

## High Light-induced Sign Change of Gravitaxis in the Flagellate *Euglena gracilis* is Mediated by Reactive Oxygen Species

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**Summary.** *Euglena gracilis* responds to abiotic stress factors (high light, salinity, heavy metals) with a sign change of its gravitactic behavior. This phenomenon is oxygen dependent and can be suppressed by the application of the reductant dithionite. It is not mediated by the photoreceptor since also blind mutants change their movement behavior upon high light exposure. It is also not mediated by the chloroplasts since the gravitactic sign change was also found in white, chloroplast-free mutants. The NO radical donor SNAP and the NO cleaver carboxy-PTIO had no obvious effects on gravitaxis or gravitactic sign change, respectively, indicating that NO radicals are not likely involved in gravitactic sign change. Gravitactic sign change was suppressed when oxygen was removed by flushing the cell suspension with nitrogen. Also, the addition of the radical scavengers Trolox, ascorbic acid or potassium cyanide abolished or reduced gravitactic sign change. Quantification of reactive oxygen species (ROS) in the cells indicated that these treatments reduced the evolution of ROS. Furthermore, addition of hydrogen peroxide induced gravitactic sign change in the absence of external stress factors. These results indicate that gravitactic sign change is triggered by ROS (most likely hydrogen peroxide) which are probably produced by cytochrome-*c*-oxidase in the mitochondria. The clear responses of *Euglena* to abiotic stress factors suggest that these cells are probably interesting model systems in the study of stress signaling.

**Key words:** *Euglena gracilis*, gravitaxis, hydrogen peroxide, reactive oxygen species, sign change, UV.

**Abbreviations:** Carboxy-PTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, DCF - 2',7'-dichlorofluorescein diacetate, DCFH(-DA) - 2',7'-dichlorodihydrofluorescein diacetate, GSC - gravitactic sign change, H<sub>2</sub>O<sub>2</sub> - hydrogen peroxide, NO• - nitric oxygen radical, NOS - Nitrogen oxygen species, PAR - photosynthetic active radiation, ROS - reactive oxygen species, SNAP - S-nitroso-N-acetylpenicillamine, Trolox - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid.

### INTRODUCTION

*Euglena gracilis* orients itself in the water column mainly by means of phototactic and gravitactic orientation (Häder and Griebenow 1988). Normally the cells are guided by negative gravitaxis towards the surface,

while negative phototaxis helps the cells to avoid regions with deleterious solar radiation, because they are relatively sensitive against excessive UV exposure (Brodhun and Häder 1995). Recently, it was found that *Euglena gracilis* reverses gravitaxis when exposed to certain environmental stressors. The effects of high light exposure and increased salinity on gravitactic sign change (GSC) was demonstrated (Richter *et al.* 2002a, b). Probably sudden changes in temperature also induce gravitactic sign change, but this was not explicitly proven yet. It was clearly demonstrated that the gravitactic sign

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change is not correlated with the photoreceptor or the chloroplasts, because this behavior also occurs in mutants, which lack these organelles. The gravitactic sign change is oxygen dependent, because in the presence of dithionite (removes oxygen chemically) the gravitactic sign change was completely suppressed. This led to the assumption that reactive oxygen species (ROS) or other radicals trigger gravitactic sign change.

ROS are unavoidably produced among others during photosynthesis or during respiration in the mitochondrion. Because of their high reactivity, ROS can exert deleterious effects on nearly every cellular level, like on the membranes, proteins or DNA. For this reason the ROS concentration is controlled by an arsenal of cellular detoxification mechanisms (e.g. catalase, superoxide dismutase). Recently it was found that ROS play an important role in cell signaling. ROS have been shown to be important transducer molecules in many organisms, which among others signal biotic and abiotic stresses, like UV (Mackerness *et al.* 2001), drought (Smirnov 1998), increased salinity (Rout and Shaw 2001), chilling stress (Lee and Lee 2000) or heavy metals (Piqueras *et al.* 1999). Among others ROS were described to be produced by NADPH oxidase activity, the activity of which is probably regulated by intracellular calcium; (Bowler and Fluhr 2000, Yang, *et al.* 2002) or *via* a phospholipase pathway (Chapman 1998).

In the unicellular ciliates *Paramecium* and *Loxodes* a strong oxygen dependency of gravitaxis was reported, (Fenchel and Finley 1986, Hemmersbach-Krause and Briegleb 1991). In *Loxodes* probably cytochrome-*c*-oxidase is the oxygen receptor, because incubation with KCN led to a loss of gravitactic orientation (Finley and Fenchel 1986). Singlet oxygen generated in high light has been suggested to trigger the reversal for phototactic reaction in *Anabaena* (Nultsch and Schuchart 1985).

The experiments presented in this paper were carried out to reveal in detail the role of ROS in the switching mechanism of gravitaxis in *Euglena gracilis*.

## MATERIAL AND METHODS

### Organisms and growth conditions

The experiments presented in this study were performed with *Euglena gracilis* Z and the colorless mutant *Euglena gracilis* 1F. Both strains were obtained from the algal culture collection of the University of Göttingen (Schlösser 1994). *Euglena gracilis* Z cells were grown in a modified (contains no EDTA) mineral medium (sodium

acetate,  $(\text{NH}_4)_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4$ ,  $\text{CaCl}_2$ ,  $\text{FeCl}_3$ , trace elements and vitamin B12 and B6) as described earlier (Starr 1964, Checcucci 1976) in stationary cultures in 100-ml Erlenmeyer flasks at about 20°C under continuous light of about 18 W m<sup>-2</sup> from mixed cool white and warm tone fluorescent lamps. *Euglena gracilis* 1F was grown in complex medium, which consists of sodium acetate,  $\text{CaCl}_2$ , tryptone, peptone and yeast extract (Starr 1964), in 100-ml Erlenmeyer flasks at about 20°C in the dark. If not otherwise indicated, the culture age was about 3 weeks, the cell density about 1 million cells per milliliter.

### Chemicals

Potassium cyanide (KCN) was purchased from Merck, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Aldrich (Taufkirchen, Germany), ascorbic acid from Sigma (Deisenhofen, Germany). The NO• donor S-nitroso-N-acetylpenicillamine (SNAP) and the NO• scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (Carboxy-PTIO) were from Tocris (Bristol, UK). The 2',7'-dichlorodihydrofluorescein diacetate (DCFH) used for detection of reactive oxygen species was obtained from Molecular Probes (Leiden, The Netherlands). Hydrogen peroxide (30 % solution) was from Aldrich and hypochloride from Roth (Karlsruhe, Germany).

### Exposure to artificial radiation

All samples used for a single experiment were taken from the same culture. 30 ml of cell suspension (each 5 ml in the case of experiments with SNAP and carboxy-PTIO) were transferred into small black plastic boxes (at least 3 parallel samples with inhibitors, control samples and corresponding dark controls) and placed into a temperature-controlled water bath (20°C). The samples were covered with a 295 nm cut-off filter foil (transmits UV-B, UV-A and PAR, Digefra, Munich, Germany) and irradiated with a Hönle lamp (Dr. Hönle, Martinsried, Germany), which produces a spectrum similar to the solar spectrum (Klisch *et al.* 2001). The irradiances were PAR 321 W m<sup>-2</sup>, UV-A 67 W m<sup>-2</sup> and UV-B 1.9 W m<sup>-2</sup> (total photon flux: 1079.6  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ , PAR: 813  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ , UV-A: 260  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ , UV-B: 6.6  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) at a distance of 65 cm from the lamp. The exposure time is indicated separately for each presented experiment. The cells were filled into a cuvette and subsequently analyzed with the image analysis software WinTrack 2000 (see below). The observed sign change phenomenon persisted for several hours (Richter *et al.* 2002a), so that the time, necessary to prepare and to perform the measurements, was sufficient. Each experiment was repeated at least three times.

### Motion analysis

After irradiation some drops of *Euglena* cell suspension were sealed between two glass slides by means of silicon (Bayer Silone, high viscous, Bayer, Leverkusen, Germany) and immediately analyzed. Motion analysis was performed with a recently developed cell tracking system (Lebert and Häder 1999b, WinTrack 2000). The system is based on a video A/D flash converter (Meteor, Matrox, Canada) connected to a PCI slot of an IBM compatible computer which digitizes the analog video images from a CCD camera mounted on a horizontally oriented microscope.

The digitized images are transferred to the computer memory. Objects are detected by brightness differences between cells and

background. The movement vectors of all motile cells on the screen are determined by subsequent analysis of five consecutive video frames (movement vectors of the objects from frame 1 to frame 5). In addition to orientation and velocity of the cells, motility, area and cell form are determined as well as several statistical parameters. The  $r$ -value indicates the precision of (gravitactic) orientation and ranges from 0 (random orientation) to 1 (precise orientation):

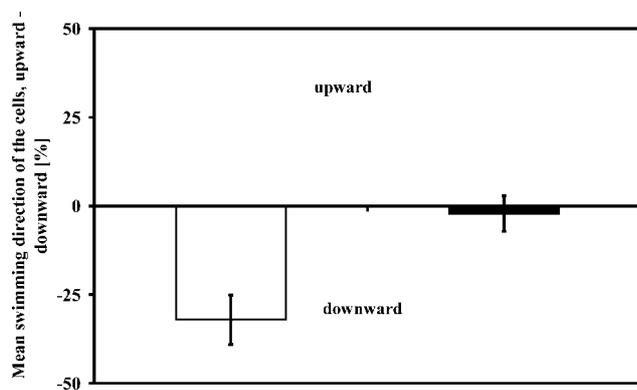
$$r = \frac{\sqrt{(\sum \sin \alpha)^2 + (\sum \cos \alpha)^2}}{n}$$

where  $\alpha$  is the deviation from the stimulus direction (here acceleration) and  $n$  the number of recorded cell tracks. The angle  $\Theta$  indicates the mean movement direction of a cell culture. The increment of the angle is clockwise (see one of the circular histograms in Fig. 6).

The measurements were performed in darkness (infrared observation of the cells) to avoid any phototactic or photophobic effects on the orientation of the cells. To exclude the evaluation of immotile cells, which sediment in the vertical cuvette, the software accepted only cells with a speed faster than the sedimentation velocity (about  $20 \mu\text{ms}^{-1}$ ). In all experiments the movement of the cells was visually monitored by the experimenters on screen in order to control data acquisition of the obtained cell tracks by the software.

### Gassing with nitrogen

Oxygen was removed by nitrogen flushing of the samples during the whole light exposure. The corresponding controls were flushed with air. Oxygen was not completely removed by this method but it was less than  $2 \text{ mg/l}$  (measurement with DO-5509 oxymeter, Conrad electronics, Hirschau, Germany).



**Fig. 1.** Effect of nitrogen flushing of the cell suspension on the movement direction of light-exposed *Euglena gracilis* 1F mutant cells. The cells have been irradiated for 120 min with artificial solar radiation in order to induce a sign change of negative gravitaxis. The pronounced gravitactic sign change of light-exposed control cells (white column) is considerably inhibited, when oxygen pressure is decreased by nitrogen (black column). The diagram shows the percentage of upward swimming cells minus the percentage of downward swimming cells. Positive values indicate upward, negative values downward mean orientation of the cells in a culture. Bars indicate SD of each four parallel control and nitrogen treated samples. The experiment was repeated three times with similar results.

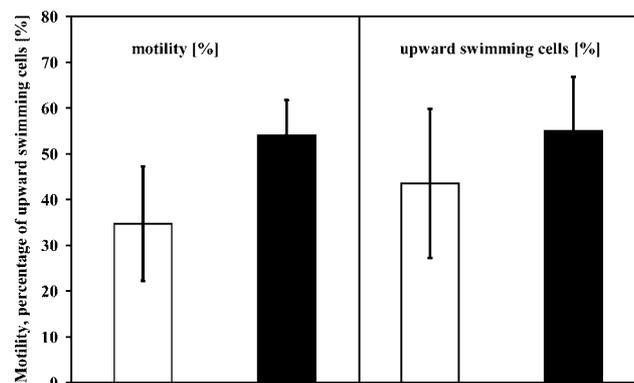
### Detection of reactive oxygen species

The ROS concentration was estimated with the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) according to He and Häder (2002a). The lipophilic DCFH-DA passes the cell membrane and is cleaved inside the cell to the non fluorescent 2',7'-dichlorodihydrofluorescein (DCFH). Upon reaction with intracellular reactive oxygen species (ROS) DCFH is oxidized to the highly fluorescent 2',7'-dichlorodifluorescein (DCF). The fluorescence of the samples was measured with a spectrofluorometer (RF-5000, Shimadzu, Kyoto, Japan) at room temperature (excitation wavelength: 485 nm, emission band between 500 and 600 nm). The integral of the signal (500-600 nm) was used for analysis. DCFH-DA was added from a 2 mM methanolic stock solution to an end concentration of  $5 \mu\text{M}$ . As DCFH-DA is sensitive against light, the samples were incubated directly after the exposure experiment (1 h incubation time in the darkness). Each one milliliter of cells was transferred into a cuvette (1.5 ml semi-micro disposable cuvettes, Brand, Wertheim, Germany), after gentle mixing of the cell culture. At least three independent fluorescence signals were measured from each sample.

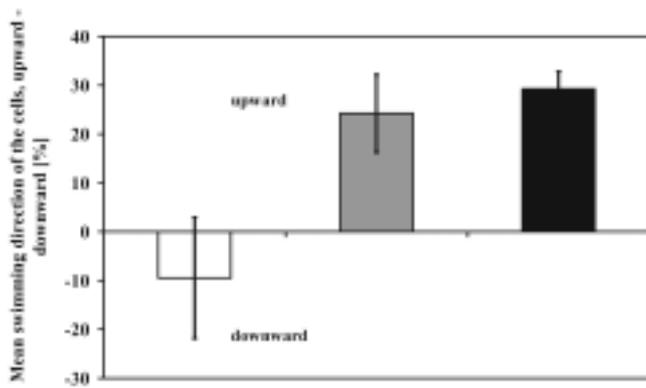
## RESULTS

### Effects of the physical replacement of oxygen with nitrogen

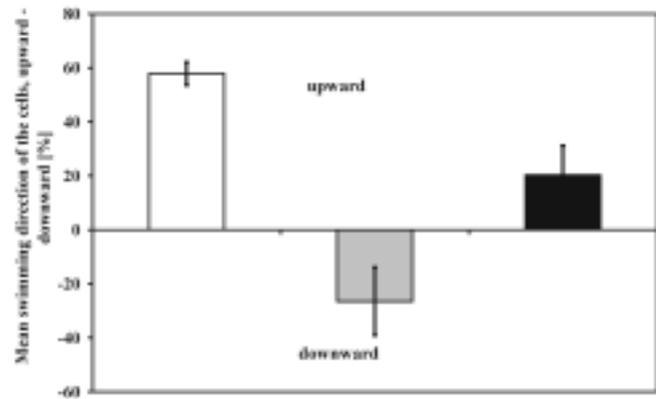
Samples from which oxygen was removed physically, showed a pronounced reduction of GSC as compared to the untreated controls (Fig. 1). The effect was not as



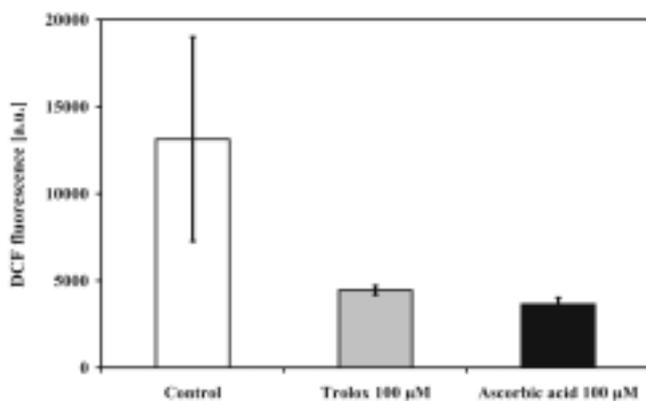
**Fig. 2.** Effect of carboxy-PTIO on motility and upward oriented movement in a *Euglena gracilis* culture after 210 min exposure to artificial solar radiation. The data indicate a slight protective role of carboxy-PTIO. White columns: control cells, black columns: samples plus carboxy-PTIO ( $200 \mu\text{M}$ ). Bars indicate SD of each four parallel control and carboxy-PTIO treated samples. The experiment was repeated several times, but the results were not consistent (see text).



**Fig. 3.** Inhibitory effect of Trolox and ascorbic acid on the light-induced gravitactic sign change in a *Euglena gracilis* strain Z. The cells were exposed to artificial solar radiation (340 min). Trolox (gray column) and ascorbic acid (black bar) considerably decreased the extent of gravitactic sign change compared to the control (white column). Considerably more cells were swimming upward in the presence of Trolox (black column) and ascorbic acid (gray column) compared to the control (white column). Bars indicate SD of four controls and each three Trolox or ascorbic acid treated samples. The experiment was repeated four times with similar results.



**Fig. 5.** Influence of potassium cyanide on gravitactic sign change in *Euglena gracilis* mutant 1F exposed to artificial solar radiation (120 min). In the presence of KCN (500 µM) gravitactic sign change was considerably inhibited, while the control cells (light only) showed a pronounced reversal of gravitaxis. White column: dim light control; gray column: light exposed control; black column: light exposure plus 500 µM KCN. Error bars indicate SD of each four parallel dim light controls, high light controls and KCN-treated samples. The experiment was repeated three times with similar results.



**Fig. 4.** ROS scavenging effect of Trolox and ascorbic acid determined with the fluorescence indicator DCFH-DA. Higher fluorescence values indicate higher ROS generation (oxidation of the non-fluorescent DCFH to highly fluorescent DCF). The addition of Trolox and ascorbic acid reduces the amount of DCF considerably. The samples shown in this diagram were exposed to artificial solar radiation (120 min) and subsequently incubated with DCFH-DA. Error bars indicate SD.

pronounced as in experiments performed in the presence of dithionite probably because the experimental setup did not allow removing intracellular oxygen completely.

#### Experiments with carboxy-PTIO and SNAP

Carboxy-PTIO is a water-soluble and stable free radical molecule that reacts stoichiometrically with NO• (NO• scavenger). SNAP releases NO• radicals under physiological conditions. SNAP (200 µM) induced

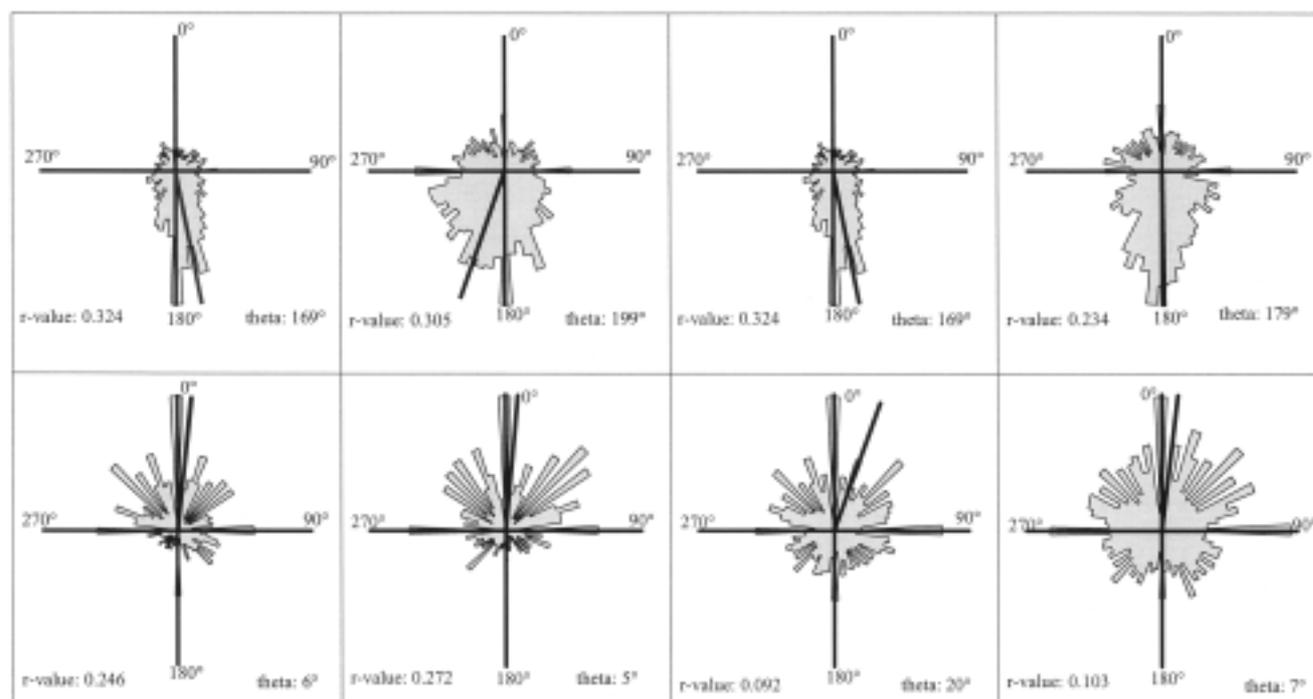
no gravitactic sign change compared to the controls (neither in darkness nor in light). Carboxy-PTIO (200 µM) did not clearly suppress GSC. The data indicate only a marginal protective effect. The proportion of motile and possibly upward swimming cells is higher in the presence of carboxy-PTIO upon light exposure than in the controls (Fig. 2), but this was not seen in all experiments. Currently, the role of NO• in GSC can not clearly be stated.

#### Effects of radical scavengers on gravitactic sign change

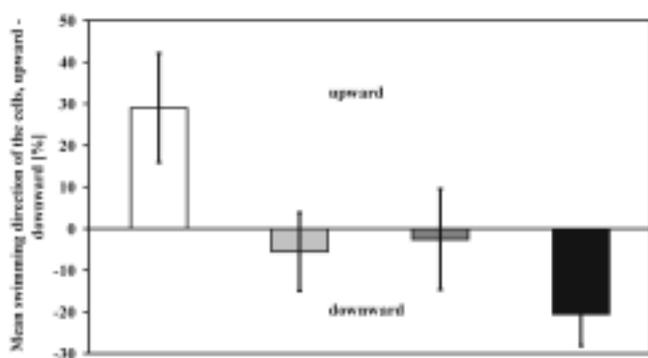
In the presence of the radical scavengers ascorbic acid and Trolox a pronounced reduction in GSC was detected at low concentrations (Fig 3.). Additionally, a decrease in the DCF-fluorescence in scavenger-treated cells was obvious indicating a loss in ROS concentration (Fig. 4). High Trolox concentrations (> 500 µM) impair the cells upon high light exposure, while dark controls were not affected (data not shown). Trolox is most likely cleaved by UV and blue light, and the products have a toxic effect on the cells at high concentrations, superimposing the positive effect of radical scavenging.

#### Effects of potassium cyanide on gravitactic sign change

Potassium cyanide considerably reduced GSC in *Euglena gracilis*. Although gravitactic orientation was

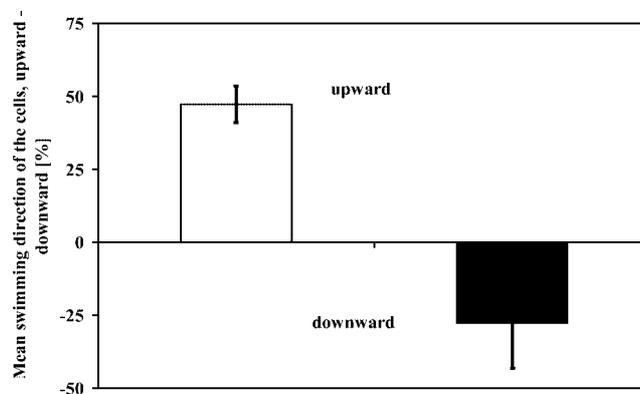


**Fig. 6.** Circular histograms of the movement behavior of the cells in Fig. 5. Upper row: untreated light-exposed controls; lower row: light-exposed samples plus 500  $\mu\text{M}$  KCN. The length of a single sector indicates the fraction of cells swimming in this direction (details see text). The r-value indicates the precision of orientation, theta (solid black line) indicates the mean movement direction of cell culture (details see text).



**Fig. 7.** Influence of hydrogen peroxide on gravitactic sign change in *Euglena gracilis* mutant 1F. The cells were incubated in  $\text{H}_2\text{O}_2$  without external stresses. In the presence of hydrogen peroxide gravitactic sign change was induced, while the control cells showed negative gravitaxis. White column: untreated control; light gray column: 0.43 mM  $\text{H}_2\text{O}_2$ ; dark gray column: 0.61 mM  $\text{H}_2\text{O}_2$ ; black column 0.84 mM  $\text{H}_2\text{O}_2$ . Incubation time about 30 min. Bars indicate SD of each six independent samples (control and  $\text{H}_2\text{O}_2$ -treated).

impaired compared to dark or dim light (presented data) controls, the mean direction of the cells in the culture was still upward (Figs 5, 6). In contrast, untreated light controls (no KCN added) showed GSC or at least a loss of gravitactic orientation (Figs 5, 6).



**Fig. 8.** Influence of hydrogen peroxide on gravitactic sign change in *Euglena gracilis*. In the presence of hydrogen peroxide gravitactic sign change was induced, while the control cells showed negative gravitaxis. White column: untreated control; black column 2.1 mM  $\text{H}_2\text{O}_2$ . Incubation time about 30 min. Bars indicate SD of six independent controls and four  $\text{H}_2\text{O}_2$ -treated samples.

### Effects of hydrogen peroxide and hypochloric acid

The addition of hydrogen peroxide at low concentrations induced GSC or at least increased the percentage of downward swimming cells in *Euglena gracilis* cul-

tures in the absence of other external stimuli. Colorless strains are more sensitive against  $H_2O_2$ . In the *Euglena gracilis* mutant 1F the addition of 0.8 mM  $H_2O_2$  to the medium induced a pronounced GSC after about 30 min of incubation (Fig. 7). In the green strain Z the addition of 2 mM of hydrogen peroxide to the medium was found to invert gravitaxis (Fig. 8). In old cultures of strain Z (>3 month) in which the cells had a drop-like cell shape GSC was not inducible, although a loss of gravitactic orientation was detected (data not shown).

In the presence of hypochloric acid (various concentrations tested, from non-effective to lethal concentrations) no GSC was detected (data not shown).

## DISCUSSION

Recent experiments revealed that *Euglena gracilis* changes the sign of gravitaxis upon abiotic stress. This effect is oxygen-dependent (see introduction). The question was which mechanism triggers this phenomenon. Samples in which oxygen was reduced by nitrogen flushing, the extent of gravitactic sign change was remarkably decreased, which confirms results with dithionite obtained in a previous study (Richter *et al.* 2002a). With the present study, where oxygen was removed physically, a non-specific effect of dithionite leading to GSC can be ruled out and the oxygen dependency of GSC can be clearly stated. The suppression of GSC in nitrogen-flushed cells was not complete, most likely due to the fact that some oxygen remained within the cells, which gives rise to the evolution of oxygen radicals.

Recent studies revealed that  $NO\bullet$  radicals and other nitrogen species (NOS) play, in combination with ROS, an important role in mitochondrial signaling (Brookes *et al.* 2002). Among others,  $NO\bullet$  radicals interact with the cytochrome-c-oxidase and hereby control the evolution of  $H_2O_2$ , which probably plays an important role in cell signaling (see below). To elucidate a possible role of  $NO\bullet$  radicals in GSC, experiments with SNAP and carboxy-PTIO were performed, which did not clearly indicate an involvement of  $NO\bullet$  radicals. Neither the production of  $NO\bullet$  radicals by SNAP nor the scavenging of  $NO\bullet$  radicals by carboxy-PTIO had any clear effect on GSC except a slightly inhibitory effect on GSC under high light exposure in the presence of carboxy-PTIO.

To test whether radicals or hydrogen peroxide are possibly involved in GSC, the known radical scavengers Trolox and ascorbic acid were applied. Among others,

these chemicals have been shown to have a protective effect against UV-B exposure. For example Trolox was found to decrease intracellular hydrogen peroxide generation in human keratinocytes in the presence or absence of UV-B radiation (Peus *et al.* 2001). This study clearly demonstrates that Trolox incubation significantly increases cell survival upon UV-B. Ascorbic acid was shown to reduce the ROS concentration in cyanobacteria and to increase their survival (He and Häder 2002a). In *Euglena* Trolox and ascorbic acid clearly reduce GSC, and in parallel, the amount of ROS was significantly reduced. This indicates that probably ROS trigger GSC.

A very important observation was that hydrogen peroxide induces GSC in the absence of external stressors. The GSC-inductive effect of hydrogen peroxide makes its involvement as key signaling molecule of abiotic stress in *Euglena* very likely. It can not be excluded, that hydrogen peroxide itself acts as stressor, so that the observed GSC is a more indirect effect. But hypochloric acid, which is chemically similar to hydrogen peroxide, does not induce GSC. This indicates that the effect obtained in the presence of hydrogen peroxide is specific. The important role of hydrogen peroxide and other ROS in cell signaling was detected in the recent years (reviewed by Neill *et al.* 2002). Intracellular hydrogen peroxide levels are reported to be controlled by regulation of antioxidant enzymes like ascorbate peroxidase or catalase, which decrease the hydrogen peroxide concentration (Neill *et al.* 2002, He and Häder 2002b). A variety of  $H_2O_2$  downstream signaling events were described, among others membrane channel activation or modulation of gene expression (probably via oxidation of  $H_2O_2$ -sensitive transcription factors) and modulation of other signaling molecules (Neill *et al.* 2002 and literature cited therein). Among others the role of hydrogen peroxide in root gravitropism was demonstrated (Joo *et al.* 2001).

The suppressive role of KCN, a known blocker of the cytochrome-c-oxidase in mitochondria, is a hint that mitochondria are a possible source of hydrogen peroxide upon abiotic stress in *Euglena*.

But KCN also interacts with other cellular metalloproteins, like e.g. catalase. The blockage of the mitochondrial respiratory chain by KCN did not kill the cells, because they also survive for hours under hypoxia (as seen in the nitrogen and dithionite experiments, which both led to microaerobic conditions). However, dramatic metabolic changes do occur even in these hypoxia-tolerant cells and may have an influence on the experiment: e.g. reduced protein synthesis and ion pump

activities, and arrest of membrane channels (see review by Boutilier and St-Pierre 2000).

It is unknown in which way ROS may induce GCS, because up to now the physiological mechanisms of gravitaxis are still not fully understood. According to a present model, gravitaxis is most likely triggered by mechano-sensitive membrane channels followed by a series of subsequent physiological mechanism, among others membrane potential changes, increased calcium and cAMP concentration, involvement of calmodulin (Lebert and Häder 1999a, Streb *et al.* 2002). An additional physical alignment component caused by body asymmetry is possible (Roberts and Deacon 2002, Richter *et al.* 2002c).

In earlier experiments performed with fluorescent calcium indicators increased intracellular calcium was observed upon cell stress (drought, high light exposure). But because this has not been the focus of these experiments the observations were not quantified or studied in more detail. From these observations it is not possible to say if calcium acts as stress signal in *Euglena gracilis* or if there is any interaction between calcium and the presumed ROS. In other systems an interaction between calcium and intracellular H<sub>2</sub>O<sub>2</sub> level was described (Yang and Poovaiah 2002).

The clear response of *Euglena* cells to unfavorable environmental factors makes these cells potential model organisms in the study of stress and redox signaling.

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