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CIRCADIAN PROTECTION AGAINST OXIDATIVE STRESS IN MARINE ALGAE

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Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPX, gluthathione peroxidase; GSNO, *S*-nitrosoglutathione; APX, ascorbate peroxidase; PS, photosystem; GSH, reduced glutathione; MT, metallothionein; PC, phytochelatin

ABSTRACT

The aspects relating molecular control of the biological clocks to the cellular toxicity and the generation of reactive oxygen species (ROS) in marine algae are presented here in the following topics: (i) quantification data of the major antioxidant enzyme activities, such as superoxide dismutase, catalase and ascorbate peroxidation, aiming to gain a better understanding of the oscillatory levels of oxidative modifications in algal cells, (ii) correlation is made of the biosynthesis of low molecular weight antioxidants, such as carotenoids, melatonin, reduced gluthatione, and (iii) damage to the ROS targets, such as polyunsaturated fatty acids in membranes, proteins and nucleic acids.

Key words: Algae, antioxidant, superoxide dismutase, biological clock, oxidative stress, phytoplankton

Metabolic Generation of ROS

Aerobic cells generate a large amount of reactive oxygen species (ROS), including the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen [O_2 ($^1{}_g$)] and the hydroxyl radical ($^{\bullet}OH$). These species are normal by-products of oxidative metabolism and pose a constant threat to all aerobic organisms (Figure 1). Although some of them may function as important signaling molecules that alter gene expression and modulate the activity of specific defense proteins (Vranová et al., 2002), all ROS, in high concentrations, can be extremely harmful to organisms. ROS can oxidize proteins, lipids and nucleic acids, often leading to organelle dysfunction, alterations in cell structure and mutagenesis (Halliwell and Gutteridge, 1999).

Production of ROS constitutes a particularly severe threat to photosynthetic organisms, as a common biological source of $O_2^{\bullet -}$ is the single-electron reduction of molecular oxygen by electron transport chains. Indeed, due to the intense electron flux in an elevated oxygen and metal ion microenvironment, the mitochondria and chloroplasts of photosynthetic organisms are cell compartments highly susceptible to oxidative injury (Halliwell and Gutteridge, 1999; Augusto et al., 2002). Paradoxically, trace metals play key roles in photosynthetic electron transport in thylakoids of O_2-evolving organisms, participating in enzymes that remove ROS, such as ascorbate peroxidase (Fe-dependent APX), Fe-superoxide dismutase (FeSOD), and CuZn-superoxide dismutase (CuZnSOD). In addition, they are part of the components essential to photosystems (Fe) or mobile electron carriers such as the iron-containing cytochrome c_6 and the copper-containing plastocyanin (Raven et al., 1999).

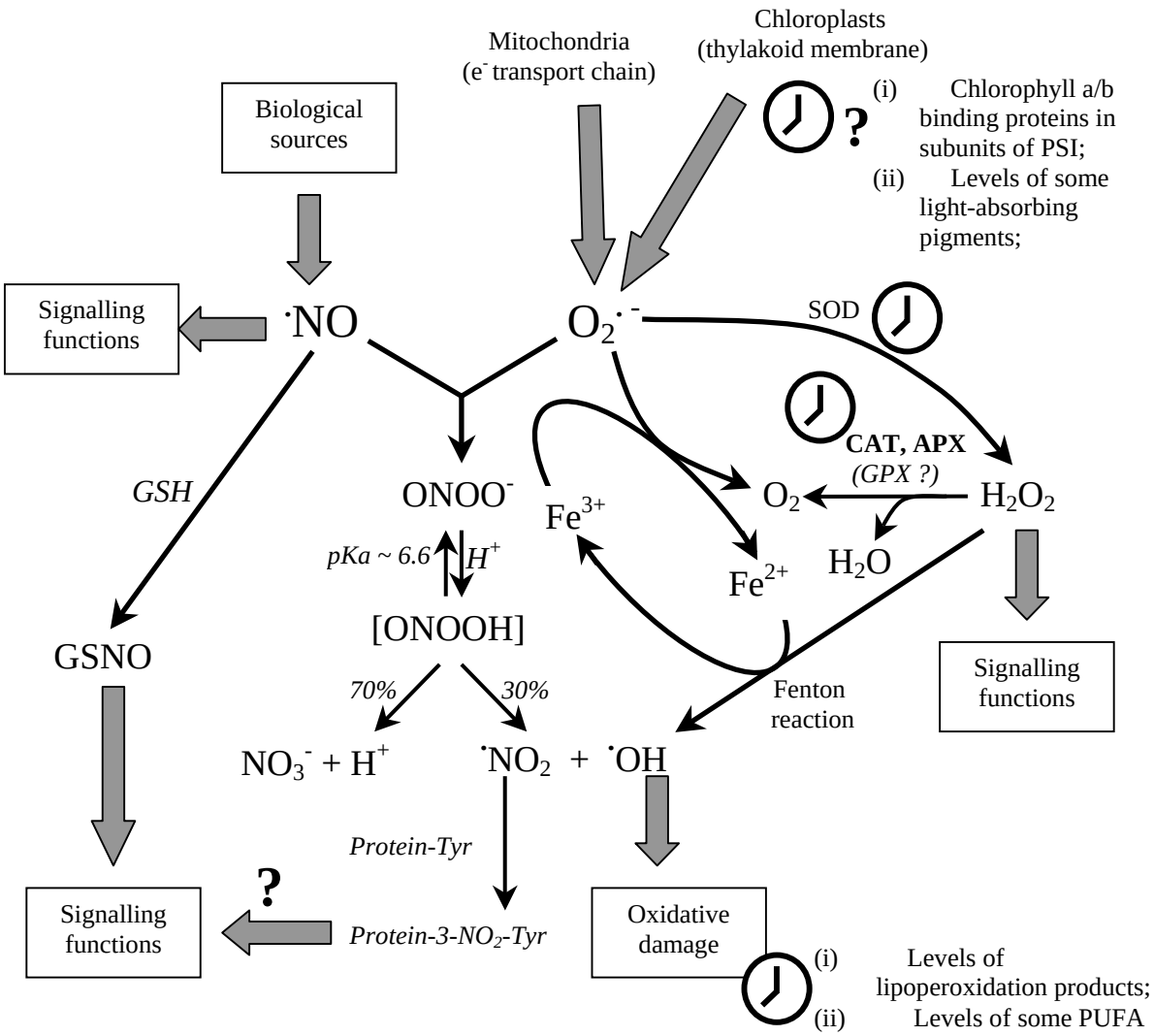

Figure 1. Possible circadian rhythms controlling the generation and interconversion of reactive oxygen/nitrogen species in algal cells. The "clock" symbol refers to already identified circadian rhythms of enzyme activities or oxidized product. SOD, superoxide dismutase; CAT, catalase; GPX, gluthathione peroxidase; GSNO, *S*-nitrosoglutathione; APX, ascorbate peroxidase; NO, nitric oxide; ONOO, peroxynitrite; PUFA, poly unsaturated fatty acids

The effect of ROS in photosynthetic organisms is exacerbated by excessive illumination. Excessive light energy input may, for instance, increase the levels of excited molecules such as triplet chlorophyll and singlet state O_2 ($^1{}_g$), the latter being highly electrophylic and capable of oxidizing many other molecules. Moreover, photochemical production of $O_2^{\bullet-}$ generated by oxygen reduction in PS I (the Mehler reaction) results in the diffusion of $O_2^{\bullet-}$ into the stroma, where it is disproportionated to O_2 and H_2O_2. The reaction of H_2O_2 with Fe^{2+} or Cu^+ ions produces $^{\bullet}OH$ *in situ*, a powerful oxidant that can

immediately react ($k_1 \sim 10^9\ M^{-1}s^{-1}$) with and damage biomolecules (Takeda et al., 1995). To add to the problem, chloroplasts have a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation (Halliwell and Gutteridge, 1999). Thus, while many ROS generating-processes are slow under normal conditions, environmental factors such as high light or UV exposure can accelerate them. Higher levels of antioxidants would be critical to withstand photo-oxidative stress elicited by a reduced energy-utilizing capacity (Okamoto et al., 2001a).

Algae defense mechanisms against high levels of ROS

Organisms have developed a wide range of protective mechanisms that serve to remove ROS before they can damage sensitive parts of the cellular machinery. These can be conveniently divided into low molecular weight compounds (LMWC; Figure 2) and enzymatic catalysts of high molecular weight (Table 1).

Table 1. Cellular Antioxidant Enzymes

Enzyme	Reaction catalysed
Superoxide Dismutase	$2\,O_2^{\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$
Glutathione Peroxidase	H_2O_2 or ROOH + 2 GSH $\rightarrow$ 2 H_2O or ROH + GSSG
Ascorbate Peroxidase	H_2O_2 + Ascorbate $\rightarrow$ H_2O + Monodehydroascorbate
Thioredoxin	Prot-S_2 + Prot'$(SH)_2$ $\rightarrow$ Prot$(SH)_2$ + Prot'-S_2
Peroxiredoxin	ROOH + R'$(SH)_2$ $\rightarrow$ ROH + R'S_2 + H_2O
Glutathione Reductase	GSSG + NAD(P)H + H^+ $\rightarrow$ 2 GSH + $NAD(P)^+$

Among the LMWC (Figure 2), carotenoids are known to be the most important antioxidants. They are widely distributed, naturally occurring pigments found in bacteria, yeast, algae, plants, animals and humans (Britton et al., 1995). Fucoxanthin and peridinin are abundant in the aquatic environment, and are present principally in the chloroplasts of many phytoplanktonic species. The role of these carotenoids is two-fold; not only do they aid in broadening the spectrum of photosynthetically active radiation but they also protect the light-harvesting pigments in the antenna complexes against photochemical

damage caused by excited triplet states (Frank and Cogdell, 1996; Krinsky, 1989; Pinto et al., 2003a) and other ROS (Woodall et al., 1997). Peridinin suppress electronically excited molecules such as O_2 ($^1{}_g$), which has been shown to be capable of inducing DNA damage and to be mutagenic (Di Mascio et al., 1990; Hollnagel et al., 1996). In general, quenching efficiency is directly proportional to the number of conjugated double bonds (Foote et al., 1970), and this holds true for peridinin, whose quenching efficiency is roughly ten-fold lower than ß-carotene. However, HPLC analysis of pigments in the dinoflagellate *Lingulodinium polyedrum* indicates that peridinin is much more abundant than ß-carotene (Pinto et al., 2000), suggesting that, despite its lower efficiency, it may contribute substantially to quenching. Fucoxanthin, a carotenoid with a structure similar to peridinin, is reported to be roughly 10 fold less effective as a quencher than ß-carotene, and it is therefore in the same range as peridinin (Barros et al., 2001; Yan et al., 1999).

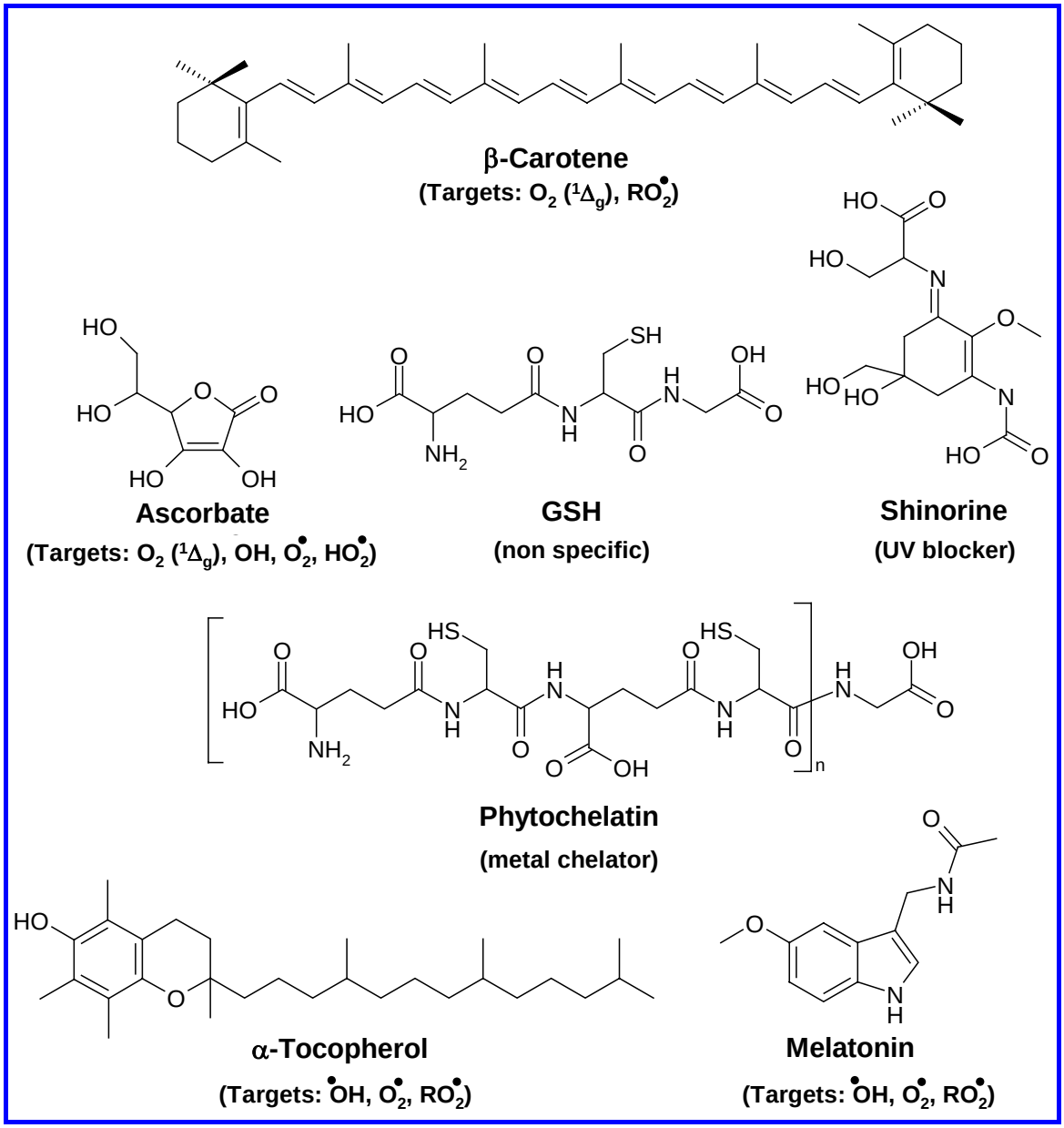

Figure 2. Cellular targets of some natural low molecular weight compounds. Chemical structures of β-carotene, ascorbate, glutathione (GSH), shinorine, α-tocopherol, melatonin and phytochelatin are shown. In parenthesis the main ROS

Other LMWC comprise melatonin, tocopherols (including vitamin E), phytochelatin, phenols, ascorbate and GSH (Table 1). Melatonin is a potent scavenger of several ROS and is believed to be present in all organisms (Hardeland et al., 1995; Rodriguez et al., 2004; Hardeland et al., 2003). Ascorbate is of particular interest as an electron donor for $^{\bullet}OH$ radicals (for which there are no enzymatic detoxification mechanisms) and as a substrate for ascorbate peroxidase. The tripeptide GSH is composed of glutamate, cysteine and glycine, and acts as a non-specific reductant and also as a substrate/cofactor for enzyme-catalyzed reactions. It serves as an aid in the rearrangement of protein disulfide bonds and in the intracellular removal of H_2O_2 and organic peroxides (ROOH). The role of GSH as

a reductant is extremely important, particularly in the highly oxidizing environment of photosynthetic cells. The sulfidryl of GSH can be used to reduce peroxides formed during partial reduction of oxygen. The resulting oxidized form of GSH consists of two disulfide molecules bonded together (abbreviated GSSG). The enzyme glutathione reductase utilizes NADPH as a cofactor to reduce GSSG back to two molecules of GSH.

With regard to the antioxidant enzymes, the aerobic organisms express a natural battery of proteins such as SOD, CAT, GPX, APX, lipid peroxidase glutathione reductase (LpxGR), thioredoxin (TR) and peroxiredoxin (PRX) (Table 1), which contribute to the control of cellular ROS levels (Rice-Evans et al., 1996; Asada 1999; Pinto et al., 2003a). PRX catalyzes the breakdown of alkyl hydroperoxides into water and their corresponding alcohols (Rouhier and Jacquot 2002). CAT and GPX catalyze the production of H_2O from the degradation of H_2O_2 and ROOH, respectively, while APX reduces H_2O_2 to H_2O using ascorbate as electron donor (Fridovich, 1997). A growing body of data has testified to the efficiency of ascorbate-dependent enzymatic systems in preventing hazardous H_2O_2 accumulation in phototsynthetic organisms (Barros et al., 2003a; 200b).

SOD, which catalyzes the disproportionation of $O_2^{\bullet-}$ to O_2 and H_2O_2, has been called the cell's first line of defense against ROS (Hassan and Scandalios, 1990; Collen et al., 2003; Leitão et al., 2003). This is due to the fact that $O_2^{\bullet-}$ is a precursor to several other highly reactive species, so that control over the steady-state $O_2^{\bullet-}$ concentration by SOD constitutes an important protective mechanism (Fridovich, 1997). There are three major SOD isoforms which have been described in eukaryotic photosynthetic organisms (Asada, 1999): a CuZnSOD located in the thylakoid membranes and cytosol of higher plants, certain dinoflagellates and charophycean green algae, a MnSOD isoform found within mitochondria, and a FeSOD isoform in the chloroplast stroma. Indeed, FeSOD is considered the major $O_2^{\bullet-}$ scavenger in chloroplasts, while MnSOD is the most active scavenger in mitochondria (Fridovich, 1997). Interestingly, SOD is induced by its substrate (Allen and Tresini, 2000) and, thus, activation of specific SOD isoforms can serve as an indicator of the cell compartment experiencing pollutant-induced $O_2^{\bullet-}$ levels. Recently, Okamoto et al., (2001b) reported the isolation and molecular cloning of the FeSOD isoform from the marine dinoflagellate *L. polyedrum*, and changes in the expression of this enzyme during algal growth have been described (Sigaud-Kutner et al., 2002).

ROS Generation and Clock Control in Algae

Algae are the basis of the food web in all aquatic ecosystems. Among the major primary producers, marine microalgae are responsible for about half of the O_2 production and most of the dimethylsulfide released into the atmosphere (Gibson et al., 1990; Stefels and van Baekel, 1993) and constitute the main food source for bivalve mollusks in all their growth stages, for zooplankton (rotifers, copepods and brine shrimps) and for the larval

stages of some crustacean and fish species. The nutritional value of an algal species is dependent on diverse characteristics including shape, size, digestibility and toxicity. However, the primary determinant in establishing the food quality transferred to the other trophic levels of the food web appears to be the biochemical composition of the algae (fatty acids, sterols, amino acids, sugars, minerals and vitamins) (Brown and Miller, 1992; Pinto et al., 2002; Pinto et al., 2003b).

Living organisms possess internal biological clocks that control the time of day at which different physiological processes occur (Wever, 1979; Moore-Ede et al., 1982; Hastings et al., 1991). Biological rhythms are therefore considered essential components of life. There are evidences that clock systems in photosynthetic organisms have a complex nature. In other words, more than one rhythm can be controlled by a single oscillator, as well as multiple rhythms may be driven by different oscillators (Johnson, 2001). In the marine unicellular alga *Lingulodinium polyedrum* numerous processes are clock-controlled: bioluminescence (BL) occurs during night phase, cell aggregation and photosynthesis peak during the day, while cell division occurs at the transition between night and day. The circadian changes in BL are correlated with daily changes in the cellular amounts of both luciferase and luciferin (substrate) binding protein (LBP), and there is a daily pulse in the synthesis of LBP that is regulated at the translational level (Morse et al., 1989). In *L. polyedrum*, it was described the presence of at least two clocks controlling distinct rhythms (Roenneberg and Morse, 1993). Using the oscillatory nitrate reductase system Lillo et al. (2001) have discussed the clock organization in photosynthetic organisms.

Although the endogenous circadian and exogenously driven daily rhythms of antioxidative enzyme activities and of low molecular weight antioxidants are described in different organisms, the rhythmicity of the antioxidant system in micro and macroalgae has only recently received any significant attention (Figure 1). The activity of SOD in cell-free extracts of *L. polyedrum* monitored at different times of the day and night was found to be three to fourfold higher during the day. This rhythm continued in cells kept in constant light, indicating that the regulation is attributable to the cellular circadian clock (Colepicolo et al., 1992). Using Western blot techniques we decribed that the extractable levels of SOD protein change in parallel with its activity (Okamoto and Colepicolo, 2001). These experiments thus show that the protein is actually synthesized and destroyed each day, as opposed to what might seem a more economical alternative to the same end, such as inhibition and activation, by phosphorylation, for example. Similar results have been previously obtained for two other proteins (luciferase and luciferin binding protein), but they differ in that they are present and active during the night phase and absent by day. Different antioxidant enzymes and low molecular weight compounds have been studied in

L. polyedrum, focusing on the circadian activity and levels (Okamoto et al., 1999; Sankievicz and Colepicolo, 1999; Hardeland et al., 2003).

In *G. tenuifrons* (Rhodophyta), SOD diurnal rhythm is 2-fold higher than at night, which strongly suggests a light regulation of SOD (Rossa et al., 2002). Prior studies showed that isoforms of SOD were differently regulated during photosynthesis and periods of environmental stress (Figure 1). These conditions increase the exposure of organisms to high concentrations of oxyradicals or high light intensities. SOD is well known to vary substantially under different light regimes, displaying circadian oscillations with wavelength, intensity and photoperiod (Asano et al., 1998). Furthermore, the APX1 mRNA level – a transcript that encodes for the peroxide-/H_2O_2-scavenging enzyme APX – has been found to increase during the day and decrease at night (Kubo et al., 1995).

The variations of low weight molecular compounds and photo-protecting compounds in algae may be related to the circadian rhythm in photosynthesis since, during photosynthetic electron flux, electrons can leak and react with molecular oxygen, producing ROS which attack biomolecules (Roenneberg and Mittag, 1996; Hardeland et al., 2003). Also, ultraviolet (UV) radiation is another source of oxidative stress and can induce rhythm in algal species (Sinha et al., 2001; Sinha et al., 2003). The adaptive response to compensate for these environmental stressful conditions may be the rhythm or oscillation of carotenoids, fatty acids, phenolics and other LMWC.

Some reports reveal daily rhythms in the biosynthesis of carotenoids (Di Mascio et al., 1995; Hollnagel et al., 1996). It was demonstrated that the ß-carotene level in *L. polyedrum* exhibited a peak in the middle of the day, which was twice the nocturnal peak. In addition, peridinin, an oxi-carotenoid, also found in *L. polyedrum*, did not show daily variation (Knoetzel and Rensing, 1990; Hollnagel et al., 2002).

Another mechanism related with carotenoid variations during photosynthesis in plants and algae is the interconversion among the xanthophyll pigments, called the xanthophyll cycle (Yamamoto, 1985). The xanthophyll cycle consists of the reversible conversion of violaxanthin into zeaxanthin via antheraxanthin (plants and green algae) or diadinoxanthin into diatoxanthin (Chromophytes), which dissipates excess energy in the photosynthetic apparatus (Lohr and Wilhelm, 1999). Results obtained *in loco* with *Sargassum natans* (Phaeophyceae) in the Gulf of Mexico suggest that the flux of light induces the violaxanthin cycle. The daily rhythm of violaxanthin/zeaxanthin is dissociated of an endogenous control (Schofield et al., 1998). Therefore, further studies on the enzyme regulation related to the carotenoids biosynthesis would be of interest to better understand the clock synchronization of these pathways.

The oscillations of fatty acid and malondialdehyde (MDA), a product of lipoperoxidation, were found in *L. polyedrum* grown under light:dark cycle conditions. The *cis*-linolenic acid showed a peak at midday, and its level was minimal during the mid to

late night phase. In addition, there was an evident diurnal oscillation in the MDA content. The higher rates of lipid peroxidation occurring during the day may be counterbalanced by higher daily PUFA levels (Cardozo et al., 2002).

A pronounced circadian rhythm of photosynthesis is correlated with circadian changes in transcript abundance of relevant genes such as phycoerythrin and subunits and large and small subunits of Rubisco in *Kappaphycus alvarezii* (Rhodophyta). Both transcripts exhibited diurnal regulation under light:dark conditions, with maximum transcript abundance during early daytime to midday (Jacobsen et al., 2003). *K. alvarezii* also showed variable rhythmicity rates of oxygen evolution upon different monochromatic light irradiances: diminished and increased free-running rhythms upon blue and red high light exposure, respectively (Granbom et al., 2001; 2004).

In the dinoflagellate *L. polyedrum*, various treatments that cause oxidative stress result in strong suppression of melatonin and its metabolite 5-methoxytryptamine (5-MT) and to secondary effects on overt rhythmicity. The protective effect of melatonin seems to be caused by the direct action of melatonin as an antioxidant, because the major antioxidant enzymes (SOD, hemoperoxidase/CAT and GPX) were not stimulated by this indol-derivative compound, although some protective enzymes responded to millimolar H_2O_2 treatments (Antolin et al., 1997). Therefore, a general effect of oxidative stress may consist in declines of easily redox signaling molecules such as melatonin, H_2O_2, and this can have consequences on the circadian endogenous organization and expression of overt rhythms (Hardeland et al., 1999; Hardeland et al., 2003).

In *Chlamydomonas reinhardtii* (green algae), the uptake of nitrogen components takes place at the beginning of the day phase. Interestingly, the activities of enzymes and transport proteins involved in nitrogen metabolism vary temporally with an opposite phase to that of the repressor protein CHLAMY1. The modulator CHLAMY1 has strong affinity for two mRNA that encode proteins involved in CO_2 metabolism, thus apparently coordinating entire metabolic pathways in a circadian way (Mittag and Wagner, 2003). *C. reinhardtii* also has programmed UV-sensitive processes responsive to low or absent UV intensities (Nikaido and Johnson, 2000; Suzuki and Johnson, 2001) and also exhibit circadian rhythms of mRNA abundance for a small gene family encoding the chlorophyll a/b-binding proteins of PSII (CABII or lhcb) (Jacobshagen et al., 1996). Noteworthy, UV radiation exerts biochemical/physiological alterations in autotrophic organisms through radical mechanisms, which probably involve both ROS intermediates (Aguilera et al., 2002).

In this scenario, the biological clock efficiently controls the sources of ROS and the antioxidant system, such as SOD, APX, CAT and others, maintaining a fine balance of the intracellular redox state over the photosynthetic organisms subjected to the light:dark cycle. Also, anticipation controlled by the biological clock, as in the case of SOD activity in *L. polyedrum* (Okamoto and Colepicolo, 1998), should be taken into account as a fine and

synchronized mechanism in the antioxidant process against dangerous damage to biomolecules and organelles induced by ROS.

In summary, the regulation and induction of antioxidants takes place in response to different kinds of environmental stress, such as changes in daylight intensity, nitrate pulses and UV radiation. Levels of antioxidant enzymes and other compounds may vary over the course of a 24 h light-dark cycle. Cellular prediction, orchestrated by the biological clock, of a higher level of oxidative status and, therefore, prompt induction of antioxidant enzymes expression must be critical to control the steady-state levels of ROS, thereby preventing the ensuing oxidative/nitrosative damage. This type of defense mechanism is especially important within subcellular sites highly prone to oxidative stress such as chloroplasts and mitochondria. Consequently, the biological clock mechanisms that control the production of ROS are conceivably of importance in understanding several aspects of susceptibility and temporal cell behavior.

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