm) guard cartridge. Mannitol was eluted with water at a flow rate of 1.0 ml min^{-1} at 70°C, identified by comparison of the retention time with those of a commercial standard prepared as 1 mM aqueous solution, and quantified by peak area.

Urea was determined following the protocol outlined by Beale and Croft (1961). Thirty mg (dw) of sample were resuspended in 500 µl of zinc/sodium sulphate solution (9 mM $ZnSO_4$, 84 mM Na_2SO_4). Then 7.5 µl of 1 M NaOH were added, and the mixture was thoroughly vortexed for two minutes before centrifugation for 10 minutes at 20,000 g. Two times 120 µl (technical replicates) of supernatant were mixed with 1 volume of DAM-PAA reagent (1 volume of 1% w/v diacetylmonoxime in 0.02 % acetic acid, 1 volume of phenylanthranilic acid in 20 % v/v ethanol with 120 mM $NaCO_3$). One ml of activated acid phosphate (1.3 M NaH_2PO_4, 10 mM $MnCl_2$, 0.4 mM $NaNO_3$, 0.2M HCl, in 31 % v/v H_2SO_4) was added before incubation in boiling water for 12 minutes. The tubes were left to cool at room temperature and centrifuged for 1 min at 20,000g before measuring absorption at 535 nm using a spectrophotometer. A standard curve was created using 0, 50, 100, 250 nmol urea dissolved in 10 µl of distilled water instead of the freeze-dried samples. Some *Ectocarpus* samples spiked with 50, 100, and 250 nmol urea were also included as positive controls to test for possible urease activity in the extract.

For the determination of **total fatty acids**, approximately 400 mg (fresh weight) of sample were ground in liquid nitrogen and extracted with 2 ml of ethyl acetate as

previously described (Küpper et al. 2006). 250 ng of 12-OH-lauric acid was added as internal standard. Extracts were evaporated under a stream of nitrogen, resuspended in 100 µL ethanol and analyzed by GC-MS as previously described by Ritter et al. (2008). Fatty acids were measured only for the 6 h data point.

All metabolite concentrations were calculated per gram dry weight, except fatty acids which were determined as percentage of total fatty acids. As *E. siliculosus* is known to accumulate high concentrations of NaCl in response to salt stress, dry weights were corrected for the quantity of NaCl in each sample. This quantity was calculated based on the ratio dry weight to fresh weight (on average 20.1 %) and the intracellular Na^+ concentrations determined by flame photometry as described in our previous study (Dittami et al. 2009). We assumed that changes in Na^+ also correspond to changes in Cl^-. All analyses were performed both with and without this correction. Although the observed differences between the two analyses were only quantitative and not qualitative, we chose to show the corrected data as we believe it to be biologically more relevant.

Gene expression data

Gene expression data for enzymes possibly explaining the observed changes in metabolite concentrations were obtained from previously published transcriptomic experiments carried out under the same stress conditions (Dittami et al. 2009). This data set is publicly available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-TABM-578.

Statistical analysis

An Analysis of Variance (ANOVA) with stress and time as categorical predictors was applied to the non-structural carbohydrate and the amino acid data. The latter data was square root transformed prior to the ANOVA in order to comply with a normal distribution. A separate ANOVA was performed to compare each of the three stress conditions to the control condition and a Bonferroni correction was applied. Fatty acid data was analyzed using a t-test instead of the ANOVA, as only one time point (6 h) was measured. All tests were performed using Statistica 7 (Statsoft, USA). Microarray data were analyzed as previously described (Dittami et al. 2009), using t-tests and log2-transformed data.

Results

Non-structural carbohydrate concentrations except mannitol are stable

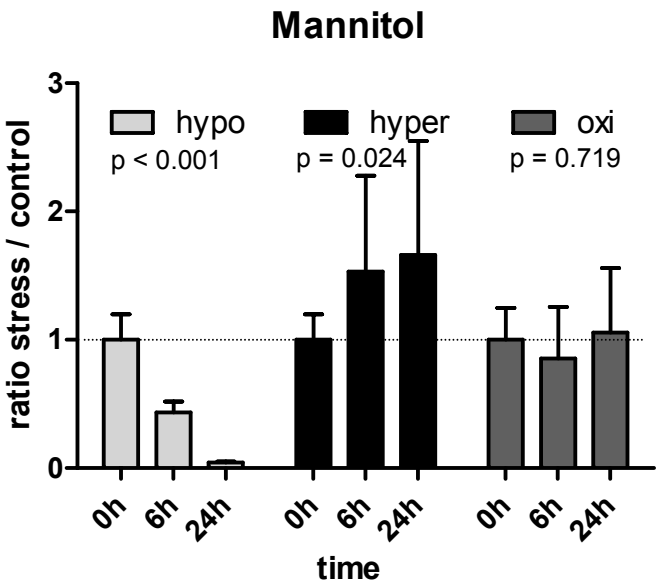

Figure 1: Changes in the mannitol concentration in response to abiotic stress. The graph shows the ratio of mannitol in the stress to the control condition (mean of three replicates ± SD). Changes can be considered significant with an overall alpha-error < 0.05 if p < 0.017. Hypo = hypoosmotic stress, hyper = hyperosmotic stress, oxi = oxidative stress.

We were able to reliably quantify glycolate, glycerol, succinate, glycerate, fumarate, malate, citrate, isocitrate, and glucose. The concentration of these compounds ranged from a few nmol g^{-1} (DW) for malate and fumarate to over 22 µmol g^{-1} (DW) for citrate. None of these compounds exhibited significant changes in response to stress. Their concentrations are listed in supplementary file 1. The concentration of mannitol varied between 15 and 300 µmol g^{-1} (DW) in all experimental conditions: it decreased strongly (by 95 % after 24 h, Figure1) under hyposaline stress, and increased, under hypersaline stress (approximately 60 % after 24 h, Figure 1). These changes in mannitol concentration are in agreement with the 6-fold down-regulation of the strongly expressed mannitol 1-phosphate dehydrogenase 1 (Esi0017_0062) under hyposaline stress and the 5-fold up-regulation of the mannitol 1-phosphate dehydrogenase 2 (Esi0020_0181) under hypersaline stress reported previously (Dittami et al. 2009).

Change in the n-3 to n-6 ratio of polyunsaturated fatty acids in hypersaline conditions

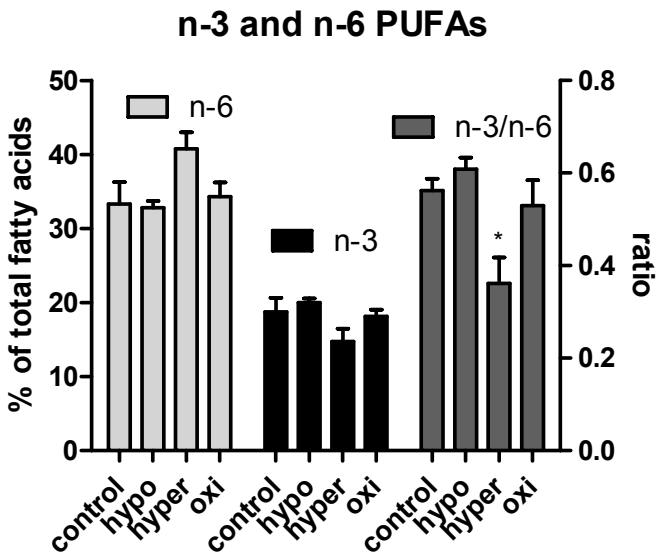

Figure 2: Changes in the ratio of n-3 to n-6 fatty acids in response to abiotic stress (6 h incubation time). The graph shows the proportion of n-3 and n-6 PUFAs of total fatty acids (mean of three replicates ± SD). * = overall alpha error < 0.05, hypo = hypoosmotic stress, hyper = hyperosmotic stress, oxi = oxidative stress.

None of the fatty acids examined changed significantly between control and stress conditions after correcting for mutliple testing (see supporting information file 2 for a complete profile of fatty acids). However, when considering the total amount of n-3 and n-6 polyunsaturated fatty acids (PUFAs), we observed a significant decrease in the ratio of n-3 to n-6 PUFAs in hypersaline conditions (Figure 2).

The *Ectocarpus* genome contains four genes possibly involved in these changes. Two of them are delta-12 desaturases, one microsomal (Esi0207_0012) and one chloroplastic (Esi0393_0016), as well as two delta-15 desaturases, again one microsomal (Esi0231_0023) and one chloroplastic (Esi0073_0116). Delta-12 desaturases were generally up-regulated under stress, while delta-15 desaturases were generally down-regulated (Table 1). Interestingly, the genes coding for microsomal desaturases were regulated exclusively under hypersaline stress, while chloroplast copies were regulated in all stress conditions.

Almost all examined amino acids change concentrations

The summarized results for the amino acid profiling are shown in Table 2. More detailed information is available for most amino acids in the context of metabolic pathways and the expression profiles of the genes involved in these pathways in Figures 4 – 7. Complete names of the enzymes in these figures as well as more detailed expression profiles can be found in Table 1.

Table 1: Expression profiles of genes of interest after 6 h of exposure to hyposaline (hypo), hypersaline (hyper), and oxidative (oxi) stress. All values are means of four replicates; "x" = relative fold-change between control and stress condition, "u" = up-regulated, "d" = down-regulated. "-*" = not regulated (< 2-fold change compared to control and p > 0.05), "**" = signal below detection limit

Fatty acid metabolism

	Enzyme	Genome	Array	Hypo	Hyper	Oxi
	microsomal Δ15 desaturase	Esi0231_0023	CL527Contig1	-*	4.35 X d, p=0.016	-*
	chloroplastic Δ15 desaturase	Esi0073_0116	CL281Contig1	15.90 X d p= 0.013	4.09 X d p = 0.009	2.77 X d, p = 0.002
	microsomal Δ12-desaturase	Esi0207_0012	LQ0AAB83YE13RM1.SCF	-*	*2.08 X u, p=0.069*	-*
	chloroplastic Δ12-desaturase	Esi0393_0016	CL69Contig1	2.93 X u, p = 0.029	17.5 X u, p = 0.004	*2.44 X u, p = 0.096*

Primary amino acids

	Enzyme	Genome	Array	Hypo	Hyper	Oxi
a	Glutamate dehydrogenase (EC 1.4.1.2)	Esi0141_0079	LQ0AAB28YN11FM1.SCF	*9.72 X d, p = 0.141*	*2.88 X d, p = 0.150*	2.97 X d, p = 0.008
b	Glutamate dehydrogenase (EC 1.4.1.4)	Esi0028_0164	CL1656Contig1	93.8 X d, p < 0.001	*2.94 X d, p = 0.129*	9.33 X d, p = 0.031
			CL2561Contig1	39.7 X d, p = 0.001	*1.37 X d, p = 0.042*	3.75 X d, p < 0.001
		Esi0520_0002	not on array			
c	Alanine transaminase (EC 2.6.1.2)	Esi0298_0027	CL5054Contig1	-*	*2.25 X d, p = 0.104*	3.72 X d, p = 0.041
			LQ0AAB85YA11FM1.SCF	*6.36 X d, p = 0.088*	2.34 X d, p = 0.017	2.13 X d, p = 0.024
			LQ0ACA20YC15RM1.SCF	2.45 X d, p = 0.002	-*	*1.72 X d, p = 0.016*
d	Aspartate transaminase (EC 2.6.1.1)	Esi0298_0018	CL6772Contig1	7.76 X d, p < 0.001	*1.87 X d, p = 0.008*	-*
			CL1725Contig1	*7.28 X d, p = 0.054*	4.74 X d, p = 0.010	-*
		Esi0128_0018	CL2218Contig1	3.86 X d, p < 0.001	4.25 X d, p = 0.011	2.24 X d, p = 0.011
			LQ0AAB97YD02FM1.SCF	**	**	**
		Esi0002_0157	CL2216Contig1	6.92 X d, p = 0.037	-*	-*
			CL5691Contig1	4.84 X d, p = 0.021	-*	-*
e	Glutamine synthetase (EC 6.3.1.2)	Esi0072_0092	not on array			
		Esi0188_0004	CL44Contig1	3.33 X d, p = 0.003	-*	-*
			LQ0AAB88YP13FM1.SCF	**	**	**
		Esi0188_0005	CL44Contig3	7.11 X d, p < 0.001	3.42 X d, p = 0.001	2.66 X d, p = 0.002
			CL44Contig4	**	**	**
f	Glutamate synthase	Esi0349_0025	not on array			
	Glutamate synthase (ferredoxin) (EC 1.4.7.1)	Esi0000_0281	CL2669Contig1	34.07 X d, p = 0.006	-*	7.64 X d, p = 0.029
	Glutamate synthase (NADH/NADPH) (EC 1.4.1.14/13)	Esi0029_0131	CL5303Contig1			
			CL4828Contig1			
			LQ0AAB39YD12FM1.SCF	6.34 X d, p = 0.007	-*	-*
	Glutamate synthase (NADH/NADPH) (EC 1.4.1.14/13)	Esi0103_0010+	CL598Contig1	-*	2.51 X u, p = 0.022	2.05 X u, p = 0.034
g	Asparagine synthase (glutamine-hydrolyzing) (EC 6.3.5.4)	Esi0156_0013	CL7424Contig1	46.3 X d, p = 0.003	*1.66 X d, p = 0.003*	-*
			LQ0AAB80YJ06FM1.SCF	**	**	**
h	Nitrate reductase (NADH) (EC 1.7.1.1)	Esi0171_0046	LQ0AAB91YI21FM1.SCF	4.68 X d, p = 0.016	2.88 X d, p = 0.068	-*
i	Nitrate reductase (NADH) (EC 1.7.1.1)	Esi0006_0124	CL3329Contig1	1.73 X u, p = 0.024	3.50 X d, p = 0.002	3.36 X d, p = 0.001
j	Nitrite reductase (ferredoxin) (EC 1.7.7.1)	Esi0003_0129	CL3736Contig1	*2.17 X d, p = 0.062*	*1.70 X u, p = 0.004*	3.75 X d, p = 0.014
			CL5351Contig1	-*	-*	*3.07 X d, p = 0.062*
k	Nitrite reductase (NAD(P)H) (EC 1.7.1.4)	Esi0249_0028	CL2999Contig1	*7.28 X d, p = 0.050*	2.09 X d, p = 0.016	2.74 X d, p < 0.001
			LQ0AAB8YN04FM1.SCF	3.68 X d, p < 0.001	*1.36 X d, p = 0.027*	2.85 X d, p = 0.003

Branched-chain amino acids

	Enzyme	Genome	Array	Hypo	Hyper	Oxi
1	Homoserine kinase (EC 2.7.1.39)	Esi0031_0156	CL2119Contig1	16.75 x d, p < 0.001	3.84 x d, p = 0.028	5.68 x d, p < 0.001
2	Threonine synthase (EC 4.2.3.1)	Esi0427_0005	LQ0AAA8YI24FM1.SCF	**	**	**
			LQ0AAB41YH24FM1.SCF	2.99 x d, p = 0.015	5.68 x u, p = 0.006	*2.01 x d, p = 0.096*
3	Threonine desaminase (EC 4.3.1.19)	Esi0028_0036	CL2987Contig1	*2.53 x d, p = 0.264*	2.34 x u, p = 0.012	-*
4	Pyruvate dehydrogenase (EC 1.2.4.1)	Esi0022_0141	CL753Contig1	-*	-*	-*
5	Acetolactate synthase (EC 2.2.1.6)	Es_cpDNA_44	not on array			
6	Ketol acid reductoisomerase (EC 1.1.1.86)	Esi0021_0135	CL2264Contig1	3.2 x d, p = 0.004	-*	*1.79 x d, p = 0.003*
			CL3085Contig1	5.58 x d, p < 0.001	*1.36 x d, p = 0.048*	-*
			LQ0AAB71YG03FM1.SCF	**	**	**
7	Dihydroxy-acid dehydratase (EC 4.2.1.9)	Esi0027_0145+	CL3439Contig1	260.9 x d, p < 0.001	4.67 x d, p < 0.001	-*
		Esi0027_0149	not on array			
8	Isopropylmalate synthase (EC 2.3.3.13)	Esi0009_0202	CL7572Contig1	3.64 x d, p = 0.009	6.44 x d, p = 0.005	2.53 x d, p = 0.002
			LQ0AAB2YH21FM1.SCF	15.89 x d, p = 0.031	42.57 x d, p < 0.001	3.33 x d, p = 0.021
		Esi0020_0048	CL1050Contig1	2.99 x d, p < 0.001	*1.59 x d, p = 0.008*	-*
			LQ0AAB58YL15FM1.SCF			
9	3-isopropylmalate dehydratase (EC 4.2.1.33)	Esi0069_0026	CL1061Contig1	6.03 x d, p < 0.001	-*	2.08 x d, p < 0.001
10	3-Isopropylmalate dehydrogenase (EC 1.1.1.85)	Esi0047_0054	CL1186Contig1	2.64 x d, p = 0.020	-*	*1.75 x d, p = 0.013*
			LQ0AAB23YA11FM1.SCF	-*	3.6 x d, p = 0.006	-*
			LQ0AAB35YI12FM1.SCF	-*	2.58 x d, p = 0.053	-*

Table 1 (continued)

11	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	Esi0081_0023	CL583Contig1	-*	-*	-*
			CL892Contig1	2.57 x d, p = 0.007	-*	-*
		Esi0023_0001	not on array			
		Esi0081_0028+	LQ0AAB43YF24FM1.SCF	3.81 x d, p = 0.032	4.8 x d, p < 0.001	-*
			LQ0AAB91YD06FM1.SCF	**	**	**
12	Leucyl-tRNA Synthetase (EC 6.1.1.4)	Esi0087_0038	CL900Contig1	76.89 x d, p = 0.002	-*	4.57 x d, p = 0.007
13	Isoleucyl-tRNA Synthetase (EC 6.1.1.5)	Esi0252_0019	LQ0AAB83YG21FM1.SCF	-*	*3.26 x d, p = 0.055*	*2.17 x u, p = 0.254*
		Esi0479_0014	CL2581Contig1	7.48 x d, p = 0.002	-*	2.78 x d, p = 0.042
14	Valine--tRNA ligase (EC 6.1.1.9)	Esi0078_0096	LQ0ADA22YH06RM1.SCF	3.62 x d, p = 0.027	10.33 x d, p < 0.001	*3.01 x d, p = 0.107*
		Esi0287_0021	CL2779Contig1	9.47 x d, p < 0.001	3 x d, p = 0.002	-*
15	3-methyl-2-oxobutanoate dehydrogenase (EC 1.2.4.4)	Esi0006_0214	CL3651Contig1	4.37 x d, p = 0.027	19.39 x u, p = 0.005	*2.7 x u, p = 0.099*
			LQ0AAB7YC16FM1.SCF	**	**	**
		Esi0358_0029	LQ0AAB58YB01RM1.SCF	13.45 x u, p < 0.001	22.84 x u, p < 0.001	*2.42 x u, p = 0.058*
			CL4476Contig1	**	**	**
			LQ0AAB58YB01FM1.SCF	**	**	**
			LQ0AAB67YK04FM1.SCF	**	**	**
16	Dihydrolipoyl transacylase (EC 2.3.1.168)	Esi0358_0027	CL5443Contig1	7.51 x u, p < 0.001	15.26 x u, p < 0.001	3.04 x u, p = 0.009
17	Isovaleryl-CoA dehydrogenase (EC 1.3.99.10)	Esi0063_0084	LQ0AAB43YD20FM1.SCF	**	**	**

Aromatic Amino acids

	Enzyme	Genome	Array	Hypo	Hyper	Oxi
I	3-deoxy-7-phosphoheptulonate synthase (2.5.1.54)	Esi0145_0051	CL686Contig1	2.18 x d, p < 0.001	3.93x d, p = 0.001	-*
II	3-Dehydroquinate Synthase (EC 4.2.3.4)	Esi0013_0196	LQ0AAB94YN08FM1.SCF	**	**	**
			LQ0ADA18YA11RM1.SCF	8.08 x d, p = 0.019	-*	3.20 x d, p = 0.002
III	Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase	Esi0082_0083	not on array			
IV	Shikimate Kinase (EC 2.7.1.71)	Esi0082_0019	CL506Contig1	6.58 x d, p = 0.011	-*	2.48 x d, p < 0.001
V	3-Phosphoshikimate 1-Carboxyvinyltransferase (EC 2.5.1.19)	Esi0137_0084	CL760Contig1	2.08 x d, p = 0.018	-*	*1.89 x d, p = 0.025*
VI	Chorismate Synthase (EC 4.2.3.5)	Esi0031_0082	CL2988Contig1	6.76 x d, p = 0.007	-*	2.06 x d, p = 0.011
			CL5658Contig1	**	**	**
VII	Anthranilate synthase b (EC 4.1.3.27)	Esi0346_0021	CL6510Contig1	2.44 x d, p = 0.034	-*	-*
			CL6004Contig1	**	**	**
			LQ0AAA10YG05FM1.SCF	**	**	*
VIII	Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	Esi0003_0266	CL3265Contig1	*1.97 x d, p = 0.012*	2.18 x d, p < 0.001	*1.73 x d, p = 0.009*
			CL6929Contig1	*6.89 x d, p = 0.055*	4.76 x d, p = 0.044	5.47 x d, p = 0.007
IX	Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	Esi0281_0043f	not on array			
X	Indole-3-glycerol phosphate synthase (EC 4.1.1.48)	Esi0000_0449	LQ0ACA17YC18RM1.SCF	6.46 x d, p = 0.032	*1.19 x u, p = 0.036*	2.1 x d, p = 0.005
		Esi0048_0063	not on array			
		Esi0107_0059	*CL7372Contig1*	10.5 x d, p < 0.001	-*	3.17 x d, p = 0.007
			CL6576Contig1	81.58 x d, p = 0.003	-*	*2.22 x d, p = 0.161*
		Esi0281_0043	not on array			
XI	Tryptophan synthase (EC 4.2.1.20)	Esi0060_0108	not on array			
		Esi0036_0051	CL5627Contig1	5.21 x d, p = 0.003	3.22 x d, p = 0.011	-*
			CL7646Contig1	**	**	**
XII	Trifunctional Chorismate Mutase (EC 5.4.99.5) /	Esi0000_0583	CL3990Contig1	*1.37 x d, p = 0.037*	*1.88 x d, p = 0.008*	-*
	Prephenate Dehydratase (EC 4.2.1.51) /		LQ0AAB89YC12FM1.SCF	6.21 x d, p = 0.025	5.74 x d, p = 0.038	*2.36 x d, p = 0.083*
	Prephenate Dehydrogenase (EC 1.3.1.12)	Esi0000_0583	not on array			
		Esi0000_0583	not on array			
XIII	Aspartate Aminotransferase (EC 2.6.1.1)	Esi0002_0157	CL2216Contig1	6.92 x d, p = 0.037	-*	-*
			CL5691Contig1	4.85 x d, p = 0.022	-*	-*
		Esi0298_0018	CL6772Contig1	7.76 x d, p = 0.004	*1.87 x d, p = 0.008*	-*
			CL1725Contig1	*7.28 x d, p = 0.054*	4.74 x d, p = 0.010	-*
			LQ0AAB56YO18RM1.SCF	3.15 x d, p = 0.002	-*	-*
		Esi0128_0018	CL2218Contig1	3.85 x d, p < 0.001	4.25 x d, p = 0.002	2.24 x d, p = 0.011
			LQ0AAB97YD02FM1.SCF	**	**	**
XIV	Phenylalanine--tRNA ligase (EC 6.1.1.20)	Esi0036_0043	CL1330Contig1	-*	-*	-*
		Esi0177_0023	CL3236Contig1	**	**	**
			LQ0AFA6YM10RM1.SCF	-*	-*	*2.07 x d, p = 0.197*
		Esi0028_0168	LQ0AAB104YE19FM1.SCF	*16.99 x d, p = 0.057*	4.37 x d, p = 0.003	3.29 x d, p = 0.016
			LQ0AAB82YF17FM1.SCF	13.62 x d, p = 0.012	6.7 x d, p < 0.001	5.14 x d, p = 0.001
XV	Tyrosine--tRNA ligase (EC 6.1.1.1)	Esi0203_0047	CL6318Contig1	-*	2.42 x d, p = 0.043	-*
XVI	Tryptophan--tRNA ligase (EC 6.1.1.2)	Esi0005_0190	LQ0AAB2YD23FM1.SCF	-*	56.09 x d, p < 0.001	-*
			LQ0AAA21YE03FM1.SCF	**	**	**

Table 1 (continued)

Photorespiration

	Enzyme	Genome	Array	Hypo	Hyper	Oxi
RUBISCO	Ribulose-bisphosphate carboxylase (EC 4.1.1.39)	Esi0159_0007	CL2899Contig1	2.89 X d, p =0.006	*1.67 X d, p = 0.011*	3.85 X d, p = 0.002
		Esi0038_0042	not on array			
PGP	phosphoglycolate phosphatase (EC 3.1.3.18)	Esi0130_0079	CL1132Contig1	23.46 X d, p = 0.044	*1.64 x u, p = 0.048*	8.20 X d, p= 0.022
GOX	Glycolate Oxidase (EC 1.1.3.15)	Esi0012_0104	CL317Contig1	19.72 X d, p = 0.002	5.73 X u, p < 0.001	-*
		Esi0017_0040	LQ0AAB30YI03FM1.SCF	**	**	**
ALT	peroxisomal alanine aminotransferase (EC 2.6.1.2)	Esi0008_0209	CL1607Contig1	14.29 X d, p < 0.001	7.01 X u, p < 0.001	2.89 X d, p = 0.004
			CL1579Contig1	**	**	**
GCS	Glycine cleavage system H protein	Esi0046_0032	CL352Contig1	4.34 X d, p = 0.022	2.30 X u, p = 0.001	-*
			LQ0AAB66YG13FM1.SCF	-*	5.88 X u, p = 0.010	-*
	Glycine-cleavage system T-protein (EC 2.1.2.10)	Esi0046_0033	CL651Contig1	19.83 X d, p = 0.038	3.23 X u, p = 0.004	-*
			CL873Contig1	**	**	**
	Glycine cleavage system P-protein (EC 1.4.4.2)	Esi0221_0023	CL815Contig1	-*	-*	-*
			CL7429Contig1	**	**	**
			CL5922Contig1	**	**	**
			LQ0AAB42YO08FM1.SCF	**	**	**
			LQ0AAB52YM08FM1.SCF	**	**	**
			LQ0AAB90YF22FM1.SCF	-*	6.70 X u, p = 0.016	*2.29 X d, p = 0.240*
	Glycine-cleavage system L-protein (EC 1.8.1.4)	Esi0322_0005	CL4512Contig1	**	**	**
			LQ0AAA14YI06FM1.SCF	**	**	**

Urea cycle, GABA and proline

	Enzyme	Genome	Array	Hypo	Hyper	Oxi
A	Ornithine carbamoyltransferase (EC 2.1.3.3)	Esi0361_0020	CL594Contig1	11.82 X d, p < 0.001	-*	2.19 X d, p = 0.016
B	argininosuccinate synthase (EC 6.3.4.5)	Esi0127_0062	LQ0AAB34YG09FM1.SCF	25.58 X d, p = 0.002	3.37 X d, p = 0.006	-*
			CL5673Contig1	19.75 X d, p = 0.032	1.86 X d, p = 0.026	-*
C	Argininosuccinate lyase (EC 4.3.2.1)	Esi0081_0083	LQ0ADA3YJ12RM1.SCF	3.33 X d, p < 0.001	3.45 X d, p < 0.001	-*
D	arginine--tRNA ligase (EC 6.1.1.19)	Esi0003_0287	CL497Contig1	*2.53 X d, p = 0.098*	4.22 X d, p = 0.005	1.49 X u, p = 0.006
			CL7349Contig1	*3.41 X d, p = 0.059*	11.28 X d, p = 0.008	4.86 X u, p = 0.012
			LQ0AAB86YK18FM1.SCF	*2.33 X d, p = 0.257*	4.49 X d, p = 0.041	8.29 X u, p = 0.023
E	Arginase (EC 3.5.3.1)	Esi0073_0060	LQ0AAB28YG22FM1.SCF	19.54 X u, p < 0.001	4.01 X u, p = 0.004	4.92 X u, p < 0.001
F	Urease (EC 3.1.5.1)	Esi0000_0248	LQ0AAB14YK16FM1.SCF	*2.25 X d, p = 0.097*	3.73 X u, p = 0.031	-*
G	Ornithine decarboxylase (EC 4.1.1.17)	Esi0048_0123	not on array			
H	diamin oxidase (EC 1.4.3.22)	Esi0076_0063	CL5550Contig1	3.58 X d, p = 0.017	14.49 X u, p < 0.001	-*
		Esi0076_0061	not on array			
I	Aldehyde dehdrogenases	Esi0010_0069	CL5294Contig1	7.84 X d, p = 0.004	3.44 X u, p = 0.002	-*
	(highly homologous to	Esi0451_0004	LQ0AAB34YA24FM1.SCF.LQ	-*	6.69 X u, p = 0.027	-*
	aminobutyraldehyde dehydrogenase)	Esi0003_0206	CL6453Contig1	**	**	**
J	ornithine--oxo-acid transaminase (EC 2.6.1.13)	Esi0072_0109	LQ0AAB88YC13RM1.SCF	5.14 X u, p = 0.004	*3.40 X u, p = 0.057*	3.45 X u, p = 0.013
			LQ0AAB10YP23FM1.SCF	4.63 X u, p = 0.016	5.63 X u, p = 0.013	4.99 X u, p = 0.018
K	pyrroline-5-carboxylate reductase (EC 1.5.1.2)	Esi0075_0064	CL6298Contig1	-*	2.58 X d, p = 0.001	4.86 x d, p = 0.002
			LQ0AAB36YB24FM1.SCF	10.47 X u, p = 0.006	*4.01 X u, p = 0.098*	-*
			LQ0AAB96YG16FM1.SCF	-*	4.16 X d, p = 0.003	13.69 X d, p = 0.002
L	proline dehydrogenase (EC 1.5.99.8)	Esi0269_0010	LQ0AAB31YD17FM1.SCF	80.43 X u, p < 0.001	-*	-*
M	1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12)	Esi0418_0003	CL2518Contig1	-*	11.78 X u, p < 0.001	-*
N	1-pyrroline-5-carboxylate synthase	Esi0663_0002	CL1823Contig1	52.81 X d, p = 0.003	*1.44 X d, p = 0.029*	3.82 X d, p = 0.001

* not regulated (< 2-fold change compared to control and p > 0.05)

** signal below detection limit

Generally, we observed that most amino acids (with a few exceptions) exhibit similar profiles in response to all of the tested stress conditions, although the most pronounced changes in amino acid abundance were clearly observed in the hypo- and hypersaline conditions. Under oxidative stress, especially after 24 h, only minor changes were observed.

Decrease of the predominant amino acids in response to stress

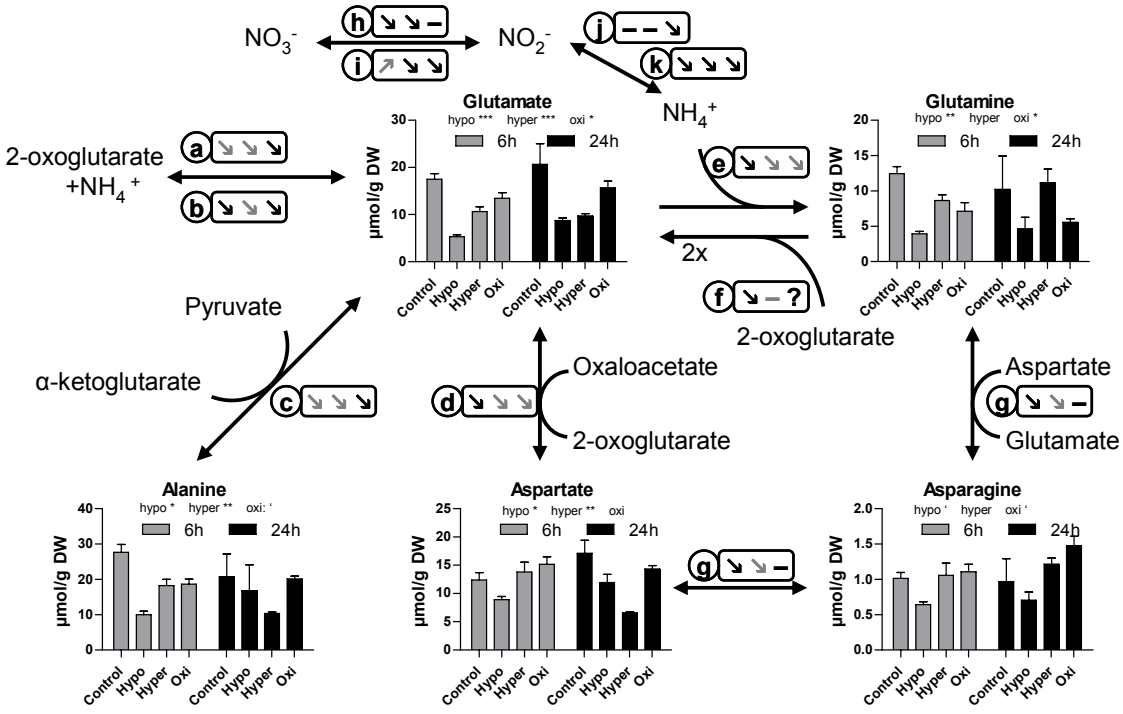

Figure 3: Synthesis of primary amino acids (and asparagine). Bars represent the mean quantity of an amino acid ± SD. Letters correspond to enzymes, as indicated in Table 1.The three symbols next the letters summarize the relative expression of this gene after 6 h of hyposaline (hypo), hypersaline (hyper), and oxidative stress (oxi) in this order. "↗" indicates that a gene was up-regulated, "↘"that a gene was down regulated, "–" that there was no detectable regulation on a transcript level (< 2-fold change and $p > 0.05$), and "?" that no conclusion can be drawn (see Table 1). Grey arrows indicate a tendency of this gene to be regulated, which was either not significant (change < 2-fold or $p > 0.05$), or not all probes followed the same profile. The statistical significance of metabolite changes (after a Bonferroni correction) is indicated for each stress condition under the name of the metabolite (' = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

The predominant amino acids (alanine, glutamate, glutamine, and aspartate), together accounting for 77.3 % of the detected amino acids under control conditions, decreased in response to all of the different stressors (Figure 3), but most strongly in response to hyposaline stress. They were the main reason for the decrease in the total free amino acid concentration in response to hyposaline stress (Table 2), where glutamate and glutamine, for example, decreased by 70 % after 6 hours. The changes in the

concentration of the predominant amino acids correspond well to the expression profiles of the genes involved in their synthesis or conversion: practically all of these genes were also down-regulated (Table 1).

Table 2: Changes in the contents of amino acids and related compounds. This graph shows the relative variations in amino acid concentration as percentage with the control condition (mean of three replicates ± SD). 6 h and 24 h samples were tested in the same ANOVA with time (6 h, 24 h) and treatment (stress, control) as predictors. Significant effects of each treatment are marked in boldface ($p < 0.05$, after Bonferroni correction).

		hypersaline stress		hypersaline stress		oxidative stress	
	% of AAs	6h	24h	6h	24h	6h	24h
Total AAs	100.0%	**51% ± 3%**	**71% ± 15%**	78% ± 7%	83% ± 5%	80% ± 7%	82% ± 3%
Alanine	30.5%	**36% ± 4%**	**81% ± 36%**	**66% ± 6%**	**50% ± 2%**	67% ± 5%	97% ± 4%
Glutamate	19.3%	**30% ± 2%**	**42% ± 3%**	**61% ± 6%**	**56% ± 3%**	**77% ± 7%**	**76% ± 7%**
Glutamine	13.8%	**32% ± 3%**	**46% ± 15%**	69% ± 7%	90% ± 15%	**57% ± 10%**	**54% ± 5%**
Aspartate	13.7%	**72% ± 5%**	**70% ± 8%**	**111% ± 14%**	**53% ± 2%**	123% ± 11%	84% ± 4%
Asparagine	1.1%	63% ± 4%	73% ± 12%	104% ± 17%	120% ± 8%	109% ± 10%	152% ± 14%
Threonine	0.9%	**173% ± 10%**	**184% ± 54%**	**129% ± 13%**	**176% ± 10%**	125% ± 4%	95% ± 3%
Valine	1.7%	**123% ± 8%**	**165% ± 25%**	117% ± 8%	139% ± 20%	108% ± 6%	98% ± 9%
Leucine	0.4%	**247% ± 18%**	**179% ± 5%**	**232% ± 19%**	**343% ± 21%**	**159% ± 5%**	**102% ± 10%**
Isoleucine	0.3%	**192% ± 12%**	**152% ± 2%**	**210% ± 18%**	**283% ± 18%**	**150% ± 5%**	**97% ± 8%**
Phenylalanin	0.6%	**120% ± 12%**	**131% ± 8%**	**146% ± 23%**	**176% ± 11%**	**130% ± 10%**	**104% ± 4%**
Tyrosine	0.3%	203% ± 38%	108% ± 6%	**167% ± 23%**	**278% ± 40%**	165% ± 34%	117% ± 27%
Tryptophane	0.2%	**145% ± 15%**	**153% ± 2%**	**139% ± 27%**	**173% ± 15%**	118% ± 19%	95% ± 12%
Arginine	0.1%	188% ± 14%	81% ± 8%	**323% ± 36%**	**526% ± 46%**	**163% ± 4%**	**100% ± 11%**
Ornithine	0.1%	218% ± 208%	73% ± 16%	109% ± 43%	241% ± 91%	128% ± 9%	52% ± 12%
GABA	0.0%	130% ± 91%	71% ± 28%	**365% ± 125%**	**451% ± 49%**	117% ± 26%	80% ± 25%
Proline	1.9%	68% ± 6%	118% ± 54%	**310% ± 19%**	**1047% ± 94%**	99% ± 6%	78% ± 5%
Serine	4.7%	114% ± 9%	125% ± 44%	58% ± 8%	119% ± 15%	**71% ± 7%**	**74% ± 4%**
Glycine	7.7%	**35% ± 2%**	**64% ± 21%**	**19% ± 3%**	**26% ± 4%**	**57% ± 8%**	**59% ± 3%**
Homoserine	0.7%	**83% ± 8%**	**63% ± 11%**	95% ± 16%	73% ± 7%	**83% ± 3%**	**75% ± 5%**
Lysine	0.5%	151% ± 16%	98% ± 20%	215% ± 11%	272% ± 11%	**174% ± 15%**	**109% ± 4%**
Cysteine	0.4%	103% ± 13%	92% ± 121%	66% ± 55%	124% ± 14%	130% ± 16%	244% ± 25%
Methionine	0.4%	63% ± 26%	48% ± 16%	80% ± 31%	152% ± 36%	92% ± 32%	90% ± 44%
Histidine	0.1%	169% ± 227%	15% ± 1%	192% ± 137%	52% ± 6%	36% ± 4%	44% ± 55%

Accumulation of aromatic and branched chain amino acids in response to stress

Aromatic (phenylalanine, tyrosine, and tryptophane), and branched chain amino acids (valine, leucine, isoleucine), constitute only 1.1 and 2.4 % of the total amino acids, respectively. Unlike the major amino acids, their intracellular concentration, together with that of threonine, consistently increased in response to the applied stress (Figure 4, Figure 5). This increase was strongest under hyposaline and hypersaline stress where their concentration in response to stress ranged from more than 108 % to over 300 % of the concentration in the control condition.

This is particularly interesting as only a single gene involved in aromatic and branched chain amino acid synthesis (a threonine desaminase (3), Figure 4) was up-

regulated under hypersaline stress, and most other genes were down-regulated (Figure 4, 5; Table 1). Moreover, genes involved in the catabolism of branched chain amino acids were clearly induced. T-RNA-ligases were generally down-regulated in response to stress for all amino acids.

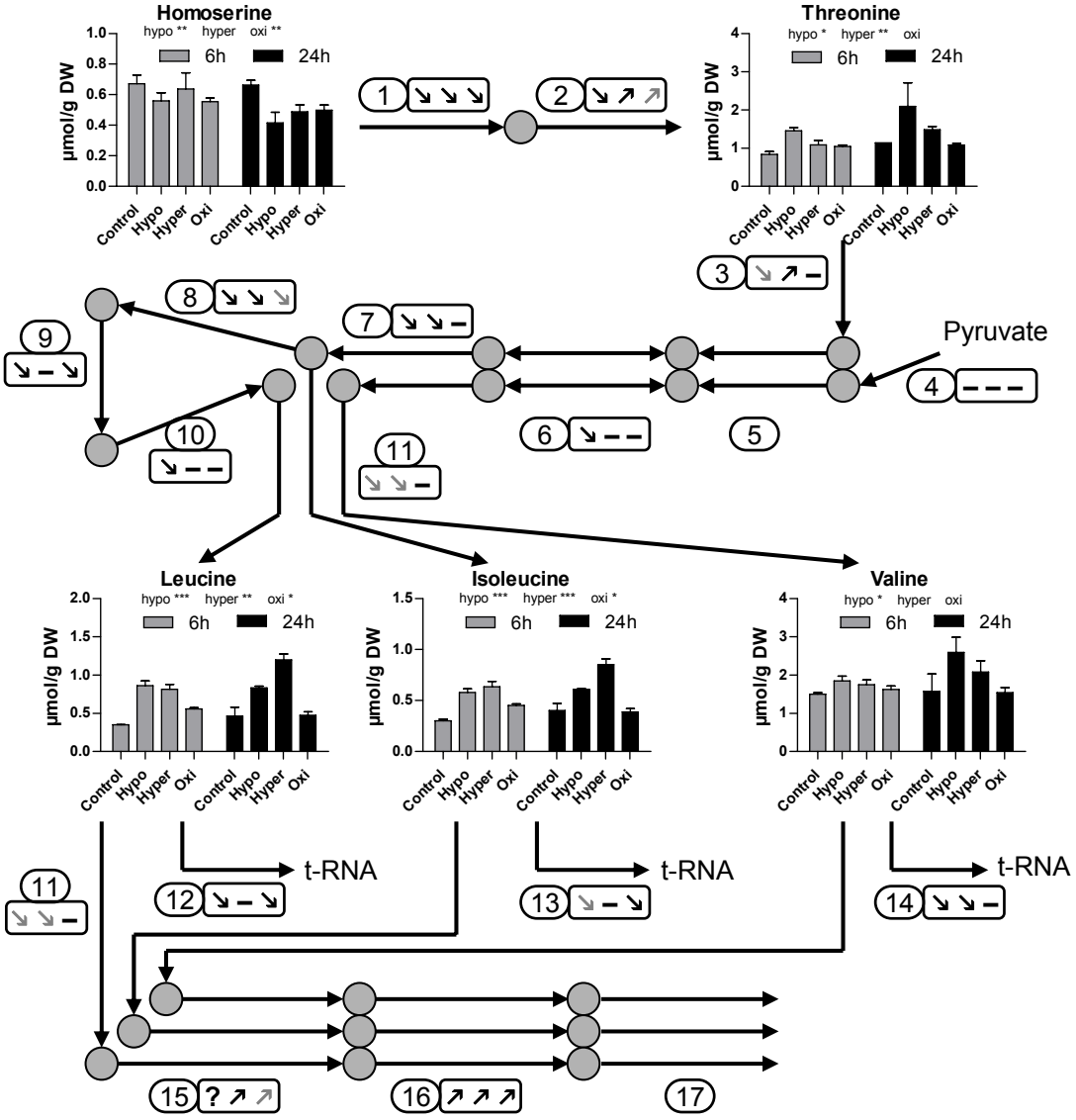

Figure 4: Biosynthesis and degradation of branched chain and related amino acids. Please refer to Figure 3 for a detailed description of all symbols used.

Decrease of glycine concentrations in response to stress

Glycine, serine, and in particular the ratio of glycine to serine, are considered a common marker of photorespiration in terrestrial plants (Foyer et al. 2003). In our study, we observed a strong decrease in the ratio of glycine to serine under hypersaline and hyposaline stress, and a slight decrease in the oxidative stress

condition (Figure 6), which was likely to be a milder stressor than the saline stresses (Dittami et al. 2009). As for the accumulation of aromatic and branched chain amino acids, this does not correlate well with the transcriptomic data, which showed genes involved the photorespiratory pathway to be generally down-regulated in hyposaline and oxidative stress conditions, and up-regulated under hypersaline stress (Figure 6, Table 1). As glycine might serve as a substrate for glutathione synthesis, we also investigated the expression of the two gamma glutamyl-cysteine synthetases (EC 6.3.2.2) and of the single copy of a glutathione synthetase (EC 6.3.2.3) in the *Ectocarpus* genome. One of the glutamyl-cysteine synthetases (Esi0184_0033 corresponding to LQ0AAB47YA16FM1.SCF on the microarray) was 1.9 to 3.9-fold down-regulated in all stress conditions, while the second copy (Esi0250_0012 corresponding to LQ0AAB39YO20FM1.SCF) was 2.6-fold up-regulated only in hypersaline conditions. The glutathione synthetase (Esi0066_0082, corresponding to CL1154Contig1), was not significantly regulated in any of the examined stress conditions ($p > 0.05$, fold-change < 2).

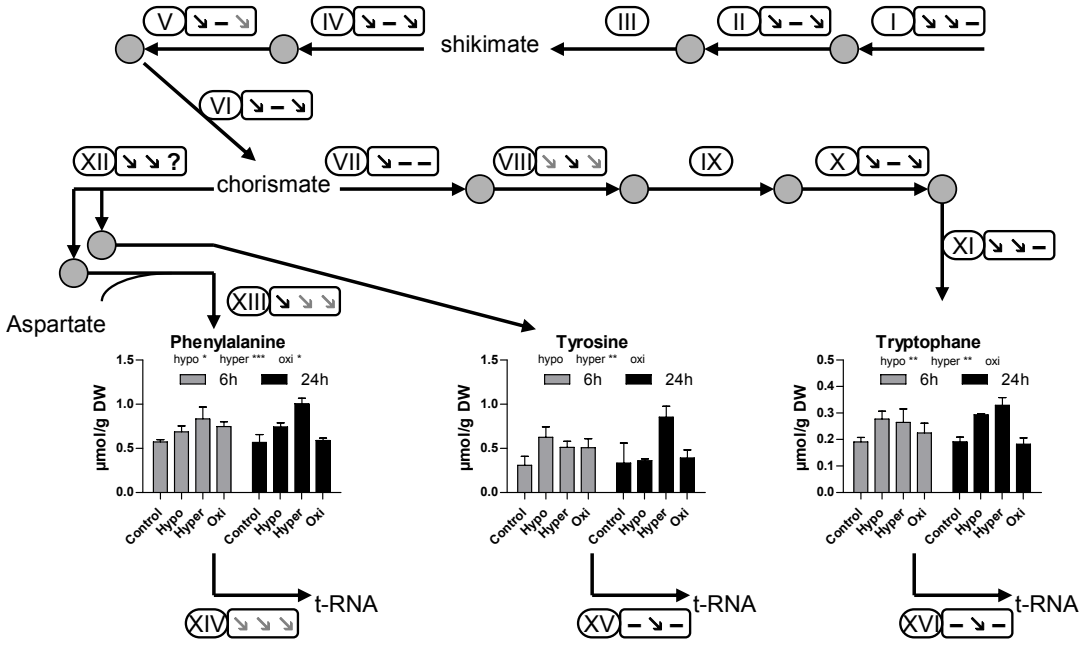

Figure 5: Biosynthesis of aromatic amino acids. Please refer to Figure 3 for a detailed description of all symbols used.

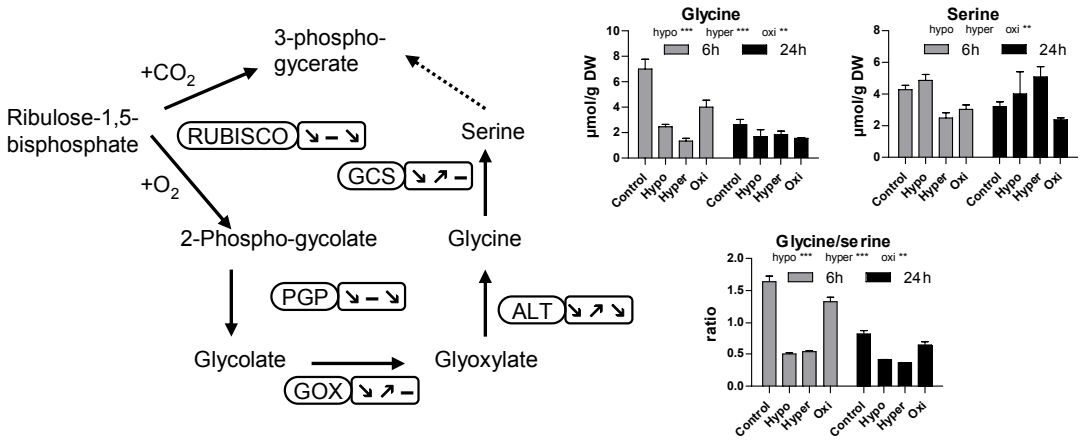

Figure 6: Genes and amino acids related to photorespiration. Please refer to Figure 3 for a detailed description of all symbols used.

GABA and proline accumulate specifically in hypersaline condition

The most marked changes in the amino acid concentrations was the increase of GABA (4.5-fold) and of proline (10-fold) under the hypersaline condition. This increase was parallel to an induction of genes involved in arginine degradation (Figure 7, Table 1). Moreover, a diamine oxidase ((H), Figure 7), potentially involved in conversion of putrescine to 4-amino-butyraldehyde (an immediate precursor for the synthesis of GABA) and an aminoaldehyde dehydrogenase ((I), Figure 7), potentially involved in the conversion of 4-amino-butyraldehyde to GABA (Sebela 2000), were significantly induced in hypersaline stress conditions.

Urea concentrations are below the detection limit

No traces of urea were detected in any of the samples, although spiked samples showed normal absorption comparable to those of our standards. We assume that our extraction method of thorough grinding in liquid nitrogen is sufficient to break the *Ectocarpus* cells – an assumption which is supported by the fact that nucleic acids are efficiently extracted using a similar protocol, and that centrifugation for non-freeze-dried but ground *E. siliculosus* leads to a separation of solid particles and intracellular medium after 5 min at 20,000 g. As our method showed clear differences between the 100 nmol standard or *Ectocarpus* samples spiked with 100 nmol urea and the blanks, we can conclude that urea, if present in *Ectocarpus*, is only present in quantities smaller than the detection limit of the method, i.e. 1.66 µmol per g dry weight.

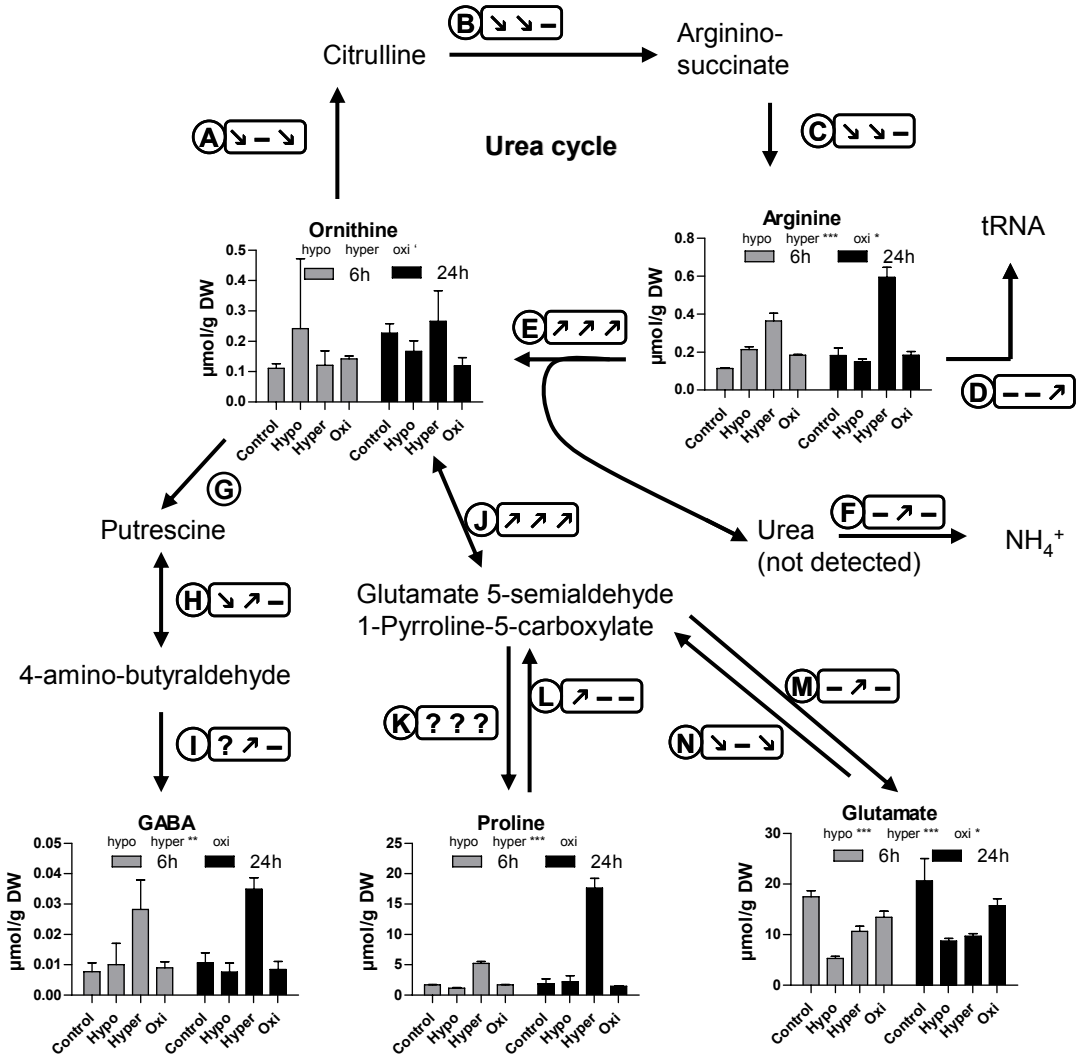

Figure 7: Concentrations of arginine, ornithine, glutamate, proline, and gamma-aminobutyric acid (GABA). Please refer to Figure 3 for a detailed description of all symbols used.

Discussion

This study demonstrates that, in response to abiotic stress, *Ectocarpus* exhibits a) no or only slight changes in the free sugars, polyols, and organic acids measured, except for mannitol, b) mild changes in fatty acid content, c) profound changes in the amino acid content and d) a strong relative increase in proline and GABA specifically in response to hypersaline stress. It is the first study of this scale in brown algae that relates metabolite changes directly to transcriptional changes, and provides an example of how, especially in complex metabolic networks, gene expression profiles might sometimes be misleading if not supported by other data on different levels (such as metabolites). This is consistent with previous studies in terrestrial plants, highlighting differences between total transcript abundance and polysomal mRNA

abundance (Branco-Price et al. 2008), enzyme activity (Gibon et al. 2004, 2006), and final metabolite content (Kaplan et al. 2007; Kempa et al. 2008). Our study, however, also shows the value of transcriptomic data for the interpretation of the observed metabolic changes, as will be demonstrated by the examples that follow.

Changes in the primary metabolism affect amino acid concentrations

Interestingly, the global down-regulation of primary metabolism in response to the tested stress conditions (Dittami et al. 2009) had no significant effect on the concentration of sugars or organic acids in our samples except mannitol, which is the main photosynthetic product in brown algae (Reed et al. 1985). As the overall concentration of these compounds in the cell was very low, it is reasonable to hypothesize that they serve mainly as intermediates in chemical reactions. Therefore, their cellular concentration would not necessarily change, even though the flow through a pathway might be affected by stress.

In contrast, we observed a decrease in the predominant amino acids (alanine, glutamate, glutamine, and aspartate), which was strongest under hyposaline stress (Table 2). These changes corresponded to the general down-regulation of genes involved in ammonia fixation and the synthesis of primary amino acids, both via the glutamate dehydrogenase- (enzymes h, i, a, b; Figure 3) and the GS/GOGAT pathway (enzymes h, i, e, f; Figure 3). Alanine, glutamate, and aspartate have been shown to be among the first amino acids to be synthesized from radio-labeled carbon in brown algae (Akagawa et al. 1972), and similar observations were made in terrestrial plants, e.g. in spinach leaves under mild salt stress (Di Martino et al. 2003). This is likely to be the consequence of a mechanism of conserving energy under stress conditions, when photosynthesis is less efficient (Kovtun et al. 2000).

With regard to aromatic and branched chain amino acids, this simple correlation did not hold true: while genes involved in synthesis of branched chain amino acids were down-regulated, the concentration of these amino acids increased in response to stress. A similar increase has been observed in terrestrial plants in response to abiotic stresses (e.g. Kaplan et al. 2004). The reason for the increase in the branched chain and aromatic amino acid concentration in spite of the down-regulation of genes involved in their synthesis is likely to be the general down-regulation of protein synthesis and an activation of autophagy and protein turnover, as previously described for these stress conditions (Dittami et al. 2009). Branched chain and aromatic amino

acids are present only in very small quantities in the cell (about 3.5 % of total free amino acids, Table 2), but likely to be comparatively abundant in the *Ectocarpus* proteome, as about 24 % of the codons in the predicted coding sequences of the *Ectocarpus* genome code for them. Thus, a reduction of their use for protein synthesis in response to stress, as indicated by a down-regulation of their corresponding aminoacryl t-RNA ligases, and their increased liberation by autophagy and protein degradation, provides a plausible explanation for their increase (Di Martino et al. 2003). The induction of the branched chain amino acid degradation pathway could represent a mechanism of keeping the intracellular levels of branched chain amino acids non-toxic, as proposed by Malatrasi et al. (2006).

Photorespiration

Photorespiration is especially evident under conditions of high light intensity, limiting carbon dioxide, and high oxygen concentrations. Under these circumstances an increased proportion of the total carbon fixed during photosynthesis flows through the glycolate pathway. Glycine is a key metabolite in this process and is synthesized in large amounts in the peroxisomes. It is rapidly oxidised and converted to serine in the mitochondria. In terrestrial plants, the ratio of glycine to serine is considered as a marker of photorespiration (Foyer et al. 2003), and in *Ectocarpus* this ratio has been shown to increase under carbon starvation and increased O_2 concentration, and to decrease in carbon-replete conditions (Gravot et al. submitted). The observed decrease in glycine and slight increase in serine in response to stress could therefore be considered an indication of a decrease in photorespiration. However, no correlation with genes involved in the photorespiratory pathway was observed. One possible explanation for these observations is that reduction of photorespiration in our study could be a direct consequence of a general reduction of photosynthesis and of the down-regulation of genes that code for parts of the light harvesting complex (Dittami et al. 2009). This would consequently reduce the amount of glycine produced by photorespiration, even if the relative rate of photorespiration stayed the same.

Another possibility is that the decrease in glycine, which accounted for most of the change in the glycine to serine ratio, was related to the synthesis of glutathione. Noctor et al. (1999) described that photorespiratory glycine can enhance gluthatione production in poplar, thus proving the existence of a metabolic link between these two pathways. Glutathione in turn is an antioxidant, which is likely to play an important

role in the response of *E. siliculosus* to several stresses (de Franco et al. 2009). Support for this hypothesis could be provided e.g. by the up-regulation of genes involved in glutathione synthesis in response to abiotic stress. Although, with the exception of one glutamyl-cysteine synthetase under hypersaline stress, such an up-regulation was not observed, further experimental evidence would be required to disprove the activation of this metabolic pathway. Furthermore, both hypotheses (glutathione synthesis and reduction of photosynthesis) could apply at the same time.

Changes in the total fatty acid composition

In parallel to these general changes in the amino acid composition, *Ectocarpus* displayed several changes specifically during hypersaline stress, such as a shift from n-3 to n-6 PUFAs (Figure 2), and corresponding changes in gene expression. One possible reason for this could be related to changes in the membrane lipid composition, as shown by Bigogno et al. (2002), who demonstrated that radioactively labeled arachidonic acid was rapidly integrated into polar lipids by the green alga *Parietochloris incisa*. Changes in the fatty acid composition of membranes are commonly observed in response to stress, mainly after temperature changes (e.g. Sanina et al. 2008). Aziz and Larher (1998), however, also reported a decrease of the overall fatty acid saturation in rape leaves after exposure to osmotic stress. It seems plausible that a change in membrane composition might be advantageous under hypersaline and hyperosmotic conditions. Furthermore, Mock et al. (2002) reported a decrease of the degree of unsaturation in the thylakoid membrane of Antarctic diatoms in response to light. This probably compensated for a parallel decrease in the number of membrane proteins, a phenomenon which could also occur in *Ectocarpus* in response to hypersaline stress, as photosynthesis was strongly affected in this condition.

The observed changes could also be related to the production of fatty acid-derived signaling compounds. Omega-6 PUFA-derived compounds are generally believed to be more effective signals than n-3 polyunsaturated fatty acid-derived ones (Sanina et al. 2008), a phenomenon well known in animals (e.g. Bagga 2003). Moreover, recent studies have reported the stress-induced production of fatty acid-derived oxylipins in the brown alga *Laminara digitata* (Ritter et al. 2008, Küpper et al. 2009), including several n-6 polyunsaturated fatty acid-derived compounds such as prostaglandins or hydroperoxy-octadecatrienoic acids. Further studies examining changes in the polar

lipid composition and oxylipin production in response to stress are needed to further decipher the biological significance of the observed changes in the fatty acid composition in *E. siliculosus*.

Proline and mannitol, but not urea, are potential osmoprotectants or local osmolytes

Other changes that occurred specifically in hypersaline stress were related to possible osmolytes. Our previous study (Dittami et al. 2009) demonstrated that the major solute responsible for the short-term adaptation of *Ectocarpus* to high salinities was NaCl. Expression profiles, however, suggested that additional osmolytes such as urea, mannitol, and proline might also play important roles.

In this study, urea was not detected in any of the examined conditions, strongly suggesting that this compound does not accumulate in *E. siliculosus* cells, and excluding a possible role as an osmolyte, at least within the first 24 h of adaptation to different salinities.

Proline, which is strongly accumulated in response to hypersaline stress, may be synthesized both from arginine via ornithine and from glutamate (Figure 7). While genes involved in the proline synthesis from arginine ((E, J), Figure 7) were induced under all stress conditions, the 1-pyrroline-5-carboxylate synthase gene ((N), Figure 7), involved in proline synthesis from glutamate, was not induced compared to the control condition in hypersaline stress, and was repressed under hyposaline and oxidative stress. Thus, our transcriptomic data do not provide clear indications as to which of these pathways is activated. However, an increased metabolic flow through the urea cycle towards proline (and GABA) under hypersaline stress would also result in an increase of urea production, providing a possible explanation for the up-regulation of the urease gene ((F), Figure 7) in the same condition.

In any case, the observed increase in proline concentrations in response to hypersaline stress would fit well with a role of this compound as osmolyte (Krell et al. 2007). Yet, even after 24 h, the overall proline concentration reaches only about 18 µmol per gram dry weight, which, considering that 1 g of dry weight corresponds roughly to 5 g of fresh weight, is equivalent to an average intracellular concentration of only about 4.5 mM. Facing changes in the extracellular osmolarity of about 2000 mOsmol L^{-1}, the contribution of proline to the adjustment of the intracellular osmolarity would be negligible, unless this osmolyte accumulates only in specific organelles. To a lesser

extent this is also true for mannitol, which, under the same assumptions, can account for about 75 mOsmol L^{-1} of the total intracellular osmolarity after 24 h of exposure to stress. Mannitol is presumably not evenly distributed throughout the cell but localized in the cytoplasm as has been proposed for marine brown algae (Davison & Reed 1985, Reed et al. 1985) and also the red macroalga *Caloglossa leprieurii* (Mostaert et al. 1995). Still, even if the cell vacuole, which usually takes up less than 50 % of the *E. siliculosus* cell volume (B. Charrier, personal communication), and the apoplast contain neither proline nor mannitol, our findings point to the conclusion that both compounds are not major compatible osmolytes during the short-term adaptation (≤ 24 h) to high salinities.

Nevertheless, the strong decrease in mannitol under hyposaline stress might be of physiological relevance during the short-term response to low salinities and both mannitol and proline might be important osmolytes for long-term adaptation (> 1 day). The latter was illustrated by Thomas and Kirst (1991), who observed mannitol concentrations of up to 100 mM after one week of exposure of *E. siliculosus* to high salinity. Furthermore, mannitol and proline might still be important in the short-term salt stress response, functioning as local osmolytes or osmoprotectors rather than global osmolytes. Both substances were reported to act as oxygen radical scavengers (Smirnoff & Cumbes, 1989), and to increase protein stability (Soderquist & Lee 2005, Takagi 2008).

GABA: a potential signaling compound

Similarly to *Arabidopsis* under saline stress (e.g. Kempa et. al. 2008), *Ectocarpus* exhibited a strong relative increase in GABA concentration under hypersaline stress compared to the control condition, although absolute levels remained low in *Ectocarpus* when compared to about 1 µmol g^{-1} DW classically observed in unstressed leaves of *Arabidopsis* (Gravot et al. submitted). GABA is closely related and structurally similar to proline, and is considered an important neurotransmitter in the mammalian central nervous system (Li & Xu 2008). It is also known to have many different functions in plants, including a central role in plant carbon and nitrogen metabolism (Fait et al. 2008), stress response (Kinnersley 2000), and signaling (Bouché & Fromm 2004). Under non-stressed conditions, GABA is present only in traces in *Ectocarpus*, and important genes of the major pathway of GABA synthesis, the GABA shunt, appear to be absent from the genome (Gravot et al.

submitted). However, in terrestrial plants, GABA can also be synthesized from polyamines (Terano 1978, Kumar & Thorpe 1989, Rastogi & Davies 1990, Petrivalský et al. 2007). Our data show that, parallel to the increase in GABA, genes likely to be involved in its synthesis from polyamines are also strongly up-regulated under hypersaline treatment.

Although the exact mechanism of GABA signaling is still unknown in terrestrial plants, the marked changes in both metabolite and transcript abundance suggest that, as in terrestrial plants, GABA plays a role in the response to (hypersaline) stress, and possibly in stress signaling in *Ectocarpus*. In this alga, however, we observe GABA induction only under hypersaline stress and not in hyposaline or oxidative stress, while in terrestrial plants GABA synthesis is induced in response to a wide variety of abiotic and biotic stresses (Kinnersley 2000).

From an evolutionary point of view, it is interesting to compare the occurrence of the pathways involved in the production of GABA in different lineages. GABA seems to have conserved similar functions in green plants, heterokonts, and to a certain extent also metazoans, although the main pathway of GABA synthesis in green plants and metazoans (the GABA shunt) has probably been lost in brown algae.

Conclusions

Our study demonstrated several metabolic changes in response to stress. While most of our results from targeted metabolite profiling correspond well to the changes observed in the transcriptome, thus generally validating the transcriptomic approach, our data also clearly demonstrate that caution needs to be taken when interpreting transcriptomic data, especially in the context of complex metabolic networks. In addition, our study invalidated the hypothesis of urea as compatible osmolyte in brown algae, and showed that, in the short-term response to salt stress, proline and mannitol are likely to function primarily as osmoprotectants rather than osmolytes. Finally, we demonstrated changes in PUFA composition and that, even though the primary pathway for GABA synthesis is not coded for in the *Ectocarpus* genome, GABA synthesis is likely to take place from putrescine in response to hypersaline stress. These findings pave the way for more detailed comparative studies of the role of these potential signaling molecules implicated in abiotic stress response in brown algae.

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Supplementary files

Supplementary file 1: concentrations of non-structural carbohydrates detected in our concentrated samples (all values given in µmol g^{-1} DW, mean of three replicates ± SE). This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

Supplementary file 2: Complete fatty acid profiles for the examined conditions. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

3.4

Stress-responsive fucoxanthin chlorophyll a/c binding proteins

Stress induced chlorophyll binding proteins in Heterokonts and *Chlamydomonas* – a relict from the past?

- preliminary manuscript -

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Abstract

Chlorophyll binding and related proteins constitute a family present in all photosynthetic organisms. In green plants, chlorophyll a/b binding proteins (CABs) function in light harvesting while three types of related proteins, namely early light induced proteins (ELIPs), high light induced proteins (HLIPs, first identified in cyanobacteria), and psbS proteins, protect from photooxidative stress and are involved in non-photochemical quenching.

Based on available transcriptomic data, ELIPs do not appear to play an important role in stress response in heterokonts, and sequenced diatom and brown genomes do not code for HLIPs or psbS. On the other hand, they do code for a large number of fucoxanthin chlorophyll a/c-binding proteins (FCPs), which are closely related to CABs. We performed a phylogenetic analysis of chlorophyll binding proteins (CBPs = CABs + FCPs) in several heterokont and plant genomes, and identified a group of stress-induced CBPs (siCBPs) present only in heterokonts and in the green microalga *Chlamydomonas reinhardtii*. Our data support the hypothesis that siCBPs might constitute an ancestral group of CBPs that has retained its original role in non-photochemical quenching – a role which was taken over by ELIPs and PsBs in most green plants.

Keywords: Fucoxanthin chlorophyll a/c-binding proteins (FCP), stress response, diatoms, brown algae, non-photochemical quenching (NPQ), photosynthesis

Introduction

The extended family of chlorophyll binding proteins (CBPs) is a large group of light harvesting antennae and stress response proteins (Green et al. 1991, Green & Kühlbrandt 1995) which comprises chlorophyll a/b binding proteins (CABs), the closely related fucoxanthin chlorophyll a/c binding proteins (FCPs, Grossmann et al. 1990, Caron et al. 2001), high light induced proteins (HLIPs), early light induced proteins (ELIPs), and the psbS subunit of photosystem II (psbS). CABs, as well as FCPs, probably evolved from cyanobacterial HLIPs as a result of two duplication events and the subsequent loss of one transmembrane helix (Green & Kühlbrandt 1995), and are considered to function as part of the light harvesting antennae. They were frequently reported to be transcriptionally repressed in response to light stress (see e.g. Tonkyn et al. 1992). PsbS proteins have a similar structure as CBPs and FCPs, but contain four transmembrane helices compared to three in CBPs and FCPs, and are involved in pH sensing as well as in the regulation of non-photochemical quenching (NPQ) in plants (Li et al. 2004). ELIPs and HLIPs are small proteins with one transmembrane helix, and were frequently observed to be induced in high light or other stress conditions in plants (e.g. Montané et al. 1997, Jansson et al. 2000), and cyanobacteria (Promnares et al. 2006). ELIPs have also been shown to protect from photooxidative stress in *Arabidopsis thaliana* (Hutin et al. 2003).

In heterokonts, recent transcriptomic studies (Hwang et al. 2008, Dittami et al. 2009) highlighted FCPs that were up-regulated in response to heat-, salt-, or oxidative stress – a surprising result considering that their primary function is thought to lie in light harvesting. Moreover, within the green lineage, similar observations were made in *C. reinhardtii*, after treatment with high light intensities (Miura et al. 2004). This raises the question whether all CABs and FCPs are really involved exclusively in light harvesting. Here we demonstrate that this is probably not the case and discuss our results in an evolutionary context.

Results and discussion

Heterokont genomes code for the highest numbers of CBPs

In order to improve our understanding about the evolution and the function(s) of CABs and FCPs, we performed a phylogenetic analysis of most of the known

heterokont FCPs and a selection of plant and green algal CABs. For heterokonts, we searched for FCPs in the available genomes of diatoms and brown algae, yielding 61 sequences from *Thalassiosira pseudonana* (Armbrust et al. 2004), 86 from *Phaeodactylum tricornutum,* (Bowler et al. 2008) and 53 from *Ectocarpus siliculosus* (Cock et al. in prep.). We also included protein sequences derived from 17 expressed sequence tags (ESTs) of the Antarctic diatom *Chaetoceros neogracile* because transcriptomic data was available for these sequences (Hwang et al., 2008). For the green lineage, we selected the sequence of the first available CAB structure from *Pisum sativum* (Kühlbrandt et al., 1994), as well as 14 CABs from the model angiosperm *Arabidopsis thaliana* and 45 from the bryophyte *Physcomitrella patens*. In addition, we included green microalgal CABs from *Micromonas sp*. RCC299 (19 sequences), *Ostreococcus tauri* (14 sequences), and *Chlamydomonas reinhardtii* (24 sequences). All sequences and accession numbers are listed in supplementary file 1. Cyanobacterial sequences as well as eukaryotic ELIPs and HLIPs were not included in this analysis due to their very low degree of sequence homology with FCPs and CBPs.

CABs and FCPs contain several subfamilies

With the exception of two *C. reinhardtii* sequences, the phylogenetic analysis of CABs and FCPs (Figure 1) resulted in a very clear separation between heterokont FCPs and plant CBPs. In addition, within both the heterokont and the plant groups, several potential subfamilies (bootstrap values ≥ 50) were identified. In the green lineage, 6 of these potential subfamilies contained proteins from at least 2 of the 3 represented phylogenetic groups (angiosperms, mosses, green algae), and 5 more consisted purely of green algal sequences. In heterokonts, 8 potential subfamilies containing both *Ectocarpus* and diatom sequences were identified. Two more contained only diatom or only *Ectocarpus* sequences.

One of these potential subfamilies was of particular interest to us, as it comprised all of the FCPs presently known to be induced by stress in the transcriptomic studies of *C. neogracile* (Hwang et. al 2008) and *E. siliculosus* (Dittami et al. 2009). Furthermore, it contained the two CABs from *C. reinhardtii* that were induced in response to high light conditions (Miura et al. 2004). These findings raise two important questions: what is the function of these stress-induced CBPs (siCBPs), and

how can their presence in heterokonts and *C. reihandtii* but not in other green algae or green plants be explained?

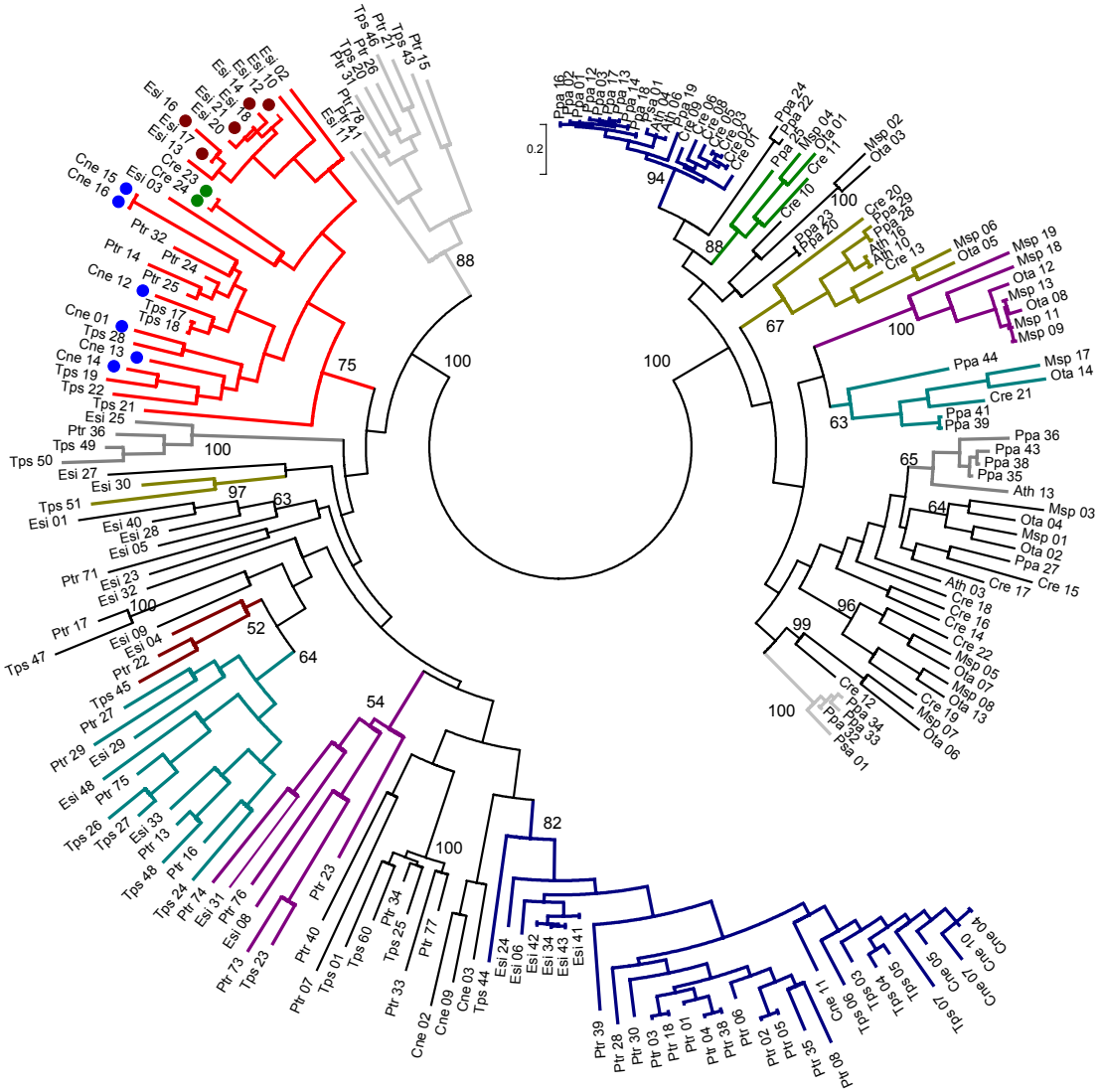

Figure 1: Phylogenetic tree of fucoxanthin a/c binding proteins (FCPs) and chlorophyll a/b binding proteins (CBPs). Colored circles after the sequence name indicate that a gene was induced in stress (blue = heat stress; green = light stress, brown = saline and oxidative stress). Groups containing genes from at least two different classes (moss, higher plants, green algae, diatoms, brown algae) and with bootstrap values ≥ 50 were plotted in the same color. Only selected bootstrap values are shown. Abbreviations: Ath = *Arabidopsis thaliana*, Cne = *Chaetoceros neogracile*, Cre = *Chlamydomonas reinhardtii*, Esi = *Ectocarpus siliculosus*, Msp = *Micromonas sp*. RCC299, Ota = *Ostreococcus tauri*, Ppa = *Physcomitrella patens*, Psa = *Pisum sativum*, Ptr = *Phaeodactylum tricornutum*, Tps = *Thalassiosira pseudonana*.

Alignments with available structures of chlorophyll binding proteins (Kühlbrandt et al., 1994; Amunts et al., 2007) demonstrated that residues thought to bind chlorophyll were equally conserved in siCBPs, non stress-induced FCPs, and plant CABs (Figure 2), suggesting that all of these proteins have similar structures and bind to pigments. On the other hand, there were several residues that were conserved specifically in

stress-induced FCPs/CABs, such as a proline residue (P, position 178, in Figure 2) in the linker region between the C-helix and the A-helix, which was absent in all other examined proteins. Although the effect of these changes on protein function still remains unknown, additional clues about the function of siCBPs may be derived from the phylogenetic position of *C. reinhardtii* CBPs.

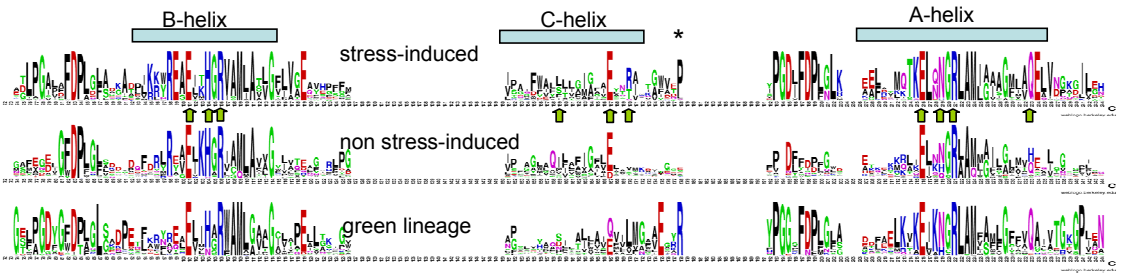

Figure 2: Sequence logos (Crooks et al., 2004) of stress-induced and non stress-induced FCPs as well as plant CABs. The size of the letter indicates the degree of conservation of a particular residue. Residues responsible for chlorophyll binding (arrows) as well as membrane-spanning helices were added manually according to Kühlbrandt et al. (1994). * indicates a highly conserved proline residue in siCBPs that was not found in any of the other examined CBPs or FCPs.

siCBPs might be close to the ancestral CBPs

The presence of two isolated green CBPs in what was otherwise a heterokont-only group of proteins, at first, seems astonishing. Although horizontal gene transfer between *C. reinhardtii* and heterokonts is one possible explanation, which is supported by the high sequence homology between *C. reinhardtii* and heterokont siCBP sequences, current theories on the evolutionary origin of CABs and FCPs could provide an alternative explanation for these findings. As described above, CBPs are thought to derive from cyanobacterial HLIPs (Dolganov et al. 1995, Green and Kühlbrandt 1995) *via* several duplication and deletion events. This hypothesis implies that the original function of CABs and FCPs was likely to be similar to that of stress-induced cyanobacterial HLIPs. The capacity to harvest and transfer light to the reaction centers most likely developed independently several times during evolution, as demonstrated by La Roche et al. (1996) for cyanobacteria and plants. Montané et al. (2000) suggested that the original function of CABs was therefore likely to have been related to NPQ, i.e. the conversion and dissipation of excess light energy into heat.

One hypothesis explaining our results is that siCBPs might have retained this role in NPQ and have not evolved to function in light harvesting. This would fit the

observation that they were transcriptionally up-regulated in response to stress – an observation which is hard to bring in line with a role in light harvesting. In this context, the *C. reinhardtii* siCBPs could be considered relicts of ancestral CBPs that have not (yet) been lost in this species. Indeed, the *C. reinhardtii* genome is peculiar in this sense, as it also contains several genes, encoding e.g. cilia and centriole proteins, derived from the last common plant-animal ancestor that were lost in angiosperms (Merchant et al. 2007).

A role of siCBPs in NPQ could also explain some of the observations made related to the gene content of heterokont genomes. *Ectocarpus* and known diatom genomes do not code for PsbS genes, which are known to function in NPQ in green plants. Moreover, these genomes also contain only one ELIP each, which, at least in the case of *E. siliculosus*, was not induced in saline and oxidative stress (Dittami et al. 2009). The absence or underrepresentation of these genes in heterokont genomes might be related to the presence siCBPs.

In conclusion, our data show that, although CBPs are highly conserved and easily annotated in genomes, several subfamilies with potentially distinct functions are likely to exist, which provides a starting point for future more targeted functional or structural studies. In addition, two nearly completed sequencing projects for red macroalgae will soon give access to several new CBP sequences which might provide further insights into the evolution of these proteins.

Methods

CBP family proteins were searched for using “blastp” and reference sequences from terrestrial plants, green algae, diatoms, and *Ectocarpus* (supplementary file 2). All sequences were aligned using MAFFT (Katoh et al. 2002). Automatic alignments were then manually refined using Bioedit 7.0.9.0 (Hall 1999), taking into consideration only the conserved regions of the proteins (supplementary file 3). In some cases, this resulted in two sequences with 100% sequence identity. In this case, one of the identical sequences was excluded for further analyses. Maximum likelihood trees were generated for the remaining sequences using phyml (Guindon & Gascuel, 2003), default parameters, and bootstrapping (100 iterations). Similar trees were also obtained using neighbor-joining and phylip (not shown). CBPs and FCPs were considered stress-induced if they were identified as such in the original

publications (Miura et al. 2004, Hwang et al. 2008), or in the case of *E. siliculosus*, if the mean log2-ratio stress/control for hyposaline-, hypersaline-, and oxidative stress reported in our previous study (Dittami et al. 2009) was greater than 1 (2-fold up-regulation).

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Supplementary files

Supplementary file 1: Accession numbers of all sequences considered for the phylogenetic tree (including those removed due to high sequence identity). For stress induced proteins, the Pubmed Id (PMID) of the corresponding publication is given. Please note that *Chaetoceros neogracile* sequences are EST sequences. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

Supplementary file 2: Aligned sequences of all CBPs / FCPs present in the phylogenetic tree. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

Supplementary file 3: The phyologenetic tree displayed in figure 1 in the MEGA format, including all bootstrap values. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

Chapter 4

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Long-term adaptation to different salinities

4. Long-term adaptation to different salinities

4.1 Preface

After having studied the short-term response to stress and in particular saline stress, this chapter examines a strain of *Ectocarpus siliculosus* that has been isolated from freshwater and therefore possesses an increased tolerance for low salinities. Similarly to the previous chapter, it is split into two sections, each represented by a manuscript to be submitted for publication (section 4.2) or a preliminary draft (section 4.3).

The primary goal in the first section was to test if and to what extent, the gene expression array designed for the marine strain of *E. siliculosus* could be used with the freshwater strain. This was examined by using this array for comparative genome hybridization experiments between strains. These experiments were not only carried out with the freshwater strain, but also with three other strains of particular interest for the laboratory. The results of these experiments formed the basis for the transcriptomic analysis described in the second section, but also highlighted differences in the degree of conservation in several genomic regions and raised questions about the species concept in *Ectocarpus siliculosus*. These experiments were carried out in collaboration with Caroline Proux and Jean-Yves Coppée (Instiut Pasteur, Paris)

The second section first examined the range of salinity tolerance of the freshwater strain, showing that it was still capable of surviving in seawater, and then compared the marine and the freshwater strain (the latter in different salinities) on a transcriptomic- and a metabolite level (metabolite profiling was carried out by Antoine Gravot, University of Rennes 1, and Sophie Goulitquer, University of Brest). All together these data hightlighted the role of transcriptomic plasticity in the adaptation to freshwater, but also showed that not all genomic adaptations necessarily translated into transcriptomic changes, and that the two strains cultured in identical media had very different metabolite-, but very similar transcriptomic profiles. The freshwater strain presents a very interesting model to study the mechanisms underlying the adaptation to drastic changes in habitat – a question rarely addressed in plants, as permanent transitions between marine and terrestrial habitats are rare and fossile records of plants scarce or non-existent.

4.2

Comparative genome hybridization of *Ectocarpus* strains

Comparative genome hybridizations of different strains and ecotypes of *Ectocarpus* reveal high intra-generic variability

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Abstract

Brown algae of the genus *Ectocarpus* can be found in diverse marine environments in all temperate regions of the world. Some strains have colonized extreme habitats such as heavily polluted sites, estuaries, and even freshwater sites close to the sea, yet not all strains are able to withstand these extreme conditions. With the establishment of *E. siliculosus* as a model for brown algae, and the possibility to carry out high throughput transcriptomic analyses, it is of interest to assess the genomic variability among strains and ecotypes of the *Ectocarpus* genus.

In this study we use a genome-wide *Ectocarpus siliculosus* expression array to assess genomic variability and feasibility of cross strain hybridizations between five strains of *Ectocarpus*: four strains of *E. siliculosus* (the male genome strain, a female strain used for outcrosses with the genome strain, a strain isolated from freshwater, and a strain with exceptionally high copper tolerance), as well as one strain of the sister species *E. fasciculatus*. Our results revealed significant genome-wide differences between different ecotypes of the same species, supporting the concept of *E. siliculosus* as a complex of cryptic species. Moreover, not all parts of the *Ectocarpus* genome seem to have evolved at equal rates, and high variability was detected particularly in transposable elements and fucoxanthin chlorophyll a/c binding proteins. Finally, we found indications of the possible presence of new *E. siliculosus* virus 1-like sequences in the genome of one of the examined strains.

Keywords: Brown algae, *Ectocarpus*, Comparative genome hybridization (CGH), cryptic species, transposable elements (TEs), fucoxanthin chlorophyll a/c binding proteins (FCPs), *Ectocarpus siliculosus* virus (ESV)

Introduction

Brown algae are multicellular and mainly marine organisms, that live along the coastlines of all continents. They are economically and ecologically important as they form the dominant vegetation in the intertidal and subtidal of rocky shores (Bartsch et al. 2008). Some species, such as giant kelps provide habitats for many other species. Being part of the chromalveolate kingdom, brown algae have evolved independently from other eukaryotic lineages, including land plants and red and green algae (Baldauf 2003, 2008). In spite of their importance, there are still many gaps in our knowledge about brown algae, such as knowledge about the mechanisms involved in their stress response, their developement, or their complex life cycle (Coelho et al. 2007).

Among brown algae, *Ectocarpus siliculosus* has a long history of research (Charrier et al. 2008), and was chosen as a genetic model (Peters et al. 2004) due to its small genome and its short life cycle. Its genome has recently been sequenced by Genoscope (France) and is the first complete genome sequence available for any seaweed (Cock et al. in prep.). As currently conceived, the genus *Ectocarpus* contains two species, *E. siliculosus* and *E. fasciculatus*. The cosmopolitan *E. siliculosus* is genetically particularly diverse and possibly contains several cryptic species (Stache-Crain et al. 1997, Peters et al in prep. a, b). In this paper we use the circumscription of *E. siliculosus* as encompassing all strains falling into clades 1-4 of the phylogeny by Stache-Crain et al. (1997).

Different strains of *E. siliculosus* have been isolated from rather extreme environments. Strain 3 (Table 1), for example, is the only well documented finding of this species in freshwater (West & Kraft 1996, McCauley & Wehr 2007), while strain 4 was isolated from a heavy metal-polluted site in Chile (Figure 1) and exhibits greatly increased tolerance to copper (Ritter et al. in press). Neither of these strains has been observed to reproduce sexually, and their sex and life cycle stage therefore still remain unknown (Table 1). However, they have been and are currently being investigated on a physiological (strain 3 and strain 4), metabolite (strain 3), and proteomic (strain 4) level in order to investigate the adaptations enabling them to tolerate the "extreme" conditions found in their original habitats.

In parallel, targeted studies of mutants have been carried out in order to examine specific aspects of *Ectocarpus* development, such as the switch in life cycle phases

(Peters et al. 2008), or early morphogenesis (Le Bail et al. in prep.). Positional cloning of mutant genes as well as the construction of the *Ectocarpus* genetic map require the use of a polymorphic outcross line such as strain 2.

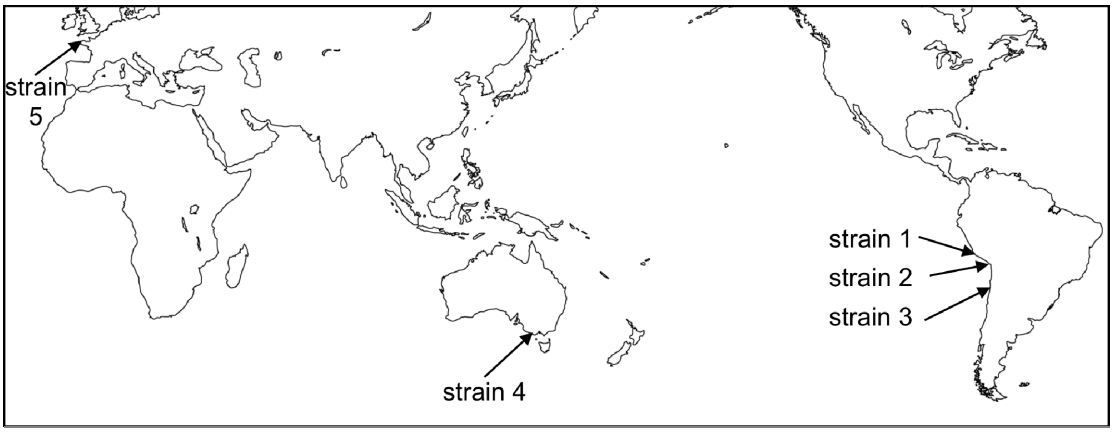

Figure 1: Map of origins of the different strains of *Ectocarpus* used in this study.

Studies of different strains as well as studies using crosses between different strains could greatly benefit from the tools developed for the sequenced strain 1 of *E. siliculosus*, including a recently developed microarray (Dittami et al. 2009). This expressed sequence tag (EST)-based expression array comprises probes for 16,888 unigenes, as well as the 231 coding sequences of the *E. siliculosus* virus 1 (ESV-1, Delaroque et al. 2001). However, in order to use this microarray to compare different strains on a transcriptomic level, the feasability of cross strain hybridizations should be assessed (Bar-Or et al., 2007). To this means, we hybridised genomic DNA of selected strains to the available microarray and compared the results to those obtaind from genomic DNA of a reference strain 1, allowing us to distinguish between well conserved probes (i.e. probes with similar signal intensities) and divergent probes (i.e. probes that exhibit changes in the signal intensities). Similar approaches have already been successfully employed in other eukaryotes, e.g. to compare two closely relates species of *Drosophila* (Ranz et al. 2003), or to perform transcriptomic experiments with a *Brassica* using an *Arabidopsis* array (Hammond et al. 2005). Our experiments, carried out with four strains of *Ectocarpus* of different genetic distance to the reference strain, and from different environments, allowed us to select a subset of highly conserved probes for each of these strains, thus facilitating future microarray experiments with the strains in question. In addition, using this technique enabled us to assess the overall degree of genomic variability between two strains and identify

genomic regions that were less conserved than others, or that had undergone changes in copy number (including deletions). Although these two processes (changes in copy number and sequence diversion) could not be distinguished by the methods employed, our observations revealed a number of important features related to physiology and evolution within the *Ectocarpus* genus.

Materials and Methods

Algal strains and culture conditions

All *Ectocarpus* strains selected (Table 1) were clonal isolates identified by ITS1 sequencing which allowed classification in the different clades of the phylogeny in Stache-Crain *et al.* (1997). They were cultivated in 10-liter plastic flasks in a culture room at 14°C using filtered and autoclaved natural seawater enriched according to Provasoli (Starr & Zeikus 1993). Cultures were irradiated by daylight-type fluorescent white light (40 μEm^{-2} s^{-1}) under a 14/10 light dark-cycle and were permanently aerated with filtered (0.22 µm) compressed air.

Table 1: Accession number, origin and description of strains and used in our experiments

Strain number in this paper	Strain characteristics	Number in Roscoff *Ectocarpus* strain collection	CCAP number[1]	Origin	Species[2]	*Ectocarpus* clade[2]	References
1	Genome-sequenced strain, male partheno-sporophyte from fully marine environment	32	1310/4	San Juan de Marcona, Peru	*E. siliculosus*	1c	Stache-Crain et al. (1997), Peters et al. (in prep. a)
2	Female partheno-sporophyte, sexually compatible with strain 1, from fully marine environment (subtidal 3m)	568	(to be submitted)	Arica, Chile	*E. siliculosus*	1c	Peters et al. (in prep. a)
3	Freshwater strain, unknown sex or life-history stage	371	1310/196	Hopkins River Falls, Victoria, Australia	*E. siliculosus*	2d	West and Kraft (1996)
4	Copper-tolerant strain, unknown sex or life-history stage	524	1310/333	Palito La Boca, Chañaral, Chile	*E. siliculosus*	1a	Peters et al. (in prep. a)
5	Outgroup, from fully marine environment (upper subtidal, epiphytic on *Himanthalia*), unknown sex or life-history stage	395	(to be submitted)	Roscoff, France	*E. fasciculatus*	5b	Peters et al. (in prep. b)

[1] Culture Collection of Algae and Protozoa, Dunstaffnage, Scotland (ccap.ac.uk)
[2] According the caldes defined by Stache-Crain et al. (1997)

DNA extraction and fragmentation

Approximately 1 g (wet weight) of tissue was harvested by filtration, dried with a paper towel, and frozen in liquid nitrogen. These samples were used for DNA extraction using CsCl-gradient purification based on the protocol described by Le Bail et al. (2008). Frozen tissues were ground in liquid nitrogen, then four ml of extraction buffer (100 mM Tris-HCl pH7.5; 1.5 M NaCl; 2% CTAB; 50 mM EDTA; 50 mM

DTT) were added, and samples were mixed vigorously at room temperature for 30 min. After addition of one volume of chlorophorm/isoamylic alcohol (24:1), samples were vortexed again for 1 min, and centrifuged at 10,000 g and room temperature for 20 min. DNA in the supernatant was precipitated with 0.8 volumes of isopropanol at 4°C overnight and centrifuged for 30 min at 18,000 g in the morning. The pellet was dissolved in 2 ml TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA), 5.4 M CsCl (density 1.66 g ml^{-1}) and 250 $\mu g.mL^{-1}$ of ethidium bromide. After 24 h of ultra-centrifugation at 90,000 g (room temperature), a band containing genomic DNA was recovered under UV and ethidium bromide was extracted 4-5 times with one volume of TE-saturated butanol (pH 8). Genomic DNA was precipitated twice with 2.5 volumes of ethanol and the final pellet was resuspended in 50 µl nuclease-free H_2O. DNA was quality checked on a 0.8 % agarose gel and quantified using a NanoDrop ND-1000 spectrophotometer before and after fragmentation by incubation at 99°C for 13 minutes, which yielded DNA with a mean fragment length of about 1,000 bp.

Hybridizations and scanning

DNA of four strains (1, 2, 3, and 4) of *E. siliculosus* and one strain of *E. fasciculatus* (5, Table 1) was hybridized to the EST-based Roche NimbleGen 4-plex expression array [ArrayExpress: A-MEXP-1445] (Dittami et al. 2009), using the sequenced genome strain (strain 1), which the array was based on, as a reference. For each sample, one µg of fragmented DNA was labeled using the Roche NimbleGen Dual-Color DNA Labeling Kit (Roche NimbleGen, Madison, WI, USA), following the manufacturer-supplied CGH Analysis protocol v5.1. Reference strain (strain 1) DNA was labeled with Cy5, other DNA with Cy3. One µg of DNA was used for each labeling reaction which yielded > 4 µg of labeled DNA. Four µg of each sample were hybridized together with 4 µg of the reference DNA (strain 1), using the NimbleGen Hybridization System 4 and following the standard Roche NimbleGen protocol (CGH Analysis protocol v5.1). Scanning was performed according to the same protocol (Roche NimbleGen CGH Analysis protocol v5.1) using a Genepix 4200AL scanner and the Genepix pro 5.0 software (Molecular Devices, Sunnyvale, CA, USA).

Normalization statistical analysis

Scanned images were imported into NimbleScan version 2.4 (Roche NimbleGen), and raw expression value (log2-ratios) were extracted for each probe. A ".pos" file

(supplementary file 1) for our microarray was generated by blasting each of the microarray probes against the entire *Ectocarpus* genome using the megablast algorithm (Zhang et al. 2000). Each genomic supercontig was treated as a chromosome. 2,676 probes (3.9%) could not be clearly assigned a position on the genome (homologous sequences were not found). These probes were assigned randomly to a "virtual" chromosome, which was later excluded from the analysis. Raw log2-ratios were normalized using the poplowess-algorithm version 1.0.2 (Staaf et al. 2007) and R [http://www.r-project.org] version 2.9.1, as this algorithm is less sensitive to copy number imbalances (or changes in sequence).

Normalized log2-ratios were then analyzed on two levels: a probe level and a gene level. For the analysis on a probe level, normalized expression values were imported into the Partek Genome Suite software version 6 (Partek Inc., St. Louis, USA), which was used for scanning for copy number alterations using circular binary segmentation (Olshen et al. 2004). Only segments with at least 30 probes and a mean log2-ratio superior to 0.5 or inferior to -0.5 were considered. We chose to apply a p-value cutoff of 7.4e-7, which corresponds to a p-value of 0.05 after a Bonferroni correction for 67,270 tests. Since we tested frames of 30 probes, the latter assumption is very conservative, as well as the Bonferroni correction in general. However, less stringent methods would not have changed the number or identity of the identified genomic regions as the p-value of the next most significant segment was three orders of magnitude above our cutoff.

Data were also analyzed on a gene level. To this means, the log2-ratios of the four probes per gene were tested against zero, using a t-test and the TIGR MeV software version 4.4 (Saeed et al. 2004). The Benjamini and Hochberg (1995) procedure was used to limit the false discovery rate to 5 %, and genes with an absolute log2-change inferior to 0.5 were not considered.

Functional analysis

Enriched GO terms were searched for within the significantly altered genes (i.e. genes for which the mean log2-ratio of all probes differed significantly from 0) using the GOLEM software (Sealfon et al. 2006) and the GO annotations generated in our previous study (Dittami et al. 2009). Transposable elements were identified by sequence homology with a database of known *E. siliculosus* transposons (Cock et al.

in prep.). Only sequences with >80% sequence similarity over >400 bp were considered.

Validation

Genomic regions with significantly different signal (Figure 5) were verified by real time quantitative PCR, as described previously (Le Bail et al. 2008). Three to four fragments were amplified and quantified per region using 4 ng of genomic DNA as template and the primer pairs listed in Table 2. Standard curves were created to calculate the reaction efficiency for each primer pair using a dilution series of 16, 8, 4, 2, 1 and 0 ng of DNA. Three biological replicates were tested. The specificity of the amplification as well as possible size differences in the amplicon were checked by a melting curve. Dyneine (Esi0298_0008 = LQ0AAB30YA12FM1) and R26S (Esi0072_0068 = CL461Contig1) were selected as reference genes because of their high degree of conservation in our study (log2-ratio < 0.17 in all *E. siliculosus* strains).

Table 2: Quantitative PCR validation of the experiments. The table shows primers and validation results for each tested genomic region (EsV-1 = region on supercontig 52 containing the *E. siliculous* virus 1 genes)

	gene/region	forward primer	reverse primer	reference strain	experimental strain	ratio (mean ± SD, n=3)	ratio for region (CGH)
ref. gene	R26S	GCTAGGCTTGCGTTTGTGTG	GGCGAGACAGAAAGATTCCG	strain 1	strain 2/3	-	-
	Dyneine	GGAACAAAGCATGGTGACAACA	CGCGTGCCTATCCAAGCT	strain 1	strain 2/3	-	-
experimental amplicons	Supercontig 16	GCGTGCGTGCTTGGAAGG	TTCGGCTGCTGAGAGTGGAG	strain 1	strain 3	0.94 ± 0.039	
	Supercontig 16	CAACCGCTCTCCACCATTCAG	GACGCCTTCACAGTATCACACC	strain 1	strain 3	0.74 ± 0.015	0.51
	Supercontig 16	AACGATAGAGCGAGACGAGAGAG	GGAAGCAGATGGACACGAGTAAC	strain 1	strain 3	0.80 ± 0.035	
	Supercontig 627	AAGGAATAGCAATTAAGCCAGCAATAC	CCATCGTAGTTCAAGCGGTTAGTC	strain 1	strain 3	0.78 ± 0.050	
	Supercontig 627	"	"	strain 1	strain 2	2.4 ± 0.87	371: 0.54
	Supercontig 627	ACTAATACCCATTGAAATCTCGTAAGAAAC	TTAATCTCAGGTTGGTCTAGTAACTCTAAG	strain 1	strain 3	0.73 ± 0.029	
	Supercontig 627	"	"	strain 1	strain 2	2.3 ± 0.75	568: 2.83
	Supercontig 627	CGTTGGGCGTATTGGGAGTTG	TTTGATAGGCGAGAAGTGTAAGAGC	strain 1	strain 3	0.93 ± 0.024	
	Supercontig 627	"	"	strain 1	strain 2	2.2 ± 0.81	
	Supercontig 68	CTCCTATCGCCCTGTGGTCTC	ACTGCCTCTATGGTCCGTCTTG	strain 1	strain 2	1.0 ± 0.12	
	Supercontig 68	GTGAGAGAAACAACAGAGCAATACAG	ATGGAACCGCAGACAACAAGC	strain 1	strain 2	0.59 ± 0.23	0.39
	Supercontig 68	TCCGACCTGACGAGCATTGG	CAGTGTGCGGTGCGAACG	strain 1	strain 2	n/a*	
	Supercontig 68	AAACACCTCCCAACCAACCAATC	AACGCAACGAGCAACCTTCC	strain 1	strain 2	n/a*	
	EsV-1	TAAGTTGATATTAGTGACAGTAGCAGGAG	GCCACGGAGGACGGAGATAC	strain 1	strain 2	n/a*	
	EsV-1	ACCACGATGCCTGTCTCCTTAC	TCCTCAGCCGCCAGAATACG	strain 1	strain 2	n/a*	0.45
	EsV-1	CTCCTCCGTAACCGTTGACATTG	CCGACCAGTAAACCCGTAAACC	strain 1	strain 2	n/a*	
	Supercontig 397	GGGTGAATGACGGGACTTGAAC	GTGGTTAAGGCAGTGGGTTGTG	strain 1	strain 2	1.5 ± 0.27	
	Supercontig 397	GCAGGTAGTCCGCATCTTCAC	TCCGACCCGCACGAAAGG	strain 1	strain 2	1.5 ± 0.28	1.5
	Supercontig 397	ATCTCCACTCACTTGCGAATGC	GTTGCGTTCCTGGCTCACC	strain 1	strain 2	1.5 ± 0.35	

* no amplification in experimental strain, or difference > 10 cycles

Data deposition

CGH-data (raw and normalized) were deposited with ArrayExpress under the accession number ArrayExpress: E-TABM-766 (Username: Reviewer_E-TABM-766, Password: 1250785653700).

Results

Strong genetic differences between most strains

As can be seen from Figure 2, strain 1 and strain 2 are genetically very close, compared to the other strains: 95 % of probes changed signal intensity less than 1.42-fold between these two strains. This is very close to values obtained for self-self hybridizations in bacteria, where 95 % of the probes varied less than 1.34-fold (standard deviation of the log2-ratio between samples = 0.215; Taboada et al. 2005). In comparison, in the freshwater- and copper-tolerant strains (strains 3 and 4), the 95 most stable percent of probes changed signal intensity up to 2.80-fold and 2.87-fold, respectively. The degree of variation in these strains was close to that of the outgroup strain (*E. fasciculatus*, strain 5), where the 95 most stable percent of the probes changed up to 3.18-fold. This latter strain (5) also differed from the others in that most of the probes averaged around a log2 fold-change of 1.1.

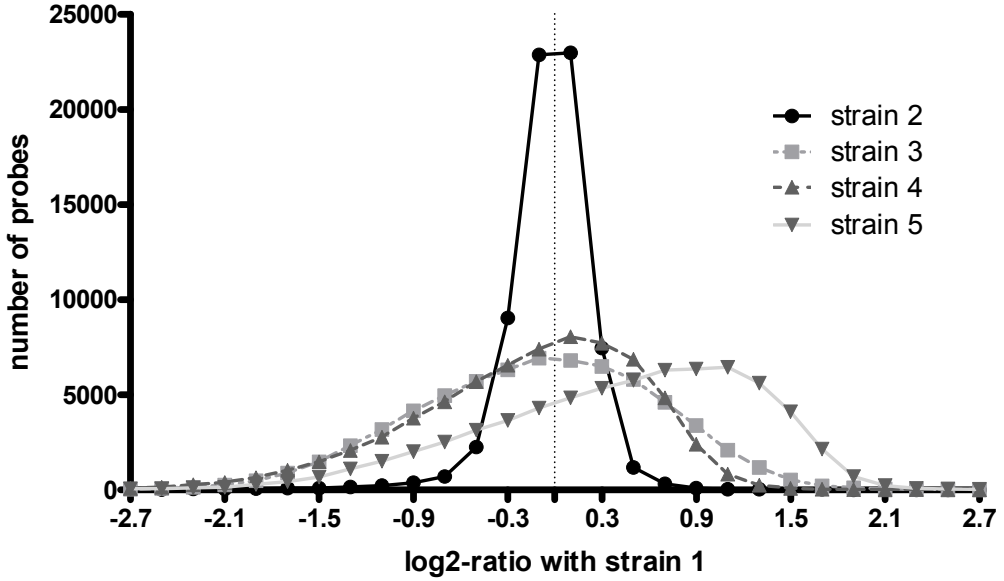

Figure 2: Distribution of log2-ratios (sample strain / reference strain) in four strains of *Ectocarpus*. Probe frequencies were calculated in intervals of 0.2.

Similar results were also obtained comparing the different strains on a gene level (Figure 3). While 97 % of all genes were stable (log2-change < 0.5) between strain 1 and strain 2, only 66 to 71 % of genes were considered stable in the freshwater- and copper-tolerant strain (strain 3 and 4, respectively). In total 10,020 genes (59 %) were defined as stable in all strains of *E. siliculosus*, although this number was subject to

slight changes, when considering only genes with all UTR (48 %) and genes with all CDS probes (64 %).

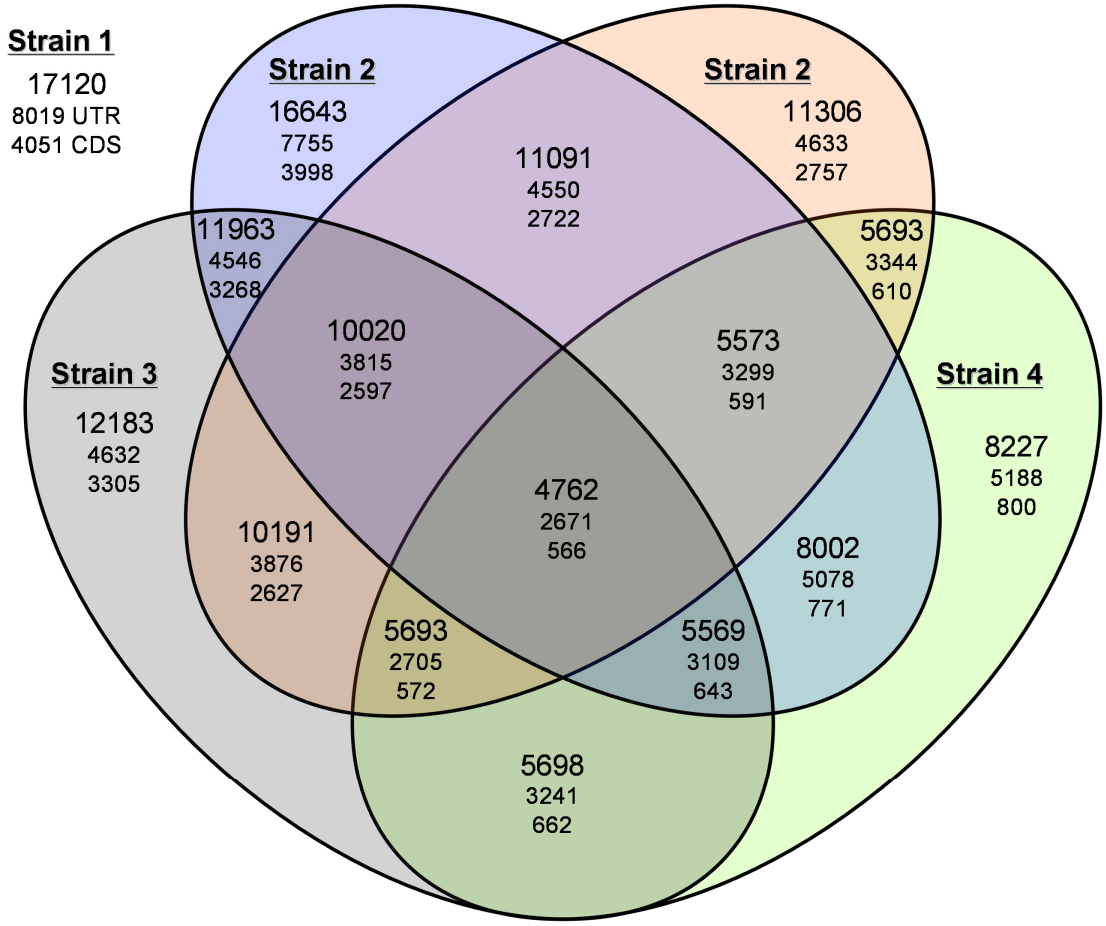

Figure 3: Venn diagram displaying the number of stable genes in the tested strains. A gene was considered stable if the mean log-ratio of all four probes for one gene ranged between -0.5 and 0.5 (i.e. the change in signal intensity compared to the reference strain was less than or equal to 1.41). In each column, the first number includes all genes, while the two numbers below correspond only to genes with 100 % of probes in the untranslated region (UTR) or the coding sequence (CDS), respectively.

Normalization introduces bias in the E. fasciculatus strain

Interestingly in *E. fasciculatus*, this bias for lower variability in the protein-coding region was inversed (64.7 % of all UTR genes and only 19.7 % of CDS were considered stable). Both this and the peak in distribution around log2-ratio of 1.1 are likely to be linked to an artifact from the normalization procedure. For DNA hybridizations from two different strains, we would ideally expect a skewed distribution with a peak at zero (perfectly matching probes) and a larger tail towards negative ratios due to probe mismatches. While classical normalization procedures, such as the qspline fit normalization (Workman et al. 2002), will shift skewed distributions (as for strains 3, 4, and 5) towards positive ratios to maintain a constant

mean (data not shown), the popLowess method searches for a stable population of probes and uses it as a basis for normalization. This worked well for the strains of *E. siliculosus* (2-4), but with *E. fasciculatus* (strain 5), sequence differences were probably too substantial (Figure 2). Assuming that this shift corresponds to a log2-ratio of 1.1 could explain why conserved CDS probes (expected log2-ratio close to 0) would appear less conserved (log2-ratios close to 1.1). For this reason, *E. fasciculatus* (strain 5) was excluded from analyses relying on the absolute ratio of signal intensity with the reference strain.

The microarray developed for the genome-sequenced strain works with other strains of E. siliculosus

In spite of strong genetic differences, microarray experiments using the existing chip remain possible at least for the three examined strains of *E. siliculosus*, when selecting only conserved probes for the analysis (Hammond et al. 2005). For instance, by selecting only probes that show log2-changes inferior to 0.5 (1.4-fold change in signal intensity), we would select 95, 48, and 53 % of the probes on the array for strains 2, 3, and 4, respectively. However, because each gene is represented by four probes, we could still obtain expression profiles from one or more probes for 98, 85, and 88 % of all genes. To facilitate the selection of probes, we have created a Java application which can be used to remove a list of probes from raw pair files, prior to normalization using the NimbleScan software (supplementary file 2). Along with this program, we also provide a list of all probes with log2-changes superior to 0.5. However, 0.5 is a rather conservative cutoff, based merely on previous experiments of Taboada et al. (2005), showing that in a self-self experiment, 96 % of the probes had log2-ratios between -0.43 and 0.43. We would recommend adjusting this value depending on the experiment (e.g. comparison of two different strains or comparison of different conditions within the same strain) and the biological question.

Conserved and variable genomic areas correlate between strains

Table 3: Pearson correlation coefficients between CGH profiles of different strains. Correlations were calculated for all probes, and separately for probes in the CDS and the UTR of a gene. All correlations were statistically significant ($p < 0.001$).

all probes			
Strain:	**2**	**3**	**4**
3	0.38		
4	0.42	0.83	
5	0.41	0.82	0.85
probes in UTR			
Strain:	**2**	**3**	**4**
3	0.35		
4	0.39	0.8	
5	0.39	0.8	0.83
probes in CDS			
Strain:	**2**	**3**	**4**
3	0.39		
4	0.42	0.8	
5	0.4	0.76	0.8

Overall, the CGH profiles of strains 2, 3, 4, and also *E. fasciculatus* strain 5 correlated well (correlation coefficient 0.38 to 0.85, table 3). One explanation for this could be a "UTR-bias", i.e. the fact that UTRs in all strains were more variable than CDSs. Therefore, correlations were also calculated separately for probes within CDSs and probes within UTRs. In this analysis, coefficients were only slightly lower than in the separated analysis (0.35 to 0.83), indicating that the position of probes only had a minor effect on their degree of conservation. The high correlation coefficient between strains could therefore either be explained by close relationship of the examined strains or by general differences in the degree of conservation throughout their genomes.

In order to identify potential differences in the degree of conservation between different genomic regions and different functional groups of genes, two approaches were taken: a gene-based analysis, evaluating the correlations between function and degree of conservation, and a probe-based analysis, examining the correlation between location of the probes on the different supercontigs and degree of conservation. Due to the possible bias introduced by the normalization procedure the *E. fasciculatus* strain (5) was excluded from these analyses.

Transposable elements and fucoxanthin-chlorophyll a/c binding proteins are among the most variable genes

As a result of the gene-based analysis in the female strain 2 compared to the reference strain 1, we found only 22 genes that had a higher signal in one or the other strain (Table 4). Among the genes with a higher signal in strain 2, there was one integrin alpha chain, two chloroplastic proteins, three mitochondrial proteins, four (conserved)

hypothetical proteins, and one repeated element. Among the genes with lower signals in strain 2, we found four repeated elements in addition to two transposable elements (i.e. repeated elements that were included in the database of known *Ectocarpus* transposable elements, Cock et al. in prep.) and five (conserved) hypothetical proteins.

Table 4: Genes with significantly different signals between strain 2 and the reference strain 1.

	Sequence	Genome ID	log2Ratio	Description
higher signal in strain 2	CL2390Contig1	-	1.02	repeated element (3 / 2 sctgs)
	LQ0AAB35YM06FM1.SCF	chloroplast	0.75	chloroplastic photosystem II reaction center
	CL1483Contig1	mitochondrion	1.44	mitochondrial NADH dehydrogenase
	LQ0AAB45YA08FM1.SCF	Esi0137_0050	1.61	hypothetical protein
	LQ0AAB29YN14FM1.SCF	Esi0021_0150	1.57	conserved hypothetical protein (heterokonts)
	LQ0AAB12YL22FM1.SCF	Esi0696_0005	0.67	hypothetical protein
	CL5728Contig1	Esi0623_0002	0.83	Integrin alpha chain
	CL365Contig1	mitochondrion	1.58	mitochondrial rRNA
	LQ0AAB64YN17FM1.SCF	chloroplast	0.73	chloroplastic cytochrome c biogenesis protein
	LQ0AAB27YJ04FM1.SCF	Esi0009_0049	0.78	conserved hypothetical protein (zinc finger domain)
	CL294Contig1	mitochondrion	1.51	mitochondrial rRNA
higher signal in strain 1	LQ0AAB28YK21FM1.SCF	Esi0595_0003	-0.72	conserved hypothetical protein (heterokonts)
	CL463Contig1	-	-0.94	repeated element (1 / 4 sctgs)
	CL4504Contig1	-	-1.56	repeated element (5 / 0 sctgs)
	LQ0AAB103YF03FM1.SCF	Esi0256_0018	-0.54	conserved hypothetical protein (peptidase domain)
	CL188Contig4	-	-1.65	transposable element (5 / 17 sctgs)
	LQ0AAB58YL14RM1.SCF	-	-0.66	repeted element (7 / 0 sctgs)
	LQ0AAB58YK05FM1.SCF	Esi0068_0025	-2.74	conserved hypothetical protein (unkown domain)
	LQ0ACA3YO14RM1.SCF	Esi0999_0003	-1.84	hypothetical protein
	LQ0AAB92YC13FM1.SCF	-	-1.13	transposable element (16 / 0 sctgs)
	LQ0AAB72YF01FM1.SCF	Esi0480_0007	-0.55	hypothetical protein
	LQ0AAB47YB22FM1.SCF	-	-0.60	repeated element (2 / 0 sctgs)

In the strains 3 and 4, we identified 973 and 817 genes with significantly different signals compared to the reference strain, respectively. Due to the high number of sequences, we used automatic gene ontology (GO) annotations to identify groups of proteins overrepresented among these genes. Allowing a false discovery rate of 5 %, this analysis yielded only a single GO-category, fucoxanthin-chlorophyll a/c binding proteins (FCPs), which was overrepresented among the genes with lower signal intensities in the freshwater strain (strain 3). Since probe position within or outside the UTR might have introduced a slight bias in this analysis (see above), we first checked if FCPs contained more probes in the UTRs than in the CDSs compared to the background (all proteins). The difference was minimal: 36 % of the probes representing FCPs were located within the CDS, while the average over all genes was 38 %. UTR-bias is therefore not likely to be a factor for FCPs. We then completed the list of FCPs used in our analysis by adding those FCPs that were not identified by the automatic analysis (36 probe sets in total), and examined their signal intensities in all

strains (Figure 4A, Supplementary file 3). Data from this analysis showed that, although FCPs were picked up by the automatic analysis only in strain 3, their average signal compared to the reference strain 1 (light grey bars in Figure 4) is significantly lower (t-test, $p < 0.001$) than that of the background (dark grey bars in Figure 4) in all strains, except strain 2.

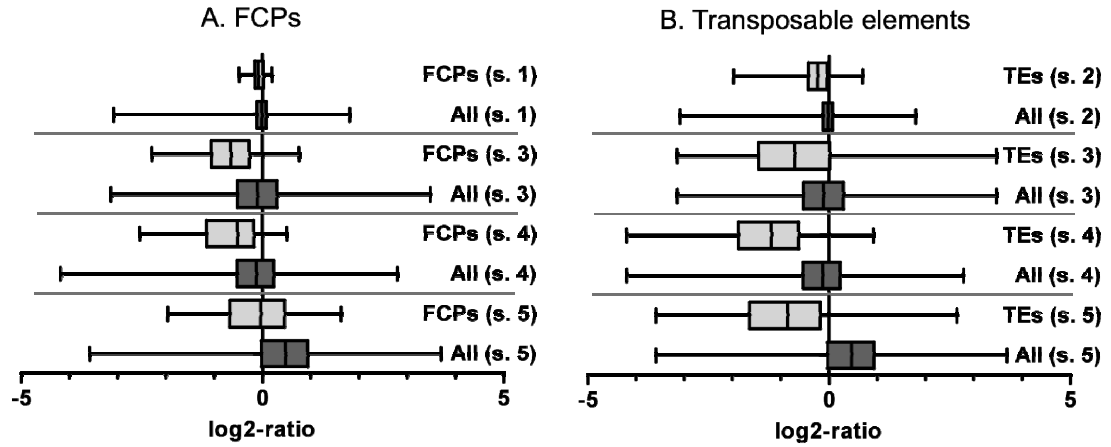

Figure 4: Box plot of log2-ratios for Fucoxanthin chlorophyll a/c binding proteins (FCPs, Panel A) and transposable elements (TEs, Panel B). The log2-ratios for all genes of each strain (s. 2-5) are shown in dark grey, while those of TEs and FCP genes are shown in light grey. A shift towards negative log2-ratios for FCPs or TEs compared to all genes in the same strain indicates a lower degree of conservation or changes in copy number.

In order to determine if this was a feature common to other multigene families, which merely remained undetected due to the lack of high quality automatic annotations for some of them, we also performed the same analysis for 25 manually annotated probe sets corresponding to glutathione-S-transferases (GSTs, de Franco et al. 2009, Supplementary files 3 and 4). Our analysis did not reveal any significant differences between GSTs and the rest of the genes (t-test, $p > 0.25$ in all strains). This shows that not all multigene families are subject to high variability in different strains of *Ectocarpus*, although this still might be true for some families.

As automatic GO annotations did not include annotations for transposable elements, but since they were highly represented among the genes found most variable in strain 2 (Table 4), they were analyzed separately. This was done to determine if high variability among transposons was a feature common to all strains. The 284 transposable elements identified according to our definition (see materials and methods) were significantly overrepresented among the probe sets with significant differences between strains: 3.6 % (strain 3) and 9 % (strain 4) of the most variable

genes were known transposable elements, while they only represented 1.7 % of all probe sets. Moreover, the mean log2ratios of transposons with the reference was significantly lower (t-test, $p < 0.001$) than that of the background (Figure 4B, Supplementary file 3).

For *E. fasciculatus* (strain 5), a search for overrepresented GO terms yielded a number of functions but was not included in this publication due to high risk of including artifacts due to normalization.

Possible deletions / duplications were detected mainly in strain 2

A probe-based analysis of the *E. siliculosus* strains 2, 3, and 4 compared to the reference strain 1 detected five regions, covered by at least 30 probes, with altered signal intensities in at least one of the sample strains (Figure 5; see also Table 2). Two of these regions were the mitochondrial and the chloroplast genome. The mitochondrial genome exhibited a 2.8-fold increased signal in strain 2 (compared to strain 1; 2.3-fold by quantitative PCR) and a 1.8-fold lower signal in strain 3 (1.2-fold by quantitative PCR). The average signal intensity of the chloroplastic genome in strain 2 was a 1.5-fold higher than in strain 1.

In strain 3, a small region on supercontig 16, containing mainly transposable elements, had significantly lower signals compared to the reference strain 1 (2-fold in the CGH experiment, 1.2-fold in the quantitative PCR validation). The clearest differences in the nuclear genome, however, were detected in strain 2. Here probes corresponding to the inserted *E. siliculosus* virus 1 (Esv-1, Delaroque et al. 2001), with the exception of one viral gene, had 2.2-fold lower signal intensities. These differences were even clearer in the quantitative PCR validation (Table 2), where all three primer pairs amplified normally in strain 1, but failed to amplify in strain 2. Finally, the entire supercontig 68 exhibited a significantly lower signal in strain 2 (2.5-fold, on average). In the quantitative PCR validation two of four primer pairs amplified only in strain 1, while two others indicated no or only a 1.7-fold decrease (factor 0.59, Table 2) in template quantity in strain 2. Supercontig 68 is predicted to code for 21 proteins, 14 of which are (conserved) hypothetical proteins with unknown function.

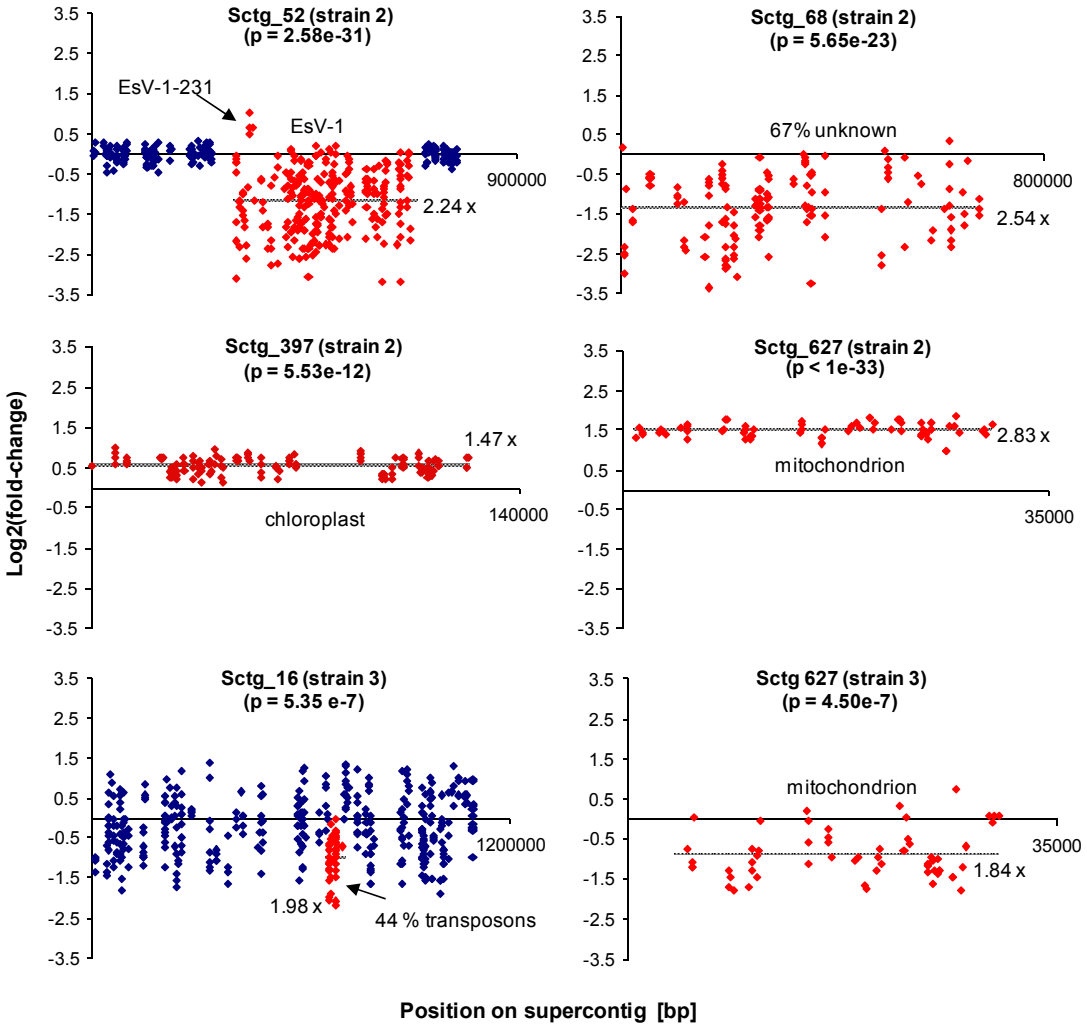

Figure 5: Genomic regions with significant changes in probe intensity in the examined *E. siliculosus* strains. The graphs display the log2-ratio between sample and reference strain (1) for each probe. A log2-ratio of 0 would indicate that there was no difference between the examined strains. Red dots indicate probes in regions with significant differences between sample- and reference strain (1), blue dots the surrounding probes (if present). The grey bar indicates the mean log2-ratio over the highlighted area; the corresponding fold-change is given followed by an "x". *E. fasciculatus* (strain 395) was not considered for this analysis (sctg = supercontig, EsV = *Ectocarpus siliculosus* virus).

Discussion

This study is the first genome-wide comparison of different algal strains, and provides a solid basis for the analysis of future comparative transcriptomic studies with the examined stains of *E. siliculosus* (2, 3, and 4), by allowing the selection of conserved probes between these strains and the reference strain (1). In addition, although the conclusions that can be drawn from CGH experiments with different species are limited by the lack of reliable means of normalization, three main findings can be reported for the examined strains of *E. siliculosus*: i) two of the three examined strains (3, 4) diverged rather strongly from the genome strain; ii) transposable elements and

FCPs are subject to particularly strong changes in sequence and / or copy number; and iii) differences between the male and the female strains (1 and 2) of *E. siliculosus* are concentrated in a few regions including the EsV-1 integration site and a largely unknown supercontig.

Different strains are almost as different as different species

Our data revealed rather strong differences in the degree of conservation between the examined strains. Overall, strains 3 and 4 were more similar to the reference strain (1) than the *E. fasciculatus* strain (5), but at the same time, a lot more divergent than strain 2, which is known to produce fertile offspring when crossed with strain 1 (Peters et al. in prep. a). From our data, it is not possible to conclude whether these differences are related to phylogenetic distance or to the capability of adapting to comparatively extreme environments such as freshwater (West & Kraft, 1996) or copper polluted sites (Ritter et al, in press). However, although transposons and FCPs were particularly variable between strains, deviations were found in the entire genome, supporting the idea that there has not been any genetic exchange between these strains for a long time. At least in the case of the freshwater strain (3), these differences were probably also related to a change in habitat, i.e. the colonization of freshwater. Overall, these data support the idea that *E. siliculosus* may be a complex of several (cryptic) species (Stache-Crain et al. 1997, Peters et al. in prep. a, b).

The most variable regions include FCPs and Transposons

The high correlation coefficients between the profiles of the more distant strains (3 and 4) further demonstrate that, among the examined strains, some regions of the *Ectocarpus* genome are less conserved than others. One possible explanation could be that the examined strains are more closely related to each other than to the reference strain. However, considering the fact that this correlation was also found for the *E. fasciculatus* strain 5, a strain of a different species, this is unlikely. A much more probable explanation is that different groups of genes or genomic regions are subject to different selective pressures and thus to different evolutionary rates. This could lead to both differences in copy number and / or differences in sequence identity. Such differences have been observed in several organisms. Sharp et al. (1989) demonstrated that, in bacteria, the mutation rate of lowly expressed genes is positively correlated with the distance from the origin of replication. Ten years later, a comparison of

mutation rates in mouse and human sequences (Matassi et al. 1999) confirmed that different chromosomal regions also evolved at different rates in mammals. In vascular plants, a targeted study of genes involved in the anthocyanin biosynthetic pathway showed that the evolutionary rate of genes of this pathway varied according to function: genes involved in several different pathways were more conserved, while regulatory genes exhibited higher variability (Rausher et al. 1999). All three aforementioned studies were based on sequence comparisons between different species. In this study, we employed a different approach, comparative genome hybridization, for different strains of the same or closely related species, and detected two functional groups of genes subjected to particularly high variability in copy number- or sequence variation: transposable elements and FCPs.

Transposable elements are a major component of many eukaryotic genomes, which, in some organisms, can account for over 50 % of the genome (reviewed in Gogvadze & Buzdin 2009). They are usually silenced by methylation or RNAi-like mechanisms. Our findings demonstrate that transposable elements were among the most variable components of the *Ectocarpus* genome, and that even very closely related strains (strains 1 and 2) differed with respect to transposons. This is in agreement with the finding that transposable elements in *Ectocarpus* are both highly expressed and lack methylation (Cock et al. in prep.), and underlines their potentially important role as a driving force in the evolution of the *Ectocarpus* species complex.

The second group of genes that was identified to be highly variable among the tested strains of *Ectocarpus* consisted of FCPs. The *Ectocarpus* genome contains a total of 53 FCPs (Cock et al. in prep.). Many of these FCPs have a high degree of sequence similarity, and some are located in close proximity on the same supercontig, both observations suggesting recent gene duplications within this family. As a part of the heterokont light harvesting complex, FCPs are classically thought to function primarily in the transmission of light energy to chlorophyll, although recent transcriptomic studies in *Chaetoceros* and in *Ectocarpus* showed some FCPs to be transcriptionally induced in response to stress (Hwang et al. 2007, Dittami et al. 2009). The recent expansion of the FCPs family in brown algae, as well as the evidence for high variation between different strains of *Ectocarpus* presented in this study, would fit well with the hypothesis that FCPs have evolved or are evolving to serve different functions within the chloroplast (chapter 3.4).

Mitochondrial and chloroplastic DNA

While the previous results relate to all of the examined strains, more specific differences were obtained by the segmentation analysis. One of the most striking differences identified by this analysis was the difference in content of mitochondrial and chloroplastic DNA in the strains 2 and 3 compared to the reference strain 1. With respect to strain 2, one explanation for this difference could be an increased number of mitochondria or increased content of mitochondrial DNA, which could potentially be related to sex. In strain 3, we observed a decrease in signal intensity; hence sequence variations might provide an additional explanation. However, chloroplastic and mitochondrial DNA contents are known to be influenced by several factors. Chloroplastic DNA content, for example, is known to fluctuate during plant development (Lamppa & Bendich 1979; Miyamura et al. 1986). In yeast, high variations in DNA content per mitochondrion were detected, depending on culture conditions (Miyakawa et al. 2004). This was also observed in different human oocytes, even if they originated from the same person (Reynier et al. 2001). In addition, both mitochondrial and chloroplastic DNA content varied strongly during different cell cycle phases and light phases in the green alga *Chlamydomonas reinhardtii* (Hiramatsu et al. 2006). In the light of these findings, and considering the fact that cultures were kept under constant culture conditions, but that not all cultures were harvested the same day, we prefer not to speculate about possible differences between the strains regarding cell organelles. More targeted studies examining the organellar DNA content under various different culture conditions would be required to address this question and to ensure that neither culture conditions nor differences in the adaptation of the different strains to various conditions introduce a bias.

Differences in the nuclear genome between strains 1 and 2 related to viral infection

In addition to the organellar genomes, three regions of the nuclear genome were found to differ significantly between the strains. One of these regions was a transposon-rich region in strain 3 (see above). The other two regions were both found to differ between strains 1 and 2, and concerned the *E. siliculosus* virus 1 (EsV-1) and a rather small genomic supercontig, both of which will be discussed in the following sections.
In strain 1, EsV-1 (Delaroque et al. 2001) is inserted mainly into the genomic supercontig 52. In spite of the presence of this virus in the genome, symptoms of a

viral infection have not been observed in this strain, and transcriptomic data suggested that viral genes are not transcribed (Cock et al. in prep.). As strains 3 and 4 showed similar signal intensity in this region compared to the reference strain 1, both strains may also contain EsV-1, although, just as in the reference strain 1, viral infection was not observed.

In strain 2, the region of the viral insertion on supercontig 52 exhibited 2.2-fold lower signal intensities compared to strain 1 (Figure 5). As EsV-1 is present in a single copy in the reference strain, this difference could be due either to the absence of EsV-1 in the genome of strain 2, in which case the remaining signals for strain 2 could be explained either by unspecific binding, or by the presence of highly divergent EsV-1-like sequences. Although the average signal in this region was lower in strain 2, in several cases the log2-ratio between the two strains reached zero or, in one case of one unknown viral gene (EsV-231, Figure 5), even positive values. Altogether, these observations favor the hypothesis of EsV-1-like sequences in the genome of strain 2.

Since symptoms of viral infection have not been observed in strain 2 (like in the other strains of *Ectocarpus* used in this experiment), one hypothesis could be that strain 2 contains only a degenerated version of EsV-1. An alternative explanation can be provided by an observation made in a previous study: Müller et al. (2000) detected amplification of a viral gene in a population of *Ectocarpus sp.* at different annealing temperatures depending on the individual, suggesting the presence of several distinct, but genetically similar, viruses within the same population. The hypothesis that strain 2 contains such a related *E. siliculosus* virus would agree with the profiles in our experiment. Further information on the viral genes potentially present in strain 2, including their insertion site, might provide clues on which common features could be responsible for the silencing of viral gene expression.

The role of supercontig 68 in Ectocarpus

Another interesting result of this study was the significantly lower signal for probes on supercontig 68 in strain 2, which could be due to two reasons: deletion(s), or very high variability of this region in strain 2. Considering the fact that the differences in signal intensity on supercontig 68 range from log2-ratios of -3.36 to 0.36, and the validation of this observation by qPCR experiments, the first hypothesis, i.e. a complete deletion of this region, seems unlikely. Regarding the second hypothesis, i.e. low sequence identity between the strains, sex related differences could provide one

possible explanation. Extensive work is currently being carried out to test this latter hypothesis (Coelho & Cock pers. comm.).

Conclusion

In this study, we assessed the genomic differences between four strains of *E. siliculosus* and one strain of *E. fasciculatus,* thus providing a basis for transcriptomic comparisons of *Ectocarpus* strains using the existing microarray *via* the selection of conserved probes. Our findings also underline the value of CGH experiments for the comparison of different strains or species: for instance, we detected significant genomic differences between different ecotypes thought to belong to the same species, suggesting the presence of several cryptic species in *E. siliculosus*. In addition, our data indicated strong differences in the content of organellar DNA, and more importantly demonstrated that different functional elements of the *Ectocarpus* genome were likely to evolve at different rates. Both transposable elements and FCPs were identified as part of the most variable elements in terms of copy number and / or sequence identity, and could play important roles in the evolution of different strains of *Ectocarpales*. Moreover, we provide first indications of the presence of ESV-1-like sequences in the genome of one of the examined strains, paving the way for further analyses, for instance of viral diversity as well as differences in its integration sites.

Acknowledgments

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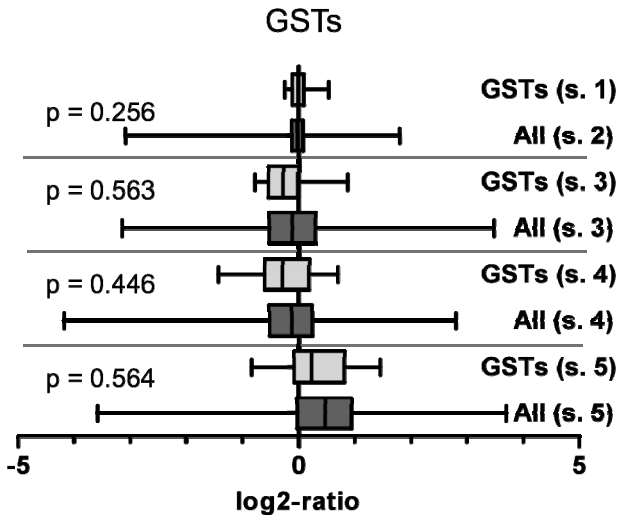
Supplementary files

Supplementary file 1: ".pos" file generated for the *E. siliculosus* gene expression array version 1. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/. An updated version of this file may also be available in the "array design" section of our transcriptomics homepage [http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/]

Supplementary file 2: List of probes with absolute log2-ratios > 0.5 for all examined strains of *E. siliculosus*. The included Java program "ProbeRemover" can be used to easily remove these probes from raw pair files generated by NimbleScan before normalization and probe averaging. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/. An updated version of "ProbeRemover" may also available in the "tools" section of our transcriptomics homepage [http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/]

Supplementary file 3: Log2-ratios of signal intensity between sample strain (2-5) and reference strain (1) of transposable elements, Fucoxanthin-chlorophyll a/c binding proteins (FCPs), and glutathione-S-transferases (GSTs) represented on the array. This file is available online at: http://www.sb-roscoff.fr/UMR7139/ectocarpus/transcriptomics/Supplementary_files_thesis/

Supplementary file 4: Box plot of log2-ratios for glutathione-S-transferases (GSTs). The Log2-ratios for all genes of each strain (s. 2-5) are shown in dark grey, while those of GSTs are shown in light grey. Corresponding p-values were determined using a t-test. A shift towards negative log2-ratios for GSTs compared to all genes in the same strain indicates a lower degree of conservation or changes in copy number.



4.3
The *Ectocarpus* freshwater strain

Transcriptomic and metabolite changes in a freshwater ecotype of *Ectocarpus* (Phaeophycaeae) provide indications on mechanisms underlying its transition from marine to freshwater habitats

- preliminary manuscript -

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Abstract

The invasion of freshwater by marine species is a rare event, which in the case of green algae preceded the colonization of land, yet very little is known about this process. Brown algae represent an independent lineage of photosynthetic, multicellular, and primarily marine organisms, although a few species have colonized freshwater. *Ectocarpus siliculosus*, a new genetic model for brown algae, is of particular interest for studying the transition to freshwater, as at is considered a marine species, which at least on one occasion has been isolated from freshwater.

In this report, we examine the salinity tolerance of this freshwater isolate and its adaptations to low salinities on a transcriptomic and a metabolite level. Compared to the sequenced marine strain, the freshwater isolate differed strongly in its tolerance of low but not of high salinities. It exhibited profound, but reversible, morphological and transcriptomic changes when transferred from strongly diluted to undiluted seawater, where gene expression profiles were similar to those of the marine strain. The biological relevance of many of the observed changes still remains unknown, although some of the variations in gene expression were related to cell wall modifications. Transcriptomic changes between different growth conditions were paralleled by strong changes in metabolite concentrations. Unlike the transcriptomic changes, metabolite profiles also differed between the marine strain and the freshwater isolate even under identical culture conditions. This shows that evolutionary adaptations, may, to a certain degree, bypass transcription.

These findings are discussed in an evolutionary context and present a first step towards answering the important question of how this brown alga might have colonized freshwater.

Introduction

Freshwater colonizations are rare events and frequently the cause of rapid evolutionary radiations (Lee & Bell 1999). Even within microbes, which, due to their short generation time, their high dispersal potential, and their high genetic diversity would be considered ideal candidates to master such harsh transitions, freshwater colonizations are infrequent (Logares et al. 2009). Within the plant kingdom, it is generally believed that all vascular plants have originated from a group of freshwater charophyte green algae – a sister group of chlorophyte green algae (McCourt et al. 2004). However, very little is known about the evolutionary events that led to the colonization of freshwater by green algae in the first place – a gap in knowledge partially due to the lack of fossil records of green algae (Coleman 1996).

While vascular plants, green algae, and, to a lesser extent, red algae have become the dominant macrophytes in freshwater environments, only about one percent of brown algal species (five genera) have colonized this habitat (Bold & Wynne 1985, Sigee 2005). These species are mainly small filamentous or crust-forming species belonging to the *Ectocarpales* or closely related orders. Some of them are found exclusively in freshwater habitats such as *Bodanella lauterborni* (Zimmerman) or *Heribaudiella fluviatilis* (Areschoug) Svedelius, while others, such as *Porterinema fluviatile* (Porter) Waern can also be found in brackish or marine environments (McCauley & Wehr 2007).

Ectocarpus siliculosus (Dillwyn) Lyngbye is considered a marine and brackish water species or possibly a complex of several sub-species (Stache-Crain et al. 1997, Peters et al. in prep., chapter 4.2), and is known for its great morphological (Ravanko, 1970) and transcriptomic plasticity (Dittami et al. 2009). It occurs world-wide in oceans of the temperate regions (Charrier et al. 2008). Different ecotypes of *E. siliculosus* have been shown to have different salinity optima (Russell & Bolton 1975), and there are a few reports of *E. siliculosus* strains isolated from freshwater. Geissler (1983), for example, reported a finding of *E. siliculosus* in a heavily salt-contaminated section of the Werra River in Germany, and West and Kraft (1996) isolated a strain of *E. siliculosus* from a true freshwater habitat in Australia. The latter strain has been deposited in public culture collections and its original classification as *E. siliculosus*

has been confirmed by sequencing of the RUBISCO large subunit (McCauley & Wehr 2007).

E. siliculosus has been established as a new model for brown algae (Peters et al. 2004a), and several genomic resources including a nearly genome-wide EST-based microarray (Dittami et al. 2009) and the 10.5-fold coverage of the genome sequence (Cock et al. in prep.), are now available. Furthermore, the suitability of comparative microarray studies between strains including the freshwater strain has been assessed (chapter 4.2), making *E. siliculosus*, and in particular the freshwater strain isolated by West and Kraft (1996), an excellent model to study the adaptation of a primarily marine photosynthetic organism to freshwater.

In this study we explore some of the adaptations possibly underlying this drastic change in habitat. We investigate the salt tolerance of the freshwater strain and perform extensive transcriptomic and metabolic profiling in low and high salinity conditions. Our results demonstrate that, in the examined strain of *E. siliculosus*, the transition to freshwater is a reversible process accompanied by fundamental morphological, transcriptomic, and metabolic changes, and highlight some of the processes possibly involved.

Materials and Methods

Culture conditions and experimental setup

Two strains of *E. siliculosus* (Dillwyn) Lyngbye (Ectocarpales, Phaeophyceae), the marine strain (MS) sequenced in the *Ecotcarpus* genome project (accession CCAP 1310/4, origin San Juan de Marcona, Peru) and a freshwater strain (FWS, accession CCAP 1310/196, origin Hopkins River Falls, Victoria, Australia) were cultured in 10 L Nalgene flasks in a culture room at 14 °C. Lighting was provided at an intensity of 40 µE m^{-2} s^{-1} for 14 hours per day. Cultures were aerated with filtered (0.22 µm) compressed air to avoid CO_2 depletion. The freshwater strain was cultured in both natural seawater with a salinity of ~32 ppt (32 ppt medium) and a mixture of 5 % seawater and 95 % distilled water with a salinity of ~ 1.6 ppt (1.6 ppt medium) for at least 6 month prior to the experiments. The marine strain was cultured only in 32 ppt medium. All culture media were enriched with Provasoli nutrients as described by Starr and Zeikus (1993).

Experiments to test the salinity tolerance of both strains were performed in 14 cm sterile petri dishes under identical light conditions. Media required for these experiments were mixed as described above, by diluting natural seawater with distilled water (for 0 ppt to 16 ppt media) or by adding appropriate amounts of NaCl (for 64 ppt to 160 ppt media). Strains were incubated in each of these media for one week prior to measurements of photosynthesis. All experiments were carried out in biological triplicates (i.e. three independent cultures).

Measurements of photosynthesis

To test the salinity tolerance, quantum yield (F_v/F_m) was measured fluorometrically on small sub-sample, using a Walz Phyto-PAM (Waltz, Germany) and default parameters (actinic light intensity 3, ~ 90 µE m^{-2} s^{-1}; saturation pulse intensity 10, ~ 2,000 µE m^{-2} s^{-1}, 200 ms). Quantum yield is a marker of photosynthetic efficiency (Björkman & Demming 1987) and thus of the physiological state of the alga.

Nucleic acid extraction and ITS1 sequencing

Samples for nucleic acid extraction (approximately 100 mg, wet weight) were harvested by filtration two hours after beginning of the light phase, quickly dried with a paper towel and immediately frozen and ground in liquid nitrogen. Nucleic acids were then extracted following the protocol outlined by Apt et al. (1995) with modifications as described in Le Bail et al. (2008). This protocol uses a cetyltrimethylammonium bromide (CTAB)-based buffer for extraction and subsequent phenol-chloroform purification. After precipitation with LiCl, RNA was subjected to a DNAse (Turbo DNAse, Ambion, Austin, TX, USA) treatment according to the vendor's recommendations. Nucleic acid quantity and quality was then verified using a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA) and on agarose gel.

DNA, contained in the supernatant of the LiCl precipitation, was used for the sequencing of the ITS1 region. To this means, DNA was precipitated with one volume of isopropanol and purified using Chromaspin 1000 columns (Clontech, Mountain View, CA, USA) before amplifying the region of interest by PCR as previously described (Peters et al. 2004b). The fragment obtained was cloned in the

pGEM-T® Easy Vector System (Promega, Madison, WI, USA) before sequencing. The resulting ITS1 sequence was deposited under accession number [gb|GQ351370].

Microarray experiments

Double strand cDNA for microarray experiments was synthesized and amplified from 100 ng of total RNA as previously described (Dittami et al. 2009) using the SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA) and the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Microarray experiments were carried out as a service by Roche Nimblegen using the *Ectocarpus siliculosus* 72k Gene Expression Array v1 [ArrayExpress: A-MEXP-1445]. This nearly genome-wide EST-based microarray contains probes for 17,119 unigenes, represented by four probes each. Microarray data was deposited under the accession number [ArrayExpress: E-TABM-731] (Reviewer's username: Reviewer_E-TABM-731, password: 1246282263528)

Total free fatty acids

The determination of total free fatty acids was performed as described in chapter 3.3. Approximately 400 mg (fresh weight) of sample were ground in liquid nitrogen and extracted with 2 ml of ethyl acetate as previously described (Küpper et al., 2006). Two hundred and fifty ng of 12-OH-lauric acid were added as internal standard. Extracts were evaporated under a stream of nitrogen, resuspended in 100 µL ethanol and analyzed by gas chromatography / mass spectrometry (GC-MS) as previously described by Ritter et al. (2008).

Amino acids and non-structural carbohydrates

For amino acid-, sugar-, polyol-, and organic acid determination, samples were ground in liquid nitrogen, freeze-dried, extracted, and quantified as described in chapters 2.2 and 3.3, with modifications for the GC-MS analysis. Approximately 10 mg of freeze-dried sample, corresponding to roughly 50 mg fresh weight, were weighted and suspended in 400 µL of a methanolic solution containing 100 µM of DL-3-aminobutyric acid and 200 µM ribitol. After 15 min of agitation at room temperature, 200 µL of chloroform were added followed by another 5-min agitation step. Finally, 400 µL of water were added, and samples were vortexed vigorously before centrifugation at 13,000*g* for 5 min. Two 50 µL and one 400 µL aliquots of the

upper phase, which contained polar metabolites including amino acids, polyols and sugars, were transferred to clean vials and vacuum-dried for subsequent chromatographic analysis.

For amino acid profiling, 50 µL vacuum-dried polar phase aliquots were resuspended in 50 µL of ultra-pure water and 10 µL were used for the derivatization using the AccQ-Tag Ultra derivatization kit (Waters). Derivatized amino acids were analyzed using an Acquity UPLC system (Waters) according to Jubault et al. (2008), using DL-3-aminobutyric acid as internal standard.

Non-structural carbohydrates (NSCs) were determined both by gas chromatography-flame ionization detection (GC-FID; mannitol, glucose, citrate) and gas chromatography-mass spectrometry (GC-MS). GC-FID analysis was carried out according to Adams et al. (1999) and Lugan et al. (2009): vacuum-dried polar phase aliquots (50 µL) were dissolved in 50 µL of 20 mg mL^{-1} methoxyaminehydrochloride in pyridine at 30 °C for 90 min under orbital shaking. Fifty µL of N,O-bis(trimethylsisyl)trifluoroacetamide (BSTFA) were added and samples were incubated at 37 °C for 30 min, then at room temperature overnight before injection. One µL of the mixture was injected in a Trace 2000 GC-FID (Thermo-Fisher Scientific, Waltham, CA, USA) equipped with an AS2000 Autosampler (Thermo-Fisher Scientific), a split/splitless injector (split mode set to 1:25) at 230 °C, a J&W DB5 30 m x 0.32 mm x 0.25 mm column, and an FID detector at 250 °C. The temperature gradient was: 5 min at 70 °C, 5 °C min^{-1} until 220 °C, 2 °C min^{-1} until 260 °C, 20 °C min^{-1}until 300 °C and finally 5 min at 300 °C. Ribitol was used as internal standard.

For GC-MS profiling of non-structural carbohydrates and organic acids, the 400 µL vacuum-dried polar phase aliquots were resuspended in 50 µL of 20 g L^{-1} methoxyamine-hydrochloride (SIGMA) in pyridine before incubation under orbital shaking at 30°C for 1h30. After addition of one volume of N,O-bis(trimethylsisyl)trifluoroacetamide (Sigma-Aldrich, St. Louis, MO, USA), samples were incubated 37 min at 30°C, transferred to glass vials, and incubated at room temperature overnight before injection. GC-MS analysis was performed according to Roessner et al. (2001). Ionization was interrupted during elution of mannitol, to preserve the source and avoid detector saturation. The GC-MS system consisted of a TriPlus auto sampler, a Trace GC Ultra chromatograph and a Trace DSQII

quadrupole mass spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). Chromatograms were deconvoluted using the AMDIS software v2.65 [http://chemdata.nist.gov/mass-spc/amdis/] associated with NIST libraries. Metabolite levels were quantified using ribitol as internal standard and by comparison with external standards for several metabolites.

Amino acids and non-structural carbohydrates were determined in µmol per gram dry weight. The dry weight used to calculate the final metabolite concentrations was corrected to account for differences in the amount of salt in our samples. This calculation was based on the observation that 1 mg dry weight corresponds to 5 mg fresh weight and previous observations that in *Ectocarpus*, the intracellular salt concentration increases linearly with the extracellular salt concentration (Dittami et al. 2009). The effect of salt according to this model would have led to a 20 % under-estimation of the relative amino acid contents in 32 ppt medium. Unless stated otherwise, this correction did not alter the results qualitatively. Precise measurements of the intracellular ion concentrations in the freshwater samples are currently underway.

Glutathione

The sum of glutathione and glutathione disulfide was determined spectrophotometrically measuring the rate of formation of 5-thio-2-nitrobenzoate (TNB) via the glutathione reductase catalyzed reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Akerboom & Sies 1981). Approximately 300 mg (wet weight) of sample were weighed, suspended in 3 ml of 5 % (m/v) 5-sulfosalicylic acid, and centrifuged for 15 min at 5,000 g (4°C). Four µl of supernatant were diluted with 996 µl of reaction buffer (0.1 M potassium phosphate and 1 mM EDTA, pH 7.5; 15 mM NADPH; 60 µM DTNB; and 0.66 units ml^{-1} glutathione reductase). The rate of TNB formation was determined in the linear phase of the reaction as difference in optical density (412 nm), after 10 and 30 min of incubation at 37 °C, and compared to a standard curve with 0 to 0.2 nmol reduced l-glutathione (Sigma-Aldrich, St. Louis, MO, USA).

Data analysis

The freshwater strain ITS1 sequence was aligned with previously published ITS1 sequences from different species of *Ectocarpus* (Stache-Crain et al. 1997) as well as

one sequence of *Laminaria digitata* using MAFFT (Katoh et al. 2002). Alignments were manually refined using Bioedit 7.0.9.0 (Hall 1999), and used for phylogenetic analysis with the phylogeny.fr platform (Dereeper et. al. 2008) using PhyML (Guindon & Gascuel 2003) and the approximate likelihood-ratio test (Anisimova & Gascuel 2006).

For microarray analysis, raw expression data was obtained directly from Roche NimbleGen. Only well conserved probes according to our previous comparative genome hybridization (CGH) experiments (chapter 4.2) were considered for further analysis (i.e. probes with an absolute log2-ratio inferior to 0.5 in our CGH experiment between the marine and the freshwater strain), resulting in the retention of 14,552 of 17,119 genes. Normalization and probe averaging was performed using the NimbleScan 2.4 software (Roche NimbleGen, Madison, WI, USA) and the Quantile Normalization / Robust Multichip Average algorithms (Irizarry et al. 2003ab). Only genes with expression values over 450 were considered for further analysis (Dittami et al. 2009), leaving 11,256 genes for the final analysis. Differentially expressed genes were identified between strains and conditions using a t-test and TigrMEV 4.4 (Saeed et al. 2003) with subsequent calculation of the FDR according to Benjamini and Hochberg (1995). Enriched gene ontology terms were searched for using the GO Local Exploration Map (GOLEM) software (version 2.1) (Sealfon et al. 2006) and the publicly available GO annotations [http://www.sb-roscoff.fr/UMR7139/transcriptomics/ectocarpus].

Metabolite data were compared by means of a t-test. As three different conditions (freshwater strain in 1.6 ppt medium, freshwater strain in 32 ppt medium, and marine strain in 32 ppt medium) were compared, a Bonferroni correction was applied.

Results

Ribosomal sequences confirm classification as E. siliculosus

In order to complete the molecular characterisation of the freshwater strain, our first step was to verify the classification by sequencing of the internal transcribed spacer 1 of the nuclear ribosomal cistron (ITS1) (Peters et al. 2004b). Despite the genomic differences detected in our previous CGH experiment (chapter 4.2) between the marine strain and the freshwater strain, the phylogenetic analysis of ITS1 sequences presented in Figure 1 confirmed the original classification as *E. siliculosus* by West

and Kraft (1996) and McCauley and Wehr (2007). However, experimental proof of the species concept, i.e. crosses with other strains of *E. siliculosus*, was not obtained, as only asexual reproduction was observed in this strain so far (A. Peters, pers. comm.), and it needs to be kept in mind that *E. siliculosus* is likely to be a complex of several cryptic subspecies (Stache-Crain et. al. 1997, Peters et al. in prep., chapter 4.2).

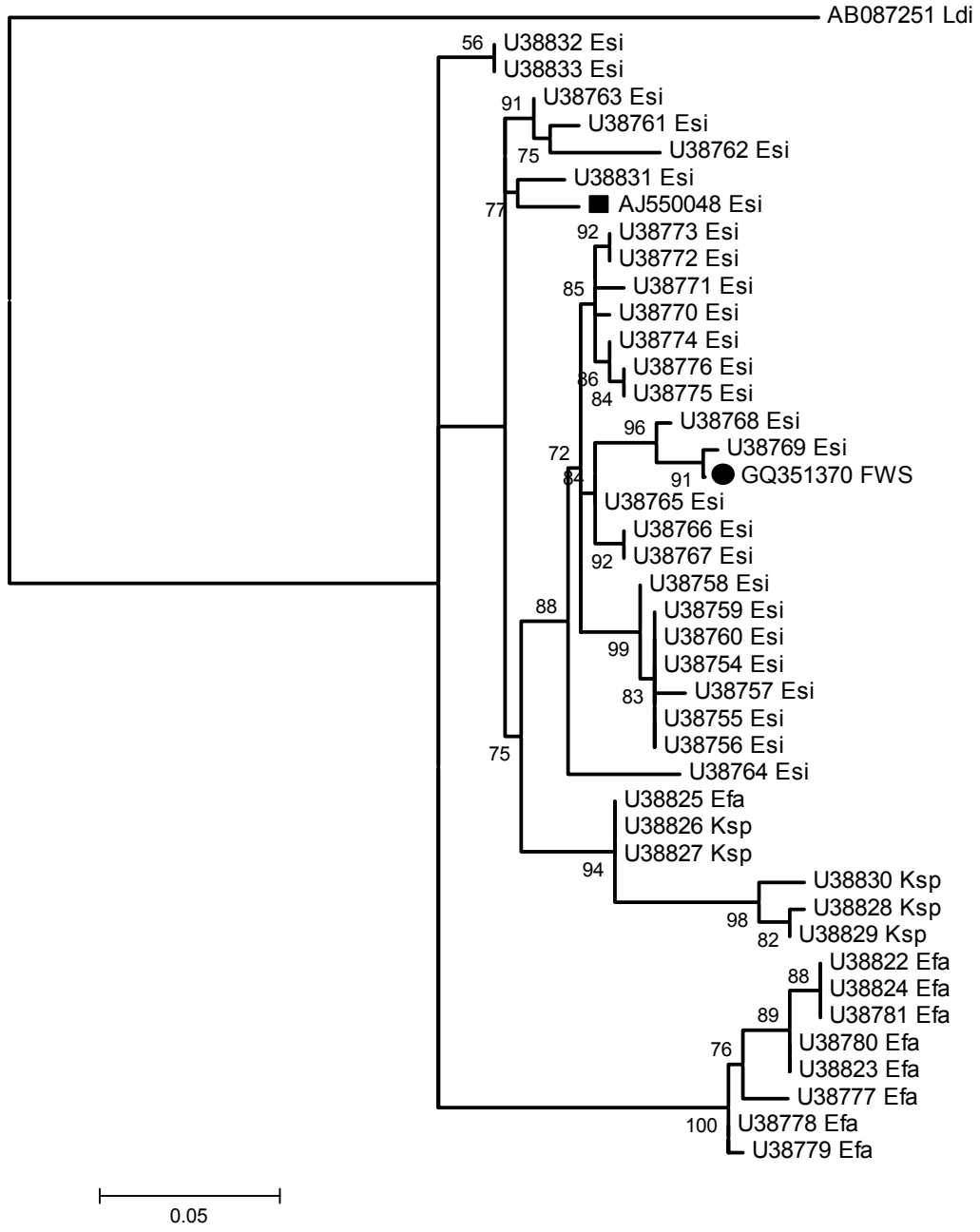

Figure 1: Maximum likelihood tree of sequences of 43 brown algal internal transcribed spacer sequences (ITS1) using PhyML and approximate likelihood-ratio testing. Only likelihood-values > 50 are displayed in the tree. Sequence names consist of the Genbank accession number followed by the species: Esi = *Ectocarpus siliculosus*, Efa = *E. fasciculatus*, Ksp = *Kuckuckia sp.*, Ldi = *Laminaria digitata*, FWS = freshwater strain of *Ectocarpus* (closed circle). The marine strain used in this study (AJ550048) is marked by a square.

The freshwater strain tolerates a large range of salinities

While the freshwater strain was able to reestablish a reasonable photosynthetic efficiency (F_v/F_m>0.3) after direct transfer to salinities ranging from 0.6 to 64 ppt, the marine strain performed equally well only in salinities from 6.4 to 64 ppt (Figure 2). Thus, the freshwater strain differed from the marine strain only in its ability to quickly adapt to low salinities, and has retained its ability to grow under high salinities. Even when adapted gradually, the marine strain tolerates only salinities down to about 2 ppt (supplementary file 1), while the freshwater strain can still grow at 0 ppt (West and Kraft, 1996).

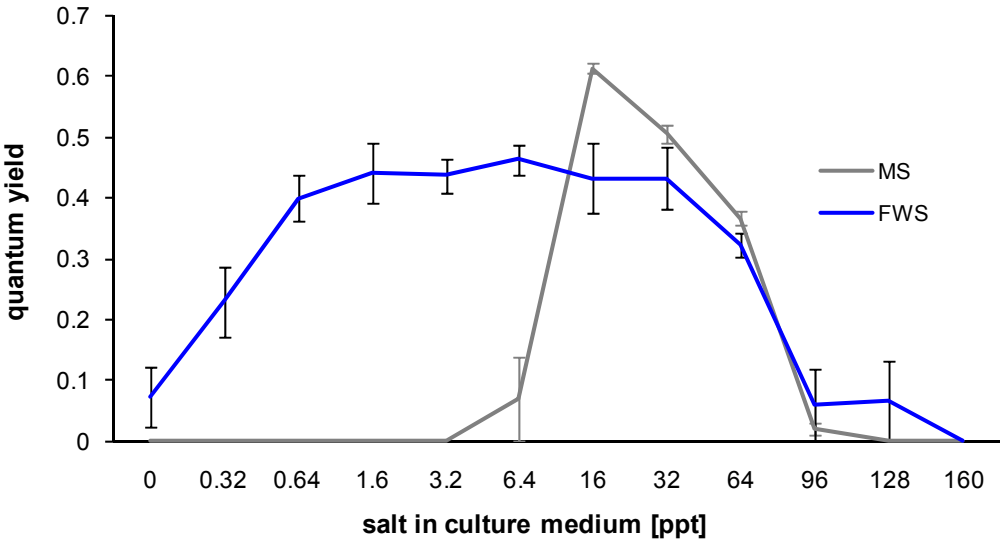

Figure 2: Efficiency of photosynthesis (quantum yield) of the freshwater strain (FWS) and the marine strain (MS) of *E. siliculosus* 7 days after a direct transfer from 32 ppt medium to different salinities (mean of three replicates ± SE).

Salinity-dependent phenotypic and transcriptomic plasticity

Interestingly, the freshwater strain exhibited very different morphotypes in 1.6 ppt and in 32 ppt culture medium (Figure 3). In 1.6 ppt medium, it formed long sticky filaments and patches, while in normal seawater (32 ppt medium) it produced detached, star-like structures. The latter much resembled the phenotype of the marine strain, which did not exhibit any apparent changes in morphology in response to salinity. These changes took a few weeks to fully develop (Figure 3), and were fully reversible.

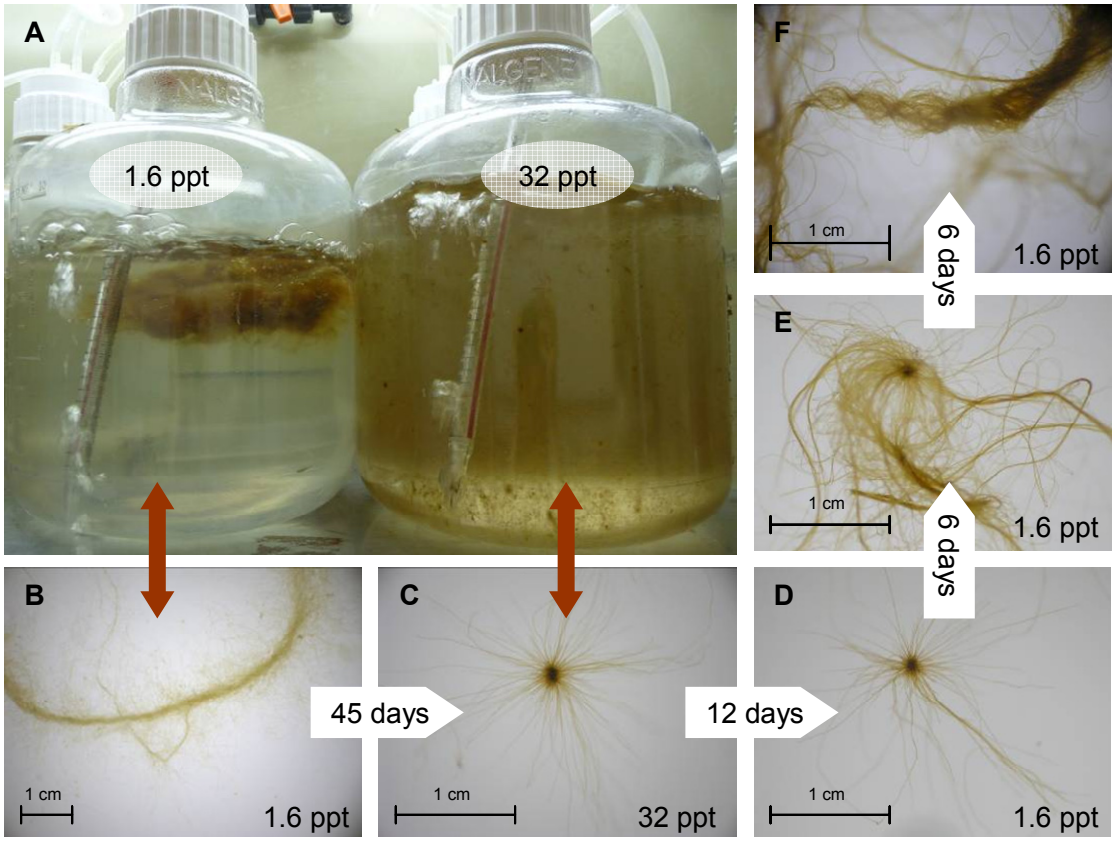

Figure 3: Morphological changes of the freshwater strain under different salinities. Panel A shows large-scale cultures in 1.6 ppt medium (left) and 32 ppt medium (right). Panels B-F display a culture in 1.6 ppt medium (B), 45 days after transferring it to 32 ppt medium (C), and 12, 18, and 24 days after transferring it back to 1.6 ppt medium (D-F).

These morphological changes were accompanied by massive changes in the transcriptome (Figure 4, Figure 5). We observed differential regulation of 3,004 genes (27 %) at an FDR of 5 % and differential regulation of 5,842 genes (52 %) at an FDR of 10 %. Although these numbers seem comparable with our previous experiments regarding the short-term response to abiotic stress (Dittami et al. 2009), this comparison is biased because only three replicates were tested in this study (vs. four in our previous report) and because some genes were represented by fewer probes (see "data analysis" section in materials and methods), which increased the variance. Therefore, Figure 4 shows the distribution of log2-ratios in this study and the saline stress conditions in our previous experiment without considering p-values. While the number of genes changing expression <2 fold (log2-ratio between -1 and 1) was lower in this experiment compared to our previous one (29 % vs. 52-60 %), the number of genes with expression changes larger than 4-fold (log2-ratio > 2 or <-2) was higher (47 % vs. 15 – 23 %).

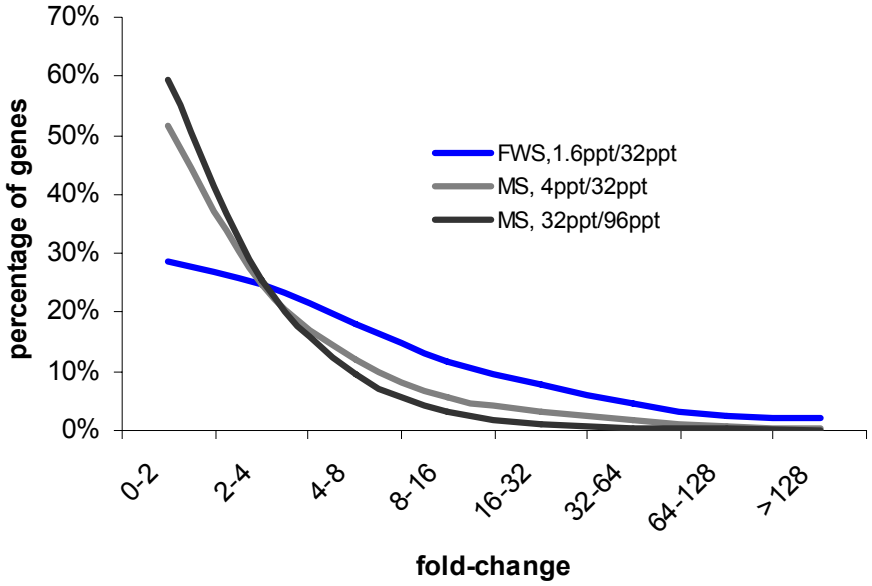

Figure 4: Distribution of fold-changes between the freshwater strain (FSW) in 1.6 ppt and 32 ppt medium (blue), and those previously observed in the marine strain (MS) during the short-term response to saline stress (Dittami et al. 2009).

Furthermore, the changes between the freshwater strain adapted to 1.6 ppt and 32 ppt medium did not seem to be focused on specific pathways. Effectively, an analysis based on the automated GO annotations did not yield any significantly overrepresented GO terms among the genes that were differentially expressed between both conditions tested.

Generally, the gene expression profile of the freshwater strain in 32 ppt medium very much resembled that of the marine strain in the same medium (Figure 5). The Pearson correlation coefficient between gene expression profiles of the two strains in the same conditions was 0.82, while that of the freshwater strain in 32 ppt medium and the freshwater strain in 1.6 ppt medium was only 0.38. Furthermore, a t-test with FDR-correction (5 %) did not identify any genes that were significantly differentially expressed between the marine strain and the freshwater strain in seawater.A direct comparison of transcriptomic changes in the freshwater strain with those previously obtained for the marine strain during the short-term hyposaline, hypersaline, and oxidative stress response (Dittami et al. 2009) showed very few similarities. Correlation coefficients observed between the results of the two sets of experiments (0.018 to 0.085) were much lower than those observed within the experiment on the short-term response to different stresses (0.29 to 0.65) (Table 1).

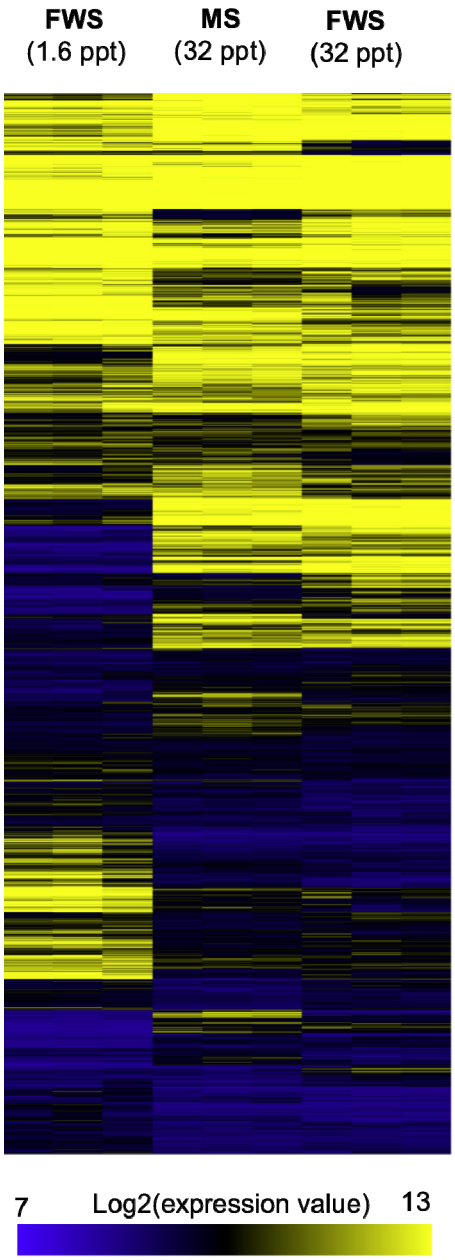

Figure 5: Expression profiles of the marine strain (MS) in 32 ppt medium and the freshwater strain in both 32 ppt and 1.6 ppt medium (three replicates each). This graph is strongly compressed in the vertical axis, which corresponds to 11,256 genes.

Table 1: Pearson correlation coefficients between transcriptomic changes in the freshwater strain cultivated in 1.6 ppt and 32 ppt medium, and between previous experiments on the short term (6h) response to saline stress and oxidative stress in the marine strain (Dittami et al. 2009). All conditions were compared to the control condition with a salinity of 32 ppt. Hypo = 4 ppt / 32 ppt, Hyper = 96 ppt / 32 ppt, Oxi = 32 ppt + 1 mM H2O2 / 32 ppt

	1.6/32ppt	**Hypo**	**Hyper**	**Oxi**
1.6/32ppt	1.00	**0.085**	**0.018**	**0.034**
Hypo		1.00	0.25	0.67
Hyper			1.00	0.29
Oxi				1.00

Changes in gene expression related to cell wall synthesis and modification

Although an automatic analysis of the transcriptomic data did not yield insights on the exact processes involved in the switch from freshwater to seawater, a manual analysis of specific genes of interest allowed us to highlight some interesting trends (Table 2). Notably, formylglycine-dependent sulfatases, enzymes involved in the desulfatation of polysaccharides, were generally induced in 1.6 ppt medium, while two sulfotransferases likely to be involved in the synthesis of the sulfated cell wall polysaccharide fucan were induced in seawater.

We also examined C5-epimerases and found three of them to be strongly induced in 32 ppt medium (74-fold, 244-fold, and 1124-fold, Table 2). C5-epimerases catalyze the conversion of mannuronic acid (M) to guluronic acid (G), which the building blocks of alginates, important brown algal cell wall polysaccharides. C5-epimerases therefore control the texture of the cell wall matrix (Nyvall et al., 2003), and their

induction in 32 ppt medium could result in an increase in the proportion of poly-G chains in the cell wall.

Table 2: Changes in gene expression profiles of selected genes in the freshwater strain in 32 ppt (SW) compared to 1.6 ppt medium (FW). The table shows relative changes (means of three replicates); non-significant or small (< 2-fold) changes are shown in italics."-*" indicates that there was no significant change in transcriptomic regulation, and "**" indicates that the expression level was below the detection limit. MAN = mannitol synthesis; FA-des. = fatty acid desaturase; GABA = gamma-aminobutyric acid; vBPO = vanadium-dependant bromoperoxidase; # = gene likely to be involved in fucan synthesis. "

	name	genome	unigene	profile
C5-epimerases	Mannuronan C-5-epimerase	Esi0010_0210	CL31Contig1	1126.4 x induced in SW, p = 0.001
	Mannuronan C-5-epimerase	Esi0882_0001	CL88Contig1	**
	Mannuronan C-5-epimerase	Esi0017_0043	not on array	
	Mannuronan C-5-epimerase	Esi0019_0126	not on array	
	Mannuronan C-5-epimerase	Esi0019_0127	not on array	
	Mannuronan C-5-epimerase	Esi0030_0144	not on array	
	Mannuronan C-5-epimerase	Esi0039_0001	not on array	
	Mannuronan C-5-epimerase	Esi0045_0025	CL4174Contig1	6.57 x induced in SW, p = 0.002
			LQ0AAB12YH01FM1.SCF	*5.94 x induced in SW, p = 0.123*
	Mannuronan C-5-epimerase	Esi0045_0027	not on array	
	Mannuronan C-5-epimerase	Esi0052_0064	not on array	
	Mannuronan C-5-epimerase	Esi0069_0050	not on array	
	Mannuronan C-5-epimerase	Esi0069_0059	not on array	
	Mannuronan C-5-epimerase	Esi0071_0098f	CL313Contig1	-*
	Mannuronan C-5-epimerase	Esi0113_0051	not on array	
	Mannuronan C-5-epimerase	Esi0116_0047	not on array	
	Mannuronan C-5-epimerase	Esi0123_0084	not on array	
	Mannuronan C-5-epimerase	Esi0218_0013	not on array	
	Mannuronan C-5-epimerase	Esi0241_0023	CL602Contig1	-*
	Mannuronan C-5-epimerase	Esi0303_0025	not on array	
	Mannuronan C-5-epimerase	Esi0307_0015	not on array	
	Mannuronan C-5-epimerase	Esi0315_0022	not on array	
	Mannuronan C-5-epimerase	Esi0326_0043	CL466Contig1	**
	Mannuronan C-5-epimerase	Esi0326_0044	not on array	
	Mannuronan C-5-epimerase	Esi0340_0006	LQ0AFA5YK19RM1.SCF	8.96 x induced in FW, p = 0.029
	Mannuronan C-5-epimerase	Esi0367_0009	LQ0AAB32YL12FM1.SCF	74.39 x induced in SW, p = 0.001
	Mannuronan C-5-epimerase	Esi0367_0010	CL4717Contig1	5.09 x induced in FW, p = 0.003
	Mannuronan C-5-epimerase	Esi0466_0003	LQ0AAA13YK20FM1.SCF	-*
	Mannuronan C-5-epimerase	Esi0466_0009	CL2039Contig1	244.01 x induced in SW, p = 0.001
	Mannuronan C-5-epimerase	Esi0495_0002	not on array	
	Mannuronan C-5-epimerase	Esi0495_0003	CL1214Contig1	**
			LQ0AAB57YD17FM1.SCF	**
			LQ0AEA5YO08RM1.SCF	-*
v-BPO	vanadium-dependant bromoperoxidase	Esi0009_0080	CL83Contig1	50.55 x induced in SW, p = 0.013
			LQ0AAB24YG07FM1.SCF	-*
Formylglycine-dependent sulfatases	Formylglycine-dependent sulfatase	Esi0069_0045	CL7144Contig1	10.78 x induced in FW, p = 0.041
	Formylglycine-dependent sulfatase	Esi0086_0031	not on array	
	Formylglycine-dependent sulfatase	Esi0144_0037	LQ0AAB16YN18FM3.SCF	*3.81 x induced in FW, p = 0.22*
	Formylglycine-dependent sulfatase	Esi0160_0027	CL3472Contig1	-*
			LQ0AAB92YH04FM1.SCF	-*
	Formylglycine-dependent sulfatase	Esi0160_0032	LQ0AAB32YO05FM1.SCF	14.22 x induced in SW, p = 0.032
	Formylglycine-dependent sulfatase	Esi0160_0043	CL5654Contig1	4.73 x induced in FW, p = 5.5e-3
	Formylglycine-dependent sulfatase	Esi0160_0052	LQ0AAB56YK20RM1.SCF	3.34 x induced in FW, p = 0.026
			LQ0AAB61YC18FM1.SCF	*54.79 x induced in FW, p = 0.064*
	Formylglycine-dependent sulfatase	Esi0280_0019	LQ0AFA6YH11RM1.SCF	6.89 x induced in FW, p = 0.012
	Formylglycine-dependent sulfatase	Esi0444_0009	not on array	
Aryl sulfo-transferases	Aryl sulfo-transferase	Esi0028_0011	LQ0AAB64YH02FM1.SCF	*9.01 x induced in FW, p = 0.071*
	Aryl sulfo-transferase	Esi0442_0008	not on array	
	Aryl sulfo-transferase	Esi0197_0023	not on array	
	Aryl sulfo-transferase	Esi0411_0021	not on array	
	Aryl sulfo-transferase	Esi0028_0006	not on array	

unknown sulfotransferases	sulfotransferase	Esi0289_0025	CL1841Contig1	42.84 x induced in FW, p = 0.017
	sulfotransferase	Esi0032_0064	not on array	
	sulfotransferase	Esi0057_0043#	LQ0AAA1YO14FM1.SCF	28.81 x induced in SW, p = 6.2e-4
	sulfotransferase	Esi0118_0049	not on array	
	sulfotransferase	Esi0283_0018	not on array	
	sulfotransferase	Esi0210_0041	CL913Contig1	-*
	sulfotransferase	Esi0312_0029#	LQ0AAA1YL18FM1.SCF	56.4 x induced in SW, p = 0.018
FA-des.	microsomal Δ15 desaturase	Esi0231_0023	CL527Contig1	6.14 x induced in SW, p = 0.012
	chloroplastic Δ15 desaturase	Esi0073_0116	CL281Contig1	6.72 x induced in SW, p = 0.019
	microsomal Δ12-desaturase	Esi0207_0012	LQ0AAB83YE13RM1.SCF	3.94 x induced in SW, p = 0.031
	chloroplastic Δ12-desaturase	Esi0393_0016	CL69Contig1	-*
nitrogen assimilation	Nitrate reductase (NADH) (EC 1.7.1.1)	Esi0171_0046	LQ0AAB91YI21FM1.SCF	**
	Nitrate reductase (NADH) (EC 1.7.1.1)	Esi0006_0124	CL3329Contig1	45.57 x induced in SW, p = 0.023
	Nitrite reductase (ferredoxin) (EC 1.7.7.1)	Esi0003_0129	CL3736Contig1	-*
			CL5351Contig1	*2.65 x induced in SW, p = 0.123*
	Nitrite reductase (NAD(P)H) (EC 1.7.1.4)	Esi0249_0028	CL2999Contig1	51.07 x induced in SW, p < 0.001
			LQ0AAB8YN04FM1.SCF	-*
	Glutamate dehydrogenase (EC 1.4.1.2)	Esi0141_0079	LQ0AAB28YN11FM1.SCF	-*
	Glutamate dehydrogenase (EC 1.4.1.4)	Esi0028_0164	CL1656Contig1	-*
			CL2561Contig1	*1.33 x induced in FW, p = 0.039*
	Glutamate dehydrogenase (EC 1.4.1.4)	Esi0520_0002	not on array	
	Glutamine synthetase (EC 6.3.1.2)	Esi0072_0092	not on array	
	Glutamine synthetase (EC 6.3.1.2)	Esi0188_0004	CL44Contig1	*2.11 x induced in SW, p = 0.129*
			LQ0AAB88YP13FM1.SCF	**
	Glutamine synthetase (EC 6.3.1.2)	Esi0188_0005	CL44Contig3	-*
			CL44Contig4	-*
	Glutamate synthase	Esi0349_0025	not on array	
	Glutamate synthase (ferredoxin) (EC 1.4.7.1)	Esi0000_0281	CL2669Contig1	2.72 x induced in FW, p = 0.033
	Glutamate synthase (NADH/NADPH) (EC 1.4.1.14/13)	Esi0029_0131	CL5303Contig1	*9.49 x induced in FW, p = 0.081*
			CL4828Contig1	**
			LQ0AAB39YD12FM1.SCF	*2.17 x induced in FW, p = 0.254*
	Glutamate synthase (NADH/NADPH) (EC 1.4.1.14/13)	Esi0103_0010+	CL598Contig1	*2.57 x induced in FW, p = 0.318*
proline	Argininosuccinate lyase (EC 4.3.2.1)	Esi0081_0083	LQ0ADA3YJ12RM1.SCF	*15.42 x induced in SW, p = 0.081*
	Arginase (EC 3.5.3.1)	Esi0073_0060	LQ0AAB28YG22FM1.SCF	15.7 x induced in FW, p = 0.04
	Urease (EC 3.1.5.1)	Esi0000_0248	LQ0AAB14YK16FM1.SCF	72.69 x induced in FW, p = 0.004
	ornithine--oxo-acid transaminase (EC 2.6.1.13)	Esi0072_0109	LQ0AAB88YC13RM1.SCF	-*
			LQ0AAB10YP23FM1.SCF	72.15 x induced in SW, p = 0.001
	pyrroline-5-carboxylate reductase (EC 1.5.1.2)	Esi0075_0064	CL6298Contig1	152.81 x induced in FW, p = 0.002
			LQ0AAB36YB24FM1.SCF	-*
			LQ0AAB96YG16FM1.SCF	31.6 x induced in FW, p = 0.029
	proline dehydrogenase (EC 1.5.99.8)	Esi0269_0010	LQ0AAB31YD17FM1.SCF	**
	1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12)	Esi0418_0003	CL2518Contig1	**
	1-pyrroline-5-carboxylate synthase	Esi0663_0002	CL1823Contig1	220.52 x induced in SW, p < 0.001
GABA	Ornithine decarboxylase (EC 4.1.1.17)	Esi0048_0123	not on array	
	diamin oxidase (EC 1.4.3.22)	Esi0076_0063	CL5550Contig1	275.12 x induced in SW, p = 0.001
	diamin oxidase (EC 1.4.3.22)	Esi0076_0061	not on array	
	Aldehyde dehdrogenase	Esi0010_0069	CL5294Contig1	7.31 x induced in FW, p = 0.018
	Aldehyde dehdrogenase	Esi0451_0004	LQ0AAB34YA24FM1.SCF.LQ	**
	Aldehyde dehdrogenase	Esi0003_0206	CL6453Contig1	*5.4 x induced in FW, p = 0.065*
MAN	mannitol 1-phosphate dehydrogenase	Esi0017_0062	CL200Contig2	**
		Esi0020_0181	CL2843Contig	*2.23 x induced in SW, p = 0.078*
Glu-tatathione synthesis	Glutamate cystéine ligase (EC 6.3.2.2)	Esi0184_0033	LQ0AAB47YA16FM1.SCF	**
	Glutamate cystéine ligase (EC 6.3.2.2)	Esi0250_0012	CL4940Contig1	-*
			CL902Contig1	**
	Glutathion synthétase (EC 6.3.2.3)	Esi0066_0082	CL1154Contig1	*2.13 x induced in SW, p = 0.076*
			LQ0AAA4YA09FM1.SCF.LQ	**
Glutathione degradation	Gamma-glutamyl transpeptidase (EC 2.3.2.2)	Esi0014_0172	not on array	
	Peptidase (EC 3.4.11.2)	Esi0366_0009	CL2158Contig1	**
			LQ0AAA7YD13FM1.SCF	**
			LQ0AAB14YF12FM1.SCF	**
	5-oxo-prolinase(EC 3.5.2.9)	Esi0018_0028	CL6899Contig1	*4.58 x induced in SW, p = 0.066*
			LQ0AAA1YN14FM1.SCF	**
	5-oxo-prolinase(EC 3.5.2.9)	Esi1350_0001	not on array	
	Cyclotransférase(EC 2.3.2.4)	Esi0037_0100	CL3195Contig1	-*
one me	Glutathion reductase (EC 1.8.1.7)	Esi0019_0176	CL789Contig1	*2.13 x induced in FW, p = 0.26*

		CL3217Contig1	58.6 x induced in FW, p = 0.002
		LQ0AAB33YP03FM1.SCF	**
		LQ0AAB61YK20RM1.SCF	-*
		LQ0AAB27YH11FM1.SCF	**
Glutathion reductase (EC 1.8.1.7)	Esi0888_0002	CL2921Contig1	-*
		LQ0AAB92YP17RM1.SCF	**
Glutathion péroxydase (EC 1.11.1.9)	Esi0152_0006	CL4848Contig1	**
		LQ0AAB74YM01FM1.SCF	**
		LQ0AAB92YN18FM1.SCF	**
		LQ0AAA12YN24FM1.SCF	**
Glutathion péroxydase (EC 1.11.1.9)	Esi0099_0085	CL2115Contig1	-*
		CL5055Contig1	*3.03 x induced in SW, p = 0.086*
Glutathion péroxydase (EC 1.11.1.9)	Esi0192_0041	CL909Contig1	4.26 x induced in FW, p = 0.006
		LQ0AAB70YN20RM1.SCF	**
		LQ0AAB91YE11RM1.SCF	**
Glutathion péroxydase (EC 1.11.1.9)	Esi0152_0008	LQ0AAB51YM17FM1.SCF	**
		CL6396Contig1	**
Glutathion péroxydase (EC 1.11.1.9)	Esi0049_0025	CL3045Contig1	-*
		LQ0AAA5YG17FM1.SCF	**
Glutathion péroxydase (EC 1.11.1.9)	Esi0509_0009	CL5797Contig1	*2.57 x induced in FW, p = 0.10*
Glutathion péroxydase (EC 1.11.1.9)	Esi0007_0074	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi1076_0002	CL457Contig1	5.19 x induced in SW, p = 0.019
Glutathion S- transferase (EC 2.5.1.18)	Esi0199_0061	CL703Contig1	6.66 x induced in SW, p = 0.023
Glutathion S- transferase (EC 2.5.1.18)	Esi0019_0095	CL1013Contig1	*2.17 x induced in FW, p = 0.052*
Glutathion S- transferase (EC 2.5.1.18)	Esi0048_0023	CL1978Contig1	10.93 x induced in SW, p = 0.008
		LQ0AAA20YP01FM1.SCF	**
		LQ0AAB11YI02FM1.SCF	**
Glutathion S- transferase (EC 2.5.1.18)	Esi0017_0101	LQ0AAB57YP06FM1.SCF	-*
Glutathion S- transferase (EC 2.5.1.18)	Esi0047_0117	LQ0AAB5YN22FM1.SCF	*3.19 x induced in SW, p = 0.10*
		CL2600Contig1	3.61 x induced in FW, p = 0.003
		CL1604Contig1	*7.55 x induced in FW, p = 0.16*
Glutathion S- transferase (EC 2.5.1.18)	Esi0648_0004	CL5715Contig1	2.09 x induced in SW, p = 0.030
		LQ0AAB34YN03FM1.SCF	2.17 x induced in SW, p = 0.023
Glutathion S- transferase (EC 2.5.1.18)	Esi0004_0261	CL4549Contig1	**
Glutathion S- transferase (EC 2.5.1.18)	Esi0648_0003	CL1143Contig1	-*
		CL5628Contig1	59.5 x induced in SW, p < 0.001
		LQ0AAB69YN15FM1.SCF	2.95 x induced in SW, p = 0.037
Glutathion S- transferase (EC 2.5.1.18)	Esi0023_0103	LQ0AAB16YD20FM3.SCF	**
		LQ0AAB44YN04FM1.SCF	52.19 x induced in FW, p = 0.032
		LQ0AAB16YD20FM1.SCF	**
Glutathion S- transferase (EC 2.5.1.18)	Esi0386_0004	CL4350Contig1	-*
Glutathion S- transferase (EC 2.5.1.18)	Esi0384_0026	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi0386_0005	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi0617_0002	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi0617_0003	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi0617_0004	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi0028_0122	not on array	
Glutathion S- transferase (EC 2.5.1.18)	Esi0191_0054	LQ0AAB18YG11FM1.SCF	-*
		LQ0ADA7YK23RM1.SCF	37.76 x induced in SW, p = 0.006
Glutathion S- transferase (EC 2.5.1.18)	Esi0021_0028	not on array	
microsomal GST	Esi0122_0054	CL523Contig1	6.61 x induced in SW, p = 0.044
		CL4027Contig1	-*
microsomal GST	Esi0122_0055	CL523Contig3	*1.71 x induced in SW, p = 0.018*
microsomal GST	Esi0122_0061	CL523Contig2	26.53 x induced in SW, p = 0.007
Glutathion/Ascorbate dehydrogenase (EC 1.8.5.1)	Esi0041_0089	CL1920Contig1	4.7 x induced in FW, p = 0.002
Glutaredoxine (EC 1.20.4.1)	Esi0010_0089	CL204Contig1	9.38 x induced in SW, p = 0.001
Glutaredoxine (EC 1.20.4.1)	Esi0197_0012	CL578Contig1	-*
		LQ0AAB26YC03FM1.SCF	?
Glutaredoxine (EC 1.20.4.1)	Esi0083_0070	CL845Contig1	5.47 x induced in SW, p = 0.012
Glutaredoxine (EC 1.20.4.1)	Esi0395_0013	CL839Contig1	-*
Glutaredoxine (EC 1.20.4.1)	Esi0072_0054	CL6857Contig1	89.12 x induced in FW, p = 0.030
		LQ0AAB75YF24RM1.SCF	-*
Glutaredoxine (EC 1.20.4.1)	Esi0050_0061	CL517Contig1	**
		LQ0AAB65YJ24FM1.SCF	**
Glutaredoxine (EC 1.20.4.1)	Esi0036_0002	LQ0AAB36YA05FM1.SCF	13.85 x induced in SW, p = 0.005
		LQ0AAA14YO04FM1.SCF	-*
Phytochelatine synthase	Esi0399_0022	not on array	**

Finally, the only vanadium-dependant bromoperoxidase coded for in the *Ectocarpus* genome followed an expression profile similar to that of the genes encoding C5-epimerases. Vanadium-dependent bromoperoxidases are enzymes involved in the uptake and metabolism of halogens (Colin et al., 2003), which, in the brown alga *Laminaria digitata*, are stored mainly in the apoplast (Verhaeghe, et al. 2008), and are generally thought to constitute a defense mechanism (Wever et al. 1991) or to function as inorganic antioxidants (Küpper et al. 2008). They might, however, also be involved in the cross-linking of phenolic polymers (Berglin et al. 2004) and cell adhesion. Thus, the up-regulation of the vanadium-dependant bromoperoxidase gene might be interpreted as an indication of stress, but could also be related to the observed morphological changes.

Lower degree of fatty acid desaturation in freshwater

The fatty acid profile of the freshwater strain in the 32 ppt medium most closely resembled the profile of the marine strain in the same medium (r = 0.97), rather than that of the freshwater strain in 1.6 ppt medium (r = 0.93). All measured fatty acids exhibited slight but significant changes in relative concentrations, either between strains, between culture media, or both (Figure 6A). The most striking differences were observed in the ratio of n-3 to n-6 polyunsaturated fatty acids (PUFAs), which was higher in the freshwater strain in 1.6 ppt medium compared to 32 ppt medium (36 %) and the marine strain in 32 ppt medium (61 %, Figure 6B). The increase in the n-3 to n-6 ratio was mainly due to an increase in n-3 PUFAs (mainly α-linolenic acid: C18:3 n-3), and paralleled by a decrease in saturated fatty acids (mainly palmitic acid: C16:0). Overall the degree of free fatty acid desaturation increased from the marine strain to the freshwater strain and from 32 ppt medium to 1.6 ppt medium. These data are in agreement with our previous report (chapter 3.3), where we observed a decrease in the n-3/n-6 ratio in response to hypersaline stress. In chapter 3.3 these changes correlated well with the expression profiles of Δ12 and Δ15 desaturases. Here, the expression profiles for the same genes (Table 2) are also in loose agreement with the observed changes, as only one of two Δ12-desatureases was 3.9-fold up-regulated in seawater, while both Δ15 desaturases (responsible for the production of n-3 PUFAs) were 6.1 and 6.7-fold up-regulated). The general trend in *Ectocarpus* therefore seems to be: higher salinities result in lower n-3/n-6 ratio and lower overall degree of

desaturation.

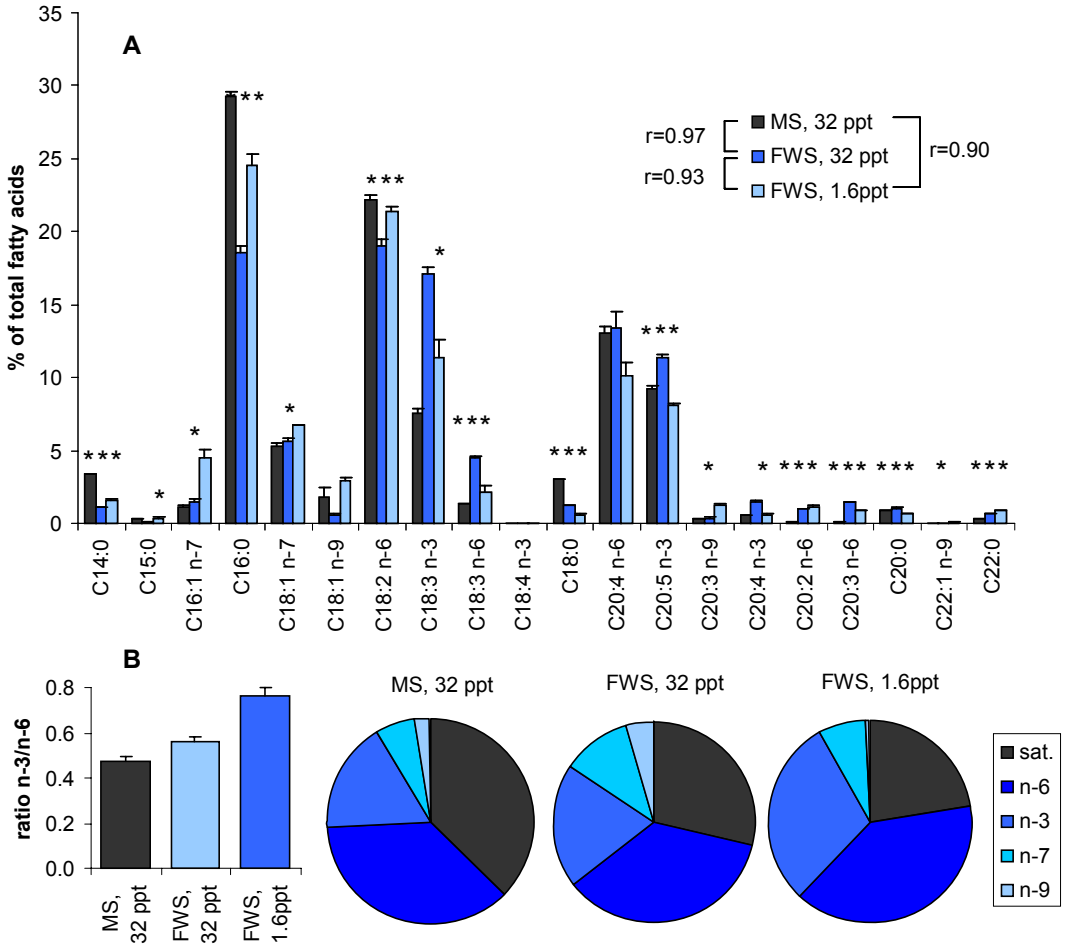

Figure 6: Fatty acid composition of the freshwater strain (FWS) in 1.6 ppt and 32 ppt medium as well as the marine strain (MS) in 32 ppt medium. Panel A displays the composition of individual fatty acids, panel B the ratio of n-3 to n-6 polyunsaturated fatty acids, and panel C the composition of n-3, n-6, n-7, and n-9 polyunsaturated fatty acids. All values are means of three replicates (if applicable ± SE). An asterisk above the column indicates that a value differed significantly from the other two columns ($p < 0.05$).

Highest concentrations of total amino acids were found in the freshwater strain

The concentrations of all of the measured amino acids significantly changed in at least one of the three examined samples, and in every case contents were highest in one of the freshwater strain samples. In many cases (glutamine, serine, threonine, valine, phenylalanine, leucine, isoleucine) amino acid concentrations in the freshwater strain in seawater ranged between those of the freshwater strain in 1.6 ppt medium and those of the marine strain, although in some cases only strain (alanine, asparagine, lysine, tyrosine, histidine) or only medium (aspartate, ornithine, tryptophan) had significant influence on the amino acid concentration (Figure 7).

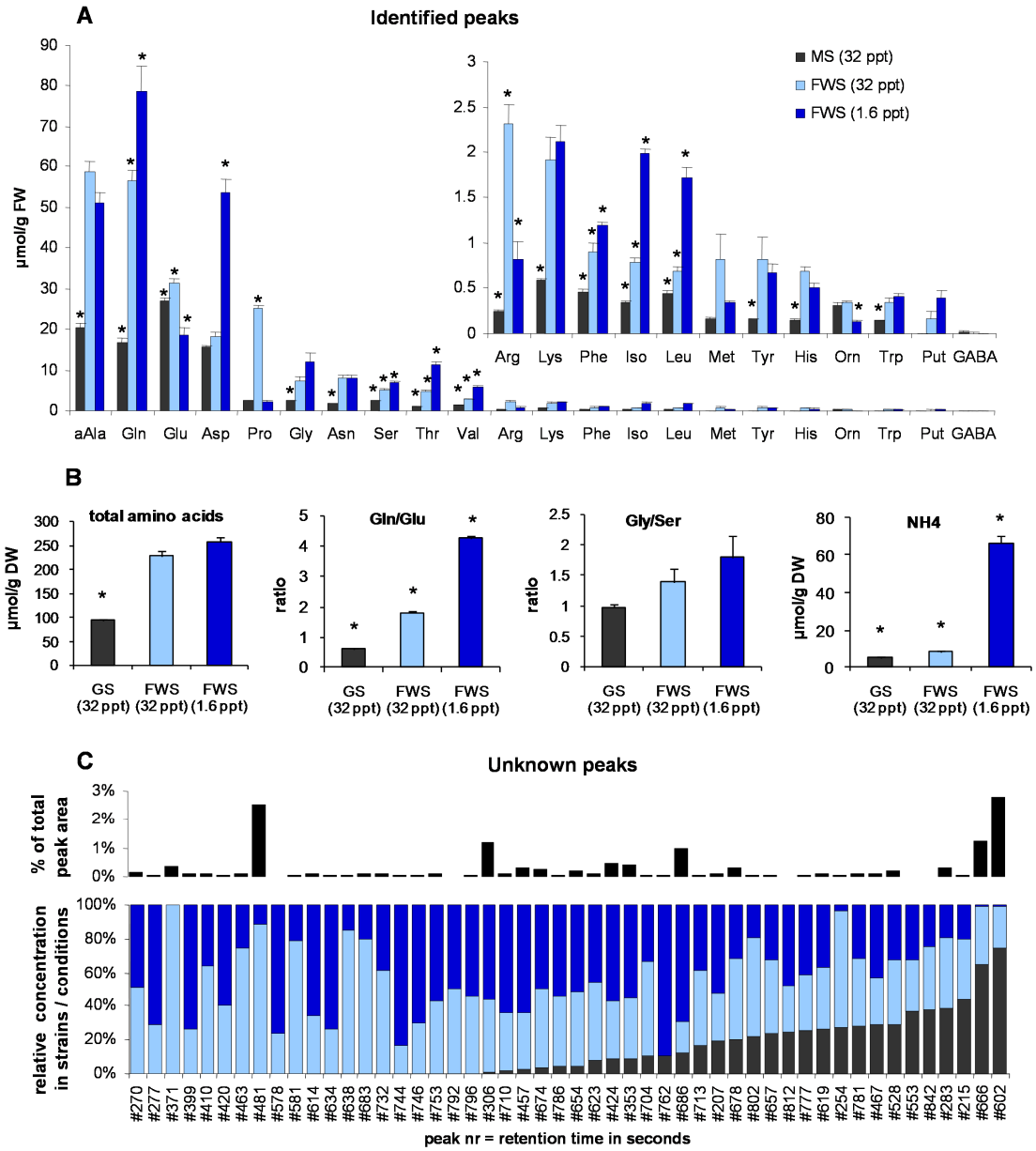

Figure 7: Panels A and B shown the amino acid composition of the freshwater strain (FWS) in 1.6 ppt and 32 ppt medium as well as the marine strain (MS) in 32 ppt medium. The graph on the right side of panel A shows a more detailed view of amino acids present in lower quantities. Panel C displays the absolute and relative peak areas of unknown peaks from the UPLC analysis. Absolute peak areas were calculated as the sum of all three tested strains / conditions. Each peak is named according to its retention time in seconds. All values are means of four replicates (if applicable ± SE). An asterisk above a column indicates that a value differed significantly from the other two columns ($p < 0.05$).

The observed strong increase in the overall amino acid content of the freshwater strain was roughly correlated with the intracellular concentration of ammonium, the ratio of glutamine to glutamate (Figure 7B), and the ratio of glycine to serine. The ratio of glutamine to glutamate is considered as a marker of nitrogen status (Flynn et al. 1989) in algae, high ratios indicating nitrogen-replete and low ratios nitrogen-deplete conditions. Similarly, the ratio of glycine to serine was shown to be related to

photorespiration and carbon availability in *Ectocarpus* (chapter 2.2), high ratios indicating high photorespiration and low carbon availability. Although the increase in these markers would fit well with a change in the ratio of available carbon to nitrogen, it needs to be kept in mind that in our experiments, both 1.6 ppt and 32 ppt medium were supplemented with nitrate in equally high concentrations via the Provasoli nutrients.

Side note:
***Ectocarpus* from a human perspective**

In humans, elevated glutamine to glutamate ratios are thought to be related to the capacity to recover from brain injury (Richards et al. 2003), and to increase in cerebrospinal fluid of patients suffering from schizophrenia (Hashimoto et al., 2005). Although, so far, no experimental evidence for the presence of a brain exists in *Ectocarpus*, one hypothesis could be that a trauma associated to the transition to freshwater was the starting point for a psychotic personality disorder in the freshwater strain, explaining both the increased glutamine to glutamate ratio and the morphological and transcriptomic changes.

On a transcriptional level, we observed that there was a general trend for nitrate and nitrite reductases, which are responsible for the production of ammonium from nitrate and nitrite, to be transcriptionally activated in seawater, while the genes coding for the glutamate synthase (GS) / NADPH-dependent glutamine:2-oxoglutarate aminotransferase (GOGAT) system, the major pathway of nitrogen assimilation in vascular plants (Lea & Miflin 1974), were marginally down-regulated (though most of the changes were not statistically significant, Table 2).

Markers of salt stress in the freshwater strain in 32 ppt medium

A very clear exception to the general trend that amino acids reached highest concentrations in the freshwater strain in 1.6 ppt medium were proline and arginine, which were both strongly accumulated only in the freshwater strain cultured in 32 ppt medium (10-fold for proline, 3 to 9-fold for arginine, Figure 7). These results correspond to our findings in chapter 3.3, where both amino acids were shown to accumulate in response to hypersaline stress. In addition, in this experiment, we observed strong transcriptomic activation (220-fold) of the 1-pyrroline-5-carboxylate synthase gene (Table 2) involved in the synthesis of proline from glutamate, suggesting the importance of this pathway in proline synthesis. Proline has been suggested to be a compatible osmolyte in vascular plants (Yoshiba et al. 1997) and diatoms (Krell et al. 2007), and is thought to function as osmoprotectant in *Ectocarpus* (chapter 3.3).

Gamma-aminobutyric acid (GABA), which was correlated with proline in our previous experiments, was not detected in the 1.6 ppt medium but found at very low

levels (0.007 µmol/g DW) in 32 ppt medium. Correspondingly, the diamine oxidase thought to be involved in GABA synthesis in *Ectocarpus* was 275-fold induced in 32 ppt medium; this was, however, not true for the two putative aminobutyraldehyde dehydrogenases.

In addition to these drastic changes in the concentration of known amino acids, the freshwater strain contained 20 unknown peaks which could not be detected in the marine strain. Some of these compounds were present only in traces, but two of them accounted for over 1 % of the total peak area (known and unknown). Although an exact quantification was not possible due to the lack of standards, a rough estimate of the quantity of these compounds can be obtained when considering that, on average, valine corresponded to 1.3 % of the total peak area, and arginine to 0.4 %.

Glutathione is produced by the freshwater strain in 32 ppt medium

Glutathione (GSH) is a tripeptide that is thought to play an important role in the protection of cells against oxidative stress both directly and as a cofactor of glutathione peroxidases (Pompella et al. 2003). Our GSH measurements showed that the intracellular GSH concentration in the freshwater strain increased dramatically from about 30 to over 700 nmol g^{-1} fresh weight (FW) when the strain was acclimated to seawater (32 ppt medium, Figure 8). In comparison, the marine strain contains about 100 nmol g^{-1} FW glutathione in control conditions and up to about 200 nmol g^{-1} (FW) in response to different stressors (P-O de Franco, S. Rousvoal, pers. comm.). Generally, genes involved in glutathione metabolism were also induced in the 32 ppt medium (Table 2): one of two genes involved in glutathione synthesis (a glutamate cysteine ligase) and one of two genes involved in its degradation were up-regulated in the 32 ppt medium. Furthermore, among the glutathione-S-transferases (GSTs, de Franco et al. 2009)

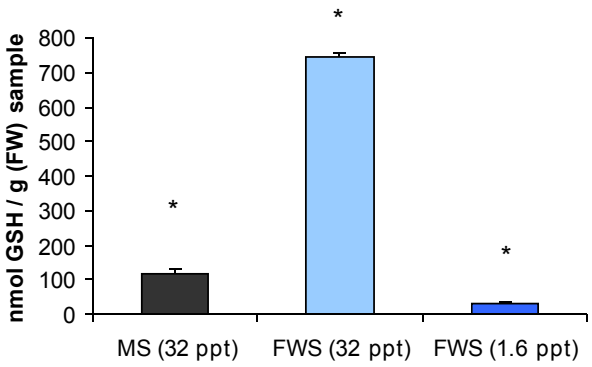

Figure 8: Glutathione (GSH) concentration of the marine strain in 32 ppt medium as well as the freshwater strain (FWS) in 32 ppt and 1.6 ppt medium (mean of four replicates ± SE). An asterisk above the column indicates that a value differed significantly from the other two columns ($p < 0.05$).

represented on the array, three of four microsomal GSTs and seven of twelve cytosolic GSTs were up-regulated in 32 ppt medium (only two were down-regulated).

Predominant soluble carbohydrates are inversely correlated to total amino acids

Mannitol, citrate, and glucose were the predominant non-structural carbohydrates in our samples and could be determined by GC-FID. They were found in highest concentrations in the marine strain, and were least concentrated in the freshwater strain in 1.6 ppt medium, with intermediate concentrations in the freshwater strain in 32 ppt medium (Figure 9).

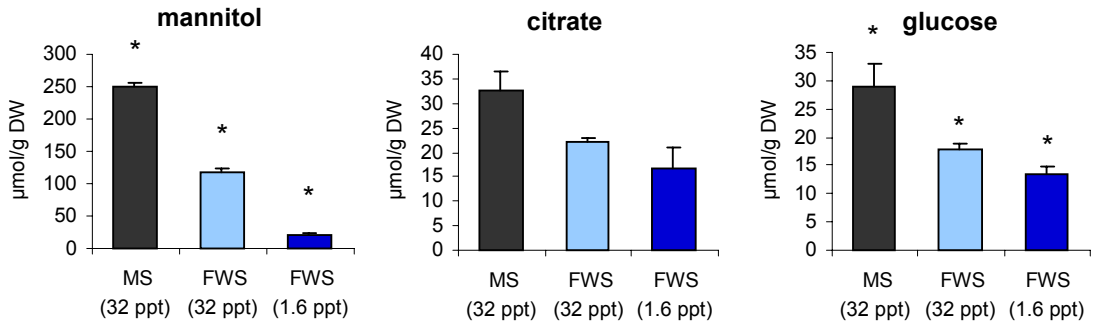

Figure 9: Mannitol, citrate, and glucose concentrations in the freshwater strain (FWS) in 1.6 ppt and 32 ppt medium as well as the marine strain (MS) in 32 ppt medium (means of four replicates ± SE). An asterisk above the column indicates that a value differed significantly from the other two columns ($p < 0.05$).

With respect to mannitol, we examined the expression profile of the only mannitol 1-phosphate dehydrogenase gene (MPDH) considered expressed this experiment. Unlike during the short-term response to hypersaline stress, where MPDH was up-regulated (Dittami et al. 2009), in this study MPDH exhibited 2.2-fold lower mean expression levels in 32 ppt medium compared to the 1.6 ppt medium. This difference was, however, not statistically significant (Table 2).

Interestingly, the decrease in mannitol was inversely correlated with the overall increase in total amino acids described above. Indeed, sugar from photosynthesis may be used either for nitrogen assimilation (amino acid production) or for polysaccharide synthesis, depending on the ratio of available carbon to nitrogen. In many terrestrial plants, the accumulation of sugar or starch is a known effect of nitrogen starvation (Veith & Komor 1993, Wang & Tillberg 1996). Although in our experiments, nitrate was provided in equally high concentrations in all conditions, this relationship should

be kept in mind for future experiments as well as for the interpretation of the data on intracellular nitrate concentrations, which are currently in preparation.

Carbohydrates at low concentrations

In addition to the GC-FID analyses, GS-MS analyses were carried out on concentrated samples from both strains. These analyses revealed a number of compounds present in *Ectocarpus* only at very low concentrations (Table 3). Many of the detected compounds (galactosylglycerol, gluconic acid, sorbitol, sucrose, melezitose, and maltose) were present in highest concentrations in the freshwater strain in 32 ppt medium. Galactosylglycerol was suggested to be involved in maintaining osmotic balance in the Chrysophyte *Ochromonas malhamensis* (Kauss 1973) and might be an osmoprotectant in red algae (Reed et al. 1980). Similarly, sorbitol is known to be involved in the osmotic adaptation of some ecotypes of the red alga *Bostrychia radicans* (Karsten et al. 1994). The presence of sucrose in the freshwater strain samples was a surprise. Sucrose is produced from UDP-glucose in response to salt stress in cyanobacteria *via* the activity of the sucrose phosphate synthase (EC 2.4.1.14) and sucrose-phosphate phosphatase (EC 3.1.3.24) enzymes (Hagemann & Marin 1999), but in *Ectocarpus* neither of these enzymes nor a sucrose synthetase (EC 2.4.1.13) was found. Similarly, the accumulation of gluconic acid (a C-6 acid that naturally occurs in fruit), melocitose (a trisaccharide best known to be synthesized by aphids), maltose (a common disaccharide in many plants), and ethanolamine (generally considered a toxic compound that is generated by the reaction of ammonium with ethylene oxide) still needs to be examined in greater detail.

More information will also be required to interpret the fact that scyllo-inositol was found at highest concentrations in the freshwater strain in 1.6 ppt medium, while myo-inositol, reached higher concentrations in seawater (both strains). Myo-inositol and scyllo-inositol are both isomers of inositol, the presence of which has been reported in several algal species including brown algae (Ikawa et al. 1968). However, no information on differences in the biological significance of these two isomers exists so far in algae. In addition to these compounds, our samples contained several yet unknown compounds, only some of which had been previously detected in other organisms, and many of which varied strongly between the strains and culture media (data not shown).

Table 3: Known compounds identified by GC-MS analysis. Where standards were available concentrations are given in µmol per g^{-1} DW, otherwise in arbitrary units. The table shows the mean of four replicates ± SD. "*" indicates that a mean differed significantly from both other means at $p \leq 0.05$ after a Bonferroni correction ("**", $p \leq 0.01$; "***" $p \leq 0.001$). If significant differences were detected, boldface formatting highlights the condition(s) with the highest concentration(s) of each metabolite. MS = marine strain, FWS = freshwater strain.

	Standard	MS, 32 ppt	FWS, 32 ppt	FWS, 1.6 ppt
Galactosylglycerol	NO	0.42 ± 0.12	**22.74 ± 1.85*** **	0.8 ± 0.18
Gluconic acid	YES	3.93 ± 0.98*	**15.75 ± 0.96*** **	0.78 ± 0.19*
Sucrose	YES	0 ± 0*	**5.86 ± 0.28*** **	0.3 ± 0.09*
Sorbitol	NO	0.09 ± 0.01*	**0.15 ± 0.01****	0.03 ± 0.01*
Melezitose	YES	0 ± 0	**0.26 ± 0.05****	0 ± 0
Maltose	YES	0.36 ± 0.12	**1.04 ± 0.08***	0.57 ± 0.12
Erythronic acid	NO	0.09 ± 0.02*	**0.5 ± 0.13**	**0.2 ± 0.03**
Myo-inositol	YES	**0.75 ± 0.06**	**0.89 ± 0.06**	0.16 ± 0.01***
Scyllo-inositol	NO	5.14 ± 0.18*	2.67 ± 0.11*	**16.19 ± 3.63***
Ethanolamine	NO	0.11 ± 0.05	0.61 ± 0.17	0.15 ± 0.02
Succinate	YES	0.05 ± 0.03	0.62 ± 0.34	0.51 ± 0.19
Glycerol	NO	0.53 ± 0.07	1.85 ± 0.98	0.97 ± 0.07
Glucose	YES	11.23 ± 2.83	20.35 ± 2.68	18.04 ± 2.47
Xylitol	NO	0.15 ± 0.03	0.11 ± 0.01	0.12 ± 0.04
Arabitol	NO	0.17 ± 0.03	0.09 ± 0.01	0.1 ± 0.02

Discussion

In this study we examined the salinity tolerance as well as metabolite and transcript profiles of a freshwater strain of *E. siliculosus* and compared them to those of the sequenced marine strain, revealing several interesting features about the adaptation of this strain to freshwater.

Two distinct transcriptomic programs

Our first result was that the freshwater strain of *E. siliculosus* was able to grow both in freshwater and in seawater. Although several organisms such as mangrove trees or other estuary species tolerate strong temporary changes in salinity (den Hartog 1967), the capacity to permanently live and reproduce both in freshwater and marine habitats is rare among multicellular organisms. Comparable adaptations are known, for example, in migratory fish such as salmon and eel, which spend most of their life in marine (salmon) or freshwater (eel) habitats, and return to their original freshwater or marine habitat to spawn. These fish undergo profound physiological and morphological changes in preparation of and during migration. The transcriptomic

changes preceding the migration have been examined in both eel and salmon (Kalujnaia et al. 2007, Seear et al. 2009), revealing that only very few genes significantly changed expression. Most of them were involved in osmoregulation as well as growth, metabolism, and oxygen transport.

In *Ectocarpus*, the situation was different. Although it also displayed changes in morphology between normal seawater and highly diluted seawater, two very distinct transcriptomic profiles were observed in the two conditions. *E. siliculosus* is known for its high transcriptomic plasticity (Dittami et al. 2009), but the intensity of the observed transcriptomic changes by far exceeded those previously observed in the marine strain during the short-term response to saline stress. Furthermore, the changes here had very little in common with the short-term response of the marine strain and did not seem to be focused on specific groups of well-known genes, so that the biological significance of these changes still remains largely unknown. Nevertheless we can conclude that the freshwater strain of *Ectocarpus* possesses a transcriptomic "freshwater program", which is active in low salinities, but at the same time retained the "marine program". Despite the genomic differences between the freshwater and the marine strains, the "marine program" of the freshwater strain was highly similar to that of the strictly marine strain, demonstrating that, should the freshwater strain be on the verge of becoming a new species, it is still very close to its marine relatives.

Adaptations to freshwater

Regarding the physiological adaptations to freshwater and seawater, the data presented above provide indications on a few of the mechanisms possibly involved. For example, the morphological plasticity, i.e. the formation of longer, stickier filaments, might present a necessary adaptation to life in flowing waters, as those above the Hopkins river falls, Victoria, Australia, where the freshwater strain was isolated (West & Kraft 1996). These changes might be in part related to the strong transcriptional changes in genes involved in cell wall modifications, such as C5-epimerases or the vanadium bromoperoxidases.

Another related process considered important for the adaptation to marine environments, possibly via a role in regulating intracellular ion concentrations, is sulphatation of cell wall polysaccharides. All marine algae (Kloareg & Quatrano 1988), as well as marine vascular plants (Aquino et al. 2005), produce sulfated polysaccharides, a feature which has never been observed in freshwater plants. In the

freshwater strain, transcriptional regulation related to the sulphatation of polysaccarides, i.e. the activation of sulfatases in 1.6 ppt medium and the activation of two sulfonotransferases likely to be involved in fucan biosynthesis in 32 ppt medium might constitute a way of controlling the degree of sulphatation.

An additional adaptation might be a decrease in mannitol concentrations in the freshwater strain and in 1.6 ppt medium. Mannitol is considered an important compatible osmolyte in brown algae (Davison & Reed 1985, Reed et al. 1985). Similarly, the change in the ratio n-3 to n-6 PUFAs might be of biological significance by mediating changes in membrane fluidity, and possibly compensating for changes in the concentration of transmembrane proteins, as previously proposed by Mock et al. (2002) for the thylakoid membrane of an Antarctic diatom. This hypothesis was discussed in more detail in chapter 3.3

Regulation of transcriptomic changes

As the freshwater strain shows reversible morphological and transcriptomic changes, one question that immediately comes to mind is related to the regulation of these changes. Very little is known about the regulation of the transcriptome in *Ectocarpus* (Cock et al. in prep.). Although we previously highlighted some interesting transcription factors, which were up-regulated in response to saline stress (Dittami et al. 2009), samples were only examined after six hours of exposure to stress. First transcriptomic changes may, however, occur only a few minutes after exposure to stress or changes in the culture medium. On the other hand, after only 6 hours in hyposaline medium (4 ppt) in our previous experiment, the marine strain was still in a "stress response" phase, whereas in this experiment, the freshwater strain had entirely adapted to 1.6 ppt medium. This could explain the relatively low correlation coefficients between the changes in expression in this and in our previous experiment.

Regardless of the identity of the genes responsible for this regulation, the reversible nature of the transcriptomic changes renders the hypothesis that the capacity of the freshwater strain to grow in freshwater is due to a mutation of one or several master regulators fairly unlikely. Moreover, many of the observed changes might also be a direct response to changes in the culture medium. An excellent example for this are genes involved in cell wall modifications. C5-epimerases, for example convert M to G, which in turn form rigid egg-box like structures in the presences of Ca^{++} (Grant 1973). In 1.6 ppt medium, Ca^{++} concentrations were 20-fold lower compared to 32 ppt

medium, possibly eliminating the benefit of high G contents in cell wall polysaccharides and providing a cue for the regulation of C5-epimerases. Similarly, the fact that genes thought to be involved in the synthesis of sulfated polysaccharides were transcribed at a lower level in low salinities, might be simply connected to the fact that sulfate concentrations were 20-fold lower compared to the 32 ppt medium. This hypothesis would, however, not explain the activation of sulfatases in 1.6 ppt medium.

Based on our observations, we can presently only present first indications and ideas about regulatory mechanisms possibly involved in the adaptation of the freshwater strain to freshwater. One way of starting to decipher the signaling mechanisms at the basis of these very profound transcriptomic changes would be a high resolution time-series of transcriptomic experiments, which could highlight early changes in the transition between the two culture media. Furthermore, targeted transcriptomic experiments with media containing controlled sulfate- or Ca^{++}-concentrations might provide further information on the role of these compounds as potential environmental signals.

An apparent paradox between transcriptomic and metabolite profiles

An important finding of our study was the apparent paradox between gene expression and metabolite profiles of the freshwater strain in 32 ppt medium. While on a transcriptomic level the "marine program" in both freshwater strain and marine strain were very similar, on a metabolite level the freshwater strain in 32 ppt usually exhibited intermediary metabolite concentrations between the two other strains / conditions (Figure 10).

In the absence of pronounced transcriptomic differences and under identical culture conditions, the most plausible explanation for the differences in metabolite concentrations between the strains lies in the observed genomic differences. In our previous study (chapter 4.2) we detected genomic differences between the freshwater strain and the marine strain over almost the entire genome. These differences may result in different enzymatic activities and thus account for different metabolite concentrations. In most cases, the differences on a metabolites level between strains were directed the same way as changes related to the switch between the two culture media. For example mannitol concentrations were lower in the freshwater strain in 32 ppt medium compared to the marine strain (effect of genomic differences), but within

the freshwater strain it was also lower in 1.6 ppt medium compared to 32 ppt medium (effect of culture medium and / or transcriptomic differences, Figure 9). This shows that not all adaptations are regulated by transcriptomic changes. In the freshwater strain, both permanent genomic and reversible transcriptomic adaptations currently act together in enabling the freshwater strain to grow in freshwater, but reversible transcriptomic adaptations may be replaced by genomic changes in the course of evolution.

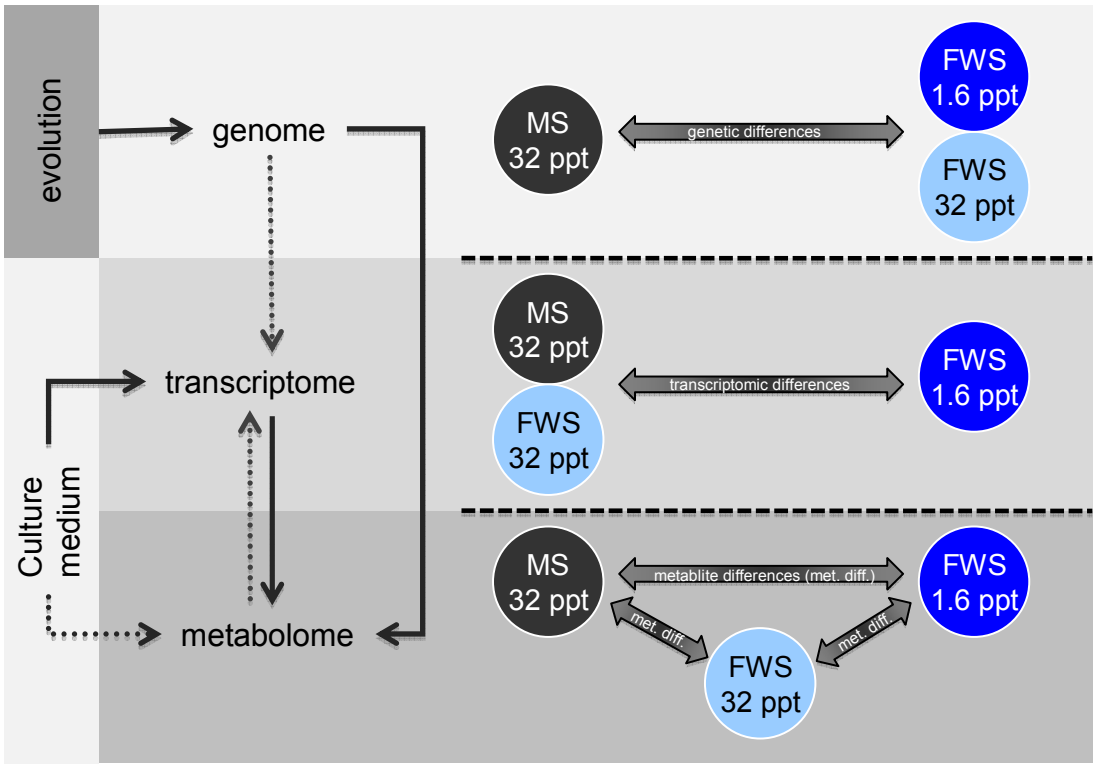

Figure 10: General overview of differences between the marine strain (MS) and the freshwater strain (FWS) in 1.6 and 32 ppt medium on a genome-, transcriptome-, and metabolite level. While the freshwater strain cannot change its genome depending on the culture medium, its transcriptome in 32 ppt medium was very similar to that of the marine strain. On a metabolite level the freshwater strain in 32 ppt medium assumed an intermediary position, indicating that genomic differences might translate directly into metabolite differences without profound changes in transcript quantity. This is visualized by the solid and dotted arrows in the left part of the figure. Solid arrows designate a strong effect observed in our data, while dotted arrows stand for possible or weak effects.

A number of stress-related changes

Nevertheless, the freshwater strain was capable of surviving and growing in 32 ppt medium and had a similar salt tolerance towards hypersaline media as the marine strain. Moreover the quantum yield of the freshwater strain was similar in both 32 and 1.6 ppt medium. In spite of this, we observed accumulation of a few stress markers in the freshwater strain in 32 ppt medium, most importantly the accumulation of proline

and glutathione. In particular the activation of proline indicates that the salt stress response in the freshwater strain was activated in 32 ppt medium. This probably allowed the freshwater strain to compensate for genomic adaptations to freshwater, and illustrates how initially reversible transitions may gradually become irreversible in the course of evolution.

The role of associated bacteria

One important factor that needs to be considered when examining the transition between marine and freshwater environments is symbiotic bacteria. Many algae depend on symbionts to produce certain substances or signals that are important for growth and development. A classical example for this is a bacteria-produced compound termed thallusin, which is necessary to maintain species-typical morphology and growth in the green alga *Monostroma oxyspermum* (Matsuo et al. 2005). In *Ectocarpus*, similar effects were observed: axenic cultures of *E. fasciculatus* exhibit poor growth and an atypical habitus, and the closely related species *Pylaiella litoralis* does not grow at all when axenic (Pedersén 1968). Growth and habitus of axenic cultures could be restored by adding natural seawater, kinetin, or cytokinin (Pedersén 1968, 1973).

Drastic changes in the environment, such as marine-freshwater transitions, could certainly be expected to have an impact on the bacteria associated to an alga, in particular considering that such transitions have been shown to be infrequent in the microbe-world (Logares et al. 2009). One indication of bacterial activity in our samples was the accumulation of sucrose in the freshwater strain in 32 ppt medium. Sucrose is a known osmolyte in bacteria (Csonka 1989), and the absence of genes important for its synthesis in *Ectocarpus* could give rise to the hypothesis that it is produced by associated bacteria in response to salt stress. Our present data do not allow us to draw conclusions about whether the freshwater- and the marine strain contain the same associated bacteria and how and to what degree these bacteria might be involved the observed transcriptomic, morphological and metabolite changes. These questions present an important challenge for future research. One way of starting to address them could be by examining axenic cultures of both strains, where supplementation with growth hormones might help to overcome problems of low growth rates.

Conclusions

The aim of this study was to explore the mechanisms enabling the transition and adaptation of *E. siliculosus* to freshwater. From the literature we know that *Ectocarpus* is a genus, which naturally inhabits a wide range of salinities and is known for its high overall plasticity. In addition, our data demonstrated that the freshwater strain is a particularly stress tolerant strain, a trait which could have been acquired after the transition to freshwater, but which might also have assisted this transition.

Presently not enough sequence information is available to reliably estimate when the freshwater strain diverged from its marine relatives, but we were able to show that it has retained its ability to grow in seawater as well as a transcriptomic program that is very close to that of the marine strain. With respect to metabolites, our data revealed two components of the adaptation to freshwater: a reversible and a permanent component. Our hypothesis is that the reversible part of the changes is related to transcriptomic changes (e.g. of genes related to cell wall modifications), but that in the course of evolution, parts of these reversible changes have been replaced or added to by permanent genomic changes that have similar effects.

What makes this model particularly interesting is the fact that, despite certain permanent changes in the metabolite profiles, the transition to freshwater has not yet become irreversible. In this sense the freshwater strain represents a snapshot of a rare evolutionary event that is still in progress, and thus an excellent opportunity to study the processes involved. Here we presented a first look at this "evolutionary snapshot" from a transcriptomic as well as a metabolic point of view. We pointed out several interesting features, yet many questions still remain unanswered: When did the freshwaters strain separate from marine strains? What are the identity, origin, and function of the unknown metabolites? What is the role of bacteria in the adaptation to freshwater? What are the regulatory elements at the basis of the transcriptomic changes? These and many more questions still await answers and promise novel insights on the mechanisms of speciation and evolution.

Acknowledgements

We thank Gurvan Michel for providing annotations of genes related to cell wall modifications and for helpful discussions and P.-O. de Franco for providing annotations of genes related to glutathione metabolism.

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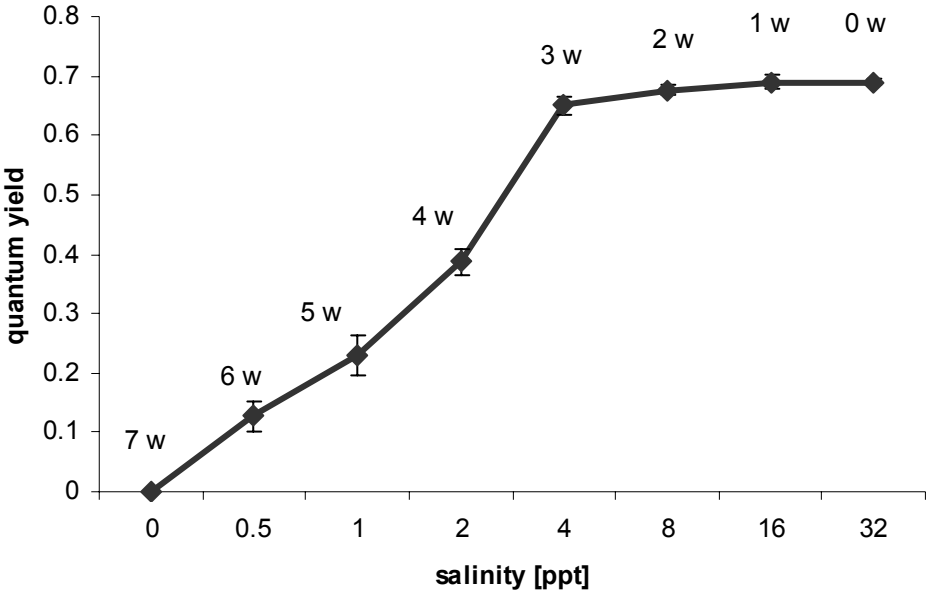
Supplementary files



Supplementary file 1: Gradual adaptation of the marine strain to freshwater: the salinity was reduced by 50 % every week (w), starting from 32 ppt medium (top right). After the 6th week the salinity was adjusted to 0 ppt (bottom right). The graph shows the mean quantum yield (F_v/F_m) of three replicates ± SE

5.

Conclusion and outlook

5. Conclusion and outlook

The main objective of my thesis was to study the abiotic stress response of *Ectocarpus siliculosus* employing a global approach, where mechanisms that enabled the exceptional stress tolerance of this seaweed were of particular interest to me. The experiments presented in the previous chapters highlighted several pathways, genes, gene families, and metabolites that are likely to be involved in the stress response in this alga. However, looking back on the results, there is a recurring theme in all of the experimental results presented – plasticity: examples of plasticity are frequent both in my thesis in and the literature about *Ectocarpus* in general. Some illustrations are:

- During the diurnal cycle and under controlled laboratory conditions, metabolite concentrations fluctuated 2-fold and more.
- During the short-term response to stress, almost 70 % of all genes changed expression in one of the three examined stress conditions.
- In the same experiment, almost all examined metabolites showed significant changes compared to the control.
- Intracellular sodium concentrations changed > 10-fold depending on the salinity of the culture medium.
- The freshwater strain of *Ectocarpus* has two entirely different transcriptional programs, depending on the culture medium, and can survive in salinities from 0 ppt to about 90 ppt; growh, as well formation of mitospores, was sill observed at 32 ppt.
- The same strain exhibits strong morphological changes in response to different media. Currently, 410 different species of *Ectocarpus* have been described, but only 110 are currently accepted [www.algaebase.org, September 14th, 2009], underlining the degree of morphological plasticity.

In *Ectocarpus*, this plasticity is combined with simple organization, which is demonstrated, for instance, by the fact that each cell can regenerate the entire organism. In my opinion, this is likely to be part of the "secret" of stress tolerance in *E. siliculosus*: while many terrestrial plants attempt to maintain controlled conditions in response to stress, the strategy of *Ectocarpus* seems to be to "sit it out", as demonstrated for example by the strong downregulation of growth in response to saline stress.

This hypothesis raises several new questions, such as which traits allow *Ectocarpus* enzymes to remain functional during- or at least to regain functionality after stress. Moreover, it will be interesting to study which regulatory mechanisms underlie the observed transcriptomic and morphological changes. Understanding the basis of plasticity in *Ectocarpus* might some day help understand why these mechanisms do not work in other, more complex organisms.

The results presented in the previous chapters will hopefully form a starting point for several more targeted studies addressing some of the unresolved questions of my thesis. One of the major points that need to be addressed and that will certainly continue to occupy researchers in the years to come is the large number of unknown genes – many of which were strongly regulated in response to stress. Targeted functional studies are still difficult in *Ectocarpus*, as important tools such as RNAi and transformation protocols have not yet been established. However, other approaches such as TILLING (Targeting Induced Local Lesions in Genomes, Cock, pers. comm.) are currently being developed and might soon provide a way of rapidly screening thousands of mutants for mutations in a specific gene of interest, and then performing more detailed functional studies with the identified mutants.

Another interesting approach that could be taken while the aforementioned protocols are being developed would be to use the available tools from diatoms. Diatoms are the closest relatives to brown algae and share numerous genomic features (e.g. absence of the GABA shunt, stress-responsive CBPs). In addition and their genomes code for homologs of several of the unknown stress response genes found in *Ectocarpus*. A number of genomic tools, including transformation protocols, are available for these organisms. I believe that closer ties between the diatom and the *Ectocarpus* research communities could be of great benefit for research in both areas.

6. Appendices

6. Appendices

6.1 Appendix 1: Oral presentations

Meetings I attended are underlined; the presenting author is highlighted in boldface:

- S. Dittami and **T. Tonon** (May 2008). Etude transcriptomique de la réponse aux stress abiotiques chez l'algue brune modèle Ectocarpus siliculosus. Oral communication presented at the Platform Transcriptome OUEST-Genopole® users day, Rennes, France.
- **S. Dittami,** T. Tonon, P. Wincker, E. Corre, D. Scornet, J.M. Cock, C. Boyen: Transcriptomic study of abiotic stress in Ectocarpus. 3rd Ectocarpus meeting, June 2008, Oban, Scotland, United-Kingdom
- **Charrier B**, Le Bail A, Dittami S, Maisonneuve C, Gicquel M, de Franco P-O, Rousvoal S, Cock J. M, Tonon T: Additional Ectocarpus post-genomic tools: a mini-library of mutants and identification of normalisation genes for expression analyses. 3rd Ectocarpus meeting 2008, Oban, Scotland, United-Kingdom
- **S. Dittami**, A. Gravot, S. Goulitquer, J. Collén, E. Corre, P. Wincker, M. Cock, A. Bouchereau, C. Boyen, T. Tonon: Abiotic stress response in the model brown alga *Ectocarpus siliculosus* (Phaeophycaea). Session P3 – developments in plant biology, SEB annual meeting 2008, Marseille. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 150(3): S191-S191.
- **S. Dittami**, A. Gravot, S. Goulitquer, J. Collén, E. Corre, P. Wincker, M. Cock, A. Bouchereau, C. Boyen, T. Tonon: Transcriptomic study of abiotic stress in *Ectocarpus*. OUEST-Genopole® Stress meeting, Plouzané, September 2008
- **T. Tonon**, S. Dittami, A. Gravot, A. Bouchereau, C. Boyen: Transcriptomic and Metabolomic Response to Saline and Oxidative Stress in the Brown Alga *Ectocarpus siliculosus.* Plant Abiotic Stress Tolerance, Vienna, February 2009
- **S. Dittami**, A. Gravot, S. Goutlitquer, S Rousvoal, S. La Barre, J. Collén, E. Corre, A. Eggert, M. Cock, A. Bouchereau, C. Boyen, T. Tonon: Abiotic stress response in the brown alga *Ectocarpus siliculosus* - a transcriptomic and metabolomic approach. Geochemical Luncheon Club, Faculty of science, Environmental sciences, University of East Anglia, Norwich, May 2009
- **Stephane LaBarre**, N. Keravec, B. Charrier, P. Potin, A. Ritter, T. Tonon, S. Dittami, S. Salaun: Prokaryote – eukaryote interactions: spectroscopic fingerprints of marine algae and of their associated microflora. 6th European Conference on Marine Natural Products, Porto, July 2009

6.2 Appendix 2: Posters

Meetings I attended are underlined, the presenting author and creator of the poster is highlighted in boldface.

Tonon T., Goulitquer S., Ritter A., Dittami S.M., Salaün J.-P., Cock J.M., Potin P., and Boyen C. (July 2008) From the shore: Molecular changes in the fatty acid metabolism of brown algae under abiotic stress conditions. Poster presented at the 18th International Symposium on Plant Lipids, Bordeaux, France.

From the shore: Molecular changes in the fatty acid metabolism of brown algae under abiotic stress conditions

Tonon T.[1,2], Goulitquer S.[3], Ritter A.[1,2,4], Dittami S.[1,2], Cock J.M.[1,2], Salaün J.-P.[3], Correa J.A.[4], Potin P.[1,2], and Boyen C.[1,2]

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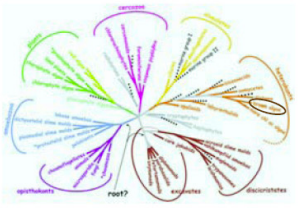
[4]Center for Advanced Studies in Ecology & Biodiversity, Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile.

Introduction

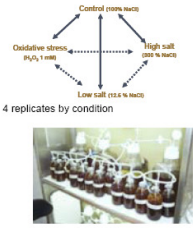
Intertidal seaweeds are subject to a number of physical changes related to their habitat and as a result frequently exhibit remarkable levels of tolerance to abiotic stresses. In their coastal environment, they should also tolerate increasing human impacts, including heavy metal pollution. However, little is known about the mechanisms underlying this tolerance to stresses, making intertidal seaweeds interesting models with large potentials for new discoveries. Among these organisms, brown algae have evolved independently from green plants more than a billion year ago [1, see eukaryotes tree of life on the right side] and provide several relevant models.

Over the last five years, the cosmopolitan filamentous alga *Ectocarpus siliculosus* has been established as a genetic and genomic model for brown algae [2,3]. It is closely related to the kelp *Laminaria digitata* which forms dense populations on rocky shores, with a major role in the marine coastal biodiversity, commercial applications, and well suited as an ecological model [4].

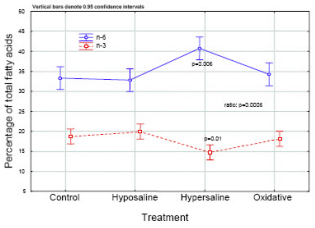


Integrative approach in *Ectocarpus siliculosus*

We have developed an EST-based pilot microarray, based on the NimbleGen technology, to study the transcriptomic response of *E. siliculosus* to three abiotic stresses (hypersaline, hyposaline, oxidative) after 6h00 of incubation (tidal cycle). We have also determined the total fatty acids content in these samples.



Samples



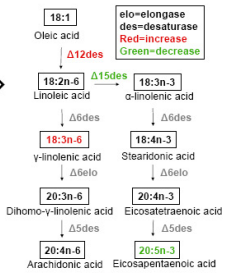
Total fatty acids content

5 cDNA libraries → 91,396 ESTs → 17,039 unigenes → 231 EsV-1 sequences → 4 probes (60^{mer}) by sequence → Chips

samples → mRNA → Reverse transcription → Amplification by PCR → Double strand cDNA → Single dye labeling

NimbleGen Iceland → Roscoff

Transcriptomic approach

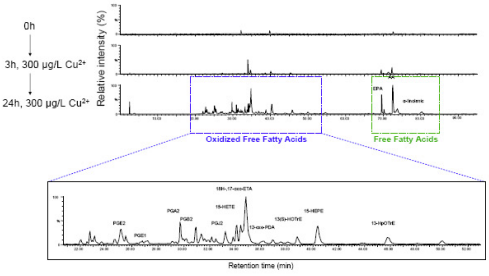


Model

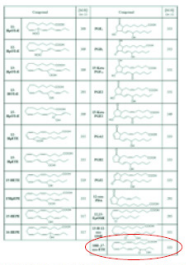
Most changes in FA metabolism occur under high salt conditions. We observed a correlation between the changes in fatty acids content and the variations of the level of expression of the putative Δ12- and Δ15-desaturase genes annotated in the genome. These changes may affect membrane stability/fluidity and be related to the synthesis of signaling compounds (oxylipins?) under the hypersaline stress.

Oxylipins synthesis in *Laminaria digitata* under copper stress

LC-MS and GC-MS analysis of copper treated *L. digitata* shows that late stress (24h) induces the release of free fatty acids, concomitant with the biosynthesis of octadecanoid (plant-like) and eicosanoid (mammal-like) oxygenated derivatives. To our knowledge, this is the first time that production of prostaglandins is reported in brown algae. In addition, a new compound, the 17-oxo,18-hydroxy-eicosatetraenoic acid, was identified.

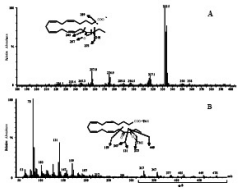


LC-MS profiling



structure of oxylipins identified in copper stressed *L. digitata*

LC-MS chromatogram of 17-oxo,18-hydroxy-eicosatetraenoic acid (17oxo,18H-ETE, *m/z* 333 [M-H](-)). Spectrum are presented in negative APCI mode (A) and EI mode as a TMS derivative (B).



A new molecule: 17oxo,18H-ETE

Conclusion

The production of C18 and C20 oxygenated fatty acid derivatives in stressed *L. digitata*, including the identification of new oxylipins, raise interesting questions on the role(s) of these molecules in these organisms, and the enzymes involved in their biosynthesis. It is tempting to suggest that these oxylipins act as signaling molecules under stress condition in brown algae.

The emergence of the model *E. siliculosus*, with the development of several genetic and genomic tools, should allow to understand the physiological functions of the fatty acids and their derivatives, and also to decipher the pathways leading to the production of these molecules.

[1] Baldauf S., 2003. The deep root of eukaryotes. Science 300, 1703-1706.
[2] Peters A. et al., 2004. Proposal of *Ectocarpus siliculosus* (Ectocarpales, Phaeophyceae) as a model organism from brown algal genetics and genomics. J. Phycol. 40, 1079-1088.
[3] Charrier C. et al., 2008. Development and physiology of the brown alga *Ectocarpus siliculosus*: two centuries of research. New Phytol. 177, 319-322.
[4] Bartsch I. et al., 2008. The genus Laminaria sensu lato: recent insights and developments. Eur. J. Phycol. 43, 1-86.

Dittami. S.M., Boyen C., Tonon T., Saline Stress in *Ectocarpus siliculosus.* Poster presented at the EST monitoring session, Paris, February 2009

Saline stress in *Ectocarpus siliculosus*

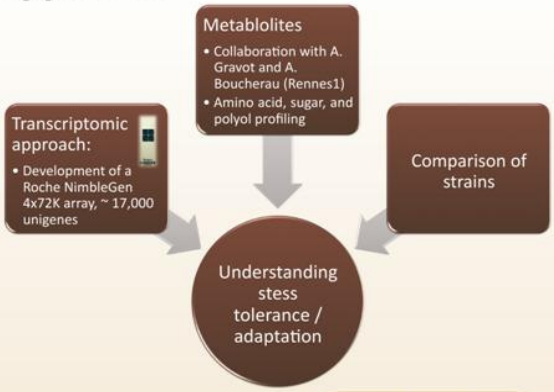
SM Dittami (dittami@sb-roscoff.fr), UMR 7139, Station Biologique, 29680 Roscoff, France
Thesis directed by C. Boyen an T. Tonon

Background

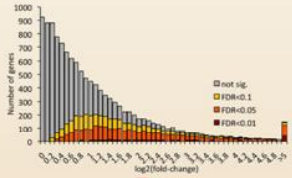
Brown algae are phylogenetically distant from red algae, green algae, and land plants, and an important component of the coastal ecosystem. They have developed unique mechanisms that allow them to inhabit the intertidal zone, an environment in which there are high levels of abiotic stress. *Ectocarpus siliculosus* is a small, cosmopolitan, filamentous brown alga that has been established as a genetic and genomic model for the brown algal lineage, but little is known about the mechanisms underlying its stress tolerance.

Young *E. siliculosus* cells

Approach

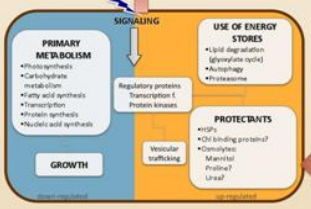


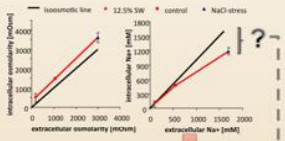
Short-term response to saline stress



Global transcriptomic reprogramming
In total, 70 % of all expressed genes are regulated under hyposaline, hypersaline, or oxidative stress (t-test, FDR 10 %)

Many regulated genes participate in known pathways (see below), but **67 % of the regulated genes are unknown** and 54 % have no known homologs



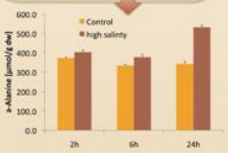


- Influx of NaCl is essential for survival
- There are other osmolytes
- Transcriptomic analysis highlights candidates

WORK IN RPGRESS: Metabolite studies

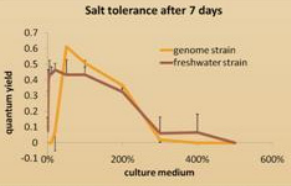
→Verify hypothesis generated from transcriptomic studies

→Additional questions, e.g.: what is he role of alanine in the stress response?



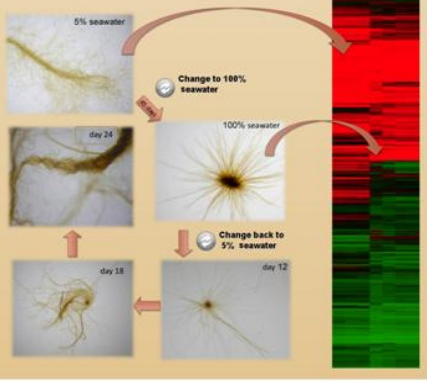
Long-term adaptation to freshwater

- Within brown algae, only very few species (mainly Ectocarpales) have colonized freshwater
- Strain 371 was isolated from freshwater, but survives and grows in freshwater and in seawater (West & Kraft, Muelleria, 1996)

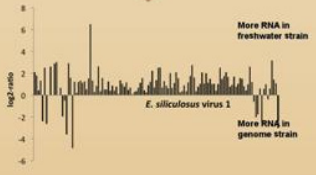


- Classification as *Ectocarpus siliculosus* confirmed by sequencing of the RUBISCO large subunit (McCauley et al. Phycologia, 2007), and by sequencing of the ribosomal internal transcribed spacer region (our lab)

- The freshwater strain exhibits fundamental changes in morphology and transcriptome in different culture media



- We have identified regions of the genome that are more strongly transcribed in the freshwater strain → possible duplications?
- One of these regions contains genes coding for a virus (to be confirmed)



→ **model for evolutionary "jumps"?**
→ **WORK IN PROGRESS** (Comparative Genome Hyridization, metabolomics …)

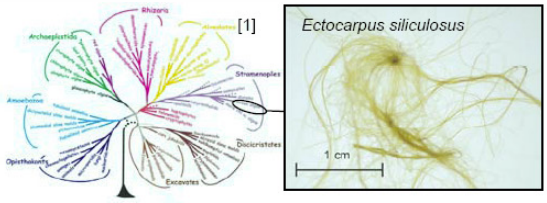
Dittami, S.M., Gravot A., Rousvoal S., Bouchereau A., Boyen C., Tonon T.: Genome-based Integrative Analysis of Primary Metabolism and Short-Term Abiotic Stress Response in the Model Brown Alga *Ectocarpus siliculosus*. Plant genomics and beyond, Evry, July 2009

Genome-based Integrative Analysis of Primary Metabolism and Short-Term Abiotic Stress Response in the Model Brown Alga *Ectocarpus siliculosus*

SM Dittami[1], A Gravot[2], S Rousvoal[1], A Bouchereau[2], C Boyen[1], T Tonon[1]

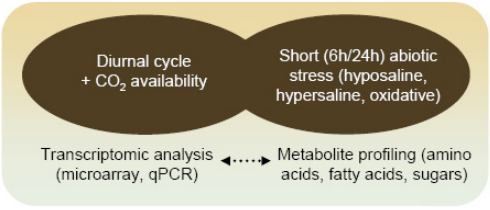
[1]Station Biologique de Roscoff, CNRS-UPMC, France [2]INRA-Agrocampus, Université Rennes 1, France

Background



Brown algae are an important component of the coastal ecosystem. They have evolved independently from red and green algae [1] and have developed unique mechanisms allowing them to inhabit the intertidal zone. *Ectocarpus siliculosus* is the first brown alga whose genome has been sequenced. However, little is known about molecular basis of its primary metabolism and its response to abiotic stress.

Integrative approach



Analysis of metabolic and transcriptomic changes in the diurnal cycle and in response to stress can provide insights in primary metabolic processes and how they are affected by stress.

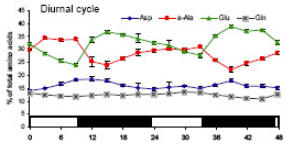
→ **Long-term objective: Discover mechanisms underlying the brown algal capacity to live in the intertidal zone**

Diurnal cycle experiment

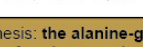
µmol/gDW	Ectocarpus	SE	Arabidopsis	SE
Amino acids	**88,1**		**82,4**	
Aspartate	14,5	2,0	10,9	0,9
Asparagine	0,7	0,1	6,1	0,2
Methionine	0,3	0,1	0,2	0,0
Isoleucine	0,2	0,1	0,2	0,0
Lysine	0,3	0,1	0,3	0,0
Threonine	0,7	0,1	4,5	0,3
Glutamate	23,9	4,6	23,6	1,1
Glutamine	10,8	2,1	16,0	1,4
Arginine	0,1	0,0	0,2	0,0
GABA	0,0	0,0	1,3	0,4
Proline	1,3	0,2	3,2	0,5
Ornithine	0,1	0,0	0,2	0,1
β-alanine	29,8	5,1	6,8	0,3
Valine	0,7	0,4	0,9	0,0
Leucine	0,3	0,1	0,3	0,0
Tyrosine	0,0	0,0	0,2	0,0
Phenylalanine	0,2	0,1	0,3	0,1
Tryptophane	0,0	0,0	0,1	0,0
Serine	3,1	1,0	6,8	0,6
Glycine	1,7	0,7	1,4	0,2
Gly/Ser	0,6	0,0	0,2	
Organic acids	**18,4**		**178,2**	
Fumarate	ND	0,0	66,9	31,9
Malate	ND	0,0	34,7	6,4
Citrate	16,7	2,1	66,7	7,1
Succinate	1,7	0,2	?	
Sugars and polyols	**334,7**		**60,6**	
Fructose	0,0	0,0	11,4	0,7
Glucose	3,3	0,7	21,7	0,7
myo-inositol	0,0	0,0	5,5	0,7
Sucrose	0,0	0,0	12,0	0,1
Mannitol	331,4	68,7	ND	
Total organic solutes	**441,1**		**311,8**	

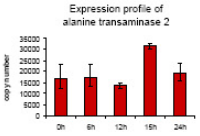
→ Amino acid and sugar profiles differ from those in *Arabidopsis*
→ No malate found in *Ectocarpus*

Profiles of most abundant amino acids



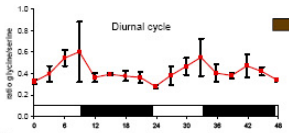
- Inverse correlation of alanine and glutamate
- The conversion of alanine and glutamate could be catalyzed by an alanine transaminase



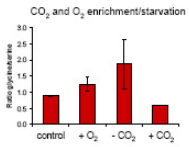


→ Hypothesis: **the alanine-glutamate system** might serve as a **short-term system for photosynthetically produced pyruvate**

Ratio of glycine to serine -a marker of Photorespiration?

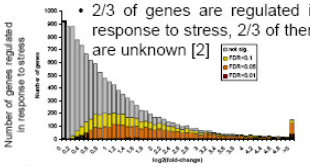


- Increases during light phase
- Confirmation by carbon starvation experiment



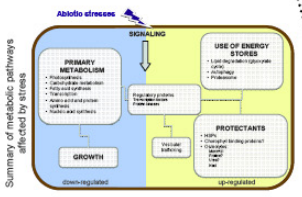
Implications for possible **carbon concentrating mechanisms (CCMs)** in *Ectocarpus*
→ If *Ectocarpus* uses a CCM, it is not very efficient and does not involve malate

Stress experiment



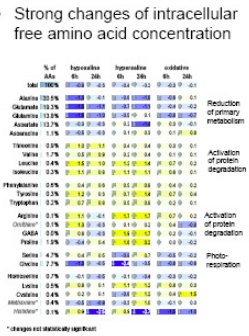
- 2/3 of genes are regulated in response to stress, 2/3 of them are unknown [2]

High transcriptomic plasticity



Primary metabolism strongly affected [2]

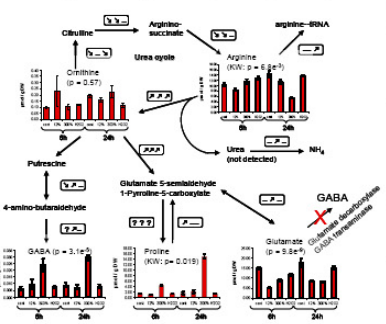
- Strong changes of intracellular free amino acid concentration

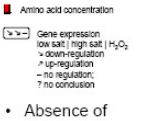


Changes in amino acid concentrations in response to stress (log2 of ratio: stress/control). Blue represents a decrease of an amino acid in response to stress, yellow an increase

High metabolic plasticity

Targeted analysis of specific pathways





- Absence of GABA-shunt, but **stess-induced GABA synthesis via putrescine**
- Urea is not an osmolyte in Ectocarpus
- **Proline** is a **potential osmoprotector**

→ Both global transcriptomic / metabolic changes, and specific protectors and signals, are part of the *Ectocarpus* stress response
→ Although some of these compounds are also known in terrestrial plants (e.g. GABA), their synthesis pathways may differ, making *Ectocarpus* an interesting model to study the evolution of primary metabolism and stress response in an environment where organisms are submitted to terrestrial and marine constraints

[1] Baldauf S., 2008. An overview of the phylogeny and diversity of eukaryotes. J. Syst. Evol. 46:263.
[2] Dittami S.M. et al., 2009. Global expression analysis of the brown alga Ectocarpus siliculosus (Phaeophyceae) reveals large-scale reorganization of the transcriptome in response to abiotic stress. Genome Biology 10:R66

6.3 Appendix 3:
Normalisation genes in *Ectocarpus*.

6.4 Appendix 4:

VU
Le Directeur de Thèse

VU
Le Responsable de l'Ecole Doctorale

VU pour autorisation de soutenance

Rennes, le

Le Président de l'Université de Rennes 1

Guy CATHELINEAU

VU après soutenance pour autorisation de publication :

Le Président de Jury,

Summary - Résumé

Abiotic stress response in *Ectocarpus siliculosus*: a global approach

Brown algae are an important component of the coastal ecosystem and have developed unique mechanisms enabling them to inhabit the intertidal zone, an environment with high levels of abiotic stress. The primary objective of this thesis was to study the mechanisms underlying the abiotic stress tolerance of brown algae in the new genetic and genomic model *Ectocarpus siliculosus*. To reach this objective, efforts were concentrated on three sub-tasks: The study of primary metabolism in *Ectocarpus*, of the short term response to saline and oxidative stress, and of the long term adaptation in a strain isolated from freshwater. Each of these sub-tasks was addressed by a combination of several approaches, including physiological measurements, metabolite- and transcriptional profiling, and genome annotation. The results presented illustrate great transcriptomic and metabolic plasticity of *E. siliculosus*, and highlight several interesting features, such as the probable absence of an efficient organic carbon concentrating mechanism, the stress induced production of the neurotransmitter and plant stress hormone gamma-aminobutyric acid (in spite of the absence of its primary biosynthetic pathway, *i.e.* the GABA shunt), the presence of a family of stress responsive fucoxanthin chlorophyll a/c binding proteins, and the use of NaCl as primary osmolyte during salt stress.

La réponse au stress abiotique chez *Ectocarpus siliculosus* : une approche globale

Les algues brunes représentent une composante importante de l'écosystème côtier, au sein duquel elles ont développé des mécanismes uniques leur permettant de vivre dans la zone intertidale, habitat caractérisé par des fluctuations fréquentes des conditions environnementales (stress biotique et abiotique). L'objectif de cette thèse était l'exploration des mécanismes permettant l'acclimatation et l'adaptation des algues brunes à certains stress abiotiques, en utilisant le modèle génétique et génomique *Ectocarpus siliculosus*. Dans ce but, trois axes de recherche ont été suivis: le métabolisme primaire chez *Ectocarpus*, la réponse à court terme à des stress salins et oxydatif, et l'adaptation à long terme d'une souche isolée dans de l'eau douce. Ils ont été abordés en combinant plusieurs approches : la détermination de paramètres physiologiques, des expériences de profilage métabolique et transcriptomique, et l'annotation du génome d'*Ectocarpus*. Les résultats obtenus démontrent une grande plasticité transcriptomique et métabolique chez *E. siliculosus*, et mettent en évidence plusieurs caractéristiques intéressantes: l'absence probable d'un mécanisme classique de concentration du carbone, l'induction de la production de l'acide gamma-aminobutyrique (un neurotransmetteur chez les animaux et une hormone de stress chez les plantes) en conditions de stress (malgré l'absence des enzymes du GABA shunt, sa voie principale de synthèse), la présence d'un groupe de FCP (Fucoxanthin/Chlorophyll a/c Binding Protein) induit par les stress, et l'utilisation de NaCl comme osmolyte primaire dans les conditions de stress salins.