Defense Genes as Indicators of Toxicity

From Basic Research to Practical Application

As a result of exposure to certain pollutants, various toxic oxygen derivatives form in the cells of plants and animals, including the so-called "singlet oxygen". Fortunately, most cells have specific defense mechanisms against the toxic effects of these derivatives. Currently, research at EAWAG is examining in detail how the unicellular green alga *Chlamydomonas reinhardtii* reacts to the presence of singlet oxygen. The long-term goal of the work is to develop a biosensor for the detection of singlet oxygen, which would give us an indirect indicator of pollutant levels.

Since aerobic respiration provides the energy required for growth and metabolism in plants and animals, molecular oxygen (O_2) is required by all higher life forms. Oxygen may, however, be life-threatening; namely when reactive, strongly-oxidizing oxygen species are formed inside the cells (see box and Fig. 1) [1]. If the cell cannot successfully defend against this oxidative stress, vital components of the cell, such as lipids, proteins and DNA, can be damaged, and the cell will die. The formation of reactive oxygen species is enhanced when organisms are exposed to heavy metals or certain organic contaminants, such as herbicides or halogenated organic compounds. This toxic effect is enhanced by intense solar radiation. Most cells, however, have developed specific defense mechanisms against reactive oxygen species [2].

Molecular Defense Scenarios

Briefly, the molecular defense mechanism works as follows [3]: the cell possesses sensors that allow it to detect stress situations, such as the intracellular formation of reactive oxygen species. These sensors, in turn, activate so-called "stress genes" that are read by the transcription apparatus, leading to the production of gene copies (messenger RNA) in quantities proportional to the strength of the activating stress. Finally, the corresponding stress proteins are synthesized. These proteins have one of two functions: (1) they remove the source of the stress itself (i.e., they convert reactive oxygen species into non-toxic species), or (2) they repair cell components that have already been damaged. The cell produces general stress proteins that are formed regardless of the stress source, as well as specific stress proteins that are able to target and efficiently remove specific stress factors [4].

The genetic activation pattern may provide valuable clues about the nature and intensity of the stress within the cell. Expression (activation levels) of specific genes has already been used successfully to detect and quantify environmental pollutants, such as in the biosensor for arsenic that was developed at EAWAG (see article by J.R. van der Meer, p. 12). The goal of the project described here is to develop a biosensor for the detection of water contaminants that cause the formation of reactive oxygen species inside cells. Since very little is known about the defense mechanisms against singlet oxygen (see box and Fig. 1), we have decided to focus our work on this reactive species.

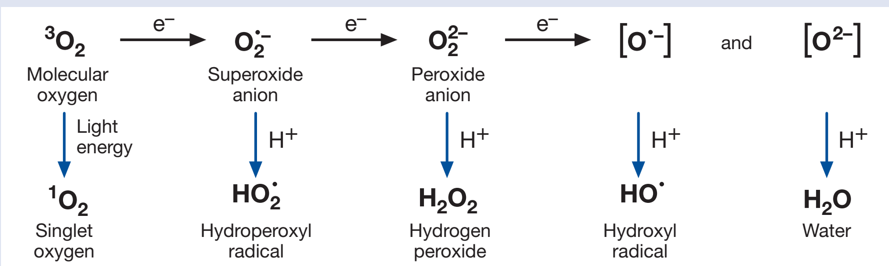


Fig. 1: Formation of reactive oxygen species from molecular oxygen by incomplete reduction or transfer of light energy. Oxygen species in parentheses are not stable and immediately transform to their protonated forms.

Reactive Oxygen Species

Due to its electron configuration, naturally occurring molecular oxygen is relatively inert and not harmful to living organisms. It can, however, be activated physically by energy transfer or chemically by electron transfer. Such activated oxygen molecules are called reactive oxygen species. They are highly reactive and can be formed in anaerobic organisms even under normal physiological conditions. Transfer of electrons, e.g., from the respiratory chain in the mitochondria, can lead to the formation of the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) (Fig. 1) [1]. If light is the energy source causing the electron transfer, singlet oxygen is formed (Fig. 1). Singlet oxygen is chemically identical to molecular oxygen; only the electronic configuration has changed. It is, however more reactive, and reacts rapidly with cellular components forming organic hydroperoxides. Unsaturated fatty acids in cell membranes react particularly rapidly with singlet oxygen, which results in the formation of lipidperoxides and may cause damage to the membrane. It is, therefore, extremely important for all organisms to have a specific defense mechanism against singlet oxygen and the damage it causes.

Cellular Stress	Contaminant	Induction Factor
Hydrogen peroxide	Hydrogen peroxide (2 mM)	2.8
Superoxide anion	Menadione (5 µM)	6.4
Organic hydroperoxides	Tert-butylhydroperoxide (0,1 mM)	4.7
Singlet oxygen	Rose bengal in light (5 µM)	78.9
Rose bengal (control)	Rose bengal in the dark (5 µM)	3.1
Photosynthetic inhibition	DBMIB (herbicide) (1 µM)	9.5
Heat shock	25 °C → 40 °C shift	1.3
Salt/osmotic stress	NaCl (200 mM)	1.5

Tab. 1: Activation of the *Gpxh* gene in *Chlamydomonas* under various stress conditions. *Chlamydomonas* cultures were exposed to various stress factors for 60 minutes. Induction factor = *Gpxh* expression in stressed culture divided by *Gpxh* expression in control cultures. *Gpxh* expression was measured as the amount of mRNA produced by the transcription process.

DNA Chips Yield First Clues

Photosynthetic organisms are particularly affected by oxidative stress [2], since the photosynthetic apparatus itself is an important source of reactive oxygen species. For this reason, we have selected a photosynthetic model organism for our studies: the unicellular, flagellated green alga *Chlamydomonas reinhardtii.* This organism has the same cellular structure as higher algae and plants, but is easier to cultivate and very suitable for the use of molecular techniques [5]. A particular advantage is the fact that DNA chips with the genetic information for *C. reinhardtii* have very recently become available. These devices allow us to examine expression patterns for the majority of the genes of a given organism [3, 6]. In our case, this will enable us to detect specific defense genes that are as yet unknown.

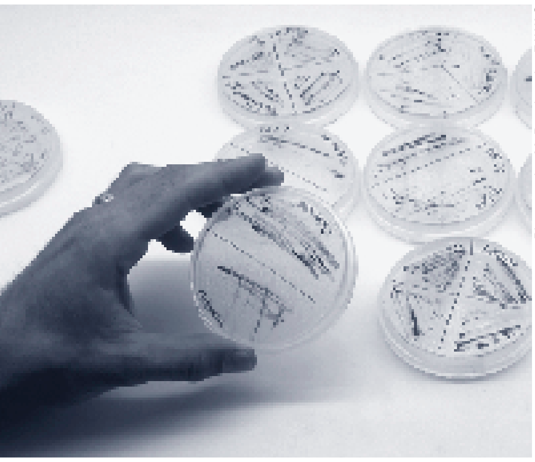
The DNA chips we are using contain a total of 2792 genes of *C. reinhardtii.* In order to differentiate between defense genes specifically responding to singlet oxygen and nonspecific genes responding to oxidative stress, the DNA chip experiments were carried out either with singlet oxygen or hydrogen peroxide (H_2O_2), another commonly occurring reactive oxygen species. A comparison of the two expression patterns indicated distinct differences. There were several genes that were activated by singlet oxygen, but not by H_2O_2 (see image of DNA chip on the front cover page; each point represents a *Chlamydomonas* gene; induced genes are red, suppressed genes are green, and genes with unaltered expression are yellow).

Specific Induction by Singlet Oxygen

The genes that were most strongly and specifically induced by the presence of singlet oxygen were examined further for their possible expression and function in other stress situations. One gene was found to be particularly interesting. The gene in question is a homologue of glutathione-peroxidase *(Gpxh)*. Related gene products in other organisms are responsible for the degradation of organic hydroperoxides [7]. Although the *Gpxh* gene is also induced by other oxygen species, expression is strongest when exposed to singlet oxygen (Tab. 1) [8], indicating that in the case of *Chlamydomonas,* singlet oxygen is detected by a specific sensor which then activates specific defense genes against it.

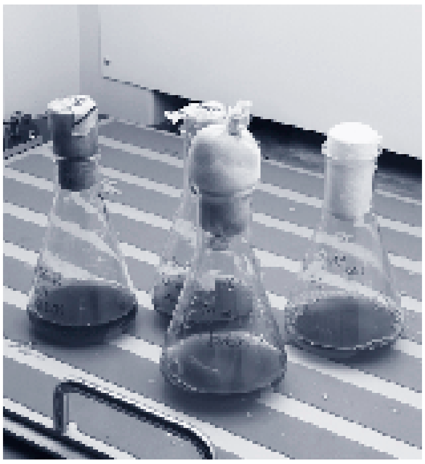
The *Gpxh* Promoter

In order to gain a better understanding of the induction mechanism of the *Gpxh* gene, we are currently examining the promoter region of the gene in more detail. A promoter region is a section of DNA in front of the actual coding sequence that controls the expression of that particular gene. It contains special regulatory elements that allow the binding of specific transcription factors which would lead to an activation of the gene. These regulatory elements have a specific base sequence that is recognized only by the associated transcription factor (Fig. 2). Through comparison with other genes, we have been able to identify such a regulatory element in the promoter region of the *Gpxh* gene. We think that this element plays an important role in the induction by singlet oxygen, since our experiments showed that after removal of the *Gpxh* regulatory element from the promoter region, the gene was no longer activated by singlet oxygen [8]. In a next step, the *Gpxh* regulatory element was introduced into the pro-

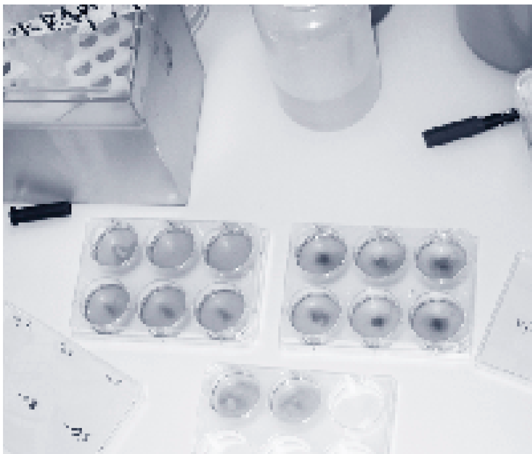


Photographs: M. Bauchrowitz, EAWAG

Growth of Chlamydomonas on solid medium in Petri dishes …



… or in liquid medium unter constant temperature and light conditions.



Induction experiments with algal cells will be carried out in multiwell cell culture plates.

moter region of a gene that is not normally induced by singlet oxygen. We used the β-tubulin gene of *Chlamydomonas,* a gene that encodes the structural protein tubulin which occurs in the flagella of a number of microalgae, including *Chlamydomonas.* Results confirmed that the transgenic β-tubulin gene becomes weakly induced by singlet oxygen after the *Gpxh* regulatory element has been inserted into the promoter region of the gene. So far, these experiments suggest that the *Gpxh* regulatory element plays a significant role in gene activation by singlet oxygen. The regulatory element found in *Chlamydomonas* has two closely related and well described homologues in mammals. Despite intensive efforts, it has not yet been correlated with one of these two mammalian regulatory elements. It is possible that the sequence was not completely conserved between mammals and algae or that the *Gpxh* regulation element in *Chlamydomonas* may be a new, as yet undescribed, regulatory element. Once the corresponding transcription factor has been isolated and characterized, it should be possible to answer these questions.

Additional research is also needed in order to determine whether or not there is a specific mechanism for the induction of *Gpxh* by singlet oxygen in *Chlamydomonas.* If this were the case, the *Gpxh* regulatory element could be used as an element in a molecular contamination sensor. Such a biosensor would allow us to detect contaminants that cause the formation of singlet oxygen in cells.

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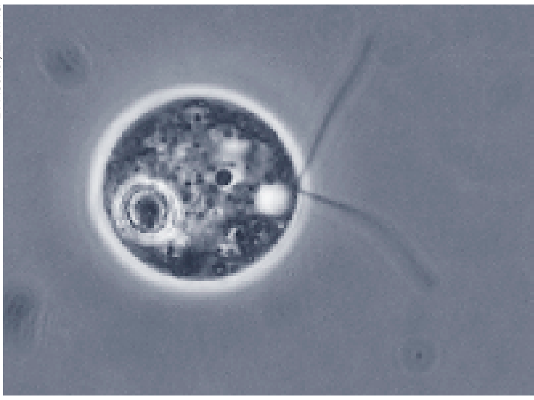

The biflagellated alga *Chlamydomonas reinhardtii* under the microscope (100-times magnified).

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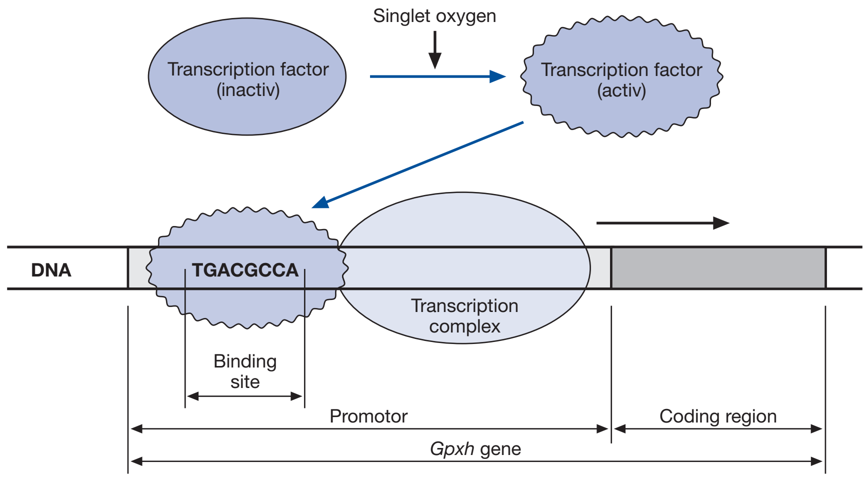

Fig. 2: Hypothetical activation path of the *Gpxh* gene by singlet oxygen. In the presence of singlet oxygen in the cell, the inactive form of the *Gpxh* transcription factor changes to the active form and binds to the *Gpxh* regulation element. Only then can the transcription complex bind to the *Gpxh* gene and read it.

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