Diss. ETH No. 15606

Photooxidative Stress Responses in the Green Alga *Chlamydomonas reinhardtii*

Dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
Doctor of Natural Sciences

Presented by
Beat Fischer
Dipl. Biol., University of Basel
Born on February 2, 1974
Citizen of Merenschwand AG

Accepted on the recommendation of
Prof. Dr. Alexander J. B. Zehnder, examiner
Dr. Rik I. L. Eggen, co-examiner
Dr. Anja Krieger-Liszkay, co-examiner
Prof. Dr. René Schwarzenbach, chairman

Zürich, 2004

Herzlichen Dank an alle, die diese Doktorarbeit ermöglicht und mich während dieser Zeit an der EAWAG unterstützt haben. Danke Rik! Er hat mir die Freiheit gegeben, alle meine mehr oder weniger genialen Ideen experimentell umzusetzen und hat mich dabei mit seiner Erfahrung und Motivation voll unterstützt. Danke Karin! Sie sorgte für die gute Atmosphäre, die mich immer gerne im Labor arbeiten liess und unterstützte mich wann immer nötig mit ihrer Hilfe. Danke Katrin, Barbara L., Urs, Nina, Barbara R.! Ihre Impulse, Ideen und Hilfe haben mir während meiner Arbeit sehr geholfen. Danke Sascha und Anja! Sie haben mich mit ihrem Fachwissen und ihrer Zusammenarbeit unterstützt und so viel zum Gelingen dieser Arbeit beigetragen. Danke MIX! Die gute Stimmung, die Hilfsbereitschaft und die Zusammenarbeit in dieser Abteilung haben mir vieles erleichtert und mir eine interessante und angenehme Zeit beschert.

Ich möchte auch Herrn Kurt Siegenthaler von der Firma Emhart Glass SA in Cham für die Finanzierung dieser Arbeit danken und hoffe, dass sie sein Interesse weckt, obwohl sie nicht genau das ursprünglich geplante Thema beschreibt.

Ganz speziell möchte ich auch meiner Frau Tina danken für die Geduld, die Unterstützung und die Liebe, die sie mir immer schenkt.

Beat Fischer

The following parts of this dissertation have been submitted for publication:

Chapter 2

Oxidative Stress Induced by the Photosensitizers Neutral Red (Type I) or Rose Bengal (Type II) in the Light Causes Different Responses in *Chlamydomonas reinhardtii* (Beat B. Fischer, Anja Liszkay-Krieger, Rik I.L. Eggen) Plant Science, accepted for publication

Chapter 3

The Photosensitizers Neutral Red (Type I) and Rose Bengal (Type II) Cause Light-dependent Toxicity in *Chlamydomonas reinhardtii* and Induce the *Gpxh* Gene via Increased Singlet Oxygen Formation (Beat B. Fischer, Anja Krieger-Liszkay, Rik I.L. Eggen) Environmental Science & Technology, accepted for publication

Chapter 4

The main part of chapter 4 has been submitted for publication in: The Glutathione Peroxidase Homologous Gene *Gpxh* in *Chlamydomonas reinhardtii* is Upregulated by Singlet Oxygen Produced in Photosystem II Upon Photoinhibition (Beat B Fischer, Rik Eggen, Achim Trebst, and Anja Krieger-Liszkay)

TABLE OF CONTENTS

ABBREVIATIONS

AP-1: Activator protein 1
APX: Ascorbate peroxidase
Ars: Arylsulfatase
ATF: Activating transcription factor
b-ZIB: Leucine zipper DNA binding domain
CBP: CREB-binding protein
CRE: cAMP responsive element
CREB: CRE-binding protein
DABCO: 1,4-Diazabicyclo[2.2.2]octane
DCMU: 1-(3,4-Dichlorophenyl)-3,3-dimethylurea
DT: Dinoterb
EST: Expressed sequence tag
GPX: Glutathione peroxidase
Gpxh: Glutathione peroxidase homologous gene
GSH: Glutathione, reduced
GSSG: Glutathione disulfide
GST: Glutathione-S-transferase
HO-1: Heme oxygenase 1 gene
H_2O_2: Hydrogen peroxide
HSP: Heat shock protein
ICAM-1: Intracellular adhesion molecule-1
LHC: Light harvesting complex
L-His: L-histidine
MAP: Mitogen-activated protein
MB: Methylene blue
NPQ: Non photochemical quenching
NR: Neutral red
$^1O_2$: Singlet oxygen
$O_2^{\cdot-}$: Superoxide radical anion
$OH^{\cdot}$: Hydroxyl radical
PAR: Photosynthetically active radiation
Pheo: Pheophytin
PHGPX: Phospholipid hydroperoxide glutathione peroxidase
PSI/PSII: Photosystem I/II
Q_A: Primary quinone
Q_B: Secondary quinone
Rbcs2: Ribulose bisphosphate carboxylase/oxygenase, gene of the small subunit
qE: ΔpH-dependent fluorescence quenching
RB: Rose bengal
ROS: Reactive oxygen species
SOD: Superoxide dismutase
UV-A: Ultraviolet A radiation
TAP: Tris-acetat-phospate
t-BOOH: *tert*-butylhydroperoxide
Tub2B: β-Tubulin gene

SUMMARY

During evolution, plants and algae have optimized the conversion of light into chemical energy: Photosynthesis results in the production of reducing power used for CO_2 fixation and in the synthesis of chemical energy in the form of ATP. Balancing the electron transport in the two photosystems is a very complex and delicate process, and photosynthetic organisms have evolved various adaptive mechanisms which allow reacting to changing environmental conditions. Nevertheless, harsh environmental conditions, such as high light intensities, can disturb the photosynthetic activity and lead to an increased production of the reactive oxygen species (ROS) superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). When the production of ROS exceeds the capacity of the cellular defense systems, cells encounter a so-called oxidative stress with subsequent damage to cellular components. One of the primary effects caused by high light illumination is the $^1O_2$-dependent degradation of the central chlorophyll binding protein D1 and subsequent dissembling of photosystem II resulting in a block of linear electron flow in photosynthesis called photoinhibition. Pollutants and herbicides, which interact with the photosynthetic activity, can also stimulate the production of ROS in the chloroplasts and thus provoke an oxidative stress under normal light conditions.

Additionally, ROS can be directly produced by exogenous chemical compounds which act as photosensitizers. These substances absorb light energy and by that enter an excited state, with subsequent uncontrolled redox reactions (type I) or the formation of $^1O_2$ (type II). Thus, high levels of photosensitizers cause a photooxidative stress in cells illuminated by light. Photosynthetic organisms have evolved efficient defense mechanisms to protect themselves from high levels of ROS and to avoid that they enter the oxidative stress state. Defense systems involve general stress responses, which may also be induced by heat shock or other types of stress conditions, as well as specific defense

systems, of which the expression is often controlled directly and specifically by ROS levels. The genetic response of an organism to a stress can thus give a lot of information about the type of stress cells encounter. In ecotoxicology this expression of defense genes is often used as an indicator for the stress condition of an organism in the environment, but for a solid interpretation of these data the mechanisms behind these responses as well as the specificity of the responses have to be known. The green unicellular alga *Chlamydomonas reinhardtii* is a widely used photosynthetic model organism for which many molecular methods are well established. *C. reinhardtii* thus is a very suitable model organism, and was used by us, to study photooxidative stress responses in photosynthetic organisms.

In this thesis we studied the genetic response of *C. reinhardtii* to photooxidative stress caused either by the presence of exogenous photosensitizers or by environmental conditions causing photoinhibition. In particular, we focused on the overall genetic responses to either type I or type II photosensitizers (chapter 2) and investigated the induction mechanism of the glutathione peroxidase homologous gene *Gpxh* in more detail. *Gpxh* turned out to be specifically induced be increased levels of $^1O_2$ (chapter 3). The *Gpxh* gene was also upregulated by $^1O_2$ produced during photosynthesis under high light illumination (chapter 4). A regulatory element, with homology to the well-known CRE/AP-1 regulatory element in various oxidative stress response genes of other organisms, was identified in the *Gpxh* promoter region and was examined in greater detail (chapter 5). This research showed that indeed the CRE/AP-1 element was essential for $^1O_2$-induced *Gpxh* expression, but that most probably additional gene regulation pathways are involved that lead to increased *Gpxh* transcript levels.

Type I and Type II Photooxidative Stress Response

The genetic response of *C. reinhardtii* to the type I photosensitizer neutral red (NR) and the type II photosensitizer rose bengal (RB) was analyzed with DNA-microarrays. Upon exposure to NR, several general and oxidative stress genes were upregulated, whereas most photosynthetic genes were downregulated by NR. Only one gene, the *Gpxh* gene, was strongly induced by RB. Analysis of the expression profiles of these NR and RB-induced genes under various oxidative stress conditions indicated the presence of a common gene regulation mechanism for most NR-induced genes responding to various oxidative stress conditions. Alternatively, a second, unrelated mechanism seems to regulate the NR-induction of the *Gpxh* which is also activated by RB, probably due to the formation of $^1O_2$. Indeed, EPR-spin trap measurements with isolated spinach thylakoids showed that NR stimulated the production of $^1O_2$ in the chloroplasts suggesting that the generation of $^1O_2$ might be the common signal for the *Gpxh* induction by NR and RB.

Induction of the *Gpxh* Gene

The toxicity in *C. reinhardtii* and the response of the *Gpxh* gene caused by NR and RB were shown to be dependent on both the concentration of the chemicals and the intensity of light illumination which is in agreement with an effect caused by photosensitizers. However, different modes of action are responsible for the toxicity of NR and RB. Addition of the $^1O_2$ quenchers DABCO (1,4-diazabicyclo[2.2.2]octane) and L-histidine to cultures of *C. reinhardtii* reduced the toxicity of the type II photosensitizer RB, showing that the lethal effect was caused by the formation of $^1O_2$, probably by modification of a component in the cell membrane. On the other hand, these quenchers could not protect the cells from the toxic effect of the type I photosensitizer NR, indicating

that other effects than $^1O_2$ production were toxic for the cell or that $^1O_2$ is generated at a cellular site where DABCO and L-histidine are absent.

The *Gpxh* expression is induced by $^1O_2$ with both photosensitizers NR and RB. This was shown using D_2O-containing growth medium, which increases the frequency of $^1O_2$-reactions with cellular components and which stimulated the induction of the *Gpxh* gene by NR and RB about two fold. Furthermore, NR caused an increased production of $^1O_2$ in isolated spinach thylakoids in a concentration and light intensity dependent manner, indicating that the *Gpxh* induction specifically responded to the production of $^1O_2$ in the chloroplast. In agreement with that, the *Gpxh* expression also increased under high light illumination, as a result of increased formation of $^1O_2$ by charge recombination during photoinhibition. This *Gpxh* response to high light intensity could be further stimulated by the phenolic herbicide dinoterb, lowering the redox potential of Q_A^- and by this increasing the frequency of charge recombination and the production of $^1O_2$. DCMU (1-(3,4-dichlorophenyl)-3,3-dimethylurea), which increases the redox potential of Q_A^- and probably reduces charge recombination and photoinhibition, also reduced the *Gpxh* induction by high light illumination. These results showed that the *Gpxh* gene is specifically upregulated by increased levels of $^1O_2$ in the thylakoids generated either by exogenous photosensitizers or during photoinhibition.

Under high light illumination, a *Gpxh*-arylsulfatase reporter gene construct showed a different expression profile compared to *Gpxh*. We therefore hypothesized a second signal beside $^1O_2$ to be involved in the response of the *Gpxh* gene to high light treatment, which does not influence the expression of the reporter gene construct. This second unknown signal seems to be produced only after 60 to 80 min of exposure to strong light, when the $^1O_2$-induction decreases again. However, this signal is speculated to be responsible for the ongoing upregulation of the *Gpxh* gene after prolonged illumination with high light intensities. This probably requires a regulation mechanism involving either

transcription factor binding site or an mRNA stabilization element which are absent in the *Gpxh*-arylsulfatase reporter gene construct.

Characterization of the *Gpxh* Promoter

A CRE/AP-1 homologous regulatory element in the promoter region of the *Gpxh* gene is required for the induction by $^1O_2$, whereas a role of a 16 bp palindrome in this response could be excluded. In addition was this 8 bp element sufficient to introduce the $^1O_2$ response in a β-tubulin promoter and the specific formation of a DNA binding complex on a *Gpxh* promoter fragment suggested that this 8 bp element functions as an active transcription factor binding site. A further differentiation between a CRE and an AP-1 element, using specific nucleotide mutations, failed, but the mutations suggested that a widespread TGAC motif, found in many homologous elements, is crucial for the function of the element. The addition of cyclic AMP and cyclic GMP analogs to the growth medium showed an inhibitory effect of the cyclic nucleotide signaling pathway on *Gpxh* expression. This is rather unusual and in contradiction to the effect of cAMP on typical CRE-dependent genes. However, this shows that different signal transduction pathways can affect the regulation of the *Gpxh* expression either positively or negatively.

ZUSAMMENFASSUNG

Im Verlauf der Evolution haben Pflanzen und Algen die Umwandlung von Licht in chemische Energie durch die Photosynthese laufend verbessert, um so die nötigen Reduktions-äquivalente und chemische Energie in Form von ATP für die CO_2 Fixierung bereitstellen zu können. Um ein optimales Funktionieren der Photosynthese zu gewährleisten, besitzen photosynthetische Organismen verschiedene Anpassungsmechanismen, die ihnen erlauben den Elektronentransport in den Photosystemen zu kontrollieren und den ständig wechselnden Umweltbedingungen anzupassen. Trotzdem können extreme Umweltbedingungen, zum Beispiel hohe Lichtintensität, die Photosyntheseaktivität stören und dadurch die Entstehung von reaktiven Sauerstoffspezies (ROS) wie Superoxid ($O_2^{\cdot-}$), Wasserstoffperoxyd (H_2O_2) und Singulett-Sauerstoff ($^1O_2$) fördern. Wenn nun die Produktion von ROS die Kapazität der zellulären Abwehrsysteme übersteigt, entsteht in den Zellen ein so genannter oxidativer Stress und eine Schädigung von verschiedenen Zellkomponenten. Einer der häufigsten Effekte von hohen Lichtintensitäten ist der $^1O_2$-abhängige Abbau des Chlorophyll bindenden D1 Proteins und der daraus folgende Zerfall des Photosystems II, ein Prozess, der als Photoinhibition bezeichnet wird. Die Entstehung von ROS in den Chloroplasten kann auch durch Schadstoffe und Herbizide in der Umwelt, welche die Photosynthese hemmen, stimuliert werden, was einen oxidativen Stress unter normalen Lichtbedingungen verursacht.

Weiter können ROS auch direkt durch phototoxische Substanzen (Photosensibilisator) produziert werden, welche durch Lichtabsorption angeregt werden und dadurch unkontrollierte Redoxreaktionen (Typ I) oder $^1O_2$-Entstehung (Typ II) bewirken können. Als Folge davon verursachen grosse Mengen dieser Substanzen in belichteten Zellen einen photooxidativen Stress,

aber Pflanzen und Algen haben effiziente Abwehrstrategien entwickelt, um sich vor ROS und oxidativem Stress zu schützen. Diese Abwehrmechanismen bestehen aus einer allgemeinen Stressantwort, die auch durch Hitzeschock und andere Stressbedingungen induziert werden kann, und einer spezifischen Abwehrreaktion, die meist direkt und spezifisch durch die Entstehung von ROS reguliert wird. Die genetische Reaktion eines Organismus auf eine Stresssituation gibt daher oft Aufschluss über den in der Zelle herrschenden Stress, was in der Ökotoxikologie als Indikator für den Stresszustand eines Organismus verwendet werden kann, vorausgesetzt, die Mechanismen und Spezifität der Reaktion sind bekannt. Aus diesem Grund verwendeten wir die Grünalge *Chlamydomonas reinhardtii*, ein oft gebrauchter photosynthetischer Modellorganismus für den viele molekulare Methoden bestens etabliert sind, um die photooxidative Stressreaktion von photosynthetischen Organismen zu studieren und besser zu verstehen.

In dieser Arbeit wurde die genetische Antwort von *C. reinhardtii* auf photooxidativen Stress untersucht, welcher entweder durch exogene phototoxische Substanzen oder durch bestimmte extreme Umweltbedingungen verursacht wurde. Speziell konzentrierten wir uns auf die gesamte genetische Reaktion der Zelle auf einen Typ I oder Typ II Photosensibilisator (Kapitel 2) und untersuchten dann genauer den Induktionsmechanismus des Glutathion-Peroxidase homologen Gens *Gpxh*. Es konnte gezeigt werden, dass die Expression des *Gpxh* Gens spezifisch durch $^1O_2$ hochreguliert wird (Kapitel 3) und auch durch $^1O_2$ aus der stark belichteten Photosynthese induziert wird (Kapitel 4). Weiter wurde eine Regulationselement in der Promoterregion des *Gpxh* Gens genauer untersucht, das Homologie zu bekannten CRE/AP-1 Elementen in oxidativen Stressgenen von anderen Organismen aufweist (Kapitel 5). Wir konnten zeigen, dass dieses CRE/AP-1 Element wirklich notwendig ist für die *Gpxh* Induktion durch $^1O_2$ und dass höchst wahrscheinlich noch andere Regulationswege die *Gpxh* Expression beeinflussen.

Typ I und Typ II Photooxidative Stressantwort

Mit Hilfe von DNA-Mikrochips wurde die genetische Reaktion von *C. reinhardtii* auf den Typ I Photosensibilisator Neutralrot (NR) und den Typ II Sensibilisator Bengalrosa (BR) analysiert. Mehrere allgemeine und oxidative Stressgene wurden stark durch NR hochreguliert, im Gegensatz zu den meisten photosynthetischen Genen, deren Expression durch NR abreguliert wurde. Nur ein einziges Gen konnte als stark BR-induziert identifiziert werden. Für die NR- und BR-induzierten Gene wurden zusätzlich Expressionsprofile bei mehreren oxidativen Stressbedingungen erstellt, die aufzeigten, dass die meisten dieser Gene durch einen gemeinsamen Mechanismus, der allgemein durch oxidativen Stress aktiviert wird, reguliert zu sein scheinen. Im Gegensatz dazu wird die NR-induzierte Expression des *Gpxh* Gens wahrscheinlich durch einen anderen, unabhängigen Mechanismus reguliert, der möglicherweise durch die Bildung von $^1O_2$ stimuliert wird. Darauf deutet auch die Hochregulierung von *Gpxh* durch BR hin. Tatsächlich konnte mit EPR-spin trap Messungen eine Stimulierung der $^1O_2$-Bildung durch NR in isolierten Spinatthylakoiden nachgewiesen werden, was die Möglichkeit einer $^1O_2$-Bildung als gemeinsames Signal für die *Gpxh* Induktion durch NR und BR unterstützt.

Induktion des *Gpxh* Gens

Die durch NR und BR verursachte Toxizität und *Gpxh* Induktion in *C. reinhardtii* waren abhängig von der Konzentration der Chemikalie und der Lichtintensität, was für von Photosensibilisatoren verursachte Effekte üblich ist. Trotzdem sind verschieden Wirkungsweisen verantwortlich für die Toxizität von NR und BR, aufgezeigt durch den unterschiedlichen Einfluss von $^1O_2$-abfangenden Substanzen wie DABCO (1,4-Diazabicyclo[2.2.2]octan) und Histidin auf das Wachstum von NR- und BR-gestressten Kulturen. Beide

Substanzen reduzierten die toxische Wirkung von BR auf die Zellen, was klar auf einen $^1O_2$-Effekt, möglicherweise auf eine Komponente der Zellmembran, hinweist. Die NR-Toxizität wurde dagegen kaum durch die $^1O_2$-abbauenden Substanzen beeinflusst und kann daher nicht auf einen $^1O_2$-Effekt zurückgeführt werden oder nur auf einen lokalen Effekt im Zellinnern, wo DABCO und Histidin nicht hingelangen.

Die *Gpxh* Induktion durch NR und BR wird hingegen von beiden Substanzen durch die Produktion von $^1O_2$ bewirkt, was durch eine Verdoppelung dieser Induktion in D_2O versetztem Medium, welches die Reaktionshäufigkeit von $^1O_2$ erhöht, gezeigt wurde. Zusätzlich konnte eine konzentrations- und lichtabhängige Produktion von $^1O_2$ durch NR in isolierten Spinatthylakoiden gemessen werden. Dies deutet auf eine Induktion von *Gpxh* durch die gezielte Bildung von $^1O_2$ in den Chloroplasten hin, was durch die erhöhte Expression von *Gpxh* bei hoher Lichtintensität, bekannt für die Bildung von $^1O_2$ durch Ladungsrekombination im Photosystem II, bestätigt wurde. Phenolische Herbizide wie Dinoterb erniedrigen das Q_A^- Redoxpotential, stimulieren dadurch die Ladungsrekombinationsfrequenz und die Bildung von $^1O_2$ während der Photosynthese und bewirkten daher auch eine zusätzliche Erhöhung der *Gpxh* Induktion durch hohe Lichtintensität. Im Gegensatz dazu reduzierte DCMU (1-(3,4-Dichlorophenyl)-3,3-dimethylurea) die Starklichtinduktion, weil DCMU das Redoxpotential von Q_A^- erhöht und damit wahrscheinlich die Häufigkeit der Ladungsrekombination und Photoinhibition verringert. Diese Resultate zeigen klar auf, dass *Gpxh* spezifisch durch die Bildung von $^1O_2$ in den Thylakoiden, entweder durch exogene Photosensibilisatoren oder während der Photo-inhibition, hochreguliert wird.

Da ein *Gpxh*-Arylsulfatase Reportergen Konstrukt ein anderes Expressionsprofil bei hoher Lichtintensität als das *Gpxh* Gen aufwies, stellten wir die Hypothese auf, dass ein zweites Signal neben $^1O_2$ in die Induktion des *Gpxh* Gens involviert sein muss, welches die Expression des Reportergens nicht beeinflusst. Dieses

zweite, unbekannte Signal scheint aber erst nach einer 60 bis 80-minütigen Belichtung mit hoher Lichtintensität zu entstehen, wenn die Produktion von $^1O_2$ wieder absinkt, und wäre dann für die andauernde Induktion von *Gpxh* nach über 80 Minuten Exponierung verantwortlich. Dazu wäre eine zusätzliche Transkriptionsfaktorbindungsstelle oder ein mRNA stabilisierendes Element im *Gpxh* Gen erforderlich, welche(s) dann verständlicherweise im *Gpxh*-Arylsulfatase Reporterkonstrukt fehlen sollte.

Charakterisierung des *Gpxh* Promotors

Wir konnten zeigen, dass ein CRE/AP-1-homologes Regulationselement in der *Gpxh* Promoterregion absolut notwendig für die Induktion durch $^1O_2$ ist, im Gegensatz zu einem 16 bp Palindrom, das nicht in diesen Prozess involviert ist. Dieses 8 bp Element reichte sogar aus, einen $^1O_2$-regulierbaren β-Tubulin-promotor zu konstruieren, und die spezifische Bildung eines DNA bindenden Komplexes an einem *Gpxh* Promoterfragment deutete klar auf eine Funktion dieses Elements als aktive Transkriptionsfaktorbindungsstelle hin. Leider konnte mit spezifischen Punktmutationen keine genauere Unterscheidung zwischen einem CRE und AP-1 Element erreicht werden, aber diese Mutationen zeigten auf, dass ein weit verbreitetes TGAC-Motiv, das in vielen homologen Elementen gefunden wird, wichtig für die Funktion der Bindungsstelle ist. Des Weiteren konnte mit Hilfe von zyklischen AMP- und zyklischen GMP-analogen Substanzen eine hemmende Wirkung des zyklischen Nukleotid-Signalweges auf die *Gpxh* Expression gemessen werden, was eher selten ist und nicht mit dem bekannten Effekt von zyklischem AMP auf CRE-abhängige Gene vereinbar ist. Doch dieses Beispiel zeigt, dass verschiedene Signalübertragungswege die *Gpxh* Expression entweder positiv oder negativ beeinflussen können.

Seite Leer /
Blank leaf

1. General Introduction

The Origin of Reactive Oxygen Species

When the first photosynthetic organisms, the cyanobacteria, started to produce molecular oxygen (O_2) about 2.5 billion years ago, a powerful alternative to produce energy emerged for living organisms in the previously reduced or neutral Earth atmosphere. Due to the strong oxidizing power of oxygen it became the major terminal electron acceptor in all aerobic organisms including animals, plants and many microorganisms. These organisms use oxygen to produce a proton gradient across the membrane by the respiration chain, which then gives rise to energy equivalents in the form of ATP. Molecular oxygen ($^3O_2$) is not very reactive due to its electron configuration: it has two electrons with parallel spins in the different π^* antibonding orbitals (Fig. 1) which reduces its reaction kinetics due to the spin restriction. With other molecules, it mainly forms covalent bonds with antiparallel spins of the bonding electron pair [13, 15]. Molecular oxygen can be activated by stepwise one electron reductions to superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) [13, 15, 35]. Additionally, excitation of $^3O_2$ by photodynamic processes or

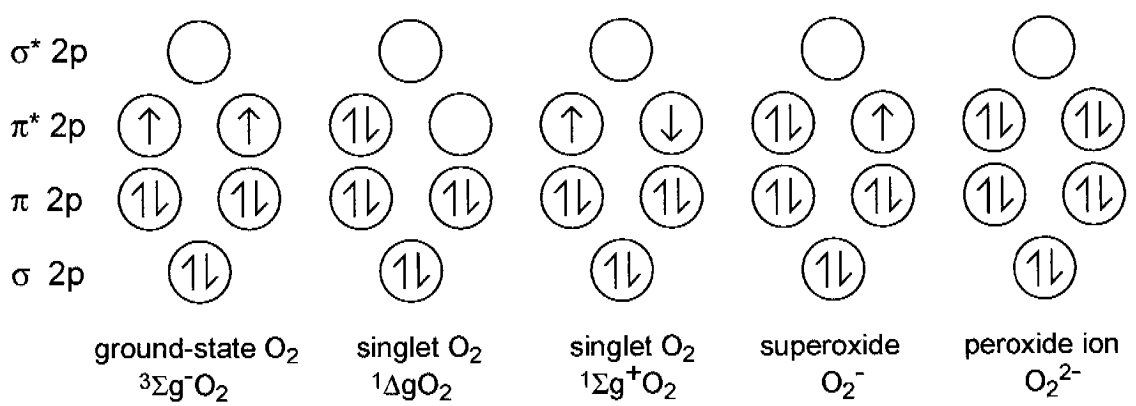


Figure 1: electron configuration of different oxygen derivatives according to Halliwell and Gutteridge [15].

during chemical reactions results in the generation of singlet oxygen ($^1O_2$), containing two electrons with antiparallel spins either in the same ($^1\Delta gO_2$) or in the different ($^1\Sigma g^+O_2$) π^* antibonding orbitals (Fig. 1) [15, 33, 37]. For both, the reduced and the excited forms of oxygen, the spin restriction is removed making these oxygen derivatives very reactive to biomolecules.

In aerobic organisms the main sources for such reactive oxygen species (ROS) are the electron transport chains of the mitochondria, the chloroplasts and the endoplasmatic reticulum [15]. The electron leakage in mitochondria occurs predominantly via the NADH-ubiquinone reductase complex [47] and via the reduced form of ubiquinone itself [48]. In the endoplasmatic reticulum, electron transfer from the cytochrome P_{450}, involved in the hydroxylation of xenobiotics and fatty acids, and the flavoprotein enzyme NADPH-cytochrome-P_{450} reductase to O_2 may result in the formation of $O_2^{\cdot-}$ [13, 55]. H_2O_2 is produced in the cell by the enzymatic disproportionation of $O_2^{\cdot-}$, but it can also evolve directly by the action of some flavin-containing oxidases, like the fatty acyl-CoA oxidase, required for peroxisomal β-oxidation, or the glycollate oxidase, involved in photorespiration in plants [7, 28, 52]. Most of the damages caused by ROS occurs upon conversion of $O_2^{\cdot-}$ and H_2O_2 to $OH^{\cdot}$ radicals in the Fenton reaction [13, 55]. $OH^{\cdot}$ radicals are highly reactive with typical second order rate constants between 10^9-10^{10} $M^{-1}s^{-1}$ with organic substrates [15].

The formation of the excited oxygen form $^1O_2$ occurs by chemi-excitation, for instance by the reaction of H_2O_2 with hypochlorite and peroxinitrite, or reactions catalysed by several peroxidases (myeloperoxidase) and oxygenases (lipoxygenase) [6, 32]. Alternatively, $^1O_2$ can be produced by photosensitized reactions via the absorption of visible or ultraviolet light by cellular photosensitizers, e.g. porphyrins, cytochromes or flavins, and the subsequent transfer of the excitation energy to $^3O_2$ [6, 37]. In addition can the formation of ROS during normal cellular metabolism be further stimulated by harsh

environmental conditions, such as the exposure to pollutants, extreme temperatures, strong illumination and other stress conditions.

ROS can react with proteins, lipids and DNA, depending on their site of generation. Lipid modification to lipid hydroperoxides can cause membrane disturbance and concomitant loss of membrane dependent functions such as respiration and photosynthetic activity, or barrier functions [14, 25]. Many intracellular proteins are very sensitive to ROS because they are only active in the reducing environment of the cytosol and often require the reduced form of sensitive amino acids for the proper folding or function [15]. Thus, the oxidation of specific amino acids can disturb the activity of various enzymes. Modification of the DNA occurs via the production of $OH^{\cdot}$ in the Fenton reaction and result in DNA strand breaks and mutations [55]. $^1O_2$ has also been shown to react with certain nucleotides of the DNA and there is strong evidence that the genotoxicity of ultraviolet A radiation is mediated by the formation of $^1O_2$ in the cell [49]. High intracellular levels of ROS can thus damage essential components of the cell, inhibiting their function and result in an oxidative stress or even cell death.

To minimize negative effects of ROS, aerobic organisms have evolved various defense mechanisms, aimed at preventing the production of ROS, removing ROS or repairing damaged molecules. To prevent the formation of ROS in the cell, transition metals, catalyzing the Fenton reaction, are tightly bound in complexes [13, 15]. Another preventive mechanism involves the synthesis of absorbing pigments which inhibit the transmission of light into the cell and thus prevent light induced oxidative damage. Inside the cell, enzymatic and nonenzymatic defense mechanisms remove ROS by catalyzing their decomposition or by directly scavenging the reactive molecule. Most prominent cellular scavengers are vitamin C and E and carotenoids [36]. Efficient enzymes involved in the removal of ROS are several superoxide dismutases (SOD), catalases and different types of peroxidases. Three different types of SOD are

known at present which catalyze the reaction of $O_2^{\cdot-}$ to H_2O_2 and O_2 [13]. The CuZnSOD is found mainly in the cytosol of eukaryotes and few prokaryotes whereas the MnSOD and the FeSOD are present predominately in prokaryotes or some organelles of the eukaryotic cells [13]. Catalases, splitting two H_2O_2 molecules into one molecule of oxygen and two molecules of water, are most abundant in the peroxisomes, where high levels of H_2O_2 are produced by oxidases [28]. In other compartment, H_2O_2 is reduced by various peroxidases using different cofactors as electron donor including reduced gluathione (GSH), ascorbate or thioredoxin [13, 15].

The glutathione peroxidases (GPX) catalyze the reduction of H_2O_2 or organic hydroperoxides to water or alcohols by reduced GSH. One of the first GPX characterized was the cytosolic GPX1 from mammalians, a homotetrameric enzyme with a selenocysteine residue in the active site [50]. However, many GPX homologous enzyme found in plants and microorganisms do not contain the selenocysteine but rather a normal cysteine in the active site, strongly reducing the peroxidase activity [12, 16, 27, 54]. Some of these nonselenocysteine GPXs belong to the family of phospholipid hydroperoxide glutathione peroxidases (PHGPX) [1]. They are monomeric and more active against organic hydroperoxides than H_2O_2. Some can use different cofactors than glutathione as reducing power, such as thioredoxin or NADPH [8, 12, 40, 55]. They were shown to be involved in the removal of lipid hydroperoxides in the membranes of oxidative damaged cells [53]. Thus, these enzymes may be specifically involved in the defense against oxidative stress in cellular membranes, especially the mitochondrial and the chloroplast membranes, where most uncontrolled ROS formation takes place in the cell.

The protection against $^1O_2$-caused damages mainly involves the function of highly efficient quenchers like α-tocopherol and carotenoids, but little is known about enzymatic defense systems [4, 45]. Due to the fast reaction of $^1O_2$ with

biomolecules, enzymatic removal of $^1O_2$ may not be required but rather the decomposition of $^1O_2$ modified components, like proteins and lipids. Thus, enzymes like the peroxidases, removing organic hydroperoxides, might also be important in the defense against $^1O_2$-caused oxidative stress [24, 53].

Oxidative Stress in Photosynthetic Organisms

In photosynthetic organisms the major sites for ROS production are the chloroplasts. There, the splitting of water, the transport of electrons in the photosystems and the high local concentration of oxygen increase the chance for partial reduction of oxygen and the formation of $O_2^{\cdot-}$ and H_2O_2 [7, 29]. Often, the rate of CO_2 fixation in the Calvin Cycle limits the reduction of $NADP^+$ by the photosystem I (PSI) and alternative electron acceptors are used, including oxygen. Several components of the PSI including the Fe-S centers and the ferredoxin are autooxidized under condition of limited $NADP^+$ and $O_2^{\cdot-}$ is formed during a process called the Mehler reaction [7, 29]. Another source of ROS in the chloroplasts is the oxygen evolving complex, where H_2O is split and successively oxidized to oxygen by a Mn-complex [5, 9].

Photosynthesis in the thylakoid membranes is the main source for the formation of $^1O_2$ in plants and algae [23]. The presence of high concentrations of endogenous photosensitizers, mainly chlorophyll a and b, is required to efficiently drive the photosynthetic electron transport, but also increases the probability of uncontrolled energy transfer from the exited photosensitizer to $^3O_2$ leading to the formation of $^1O_2$. Under normal light conditions the excited chlorophylls of the antenna transfer its excitation energy to the reactive center P_{680} in the PSII, followed by an electron transfer from the excited P_{680}* to the primary electron acceptor pheophytin (Pheo) in the charge separation reaction. The oxidized P_{680}^+ is subsequently neutralized again by an electron from the oxygen evolving complex via reduction of the redox active tyrosine TyrZ of the

PSII. The electron from the reduced $Pheo^-$ is passed further through the electron transport chain via the primary quinone Q_A to the secondary quinone Q_B which is, once it has bound two electrons, exchanged by an oxidized quinone from the plastoquinone pool (Fig. 2).

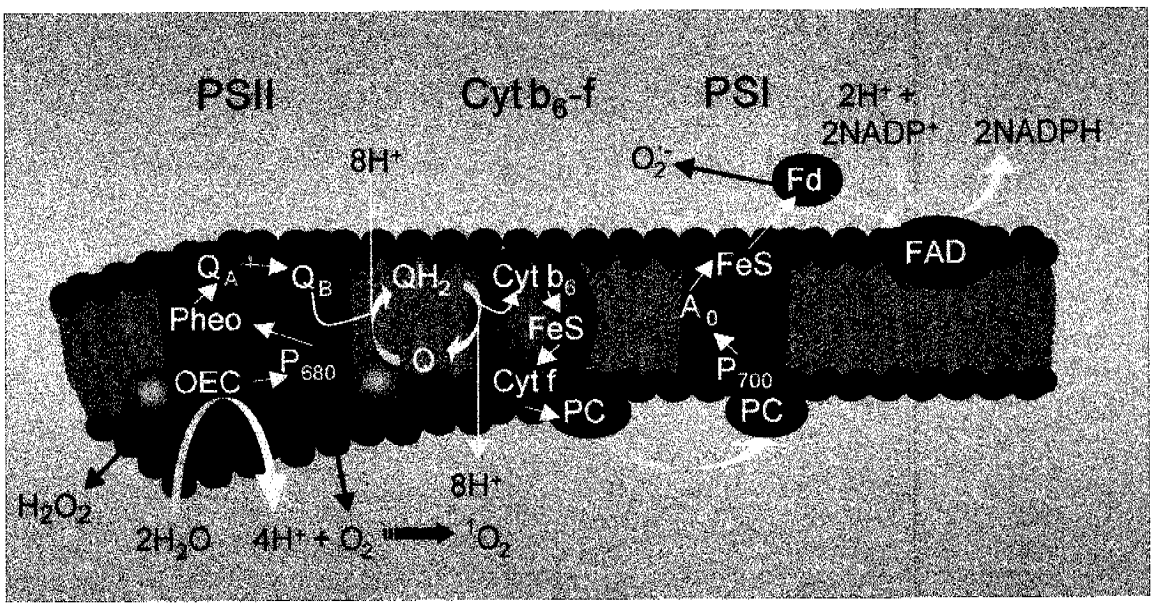


Figure 2: schematic drawing of the electron transport chain and the source of ROS during photosynthesis. PSI, photosystem I; PSII, photosystem II; Cyt b_6-f, cytochrome b_6-f complex; P_{680}, reactive center of PSII; OEC, oxygen evolving complex; Pheo, pheophytin; Q_A, primary quinone; Q_B, secondary quinone; QH_2/Q, plastoquinone pool; Cyt b_6, cytochrome b_6; FeS, iron-sulfur cluster; Cyt f, cytochrome f; PC, plastocyanine; P_{700}, reactive center of PSI; A_0 primary acceptor of PSI; Fd, ferredoxin, FAD, ferredoxin-$NADP^+$ oxidoreductase

Under conditions, where the absorption of light and the charge separation exceed the capacity of the electron transport chain, the electron flow in the PSII is blocked, resulting in a stabilized Q_A^- (closed state of reaction center) [23]. Electron transfer from $Pheo^-$ to Q_A^- is inhibited and charge recombination between the $Pheo^-$ and the P_{680}^+ occurs which can result in the formation of the excited triplet state of P_{680} ($^3P_{680}$) [51]. The $^3P_{680}$ state has a relatively long lifetime and can react with $^3O_2$ to form $^1O_2$ [17, 34, 42]. Efficient quenchers of the chlorophyll triplet state or $^1O_2$ such as carotenoids are in close proximity to

the reaction center in the PSII to minimize the production of $^1O_2$ [4, 41]. Thus, $^1O_2$ generation in the chloroplast is usually low. However, under harsh environmental conditions like high light illumination, drought and cold stress or the presence of certain PSII-inhibiting herbicides, the electron transport is blocked and charge recombination is enhanced [7, 34]. This results in the increased formation of $^1O_2$ and in a concomitant photooxidative stress [11, 17, 18]. In the PSII, $^1O_2$ can react with the D1 protein and by that initiates its proteolytic degradation [3, 23]. As a consequence, the PSII complex disassembles and the photosynthetic activity is lost, referred to as photoinhibition. Rapid *de novo* synthesis of the D1 protein and reassembling of the PSII is required to restore a functional photosynthetic apparatus, a limiting process when photoinhibition occurs. Additionally, $^1O_2$ can modify lipids by lipid peroxidation and thus disturb the membrane integrity [15, 39]. In the chloroplast this may influence the structure and function of the photosynthetic apparatus.

Photosynthetic organisms have evolved specific defense mechanisms to prevent photoinhibition. The distribution of the light harvesting complex (LHC) between the PSI and PSII can be regulated by state transition to optimize the electron flow in the electron transport chain and to reduce the absorbed light intensity by the PSII [23]. In addition, several non photochemical quenching (NPQ) mechanisms prevent the formation of $^1O_2$. This includes the synthesis of carotenoids such as zeaxanthin, violaxanthin and lutein in the xanthophyll-cycle which quench the singlet or triplet excited state of chlorophylls and $^1O_2$, dissipating the energy as heat [4]. The synthesis of the xanthophylls is dependent on a strong pH gradient across the thylakoid membrane and is thus termed ΔpH-dependent quenching (qE), including also xanthophyll independent mechanisms [30]. Another efficient scavenger of $^1O_2$ is α-tocopherol, which is

abundant in the thylakoid membrane and also protects the photosynthetic apparatus from photoinhibition [44, 45].

All these quenching mechanisms cannot prevent the formation of ROS in the chloroplasts during environmental stress. Thus, when the level of ROS raises in cells exposed to harsh environmental conditions, a cellular defense system is induced, including specific oxidative stress response genes to remove ROS. The combination of genes which are induced is very much dependent on the nature and location of the oxidative stress and involves complex regulation mechanisms.

Regulation of the Oxidative Stress Response

Optimal protection against oxidative stress requires the temporally and spatially controlled expression of defense genes. Whereas genes involved in general stress response, such as many heat shock proteins and proteases, are expressed during different type of stresses, many specific defense genes, involved in the removal of ROS, are only needed when the level of ROS rise. Different mechanisms have been identified to control the expression of stress genes due to the increased ROS formation. One of them involves the direct induction of the gene expression by the ROS formed. In many organisms, including microorganism, animals and plants the expression of SODs is increased by elevated oxygen concentrations and the presence of paraquat, leading to the increased formation of $O_2^{\cdot-}$ [55]. In *E. coli,* the redox sensitive transcription factor SoxR is directly modified by $O_2^{\cdot-}$ resulting in a transcriptional activation of the target genes, including SOD, together with SoxS [38]. Redox sensitive transcription factor are also found in eukaryotes, including the activator protein-1 (AP-1) or the H_2O_2-specific NFκB in mammalians and the yeast AP-1 homologous YAP-1, known to regulate the expression of several oxidative stress response genes [20, 38, 43]. Interestingly, the H_2O_2 dependent activation of

YAP-1 was shown not to be a direct oxidation by H_2O_2 but to be mediated by the glutathione peroxidase-like gene Gpx3 [8].

In plants, the expression of oxidative stress response genes is often upregulated by several stress conditions, because various abiotic stresses cause an increased cellular ROS production. Thus, catalases and SODs are induced by ROS and by exposure to high light illumination, known to increase $O_2^{\cdot-}$ levels inside the chloroplasts [7, 46]. In addition are SODs and other oxidative stress response genes also induced by drought, salt stress, high or low temperature or the presence of heavy metals [7]. But in *Arabidopsis* exposed to high light intensities the induction of the APX1 and APX2 genes could directly be linked to the increased formation of ROS, even though an additional signal from the active photosynthesis was required for induction [22]. A possible effect of the redox state of the plastoquinone pool on the APX expression was observed [21, 22]. A role of the plastoquinone pool in the signaling of light intensity dependent gene expression was also established for other photosynthetic genes [31]. Additionally, the ratio of oxidized to reduced glutathione (GSSG/GSH) in the cell, which may be directly linked to the production of ROS, was suggested to play a key role in the gene expression during excess light stress [2, 19, 21]. These examples show the complexity of the regulation of the oxidative stress response, required for an optimal protection of photosynthetic organisms.

In contrast to $O_2^{\cdot-}$ and H_2O_2 induced stress response, little is known about the genetic response to increased levels of $^1O_2$, even though the production of this ROS is a well known effect of high light exposure in photosynthetic organisms [11, 17, 18]. Increased formation of $^1O_2$ in plants may be mainly caused by photosensitation processes involving either the cellular accumulation of an exogenous or endogenous photosensitizer or a disturbance of the photosynthetic electron flux causing photoinhibition. Recently, Leisinger et al. (2001) have identified a glutathione peroxidase homologous gene (*Gpxh*) in the green alga

Chlamydomonas reinhardtii, which was shown to be strongly upregulated by exposure to the exogenous photosensitizers neutral red (NR), rose bengal (RB) and methylene blue (MB) under illumination [26]. This response was hypothesized to be caused by $^1O_2$, even though only RB and MB produce $^1O_2$ in the presence of light (type II photosensitizer), whereas NR is rather a type I photosensitizer, reacting with its substrate in a direct electron transfer reaction [10]. However, since the expression of the *Gpxh* gene is induced by other ROS to a much lower extent, the response of this gene seems to be highly specific for photosensitized processes. This makes the *Gpxh* expression a very interesting system to study photooxidative stress in photosynthetic organisms, in particular $^1O_2$-induced stress, and the *Gpxh* gene a possible candidate to measure the effect of environmental pollutants on photosynthesis. But before such a practical application can be considered, several questions have to be answered concerning the *Gpxh* induction:

Which is the common signal of the photosensitizer-induced response of *Gpxh*?

How is the *Gpxh* expression regulated and which signaling mechanisms are involved?

Is the *Gpxh* expression also induced by environmental conditions causing a photooxidative stress?

In this thesis we investigated the molecular mechanisms triggering the *Gpxh* response to unravel the signal and components of the signaling pathway responsible of *Gpxh* induction. First the total genetic response of *C. reinhardtii* upon exposure to RB was measured and compared to the response caused by NR in the light to find additional genes induced by the two photosensitizers and to reveal similarities and differences in the response to type I and type II sensitizers (chapter 2). Surprisingly, the *Gpxh* was the only gene induced by both photosensitizers to similar degree indicating that a common signal of the two stress conditions cause the *Gpxh* upregulation. An increased formation of $^1O_2$ in

isolated thylakoids exposed to NR could be detected by EPR spin trap measurements and indeed, the induction of the *Gpxh* expression by the two photosensitizers could be directly linked to the formation of $^1O_2$ in the chloroplasts, whereas the induction of other genes by NR was probably caused by a type I oxidative stress (chapter 3). In addition, we could show an upregulation of the *Gpxh* gene by environmental stress conditions causing increased $^1O_2$ production, such as high light illumination and the presence of phenolic herbicides (chapter 4). We have also strong evidence that additional signals caused by high light exposure enhance the *Gpxh* expression after prolonged illumination. A single transcription factor binding site containing a widespread TGAC motif, identified in the promoter region of the *Gpxh* gene, was shown to be required and sufficient for gene induction by $^1O_2$ (chapter 5). Thus, in this study we could show for the first time the specific upregulation of a nuclear gene, the *Gpxh* gene, by the production of $^1O_2$ in the chloroplast involving a common cis element in the promoter.

REFERENCES

1. Avery AM, Avery SV: Saccharomyces cerevisiae expresses three phospholipid hydroperoxide glutathione peroxidases. J Biol Chem 276: 33730-5 (2001).
2. Baena-Gonzalez E, Baginsky S, Mulo P, Summer H, Aro EM, Link G: Chloroplast transcription at different light intensities. Glutathione-mediated phosphorylation of the major RNA polymerase involved in redox-regulated organellar gene expression. Plant Physiol 127: 1044-52 (2001).
3. Barber J, Andersson B: Too much of a good thing: light can be bad for photosynthesis. Trends Biochem Sci 17: 61-6 (1992).
4. Baroli I, Niyogi KK: Molecular genetics of xanthophyll-dependent photoprotection in green algae and plants. Philos Trans R Soc Lond B Biol Sci 355: 1385-94 (2000).
5. Bradley RL, Long KM, Frasch WD: The involvement of photosystem II-generated H2O2 in photoinhibition. FEBS Lett 286: 209-13 (1991).
6. Briviba K, Klotz L-O, Sies H: Toxic and signalling effects of photochemically or chemically generated singlet oxygen in biological systems. Biol Chem 378: 1259-1265 (1997).
7. Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F: Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci 57: 779-95 (2000).
8. Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB: A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell 111: 471-81 (2002).
9. Fine PL, Frasch WD: The oxygen-evolving complex requires chloride to prevent hydrogen peroxide formation. Biochemistry 31: 12204-10 (1992).
10. Foote CS: Definition of Type-I and Type-II Photosensitized Oxidation. Photochemistry and Photobiology 54: 659-659 (1991).
11. Fufezan C, Rutherford AW, Krieger-Liszkay A: Singlet oxygen production in herbicide-treated photosystem II. FEBS Lett 532: 407-10 (2002).
12. Gaber A, Tamoi M, Takeda T, Nakano Y, Shigeoka S: NADPH-dependent glutathione peroxidase-like proteins (Gpx-1, Gpx-2) reduce unsaturated fatty acid hydroperoxides in Synechocystis PCC 6803. FEBS Lett 499: 32-6 (2001).
13. Gille G, Sigler K: Oxidative stress and living cells. Folia Microbiol 40: 131-52 (1995).
14. Girotti AW: Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B 63: 103-13 (2001).
15. Halliwell B, Gutteridge JMC: Free radicals in biology and medicine. Clarendon Press ; Oxford University Press, Oxford New York (1999).

16. Hazebrouck S, Camoin L, Faltin Z, Strosberg AD, Eshdat Y: Substituting selenocysteine for catalytic cysteine 41 enhances enzymatic activity of plant phospholipid hydroperoxide glutathione peroxidase expressed in Escherichia coli. J Biol Chem 275: 28715-21 (2000).

17. Hideg E, Kalai T, Hideg K, Vass I: Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry 37: 11405-11 (1998).

18. Hideg E, Kalai T, Hideg K, Vass I: Do oxidative stress conditions impairing photosynthesis in the light manifest as photoinhibition? Philos Trans R Soc Lond B Biol Sci 355: 1511-6 (2000).

19. Irihimovitch V, Shapira M: Glutathione redox potential modulated by reactive oxygen species regulates translation of Rubisco large subunit in the chloroplast. J Biol Chem 275: 16289-95 (2000).

20. Karin M, Takahashi T, Kapahi P, Delhase M, Chen Y, Makris C, Rothwarf D, Baud V, Natoli G, Guido F, Li N: Oxidative stress and gene expression: the AP-1 and NF-kappaB connections. Biofactors 15: 87-9 (2001).

21. Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM: Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell 9: 627-40 (1997).

22. Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux P: Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science 284: 654-7 (1999).

23. Keren N, Ohad I: State transition and photoinhibition. In: Rochaix J-D, Michel G-C, Sabeeha M (eds) The molecular biology of chloroplasts and mitochondria in chlamydomonas, pp. 569-596. Kluwer academic publishers, Dordrecht (1998).

24. Kim SY, Kim EJ, Park JW: Yeast thioredoxin peroxidase expression enhances the resistance of Escherichia coli to oxidative stress induced by singlet oxygen. Redox Rep 7: 79-84 (2002).

25. Kowaltowski AJ, Vercesi AE: Mitochondrial damage induced by conditions of oxidative stress. Free Radic Biol Med 26: 463-71 (1999).

26. Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL: The glutathione peroxidase homologous gene from Chlamydomonas reinhardtii is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology 46: 395-408 (2001).

27. Li WJ, Feng H, Fan JH, Zhang RQ, Zhao NM, Liu JY: Molecular cloning and expression of a phospholipid hydroperoxide glutathione peroxidase homolog in Oryza sativa. Biochim Biophys Acta 1493: 225-30 (2000).

28. Mittler R: Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405-10 (2002).

29. Mullineaux P, Ball L, Escobar C, Karpinska B, Creissen G, Karpinski S: Are diverse signalling pathways integrated in the regulation of arabidopsis antioxidant defence gene expression in response to excess excitation energy? Philos Trans R Soc Lond B Biol Sci 355: 1531-40 (2000).

30. Niyogi KK: Safety valves for photosynthesis. Curr Opin Plant Biol 3: 455-60 (2000).

31. Pfannschmidt T: Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci 8: 33-41 (2003).

32. Pierlot C, Aubry JM, Briviba K, Sies H, Di Mascio P: Naphthalene endoperoxides as generators of singlet oxygen in biological media. Methods Enzymol 319: 3-20 (2000).

33. Punjabi PB, Kabra BV, Pitliya RL, Vaidya VK, Ameta SC: The chemistry of singlet molecular oxygen. Journal of the Indian Chemical Society 78: 175-184 (2001).

34. Rutherford AW, Krieger-Liszkay A: Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 26: 648-53 (2001).

35. Scandalios JG: Molecular biology of free radical scavenging systems. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1992).

36. Scandalios JG: The rise of ROS. Trends Biochem Sci 27: 483-6 (2002).

37. Smith KC: The Science of photobiology. Plenum Press, New York (1981).

38. Storz G, Polla BS: Transcriptional regulators of oxidative stress-inducible genes in prokaryotes and eukaryotes. Exs 77: 239-54 (1996).

39. Stratton SP, Liebler DC: Determination of singlet oxygen-specific versus radical-mediated lipid peroxidation in photosensitized oxidation of lipid bilayers: effect of beta-carotene and alpha-tocopherol. Biochemistry 36: 12911-20 (1997).

40. Takeda T, Miyao K, Tamoi M, Kanaboshi H, Miyasaka H, Shigeoka S: Molecular characterization of glutathione peroxidase-like protein in halotolerant Chlamydomonas sp. W80. Physiol Plant 117: 467-475 (2003).

41. Telfer A: What is beta-carotene doing in the photosystem II reaction centre? Philos Trans R Soc Lond B Biol Sci 357: 1431-39; discussion 1439-40, 1469-70 (2002).

42. Telfer A, Bishop SM, Phillips D, Barber J: Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. J Biol Chem 269: 13244-13253 (1994).

43. Toone WM, Morgan BA, Jones N: Redox control of AP-1-like factors in yeast and beyond. Oncogene 20: 2336-46 (2001).

44. Trebst A: Function of beta-carotene and tocopherol in photosystem II. Z Naturforsch [C] 58: 609-20 (2003).

45. Trebst A, Depka B, Hollander-Czytko H: A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in Chlamydomonas reinhardtii. FEBS Lett 516: 156-60 (2002).

46. Tsang EW, Bowler C, Herouart D, Van Camp W, Villarroel R, Genetello C, Van Montagu M, Inze D: Differential regulation of superoxide dismutases in plants exposed to environmental stress. Plant Cell 3: 783-92 (1991).

47. Turrens JF, Boveris A: Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 191: 421-7 (1980).

48. Turrens JF, Freeman BA, Crapo JD: Hyperoxia increases H2O2 release by lung mitochondria and microsomes. Arch Biochem Biophys 217: 411-21 (1982).

49. Tyrrell RM: Role for singlet oxygen in biological effects of ultraviolet A radiation. Methods Enzymol 319: 290-6 (2000).

50. Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D, Flohe L: Diversity of glutathione peroxidases. Methods Enzymol 252: 38-53 (1995).

51. Vass I, Styring S, Hundal T, Koivuniemi A, Aro E, Andersson B: Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced QA species promote chlorophyll triplet formation. Proc Natl Acad Sci U S A 89: 1408-12 (1992).

52. Vranova E, Inze D, Van Breusegem F: Signal transduction during oxidative stress. J Exp Bot 53: 1227-36 (2002).

53. Wang HP, Qian SY, Schafer FQ, Domann FE, Oberley LW, Buettner GR: Phospholipid hydroperoxide glutathione peroxidase protects against singlet oxygen-induced cell damage of photodynamic therapy. Free Radic Biol Med 30: 825-35 (2001).

54. Yamada K, Nakagawa CW, Mutoh N: Schizosaccharomyces pombe homologue of glutathione peroxidase, which does not contain selenocysteine, is induced by several stresses and works as an antioxidant. Yeast 15: 1125-32 (1999).

55. Yu BP: Cellular Defenses Against Damage From Reactive Oxygen Species. Physiological Reviews 74: 139-162 (1994).

Seite Leer /
Blank leaf

2. Oxidative Stress Induced by the Photosensitizers Neutral Red (Type I) or Rose Bengal (Type II) in the Light Causes Different Genetic Responses in *Chlamydomonas reinhardtii*

ABSTRACT

The molecular defense mechanisms against photooxidative stress in photosynthetic organisms are essential to protect cells from damaging effects of high light illumination and photoinhibition but also to protect against effects by endogenous and exogenous photosensitizers. Here we analyzed the genetic response of *Chlamydomonas reinhardtii* to the model type I photosensitizer neutral red (NR) and the type II photosensitizer rose bengal (RB) using DNA-microarrays. Many oxidative and general stress response genes, which were also induced by other oxidative stress conditions, were strongly induced by NR. Only one gene was upregulated by RB, the glutathione peroxidase homologous gene *Gpxh*, which was also induced by NR. In addition NR exposure resulted in the reduced expression of most nuclear photosynthetic genes and subunits of the light harvesting complex indicating an inhibition of photosynthetic activity. This is supported by a stimulation of singlet oxygen generation in NR-treated thylakoids. Thus, in *C. reinhardtii* the *Gpxh* expression is most probably induced by the formation of singlet oxygen in both the NR and RB-treated cells via the activation of a very sensitive and specific sensor, whereas general oxidative stress response mechanisms seem to be involved in the response of most other genes to the type I photooxidative stress.

INTRODUCTION

In photosynthetic organism, the increased formation of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$) is a major source for oxidative stress and cellular damages. Especially in the thylakoid membranes, where the photosynthetic light harvesting complex (LHC) absorbs light energy and drives the electron transport chain in the photosystem I and II (PSI and PSII), increased amounts of ROS are generated by uncontrolled electron transfer reactions [11]. High light intensities enhance the rate of charge recombination and triplet chlorophyll formation in the PSII, resulting in the increased production of $^1O_2$ and photoinhibition [2, 29, 33, 52]. Thus, the strict regulation of the excitation of the photosystems or other endogenous photosensitizers, such as porphyrins, and the fast induction of defense mechanisms against oxidative stress is crucial for photosynthetic organisms. Several mechanisms have been described to be involved in the regulation of the responses due to light induced stresses. Some ROS such as O_2^- and H_2O_2 have been shown to directly act as second messenger to regulate the expression of defense genes, including glutathione peroxidases, glutathione-S-transferases and ascorbate peroxidases [11]. For other responses the redox status of the glutathione (GSH) or the plastoquinone pool was identified to trigger the response, including the control of gene expression [25, 32, 50]. However, unraveling the specific mechanism responsible for the induction of a gene is in many cases difficult because some of the potential signals, e.g. ROS production and redox status of the plastoquinone pool, are tightly linked during photosynthesis.

By using exogenous photosensitizers the problem of such linked signals may be partially eliminated. Upon absorption of visible light and entering an excited state, such photosensitizers induce a photooxidative stress independent of the

photosynthetic activity. In the presence of oxygen, excited photosensitizers can react in two ways: direct electron transfer to the substrate usually results in a semi-reduced form of the photosensitizer and a semi-oxidized form of the substrate, which becomes fully oxidized upon binding molecular oxygen (type I) [16, 62]. Neutral red (NR), a phenazine-based dye widely used for staining cellular particles and as an intracellular pH indicator, has been used as a photosensitizer in phototherapy and was reported to interact with several substrates in a type I reaction [43, 60, 61]. The second reaction type involves the transfer of the excitation energy from the photosensitizer to molecular oxygen, resulting in the formation of $^1O_2$ (type II) [16]. Rose bengal (RB) and methylene blue are two typical type II photosensitizers, known to mainly generate $^1O_2$ when excited by light in the visible range [38, 40, 68]. Thus, exposure of organisms to RB in the light may specifically induce the genetic response to increased generation of $^1O_2$, whereas NR rather activates the response to free radicals-induced oxidative stress.

Recently, we have shown, that the glutathione peroxidase homologous gene *Gpxh* from *Chlamydomonas reinhardtii* is transcriptionally upregulated by the exogenous photosensitizers RB and methylene blue in the light, most probably through the formation of $^1O_2$ [41, 42]. Interestingly, *Gpxh* expression is also strongly induced by the addition of NR under illumination with kinetics that are similar to the RB-induced response, suggesting the presence of a common mechanism for type I and type II photosensitizer-induced *Gpxh* expression in *C. reinhardtii*. However, exposure to O_2^-, H_2O_2 or organic hydroperoxides only caused a slightly induced *Gpxh* expression, indicating that a specific rather than a general oxidative stress regulates the *Gpxh* transcription. In order to understand the *Gpxh* response in the presence of type I or type II photosensitizers we wanted to study the NR and RB-induced responses in *C. reinhardtii* and compare these responses with other oxidative stress-induced effects. DNA-microarrays are a good method to compare the genetic response

caused by different conditions, enabling to measure the expression levels of large sets of genes in one experiment [7]. In *C. reinhardtii*, the collection of cDNA sequences in an expressed sequence-tag (EST) library has recently led to the development of a first series of DNA-microarrays containing 2876 spots, representing approximately 2700 unique genes of the *C. reinhardtii* nuclear genome [30, 57]. We used these microarrays to study the genetic response of *C. reinhardtii* cells exposed to either a type I (NR) or a type II (RB) photosensitizer in the light. Additionally, we compared the response to these photosensitizers with gene expression profiles caused by other oxidative stress conditions. This could give more information about the nature and specificity of the defence mechanisms and the signals, triggering the activation of genes involved in the photooxidative stress response, in particular of the *Gpxh* gene.

MATERIALS AND METHODS

Strains and Culture Conditions

C. reinhardtii strain $cw_{15}arg_7mt^-$ (CC-1618), generously provided by E. Harris, was inoculated in Tris-Acetate-Phosphate-medium (TAP) [26] in Erlenmeyer flasks and agitated on a rotatory shaker (150 rpm) under constant illumination (120 µmol m^{-2} s^{-1} PAR) at 25°C. All media were supplemented with 50 mg/l ampicillin and 50 mg/l arginine.

Chemicals

NR, RB and menadione (Fluka, Buchs SG, Switzerland) were dissolved in water and stored as 1 or 10 mM stock solutions at 4°C in the dark. H_2O_2 (Merck, Whitehouse Station, USA) and t-BOOH (Fluka, Buchs SG, Switzerland) were stored at 4°C and were diluted to 1 M stock solutions before use. All chemicals used were of PA quality.

Growth Experiments, Stress Treatment and RNA Isolation

An overnight culture of $cw_{15}arg_7$ mt^- was grown to a cell density of 1×10^7 cells/ml. Then, aliquots of 5 ml of the culture were distributed in six-well culture plates and the appropriate amount of photosensitizer was added. Cell density was analyzed 0, 1, 2, 4 and 6 h after incubation by measuring A_{750} in a one-to-five dilution with water. Growth rate was calculated for each culture out of the five time point measurements in three independent experiments. In parallel, for each condition one 20 ml culture in a 150 ml Erlenmeyer flask was incubated with the appropriate concentration of photosensitizer for 60 min and total RNA was isolated by the acid guanidine isothiocyanate-phenol-chloroform method [8] using TRIzol Reagent (Invitrogen, Basel, Switzerland) following the suppliers instructions. Sample concentration was adjusted to 3 µg/µl total RNA and RNA quality was checked by agarose gel electrophoreses and ethidium bromide staining.

For all stress treatments with three time point measurements a culture of strain $cw_{15}arg_7mt^-$ was diluted in 100 ml TAP medium to a cell density of 2×10^6 cells/ml and incubated on a rotatory shaker in the light or in the dark for about 16 h, depending on the experimental purpose. When cultures reached a cell density of about 8×10^6 cells/ml, cells were incubated under the different stress conditions. Two cultures, one grown in the light and one in the dark, were kept without treatment as standards to calculate induction factors. After 20, 60 and 120 min, cells of 30 ml culture were harvested and total RNA was isolated as described above.

Probe Preparation, DNA-Array Hybridization and Data Analysis

Construction of the microarrays is described in details in the manufacturer's protocol (http://aracyc.stanford.edu/~jshrager/lab/chlamyarray). Probe labeling, purification and DNA-array hybridization was performed according the

manufacturer's protocol with the following modifications: 30 µg total RNA was utilized for the reverse transcription in a 40 µl reaction mixture. Cy5-dUTP was used for labeling of control samples and Cy3-dUTP was used for labeling of treatment samples in all experiments. After RNA degradation and probe purification, the labeled cDNA was concentrated in a vacuum evaporator, redisolved in 17 µl of hybridization buffer (50 mM sodium phosphate pH 8.0, 50% (v/v) formamide, 6x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS) and incubated for 2 min at 95°C. The microarray slides were pre-hybridized for 30 to 60 min at 42°C and hybridized at 42°C for 16 to 20 h in a hybridization chamber (Corning, Ontario, Canada).

After post-hybridization washing and drying, the slides were scanned using a laser scanner (428™ Array Scanner, Affymetrix, High Wycombe, UK), and the spot and corresponding background signals were quantified with the Affymetrix Jaguar™ software version 2.0. Further analyses of the microarrays were performed with the program GeneSpring 4.1 from Silicon Genetics (Redwood City, USA). The treatment to control signal ratio was calculated using the Cy3 and Cy5 signal intensities of each spot. All spots with a control signal below the value of 150 were excluded from the analysis and the minimal treatment to control signal ratio was set to 0.01. After background correction a signal normalization was performed using the 50th percentile distribution of the remaining spots per replicate. Average induction factors and *t*-test *P*-value were calculated with the maximal eight replicates per gene and finally all induction factors with an average treatment signal below 150 were filtered out. Primary data and the average induction factors of all DNA-array experiments can be downloaded from the Internet (www.eawag.ch/~fischebe).

cDNA Synthesis and Real-time RT-PCR

For reverse transcription, 1 µg of DNase I-treated total RNA was incubated in a 50 µl reaction including 10 µl of a 5×reaction buffer (Invitrogen, Basel, Switzerland), 2 µl oligo$(dT)_{18}$ primer (0.1 mM), 5 µl dNTP (5 mM), 5 µl 0.1 M dithiothreitol (DTT) and 300 units SuperScript™ reverse transcriptase (Invitrogen, Basel, Switzerland) for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min and the volume was adjusted to a concentration of 20 ng/µl of original RNA quantity for each sample.

Sequences of the primers for real-time RT-PCR were designed with the Primer Express™ software (Applied Biosystems, Rotkreuz, Switzerland) using the sequence of the 3' untranslated region of each gene as a template. Real-time RT-PCR reactions were performed on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) using the SYBR® Green PCR Master Mix kit as recommended by the manufacturer. Primer concentrations were optimized for each gene. In order to evaluate unspecific amplification by primer dimers or contaminations, annealing profiles of PCR products were analyzed and control reactions without cDNA were performed. Threshold cycle (C_t) values were determined for all reactions in the logarithmic amplification phase, and the average C_t value was calculated for each sample out of two to three replicates. The C_t value of the gene coding for the Rubisco small subunit (*Rbcs2*) was used for normalization in all cases. Induction factors for the different conditions were calculated for each gene as suggested by the manufacturer, in at least two independent experiments.

Determination of total GSH and GSSG-levels

Cells of 15 ml culture of strain $cw_{15}arg_7$ mt^- were harvested, washed with 1 ml of a 10 mM sodium phosphate buffer (pH 7.0) and resuspended in 400 µl of a 5% 5-sulfosalicylic acid solution. After shock-freezing in liquid nitrogen the sample

was centrifuged for 15 min at 4°C and 300 µl of supernatant was separated for determination of GSSG levels. The amount of total GSH and GSSG were quantified as described by Griffith et al (1980) [22] and normalized to the chlorophyll content, quantified according to Arnon (1949) [1].

Spin-Trapping of $^1O_2$ by TEMP

Spin-trapping assays were performed with 10 mM 2,2,6,6-tetramethylpiperidine (TEMP) and 30 mM methanol. Samples were illuminated for 10 min with 150 or 250 µmol m^{-2} s^{-1} white light at 20°C. X-band EPR spectra were recorded in a flat cell with a Bruker ESP 300 spectrometer at room temperature with 9.7 GHz microwave frequency, modulation frequency 100 kHz, modulation amplitude 2 G. The spin trap was purified as described by Fufezan et al. (2002) [18].

Data Base Search and Clustering

Sequence analysis of specific genes was performed using the ChlamyEST database of the *Chlamydomonas* Resource Center (www.biology.duke.edu/chlamy_genome) and the *Chlamydomonas reinhardtii v1.0* genomic database at the DOE Joint Genome Institute (JGI) (www.jgi.doe.gov). To find homologous genes of other organisms, the protein database of the National Center for Biotechnology Institute (NCBI) (www.ncbi.nlm.nih.gov) was used for blast searches in a translated query to protein database search. Identity of more than 30% in a region larger than 100 amino acid residues or more than 40% in a shorter region was accepted as significant.

Hierarchical gene clustering was performed using the Cluster 2.20 software, and the results were visualized in a color based expression pattern using the TreeView 1.60 software designed by the Eisen Lab (http://rana.lbl.gov/) [14].

RESULTS

RB and NR-induced effects in *C. reinhardtii*

The stress response of *C. reinhardtii* cells, either exposed to type I or type II photosensitizers, was analyzed by determining the gene expression profiles of cultures exposed to NR or RB in the light. In order to know the type and severity of stress caused by a certain concentration of photosensitizer, different parameters were analyzed in strain $cw_{15}arg_7$ mt^- exposed to NR and RB under illumination of 120 μmol m^{-2} s^{-1}. First, lethal concentrations of NR and RB in the light were determined by measuring growth of different cultures exposed to increasing concentrations of the photosensitizers. The growth rate continuously decreased in NR-exposed cultures up to a concentration of 5 μM, lethal effects occurred at 8 μM NR (Fig. 1a). RB caused little effect up to 0.7 μM, but strongly reduced the growth rate at 1.0 μM and was lethal at 1.5 μM. In parallel, the expression of the *Gpxh* gene, known to be induced by both photosensitizers, was analyzed by real-time RT-PCR 60 min after exposure to each concentration to test the strength of the genetic response. *Gpxh* expression increased with increasing concentrations of NR and RB up to the sublethal concentrations (Fig. 1a). Thus, 5 μM NR and 1 μM RB caused a strong but not lethal cellular stress and the highest induction of the genetic stress response tested. We further measured the cellular levels of total GSH and glutathione disulfide (GSSG), two parameters indicating the cellular redox state and thus the oxidative stress [12]. Surprisingly, no effect of RB was detected neither on the total GSH nor on the GSSG concentration in cells during two hours of exposure to 1 μM RB (Fig. 1b). In the presence of 5 μM NR, however, the level of total GSH increased continuously between 20 to 120 min after exposure indicating a stimulation of GSH synthesis. Also a slight increase in the GSSG level was observed

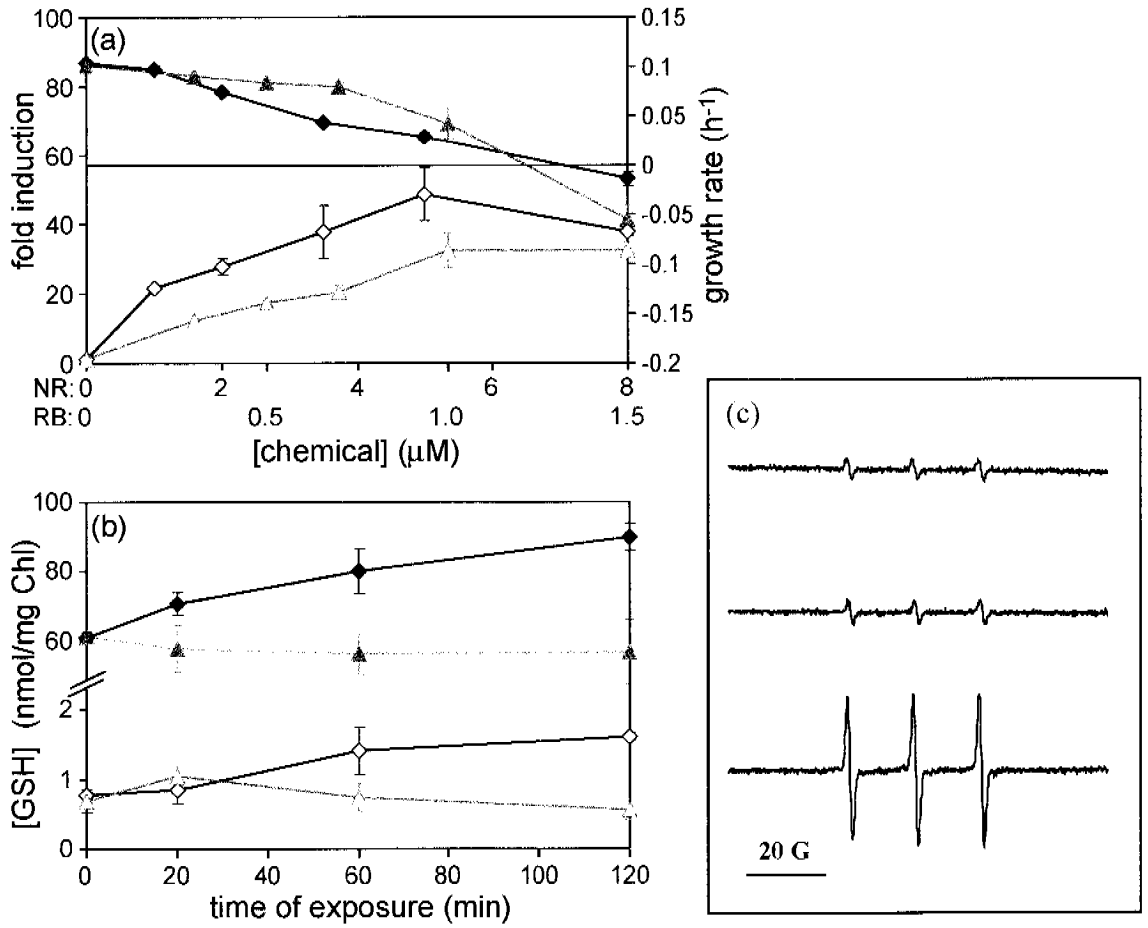


Figure 1: (a) Growth rate (closed symbols) and *Gpxh* induction (open symbols) was measured in *C. reinhardtii* cultures exposed to various concentrations of NR (diamonds) or RB (triangles) under illumination. Average growth rate over 6 h was calculated in three independent experiments. Induction of the *Gpxh* gene by the appropriate concentration of photosensitizer was measured by determining the *Gpxh* mRNA levels with real-time RT-PCR after 1 h of exposure and induction factors were calculated out of two independent experiments. (b) Total GSH (closed symbols) and GSSG (open symbols) levels were detected after incubation with 5 µM NR (diamonds) or 1µM RB (triangles) in the light in 2 to 4 independent experiments. (c) Typical EPR spectra of TEMPO as adduct of the reaction of TEMP without $^1O_2$ (top spectrum), with $^1O_2$ produced by 5 µM NR (second spectrum) or by 1 µM RB (bottom spectrum) in aqueous solutions after 10 min illumination with 150 µmol m^{-2} s^{-1} PAR.

suggesting a disturbed cellular redox balance, even though this effect was rather low compared to the effect of other stresses like high light exposure [32]. Finally, we measured the production of $^1O_2$ by NR and RB with EPR-spin trapping in an aqueous solution to test their potency as type II photosensitizers.

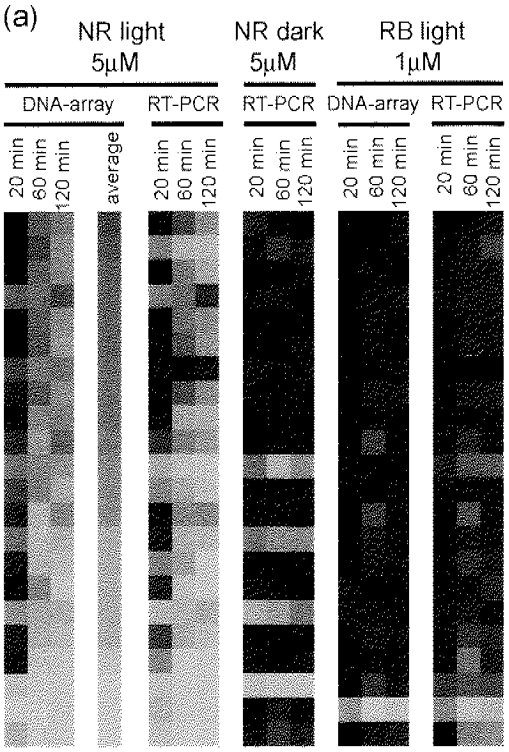
No direct formation of $^1O_2$ could be detected by 5 μM NR illuminated for 10 min with 150 μmol m^{-2} s^{-1} (Fig. 1c). On the contrary 1 μM RB, under the same light condition, resulted in a strong EPR-signal, showing that it efficiently leads to the production of $^1O_2$ as expected for a type II photosensitizer. These concentrations of photosensitizers were chosen to analyze the total gene expression profiles caused by type I and type II photosensitizers.

Identification of Genes Induced by NR and RB with DNA-Arrays

To analyze total gene expression profiles, cultures of *C. reinhardtii* cells were exposed either to 5 μM NR or 1 μM RB under continuous illumination of 120 μmol m^{-2} s^{-1}. A control culture without any treatment was incubated in parallel and total RNA was isolated from each condition at 20, 60 and 120 min after exposure in two independent experiments. Afterwards, a Cy5-dUTP labeled cDNA of each control RNA sample and a Cy3-dUTP labeled cDNA of each treatment RNA sample was synthesized and used to hybridize to the *C. reinhardtii* microarrays. Signal intensities of the two independent experiments and of four replicates per RNA sample were used to calculate the induction factors for each gene at the three time points and a replicate normalization of varying labeled cDNA levels was performed, using the median signal intensity. Genes with low signal intensities were not considered in the analyses. Only 11 genes in the RB experiment but as much as 75 genes in the NR-exposed cells were induced more than two fold in at least one time point measurement (all data on www.eawag.ch/~fischebe). Only those genes which were significantly induced more than two fold in at least two out of the three time point measurements of each stress condition (*t*-test *P*-value<0.01) were selected for a more intensive analysis by real-time RT-PCR. Only one gene (BM519216) passed this restriction in the RB exposed cultures, but 22 genes could be selected from the NR exposure, including the RB-induced gene

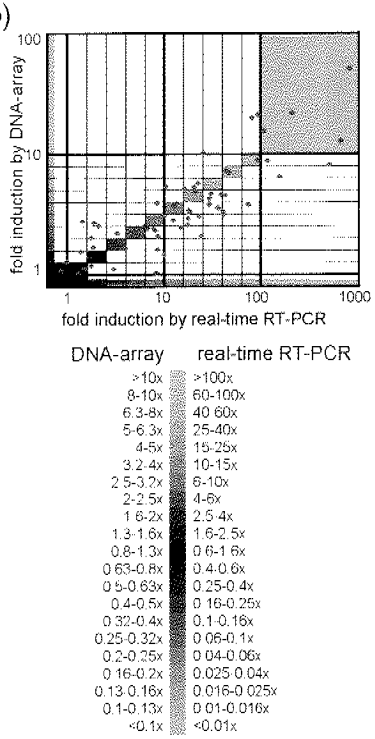
BM519216 (Fig. 2a, supplemental data). This indicates that NR causes a much broader response in *C. reinhardtii* than RB, inducing the expression, more then five fold, of 10 genes in the microarrays. With RB, only BM519216 was strongly induced (12.5 fold), whereas all other nuclear genes tested showed maximal induction of two to three fold.

(a)



clone ID	accession
963041H11	BF863196
894028F11	BE212253
894096D05	BE726915
894008C03	BE025124
894006G02	BE024890
963036A07	BF862561
894016F07	BE122014
894077F06	BE724630
894009B03	BE056399
894100A09	BM518858
963045E08	BF863629
963032A08	BF862179
894024F09	BE211922
963068A08	BF866369
963068F01	BF866418
894022H10	BE129393
894018D09	BE122244
894096A07	BE726861
963050F02	BF864288
894087F06	BE725962
963046E04	BM519216
894082A11	BE725265

(b)



(c)

accession	contig	scaffold	blast result with highest identity	identity
BF863196	20021010.2151.2	1642	Putative protein AT5g45020.1 [Arabidopsis thaliana], predicted GST	46% (178)
BE212253	20021010.7936.2	234	Carbonyl reductase-like protein [Arabidopsis thaliana]	40% (290)
BE726915	20021010.3481.2	574	20S proteasome alpha 2 subunit [Oryza sativa]	78% (123)
BE025124	20021010.1860.1	21	Quinol-to oxygen oxidoreductase [Chlamydomonas reinhardtii]	100%
BE024890	20021010.5487.1	395	20S proteasome alpha 6 subunit [Nicotiana benthamiana]	71% (149)
BF862561	20021010.3244.1	32	20S proteasome alpha subunit A2 (PAA2) [Arabidopsis thaliana]	67% (189)
BE122014	20021010.4483.1	421	No significant homology	
BE724630	20021010.8550.1	2405	Thioredoxin h [Chlamydomonas reinhardtii]	100% (113)
BE056399	20021010.5394.4		No significant homology	
BM518858	20021010.8053.1	2138	No significant homology	
BF862179	20021010.6781.1	247	No significant homology	
BE211922	20021010.5909.1	2397	Heat shock protein hsp80 [Lycopersicon esculentum]	75% (169)
BF866418	20021010.5588.1[1.]	961	18 kD heat shock protein [Hordeum vulgare subsp. vulgare]	57% (33)
BE129393	20021010.3547.1	89	Glutathione-S-transferase 2 [Nematospiroides dubius]	35% (180)
BE726861	20021010.7754.1	961[2.]	18 kD heat shock protein [Hordeum vulgare subsp. vulgare]	57% (33)
BF864288	20021010.3862.1	604	Chloroplast heat shock 22 kD protein [Chlamydomonas reinhardtii]	42% (61)
BM519216	20021010.5826.1	9	Gpxh (AF014927) [Chlamydomonas reinhardtii]	100%
BE725265	20021010.6612.1	1031	Glutathione S-transferase 1 [Ascaris suum]	57% (64)

1.) 70% identitical to EST in 720bp
2.) contig matches in a continuous stretch of 875bp out of 1374bp

The 22 genes, which passed the restriction in the NR and/or the RB experiment, were further investigated using real-time RT-PCR. Specific primer pairs for all genes were synthesized including primers for the *RbcS2* gene, which was not induced by the treatments and was used for normalization of varying mRNA levels in the reverse transcription reactions. Interestingly, all induction factors, except for those of BE122014, were much higher when determined with real-time RT-PCR than with DNA-arrays. Still, a good correlation between the expression patterns determined by the two methods was reached, and both methods gave comparable induction profiles, when the scale was adjusted to maximal induction factors (Fig. 2a and b). This shows that induction profiles determined with the same method can be compared, whereas relative induction should be used whenever comparing data analyzed by different methods. In order to exclude genes, which were induced by the addition of the chemical as such, rather than by the photosensitizing process, cells were exposed under the same conditions in the dark. Four genes (BF863629, BF866369, BE122244 and BE725962) of the NR-induced genes were also strongly upregulated by NR in the dark and were excluded from further analyses (Fig. 2a), whereas the single RB-induced gene did not respond to the chemical in the dark (data not shown).

Figure 2: (a) Expression profiles of the 22 genes, which passed the restriction of strongly induced genes in the DNA-array experiments, exposed to 5 μM NR (all) or 1 μM RB (BM519216) in the light (see results for details). Induction factors were analyzed either by DNA-arrays or real-time RT-PCR and transformed into a color based expression scale adjusted to relative induction strength (see also figure b). The genes were sorted by increasing average induction factors in NR samples analyzed by DNA-arrays. (b) Correlation of the induction factors of the 22 analyzed genes determined by DNA-arrays or real-time RT-PCR. Red fields were colored according the induction scale used for figure a. (c) Results of the data base searches including contig number, scaffold number of gene location in JGI *Chlamydomonas* genome data base and the translated-blast result with the highest identity over the indicated number of amino acid residues (number in parenthesis) of the putative gene product found for each EST.

To analyze probable functions of the selected 18 upregulated genes, blast searches were performed, aimed at finding the most homologous genes in other organisms. In order to do so, major parts of the coding sequences were extracted from the *Chlamydomonas* EST database. EST sequences, generated from 3'reads, are assembled based on sequence similarity to contigs of the mean size of 791 nucleotides. These contigs were then aligned to a pool of 5'reads to generate unique gene sets called ACEs [57]. We additionally searched the *C. reinhardtii* genome, which was recently made accessible on the web by JGI, to find the putative genomic sequences. Contigs, which best matched the EST sequences and scaffolds with the putative genomic sequences are shown in figure 2c. All ESTs except one (BE056399) nicely matched to a genomic sequence. Two clones (BF866418, BE726861), though having totally different sequences, aligned best to contigs with identical 5' parts, but for BF866418 the identity was only 70% over the full length. Nevertheless, only one of these contigs (20021010.5588.1) fully aligned to a genomic sequence (scaffold 961), whereas the other (20021010.7754.1) was identical to the sequence of scaffold 961 only in its 5' part.

For 14 of the 18 induced genes we found a most probable gene product with 35 to 100% identity in amino acid sequence to known proteins of different organisms. The gene, which was strongly induced by both stress conditions was *Gpxh* (BM519216), known to be highly upregulated by RB and NR in the light before [41]. BE725265, the strongest induced gene by NR, was predicted to code for a glutathione-S-transferase (GST) gene. The same function was found for the gene products of BF863196 and BE129393. Additionally, we found four genes homologous to different heat shock proteins (HSP) (BE211922, BF866418, BE726861, BF864288) and three genes with 67 to 78% identity to 20S proteasome subunits. Beside *Gpxh*, two other genes could be identified as already characterized *C. reinhardtii* genes: BE025124, coding for a quinol-to-oxygen oxidoreductase and BE724630, coding for the cytosolic form of

thioredoxin (*Trxh*). The sequence of BE212253 finally showed most similarity to a carbonyl reductase-like protein of *Arabidopsis thaliana.*

Since three proteasome subunit genes were induced more than two fold at 60 and 120 min by NR, we wondered whether the expression of other proteasome subunits is upregulated by NR too. Therefore, the list of genes on the DNA-array was searched for genes homologous to proteasome subunits. Sixteen additional genes were found with 39.1 to 84.5% identity in amino acid sequence to known proteasome subunits of both, the 20S proteasome catalytic core particle and the 19S proteasome regulatory subunit (Fig. 3a). Of these 16 genes, 14 are induced more than 1.8 fold at least at 120 min after exposure to NR in the light, indicating a general upregulation of subunits of the proteasome degradative complex by NR. In addition, genes coding for other proteases were analyzed. Three of them were significantly induced by NR ($P<0.01$), including a DegP, an FtsH and a cysteine proteinase homologous gene (Fig. 3b). We further searched for additional heat shock proteins and molecular chaperones to look at their expression profiles on the DNA-arrays. Only one gene, coding for the HSP70B protein, was found to be induced by NR (Fig. 3c), whereas four other putative heat shock genes did not show any changed expression compared to the control samples (data not shown). We found similar results analyzing the expression of 19 putative genes involved in oxidative stress response. Of these genes, only three, a thioredoxin peroxidase, a Fe-superoxide dismutase precursor and a glutaredoxin homologous gene, were induced by NR between 1.7 and 3.1 fold (Fig. 3d). Two GST-like genes and a Mn-superoxide dismutase gene were not altered in their expression, whereas a catalase gene was even downregulated by a factor of 1.8 (data not shown). RB did not or to only miner extent induce the expression of these genes, indicating that this substance caused much less stress response (Fig. 3, a-d).

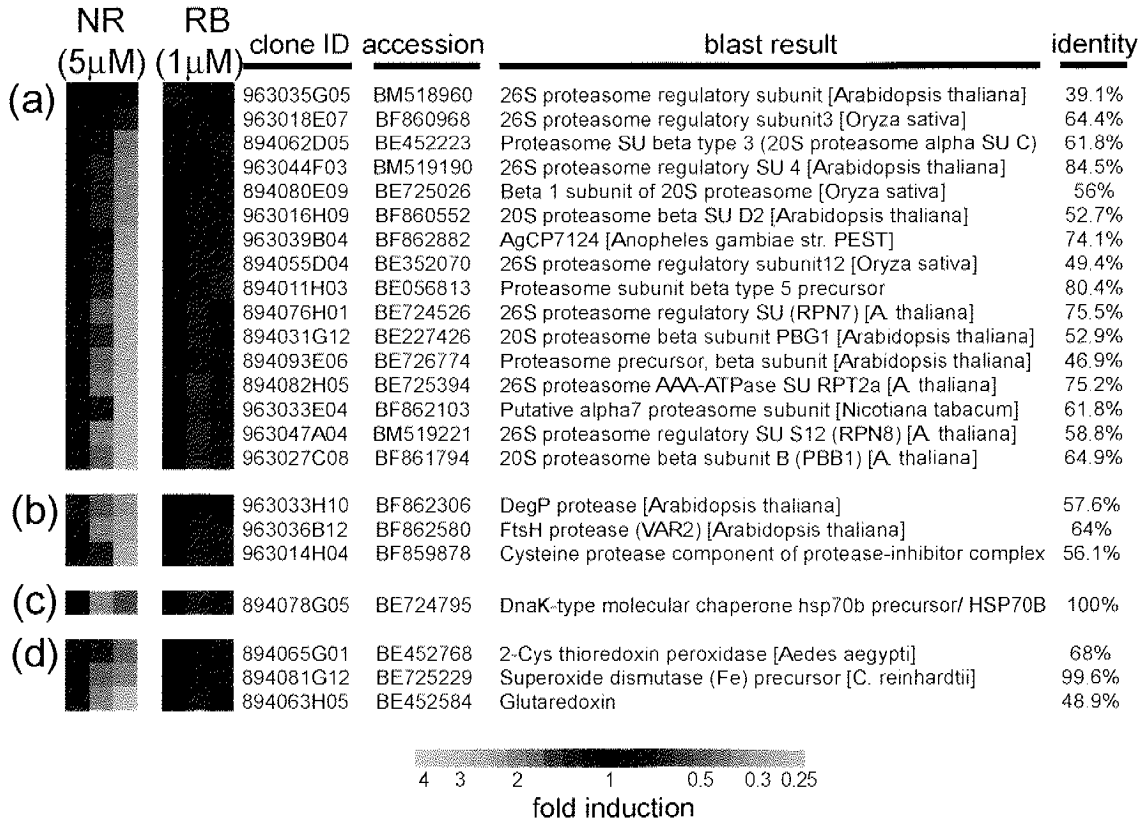


Figure 3: The expression of selected genes, identified due to their homology to known proteins involved in oxidative or general stress response, was analyzed with DNA-arrays and represented in a color based expression pattern according to the scale indicated: (a) proteasome subunits, (b) proteases, (c) heat shock proteins, (d) oxidative stress response genes. For (a) all genes with significant homology are shown, for (b), (c) and (d), only induced genes are represented.

General Oxidative Stress Response of Photooxidative Stress Induced Genes

The small number of genes induced by RB compared to NR exposure indicates a different efficiency and specificity for the type I and type II photosensitizer-induced stress response. The comparison of the induction profiles of the NR and/or RB-induced genes by other oxidative and photooxidative stress conditions may give further information about common and different mechanisms involved in the activation of these genes. To do so, cultures of strain $cw_{15}arg_7mt^-$ were exposed to sublethal concentrations of either menadione (5 µM), known to lead to the formation of O_2^-, H_2O_2 (2 mM) or tert-

butylhydroperoxide (t-BOOH) (0.1 mM), conditions known to also partially induce the *Gpxh* expression [41]. Additionally, one culture was shifted from 16 h dark to light incubation, a process known to induce the synthesis of stress proteins involved in defense against photoinhibition [36, 54]. Control samples without any treatment were incubated in parallel either in the dark, for the dark-light shift, or the light. The expression of the 18 NR and/or RB-induced genes under each stress and control condition was quantified at 20, 60 and 120 min exposure by real-time RT-PCR and induction, compared to the corresponding control sample, was calculated using the *Rbcs2* mRNA level for normalization. Many genes were strongly induced by at least 3 to 4 of the conditions tested albeit the induction kinetics were often different (Fig. 4). The response to menadione and the dark-light shift was usually early with maximum levels at 20 to 60 min after induction. All other stress conditions caused a later response, which peaked either 60 or 120 min after exposure to the chemical. In order to find genes with a similar expression pattern by the various stress conditions, a hierarchical clustering, using the induction factors of all 18 genes, was performed (Fig. 4a) [14]. The closest related induction profiles were found for BE726915 and BE024890, both coding for 20S proteasome alpha subunits. A second cluster included the genes BF866418 and BE726861, of which the deduced sequences showed highest homology to the same 18 kD HSP (Fig. 2c). The two genes showed very similar induction profiles, even though the primer pairs used for real-time RT-PCR did not cross-hybridize between the two contig sequences (Fig. 4a). Generally, most of the typical stress response genes including GSTs, *Trxh*, HSPs and proteasome subunit cluster together in a group, which is strongly induced by all conditions tested except RB-treatment and the dark-light shift. However, some genes showed an induction pattern not similar to any other expression profile: The *Gpxh* genes was 5 to 12 fold stronger induced by NR and RB than by any other stress tested, supporting the

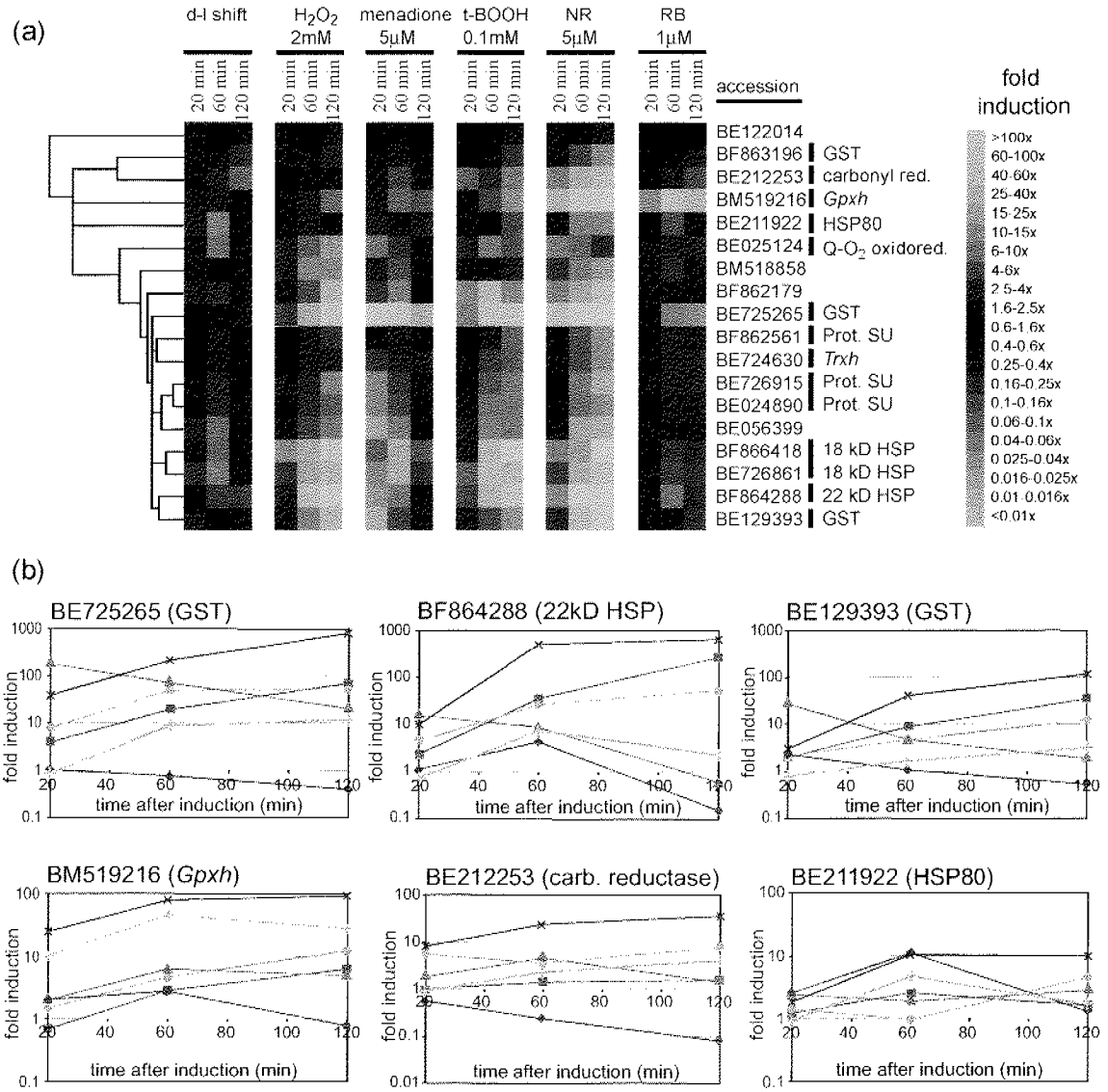


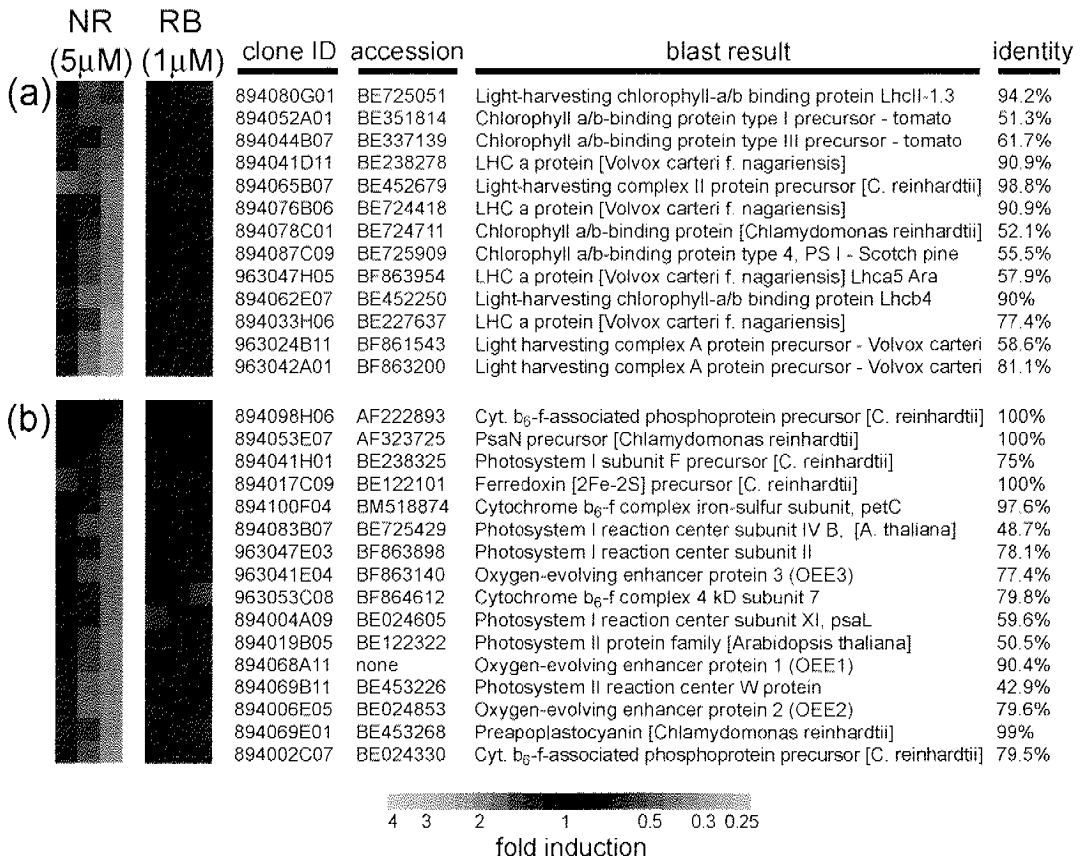
Figure 4: (a) Hierarchical clustering of the 18 analyzed genes by their expression profiles due to a dark-light shift (d-l shift) or exposure to 2 mM H_2O_2, 5 µM menadione, 0.1 mM t-BOOH, 5 µM NR or 1 µM RB for 20, 60 and 120 min determined by real-time RT-PCR. Induction factors are represented in a color based expression scale and the similarities between the expression profiles are indicated by the distances in the hierarchical tree. (b) Time profiles of the induction of six selected genes by various oxidative and photooxidative stress conditions, including 2 mM H_2O_2 (■), 5 µM menadione (▲), 0.1 mM t-BOOH (●), 5 µM NR (×), 1 µM RB (+) and a dark-light shift (♦) analyzed by real-time RT-PCR.

involvement of a common response mechanisms to these photosensitizers (Fig. 4b) [41]. Two genes, homologous to a GST and a carbonyl reductase respectively, were 12 to 35 fold induced by NR but only up to 8 fold by the

other oxidative stress conditions and were downregulated 10 fold by the shift from the dark to the light (Fig. 4a). The HSP80 homologous gene BE211922 finally was strongly upregulated only by the presence of NR in the light and the dark-light shift (Fig. 4a and b). Dark-light induced expression of genes coding for an Hsp80 and a related Hsp70 has been shown in *C. reinhardtii* before [70]. These data indicate that NR-induced genes were activated by several unrelated induction mechanisms and that NR caused multiple effects in the cell, whereas RB affected the cell more specifically only inducing few genes.

NR-Disturbed Photosynthetic Electron Flow Resulted in the Down-regulation of Photosynthetic Genes and the Formation of $^1O_2$ in the Thylakoid Membrane

The NR-dependent induction of the plastoquinol oxidizing enzyme quinol-to-oxygen oxidoreductase (BE025124), homologous to the Arabidopsis IMMUTANS gene involved in chlororespiration, and the HSP80 gene, for which a protection of PSII during photoinhibition was described [54], indicates that one effect of NR in the cell may involve an interaction with the photosynthetic apparatus. Indeed, an inhibition of the linear photosynthetic electron flow by NR above 20 μM was already observed in isolated pea chloroplasts earlier and a function of NR as uncoupler was discussed [48]. To check an effect of NR on photosynthesis, the expression of genes coding for components of the photosynthetic apparatus was analyzed in cells exposed to NR. We found 13 genes with 51.3 to 98.8% identity in amino acid sequence to LHC genes of other photosynthetic organisms on the DNA-microarrays. Interestingly, all of them were downregulated at least 1.8 fold by NR (Fig. 5a). A similar response was found for many other components of the photosynthetic apparatus, including the three oxygen-evolving enhancer-like proteins or several putative proteins of PSI and PSII, which were downregulated between 1.3 and



clone ID	accession	blast result	identity
894080G01	BE725051	Light-harvesting chlorophyll-a/b binding protein LhcII-1.3	94.2%
894052A01	BE351814	Chlorophyll a/b-binding protein type I precursor - tomato	51.3%
894044B07	BE337139	Chlorophyll a/b-binding protein type III precursor - tomato	61.7%
894041D11	BE238278	LHC a protein [Volvox carteri f. nagariensis]	90.9%
894065B07	BE452679	Light-harvesting complex II protein precursor [C. reinhardtii]	98.8%
894076B06	BE724418	LHC a protein [Volvox carteri f. nagariensis]	90.9%
894078C01	BE724711	Chlorophyll a/b-binding protein [Chlamydomonas reinhardtii]	52.1%
894087C09	BE725909	Chlorophyll a/b-binding protein type 4, PS I - Scotch pine	55.5%
963047H05	BF863954	LHC a protein [Volvox carteri f. nagariensis] Lhca5 Ara	57.9%
894062E07	BE452250	Light-harvesting chlorophyll-a/b binding protein Lhcb4	90%
894033H06	BE227637	LHC a protein [Volvox carteri f. nagariensis]	77.4%
963024B11	BF861543	Light harvesting complex A protein precursor - Volvox carteri	58.6%
963042A01	BF863200	Light harvesting complex A protein precursor - Volvox carteri	81.1%
894098H06	AF222893	Cyt. b_6-f-associated phosphoprotein precursor [C. reinhardtii]	100%
894053E07	AF323725	PsaN precursor [Chlamydomonas reinhardtii]	100%
894041H01	BE238325	Photosystem I subunit F precursor [C. reinhardtii]	75%
894017C09	BE122101	Ferredoxin [2Fe-2S] precursor [C. reinhardtii]	100%
894100F04	BM518874	Cytochrome b_6-f complex iron-sulfur subunit, petC	97.6%
894083B07	BE725429	Photosystem I reaction center subunit IV B, [A. thaliana]	48.7%
963047E03	BF863898	Photosystem I reaction center subunit II	78.1%
963041E04	BF863140	Oxygen-evolving enhancer protein 3 (OEE3)	77.4%
963053C08	BF864612	Cytochrome b_6-f complex 4 kD subunit 7	79.8%
894004A09	BE024605	Photosystem I reaction center subunit XI, psaL	59.6%
894019B05	BE122322	Photosystem II protein family [Arabidopsis thaliana]	50.5%
894068A11	none	Oxygen-evolving enhancer protein 1 (OEE1)	90.4%
894069B11	BE453226	Photosystem II reaction center W protein	42.9%
894006E05	BE024853	Oxygen-evolving enhancer protein 2 (OEE2)	79.6%
894069E01	BE453268	Preapoplastocyanin [Chlamydomonas reinhardtii]	99%
894002C07	BE024330	Cyt. b_6-f-associated phosphoprotein precursor [C. reinhardtii]	79.5%

Figure 5: The expression of genes coding for components of the photosynthetic apparatus was analyzed in *C. reinhardtii* due to exposure to NR or RB and represented in a color based expression pattern according to the scale indicated. Genes present on the DNA-array were searched for homologous to either (a) subunit of the LHC or (b) components of the PSI, PSII or the cytochrome b_6-f complex.

2.6 fold, 60 to 120 min after exposure to NR (Fig. 5b). Together with the decreased expression of four genes, homologous to components of the cytochrome b_6-f complex and of two genes, coding for a plastocyanin and a ferredoxin, members of all major components of the photosynthetic apparatus were found to be downregulated by NR. On the contrary, none of them was significantly changed in its expression by RB (Fig. 5) even though the PSII is the most important source for $^1O_2$ in photosynthetic organisms under natural conditions and negative effects of $^1O_2$ produced by exogenous photosensitizers on PSII stability has been described in isolated chloroplasts before [9, 66].

NR-caused inhibition of the photosynthetic activity may also result in enhanced charge recombination and the concomitant increased formation of $^1O_2$ in PSII as described for phenolic herbicides [18, 52]. Therefore, the formation of $^1O_2$ in NR-treated spinach thylakoids was tested with EPR spin trapping [18]. A suspension of 15 μg Chl/ml isolated thylakoid membranes exposed to 5 μM NR was illuminated for 10 min with white light of 250 μmol m^{-2} s^{-1}. Even though a significant amount of $^1O_2$ was already produced by isolated thylakoids in the absence of any chemical treatment, an increased generation of $^1O_2$ could be detected in the thylakoids exposed to NR, showing that NR indeed affected photosynthesis and enhanced the rate of charge recombination and $^1O_2$ formation in the PSII.

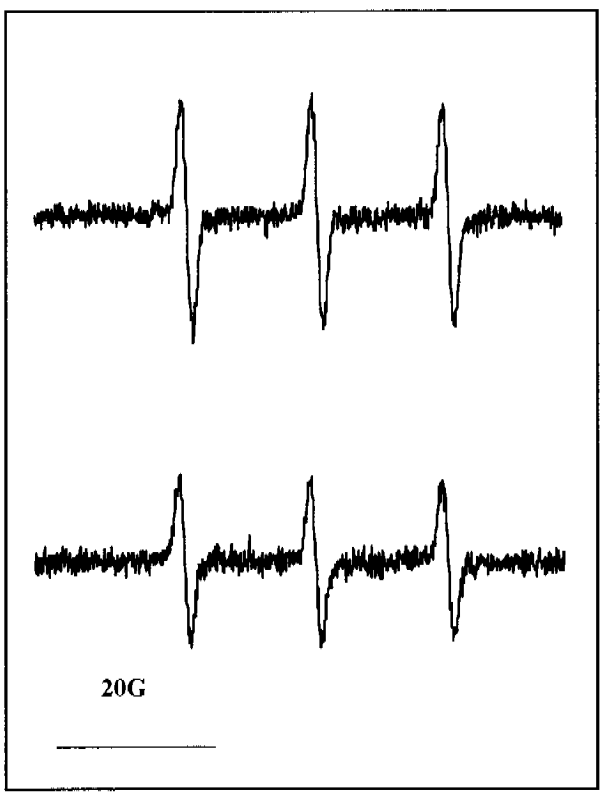


Figure 6: EPR spectra of TEMPO as adduct of the reaction of TEMP with $^1O_2$ produced by 5 μM NR in the presence of isolated spinach thylakoids (top spectrum) or by untreated isolated spinach thylakoids (bottom spectrum) after 10 min illumination with 250 μmol m^{-2} s^{-1} PAR.

DISCUSSION

RB Causes Little Stress Response but Strongly Induces the *Gpxh* Expression

In this study we investigated the genetic responses of *C. reinhardtii* to the type I photosensitizer NR and the $^1O_2$-producing photosensitizer RB to unravel the similarities and difference in the expression pattern caused by the two substances and to compare them with the profiles induced by other oxidative stress conditions. Contrary to NR effects, which not only altered the expression of genes due to oxidative stress but also due to an inhibition of photosynthesis, the response of the cell to RB-exposure was very specific. The *Gpxh* gene was the only gene found on the DNA-array to be strongly induced by 1 μM RB in the light, despite the fact that this RB concentration resulted in a strong formation of $^1O_2$ under illumination and was in the sublethal range for the cell (Fig. 1a and c). Several factors could influence the efficiency of $^1O_2$-reponse caused by RB: First, RB may not enter the cell efficiently or is not equally distributed within the cell. RB has two negative charges at pH 7 and most probably localizes near the lipid-aqueous interface of membranes and is presumably located near specific proteins resulting in a high variation in the sensitivity of different membrane function to RB [35, 37]. Thus, the toxicity of RB might be either due to a much higher concentration of RB at a certain cellular site, e. g. in the plasma membrane, due to the modification of a highly sensitive cell component at concentrations, which do not affect other molecules or by a combination of both. A low intracellular concentration of RB is supported by the absence of any oxidative stress causing an alteration in the GSH balance (Fig. 1b).

The thylakoid membrane, on the other hand, contains a variety of protective molecules like carotenoids and α-tocopherol, which can efficiently scavenge $^1O_2$

and thus might prevent damaging effects of $^1O_2$ produced by RB located in the chloroplast [4, 69]. This could explain the low effect of RB-exposure on the expression of photosynthetic genes (Fig. 5), which gives good evidence that a direct effect of $^1O_2$ and not signals from a disturbed photosynthesis are causing the genetic response by RB. Therefore, a very specific and sensitive sensor for $^1O_2$ must be present in *C. reinhardtii*, which directly activates the expression of only a few genes, including *Gpxh*. We cannot determine the exact number of these genes so far, since only a part of the nuclear genome is represented on the DNA-array used in this study. The fact that only one of the 2700 genes tested was strongly induced indicates the presence of a specific defense system against $^1O_2$-induced cellular damages probably including only a few genes.

Oxidative Stress Induced by NR Causes Multiple Stress Response

Exposure of *C. reinhardtii* cells to NR induced a strong oxidative stress response and resulted in an enhancement of oxidation and *de novo* synthesis of GSH already after 1 to 2 h. Since synthesis of GSH is controlled by feedback regulation [23], a fast decrease in cellular GSH levels is expected in the early phase of NR-treatment, either by oxidation to GSSG or by chemical binding to proteins. In agreement with that, an increased expression of putative GSH-using enzymes, the *Gpxh* and three GST, was found in NR-stressed cells already after 20 min (Fig. 2 and 4). Two main functions of GSTs in the cytoplasm are the detoxification of natural or synthetic toxins and the removal of oxidized compounds including modified proteins [13]. In *Arabidopsis thaliana,* 48 GST-like genes have been found, which belong to four different classes, of which two are plant specific [56, 71]. The high diversity of this gene family represents a multitude of functions of GSTs in the cell. The various GSTs also show different expression profiles due to a variety of stress conditions and compounds [44, 71]. We have found a similar behavior in the induction of GST-like genes by NR in

C. reinhardtii, where two GSTs were strongly induced (BE725265, BE129393), one was moderate induced (BF863196) (Fig. 2a) and two were not changed in their expression (data not shown). However, the fact that they all were only induced by NR in the light, indicates that oxidative stress caused the gene induction rather than the chemical itself. Other important defense systems against oxidative stress include the cytosolic thioredoxin (*Trxh*), the functionally related glutaredoxin and the thioredoxin peroxidase which all were found to be induced by NR in the light [31, 63]. Thioredoxin and glutaredoxin are involved in reduction of intracellular disulfides and can together with the thioredoxin peroxidase remove H_2O_2 and organic hydroperoxides from the cell [21, 45]. Typically, most of these oxidative stress response genes were also induced in *C. reinhardtii* by various oxidative stress conditions like H_2O_2, O_2^- and t-BOOH (Fig. 4).

The diversity in functions and expression of the cellular HSPs is similar to that of GSTs [65, 72]. Whereas in *C. reinhardtii* the small HSPs, including the 18 kD and 22 kD HSPs (BE726861, BF866418, BF864288), were strongly upregulated in their expression by various oxidative stress conditions, the Hsp80-like gene (BE211922) was mainly induced by NR and a dark-light shift (Fig. 4). During oxidative stress response, HSPs are thought to function as molecular chaperones, and a protection of the electron transport chains in mitochondria and chloroplasts has been described for some small HSPs [28, 39]. Additionally, several articles reported the induced expression of small HSPs by oxidative stress, and some results even provide evidence for a function as scavengers of reactive oxygen species [65]. Thus, the HSPs seem to play an important role in the defense against NR-induced oxidative stress.

Many of the oxidative damaged proteins are removed by degradation, often by the 20S or 26S proteasome [24, 58]. Therefore, the induced expression of all proteasome subunits found on the DNA-array most probably is a consequence of an increased level of oxidized proteins (Fig. 2a and 3). Since the expression of

proteasome subunits was found to occur in a concerted mechanism in various organisms, we assume the other proteasome subunits to be upregulated by NR too [17, 19]. Increased proteasome subunit levels under oxidative stress were found earlier in mouse cell lines and in maize roots [5, 20]. Thus, induction of proteasome dependent protein degradation seems to be a general mechanism to remove oxidative damaged proteins.

Clustering analysis showed that many of the NR-induced genes had a similar expression profile by all stress conditions tested (Fig. 2) suggesting that a concerted induction mechanism might be involved in their activation. Still, this mechanism would be induced by individual treatment with different strength and kinetic. However, due to the similar order of induction strength for most of these genes, e.g. BE725265, BF864288 and BE129393, we hypothesized only one single mechanism to be responsible for the induction of these genes by different oxidative stresses (Fig. 2b). Other genes, like the *Gpxh* gene (BM519216), the HSP80 (BE211922) or the carbonyl reductase gene (BE212253) had an expression profile not very closely related to any other profile tested, indicating that NR might have multiple effects on *C. reinhardtii* which are not linked to its function as type I photosensitizer.

NR Affects Photosynthesis Resulting in Downregulation of Photosynthetic Genes and Production of $^1O_2$

The induction of genes by the type I photosensitizer NR, which were not strongly induced by general oxidative stress but rather by more specific treatments like RB-exposure (*Gpxh*) or dark-light shift (HSP80) indicated a second light dependent effect of NR on *C. reinhardtii* activating a more specific genetic responses (Fig. 3). Recently, NR has been shown to inhibit the electron transport in PSII [48] and indeed, almost all photosynthetic genes found on the DNA-microarray, including many putative LHC genes and subunits of the PSI,

PSII and of the oxygen-evolving complex, were downregulated up to three fold by NR exposure, implicating a severe effect of NR on the photosynthetic apparatus (Fig. 5). This is supported by the induction of the gene coding for the chloroplast targeted HSP70B protein, known to protect and repair the PSII during photoinhibition, and for a chloroplast small HSP, for which a protective function on the photosynthetic electron transport chain and D1 protein degradation was reported during heat stress (Fig. 2a and 3c) [28, 54, 55]. A similar function may also be predicted for the HSP80 protein upregulated by NR and the dark-light shift. NR may inhibit the photosynthetic electron flow similar to phenolic herbicides and thus enhance the charge recombination reaction in the PSII reaction centre, resulting in the increased formation of $^1O_2$ and photoinhibition [52]. In agreement with that an increased formation of $^1O_2$ could be measure in NR-treated isolated spinach thylokoid membranes whereas no $^1O_2$ was detected with NR in an aqueous solution (Fig. 1c and 6). This mechanism might also be compatible with the observed downregulation of LHC genes in *C. reinhardtii* under high light illumination, where the production of a reactive oxygen species was proposed to be the initial signal for this repression [67]. Further support for the induction of photoinhibition by NR was received by the upregulation of two genes, homologous to the chloroplast located proteases FtsH and DegP (Fig. 3b). Both genes have been described to mediate the initial step in D1 cleavage during photoinhibition [3, 27, 59].

Other effects of NR on gene expression rather point at an inhibition of the photosynthetic electron transport in PSI or in the cytochrome b_6-f complex, since a reduced plastoquinol pool was reported to be a signal for the response of several photosynthetic genes. Reduced expression of genes coding for components of the PSI, PSII and the LHC and also the induction of the ascorbate peroxidase genes (APX) correlate with the redox state of the plastoquinone pool [15, 32, 49, 51]. Additionally, the induction of the plastoquinol oxidizing enzyme quinol-to-oxygen oxidoreductase (BE025124),

homologous to the *Arabidopsis* IMMUTANS gene involved in chlororespiration, might be a consequence of a reduced plastoquinol pool (Fig. 2a) [10, 46]. This protein is also involved in carotenoid biosynthesis, the precursor for the xanthophylls, which are a family of important antioxidants [6]. However, an inhibition leading to a reduced plastoquinol pool is not in contradiction to the formation of $^1O_2$ by charge recombination as measured by the cytochrome b_6-f inhibitor DBMIB in isolated thylakoid with spin trapping (data not shown).

Increased formation of $^1O_2$ in NR-treated cells is presumably also the cause for the strong induction of the *Gpxh* gene which was otherwise only highly upregulated by the type II photosensitizer RB but to a minor extent by other oxidative stress. This shows that the specific sensor for $^1O_2$, already postulated to be responsible for RB induction, can be activated by $^1O_2$ produced in the chloroplasts. A specific activation of genetic response by $^1O_2$ was also found in *Arabidopsis* and a direct function as signal molecule rather than damaging effects of $^1O_2$ were suggested to be responsible for the gene induction [47]. So far, we neither can say anything about the sensor for the *Gpxh* response to $^1O_2$ nor do we know the function of the Gpxh protein in the $^1O_2$-stressed cell. In mammalian cells, several signal transduction pathways, activating either the AP-1, AP-2 or the NF-κB transcription factor, have been induced by $^1O_2$, and different components of the cell, which can be modified by $^1O_2$, e. g. membrane lipids, are considered as probable "receptors" for $^1O_2$ [34, 53]. $^1O_2$ is known to react efficiently with phospolipids and to result in the formation of specific hydroperoxides which may activate the $^1O_2$-mediated response [64]. In this study we could show that an increased formation of $^1O_2$, in contrast to type I photooxidative stress, induced only few genes during the first two hours, most probably through activation of a very sensitive and specific sensor inside the cell.

REFERENCES

1. Arnon DI: Copper Enzymes in Isolated Chloroplasts - Polyphenoloxidase in Beta-Vulgaris. Plant Physiology 24: 1-15 (1949).
2. Aro EM, Virgin I, Andersson B: Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113-34 (1993).
3. Bailey S, Silva P, Nixon P, Mullineaux C, Robinson C, Mann N: Auxiliary functions in photosynthesis: the role of the FtsH protease. Biochemical Society Transactions 29: 455-459 (2001).
4. Baroli I, Gutman BL, Ledford HK, Shin JW, Chin BL, Havaux M, Niyogi KK: Photo-oxidative Stress in a Xanthophyll-deficient Mutant of Chlamydomonas. J Biol Chem 279: 6337-6344 (2004).
5. Basset G, Raymond P, Malek L, Brouquisse R: Changes in the expression and the enzymic properties of the 20S proteasome in sugar-starved maize roots. evidence for an in vivo oxidation of the proteasome. Plant Physiol 128: 1149-62 (2002).
6. Bennoun P: Chlororespiration and the process of carotenoid biosynthesis. Biochim Biophys Acta 1506: 133-42 (2001).
7. Brown PO, Botstein D: Exploring the new world of the genome with DNA microarrays. Nat Genet 21: 33-7 (1999).
8. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-9 (1987).
9. Chung SK, Jung J: Inactivation of the Acceptor Side and Degradation of the D1 Protein of Photosystem-Ii by Singlet Oxygen Photogenerated from the Outside. Photochemistry and Photobiology 61: 383-389 (1995).
10. Cournac L, Redding K, Ravenel J, Rumeau D, Josse EM, Kuntz M, Peltier G: Electron flow between photosystem II and oxygen in chloroplasts of photosystem I-deficient algae is mediated by a quinol oxidase involved in chlororespiration. J Biol Chem 275: 17256-62 (2000).
11. Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F: Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci 57: 779-95 (2000).
12. Dickinson DA, Forman HJ: Glutathione in defense and signaling: lessons from a small thiol. Ann N Y Acad Sci 973: 488-504 (2002).
13. Dixon DP, Lapthorn A, Edwards R: Plant glutathione transferases. Genome Biol 3: REVIEWS3004 (2002).

14. Eisen MB, Spellman PT, Brown PO, Botstein D: Cluster analysis and display of genome-wide expression patterns (vol 95, pg 14863, 1998). Proceedings of the National Academy of Sciences of the United States of America 96: 10943-10943 (1999).

15. Escoubas JM, Lomas M, LaRoche J, Falkowski PG: Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. Proc Natl Acad Sci U S A 92: 10237-41 (1995).

16. Foote CS: Mechanisms of Photosensitized Oxidation - There Are Several Different Types of Photosensitized Oxidation Which May Be Important in Biological Systems. Science 162: 963-& (1968).

17. Fu H, Doelling JH, Arendt CS, Hochstrasser M, Vierstra RD: Molecular organization of the 20S proteasome gene family from Arabidopsis thaliana. Genetics 149: 677-92 (1998).

18. Fufezan C, Rutherford AW, Krieger-Liszkay A: Singlet oxygen production in herbicide-treated photosystem II. FEBS Lett 532: 407-10 (2002).

19. Genschik P, Jamet E, Philipps G, Parmentier Y, Gigot C, Fleck J: Molecular characterization of a beta-type proteasome subunit from Arabidopsis thaliana co-expressed at a high level with an alpha-type proteasome subunit early in the cell cycle. Plant J 6: 537-46 (1994).

20. Gomes-Marcondes MC, Tisdale MJ: Induction of protein catabolism and the ubiquitin-proteasome pathway by mild oxidative stress. Cancer Lett 180: 69-74 (2002).

21. Goyer A, Haslekas C, Miginiac-Maslow M, Klein U, Le Marechal P, Jacquot JP, Decottignies P: Isolation and characterization of a thioredoxin-dependent peroxidase from Chlamydomonas reinhardtii. Eur J Biochem 269: 272-82 (2002).

22. Griffith OW: Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106: 207-12 (1980).

23. Griffith OW: Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med 27: 922-35 (1999).

24. Grune T, Merker K, Sandig G, Davies KJ: Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 305: 709-18 (2003).

25. Haldrup A, Jensen PE, Lunde C, Scheller HV: Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci 6: 301-5 (2001).

26. Harris EH: The Chlamydomonas sourcebook : a comprehensive guide to biology and laboratory use. Academic Press, San Diego (1989).

27. Haussuhl K, Andersson B, Adamska I: A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II. Embo J 20: 713-22 (2001).

28. Heckathorn SA, Downs CA, Sharkey TD, Coleman JS: The small, methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. Plant Physiol 116: 439-444 (1998).

29. Hideg E, Kalai T, Hideg K, Vass I: Do oxidative stress conditions impairing photosynthesis in the light manifest as photoinhibition? Philos Trans R Soc Lond B Biol Sci 355: 1511-6 (2000).

30. Im CS, Zhang ZD, Shrager J, Chang CW, Grossman AR: Analysis of light and CO2 regulation in Chlamydomonas reinhardtii using genome-wide approaches. Photosynthesis Research 75: 111-125 (2003).

31. Jacquot JP, Gelhaye E, Rouhier N, Corbier C, Didierjean C, Aubry A: Thioredoxins and related proteins in photosynthetic organisms: molecular basis for thiol dependent regulation. Biochem Pharmacol 64: 1065-9 (2002).

32. Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM: Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell 9: 627-40 (1997).

33. Keren N, Ohad I: State transition and photoinhibition. In: Rochaix J-D, Michel G-C, Sabeeha M (eds) The molecular biology of chloroplasts and mitochondria in chlamydomonas, pp. 569-596. Kluwer academic publishers, Dordrecht (1998).

34. Klotz LO, Briviba K, Sies H: Mitogen-activated protein kinase activation by singlet oxygen and ultraviolet A. Methods Enzymol 319: 130-43 (2000).

35. Kochevar IE, Lambert CR, Lynch MC, Tedesco AC: Comparison of photosensitized plasma membrane damage caused by singlet oxygen and free radicals. Biochim Biophys Acta 1280: 223-30 (1996).

36. Kropat J, von Gromoff ED, Muller FW, Beck CF: Heat shock and light activation of a Chlamydomonas HSP70 gene are mediated by independent regulatory pathways. Mol Gen Genet 248: 727-34 (1995).

37. Lambert CR, Kochevar IE: Electron transfer quenching of the rose Bengal triplet state. Photochemistry and Photobiology 66: 15-25 (1997).

38. Lambert CR, Stiel H, Leupold D, Lynch MC, Kochevar IE: Intensity-dependent enzyme photosensitization using 532 nm nanosecond laser pulses. Photochem Photobiol 63: 154-60 (1996).

39. Lee GJ, Vierling E: A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. Plant Physiol 122: 189-98 (2000).

40. Lee PC, Rodgers MA: Laser flash photokinetic studies of rose bengal sensitized photodynamic interactions of nucleotides and DNA. Photochem Photobiol 45: 79-86 (1987).

41. Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL: The glutathione peroxidase homologous gene from Chlamydomonas reinhardtii is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology 46: 395-408 (2001).

42. Leisinger U, Rüfenacht K, Zehnder AJB, Eggen RIL: Structure of a glutathione peroxidase homologous gene involved in the oxidative stress response in *Chlamydomonas reinhardtii*. Plant Science 149: 139-149 (1999).

43. Marks GT, Lee ED, Aikens DA, Richtol HH: Transient Photochemistry of Neutral Red. Photochemistry and Photobiology 39: 323-328 (1984).

44. Marrs KA: The functions and regulation of glutathione S-transferases in plants. Annual Review of Plant Physiology and Plant Molecular Biology 47: 127-158 (1996).

45. Masutani H, Yodoi J: Thioredoxin. Overview. Methods Enzymol 347: 279-86 (2002).

46. Nixon PJ: Chlororespiration. Philos Trans R Soc Lond B Biol Sci 355: 1541-7 (2000).

47. op den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M, Apel K: Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 15: 2320-32 (2003).

48. Opanasenko V, Agafonov A, Demidova R: Effects of heterocyclic and tertiary permeant amines on the electron transfer in thylakoid membranes. Photosynthesis Research 72: 243-253 (2002).

49. Oswald O, Martin T, Dominy PJ, Graham IA: Plastid redox state and sugars: interactive regulators of nuclear-encoded photosynthetic gene expression. Proc Natl Acad Sci U S A 98: 2047-52 (2001).

50. Pfannschmidt T: Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci 8: 33-41 (2003).

51. Pfannschmidt T, Schutze K, Brost M, Oelmuller R: A novel mechanism of nuclear photosynthesis gene regulation by redox signals from the chloroplast during photosystem stoichiometry adjustment. J Biol Chem 276: 36125-30 (2001).

52. Rutherford AW, Krieger-Liszkay A: Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 26: 648-53 (2001).

53. Ryter SW, Tyrrell RM: Singlet molecular oxygen ($^1O_2$): a possible effector of eukaryotic gene expression. Free Radic Biol Med 24: 1520-1534 (1998).

54. Schroda M, Vallon O, Wollman FA, Beck CF: A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. Plant Cell 11: 1165-78 (1999).

55. Schuster G, Even D, Kloppstech K, Ohad I: Evidence for Protection by Heat-Shock Proteins against Photoinhibition During Heat-Shock. Embo Journal 7: 1-6 (1988).

56. Sheehan D, Meade G, Foley VM, Dowd CA: Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J 360: 1-16 (2001).

57. Shrager J, Hauser C, Chang CW, Harris EH, Davies J, McDermott J, Tamse R, Zhang Z, Grossman AR: Chlamydomonas reinhardtii genome project. A guide to the generation and use of the cDNA information. Plant Physiol 131: 401-8 (2003).

58. Shringarpure R, Grune T, Mehlhase J, Davies KJ: Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. J Biol Chem 278: 311-8 (2003).

59. Silva P, Thompson E, Bailey S, Kruse O, Mullineaux CW, Robinson C, Mann NH, Nixon PJ: FtsH is involved in the early stages of repair of photosystem II in Synechocystis sp PCC 6803. Plant Cell 15: 2152-64 (2003).

60. Singh MK, Pal H, Bhasikuttan AC, Sapre AV: Photophysical properties of the cationic form of neutral red. Photochemistry and Photobiology 69: 529-535 (1999).

61. Singh MK, Pal H, Sapre AV: Interaction of the excited singlet state of neutral red with aromatic amines. Photochemistry and Photobiology 71: 300-306 (2000).

62. Smith KC: The Science of photobiology. Plenum Press, New York (1981).

63. Stein M, Jacquot JP, Jeannette E, Decottignies P, Hodges M, Lancelin JM, Mittard V, Schmitter JM, Miginiac-Maslow M: Chlamydomonas reinhardtii thioredoxins: structure of the genes coding for the chloroplastic m and cytosolic h isoforms; expression in Escherichia coli of the recombinant proteins, purification and biochemical properties. Plant Mol Biol 28: 487-503 (1995).

64. Stratton SP, Liebler DC: Determination of singlet oxygen-specific versus radical-mediated lipid peroxidation in photosensitized oxidation of lipid bilayers: effect of beta-carotene and alpha-tocopherol. Biochemistry 36: 12911-20 (1997).

65. Sun W, Van Montagu M, Verbruggen N: Small heat shock proteins and stress tolerance in plants. Biochim Biophys Acta 1577: 1-9 (2002).

66. Telfer A, Bishop SM, Phillips D, Barber J: Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. J Biol Chem 269: 13244-13253 (1994).

67. Teramoto H, Nakamori A, Minagawa J, Ono TA: Light-intensity-dependent expression of Lhc gene family encoding light-harvesting chlorophyll-a/b proteins of photosystem II in Chlamydomonas reinhardtii. Plant Physiol 130: 325-33 (2002).

68. Tomita M, Irie M, Ukita T: Sensitized photooxidation of histidine and its derivatives. Products and mechanism of the reaction. Biochemistry 8: 5149-60 (1969).

69. Trebst A, Depka B, Hollander-Czytko H: A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in Chlamydomonas reinhardtii. FEBS Lett 516: 156-60 (2002).

70. von Gromoff ED, Treier U, Beck CF: Three light-inducible heat shock genes of Chlamydomonas reinhardtii. Mol Cell Biol 9: 3911-8 (1989).

71. Wagner U, Edwards R, Dixon DP, Mauch F: Probing the diversity of the Arabidopsis glutathione S-transferase gene family. Plant Mol Biol 49: 515-32 (2002).

72. Waters ER: The molecular evolution of the small heat-shock proteins in plants. Genetics 141: 785-95 (1995).

Seite Leer /
Blank leaf

3. The Photosensitizers Neutral Red (Type I) and Rose Bengal (Type II) Cause a Light-dependent Toxicity in *Chlamydomonas reinhardtii* and Induce the *Gpxh* Gene via Increased Singlet Oxygen Formation

ABSTRACT

The connection between the mode of toxic action and the genetic response caused by the type I photosensitizer and photosynthesis inhibitor neutral red (NR) and the type II photosensitizer rose bengal (RB) was investigated in the green alga *Chlamydomonas reinhardtii*. For both photosensitizers, a light intensity dependent increase in toxicity and expression of the glutathione peroxidase homologous gene (*Gpxh*) was found. The toxicity of RB was reduced by the singlet oxygen ($^1O_2$) quenchers 1,4-diazabicyclo[2.2.2]octane and L-histidine, and the RB-induced *Gpxh* expression was stimulated in deuterium oxide-supplemented growth medium. These observations clearly indicate the involvement of $^1O_2$ in both toxicity and the genetic response caused by RB. NR upregulated the expression of typical oxidative and general stress response genes, probably by a type I mechanism, and also strongly induced the *Gpxh* expression. The stimulating effect of deuterium oxide in the growth medium suggested the involvement of $^1O_2$ also in the NR-induced response. Indeed, an increased $^1O_2$ formation was detected with EPR-spin trapping in NR-treated spinach thylakoids. However, none of the $^1O_2$ quenchers could reduce the light dependent toxicity of NR in *C. reinhardtii*, indicating that NR has a different mode of toxic action than RB.

INTRODUCTION

Sunlight is essential as energy source for photosynthetic organisms but it can also harm the cell [8, 26]. By absorbing visible light, exogenous or endogenous photosensitizers increase the probability of light-induced damages. Once excited, photosensitizers become highly reactive and interact with cellular components disturbing their function [14, 49]. Chloroplasts contain many potential photosensitizers, especially the chlorophylls, which are linked with chlorophyll-binding proteins to reduce their photosensitizing properties. Photosynthetic organisms have optimized defense mechanisms, e.g. efficient $^1O_2$ quenchers like carotenoids and α-tocopherol, to protect themselves from light-induced damages under normal conditions [9, 26, 54]. Under extreme situations, however, such as illumination with high light intensities or inhibition of the photosynthetic electron transport, the capacity of the defense mechanisms may be too limited [4, 7, 23, 42]. As a consequence, cells encounter an oxidative stress condition with subsequent damage to cell components. Beside these natural photosensitizers, pollutants, pesticides and their degradation products have been shown to act as photosensitizers and to be toxic to organisms in the presence of light [24, 25].

Two types of reaction mechanisms are involved in photosensitizer-caused toxicity: In type I reactions, the excited photosensitizer reacts with a substrate by direct electron transfer resulting in a semi-reduced radical form of the substrate [36]. Type II reactions involve the formation of singlet oxygen ($^1O_2$) via energy transfer from the excited photosensitizer to ground state molecular oxygen ($^3O_2$) [17]. Typical examples of type I and II photosensitizers are neutral red (NR) and rose bengal (RB). RB is known to mainly generate $^1O_2$ by absorbing light in the visible range and it is often used to study $^1O_2$ mediated responses *in vivo* [1, 29, 32, 51]. Negative effects of RB on the photosynthetic electron transport chain in

photosystem I and II (PSI and PSII) have been studied before [13, 53]. NR, a phenazine-based dye, is widely used for staining cellular particles and as an intracellular pH indicator [46, 48] and was reported to directly transfer electrons in the excited state to several substrates in a type I manner [36, 46, 47]. Additionally, NR accumulates in the thylakoid lumen of photosynthetic organisms upon illumination with concomitant changes in the chloroplast ultrastructure [40]. As a result, a leak of protons from the thylakoid lumen and an inhibition of the electron transport chain in isolated chloroplasts were observed, and different pH-dependent inhibition mechanisms were discussed [39]. NR therefore may have multiple effects on photosynthetic organisms which may result in various cellular responses.

Recently, we have investigated the genetic response of the green alga *Chlamydomonas reinhardtii* to the type I photosensitizer NR and the type II photosensitizer RB with DNA-microarrays and found many general and oxidative stress genes to be induced by NR, but only one gene, the glutathione peroxidase homologous gene *Gpxh*, was strongly induced by both photosensitizers (unpublished data). *Gpxh* was shown to be specifically induced by the action of photosensitizers with very similar kinetics, indicating that a concerted signal cascade may be involved in the activation of *Gpxh* by the different photosensitizers [34, 35]. The production of $^1O_2$ was hypothesized to be a key intermediate in the response of the *Gpxh* expression to both NR and RB, even though NR normally reacts as type I photosensitizer. However, we did not have a direct proof for the involvement of $^1O_2$ in the *Gpxh* induction, nor could we link the toxic action of these photosensitizers with $^1O_2$ or with genetic responses. Knowing these links would allow to predict the toxic potential of a chemical from the genetic response induced already at low concentrations, an approach which is becoming more important in ecotoxicology. This knowledge builds the bases for the interpretation of data obtained by toxicogenomics and for the development of molecular biomarkers or bioassays using reporter genes

[37]. But before such biomarkers can be applied for the hazard assessment of unknown chemicals or environmental samples, the responsible mechanisms for their induction must be carefully studied [16].

In this work we used RB and NR to study the toxic effects of photosensitizers, the responsible mechanisms and the links with genetic responses. The type II photosensitizer RB caused a light intensity dependent toxic effect and induction of the *Gpxh* expression which both were linked to the increased formation of $^1O_2$. NR, on the other hand, provoked a type I photo-oxidative stress response, an increased formation of $^1O_2$ and interrupted photosynthesis. As a result, NR caused a light dependent toxic action and multiple genetic stress responses.

MATERIALS AND METHODS

Strains and Culture Conditions

C. reinhardtii strain $cw_{15}arg_7mt^-$ (CC-1618) and strain $cw_{15}arg_7mt^-$ containing the plasmid pASPro1 [34] were inoculated in Tris-Acetate-Phosphate (TAP) medium [20] or TAP medium containing 50% deuterium oxide (D_2O) and agitated on a rotatory shaker (150 rpm) under constant illumination with white light of 150 μmol m^{-2} s^{-1} (except otherwise mentioned) at 25°C. All media were supplemented with 50 mg/l ampicillin and 50 mg/l arginine when required. For chlorophyll fluorescence measurements, cells were harvested during exponential growth phase, resuspended in high salt minimal medium (HSM) [52] and further incubated until they reached a density of 6×10^6 cells/ml.

Growth Experiments, Stress Treatment and Arylsulfatase Assay

An overnight culture of strain $cw_{15}arg_7$ mt^- pASPro1 was grown to a cell density of 6×10^6 cells/ml. Then, cells were washed by centrifugation at 1000 rpm for 10 min and re-suspended in fresh TAP medium to a cell density of 6×10^6 cells/ml.

Aliquots of 5 ml of the culture were distributed in 6-well culture plates and the appropriate amount of each chemical was added. Cell density was analyzed 0, 1, 3 and 5 h after incubation by measuring the optical density at 750 nm in a 1 to 5 dilution. An average growth rate over the 5 h was calculated for each culture out of four time point measurements. A decrease in optical density was expressed as negative growth rate and expected to describe a lethal effect of the treatment. After 2, 4 and 6 h incubation, a 300 µl sample was taken and the accumulation of the arylsulfatase in the medium was analyzed by an arylsulfatase assay as described [34]. The average reporter gene induction over the 6 h was calculated by dividing the arylsulfatase activity of the stressed culture by the activity of the control culture.

For experiments in D_2O-containing medium, cells were adapted to the medium for 3-5 days by serial dilution in fresh medium to ensure optimal growth [12]. Subsequently, a 10 ml culture in 100 ml Erlenmeyer flask was incubated with the appropriate concentration of each chemical for 60 min before samples were taken.

RNA Isolation and Real-time RT-PCR

For RNA isolation, cells of 10 to 20 ml of individual cultures were harvested by centrifugation and total RNA was isolated by the acid guanidine isothiocyanate-phenol-chloroform method [11] using TRIzol Reagent (Life Technologies Ltd.) following the suppliers instructions. Sample concentration was adjusted to 3 µg/µl total RNA and RNA quality was checked by agarose gel electrophoreses and ethidium bromide staining.

For reverse transcription, 1 µg of DNase I-treated total RNA was incubated in a 50 µl reaction including 10 µl of a 5× reaction buffer (Invitrogen™), 2 µl oligo$(dT)_{18}$ primer (0.1 mM), 5 µl dNTP (5 mM), 5 µl 0.1M dithiothreitol (DTT) and 300 units SuperScript™ reverse transcriptase (Invitrogen™) for 1 h at 37°C.

The reaction was stopped by heating at 95°C for 5 min and the volume was adjusted to a concentration of 20 ng/µl of original RNA quantity for each sample.

Sequences of the primers for real-time RT-PCR were designed with the Primer Express™ software (Applied Biosystems) using the sequence of the 3' un-translated region of each gene as a template. Real-time RT-PCR reactions were performed on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems) using the SYBR® Green PCR Master Mix kit as recommended by the manufacturer. Primer concentrations were optimized for each gene. In order to evaluate unspecific amplification by primer dimers or contaminations, annealing profiles of PCR products were analyzed and control reactions without cDNA were performed. Threshold cycle (C_t) values were determined for all reactions in the logarithmic amplification phase, and the average C_t value was calculated for each sample out of three replicates. The C_t value of the gene coding for the Rubisco small subunit (*Rbcs2*) was used for normalization. Induction factors for the different conditions were calculated for each gene as suggested by the manufacturer, in three independent experiments.

Preparation of Thylakoid Isolates

Chloroplasts were prepared according to Laasch (1987) from market spinach [30]. Chloroplasts were osmotically shocked prior to measurements of photosynthetic electron transport or spin trapping EPR. Measurements were performed in a medium containing 0.3 M sucrose, 50 mM KCl, 1 mM $MgCl_2$ and 25 mM Hepes (pH 7.6). The chlorophyll content was set to 20 µg Chl/ml.

Oxygen Evolution and Chlorophyll Quantification

Photosynthetic O_2 evolution in isolated spinach thylakoids and intact *C. reinhardtii* cells was measured with a Clark-type oxygen electrode

(Hansatech) using either a 20 μg Chl/ml thylakoid membrane suspension illuminated with saturating light or a 12 μg Chl/ ml *C. reinhardtii* culture at 25°C at 200 μmol m^{-2} s^{-1} light. With spinach thylakoids, 100 μM methyl-viologen was added as electron acceptor and 5 mM ammonium chloride as uncoupler. The chlorophyll content was quantified according to Arnon (1949) [3].

Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence was measured with a pulse-amplitude modulated fluorometer (PAM) 101 (Walz, Germany) [44]. The F_0-level was monitored by weak modulated measuring light (about 0.05 μmol m^{-2} s^{-1}) and fluorescence rise was induced by a 50 ms pulse of saturating white light (8000 μmol m^{-2} s^{-1}) using a xenon flash XMT 103. Before measuring, cells were exposed to the appropriate treatment and incubated for 15 min in the dark at 25°C with continuous stirring.

Spin-Trapping of $^1O_2$ by TEMP

Spin-trapping assays were performed with 10 mM 2,2,6,6-tetramethylpiperidine (TEMP) and 30 mM methanol. Samples were illuminated for 10 min with different light intensities at 20°C. X-band EPR spectra were recorded in a flat cell with a Bruker ESP 300 spectrometer at room temperature with 9.7 GHz microwave frequency, modulation frequency 100 kHz, modulation amplitude 2 G. The spin trap was purified as described by Fufezan et al. (2002) [18]. Relative $^1O_2$ levels were calculated as the difference between the signals of the treated and the untreated thylakoid sample or between the treated sample and the background, respectively.

RESULTS

Toxicity of NR and RB under Different Light Intensities

In contrast to normal toxic substances, the toxicity of photosensitizers is rather complex in that it increases with both concentration and light intensity [5, 6, 32]. Therefore, we first studied the effect of increasing concentrations of NR and RB on growth of *C. reinhardtii* under two different light intensities and compared the results to the effect caused by the PSII inhibiting herbicide Dinoterb (DT). Cells of strain $cw_{15}arg_7mt^-$ pASPro1 were exposed to light intensities of 100 or 200 μmol m^{-2} s^{-1} for 6 h and the average growth rate was calculated for each condition by measuring the cell densities. A concentration dependent decrease of growth rate could be measured for both photosensitizers, reaching the level of no growth in the lower light intensity at about 4 μM for NR and between 0.5 and 0.6 μM for RB, indicating that this may be the lethal concentration (Fig. 1a). At higher light intensities (200 μmol m^{-2} s^{-1}) this border was already between 1.5 and 2.0 μM for NR and between 0.3 and 0.4 μM for RB. On the contrary, DT did not result in a significant lower lethal concentration at the higher light intensity (Fig. 1a).

In order to know, whether the same mechanisms could underlie both the toxicity and the *Gpxh* induction by RB and NR in *C. reinhardtii*, we compared the induction of the *Gpxh* gene by RB, NR and DT at the two different light intensities (Fig. 1b). For this experiment the expression of a reporter gene construct integrated into the genome, containing a fusion of the *Gpxh* promoter to the arylsulfatase gene, was analyzed by measuring the enzyme activity of the arylsulfatase after 2, 4 and 6 h [34]. With these activities we then calculated an average induction of the *Gpxh* reporter gene over the 6 h exposure. The *Gpxh* expression was induced by all three substances with a concentration dependent increase up to the level, where the toxic effect of the substances dramatically

reduced the growth rate and thus presumably the cell viability (Fig. 1b). Up to these cytotoxic concentrations, the reporter gene expression was strongly stimulated by the elevated light intensity with both photosensitizers. Additionally, there was already a 1.5 fold increase in the ground state expression at the higher compared to the lower light intensity, indicating that there is some induction by endogenous photosensitizers under this condition. Even though we could measure an induction by the herbicide DT, there was no significant difference between the expressions under the two light conditions.

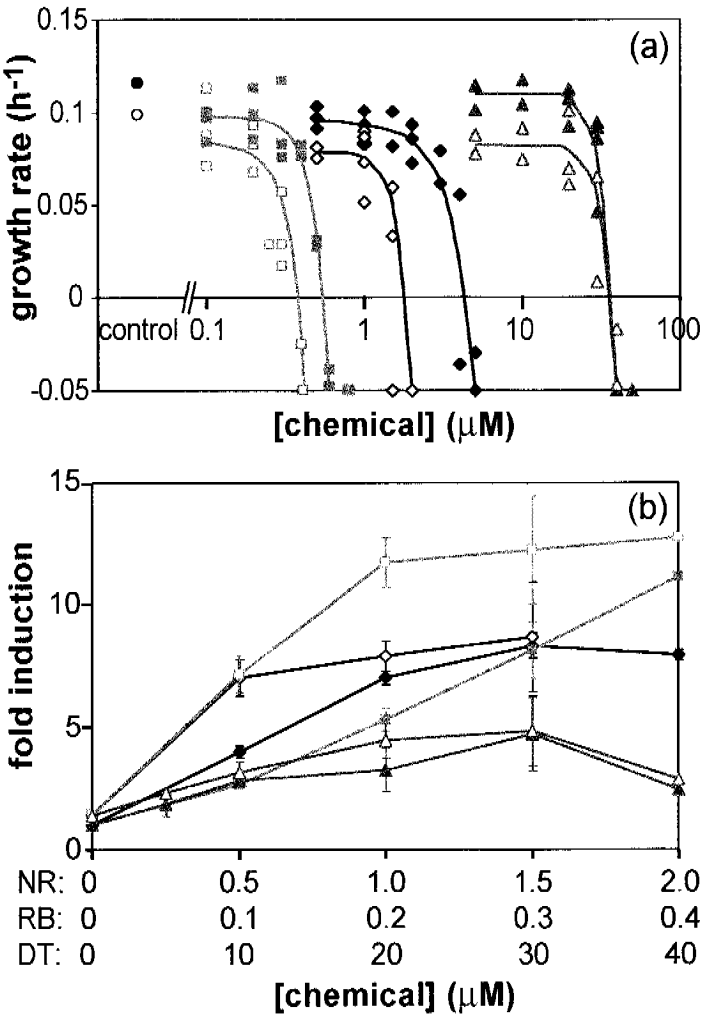


Figure 1: The effect of different light intensities and increasing concentrations of the photosensitizers RB (squares) and NR (diamonds) or the herbicide DT (triangles) on (a) the average growth rate or (b) the average induction of a *Gpxh*-arylsulfatase reporter gene construct was measured in *C. reinhardtii* cultures over 6 h. The cells were exposed to either light intensities of 100 (closed symbols) or 200 (open symbols) µmol m^{-2} s^{-1} at 25°C. Expression of the reporter gene construct was normalized for average cell numbers and induction factors compared to the untreated culture at 100 µmol m^{-2} s^{-1} light were calculated. Average induction factors and standard errors of three independent measurements are shown.

Effect of $^1O_2$ Quenchers

Formation of $^1O_2$ is one possible toxic effect caused by photosensitizers to organisms, and the addition of exogenous $^1O_2$ quenchers may have a protective effect on the cells exposed to type II photooxidative stress [15, 41, 50]. To test, whether higher rates of $^1O_2$ production are causing the cell death upon exposure to RB and NR, two widely used quenchers of $^1O_2$, 1,4-diazabicyclo[2.2.2]octane (DABCO) and L-histidine (L-His), were added to cultures of the *C. reinhardtii* strain $cw_{15}arg_7$ mt^- pASPro1 exposed to increasing concentrations of RB or NR at 100 μmol m^{-2} s^{-1} light. Even though L-His can be used by *C. reinhardtii* as a nitrogen source [22], its uptake is probably limited under the experimental conditions used [21]. The effect of these quenchers on toxicity by the photosensitizers was measured by following growth for 6 h. Without quenchers, the average growth rate decreased dramatically above 0.5 μM RB and 3 μM NR (Fig. 2). Upon addition of the $^1O_2$ quenchers DABCO (8 mM) or L-His (20 mM) no decreased growth rate was measured up to 1 μM RB (Fig. 2a). The NR-caused cytotoxicity could not be neutralized by the addition of the two quenchers. Surprisingly, even an increased toxicity of NR was detected in the presence of 8 mM DABCO (Fig. 2b).

To detect possible effects of $^1O_2$ quenching on the induction of the *Gpxh* gene by RB and NR, the expression of the *Gpxh*-arylsulfatase reporter gene was measured during the 6 h of exposure. No significant difference in the *Gpxh* expression between quencher-containing and control culture was measured at low RB and NR concentrations, where toxicity does not affect the expression of the enzyme (Fig. 2). Since DABCO has a negative effect on the growth of NR-exposed cells, the induction of *Gpxh* is reduced already at lower concentrations compared to the control, whereas no difference was seen for L-His. Addition of the quenchers to RB exposed cells reduced toxicity of the photosensitizer and positively influenced the *Gpxh* expression at higher RB levels (Fig. 2a).

Consequently, the induction of *Gpxh* further increased at concentrations above 0.5 µM RB reaching a maximum level at 0.7 to 1 µM. No direct effect of DABCO on the *Gpxh* expression could be observed at low photosensitizer concentrations and L-His only slightly reduced the *Gpxh* induction between 0.3 and 0.5 µM RB but not at concentrations below 0.2 µM RB (Fig. 2a).

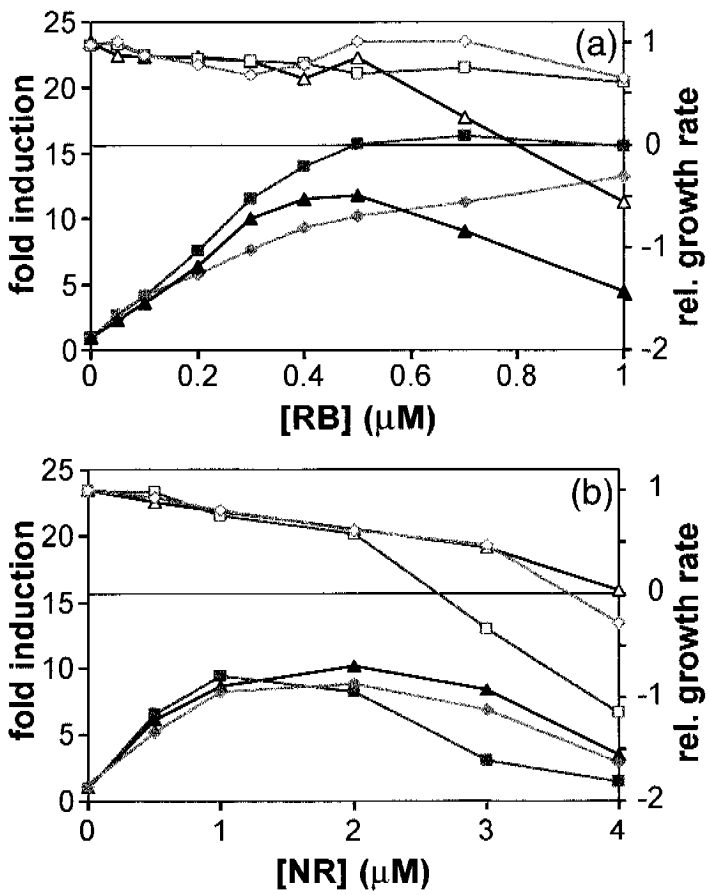


Figure 2: Average growth rate (open symbols) and the average induction of the *Gpxh*-arylsulfatase reporter gene construct (closed symbols) over 6 h affected by increasing concentrations of the photosensitizers RB (a) and NR (b) at 100 µmol m^{-2} s^{-1} light without quencher (triangles) or in the presence of 8 mM DABCO (squares) or 20 mM L-His (diamonds).

Induction Profiles by RB and NR in D_2O-Supplemented Growth Medium

Another often used method to test the involvement of $^1O_2$ in photosensitized processes is the exchange of water (H_2O) by deuterium oxide (D_2O) in the reaction medium. The presence of D_2O increases the life time of $^1O_2$ in aqueous solutions and subsequently increases the frequency of $^1O_2$-caused substrate

modifications [10, 15, 33]. We studied the effect of D_2O-supplemented growth medium on the expression of several stress genes, known to be induced by RB and/or NR and thus analyzed, whether $^1O_2$ may be involved in the response of these genes upon exposure to these photosensitizers. First, a culture of strain $cw_{15}arg_7$ mt^- was pre-grown for 3-4 days in TAP medium containing 50% D_2O to adapt the cells and allow the cells to overcome the D_2O-caused stress [55, 56]. Subsequently, the cells were exposed to a low concentration of the photosensitizer RB (0.3 μM) or NR (1 μM) and to 2 mM H_2O_2, inducing an oxidative stress not involving $^1O_2$. Total RNA was isolated after 60 min exposure and the mRNAs of the stress response genes *Gpxh*, BE129393 and BF864288 and the structural gene *Tub2B* were quantified with real-time RT-PCR and normalized using the *Rbcs2* gene as a reference. Induction factors for each gene were calculated compared to the unexposed control culture in normal TAP medium.

As shown earlier, the *Gpxh* gene was induced by both NR and RB even at low concentrations, whereas only little response was measured upon H_2O_2 exposure (Fig. 3a) [34, 35]. The induction of *Gpxh* by both RB and NR was two fold higher in the D_2O-medium, indicating that indeed $^1O_2$, with a longer life-time in D_2O, is responsible for the *Gpxh* induction by the two photosensitizers. The response of the *Gpxh* expression to H_2O_2 was not or only to a minor extent influenced by the D_2O-containing medium. Remarkably, the expression of *Gpxh* in the untreated control was already higher in the D_2O-containing medium compared to normal TAP medium. This could be either due to a higher $^1O_2$ production under the D_2O-dependent stress condition or, more likely, due to the stabilization of $^1O_2$, which is produced in normal photosynthesis and presumably involved in the basal expression of *Gpxh* in the light.

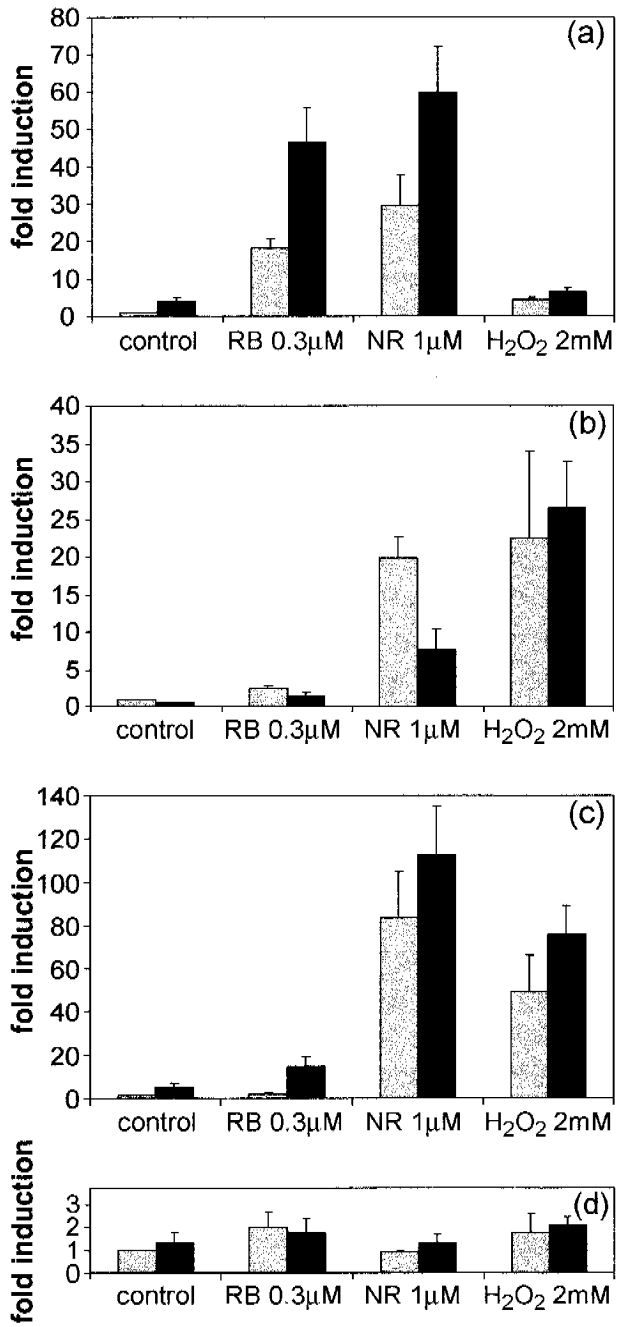


Figure 3: Expression profiles of four genes in *C. reinhardtii* cells after exposure to 0.3 µM RB, 1 µM NR or 2 mM H_2O_2 for 60 min in either a normal (gray bars) or a 50% D_2O-containing (black bars) medium. Each induction factor was calculated as an average of three independent experiments with standard error bars. (a) *Gpxh* (b) GST (BE129393) (c) HSP (BF864288) (d) *Tub2B*

Additionally, the expression of two genes which we had identified in a previous study as being strongly induced by NR but hardly by RB (unpublished data) was tested in D_2O-medium. One gene, identical to EST BE129393 in the *Chlamydomonas* EST library [45] and most probably coding for a Glutathione-S-tranferase (GST), was strongly induced in the normal medium by NR and H_2O_2, indicating that it responds to several oxidative stress conditions (Fig. 3b).

H_2O_2-induced expression was not significantly altered by the presence of D_2O, but for NR, and to a minor extent also for RB, there was rather a down-regulation by the D_2O-containing medium compared to the induction in normal medium. The expression of another stress gene, BF864288, homologous to a chloroplast heat shock protein (HSP), had a similar profile to that of the GST gene when exposed to RB, NR and H_2O_2 in normal medium (Fig. 3c). A slight upregulation was observed under all conditions in D_2O-containing medium. As control, the expression of the *Tub2B* gene, coding for a β-tubulin gene, was tested under the various conditions. *Tub2B* expression hardly varied by the exposure to the various conditions and the D_2O-medium did not cause any significant effect (Fig. 3d). In conclusion, these results clearly showed that NR induced the expression of genes both by the formation of $^1O_2$ and in another oxidative stress dependent manner whereas RB only caused a $^1O_2$ mediated stress response.

Inhibition of Photosynthetic Activity and Generation of $^1O_2$ by NR in Isolated Thylakoids

NR has been shown to inhibit the photosynthetic activity by different mechanisms [39]. To check, whether the induction of the *Gpxh* expression by NR may be due to an increased generation of $^1O_2$ in the NR-inhibited photosynthetic apparatus as found for phenolic herbicides [18], inhibition of the photosynthetic activity by NR was tested. Therefore, the reduction of photosynthetic O_2 evolution was measured both in isolated spinach thylakoid membranes and in intact *C. reinhardtii* cells. As already reported by Opanasenko et al., reduction of the electron transport by NR is maximally 50 to 60% [39]. In agreement with this we found an inhibition of photosynthetic activity of 10 to 45% between 20 to 40 μM NR both in the thylakoid suspension and the *C. reinhardtii* culture (Tab. 1). No significant reduction was found at

concentrations lower than 20 µM NR in *C. reinhardtii* but a 5% inhibition of linear electron transport was measured in the thylakoid membranes.

	spinach thylakoids		*C. reinhardtii*	
NR	photosynth. activity µmol O_2/(mg Chl*h)	inhibition	photosynth. activity µmol O_2/(mg Chl*h)	inhibition
control	422	-	24	-
10µM	399	5%	23.8	1%
20µM	312	26%	21.3	11%
40µM	234	45%	14.8	38%

Table 1: Inhibition of photosynthetic O_2 evolution by NR

Furthermore, chlorophyll fluorescence in dark-adapted *C. reinhardtii* cultures exposed to NR was analyzed and compared to the effects caused by RB (Tab. 2). Already in the presence of 1 µM NR the variable fluorescence parameter was reduced to 89% compared to the untreated sample and further decreased to 76% of the normal level at 10 µM NR. This indicates that a reduction of photosynthetic efficiency takes place already at low NR concentrations which might be even more pronounced under illumination [39]. RB, on the other hand, did not influence the variable chlorophyll fluorescence in the dark indicating that no direct interaction of RB with the photosynthetic apparatus occurs (Tab. 2).

NR	rel. F_v/F_m	RB	rel. F_v/F_m
1μM	0.89 ±0.05	0.5μM	0.92±0.03
5μM	0.81±0.04	1μM	0.97±0.04
10μM	0.76±0.05	2μM	0.98±0.05

Table 2: Effect of RB or NR exposure on the yield of the variable chlorophyll fluorescence in *C. reinhardtii*

Finally, the production of $^1O_2$ in isolated thylakoid treated either with RB or NR was directly measured by EPR-spin trapping [18]. Therefore, thylakoids were exposed to increasing concentrations of RB or NR and illuminated with white light for 10 min at 150 or 1500 μmol m^{-2} s^{-1}. As a type II photosensitizer, RB produced $^1O_2$ both in an aqueous solution and in the presence of thylakoids in a concentration dependent manner (Fig. 4a and b). In contrary, NR did not lead to the formation of any $^1O_2$ in aqueous solutions up to 20 μM (Fig. 4a and b) but it significantly rose the production of $^1O_2$ in a solution with isolated spinach thylakoids already at low concentrations compared to an untreated thylakoid solution (Fig. 4a and b). We additionally tested, whether the production of $^1O_2$ is dependent on the light intensity. As expected, in the presence of the type II photosensitizer RB the $^1O_2$ level strongly increased with higher light intensities already in the presence of 0.2 μM RB (Fig. 4c). Similarly, NR also stimulated the production of $^1O_2$ in a thylakoid suspension in a light dependent manner with a significant increase compared to the control already at light intensities between 100 and 200 μmol m^{-2} s^{-1} (Fig. 4c).

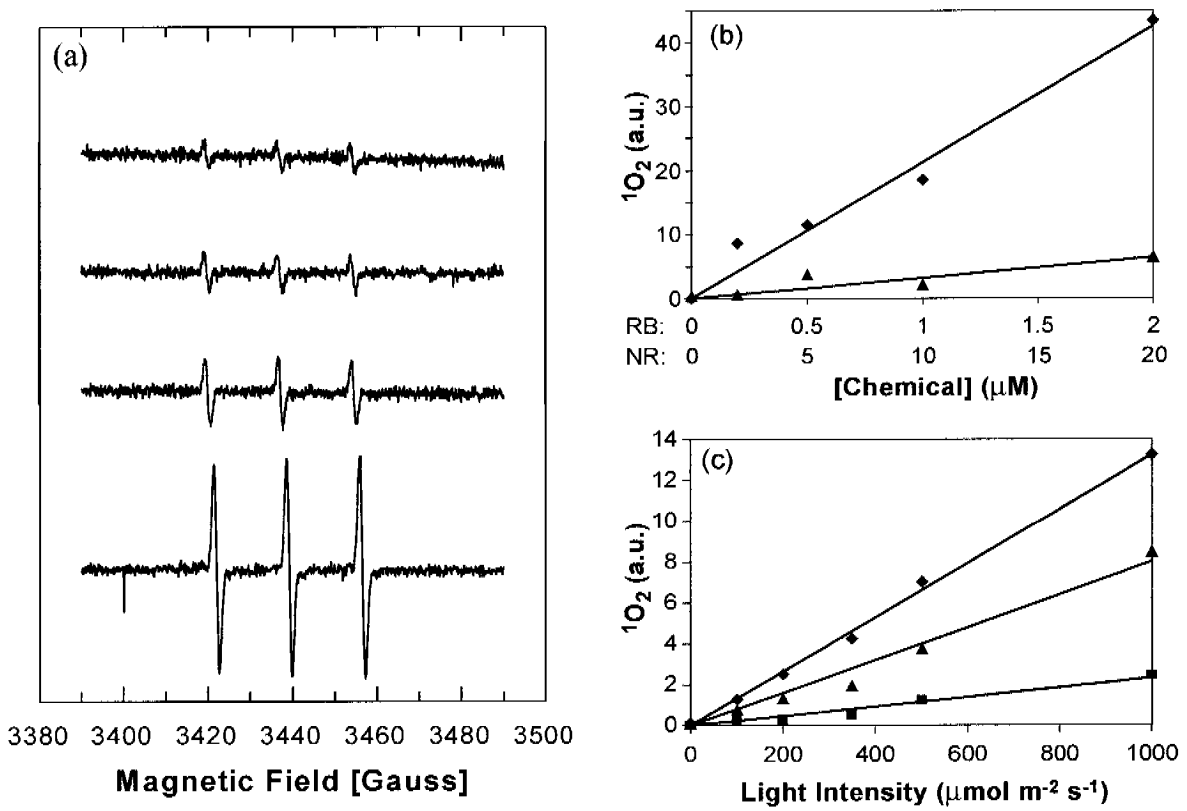


Figure 4: (a) Typical EPR spectra of TEMPO as adduct of the reaction of TEMP without $^1O_2$ (top spectrum), with $^1O_2$ produced by 5 μM NR in the absence (second spectrum) or the presence (third spectrum) of isolated spinach thylakoid membranes or with $^1O_2$ produced by 0.5 μM RB (bottom spectrum) after 10 min illumination with 150 μmol m^{-2} s^{-1} PAR. (b) Concentration dependent increase of $^1O_2$ production in isolated thylakoids in the presence of RB (diamonds) or NR (triangles) illuminated with 1500 μmol m^{-2} s^{-1} PAR. (difference between treated and untreated thylokoid signal) (c) Light intensity dependent increase of $^1O_2$ production in isolated spinach thylakoids incubated either without any treatment (squares) or with 0.2 μM RB (diamonds) or 20 μM NR (triangles) for 10 min (difference between treated thylakoids and background signal).

DISCUSSION

Exposure of *C. reinhardtii* to exogenous photosensitizers caused severe damages to the cell resulting in cell death and the induction of genetic stress response. In this work we investigated the effects of different photosensitizers on *C. reinhardtii*, using the type I sensitizer NR and the type II sensitizer RB, to study the mode of toxic action and the intermediates involved in the toxicity. Further, we linked the physiological responses caused by the two photosensitizers with genetic expression profiles and identified similarities and differences in the mechanisms of RB- and NR-induced cellular effects.

RB-Induced Cellular Response

The toxicity and the *Gpxh* induction caused by RB in the *C. reinhardtii* cultures were both dependent on the light intensity and the concentration of the photosensitizer (Fig.1). The same characteristics were found for the RB-dependent $^1O_2$ production indicating that this photosensitizing property might cause the cellular effects (Fig. 4). A role of $^1O_2$ in these response is supported by the reduced toxicity in the presence of the $^1O_2$ quenchers DABCO and L-His and the stimulated expression of *Gpxh* in the D_2O-supplemented medium (Fig. 2a and 3a). As control, another typical oxidative stress gene (GST) and a general stress response gene (HSP) which both strongly responded to oxidative stress caused by H_2O_2 and NR were not, or only to a minor extent induced by RB in both the normal and the D_2O-supplemented medium supporting the specific $^1O_2$-dependent *Gpxh* induction (Fig. 3b and c). A slightly higher induction of the HSP gene in the D_2O-medium was found for all treatments tested. This is most probably due to an increased general stress caused by the D_2O-containing medium rather than due to any specific stress caused by $^1O_2$ [55]. Earlier expression analysis with DNA-microarrays indicates that only a few genes are

induced by $^1O_2$ in *C. reinhardtii* suggesting the involvement of a specific mechanism in the response to $^1O_2$ (unpublished data).

No obvious effect of the $^1O_2$ quenchers DABCO and L-His on the expression of the *Gpxh* gene could be measured at low RB concentrations, even though the reduced toxicity and concomitantly increased expression of the reporter gene construct at high concentrations indicate that $^1O_2$ is significantly quenched by both substances (Fig. 2a). However, the rate and site of production or removal of $^1O_2$ by RB or DABCO and L-His respectively might not be homogenous inside the cell [29]. Local concentration of the photosensitizer in the cell was shown to be crucial for its mode of action and the efficiency earlier [5, 6]. RB is taken up efficiently by the cell and is thought to localize near the lipid-aqueous interface with two carboxylic acid group exposed to the aqueous phase [27, 31]. DABCO, on the other hand, was suggested to penetrate only to a low extent into the cell at physiological pH and therefore mainly protects against $^1O_2$ damages in the cell membrane [2]. Thus, it is possible that the sensing of $^1O_2$ or its effect leading to the induction of the *Gpxh* expression is locally different in the cell from the site where the toxic action of RB is taking place. Additionally, different target molecules of $^1O_2$ have very different quenching efficiencies for $^1O_2$ produced in the cell [27, 28]. Therefore, $^1O_2$ dependent activation of the cellular sensor responsible for the *Gpxh* expression might occur with a much higher efficiency than the modification of the toxic site. Thus, the quenching efficiency of DABCO and L-His for $^1O_2$ may be too low to significantly reduce the activation of the sensor, whereas it may successfully compete with a lower quenching efficiency of the component responsible for the toxic action of $^1O_2$. Different localization and/or quenching efficiency of the photosensitizer, the targets and the quenchers may explain the converse effect of DABCO and L-His on the toxicity and the *Gpxh* expression caused by RB.

NR-induced Cellular Response

NR has been shown to act as a type I photosensitizer [36, 47, 48] but also to uncouple ATP synthesis from photosynthetic electron transport and to inhibit the electron transport chain [39]. Even though the toxic effect of NR to the cell is unknown, the light dependent reduction of growth rate indicates that a photosensitized process is responsible for the NR-toxicity (Fig. 1a). Therefore, two different mechanisms may be involved; In a type I mechanism the excited NR could directly interact with cellular components in an electron transfer reaction and thus damage essential cellular functions. This is supported by the strong induction of the oxidative stress gene GST and the HSP gene, which both were also induced by H_2O_2, indicating that a strong intracellular stress caused by a disturbed redox balance at low NR concentrations occurred (Fig. 3b and c).

The second possible mechanism for a light dependent toxicity of NR could involve an increased formation of $^1O_2$ in the chloroplast, as measured in NR-treated isolated spinach thylakoids by EPR-spin trapping, with an enhanced generation of $^1O_2$ already at low NR concentrations (Fig. 4). These levels of $^1O_2$ produced in the thylakoid exposed to NR might still be underestimated, because only $^1O_2$ which diffused out of thylakoids is measured with the spin trapping. Unfortunately, we cannot measure directly the production of $^1O_2$ by NR in intact *C. reinhardtii* cells by chemical trapping techniques (data not shown). The induction of the *Gpxh* gene by NR and the stimulating effect of D_2O-medium compared to normal growth medium suggesting that $^1O_2$ is indeed produced in *C. reinhardtii* cells exposed to NR and that $^1O_2$ is mediating the concomitant response of the *Gpxh* gene (Fig. 3a). Since the expression of the *Gpxh* gene increased linearly with the concentration of RB in the non-toxic range, we assume a proportional induction of *Gpxh* to the amount of $^1O_2$ formed (Fig. 1b and 2a). As a consequence the increased expression of *Gpxh* at 200 compared to 100 μmol m^{-2} s^{-1} illumination at non-toxic NR concentrations (0.5 μM) (Fig. 1b)

is an indirect evidence for a light intensity dependent generation of $^1O_2$ by NR in *C. reinhardtii* cells. Still, the mechanism of the increased $^1O_2$ generation by NR in the thylakoids is unknown: NR might have a type II activity under certain conditions as found in the thylakoid lumen or it might increase the production of $^1O_2$ by charge recombination, as described for phenolic herbicides like DT [18, 42]. However, no significant reduction of the linear electron transport in NR-treated *C. reinhardtii* cultures or in isolated spinach thylakoids was measured with an O_2-electrode at concentrations below 20 µM (Tab. 1). Similar results were also obtained by Opanasenko et al. in isolated pea chloroplasts [39]. The authors hypothesized that a NR-caused inhibition of photosynthetic O_2 evolution may occur already at low concentrations but is compensated by a stimulating effect of NR on the electron transport chain by acting as an uncoupler similar to other amines. Indeed, an inhibitory effect of low NR concentrations was measured on the yield of variable chlorophyll fluorescence in dark adapted *C. reinhardtii* cell (Tab. 2). Interestingly, DABCO has also been shown to uncouple electron transport in thylakoids like other amines [43]. A synergistic uncoupling effect of NR and DABCO could therefore be responsible for the increased toxicity found by the combination of the two substances (Fig. 2b).

Similar to RB response, no direct effect on the *Gpxh* expression by NR could be measured by the $^1O_2$ quencher DABCO and L-His indicating that these quenchers have no access to the $^1O_2$ molecules involved in the response (Fig. 2b). This suggests that the sensor for *Gpxh* induction by $^1O_2$ is located close to the source of $^1O_2$ in NR-treated cell, i.e. in the thylakoid membrane where $^1O_2$ is produced by charge recombination and energy transfer. Further support for the localization of the $^1O_2$ sensor in the thylakoids is given by the similar induction strength achieved by the relatively low amount of $^1O_2$ produced by NR in the thylakoids compared to the high yield of $^1O_2$ produced by RB (Fig. 1 and 4). So far, no sensor for $^1O_2$ was characterized in the chloroplast, even though a strong genetic response to $^1O_2$ was also found in

Arabidopsis where a set of 70 genes was specifically induced in proto-chlorophyllide accumulating mutant exposed to light [38]. In this manuscript, the authors speculate that the $^1O_2$-dependent modification of lipids, resulting in the formation of lipid peroxides, might trigger the stress response similar to the generation of ceramide in mammalian cells causing the activation of the transcription factor AP-2 [19].

We have strong evidence that NR causes several unrelated effects to *C. reinhardtii* cells, which result in a light dependent toxicity and the strong induction of various stress response genes including the $^1O_2$-dependent *Gpxh* induction. RB on the other side, only strongly induced the *Gpxh* gene by the formation of $^1O_2$ and also its toxicity could be linked to $^1O_2$ effects. This shows that the genetic response of an organism to a pollutant already at low concentrations can give a lot of important information about the specificity and severity of its harmful action, on condition that the mechanisms responsible for the induction of the selected genes are well characterized. Unravelling these mechanisms builds the basis for the development of molecular biosensors and for the interpretation of genetic expression data.

REFERENCES

1. Allen MT, Lynch M, Lagos A, Redmond RW, Kochevar IE: A Wavelength Dependent Mechanism for Rose Bengal-Sensitized Photoinhibition of Red-Cell Acetylcholinesterase. Biochimica Et Biophysica Acta 1075: 42-49 (1991).
2. Anderson RF, Patel KB: The effect of 1,4-diazabicyclo[2.2.2.]octane on the radiosensitivity of bacteria. Photochem Photobiol 28: 881-5 (1978).
3. Arnon DI: Copper Enzymes in Isolated Chloroplasts - Polyphenoloxidase in Beta-Vulgaris. Plant Physiology 24: 1-15 (1949).
4. Aro EM, Virgin I, Andersson B: Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113-34 (1993).
5. Aveline BM, Redmond RW: Can cellular phototoxicity be accurately predicted on the basis of sensitizer photophysics? Photochem Photobiol 69: 306-16 (1999).
6. Aveline BM, Sattler RM, Redmond RW: Environmental effects on cellular photosensitization: correlation of phototoxicity mechanism with transient absorption spectroscopy measurements. Photochem Photobiol 68: 51-62 (1998).
7. Barber J: The Photosystems : structure, function, and molecular biology. Elsevier ;Sole distributors for the USA and Canada Elsevier Science Pub. Co., Amsterdam ; New York New York, NY, USA (1992).
8. Barber J, Andersson B: Too much of a good thing: light can be bad for photosynthesis. Trends Biochem Sci 17: 61-6 (1992).
9. Baroli I, Gutman BL, Ledford HK, Shin JW, Chin BL, Havaux M, Niyogi KK: Photo-oxidative Stress in a Xanthophyll-deficient Mutant of Chlamydomonas. J Biol Chem 279: 6337-6344 (2004).
10. Bilski P, Belanger AG, Chignell CF: Photosensitized oxidation of 2 ',7 '-dichlorofluorescin: Singlet oxygen does not contribute to the formation of fluorescent oxidation product 2 ',7 '-dichlorofluorescein. Free Radical Biology and Medicine 33: 938-946 (2002).
11. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-9 (1987).
12. Chorney W, Scully NJ, Crespi HL, Katz JJ: The growth of algae in deuterium oxide. Biochim Biophys Acta 37: 280-7 (1960).
13. Chung SK, Jung J: Inactivation of the Acceptor Side and Degradation of the D1 Protein of Photosystem-Ii by Singlet Oxygen Photogenerated from the Outside. Photochemistry and Photobiology 61: 383-389 (1995).
14. Davies MJ: Singlet oxygen-mediated damage to proteins and its consequences. Biochem Biophys Res Commun 305: 761-70 (2003).

15. de Mol NJ, Beijersbergen van Henegouwen GM, Mohn GR, Glickman BW, van Kleef PM: On the involvement of singlet oxygen in mutation induction by 8-methoxypsoralen and UVA irradiation in Escherichia coli K-12. Mutat Res 82: 23-30 (1981).

16. Eggen RI, Behra R, Burkhardt-Holm P, Escher BI, Schweigert N: Challenges in ecotoxicology. Environ Sci Technol 38: 58A-64A (2004).

17. Foote CS: Definition of Type-I and Type-II Photosensitized Oxidation. Photochemistry and Photobiology 54: 659-659 (1991).

18. Fufezan C, Rutherford AW, Krieger-Liszkay A: Singlet oxygen production in herbicide-treated photosystem II. FEBS Lett 532: 407-10 (2002).

19. Grether-Beck S, Bonizzi G, Schmitt-Brenden H, Felsner I, Timmer A, Sies H, Johnson JP, Piette J, Krutmann J: Non-enzymatic triggering of the ceramide signalling cascade by solar UVA radiation. Embo J 19: 5793-800 (2000).

20. Harris EH: The Chlamydomonas sourcebook : a comprehensive guide to biology and laboratory use. Academic Press, San Diego (1989).

21. Hellio C, Le Gal Y: Histidine utilization by the unicellular alga Dunaliella tertiolecta. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 119: 753-758 (1998).

22. Hellio C, Veron B, Le Gal Y: Amino acid utilization by Chlamydomonas reinhardtii: specific study of histidine. Plant Physiol Biochem 42: 257-64 (2004).

23. Hideg E, Kalai T, Hideg K, Vass I: Do oxidative stress conditions impairing photosynthesis in the light manifest as photoinhibition? Philos Trans R Soc Lond B Biol Sci 355: 1511-6 (2000).

24. Huang XD, Zeiler LF, Dixon DG, Greenberg BM: Photoinduced toxicity of PAHs to the foliar regions of Brassica napus (canola) and Cucumbis sativus (cucumber) in simulated solar radiation. Ecotoxicol Environ Saf 35: 190-7 (1996).

25. Joshi PC, Misra RB: Evaluation of chemically-induced phototoxicity to aquatic organism using Paramecium as a model. Biochem Biophys Res Commun 139: 79-84 (1986).

26. Keren N, Ohad I: State transition and photoinhibition. In: Rochaix J-D, Michel G-C, Sabeeha M (eds) The molecular biology of chloroplasts and mitochondria in chlamydomonas, pp. 569-596. Kluwer academic publishers, Dordrecht (1998).

27. Kochevar IE, Bouvier J, Lynch M, Lin CW: Influence of dye and protein location on photosensitization of the plasma membrane. Biochim Biophys Acta 1196: 172-80 (1994).

28. Kochevar IE, Lambert CR, Lynch MC, Tedesco AC: Comparison of photosensitized plasma membrane damage caused by singlet oxygen and free radicals. Biochim Biophys Acta 1280: 223-30 (1996).

29. Kochevar IE, Redmond RW: Photosensitized production of singlet oxygen. Methods Enzymol 319: 20-8 (2000).

30. Laasch H: Nonphotochemical Quenching of Chlorophyll a Fluorescence in Isolated-Chloroplasts under Conditions of Stressed Photosynthesis. Planta 171: 220-226 (1987).

31. Lambert CR, Kochevar IE: Electron transfer quenching of the rose Bengal triplet state. Photochemistry and Photobiology 66: 15-25 (1997).

32. Lambert CR, Stiel H, Leupold D, Lynch MC, Kochevar IE: Intensity-dependent enzyme photosensitization using 532 nm nanosecond laser pulses. Photochem Photobiol 63: 154-60 (1996).

33. Lee PC, Rodgers MA: Laser flash photokinetic studies of rose bengal sensitized photodynamic interactions of nucleotides and DNA. Photochem Photobiol 45: 79-86 (1987).

34. Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL: The glutathione peroxidase homologous gene from Chlamydomonas reinhardtii is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology 46: 395-408 (2001).

35. Leisinger U, Rüfenacht K, Zehnder AJB, Eggen RIL: Structure of a glutathione peroxidase homologous gene involved in the oxidative stress response in *Chlamydomonas reinhardtii*. Plant Science 149: 139-149 (1999).

36. Marks GT, Lee ED, Aikens DA, Richtol HH: Transient Photochemistry of Neutral Red. Photochemistry and Photobiology 39: 323-328 (1984).

37. Neumann NF, Galvez F: DNA microarrays and toxicogenomics: applications for ecotoxicology? Biotechnol Adv 20: 391-419 (2002).

38. op den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M, Apel K: Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 15: 2320-32 (2003).

39. Opanasenko V, Agafonov A, Demidova R: Effects of heterocyclic and tertiary permeant amines on the electron transfer in thylakoid membranes. Photosynthesis Research 72: 243-253 (2002).

40. Opanasenko VK, Semenova GA, Agafonov AV, Gubanova ON: Effect of Delta pH indicators neutral red and 9-aminoacridine on chloroplast ultrastructure. Biochemistry-Moscow 61: 1083-1087 (1996).

41. Polo L, Presti F, Schindl A, Schindl L, Jori G, Bertoloni G: Role of ground and excited singlet state oxygen in the red light-induced stimulation of Escherichia coli cell growth. Biochem Biophys Res Commun 257: 753-8 (1999).

42. Rutherford AW, Krieger-Liszkay A: Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 26: 648-53 (2001).

43. Sabat SC, Mohanty P: Characterization of aliphatic amine 1,4-diazobicyclo (2,2,2) octane-induced stimulation of beet spinach thylakoid electron transport. Indian J Biochem Biophys 29: 490-3 (1992).

44. Schreiber U, Schliwa U, Bilger W: Continuous Recording of Photochemical and Nonphotochemical Chlorophyll Fluorescence Quenching with a New Type of Modulation Fluorometer. Photosynthesis Research 10: 51-62 (1986).

45. Shrager J, Hauser C, Chang CW, Harris EH, Davies J, McDermott J, Tamse R, Zhang Z, Grossman AR: Chlamydomonas reinhardtii genome project. A guide to the generation and use of the cDNA information. Plant Physiol 131: 401-8 (2003).

46. Singh MK, Pal H, Bhasikuttan AC, Sapre AV: Photophysical properties of the cationic form of neutral red. Photochemistry and Photobiology 69: 529-535 (1999).

47. Singh MK, Pal H, Sapre AV: Interaction of the excited singlet state of neutral red with aromatic amines. Photochemistry and Photobiology 71: 300-306 (2000).

48. Singh MK, Pal H, Sapre AV: Studies on the radiolytically produced transients of neutral red: Triplet and reduced radicals. Photochemistry and Photobiology 71: 44-52 (2000).

49. Smith KC: The Science of photobiology. Plenum Press, New York (1981).

50. Song YZ, An J, Jiang L: ESR evidence of the photogeneration of free radicals (GDHB*-, O2*-) and singlet oxygen ((1)O2) by 15-deacetyl-13-glycine-substituted hypocrellin B. Biochim Biophys Acta 1472: 307-13 (1999).

51. Stiel H, Teuchner K, Paul A, Leupold D, Kochevar IE: Quantitative comparison of excited state properties and intensity- dependent photosensitization by rose bengal. J Photochem Photobiol B 33: 245-54 (1996).

52. Sueoka N: Mitotic replication of deoxyribonucleic acid in Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A 46: 83-91 (1960).

53. Telfer A, Bishop SM, Phillips D, Barber J: Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. J Biol Chem 269: 13244-13253 (1994).

54. Trebst A: Function of beta-carotene and tocopherol in photosystem II. Z Naturforsch [C] 58: 609-20 (2003).

55. Unno K, Kishido T, Morioka M, Okada S, Oku N: Increased expression of Hsp70 for resistance to deuterium oxide in a yeast mutant cell line. Biol Pharm Bull 26: 799-802 (2003).

56. Williams AJ, Morse AT, Stuart RS: Production of green algae in deuterium oxide. Can J Microbiol 12: 1167-73 (1966).

4. The Glutathione Peroxidase Homologous Gene *Gpxh* in *Chlamydomonas reinhardtii* is Upregulated During Photoinhibition Presumably by Two Different Signals

ABSTRACT

The expression of the glutathione peroxidase homologous gene *Gpxh*, known to be specifically induced by the formation of singlet oxygen ($^1O_2$), was analyzed in *Chlamydomonas reinhardtii* upon exposure to environmental conditions causing photoinhibition. Illumination with high light intensities, leading to increased formation of $^1O_2$ in the photosystem II, continuously induced the expression of *Gpxh* for at least two hours, whereas the expression of a *Gpxh*-arylsulfatase reporter gene construct only was upregulated for one hour. Phenolic herbicides like dinoterb, increasing the rate of $^1O_2$ formation in the thylakoid membranes, further stimulated the high light-induced *Gpxh* expression in *C. reinhardtii*. The expression of the reporter gene construct, on the other hand, was not increased by dinoterb in these samples. DCMU, a urea herbicide which reduces the $^1O_2$ generation in the photosystem II, also lowered the expression of both the *Gpxh* gene and the reporter gene construct, compared to untreated cells exposed to high light intensities. These data show that the *Gpxh* expression is induced by environment conditions causing photoinhibition presumably by increased formation of $^1O_2$. However, the difference between the expression of the *Gpxh* gene and the *Gpxh*-arylsulfatase reporter gene indicates the presence of a second regulatory mechanism involved in the response of *Gpxh* to high light exposure not affecting the reporter gene construct.

INTRODUCTION

Photosynthetic organisms are frequently confronted with reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). ROS can be produced during normal cell metabolisms but they emerge to increased levels by exposure to harsh environmental conditions such as high light illumination, low temperature or drought conditions [3, 22, 28]. This can result in a cellular oxidative stress and serious damage to proteins, lipids and the DNA [11]. Aerobic organisms have evolved efficient defense mechanisms to either prevent the formation of ROS, to remove them from the cell or to repair damaged components [3, 4, 16, 22, 24, 32]. Several signals have been described to trigger the response of these defense mechanisms to the various stress conditions. One mechanism involves the direct activation of the stress response by the ROS and the expression of genes coding for glutathione peroxidases or ascorbate peroxidases has been shown to be induced by H_2O_2 and $O_2^{\cdot-}$ in several plants [3, 23, 34]. Recently, several articles have reported the specific induction of genes by the formation of $^1O_2$ including several genes in *Arabidopsis* and a glutathione peroxidase homologous gene (*Gpxh*) in *Chlamydomonas reinhardtii* [21, 25]. The *Gpxh* gene from *C. reinhardtii* was strongly induced by $^1O_2$ but only to a minor extent by $O_2^{\cdot-}$, H_2O_2 or organic hydroperoxides [5]. In that study, however, $^1O_2$ was artificially produced by exogenous photosensitizers, either directly by the type II photosensitzer rose bengal (RB) or indirectly by the type I photosensitizer neutral red (NR), which affected the photosynthetic apparatus resulting in the formation of $^1O_2$ [6]. The induction of the *Gpxh* gene by environmental conditions which lead to the increased formation of ROS and oxidative stress has not been examined so far.

In nature, $^1O_2$ in photosynthetic organisms is mainly produced during high light illumination when the rate of charge separation in the reaction center of photosystem II (PSII) exceeds the capacity of the electron transport chain to accept electrons [13, 14, 30]. Back electron flow and charge recombination in the reactive center results in the formation of a triplet chlorophyll and in the generation of $^1O_2$ in the thylakoids [26, 33]. High levels of $^1O_2$ cause the degradation of the D1 protein in the PSII and the concomitant disassembling of the PSII during photoinhibition [1, 16]. Certain herbicides specifically inhibit the reduction of the second plastoquinone (Q_B) at the Q_B-site and by that block the electron transport chain already at low light intensities. This includes phenolic herbicides like bromoxynil or dinoterb which lower the redox potential of the Q_A/Q_A^- redox couple and thus enhance the charge recombination and $^1O_2$ formation [20, 26]. The urea herbicide DCMU, on the other hand, rather prevents charge recombination reactions and the formation of $^1O_2$ by raising the potential of the Q_A/Q_A^- redox couple and reducing back electron flow in the PSII. Both, phenolic and urea herbicides can strongly influence the rate of $^1O_2$ generation in the PSII under photoinhibitiory conditions as shown with EPR-spin trapping in isolated spinach thylakoids [7].

In this study we examined the induction of the *Gpxh* gene and a *Gpxh*-arylsulfatase (*Gpxh-Ars*) reporter gene construct upon exposure to high light intensities, a condition leading to the increased formation of $^1O_2$ under natural conditions. In addition the effect of the phenolic herbicide dinoterb and the urea herbicide DCMU on the expression of the *Gpxh* and the reporter gene was tested. The data clearly show that the expression of the *Gpxh* and the *Gpxh-Ars* reporter gene is regulated by the formation of $^1O_2$ during exposure to photoinhibitory treatments. Additionally, a second $^1O_2$-independent signal, not influencing the *Gpxh-Ars* expression, seems to be involved in the regulation of

the *Gpxh* mRNA level by high light exposure. A model for the response mechanism of *Gpxh* to high light conditions is proposed.

MATERIALS AND METHODS

Strains and Culture Conditions

C. reinhardtii strain $cw_{15}arg_7mt^-$ (CC-1618), containing the plasmid pASPro1 [21], was inoculated in Tris-Acetate-Phosphate-medium (TAP) [12] in Erlenmeyer flasks and agitated on a rotatory shaker (150 rpm) under constant illumination (120 µmol m^{-2} s^{-1} PAR) at 25°C. All media were supplemented with 50 mg/l ampicillin.

Stress Treatment

A culture of strain $cw_{15}arg_7mt^-$ containing the reporter gene construct pASPro1 was grown to a cell density of 6×10^6 cells/ml and divided into 50 ml subcultures. One subculture was exposed to high light intensities (3000 µmol m^{-2} s^{-1} PAR) at 25°C with either 0.1 µM DCMU, 30 µM dinoterb or without treatment and 15 ml samples were taken at the time point indicated. In parallel the other subcultures were exposed to 120 µmol m^{-2} s^{-1} white light at 25°C with the same herbicide concentrations or without treatment as a control.

RNA Isolation and Real-time RT-PCR

For RNA isolation, cells of 15 ml of individual cultures were harvested by centrifugation and total RNA was isolated by the acid guanidine isothiocyanate-phenol-chloroform method [2] using TRIzol Reagent (Life Technologies Ltd.) following the suppliers instructions. Sample concentration was adjusted to

3 µg/µl total RNA and RNA quality was checked by agarose gel electrophoreses and ethidium bromide staining.

For reverse transcription, 1 µg of DNase I-treated total RNA was incubated in a 50 µl reaction including 10 µl of a 5× reaction buffer (Invitrogen™), 2 µl oligo(dT)$_{18}$ primer (0.1 mM), 5 µl dNTP (5 mM), 5 µl 0.1M dithiothreitol (DTT) and 300 units SuperScript™ reverse transcriptase (Invitrogen™) for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min and the volume was adjusted to a concentration of 20 ng/µl of original RNA quantity for each sample.

Sequences of the primers for real-time RT-PCR were designed with the Primer Express™ software (Applied Biosystems) using the sequence of the 3` un-translated region of each gene as a template. Real-time RT-PCR reactions were performed on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems) using the SYBR® Green PCR Master Mix kit as recommended by the manufacturer. Primer concentrations were optimized for each gene. In order to evaluate unspecific amplification by primer dimers or contaminations, annealing profiles of PCR products were analyzed and control reactions without cDNA were performed. Threshold cycle (C_t) values were determined for all reactions in the logarithmic amplification phase, and the average C_t value was calculated for each sample out of three replicates. The C_t value of the gene coding for the Rubisco small subunit (*RbcS2*) was used for normalization. Average induction factors for the different conditions were calculated for each gene as suggested by the manufacturer, out of three independent experiments.

RESULTS

Induction of the *Gpxh* Gene by Photoinhibitory Treatment

The *Gpxh* gene in *C. reinhardtii* was shown to be specifically induced by photooxidative stress caused by exogenous photosensitizers in the light [5, 6, 21]. To examine the response of the *Gpxh* gene to natural photooxidative stress conditions we exposed a culture of strain $cw_{15}arg_7mt^-$ pASPro1 to high light intensities of 3000 μmol m^{-2} s^{-1}. After 20, 60 and 120 min samples were taken, total RNA was isolated and the *Gpxh* mRNA was quantified with real-time RT-PCR. In parallel, a control culture was kept at low light intensities (120 μmol m^{-2} s^{-1}), samples were taken after 20, 60 and 120 min and used to calculate induction factors for each time point. In high light samples, the level of *Gpxh* mRNA continuously increased between 20 and 120 min reaching a 70 fold higher level than the control after 120 min of exposure, whereas the *Tub2B* gene, used as a control, was not altered in its expression by the treatment (Fig. 1a).

Some herbicides can either stimulate or repress the process of photoinhibiton in photosynthetic organism exposed to high light intensities as described in the introduction [26]. Cultures of the *C. reinhardtii* strain $cw_{15}arg_7mt^-$ pASPro1 were therefore exposed to the phenolic herbicide dinoterb, known to enhance charge recombination resulting in an increased formation of $^1O_2$ [7]. In agreement with that, we found a two fold stimulation of the *Gpxh* expression in the presence of 30 μM dinoterb in cells exposed to high light intensities (3000 μmol m^{-2} s^{-1}) for 60 min (Fig. 1b). As expected, a stimulation of the *Gpxh* induction was already detected at low light intensities (120 μmol m^{-2} s^{-1}). However, this was only slightly higher than the small induction by the herbicide

observed in the dark sample and can therefore not be undoubtedly be linked to increased $^1O_2$ formation under this light condition.

The urea herbicide DCMU, which was shown to reduce charge recombination and $^1O_2$ formation in the reactive center of PSII, did not show any significant effect on the *Gpxh* mRNA level at low light intensities or under the dark condition (Fig. 1b). However, in cells exposed to high light conditions, the presence of DCMU reduced the *Gpxh* expression to less than 50% of the induction without herbicide. The expression of the *Tub2B* genes was not altered significantly by the treatments indicating that no global change in the gene expression was caused by the different conditions (Fig. 1b).

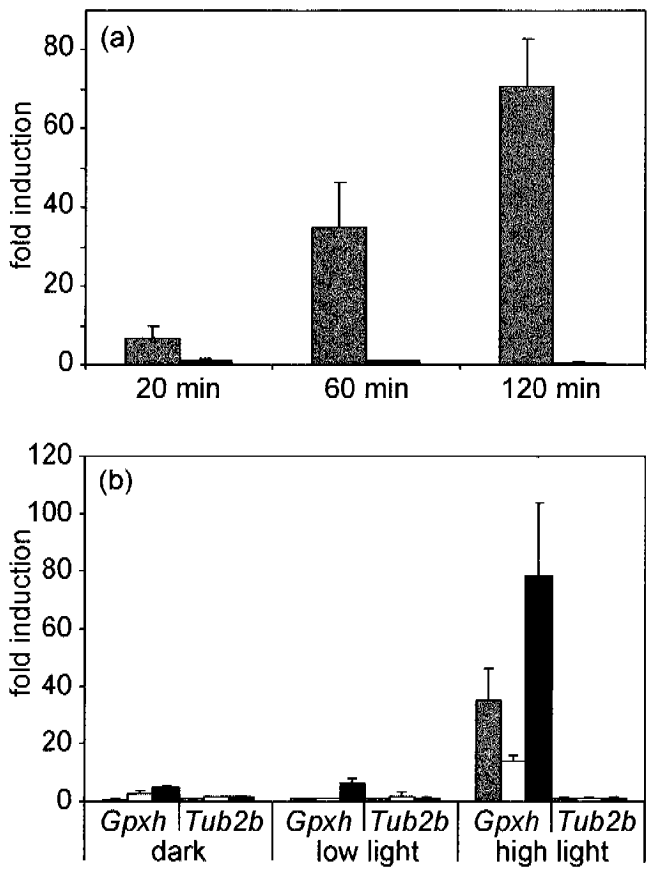


Figure 1: (a) The mRNA levels of the *Gpxh* gene (gray boxes) and the *Tub2B* gene (black boxes) in *C. reinhardtii* were analyzed by high light illumination (3000 µmol m^{-2} s^{-1}) for 20, 60 or 120 min in three independent experiments. (b) Cultures of $cw_{15}arg_7mt^-$ pASPro1 incubated either with 30 µM dinoterb (black boxes), 0.1 µM DCMU (white boxes) or without any treatment (gray boxes) were exposed to the dark, to low light (120 µmol m^{-2} s^{-1}) or to high light (3000 µmol m^{-2} s^{-1}) intensities and the expression of the *Gpxh* and the *Tub2B* gene was analyzed after 60 min with real-time RT-PCR in three independent experiments.

A *Gpxh*-arylsulfatase Reporter Gene Construct Showed an Expression Profile Different to *Gpxh* in High Light Exposed Cells

In earlier studies, a *Gpxh-Ars* reporter gene construct was used to show the transcriptional activation of the *Gpxh* promoter by $^1O_2$ produced by exogenous photosensitizers [21]. To test whether the increase of the *Gpxh* mRNA level in cell exposed to high light is also caused by transcriptional activation, the expression of the *Gpxh-Ars* was analyzed with real-time RT-PCR under the same light conditions as used before for the *Gpxh* mRNA level. Upon exposure to $^1O_2$, produced by NR or RB, the *Gpxh-Ars* mRNA level is 1.5 fold higher than the *Gpxh* mRNA level in the same RNA sample (data not shown). However, the induction factors were not significantly different between *Gpxh* and the reporter gene after 20 to 60 min exposure to high light and after 120 min the *Gpxh-Ars* was even three fold lower induced than the *Gpxh* gene (Fig. 2). Additionally, we analyzed the induction of the *Gpxh-Ars* in the herbicide treated sample. Surprisingly, the *Gpxh-Ars* reporter gene was induced only 20 fold by dinoterb after 60 min of illumination with high light, indicating a 50% lower mRNA level compared to the sample without herbicide (Fig. 2). DCMU, on the contrary, caused the same induction of the *Gpxh-Ars* and the *Gpxh* gene after 60 min of exposure to 0.1 µM DCMU (Fig. 2).

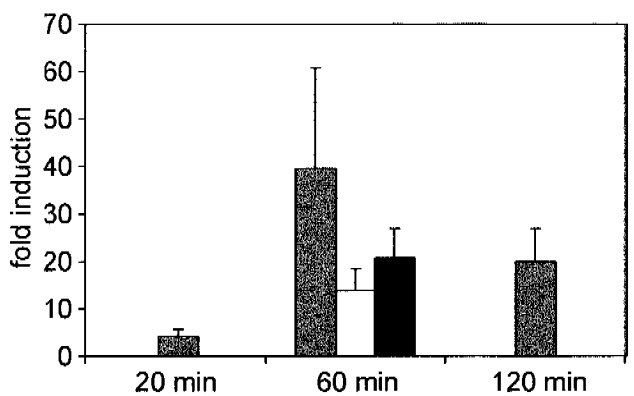


Figure 2: The expression of the *Gpxh-Ars* mRNA was analyzed in cells exposed to high light intensities for 20, 60 and 120 min (gray boxes) or in cells incubated with either 30 µM dinoterb (black box) or 0.1 µM DCMU (white box) for 60 min under high light illumination in three independent experiments.

DISCUSSION

The induction of the *Gpxh* gene, known to be specifically induced by $^1O_2$ produced by exogenous photosensitizers [6], was investigated in *C. reinhardtii* exposed to treatments causing photoinhibition. We could show that the *Gpxh* expression is induced by photooxidative stress due to harsh environmental conditions such as illumination with high light intensities (Fig. 1a). *Gpxh* expression was further stimulated by the presence of the phenolic herbicide dinoterb, known to enhance the rate of charge recombination in the reactive center of PSII and to increase the production of $^1O_2$ in the thylakoids (Fig. 1b) [26]. DCMU, a urea herbicide, lowered the $^1O_2$ generation in thylakoids exposed to high light illumination [7]. In agreement with that, DCMU-exposed cells had a significantly reduced *Gpxh* mRNA level, compared to untreated cells after 60 min of high light illumination. This strongly suggests that the generation of $^1O_2$ during photoinhibition is indeed a signal to regulate the expression of nuclear genes, in particular the *Gpxh* gene. Interestingly, the kinetics of the *Gpxh* expression by high light treatment, continuously increasing between 20 and 120 min of illumination (Fig. 1a), was different to the profiles caused by RB and NR in normal light which showed a strong increase already after 20 min and reached the maximal induction after 60 min of exposure [5]. This indicates that the formation of $^1O_2$ occurs faster in NR and RB-treated cells than in cells exposed to high light illumination. The delayed response to high light exposure compared to the photosensitizer-caused induction is in agreement with the presence of various protection mechanisms to prevent the formation of ROS in the thylakoid membrane [31, 32]. The fast activation of the xanthophyll cycle and the ΔpH depending quenching of the chlorophyll triplet state reduce the formation of $^1O_2$ in the early phase of high light illumination [19, 24]. In addition the cells regulate the absorption of light by the PSII by controlling the distribution of the

light harvesting complex between the PSI and PSII during state transition to minimize a disturbance of the electron flow in the photosystems [10, 16]. All these protective mechanisms can, however, not prevent the formation of $^1O_2$ in the PSII after prolonged illumination with high light intensities [13, 15].

Even though the response of the *Gpxh* gene to $^1O_2$ was shown to be regulated at the transcriptional level [21], the *Gpxh-Ars* mRNA level was lower in the high light samples than expected from the relationship of the *Gpxh* and *Gpxh-Ars* expression in NR and RB-treated cells (Fig. 2). The expression of the *Gpxh-Ars* even decreased again after 60 min of exposure and was lower in dinoterb-treated samples than in the high light induced samples what is in contradiction to the strong stimulation of the *Gpxh* response by dinoterb. Two scenarios are considered to explain this effect, which both predict two signals involved in the *Gpxh* expression upon high light exposure. The involvement of multiple mechanisms in the activation of one gene, e.g. the APX genes, by high light exposure was already described in *Arabidopsis*, requiring both the increased formation of ROS and a change in the plastoquinone redox state for full induction [23]. We suggest the following two models for *Gpxh* and *Gpxh-Ars* induction by the different conditions tested:

1) In cells exposed to high light illumination an increased level of $^1O_2$ is produced 10 to 20 min after exposure [13]. This induces the expression of the *Gpxh* gene and the *Gpxh-Ars* construct via activation of a specific $^1O_2$ sensor resulting in the binding of a transcription factor to a known DNA element in the *Gpxh* promoter [21]. As a consequence the mRNA levels of both the native and the reporter gene increase (Fig. 3, dark gray lines). After about 60 to 80 min, all PSII are strongly damaged and no further $^1O_2$ can be produced. However, the increase in *Gpxh* mRNA level continues (dark gray full line), suggesting the involvement of two independent signals in *Gpxh* expression. One is upregulated specifically by $^1O_2$ and would result in a *Gpxh* mRNA level (dark gray dotted

line), which is expected to be 1.5 fold lower than the *Gpxh-Ars* mRNA level (dark gray dashed line). The second, unknown signal further stimulates the *Gpxh* expression upon exposure to high light intensities by a second signaling and activating mechanism (difference between the dark gray dotted and full line) and is responsible for the ongoing increase of the *Gpxh* mRNA level after the $^1O_2$ generation has stopped. The expression of the *Gpxh-Ars* construct on the other hand, only reacts to direct formation of $^1O_2$ and is reduced again after 60 min due to lowered $^1O_2$ levels. Thus, the *Gpxh-Ars* mRNA level decreases (dark gray dashed line). This suggests the presence of additional regulatory elements in the *Gpxh* promoter, upstream from the DNA fragment cloned in the reporter gene construct, which respond to the second unknown signal. In dinoterb treated cells the formation of $^1O_2$ is strongly enhanced, reaching the critical level causing the inactivation of all PSII already after 20 to 30 min of illumination. Therefore, the expression of the *Gpxh-Ars* may be strongly induced after 30 min exposure but then decreases again to reach a residual mRNA level of only 20 fold control level after 60 min (black dashed line). The *Gpxh* expression, if induced only by $^1O_2$, would also decrease after the inactivation of all PSII (black dotted line). The *Gpxh* expression, however, further increases up to an 80 fold induction after 60 min due to the putative second and still active signal (black full line). In DCMU treated cells the production of $^1O_2$ under high light illumination is low and does not result in the loss of all active PSII. Both signals are therefore continuously active up to 120 min, inducing the expression of the *Gpxh* gene (bright gray full line) and the *Gpxh-Ars* construct (bright gray dashed line) to a similar strength.

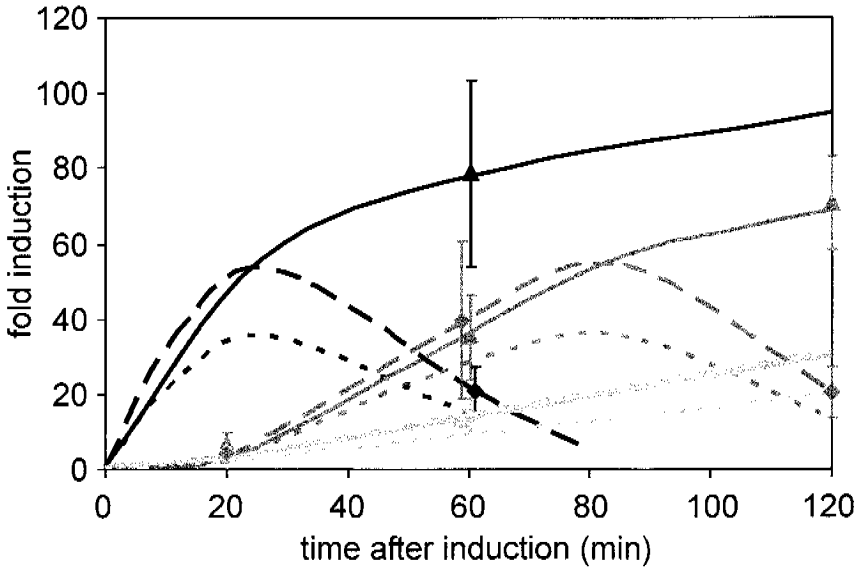


Figure 3: Model for the expression of the *Gpxh* gene (triangles) and the *Gpxh-Ars* reporter gene construct (diamonds) due to the exposure to high light intensities in the absence (dark gray) or the presence of the herbicides dinoterb (black) or DCMU (bright gray). The lines describe the hypothetical *Gpxh* mRNA level (full lines), the *Gpxh* mRNA level when the gene was only induced by the $^1O_2$ mediated signal (dotted lines) and the *Gpxh-Ars* mRNA level (dashed lines) (see discussion for further details).

2.) The second model involves the combination of a $^1O_2$-dependent transcriptional activation of the *Gpxh* promoter and a mRNA stabilization mechanism resulting in the same mRNA profiles as proposed for model 1 (Fig. 3). Therefore, a stabilization element in the *Gpxh* mRNA would prevent a fast decrease of the mRNA level after prolonged exposure to high light when the induction by $^1O_2$ is reduced again due to lower $^1O_2$ generation. However, the induction of *Gpxh* transcription does not stop completely since a small increase of the *Gpxh* mRNA level is still present after the loss of most PSII activity. The *Gpxh-Ars* mRNA is not expected to contain such a stabilization element. Fast degradation results in a net decrease of the *Gpxh-Ars* mRNA level even though the transcription may still be partially induced as considered for the *Gpxh* expression. However, to proof one of these models several additional measurements of *Gpxh* and *Gpxh-Ars* mRNA levels and stabilities at different

time points between 20 and 120 min have to be analyzed for each exposure condition. Additionally, the presence of the second regulatory mechanism, either including an enhancer element more than 1.3 kb upstream of the transcription start site in the *Gpxh* promoter or a stabilization element in the *Gpxh* mRNA has to be tested.

Because the lifetime of $^1O_2$ is very low in cellular systems (~100 ns) the sensor for the $^1O_2$-mediating transcriptional activation of the *Gpxh* promoter has to be located close to the source of $^1O_2$ in the thylakoids [17, 18, 27]. The relatively fast reduction of *Gpxh-Ars* expression after the stop of $^1O_2$ production indicates that this sensor only is active in the presence of $^1O_2$ and is immediately deactivated again when $^1O_2$ levels decrease. The second signal, on the other hand, proposed to be involved in the "late" *Gpxh* induction, might either be a direct response to high light intensities or a secondary effect of the formation of $^1O_2$ which is lasting longer then its source, the $^1O_2$ production. This might be a component of the damaged PSII or the organic hydroperoxides formed by lipid peroxidation, a well-known effect of increased levels of $^1O_2$ in the cell [8, 29]. Interestingly, the induction of *Gpxh* by organic hydroperoxides, which, similar to high light exposure, showed a rather late response compared to NR and RB exposure, was also missing for the *Gpxh-Ars* construct indicating that the same unknown mechanism could be involved in response to organic hydroperoxides and to high light exposure [21]. In mammalian cells the formation of ceramide from sphingomyelin by UVA was shown to activate the AP-2 dependent transcription [9]. Thus, lipid peroxides might be good candidates for signal molecules inducing the second, unknown activation mechanism responsible for the *Gpxh* expression during high light exposure [8, 18, 27].

REFERENCES

1. Barber J, Andersson B: Too much of a good thing: light can be bad for photosynthesis. Trends Biochem Sci 17: 61-6 (1992).
2. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-9 (1987).
3. Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F: Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci 57: 779-95 (2000).
4. Dixon DP, Lapthorn A, Edwards R: Plant glutathione transferases. Genome Biol 3: REVIEWS3004 (2002).
5. Fischer BB, Krieger-Liszkay A, Eggen RIL: Oxitative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different genetic responses in Chlamydomonas reinhardtii. (2004a) submitted.
6 Fischer BB, Krieger-Liszkay A, Eggen RIL: Unraveling the Molecular Mechanisms behind the Response of Chlamydomonas reinhardtii to Type I (Neutral Red) and Type II (Rose Bengal) Photosensitizers. (2004b) submitted.
7. Fufezan C, Rutherford AW, Krieger-Liszkay A: Singlet oxygen production in herbicide-treated photosystem II. FEBS Lett 532: 407-10 (2002).
8. Girotti AW: Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B 63: 103-13 (2001).
9. Grether-Beck S, Bonizzi G, Schmitt-Brenden H, Felsner I, Timmer A, Sies H, Johnson JP, Piette J, Krutmann J: Non-enzymatic triggering of the ceramide signalling cascade by solar UVA radiation. Embo J 19: 5793-800 (2000).
10. Haldrup A, Jensen PE, Lunde C, Scheller HV: Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci 6: 301-5 (2001).
11. Halliwell B, Gutteridge JMC: Free radicals in biology and medicine. Clarendon Press ; Oxford University Press, Oxford, New York (1999).
12. Harris EH: The Chlamydomonas sourcebook : a comprehensive guide to biology and laboratory use. Academic Press, San Diego (1989).
13. Hideg E, Kalai T, Hideg K, Vass I: Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry 37: 11405-11 (1998).
14. Hideg E, Kalai T, Hideg K, Vass I: Do oxidative stress conditions impairing photosynthesis in the light manifest as photoinhibition? Philos Trans R Soc Lond B Biol Sci 355: 1511-6 (2000).

15. Hideg E, Spetea C, Vass I: Singlet Oxygen Production in Thylakoid Membranes During Photoinhibition as Detected by Epr Spectroscopy. Photosynthesis Research 39: 191-199 (1994).
16. Keren N, Ohad I: State transition and photoinhibition. In: Rochaix J-D, Michel G-C, Sabeeha M (eds) The molecular biology of chloroplasts and mitochondria in chlamydomonas, pp. 569-596. Kluwer academic publishers, Dordrecht (1998).
17. Klotz LO: Oxidant-induced signaling: effects of peroxynitrite and singlet oxygen. Biol Chem 383: 443-56 (2002).
18. Kochevar IE: Singlet oxygen signaling: from intimate to global. Sci STKE 2004: pe7 (2004).
19. Krieger A, Moya I, Weis E: Energy-Dependent Quenching of Chlorophyll-a Fluorescence - Effect of Ph on Stationary Fluorescence and Picosecond- Relaxation Kinetics in Thylakoid Membranes and Photosystem-Ii Preparations. Biochimica Et Biophysica Acta 1102: 167-176 (1992).
20. Krieger A, Rutherford AW, Vass I, Hideg E: Relationship between activity, D1 loss, and Mn binding in photoinhibition of photosystem II. Biochemistry 37: 16262-9 (1998).
21. Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL: The glutathione peroxidase homologous gene from Chlamydomonas reinhardtii is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology 46: 395-408 (2001).
22. Mittler R: Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405-10 (2002).
23. Mullineaux P, Ball L, Escobar C, Karpinska B, Creissen G, Karpinski S: Are diverse signalling pathways integrated in the regulation of arabidopsis antioxidant defence gene expression in response to excess excitation energy? Philos Trans R Soc Lond B Biol Sci 355: 1531-40 (2000).
24. Niyogi KK: Safety valves for photosynthesis. Curr Opin Plant Biol 3: 455-60 (2000).
25. op den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M, Apel K: Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 15: 2320-32 (2003).
26. Rutherford AW, Krieger-Liszkay A: Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 26: 648-53 (2001).
27. Ryter SW, Tyrrell RM: Singlet molecular oxygen ($^1O_2$): a possible effector of eukaryotic gene expression. Free Radic Biol Med 24: 1520-1534 (1998).
28. Scandalios JG: The rise of ROS. Trends Biochem Sci 27: 483-6 (2002).
29. Stratton SP, Liebler DC: Determination of singlet oxygen-specific versus radical-mediated lipid peroxidation in photosensitized oxidation of lipid bilayers: effect of beta-carotene and alpha-tocopherol. Biochemistry 36: 12911-20 (1997).

30. Telfer A, Bishop SM, Phillips D, Barber J: Isolated phostosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. J Biol Chem 269: 13244-13253 (1994).

31. Trebst A: Function of beta-carotene and tocopherol in photosystem II. Z Naturforsch [C] 58: 609-20 (2003).

32. Trebst A, Depka B, Hollander-Czytko H: A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in Chlamydomonas reinhardtii. FEBS Lett 516: 156-60 (2002).

33. Vass I, Styring S, Hundal T, Koivuniemi A, Aro E, Andersson B: Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced QA species promote chlorophyll triplet formation. Proc Natl Acad Sci U S A 89: 1408-12 (1992).

34. Vranova E, Inze D, Van Breusegem F: Signal transduction during oxidative stress. J Exp Bot 53: 1227-36 (2002).

5. A CRE/AP-1 Homologous Element in the *Gpxh* Promoter of *Chlamydomonas reinhardtii* is a Functional Transcription Factor Binding Site Mediating the Response to Singlet Oxygen

ABSTRACT

In *Chlamydomonas reinhardtii*, the glutathione peroxidase homologous gene *Gpxh* is specifically induced by high intracellular levels of singlet oxygen ($^1O_2$), produced either by exogenous photosensitizers or by environmental conditions causing photoinhibition. A 8 bp sequence element, homologous to the consensus sequences of the cAMP responsive element (CRE) and the activator protein-1 (AP-1) binding site of mammalians, was shown to be required for activation by $^1O_2$ [33]. Here, we show that this 8 bp element is indeed a functional transcription factor binding site which is specifically recognized by a DNA binding complex and is sufficient to introduce partial $^1O_2$–dependent induction into a β-tubulin promoter. A role of a 16 bp palindrome, overlapping the 8 bp element, in the response to $^1O_2$ could be excluded. Point mutations revealed an essential cytosine at position four of the 8 bp element which is part of a strong conserved TGAC motif of many other transcription factor binding sites, whereas mutations in the 3`part of the element caused minor effects on the DNA binding affinity and activation of transcription. Experiments with chemicals affecting the cyclic AMP signaling pathway and with a reporter construct containing a CRE consensus element in the *Gpxh* promoter excluded the function of the 8 bp element as a typical CRE element. However, it is very likely that the 8 bp element in the *Gpxh* promoter is a transcription factor binding site homologous to other elements with a TGAC motif required for the induction of various oxidative stress-responsive genes.

INTRODUCTION

The production of high levels of molecular oxygen is a permanent challenge for photosynthetic organisms. Upon exposure to harsh environmental conditions, molecular oxygen can be modified to the formation of the reactive oxygen species (ROS) superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) [17, 35, 49, 51]. High levels of ROS cause a so-called oxidative stress and often result in serious damages to cell components such as proteins, lipids or DNA and finally even cell death. In photosynthetic organisms various defense mechanisms have evolved to either prevent the formation of ROS, to remove them or to repair the damaged components in the cell [17, 35, 51]. Many of these mechanisms involve general defense genes which are induced by various stress conditions in the cell. In addition are special defense genes upregulated upon the increased formation of ROS by regulation mechanisms which are often specific for the type or location of the ROS formed [35, 49]. One important regulation mechanism involves the transcriptional activation of gene expression by transcription factors which either directly or indirectly respond to elevated ROS levels. In *Escherichia coli,* the transcription factor OxyR is activated via oxidation by H_2O_2 and then binds a promoter element with subsequent gene activation, and the regulator pair SoxR/SoxS triggers the $O_2^{\cdot-}$-induced response of certain defense genes [42]. In eukaryotes, the induction of genes by ROS is mainly mediated by transcription factors of the activator protein-1 (AP-1), the activating transcription factor (ATF) or the NF-κB families, activated by various mitogen-activated protein (MAP) kinase pathways (for review see [44]). In yeasts, the AP-1 homologous YAP-1 is modified by H_2O_2 via the GPX3 protein, indicating that direct activation of transcription factors also occurs in eukaryotic cells [11]. Many of the oxidative stress response mechanisms in eukaryotes are activated by various ROS, but

little is known about specific gene regulation mechanisms in response to $O_2^{\cdot-}$, H_2O_2 or $OH^{\cdot}$. The response to increased $^1O_2$ levels has been studied even less, even though the formation of $^1O_2$ is strongly enhanced during photosensitized processes. The genetic response to increased generation of $^1O_2$ was analyzed in a protochlorophyllide accumulating mutant of *Arabidopsis thaliana* with DNA microarrays, but the direct connection between $^1O_2$ and the gene induction was not proofed [37]. Other examples are the $^1O_2$-mediated response of the heme oxygenase 1 gene (HO-1) and the intercellular adhesion molecule-1 (ICAM-1) to ultraviolet A (UV-A) radiation in human cells [21, 45]. The $^1O_2$-induced formation of ceramide was suggested to trigger the ICAM-1 response via the activation of the AP-2 transcription factor [21], whereas for the activation of HO-1 transcription the mechanism is not known.

We have recently described the specific induction of the glutathione peroxidase homologous gene (*Gpxh*) in *Chlamydomonas reinhardtii* by increased formation of $^1O_2$ upon exposure to the exogenous photosensitizers neutral red (NR) and rose bengal (RB) or by illumination with high light intensities [15, 33]. In the promoter region of the *Gpxh* gene a 8 bp sequence element (5`-TGACGCCA-3`) was identified with strong homology to the cAMP responsive element (CRE) and the AP-1 binding site identified in mammalians [1, 13]. The CRE-binding protein (CREB), containing a leucine zipper DNA binding domain (bZIP), was originally described as being activated by increasing cellular levels of cyclic AMP or by the addition of the exogenous cyclic nucleotide analog 8 Br-cAMP [8, 19, 24, 38]. Over the years many bZIP CREB-homologous transcription factor have been identified to bind to the same consensus sequence and they all belong to the ATF/CREB family [8, 24]. The AP-1 transcription factor consists of the two proteins Jun and Fos, which either form a heterodimer of Jun and Fos or a homodimer of two Jun proteins to activate gene expression [30]. In addition, the Jun protein can also form stable heterodimers with ATF2, a

member of the ATF family, which then preferentially bind to the CRE consensus sequence [23, 25].

In *C. reinhardtii,* deletion of the CRE/AP-1 element in the *Gpxh* promoter of a reporter gene construct (pASPro2ΔCRE) revealed the requirement of this element for the transcriptional upregulation of *Gpxh* upon exposure to NR and RB in the light [33]. This 8 bp sequence element is part of a 16 bp almost perfect palindrome (5`-GCGCCAACGT*TGACGC*-3`), which resembles the palindromic canonical motif 5`-CNNGAANNTTCNNG-3`of heat shock elements, HSE [4]. However, the *Gpxh* gene was only slightly upregulated by a heat shock treatment indicating that this palindromic sequence in not a functional heat shock element [33]. We could, however, not exclude a role of the palindrome in the $^1O_2$-induced *Gpxh* expression, since the deletion of the 8 bp element in pASPro2ΔCRE also disturbed the palindromic sequence of the 16 bp element. Here, we further characterized the role of the 8 bp sequence element in the *Gpxh* promoter in the $^1O_2$ induction by specifically introducing mutations in and around this element and analyzing the effect of these mutations on the reporter gene expression. Within this 8 bp element, a conserved TGAC motif was identified as being essential for $^1O_2$ induction. Gel-mobility shift assays proofed the presence of a protein complex in total *C. reinhardtii* extracts which binds to the 8 bp element with a sequence-dependent binding efficiency. Finally, we could show that the introduction of the CRE/AP-1 element into a β-tubulin promoter is sufficient to create a $^1O_2$-inducible *Tub2B* promoter.

MATERIALS AND METHODS

Strains and Culture Conditions

C. reinhardtii strains $cw_{15}arg_7mt^-$ (CC-1618) was inoculated in Tris-Acetate-Phosphate-medium (TAP) [26] in Erlenmeyer flasks and agitated on a rotatory

shaker (150 rpm) under constant illumination (120 µmol m^{-2} s^{-1} PAR) at 25°C. All media were supplemented with 50 mg/l ampicillin.

Escherichia coli DH5α [41] was used for routine cloning experiments, and the *dam-3 E.coli* strain GM119 [5] was used to obtain unmethylated pUC28. *E. coli* strains were grown on LB at 37°C.

Chemicals

The photosensitizers neutral red (NR) and rose bengal (RB) (Fluka) were dissolved in water and stored in 1 or 10 mM stock solutions at 4°C in the dark. The cyclic nucleotide analogs 8-bromoadenosine 3`:5`-cyclic monophosphate (8 Br-cAMP) and 8-bromoguanosine 3`:5`-cyclic monophosphate (8 Br-cGMP) (Sigma) (0.2M stock solution in water) and the adenylate cyclase inhibitor MDL-12 (Calbiochem) (5mM stock solution in water) were stored at –20°C.

Preparation of Crude Extract

Crude extract from *C. reinhardtii* cultures was prepared as described by Mittag et al. [34]. Cells were grown to a density of 8×10^6 cell/ml and exposed to 5 µM NR for 1 h before harvesting.

Gel Mobility Shift Assay

Radioactive DNA fragments, containing the *Gpxh* promoter and the 8 bp CRE/AP-1 element were obtained by incubating 0.2 nmol of a 50 base oligonucleotide in a 50 µl reaction mixture including 5 µl γ^{32}P ATP (370 mBq/ml) and 2 µl of Polynucleotide Kinase (Promega; 10U/µl) for 1h at 37°C. DNA was purified with a DNA purification kit (Qiagen) and annealed with 0.2 nmol of the corresponding unlabelled and complementary oligo-nucleotide by heating at 95°C for 1 min and concomitant incubation on ice.

Various amounts of crude cell extract were preincubated with 1µg of poly d(I-C) in 16 µl for 20 min at 23°C to block unspecific DNA binding. Then 0.4 µl of the radiolabeled dsDNA fragment was added and incubated for another 20 min at 23°C. After adding 3 µl of 5×loading buffer (40% glycerol, 5×TBE pH 8.3, 50 mM EDTA, 0.1% w/v bromophenolblue) the samples were separated on a 4% polyacrylamide gel containing 5% glycerol in 1×TBE [2]. The gel was transferred to Whatman paper (3MM), dried and exposed to an X-ray film for 10-48 h.

Construction and Transformation of Plasmids

Plasmids pASPro1, pASPro2 and pASPro2ΔCRE have been described before [33]. For construction of pBF2, pBF7 and pBF27 first a 700 bp *Kpn*I-*Eco*RV promoter fragment of pASPro2 was subcloned in a *Kpn*I/*Eco*RV digested vector pT7blue (Novagen) resulting in plasmid pT7Pro5. Point mutations were subsequently introduced in the 8 bp CRE/AP-1 sequence element using a site directed mutagenesis kit (Stratagene) and primers (5`-CGCCAACGTTGACGC CTGTTAGAGAA-3`), (5`-CGCCAACGTTGAGGCCAGTTAGAGAA-3`) and (5`-CGCCAACGTTGACGTCAGTTAGAGAA-3`) respectively, together with the corresponding reverse complement primer. For cloning of plasmid pBF19 and pBF20 the site directed mutagenesis kit (Stratagene) was applied with primer (5`-GCTGTGGTAGGTGTTCGAGGACGTTGACGCCAGTTA-3`) and plasmid pT7Pro5 as template or with primer (5`-GCTGTGGTAGGTGTTCG AGGACGTCCTCGAGGGTTA-3`) and a plasmid, containing the CRE deleted *Gpxh* promoter in pGEMTeasy (Promega) [33] as template to introduce the mutations. All mutations were confirmed by sequencing. Finally, *Kpn*I-*Eco*RV fragments of each mutated promoter was isolated and used to exchange the 1.4 kb *Kpn*I-*Eco*RV promoter fragment of pASPro1.

Plasmid pJD55, kindly provided by J. Davis, was used to excise the β-tubulin promoter. A 2.4 kb *Kpn*I-*Cla*I fragment of pJD55 was subcloned in to *Kpn*I/*Cla*I digested pUC28. This construct served as template for PCR based method to introduce the 8 bp CRE/AP-1 sequence element with and without flanking sequence into the β-tubulin promoter. For plasmid pBF31 the β-tubulin promoter was amplified with the forward primer (5`-GCGCCAACGTTGACG CCAGTTAGAGCTTCCCGGCGCTGCATG-3`) and the reverse primer (5`-GT GAGCGGATAACAATTTCACA-3`) complementary to the pUC28 sequence using *Pfu* DNA polymerase (Promega) to generate blunt ended fragments. The same method was used to generate the β-tubulin promoter fragments for pBF32 and pBF33 using primer (5`-TGACGCCAGAGACGGCTTCCCGGCGCTG-3`) or primer (5`-GAGACGGCTTCCCGGCGCTG-3`) together with the same downstream primer. All three PCR products were digested with *Eco*RI and subcloned into *Sma*I and *Eco*RI digested pUC28. The modified promoter fragments were isolated by digesting the constructs with *Sal*I and *Cla*I. Fragments obtained were used to exchange the *Sal*I-*Cla*I promoter fragment of pASPro1 resulting in plasmid pBF31, pBF32 and pBF33.

Strain $cw_{15}arg_7mt^-$ was cotransformed with reporter gene constructs and pARG7.8 [10] following the protocol of Kindle [32]. Transformants were selected on TAP agar plates without arginine, and the colonies were screened for arylsulfatase expression by spraying the plates with 0.05 mM X-SO_4 (5-bromo-4-chloro-3-indolyl sulfate potassium salt, Biosynth AG) dissolved in water.

Arylsulfatase Assay

Quantitative assays of arylsulfatase activity were performed essentially as described earlier [33]. Cells of individual clones were grown in TAP to a density of approximately 8×10^6 cells/ml, harvested by centrifugation and resuspended in TAP at 1×10^7 cells/ml for experiments using 5μM NR or 5×10^6 cells/ml for

experiments using 2 μM NR. The culture was distributed into different fractions for exposure experiments. Samples of 300 ml were taken after 2, 4 and 6 h of exposure and arylsulfatase activity was measured as described [33].

RESULTS

An 8 bp CRE/AP-1 Homologous Sequence Element is Responsible for the $^1O_2$-Induced *Gpxh* Expression

The promoter of the *Gpxh* gene in *C. reinhardtii* was strongly induced by NR in different *Gpxh* promoter-arylsulfatase reporter gene constructs (pASPro1-4) but not in a construct missing a 8 bp CRE/AP-1 homologous sequence element in the *Gpxh* promoter (pASPro2ΔCRE) [33]. This 8 bp sequence element is part of a 16 bp almost perfect palindrome (5`-GCGCCAACGTTGACGC-3`). Since the deletion of the 8 bp element in pASPro2ΔCRE also disturbed this palindromic structure, we could not exclude a role of this element in the $^1O_2$-induced *Gpxh* expression. To examine whether this palindrome is required for *Gpxh* induction by $^1O_2$, two new constructs were created based on the reporter gene construct pASPro2. In plasmid pBF20 only the 5`part of the palindrome was deleted without affecting the 8 bp sequence element (Fig. 1a). In pBF19, the 8 bp CRE/AP-1 element was deleted and a new artificial 16 bp palindrome was introduced in the *Gpxh* promoter, replacing the original 16 bp palindrome (pBF19). For both constructs four independent transformants were tested for the expression of the reporter gene upon exposure to 5 μM NR in the light and compared to expression of the wild-type promoter (pASPro2). Mutating the 5`part of the palindrome in pBF20 did not influence the expression of the reporter gene but when the entire palindrome was replaced by a new palindromic sequence, missing the CRE/AP-1 element (pBF19), almost no induction was measured by 5 μM NR any more (Fig. 1a). These data showed

that the 16 bp palindrome is not required for $^1O_2$-induced *Gpxh* expression, but that the 8 bp element is essential for the activation of the *Gpxh* promoter by NR. In order to know whether the 8 bp CRE/AP-1 element is sufficient for the induction of a promoter by $^1O_2$, two plasmids were constructed where this element was introduced in the β-tubulin (*Tub2B*) promoter upstream of the arylsulfatase reporter gene of plasmid pJD55 [9]. In plasmid pBF32 the 8 bp sequence element was directly introduced upstream of position -133 relative to the transcription start of the *Tub2B* promoter, at the same position as found in the *Gpxh* promoter (Fig. 1b). In plasmid pBF31, the same sequence element, flanked by 6 nucleotides up- and downstream of the wild-type *Gpxh* promoter region, was cloned again at position -133 of the *Tub2B* promoter. As a control, a construct containing a *Tub2B* promoter fragment of the same length was used (pBF33).

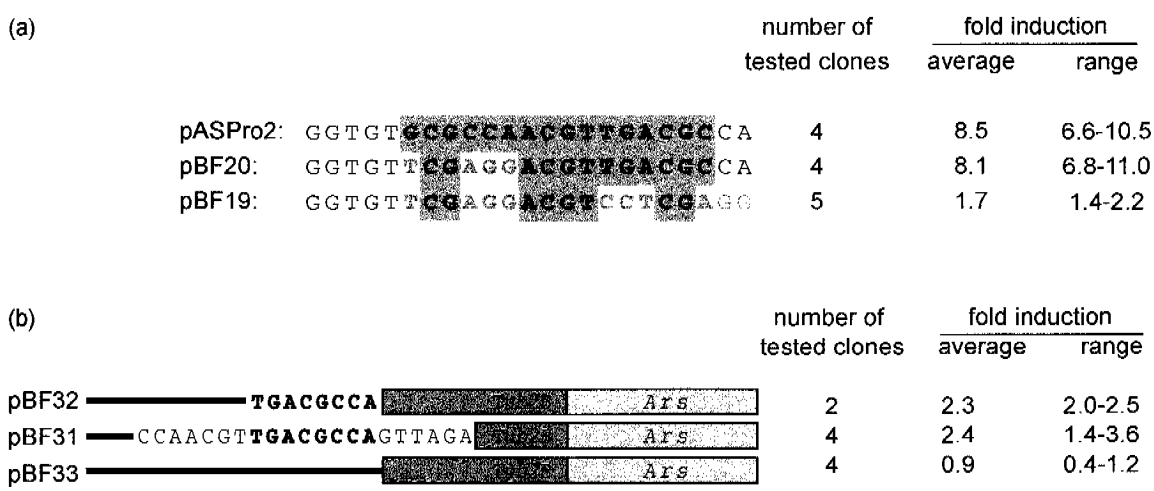


Figure 1: (a) The role of a 16 bp palindrome (bold letters) in the expression of the *Gpxh* promoter by 5 μM NR was analyzed either by mutating (gray letters) only the 5`part of the palindromic sequence (pBF20) or by introducing a new palindrome into the promoter sequence of pASPro2ΔCRE (pBF19). Identity to pASPro2 is shaded. (b) Induction of different modified β-tubulin promoter reporter gene constructs by 5 μM NR in the light. The 8 bp CRE/AP-1 element of *Gpxh* was fused to the β-tubulin promoter at position –133 relative to the transcription start with (pBF31) or without (pBF32) flanking region of the *Gpxh* promoter. A β-tubulin promoter of the same length without additional elements served as a control (pBF33).

The transformants containing plasmids pBF31, pBF32, pBF33 and pJD55 showed similar arylsulfatase expression, when grown in normal TAP medium in the light. Upon exposure to 5 μM NR, the expression of the reporter gene was induced 2.3 and 2.4 fold compared to basal expression in transformants with plasmids pBF31 and pBF32 respectively (Fig. 1b). The expression of the control strains with plasmid pBF33, on the other hand, was not changed by the addition of the photosensitzer, indicating that not the shortening of the *Tub2B* promoter was responsible for the induction of the gene in pBF31 and pBF32.

Characterization of the CRE/AP-1 Element by Analyzing the Effect of Point Mutations on Transcriptional Activation of the *Gpxh* Promoter

To further characterize the CRE/AP-1 homologous sequence element, site-specific mutations were introduced in 3 out of 8 nucleotides. The mutated nucleotides were chosen in different positions of the element to test their requirement for activation and to compare the element with the consensus CRE or AP-1 sequence. In construct pBF2 the consensus sequence of the 8 bp CRE element but not of the 7 bp AP-1 binding site was modified by an A to T transversion of the last base of the 8 bp element. This mutation reduced the average induction factor of the reporter gene by 5 μM NR more than two fold (Fig.2, lane 5) even though the range from 1.9 to 6.5 fold indicates that the effect is dependent on the position of the construct in the chromosome.

The fourth base in the AP-1 consensus sequence was described to be either a guanine or a cytosine. Therefore a G to C transversion of the fourth base of the 8 bp element was introduced in the *Gpxh* promoter of construct pBF7 which kept the homology to an AP-1 element but reduced the homology to a CRE element, usually resulting in a loss of function [13]. Indeed, the expression of the reporter gene in all transformants with the plasmid pBF7 was dramatically

reduced to an average of only 2.2 fold compared to the 8.5 fold induction of the wild-type construct (Fig.2, lane 6). Remarkable, the mutation also resulted in a reduction of basal expression in the light similar to the deletion of the element as a whole (data not shown).

In construct pBF27 the CRE/AP-1 element was exchanged with the consensus CRE sequence (C to T transition of the sixth base) simultaneously reducing the homology to an AP-1 binding site. In transformants containing pBF27, reporter gene activity was induced 4.8 fold upon exposure to 5 μM NR in the light (Fig.2, lane7). Thus, the presence of a consensus CRE element causes a 1.8 fold reduced induction compared to the presence of the wild-type 8 bp element.

		number of tested clones	fold induction	
			average	range
CRE consensus sequence:	TGACGTCA			
AP-1 consensus sequence:	TGA(C/G)TCA			
pASPro2:	TGACGCCA	4	8.5	6.6-10.5
pASPro2 delta CRE:	CCTCGAGG	4	1.7	0.8-3.2
pBF2:	TGACGCCT	7	3.4	1.9-6.5
pBF7:	TGAGGCCA	6	2.2	0.8-3.4
pBF27:	TGACGTCA	4	4.8	3.2-6.6

Figure 2: Effect of different mutations in the CRE/AP-1 element of the *Gpxh* promoter on the expression of the reporter gene construct by 5 μM NR in the light. The 8 bp wild-type element present in the *Gxph* promoter of pASPro2 was either deleted (pASPro2ΔCRE) or mutated by the insertion of different point mutations (gray letters) resulting in plasmid pBF2, pBF7 and pBF27. CRE and AP-1 consensus sequence are added for comparison of homology.

Binding of a Transcription Factor to the CRE/AP-1 Element is Differently Affected by Various Mutations in the Element

To examine whether a DNA binding protein is present in *C. reinhardtii* that can bind to the 8 bp CRE/AP-1 element, gel mobility shift assays were performed. A

radiolabeled 50 bp DNA fragment of the *Gpxh* promoter, including the 8 bp CRE/AP-1 element, was incubated with an increasing amount of crude cell extract isolated from a *C. reinhardtii* culture and subsequently separated on an 4% polyacrylamide gel. A retarded band was observed on the gel indicating the formation of a DNA binding complex bound to the labeled DNA fragment (Fig. 3a). The intensity of the bands increased with the amount of crude extract, and reached a maximum with 30 μg of extract. The labeled fragment was competed away from the DNA binding complex by adding an excess of unlabeled wild-type fragments showing the presence of a specific DNA binding complex (Fig. 3a, lane 6).

We further tested the effect of individual mutations introduced in the CRE/AP-1 element in the reporter gene assays on DNA binding affinity. Deleting the 8 bp element in the 50 bp *Gpxh* promoter fragment as in construct pASPro2ΔCRE, resulted in a total loss of binding of the putative transcription factor to the DNA fragment, indicating that the formation of the complex with the wild-type fragment is indeed dependent on the 8 bp sequence element (Fig. 3b del). Also the C to G transversion (pm2) introduced in construct pBF7, reduced the binding capacity to the *Gpxh* promoter fragment in the gel mobility shift assay to about 60% of wild-type level, indicating a reduced binding affinity. However, mutant fragments pm1 and pm3, equal to pBF2 and pBF27 promoter fragments showed no significant reduction in the binding affinity in the gel mobility shift assay compared to the wild-type fragment, even though these mutations caused reduced induction in the reporter gene assay. These results show the presence of a DNA binding complex in *C. reinhardtii* extracts, which binds to the CRE/AP-1 element, indicating that indeed a transcription factor specifically interacts with this 8 bp element in the *Gpxh* promoter.

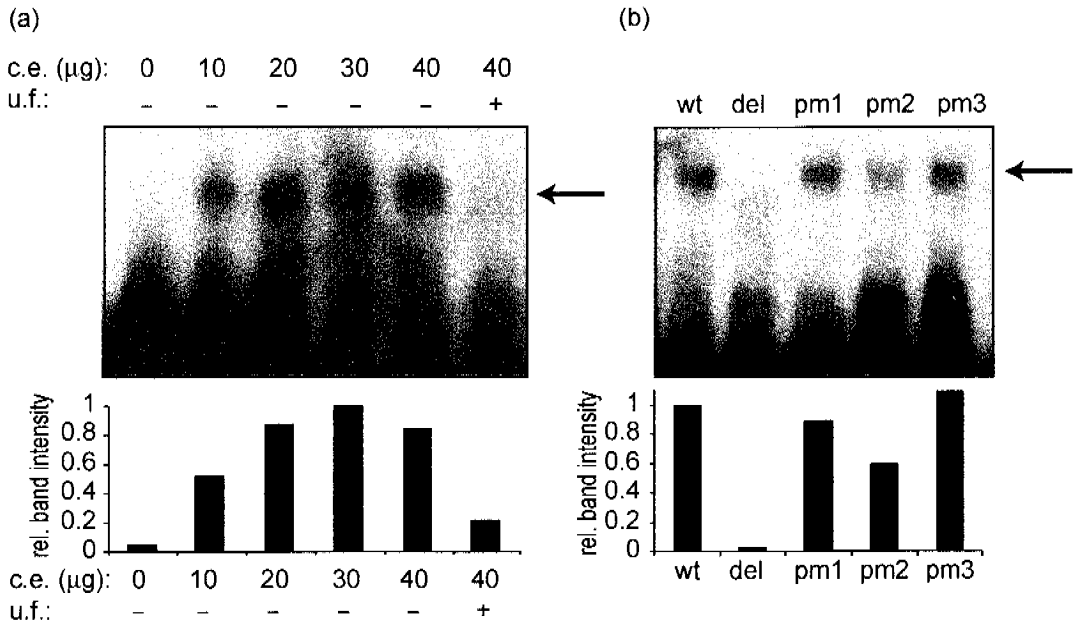


Figure 3: Gel mobility shift assays with crude cell extract from *C. reinhardtii* and a labeled 50 bp DNA fragment of the *Gpxh* promoter as template. A culture of strain $cw_{15}arg_7mt^-$ was incubated for 1 h with 5 µM NR in the light before a crude cell extract was prepared. (a) Increasing amounts of crude extract (c.e.) were incubated with radioactively labeled DNA fragments of the wild-type *Gpxh* promoter with (+) or without (-) the addition of an 20 fold excess of the same unlabeled DNA fragment (u.f.) and separated on a 4% polyacrylamide gel. Intensities of the retarded bands (arrow) were quantified with ImageJ (RSB at NIH), normalized to the most intense band and blotted in a graph below each band. (b) Binding capacity of a crude cell extract to different mutated promoter regions. Various 50 bp DNA fragments of the *Gpxh* promoter either with the wild-type sequence (wt) or including the mutations present in pASPro2ΔCRE (del), pBF2 (pm1), pBF7 (pm2) and pBF27 (pm3) were radioactively labeled and incubated with 30 µg of crude cell extract. After separation on a 4% polyacylamide gel the retarded bands (arrow) were quantified, normalized to the wt band and blotted in a graph.

Increased Levels of cAMP or cGMP Inhibit the *Gpxh* Induction by $^1O_2$

The activation of the transcription factors is mediated by a signal transduction pathway usually leading to the phosphorylation of the transcription factor by a specific kinase. The most common mechanism for activation of CRE dependent genes involves the cAMP dependent activation of the kinase A and the CRE-

binding protein CREB [8]. This pathway can artificially be induced by the addition of the exogenous cyclic nucleotide analogs 8 Br-cAMP and 8 Br-cGMP [19, 29, 38]. In order to test whether the *Gpxh* expression is dependent on a pathway in which cAMP or cGMP is involved, we added 8 Br-cAMP and 8 Br-cGMP in different concentrations to transformants with the construct pASPro1 with a full length *Gpxh* promoter fused to the arylsulfatase reporter gene in the chromosome [33]. Induction was measured by the expression of the reporter gene in the presence of 2 μM NR. The addition of 3 mM 8 Br-cAMP and 8 Br-cGMP reduced the *Gpxh* induction about 3 fold compared to the response caused by 2 μM NR alone. The addition of 15 mM of either of two substances almost completely inhibited the NR-induced *Gpxh* activation, whereas cell viability was not affected under these conditions (Fig. 4). Both cyclic nucleotide analogs did not cause any induction in the control samples.

The negative effect of increased cyclic nucleotides concentrations on *Gpxh* expression is supported by the response of pASPro1 to the addition of MDL-12, which inhibits the activity of the cellular adenylate cylase, leading to a reduced cAMP concentration in the cell [7]. At a concentration of 20 μM, MDL-12 results in a 3 fold induction of the *Gpxh* expression indicating that there is a down regulation by cAMP under normal light conditions (Fig. 4). Similarly, upon addition of MDL-12, the induction by 2 μM NR was increased. However, this effect was not strong since the addition of 20 μM MDL-12 and 2 μM NR in combination starts to become toxic to the cells at these concentrations (data not shown).

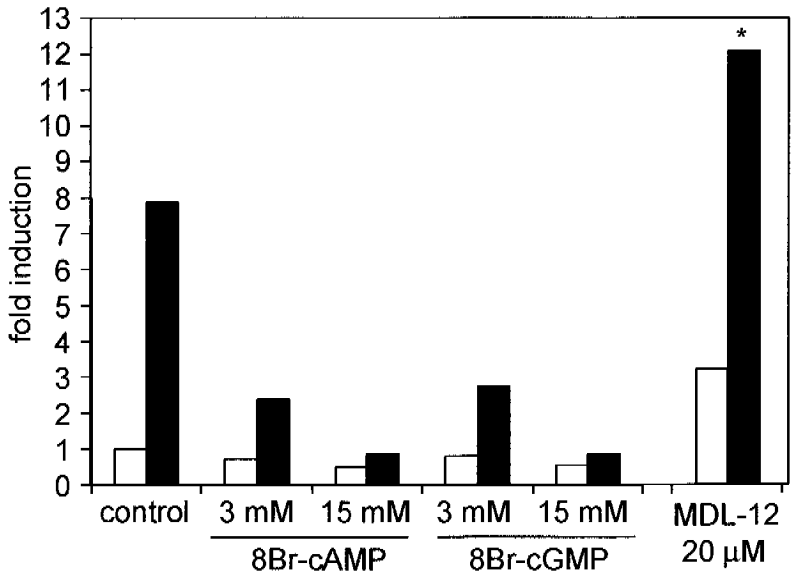


Figure 4: Induction of pASPro1, containing a *Gpxh* wild-type promoter in front of the arylsulfatase reporter gene, by different concentrations of the cyclic nucleotide analogs 8 Br-cAMP and 8 Br-cGMP or the adenylate cyclase inhibitor MDL-12 in the absence or presence of 2 µM NR. Asterisk: Combination of 20 µM MDL-12 and 2 µM NR in the light caused toxic effects to the cell resulting in a reduced number of viable cells responding to the stress.

DISCUSSION

In *C. reinhardtii,* the *Gpxh* gene is strongly and specifically induced by $^1O_2$ [15, 33]. Here, we studied the induction mechanism of *Gpxh* in more detail with focus on regulatory promoter elements and signaling pathways. We showed before that an 8 bp sequence element in the *Gpxh* promoter region is required for transcriptional activation by $^1O_2$ [33]. In this study we could show that the introduction of this element into a β-tubulin promoter (pBF32) is sufficient for at least partial induction by $^1O_2$ (Fig. 1b). This suggests a role of this 8 bp element as a transcription factor binding site, supported by the formation of a DNA binding complex, upon incubation of a *Gpxh* promoter fragment with a

C. reinhardtii crude cell extract (Fig. 3a). The binding of this protein complex was dependent on the presence of the 8 bp element, showing for the first time the specific interaction of this motif with a putative transcription factor (Fig. 3b). The reasons for the lower induction in the modified β-tubulin promoter compared to the wild-type *Gpxh* promoter by the same concentration of photosensitizer in the light might be various. A positive effect of the neighboring (contextual) bases was observed in various CRE and AP-1 dependent promoters before, but a general correlation between the flanking sequence and the response of the different promoters was not established [12, 13]. We could exclude a possible effect of immediate contextual bases in our case, because the introduction of flanking sequences (pBF31) did not influence expression levels in comparison with the 8 bp element alone (pBF32) (Fig. 1b). We could, however, not exclude additional sequence elements, which are separated from the CRE/AP-1 element by several nucleotides, to be required for full activity of the transcription factor. Such an element is found in many auxin and salicyclic acid inducible promoters were two TGACG motifs are separated by 7 nucleotides in the as-1/ocs element [6, 46]. Interestingly, two candidates for such a second element with homology to the TGACG motif (TGCGC or TGTGC) are indeed found 6 and 8 nucleotide upstream of the CRE/AP-1 element. Since both these elements were mutated in construct pBF20, which showed no reduced induction compared to the wild-type promoter, it is unlikely that these motifs are involved in *Gpxh* induction by $^1O_2$ (Fig. 1a). We additionally excluded a role of a 16 bp palindrome overlapping the 8 bp element in the $^1O_2$ response by mutating the 5`-part of this palindrome (Fig. 1a). Thus, the 8 bp element was concluded to contain the core sequence for $^1O_2$ induced response in *C. reinhardtii*. Still, additional enhancer elements in the *Gpxh* promoter, located further up- or downstream of the CRE/AP-1 element, might be required for full induction of the *Gpxh* expression by NR. Binding of a

protein to such an additional element could cooperate positively with a transcription factor and facilitate its binding to the CRE/AP-1 element [27]. Additionally, altered DNA secondary structure or the presence of repressor proteins bound to the β-tubulin promoter could also be responsible for the low induction of the modified β-tubulin promoter.

Further characterization of the CRE/AP-1 element by introducing specific point mutations did not allow to discriminate between a typical CREB or AP-1 trancription factor binding site. Two mutations which did not affect the AP-1 consensus sequence (pBF2 and pBF7) reduced the induction by 5 μM NR to 20 to 40% of wild-type expression, indicating that the element is maybe not a common AP-1 site (Fig. 2). Since both these mutations reduced the homology to the CRE consensus sequence a function of the 8 bp element as CRE element was considered. However, exchanging the CRE/AP-1 element with the consensus sequence of the CRE element (pBF27) rather reduced the induction to 50 to 60% of the wild-type expression (Fig. 2). This is in disagreement with an activation mechanism involving a CRE element. We also compared the effect of the different point mutations on DNA binding affinity, but only the mutation of the fourth base (pm2) significantly reduced the efficiency of complex formation whereas the mutation of bases six and eight (pm1 and pm3) did not affect the binding of the transcription factor. This proofed that the reduced induction of pBF7 is indeed due to a reduced DNA binding affinity, confirming a function of the 8 bp element as transcription factor binding site. For pBF2 and pBF27 the reduction of DNA binding affinity may be to small and not visible in the DNA band shift assays because these *in vitro* experiments might result in an overestimation of the binding due to a missing competition of transcription factor binding sites with different binding affinities for the same transcription factor.

Further support against an induction of the *Gpxh* expression by a CREB is given by the inhibition of the *Gpxh* expression by the cyclic nucleotide analogs 8 Br-cAMP and 8 Br-cGMP and the stimulating effect of the adenylate cyclase inhibitior MDL-12 (Fig. 4). Still, an interaction between the $^1O_2$-activated and the cAMP-mediated signal pathways seems to occur when both are activated. Such interaction are widely observed in regulation of gene expression by various signals and both activating and inhibiting effects of the same signal transduction pathway on the expression of different genes has been described [18, 39]. Recently, a crucial role for the CREB binding protein (CBP), an essential cofactor for the transcriptional activation by the CREB and many other transcription factors, in the negative regulation of genes by a second signal has been suggested [14, 22]. Guberman et al. presented a model where CBP is the limiting factor for the CREB-dependent activation of the 5-aminolevulinate synthase gene in human hepatoma cells [22]. Concomitant activation of AP-1 by a second signal results in the binding of the AP-1 transcription factor to an upstream AP-1 site and a shift of the CBP from the CREB to the AP-1 complex causing an inhibition of CREB-dependent gene expression. A similar mechanism could be involved in the cAMP-inhibited *Gpxh* expression where an activated CREB complex would sequester the CBP away from the 8 bp element bound complex. Alternatively, cAMP could regulate the nuclear localization of the *Gpxh* activating transcription factor as described for the stress regulated transcription factor Msn2 in yeast, where high cAMP levels inhibit the accumulation and thus the activity of the Msn2 in the nucleus [20].

In the last years the number of transcription factor binding site with similar consensus sequences identified in various organisms continuously increased, including the CRE and AP-1 element in mammalians, the hormone induced elements as-1 and ocs in plants, the antioxidative response element ARE and the GCN4 binding site regulating the amino acid biosynthesis in yeast [1, 13, 28, 46,

50]. Most of them contain a TGAC sequence element in their consensus sequence indicating that this element might be an essential binding site for bZIP transcription factors in different organisms. This is supported by the strong reduction of the transcriptional activation and the reduced DNA binding affinity by a mutation in the fourth base in our experiments (Fig. 2). This might also be true for an AP-1 site even though in the consensus sequence of AP-1 binding sites the fourth base is either a C or a G. It is possible that in one promoter the element requires a C for activity, whereas in another promoter a G is essential at the fourth base of the AP-1 site. The same specificity was observed already in the highly homologous GCN4 binding site in yeast [13, 28]. Adjacent bases or additional elements are often different between the members of the same or different families of element and can further specify the binding of a distinct complex [12]. Thus, it might be possible that the introduction of a consensus CRE element in the *Gpxh* promoter of pFB27 increased the binding affinity for the CREB. The bound CREB, not activating the *Gpxh* expression, competes with the active transcription factor and thus reduces the induction of the *Gpxh* gene by NR. In addition, various bZIP type transcription factors in the cell can either act as activators or inhibitors upon binding to the same element in the promoter [18]. Members of the same or different families of transcription factors can build heterodimers like several members of the ATF family with the Jun protein [3, 25]. Such cross-family dimerization between ATF and AP-1 components also alters the DNA binding specificity, so that either CRE or AP-1 depending promoters can be activated, depending on the heterodimer [23, 47]. Some heterodimers were even shown to bind to composite sites, which are built up of the half sites of individual transcription factor binding site [25]. All these examples show that the distinction between these activation mechanisms is blurred and that only the characterization of the active DNA binding complex may identify the involved components [24].

In mammalians and yeasts, the AP-1 and YAP-1 site were shown to be involved in oxidative stress response [31, 43]. Other regulatory elements containing a TGAC motif in the core sequence were identified in several glutathione-S-transferases of plants [6, 40, 48]. One of them is an auxin-sensitive element in a tobacco glutathione-S-transferase with strong homology to the known as-1 element in other auxin and salicyclic acid inducible promoters [48]. Recently, the as-1 elements in the promoters of glutathione-S-transferases were identified as oxidative stress responsive elements and the activation by salicyclic acid was found to be mediated by ROS [16]. The same element was suggested to be responsible for induction of the *Arabidopsis* phospholipase A IIA gene by paraquat and rose bengal [36]. Thus, there is strong evidence for a connection between oxidative stress induced gene expression and the activation of promoters containing transcription factor binding sites with a TGAC motif. In agreement with this we could show that the 8 bp sequence element in the *Gpxh* promoter, containing a conserved TGAC motif, is an active transcription factor binding site required for induction of the *Gpxh* gene by $^1O_2$ and sufficient to partially induce the $^1O_2$-dependent expression of a β-tubulin promoter. How the signal is transmitted from the source of $^1O_2$ to the promoter of the *Gpxh* gene and which factors and additional elements are involved in the response needs to be investigated.

REFERENCES

1. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M: Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49: 729-39. (1987).
2. Ausubel FM: Current protocols in molecular biology. John Wiley & Sons, New York (1994).
3. Benbrook DM, Jones NC: Heterodimer formation between CREB and JUN proteins. Oncogene 5: 295-302 (1990).
4. Bienz M, Pelham NRB: Heat-shock regulatory elements function as an inducible enhancer in the *Xenopus hsp70* gene and when linked to a heterologous promoter. Cell 45: 753-760 (1986).
5. Boyer HW, Roulland-Dussoix D: A complementation analysis of the restriction and modification of DNA in Escherichia coli. J Mol Biol 41: 459-72. (1969).
6. Chen W, Chao G, Singh KB: The promoter of a H2O2-inducible, Arabidopsis glutathione S-transferase gene contains closely linked OBF- and OBP1-binding sites. Plant J 10: 955-66 (1996).
7. Correia-de-Sa P, Ribeiro JA: Evidence that the presynaptic A2a-adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase. Naunyn Schmiedebergs Arch Pharmacol 350: 514-22. (1994).
8. Daniel PB, Walker WH, Habener JF: Cyclic AMP signaling and gene regulation. Annu Rev Nutr 18: 353-83 (1998).
9. Davies JP, Weeks DP, Grossman AR: Expression of the arylsulfatase gene from the β_2-tubulin promoter in *Chlamydomonas reinhardtii*. Nucleic Acids Res 29: 1959 - 2965 (1992).
10. Debuchy R, Purton S, Rochaix JD: The argininosuccinate lyase gene of Chlamydomonas reinhardtii: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. Embo J 8: 2803-9. (1989).
11. Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB: A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell 111: 471-81 (2002).
12. Deutsch PJ, Hoeffler JP, Jameson JL, Habener JF: Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. Proc Natl Acad Sci U S A 85: 7922-6. (1988).
13. Deutsch PJ, Hoeffler JP, Jameson JL, Lin JC, Habener JF: Structural determinants for transcriptional activation by cAMP- responsive DNA elements. J Biol Chem 263: 18466-72 (1988).
14. DiSepio D, Sutter M, Johnson AT, Chandraratna RA, Nagpal S: Identification of the AP1-antagonism domain of retinoic acid receptors. Mol Cell Biol Res Commun 1: 7-13 (1999).

15. Fischer BB, Krieger-Liszkay A, Eggen RIL: Unraveling the Molecular Mechanisms behind the Response of Chlamydomonas reinhardtii to Type I (Neutral Red) and Type II (Rose Bengal) Photosensitizers. (2004b).

16. Garreton V, Carpinelli J, Jordana X, Holuigue L: The as-1 promoter element is an oxidative stress-responsive element and salicylic acid activates it via oxidative species. Plant Physiol 130: 1516-26 (2002).

17. Gille G, Sigler K: Oxidative stress and living cells. Folia Microbiol 40: 131-52 (1995).

18. Giono LE, Varone CL, Canepa ET: 5-Aminolaevulinate synthase gene promoter contains two cAMP-response element (CRE)-like sites that confer positive and negative responsiveness to CRE-binding protein (CREB). Biochem J 353: 307-16 (2001).

19. Goodenough UW: Cyclic AMP enhances the sexual agglutinability of Chlamydomonas flagella. J Cell Biol 109: 247-52. (1989).

20. Gorner W, Durchschlag E, Wolf J, Brown EL, Ammerer G, Ruis H, Schuller C: Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. Embo J 21: 135-44 (2002).

21. Grether-Beck S, Bonizzi G, Schmitt-Brenden H, Felsner I, Timmer A, Sies H, Johnson JP, Piette J, Krutmann J: Non-enzymatic triggering of the ceramide signalling cascade by solar UVA radiation. Embo J 19: 5793-800 (2000).

22. Guberman AS, Scassa ME, Giono LE, Varone CL, Canepa ET: Inhibitory effect of AP-1 complex on 5-aminolevulinate synthase gene expression through sequestration of cAMP-response element protein (CRE)-binding protein (CBP) coactivator. J Biol Chem 278: 2317-26 (2003).

23. Hai T, Curran T: Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci U S A 88: 3720-4 (1991).

24. Hai T, Hartman MG: The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. Gene 273: 1-11. (2001).

25. Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U: ATF3 and stress responses. Gene Expr 7: 321-35 (1999).

26. Harris EH: The Chlamydomonas sourcebook : a comprehensive guide to biology and laboratory use. Academic Press, San Diego (1989).

27. Haus-Seuffert P, Halle JP, Sanner S, Meisterernst M: Conserved cAMP responsive element and core promoter complex are critical for specificity of the distal T-cell receptor beta chain enhancer for its native promoter. Gene 236: 209-19. (1999).

28. Hill DE, Hope IA, Macke JP, Struhl K: Saturation mutagenesis of the yeast his3 regulatory site: requirements for transcriptional induction and for binding by GCN4 activator protein. Science 234: 451-7 (1986).

29. Immenschuh S, Hinke V, Ohlmann A, Gifhorn S, Katz N, Jungermann K, Kietzmann T: Transcriptional activation of the haem oxygenase-1 gene by cGMP via a cAMP response element/activator protein-1 element in primary cultures of rat hepatocytes. Biochem J 334 (Pt 1): 141-6 (1998).

30. Karin M, Liu Z, Zandi E: AP-1 function and regulation. Curr Opin Cell Biol 9: 240-6 (1997).

31. Karin M, Takahashi T, Kapahi P, Delhase M, Chen Y, Makris C, Rothwarf D, Baud V, Natoli G, Guido F, Li N: Oxidative stress and gene expression: the AP-1 and NF-kappaB connections. Biofactors 15: 87-9 (2001).

32. Kindle KL: High-frequency nuclear transformation of Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A 87: 1228-32. (1990).

33. Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL: The glutathione peroxidase homologous gene from Chlamydomonas reinhardtii is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology 46: 395-408 (2001).

34. Mittag M: Conserved circadian elements in phylogenetically diverse algae. Proc Natl Acad Sci U S A 93: 14401-4. (1996).

35. Mittler R: Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405-10 (2002).

36. Narusaka Y, Narusaka M, Seki M, Fujita M, Ishida J, Nakashima M, Enju A, Sakurai T, Satou M, Kamiya A, Park P, Kobayashi M, Shinozaki K: Expression profiles of Arabidopsis phospholipase A IIA gene in response to biotic and abiotic stresses. Plant Cell Physiol 44: 1246-52 (2003).

37. op den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M, Apel K: Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. Plant Cell 15: 2320-32 (2003).

38. Pasquale SM, Goodenough UW: Cyclic AMP functions as a primary sexual signal in gametes of Chlamydomonas reinhardtii. J Cell Biol 105: 2279-92. (1987).

39. Robinson MJ, Cobb MH: Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9: 180-6 (1997).

40. Rodriguez Milla MA, Maurer A, Rodriguez Huete A, Gustafson JP: Glutathione peroxidase genes in Arabidopsis are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. Plant J 36: 602-15 (2003).

41. Sambrook J, Maniatis T, Fritsch EF: Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

42. Storz G, Tartaglia LA, Ames BN: Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 248: 189-94 (1990).

43. Toone WM, Morgan BA, Jones N: Redox control of AP-1-like factors in yeast and beyond. Oncogene 20: 2336-46 (2001).

44. Turpaev KT: Reactive oxygen species and regulation of gene expression. Biochemistry (Mosc) 67: 281-92 (2002).

45. Tyrrell R: Redox regulation and oxidant activation of heme oxygenase-1. Free Radic Res 31: 335-40 (1999).

46. Ulmasov T, Hagen G, Guilfoyle T: The ocs element in the soybean GH2/4 promoter is activated by both active and inactive auxin and salicylic acid analogues. Plant Mol Biol 26: 1055-64 (1994).

47. van Dam H, Wilhelm D, Herr I, Steffen A, Herrlich P, Angel P: ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. Embo J 14: 1798-811 (1995).

48. van der Zaal BJ, Droog FN, Pieterse FJ, Hooykaas PJ: Auxin-sensitive elements from promoters of tobacco GST genes and a consensus as-1-like element differ only in relative strength. Plant Physiol 110: 79-88 (1996).

49. Vranova E, Inze D, Van Breusegem F: Signal transduction during oxidative stress. J Exp Bot 53: 1227-36 (2002).

50. Wasserman WW, Fahl WE: Functional antioxidant responsive elements. Proc Natl Acad Sci U S A 94: 5361-6 (1997).

51. Yu BP: Cellular Defenses Against Damage From Reactive Oxygen Species. Physiological Reviews 74: 139-162 (1994).

6. General Discussion

The $^1O_2$ Response of the *Gpxh* Gene in *C. reinhardtii*

The *Gpxh* gene of *C. reinhardtii* has been shown to be strongly induced by the addition of the exogenous photosensitizers neutral red (NR) and rose bengal (RB) in the light but was only slightly induced upon exposure to other ROS like $O_2^{\cdot-}$ and H_2O_2 [15]. We investigated the response of the *Gpxh* gene in more details and showed that $^1O_2$ was the responsible intermediate of the *Gpxh* induction by photosensitizers in the light. We hypothesize that the following scenario is taking place in *C. reinhardtii* cells upon exposure to different photooxidative stress conditions, leading to the *Gpxh* induction (Fig.1).

Under conditions causing photoinhibition, such as high light intensities or the presence of certain PSII herbicides, charge recombination in the reactive center of the PSII is enhanced and the formation of $^1O_2$ by energy transfer increases (chapter 4) [5, 9, 21]. $^1O_2$ subsequently reacts with a currently unknown cellular component which acts as a sensor for $^1O_2$ and which initiates a signal transduction pathway, activating a specific transcription factor. Alternatively, exogenous or endogenous photosensitizers can produce $^1O_2$ directly in the presence of light and thus activate the *Gpxh* expression. Several facts point to a sensing of $^1O_2$ in the chloroplast: First, *Gpxh* is strongly upregulated by $^1O_2$ produced upon illumination with high light intensities (chapter 4; Fig. 1). Under this condition $^1O_2$ is produced by the PSII in the thylakoid membrane and has to be sensed, due to the very short lifetime of $^1O_2$, in close proximity to the photosynthetic apparatus. Second, the production of $^1O_2$ by NR, responsible for the induction of *Gpxh*, takes only place in illuminated thylakoids but not in aqueous solutions, showing that the diffusion of NR into the chloroplast may be required for the *Gpxh* response (chapter 3; Fig. 4). The third argument for a

chloroplast localized sensor comes from the different effect of the $^1O_2$ quenchers DABCO and histidine on the induction and the toxicity caused by RB, as discussed in chapter 3. Both, DABCO and Histidine reduced the toxicity of RB, probably by quenching $^1O_2$ in the cell membrane, whereas the *Gpxh* induction was not affected, indicating that $^1O_2$ is sensed at a different location. Thus, this hypothesized local difference of the toxic effect and the sensing of $^1O_2$ supports a model with a sensor present in an organelle.

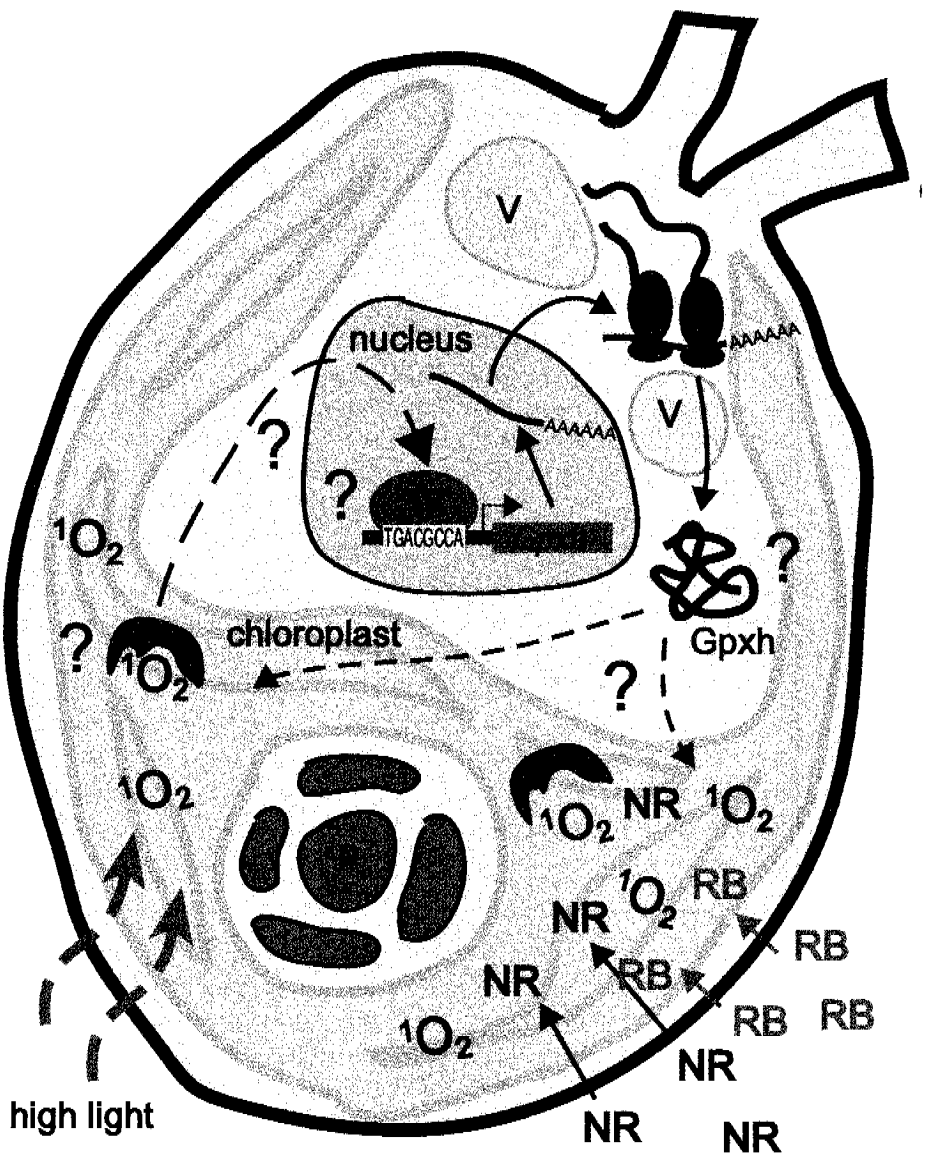


Figure 1 schematic model of the $^1O_2$ response of the *Gpxh* gene in *C. reinhardtii*

In the nucleus, the activated transcription factor binds specifically to the CRE/AP-1 homologous sequence element in the *Gpxh* promoter region and activates the transcription of the *Gpxh* gene (chapter 5). This can be inhibited by high levels of cAMP and the adenylate cyclase inhibitor MDL-12. After prolonged exposure to high light intensities the induction of *Gpxh* transcription by the $^1O_2$ signal probably decreases due to reduced $^1O_2$ generation in the damaged PSII, but a putative second and more stable signal may further stimulate the *Gpxh* expression (chapter 4). Expression of the *Gpxh* gene results in the synthesis of the Gpxh protein, which may exhibit a critical function in the response to photooxidative stress response.

In this work we could show, for the first time, the specific response of a nuclear gene to increased formation of $^1O_2$ in the chloroplast of a photosynthetic organism, as occurring during photoinhibition. Not unexpectedly are unsolved problems and open questions left, concerning the response of *C. reinhardtii* to photooxidative stress, which are discussed below in more detail.

Photooxidative Stress Response

The expression analysis of *C. reinhardtii* exposed to NR and RB with DNA-microarrays showed a remarkable difference between type I and type II photooxidative stress responses (chapter 2). A comparison of expression profiles of the induced genes by various stress conditions also showed that a general expression analysis should be performed for all genes induced by a chemical in microarray experiments. Different expression pattern of these genes indicate that more than one stress response mechanism might be activated by the chemical in the cell. In our case, these expression profiles showed that at least two different responses are induced by NR (chapter 2; Fig. 4). NR probably caused an altered expression of many genes, especially photosynthetic genes, due to the accumulation of NR in the thylakoids with the subsequent production of $^1O_2$, but

NR also induced several typical general and oxidative stress genes (chapter 2). This second stress response is most likely due to the type I photosensitising effect and indicates that the $O_2^{\cdot-}$ or H_2O_2-induced and the type I photooxidative stress response have many similarities. It would be interesting to characterize the responsible mechanisms for the induction of these genes to the various stresses and to analyse, whether one common or several unrelated mechanisms are involved in the regulation of the type I photooxidative stress induced genes by the diverse oxidative stress conditions. These mechanisms could then be compared to the specific mechanism of the *Gpxh* response to $^1O_2$ and show which differences cause the specificity of the *Gpxh* regulation.

The response to RB in the microarray experiments was surprisingly limited, with the exception of the *Gpxh* gene, even though the concentration was just below lethality (chapter 2). This shows that the toxicity of a chemical is not urgently connected to strong gene expression and that genetic response data may miss the effect caused by a chemical. In addition, this shows that the response to $^1O_2$ is restricted to a very low number of genes, at least in the nuclear genome. Since the production of $^1O_2$ occurs predominantly in the chloroplast under natural conditions, the low induction of nuclear genes may be compensated by a strong response of genes in the chloroplast genome. Still, a large part of the chloroplast proteins are encoded in the nuclear genome and it would be a big surprise, when only chloroplast genes but none of the nuclear genes coding for chloroplast proteins are induced by $^1O_2$. However, unequal localization of RB in the cell may limit a stronger $^1O_2$ response of nuclear genes in some case [13, 14]. Thus, it would be better to analyse the expression profile caused by a natural source of $^1O_2$, like the illumination with high light intensities and the presence of the herbicide dinoterb [5, 9, 21]. This expression profile could then be compared to the one of DCMU-exposed cells under high light intensities, having a reduced $^1O_2$ generation compared to dinoterb treated cultures (chapter 4) [5]. Such a

comparison could give much more information about the stress response to high level of $^1O_2$ produced at the natural site of generation.

Induction of the *Gpxh* Gene

The *Gpxh* gene in *C. reinhardtii* was partially induced by the addition of various ROS and organic hydroperoxides, but none of these ROS induced the *Gpxh* gene as strong as $^1O_2$ did (chapter 2; Fig. 4). This may be caused by a very sensitive sensor for $^1O_2$ in a specific cellular location, e.g. the chloroplast, or by the generation of high levels of $^1O_2$ in the whole cell. The mechanism of $^1O_2$ production by NR in the thylakoids is not known. The inhibition of photosynthetic electron transport and the reduction of the variable chlorophyll fluorescence indicate a stimulation of the $^1O_2$ generation in the PSII via charge recombination (chapter 3; Tab. 1 and 2). However, some observed effects by NR are not compatible with this mechanism. The expression of *Gpxh* by NR is already induced at concentrations below 1 µM (chapter 3; Fig. 1) whereas the inhibition of electron transport takes place only above 10 µM NR. An uncoupling activity of NR may partially overcome this inhibition at low concentrations, but the discrepancy is too high to explain the strong induction of *Gpxh* at 1 to 2 µM NR. The difference between the responses to NR and to dinoterb, a phenolic herbicide which also blocks the electron transport and increases $^1O_2$ production in the PSII, further questions the charge recombination mechanism. Even though dinoterb strongly inhibits the electron transport already at 30 µM (data not shown), this concentration only caused a strong stimulation of *Gpxh* expression at high light intensities (chapter 4; Fig 1), whereas NR induced the *Gpxh* gene already at low light. Furthermore, the positive effect of D_2O on the *Gpxh* induction by RB and NR suggests, that $^1O_2$ is produced by these two chemicals in an aqueous environment, where solvent quenching is the main mode of $^1O_2$ deactivation [24]. Solvent quenching by H_2O

seems not very likely for $^1O_2$ formed by charge recombination because many efficient $^1O_2$ quenchers like carotenoids and α-tocopherol and good substrates like lipids and proteins are close to the source of $^1O_2$ in the PSII in a probably rather hydrophobic environment [26, 27]. Thus, it may be that NR has a tendency to produce significant amounts of $^1O_2$ directly in the chloroplasts. A minor function as type II photosensitizer may occur for every photosensitizer (Canonica S., personal communications) and was shown for NR by Phoenix et al. [20]. This may be further stimulated by the accumulation of high NR levels in the thylakoid lumen [19], the low pH of the lumen, shifting the NR equilibrium to the protonated form, and by the high concentration of oxygen which is produced by the oxygen evolving complex during photosynthesis. However, the mechanism of $^1O_2$ production by NR in the chloroplasts is of minor importance for the $^1O_2$-induced response of the *Gpxh* gene.

Although the type and location of the signal which triggers the *Gpxh* expression are fairly clear, the nature of the sensor is still obscure. Every component in the thylakoids, which can by modified by $^1O_2$, may be a candidate, such as lipids and proteins. Lipid peroxidation may be a logical cause for a strong *Gpxh* induction because the degradation of organic hydroperoxides is an important function for glutathione peroxidases in many organisms [29, 30]. Indeed, the *Gpxh* gene is also induced by organic hydroperoxides like tert-butylhydroperoxide and cumene hydroperoxide, but the kinetics of these responses were totally different from the $^1O_2$-caused induction and are rather in agreement with the late response hypothesized for the high light illumination as described in chapter 4 [15, 16]. Protein modification, e.g. the D1 protein, is a well reported effect during photoinhibition and most likely caused by the formation of $^1O_2$ [12]. Thus, break down products of degraded PSII proteins could initiate the *Gpxh* response very specifically. Alternatively, also degradation products of bleached pigments like chlorophylls and carotenoids

could be responsible for the signal causing *Gpxh* induction. Obviously, there are still important open questions concerning the signal triggering the *Gpxh* expression, such as:

- Is the signal causing the *Gpxh* induction really and only sensed in the chloroplast?
- Which is the key component, modified by $^1O_2$, acting as sensor for *Gpxh* induction?
- How is the signal, if sensed in the chloroplast, transmitted to the nucleus?

Answering these questions, especially the identification of the $^1O_2$ sensor, would help a lot to better understand the role of $^1O_2$ mediated response in *C. reinhardtii*.

Function of the Gpxh Protein

A topic addressed but not described in this work concerns the function of the Gpxh protein during photooxidative stress response. Measurements for glutathione peroxidase (GPX) activity were performed earlier with a recombinant Gpxh protein in *E. coli*, using H_2O_2 and organic hydroperoxide as substrate [16], but no significant activity could be measured. We reinvestigated a possible peroxidase activity of the Gpxh using a modified protocol of Avery et al. [2] and could measure a low but significant activity in an *E. coli* crude extract containing the overexpressed Gpxh protein with tert-butylhydroperoxide and cumene hydroperoxide as substrate [17]. This activity is comparable with measured activities of homologous nonselenocysteine containing GPXs, but seems to be too low to exhibit any physiological relevance, especially in the presence of a second, selenocysteine containing Gpx [4, 22]. Two explanations are possible: First, it could be that the selenocysteine Gpx and the Gpxh function in different cellular compartments and that the peroxidase activity of the Gpxh protein, even though being low, is essential in an organelle where the

selenocysteine Gpx, a mitochondrial protein, is absent [4]. The second and more likely explanation suggests that the Gpxh enzyme has a different substrate range than its selenocysteine homologous counterpart. Some GPXs, especially members of the phospholipid hydroperoxide glutathione peroxidases (PHGPX) have been identified to better use NADPH or thioredoxin than glutathione as electron donor to reduce their substrates [3, 6]. This may be supported by the absence of four critical arginines residues in the amino acid sequences of the PHGPX required for proper binding of glutathione in normal GPXs [29]. Of special interest is the recently described function of the yeast GPX3 in the activation of the YAP-1 transcription factor by H_2O_2, indicating that this enzyme may be involved in signal transduction [3]. However, such a function is less likely for the Gpxh protein in *C. reinhardtii,* because the *Gpxh* gene is itself strongly induced by photooxidative stress, which is not in agreement with a regulatory protein, which are usually constitutively expressed.

The Gpxh, similar to other nonseleno GPX, rather belongs to the PHGPX family than to the normal GPX due to a missing part in the primary sequence required for tetramer formation [2, 16]. The PHGPXs have a more open structure and can bind bulk substrates [3]. They also can remove organic hydroperoxides directly from the lipid bilayer, indicating a role in the defense against lipid peroxidation [29, 30]. This may be a logical function of an enzyme which is induced by $^1O_2$ produced in the chloroplast. However, the Gpxh protein does not have a typical N-terminal chloroplast localization signal and is thus not predicted to enter the chloroplast, although it was recently reported that the import of proteins into the chloroplast does not necessarily need a localization signal [18]. Information about the localization of the Gpxh protein is of special interest for the identification of the function of Gpxh in *C. reinhardtii.* Additionally, has the expression of the *Gpxh* gene to be measured on protein level and has to be

connected to a cellular function. Only then can the physiological relevance for the strong and specific induction of the *Gpxh* gene by $^1O_2$ be explained.

Regulation of the *Gpxh* Induction

One important regulation mechanism of the *Gpxh* expression was identified to occur on transcriptional level and was shown to involve a transcription factor binding site containing a TGAC motif in the *Gpxh* promoter region (chapter 5). Similar elements with a TGAC motif were already characterized in many genes of various organisms and some of these elements are also involved in the oxidative stress response, such as the AP-1 element in mammalians and YAP-1 binding site in yeast [10, 11, 25]. However, most of the factors bound to these motifs are under redox control and can be activated by various ROS, and many of its target genes contain more than one of the transcription factor binding site triggering the oxidative stress response like the AP-1, NF-κB and CRE-binding sites [23, 28]. In addition, the active transcription factors can form different homo- and heterodimers and like that bind to different homologous elements [7, 8]. Thus, it seems rather unlikely that the specific response of the *Gpxh* gene to $^1O_2$ is mediated only by the binding of one transcription factor to such a widespread DNA element without inducing other genes to comparable strength. The partial induction of the modified *Tub2B* promoter, containing the TGAC motif element from the *Gpxh* promoter, indicates that an additional element may be required for full activation (chapter 5; Fig. 1). Two simultaneously active binding complexes would increase the specificity for induction of the *Gpxh* gene dramatically. It would therefore be interesting to further analyze the *Gpxh* promoter for additional elements most likely present in the first 180 bp upstream of the transcriptional start site, because a *Gpxh* promoter fragment of this length still showed the full response to NR [15]. This should also involve an examination of the *Gpxh* mRNA sequence for stabilization signals or of the

Gpxh promoter region upstream of the 1.3 kb fragment present in plasmid pASPro1 for additional elements, which are suggested to be involved in the high light response (chapter 4). If such an additional signaling mechanism is indeed involved in the late response of the *Gpxh* gene to high light illumination, then a further challenge enters the analysis of this response and would pose new questions:

- Which is this second signal resulting in an increased *Gpxh* mRNA level late in high light response?
- How are the two signals involved in high light response related to each other, and can both of them induce the *Gpxh* expression independently?
- Are the signaling cascades of these two signals connected?
- Do these signals have similar or different mechanisms to regulate the *Gpxh* expression?

Beside the cis-acting elements of the *Gpxh* response, also the transcription factors binding to these elements have to be identified for a better characterization of the $^1O_2$ response mechanism. As discussed in chapter 5, different combinations of proteins could be involved in the binding to the TGAC motif present in the *Gpxh* promoter and only isolating the active factor can unravel its nature and its specificity for the *Gpxh* regulatory element. The composition and complexity of this transcriptional activating complex may give more information about the number and types of signaling mechanisms which are regulating it. We already identified a negative effect of the cyclic AMP pathway on the *Gpxh* expression (chapter 5). In a more expanded biochemical approach several other activators and inhibitors of common signal transduction pathways were tested for their effect on the *Gpxh* expression (data not shown). Some of them, including the G-protein activator mastoparan, the calmodulin kinase inhibitor KN-93, the phospholipase C inhibitor U73122 and the calcium channel inhibitor verapamil, could stimulate the *Gpxh* induction to some extent.

However, the fact that the stimulation by these chemicals was always stronger in illuminated cells compared to dark grown cells indicates that either an additional, light dependent signal was needed or that most of the effect was indirect, by altering the production of $^1O_2$ in the PSII. This may be the case with mastoparan, which inhibited the photosynthetic electron transport in *Asparagus* mesophyll cells [1]. Thus, the identification of components involved in the signalling mechanism of $^1O_2$ response is rather difficult and it would probably be more successful to isolate mutants with altered *Gpxh* expression than doing biochemical approaches, except when a strong activation of the *Gpxh* expression could be achieved in the dark.

REFERENCES

1. Allen LJ, MacGregor KB, Koop RS, Bruce DH, Karner J, Bown AW: The relationship between photosynthesis and a mastoparan-induced hypersensitive response in isolated mesophyll cells. Plant Physiol 119: 1233-42 (1999).
2. Avery AM, Avery SV: Saccharomyces cerevisiae expresses three phospholipid hydroperoxide glutathione peroxidases. J Biol Chem 276: 33730-5 (2001).
3. Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB: A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell 111: 471-81 (2002).
4. Fu LH, Wang XF, Eyal Y, She YM, Donald LJ, Standing KG, Ben-Hayyim G: A selenoprotein in the plant kingdom. Mass spectrometry confirms that an opal codon (UGA) encodes selenocysteine in Chlamydomonas reinhardtii gluththione peroxidase. J Biol Chem 277: 25983-91 (2002).
5. Fufezan C, Rutherford AW, Krieger-Liszkay A: Singlet oxygen production in herbicide-treated photosystem II. FEBS Lett 532: 407-10 (2002).
6. Gaber A, Tamoi M, Takeda T, Nakano Y, Shigeoka S: NADPH-dependent glutathione peroxidase-like proteins (Gpx-1, Gpx-2) reduce unsaturated fatty acid hydroperoxides in Synechocystis PCC 6803. FEBS Lett 499: 32-6 (2001).
7. Hai T, Curran T: Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci U S A 88: 3720-4 (1991).
8. Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U: ATF3 and stress responses. Gene Expr 7: 321-35 (1999).
9. Hideg E, Kalai T, Hideg K, Vass I: Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry 37: 11405-11 (1998).
10. Karin M, Liu Z, Zandi E: AP-1 function and regulation. Curr Opin Cell Biol 9: 240-6 (1997).
11. Karin M, Takahashi T, Kapahi P, Delhase M, Chen Y, Makris C, Rothwarf D, Baud V, Natoli G, Guido F, Li N: Oxidative stress and gene expression: the AP-1 and NF-kappaB connections. Biofactors 15: 87-9 (2001).
12. Keren N, Ohad I: State transition and photoinhibition. In: Rochaix J-D, Michel G-C, Sabecha M (eds) The molecular biology of chloroplasts and mitochondria in chlamydomonas, pp. 569-596. Kluwer academic publishers, Dordrecht (1998).
13. Kochevar IE, Bouvier J, Lynch M, Lin CW: Influence of dye and protein location on photosensitization of the plasma membrane. Biochim Biophys Acta 1196: 172-80 (1994).

14. Lambert CR, Kochevar IE: Electron transfer quenching of the rose Bengal triplet state. Photochemistry and Photobiology 66: 15-25 (1997).

15. Leisinger U, Rüfenacht K, Fischer B, Pesaro M, Spengler A, Zehnder AJB, Eggen RIL: The glutathione peroxidase homologous gene from Chlamydomonas reinhardtii is transcriptionally up-regulated by singlet oxygen. Plant Molecular Biology 46: 395-408 (2001).

16. Leisinger U, Rüfenacht K, Zehnder AJB, Eggen RIL: Structure of a glutathione peroxidase homologous gene involved in the oxidative stress response in *Chlamydomonas reinhardtii*. Plant Science 149: 139-149 (1999).

17. Luchsinger B: Functional characterisation of Gpxh from Chlamydomonas reinhardtii (diploma thesis). Department of Environmental Sciences, Federal Institute of Technology Zurich (ETHZ), Zürich (2003).

18. Miras S, Salvi D, Ferro M, Grunwald D, Garin J, Joyard J, Rolland N: Non-canonical transit peptide for import into the chloroplast. J Biol Chem 277: 47770-8 (2002).

19. Opanasenko VK, Semenova GA, Agafonov AV, Gubanova ON: Effect of Delta pH indicators neutral red and 9-aminoacridine on chloroplast ultrastructure. Biochemistry-Moscow 61: 1083-1087 (1996).

20. Phoenix DA, Sayed Z, Hussain S, Harris F, Wainwright M: The phototoxicity of phenothiazinium derivatives against Escherichia coli and Staphylococcus aureus. FEMS Immunol Med Microbiol 39: 17-22 (2003).

21. Rutherford AW, Krieger-Liszkay A: Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 26: 648-53 (2001).

22. Shigeoka S, Takeda T, Hanaoka T: Characterization and immunological properties of selenium-containing glutathione peroxidase induced by selenite in Chlamydomonas reinhardtii. Biochem J 275 (Pt 3): 623-7 (1991).

23. Storz G, Polla BS: Transcriptional regulators of oxidative stress-inducible genes in prokaryotes and eukaryotes. Exs 77: 239-54 (1996).

24. Taniclian C, Mechin R, Seghrouchni R, Schweitzer C: Mechanistic and kinetic aspects of photosensitization in the presence of oxygen. Photochem Photobiol 71: 12-9 (2000).

25. Toone WM, Morgan BA, Jones N: Redox control of AP-1-like factors in yeast and beyond. Oncogene 20: 2336-46 (2001).

26. Trebst A: Function of beta-carotene and tocopherol in photosystem II. Z Naturforsch [C] 58: 609-20 (2003).

27. Trebst A, Depka B, Hollander-Czytko H: A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in Chlamydomonas reinhardtii. FEBS Lett 516: 156-60 (2002).

28. Turpaev KT: Reactive oxygen species and regulation of gene expression. Biochemistry (Mosc) 67: 281-92 (2002).

29. Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D, Flohe L: Diversity of glutathione peroxidases. Methods Enzymol 252: 38-53 (1995).

30. Wang HP, Qian SY, Schafer FQ, Domann FE, Oberley LW, Buettner GR: Phospholipid hydroperoxide glutathione peroxidase protects against singlet oxygen-induced cell damage of photodynamic therapy. Free Radic Biol Med 30: 825-35 (2001).

Table I: Fold induction (range in parenthesis) by 5 μM NR with the corresponding *t*-test *P*-value of all genes present in figure 2, 3 and 5 of chapter 2.

Figure 2:

clone ID	NR induction 20 min	*P*-value	NR induction 60 min	*P*-value	NR induction 120 min	*P*-value
963041H11	1.115 (1.025 - 1.255)	7.52E-03	2.293 (2.101 - 2.580)	1.30E-08	2.749 (1.716 - 4.138)	3.17E-04
894028F11	1.018 (0.811 - 1.492)	8.04E-01	2.049 (0.531 - 5.778)	7.82E-02	3.467 (1.419 - 7.083)	7.30E-04
894096D05	1.254 (1.121 - 1.459)	2.02E-04	2.324 (1.522 - 3.021)	2.72E-05	3.229 (1.925 - 4.733)	5.52E-05
894008C03	2.250 (1.934 - 2.798)	4.85E-07	2.532 (1.012 - 5.183)	5.48E-03	2.153 (1.284 - 3.963)	3.39E-03
894006G02	1.058 (0.827 - 1.238)	2.47E-01	2.351 (1.440 - 3.277)	6.06E-05	3.575 (1.979 - 5.247)	5.42E-05
963036A07	1.011 (0.812 - 1.100)	7.56E-01	2.215 (0.988 - 3.530)	1.70E-03	3.847 (2.535 - 6.797)	3.23E-05
894016F07	2.002 (1.453 - 2.550)	6.04E-06	2.713 (1.282 - 4.886)	6.04E-04	2.621 (1.757 - 3.522)	6.22E-05
894077F06	1.508 (1.170 - 2.210)	1.21E-03	2.271 (1.229 - 3.246)	5.65E-04	3.626 (3.012 - 4.103)	4.34E-09
894009B03	1.171 (1.047 - 1.297)	1.79E-04	3.335 (1.080 - 6.143)	1.43E-03	2.974 (1.912 - 4.629)	8.84E-05
894100A09	1.834 (1.208 - 3.255)	1.79E-03	3.820 (1.270 - 12.132)	3.35E-03	2.389 (1.213 - 4.409)	4.82E-03
963045E08	2.534 (1.562 - 4.086)	5.39E-05	3.059 (1.276 - 11.273)	5.74E-03	3.731 (1.330 - 7.318)	6.18E-04
963032A08	2.031 (1.497 - 2.561)	5.38E-05	2.859 (1.133 - 8.990)	7.76E-03	4.446 (1.954 - 7.440)	3.90E-05
894024F09	1.655 (1.374 - 2.047)	5.28E-05	5.306 (3.137 - 9.405)	1.38E-05	3.035 (2.128 - 4.602)	6.30E-05
963068A08	2.487 (2.003 - 3.494)	1.61E-06	5.598 (3.987 - 9.114)	1.37E-06	4.617 (2.798 - 6.826)	1.41E-05
963068F01	1.580 (1.127 - 2.026)	2.02E-04	5.068 (2.214 - 9.253)	1.14E-04	6.355 (3.055 - 12.871)	1.72E-04
894022H10	1.096 (0.770 - 1.508)	3.75E-01	3.260 (1.070 - 13.587)	1.22E-02	8.663 (5.589 - 11.542)	1.47E-07
894018D09	3.727 (2.658 - 6.057)	9.07E-06	4.212 (2.219 - 8.851)	1.80E-04	5.213 (4.085 - 6.687)	1.31E-07
894096A07	1.646 (1.298 - 1.968)	3.67E-05	4.770 (1.647 - 12.067)	1.07E-03	7.030 (3.277 - 12.473)	1.60E-05
963050F02	1.418 (1.171 - 1.797)	1.46E-04	7.966 (2.251 - 23.900)	5.42E-04	12.478 (4.710 - 29.040)	7.11E-05
894087F06	6.825 (2.48 - 16.969)	5.66E-05	8.647 (1.161 - 56.975)	6.28E-03	15.277 (2.776 - 30.307)	5.83E-05
963046E04	10.003 (6.628 - 13.23)	4.25E-08	19.521 (7.576 - 32.613)	1.11E-06	20.880 (13.500 - 31.317)	6.82E-08
894082A11	4.504 (2.944 - 10.160)	2.45E-05	21.489 (3.329 - 90.718)	4.91E-04	49.831 (32.353 - 61.830)	2.09E-10

Figure 3:

clone ID	NR induction 20 min	*P*-value	NR induction 60 min	*P*-value	NR induction 120 min	*P*-value
963035G05	0.947 (0.635 - 1.134)	4.95E-01	0.879 (0.441 - 1.422)	3.94E-01	1.120 (1.005 - 1.441)	3.53E-02
963018E07	0.881 (0.699 - 1.044)	3.31E-02	0.876 (0.591 - 1.149)	1.05E-01	1.337 (1.011 - 1.616)	1.58E-03
894062D05	0.946 (0.842 - 1.016)	6.14E-02	1.094 (0.505 - 1.691)	6.03E-01	1.816 (1.223 - 2.267)	1.60E-04

963044F03	1.141 (0.832 - 1.804)	2.91E-01	1.425 (1.009 - 2.064)	1.78E-02	1.907 (1.024 - 2.778)	3.32E-03
894080E09	0.945 (0.706 - 1.297)	4.01E-01	1.380 (1.035 - 1.980)	1.13E-02	1.985 (1.304 - 2.281)	2.31E-05
963016H09	0.955 (0.882 - 1.222)	2.39E-01	1.469 (0.472 - 2.778)	8.63E-02	2.038 (1.147 - 2.982)	6.52E-04
963039B04	0.985 (0.920 - 1.056)	4.36E-01	1.201 (0.668 - 1.765)	2.06E-01	2.281 (1.421 - 3.547)	4.93E-04
894055D04	1.055 (0.821 - 1.297)	2.92E-01	1.336 (0.947 - 1.864)	9.26E-03	2.377 (1.964 - 2.675)	1.87E-07
894011H03	1.006 (0.781 - 1.333)	9.30E-01	1.429 (0.736 - 2.228)	4.19E-02	2.393 (1.612 - 3.277)	1.65E-04
894076H01	0.894 (0.751 - 1.024)	3.30E-02	1.673 (1.010 - 3.008)	1.08E-02	2.507 (1.844 - 4.197)	9.52E-05
894031G12	0.914 (0.753 - 1.011)	3.88E-02	1.461 (0.969 - 2.366)	1.28E-02	2.554 (1.307 - 3.826)	3.73E-04
894093E06	0.974 (0.923 - 1.078)	1.78E-01	1.714 (1.019 - 3.123)	7.00E-03	2.586 (1.813 - 3.701)	7.45E-05
894082H05	0.897 (0.784 - 1.002)	9.10E-03	1.807 (1.188 - 3.517)	5.46E-03	2.812 (1.707 - 4.155)	9.00E-05
963033E04	1.062 (0.805 - 1.285)	3.30E-01	1.259 (1.028 - 1.726)	3.44E-03	2.915 (1.885 - 4.675)	1.43E-04
963047A04	1.142 (1.072 - 1.362)	2.06E-03	1.933 (1.313 - 3.506)	9.12E-04	2.940 (1.968 - 5.580)	1.57E-04
963027C08	1.138 (0.905 - 1.454)	4.45E-02	1.685 (1.027 - 3.441)	2.90E-02	3.117 (2.068 - 4.920)	7.36E-05
proteases						
963033H10	1.284 (1.161 - 1.490)	7.26E-05	1.646 (1.088 - 2.532)	3.72E-03	2.220 (1.375 - 3.135)	2.22E-04
963036B12	0.898 (0.816 - 0.974)	2.99E-03	1.972 (1.090 - 2.699)	1.20E-03	2.333 (1.673 - 3.194)	4.26E-05
963014H04	0.834 (0.592 - 1.015)	2.38E-02	1.383 (0.937 - 2.204)	1.89E-02	2.632 (1.858 - 3.625)	7.97E-05
HSP						
894078G05	1.053 (0.956 - 1.172)	6.87E-02	2.111 (0.992 - 3.351)	1.98E-03	1.568 (1.254 - 1.720)	1.16E-05
ox. stress genes						
894065G01	0.897 (0.695 - 1.196)	2.39E-01	1.290 (0.745 - 1.844)	7.12E-02	1.681 (1.525 - 1.966)	2.51E-06
894081G12	1.038 (0.716 - 1.380)	7.13E-01	1.711 (1.510 - 1.858)	5.98E-08	1.933 (1.680 - 2.258)	2.26E-06
894063H05	1.186 (0.898 - 1.780)	5.50E-02	1.967 (1.274 - 3.585)	3.67E-03	3.102 (2.109 - 4.478)	9.33E-06

Figure 5

LHC						
894080G01	0.933 (0.496 - 1.963)	7.61E-01	0.553 (0.459 - 0.632)	1.83E-06	0.644 (0.437 - 0.952)	1.27E-02
894052A01	0.686 (0.519 - 0.969)	2.19E-03	0.549 (0.462 - 0.686)	4.65E-06	0.532 (0.373 - 0.892)	1.36E-03
894044B07	0.692 (0.628 - 0.855)	9.74E-05	0.748 (0.636 - 0.840)	2.14E-05	0.516 (0.433 - 0.722)	1.78E-05
894041D11	0.815 (0.706 - 0.941)	4.94E-04	0.540 (0.383 - 0.742)	8.28E-05	0.470 (0.284 - 0.786)	3.37E-03
894065B07	0.488 (0.379 - 0.722)	4.92E-05	0.558 (0.488 - 0.604)	5.92E-08	0.436 (0.323 - 0.594)	6.65E-05
894076B06	0.833 (0.703 - 1.051)	2.55E-02	0.706 (0.588 - 0.873)	3.22E-04	0.435 (0.350 - 0.564)	7.10E-06
894078C01	1.055 (0.901 - 1.312)	2.83E-01	0.651 (0.595 - 0.777)	8.23E-06	0.433 (0.325 - 0.634)	6.95E-05
894087C09	0.821 (0.575 - 1.087)	4.97E-02	0.542 (0.355 - 0.853)	8.33E-04	0.429 (0.348 - 0.564)	5.47E-06

963047H05	0.901 (0.782 - 1.100)	4.97E-02	0.564 (0.484 - 0.674)	4.50E-07	0.418 (0.296 - 0.634)	1.64E-04
894062E07	0.677 (0.446 - 0.860)	1.26E-03	0.463 (0.350 - 0.658)	9.52E-05	0.402 (0.254 - 0.628)	7.08E-04
894033H06	0.955 (0.572 - 1.766)	6.99E-01	0.612 (0.464 - 0.902)	1.51E-03	0.337 (0.192 - 0.627)	1.22E-03
963024B11	0.689 (0.549 - 1.047)	2.50E-03	0.469 (0.351 - 0.661)	1.01E-05	0.329 (0.196 - 0.606)	4.54E-04
963042A01	0.798 (0.659 - 1.409)	3.29E-02	0.435 (0.313 - 0.543)	4.33E-06	0.317 (0.218 - 0.496)	5.67E-05
photosynth gene						
894098H06	0.984 (0.850 - 1.641)	8.45E-01	1.035 (0.699 - 1.207)	6.12E-01	0.783 (0.572 - 0.964)	1.27E-02
894053E07	1.130 (0.928 - 1.597)	1.86E-01	0.781 (0.731 - 0.860)	2.62E-06	0.653 (0.527 - 0.818)	4.59E-04
894041H01	1.052 (0.762 - 1.303)	4.45E-01	0.732 (0.625 - 0.864)	4.30E-04	0.594 (0.424 - 1.038)	4.83E-03
894017C09	0.626 (0.469 - 0.799)	1.40E-04	0.769 (0.633 - 0.877)	3.01E-04	0.571 (0.439 - 0.758)	6.68E-04
894100F04	1.046 (0.730 - 1.372)	5.77E-01	0.714 (0.614 - 0.884)	1.55E-04	0.498 (0.380 - 0.638)	1.44E-05
894083B07	0.854 (0.728 - 1.049)	1.05E-02	0.554 (0.390 - 0.680)	7.99E-05	0.497 (0.375 - 0.679)	2.55E-04
963047E03	1.051 (0.878 - 1.537)	4.82E-01	0.721 (0.422 - 0.871)	5.50E-03	0.491 (0.331 - 0.802)	1.27E-03
963041E04	1.043 (0.796 - 1.375)	5.58E-01	0.546 (0.508 - 0.569)	1.22E-09	0.485 (0.394 - 0.616)	2.26E-05
963053C08	0.842 (0.762 - 0.909)	1.16E-04	0.693 (0.471 - 0.861)	3.61E-03	0.477 (0.379 - 0.612)	4.03E-05
894004A09	0.838 (0.549 - 2.096)	2.92E-01	0.712 (0.559 - 0.946)	2.90E-03	0.462 (0.261 - 0.941)	7.36E-03
894019B05	0.977 (0.817 - 1.286)	6.75E-01	0.600 (0.368 - 0.962)	1.16E-02	0.437 (0.317 - 0.696)	2.62E-04
894068A11	0.790 (0.655 - 0.892)	6.43E-04	0.578 (0.435 - 0.851)	1.10E-03	0.436 (0.265 - 0.726)	2.65E-03
894069B11	0.743 (0.661 - 0.857)	5.59E-05	0.497 (0.355 - 0.771)	7.12E-04	0.421 (0.326 - 0.596)	3.56E-05
894006E05	0.748 (0.487 - 1.672)	5.99E-02	0.482 (0.414 - 0.604)	2.17E-06	0.412 (0.276 - 0.732)	5.56E-04
894069E01	0.695 (0.517 - 0.898)	7.90E-04	0.638 (0.559 - 0.830)	1.96E-05	0.405 (0.286 - 0.597)	1.66E-04
894002C07	0.773 (0.491 - 1.054)	3.55E-02	0.545 (0.448 - 0.790)	6.17E-05	0.378 (0.242 - 0.616)	6.21E-04

Table II: Fold induction (range in parenthesis) by 1 μM RB with the corresponding *t*-test *P*-value of all genes present in figure 2, 3 and 5 of chapter 2.

Figure 2:

clone ID	RB induction 20 min	*P*-value	RB induction 60 min	*P*-value	RB induction 120 min	*P*-value
963041H11	1.025 (0.851 - 1.561)	7.12E-01	0.997 (0.794 - 1.296)	9.67E-01	0.360 (0.010 - 1.211)	1.18E-01
894028F11	1.068 (1.048 - 1.095)	5.21E-06	1.149 (0.996 - 1.412)	2.24E-02	1.128 (1.057 - 1.240)	2.55E-04
894096D05	1.088 (0.986 - 1.250)	1.51E-02	1.305 (1.123 - 1.474)	4.57E-05	1.034 (0.915 - 1.114)	2.07E-01
894008C03	1.112 (1.086 - 1.145)	2.43E-06	1.357 (1.116 - 1.712)	2.12E-03	1.385 (0.992 - 2.646)	2.81E-02
894006G02	1.089 (1.006 - 1.193)	3.32E-03	1.378 (1.140 - 1.698)	1.50E-03	1.074 (0.837 - 1.244)	2.03E-01
963036A07	1.005 (0.795 - 1.127)	9.12E-01	1.313 (1.113 - 1.521)	1.46E-04	0.539 (0.010 - 1.256)	3.31E-01
894016F07	1.034 (0.980 - 1.144)	1.76E-01	1.115 (1.029 - 1.187)	6.27E-04	0.751 (0.399 - 1.079)	8.52E-02
894077F06	1.016 (0.918 - 1.118)	5.11E-01	1.458 (0.898 - 2.254)	5.04E-02	1.430 (1.088 - 1.893)	1.59E-03
894009B03	1.093 (0.992 - 1.147)	1.57E-03	1.218 (1.159 - 1.331)	1.90E-05	0.947 (0.737 - 1.088)	3.11E-01
894100A09	1.140 (1.086 - 1.244)	8.55E-05	2.036 (1.584 - 2.862)	7.08E-05	1.069 (0.974 - 1.162)	2.66E-02
963045E08	1.191 (1.078 - 1.324)	3.12E-04	1.230 (1.057 - 1.579)	5.90E-03	1.073 (0.914 - 1.185)	1.37E-01
963032A08	1.080 (1.018 - 1.211)	2.11E-02	0.990 (0.866 - 1.167)	7.83E-01	0.894 (0.668 - 1.044)	6.35E-02
894024F09	0.988 (0.936 - 1.042)	4.40E-01	2.023 (1.382 - 2.703)	5.29E-05	1.125 (0.928 - 2.172)	2.66E-01
963068A08	1.066 (0.958 - 1.229)	7.84E-02	1.604 (1.400 - 1.872)	7.04E-06	0.521 (0.010 - 1.475)	3.10E-01
963068F01	1.056 (0.960 - 1.146)	4.17E-02	1.492 (1.069 - 1.895)	2.59E-03	1.202 (0.908 - 1.604)	3.55E-02
894022H10	1.372 (0.903 - 1.810)	1.08E-02	0.967 (0.944 - 1.010)	6.98E-03	0.966 (0.890 - 1.016)	8.98E-02
894018D09	1.413 (1.329 - 1.532)	4.83E-08	1.343 (1.031 - 1.926)	1.83E-02	1.084 (0.898 - 1.191)	8.31E-02
894096A07	0.939 (0.884 - 1.012)	7.06E-03	1.497 (0.900 - 2.557)	5.89E-02	1.278 (1.014 - 1.591)	5.98E-03
963050F02	1.087 (0.950 - 1.336)	7.09E-02	1.149 (1.005 - 1.259)	1.37E-03	0.881 (0.660 - 1.071)	8.15E-02
894087F06	1.102 (1.018 - 1.235)	7.21E-03	1.826 (1.187 - 3.349)	6.37E-03	1.307 (0.747 - 2.336)	1.04E-01
963046E04	3.635 (2.455 - 4.574)	1.36E-06	12.448 (7.932 - 18.728)	4.80E-07	6.621 (3.671 - 9.083)	2.50E-06
894082A11	0.992 (0.892 - 1.064)	6.97E-01	1.478 (1.213 - 1.734)	4.55E-05	1.439 (1.051 - 3.041)	5.83E-02

Figure 3:

clone ID	RB induction 20 min	*P*-value	RB induction 60 min	*P*-value	RB induction 120 min	*P*-value
963035G05	1.233 (0.990 - 1.478)	6.69E-03	1.051 (1.002 - 1.094)	1.30E-03	1.077 (0.947 - 1.265)	5.31E-02
963018E07	1.018 (0.978 - 1.041)	4.25E-02	1.152 (1.092 - 1.226)	3.75E-05	0.967 (0.799 - 1.146)	5.38E-01
894062D05	0.905 (0.847 - 1.102)	3.60E-02	1.048 (0.968 - 1.156)	1.15E-01	1.230 (1.036 - 1.439)	5.53E-03
963044F03	0.964 (0.945 - 1.014)	6.18E-03	1.001 (0.787 - 1.196)	9.88E-01	1.188 (0.989 - 1.432)	9.08E-03

894080E09	0.958 (0.932 - 0.981)	6.40E-04	1.142 (0.976 - 1.380)	2.26E-02	1.130 (1.003 - 1.393)	5.61E-02
963016H09	1.011 (0.959 - 1.153)	6.31E-01	1.218 (1.080 - 1.386)	2.87E-04	1.177 (0.915 - 1.823)	1.07E-01
963039B04	0.996 (0.881 - 1.162)	9.18E-01	1.189 (0.924 - 1.416)	9.75E-03	0.792 (0.307 - 1.292)	2.51E-01
894055D04	1.105 (1.041 - 1.247)	6.73E-03	1.256 (1.139 - 1.375)	8.82E-05	1.259 (1.067 - 1.553)	2.72E-03
894011H03	1.085 (1.060 - 1.115)	1.59E-05	1.206 (1.046 - 1.287)	1.39E-04	1.353 (0.904 - 3.145)	1.40E-01
894076H01	0.996 (0.927 - 1.068)	8.71E-01	1.313 (1.176 - 1.432)	9.13E-06	1.024 (0.769 - 1.132)	6.14E-01
894031G12	1.019 (0.981 - 1.139)	3.95E-01	1.318 (1.221 - 1.419)	9.70E-07	1.036 (0.911 - 1.196)	3.23E-01
894093E06	1.063 (0.986 - 1.121)	1.41E-02	1.178 (1.076 - 1.300)	1.99E-04	1.072 (0.970 - 1.361)	1.63E-01
894082H05	0.978 (0.957 - 1.005)	1.94E-02	1.450 (1.057 - 1.988)	6.40E-03	1.165 (1.050 - 1.288)	4.89E-04
963033E04	0.981 (0.854 - 1.042)	4.77E-01	1.326 (1.061 - 1.650)	1.62E-03	1.075 (0.947 - 1.377)	2.04E-01
963047A04	0.982 (0.909 - 1.055)	3.28E-01	1.433 (0.964 - 1.912)	4.99E-03	1.194 (1.090 - 1.290)	5.00E-05
963027C08	0.999 (0.930 - 1.144)	9.72E-01	1.387 (1.200 - 1.680)	6.66E-05	1.201 (0.420 - 3.356)	3.88E-01
proteases						
963033H10	0.978 (0.956 - 1.023)	2.53E-02	1.179 (0.981 - 1.592)	3.20E-02	1.030 (0.955 - 1.100)	9.15E-02
963036B12	0.960 (0.903 - 1.030)	4.83E-02	1.264 (1.102 - 1.428)	1.19E-04	1.269 (1.054 - 2.261)	2.99E-02
963014H04	0.936 (0.922 - 0.954)	3.93E-06	1.265 (0.959 - 1.647)	2.33E-02	1.178 (1.011 - 1.357)	2.86E-03
HSP						
894078G05	0.903 (0.881 - 0.920)	1.76E-07	1.336 (0.934 - 1.809)	3.15E-02	1.153 (1.033 - 1.340)	4.54E-03
ox. stress genes						
894065G01	0.951 (0.858 - 1.067)	7.73E-02	1.094 (0.880 - 1.316)	2.08E-01	1.093 (1.010 - 1.177)	4.01E-03
894081G12	0.966 (0.901 - 1.081)	2.29E-01	1.106 (1.027 - 1.220)	2.82E-03	1.114 (1.006 - 1.227)	3.45E-03
894063H05	0.987 (0.877 - 1.221)	7.87E-01	1.233 (1.095 - 1.408)	6.33E-04	1.032 (0.944 - 1.137)	1.64E-01

Figure 5:

LHC						
894080G01	0.914 (0.797 - 0.999)	2.01E-02	0.724 (0.692 - 0.798)	2.95E-07	0.687 (0.603 - 0.775)	1.26E-05
894052A01	0.819 (0.791 - 0.847)	2.08E-08	0.778 (0.708 - 0.849)	2.63E-05	0.814 (0.664 - 0.974)	6.45E-03
894044B07	0.993 (0.922 - 1.050)	6.10E-01	0.854 (0.716 - 0.976)	1.05E-02	0.831 (0.742 - 0.924)	1.48E-04
894041D11	0.909 (0.858 - 1.031)	3.61E-03	0.857 (0.832 - 0.889)	1.11E-07	0.928 (0.742 - 1.098)	2.10E-01
894065B07	0.907 (0.884 - 0.949)	1.55E-05	0.756 (0.685 - 0.826)	2.28E-05	0.900 (0.744 - 1.319)	2.01E-01
894076B06	0.987 (0.925 - 1.207)	6.88E-01	0.908 (0.865 - 0.982)	6.68E-04	0.922 (0.746 - 1.134)	8.60E-02
894078C01	0.988 (0.950 - 1.017)	2.16E-01	0.916 (0.867 - 0.961)	2.54E-04	0.792 (0.763 - 0.829)	3.57E-08
894087C09	0.955 (0.927 - 1.064)	2.63E-02	0.824 (0.794 - 0.876)	3.83E-07	1.030 (0.864 - 1.380)	6.32E-01
963047H05	1.042 (1.000 - 1.107)	2.00E-02	0.833 (0.766 - 0.938)	4.00E-04	1.020 (0.825 - 1.650)	8.16E-01

894062E07	0.904 (0.840 - 1.038)	1.45E-02	0.797 (0.713 - 0.881)	5.02E-05	0.833 (0.743 - 0.926)	2.31E-03
894033H06	0.931 (0.827 - 0.991)	3.07E-02	0.781 (0.723 - 0.827)	3.11E-07	0.843 (0.734 - 0.922)	8.79E-04
963024B11	0.936 (0.866 - 1.142)	1.02E-01	0.803 (0.741 - 0.881)	3.19E-05	0.826 (0.621 - 1.038)	2.88E-02
963042A01	1.152 (0.972 - 1.314)	1.00E-02	0.739 (0.603 - 0.889)	7.44E-04	0.809 (0.606 - 1.114)	1.77E-02
photosynth genes						
894098H06	0.969 (0.929 - 1.013)	4.23E-02	1.035 (0.926 - 1.086)	1.40E-01	1.069 (0.896 - 1.453)	3.33E-01
894053E07	0.896 (0.883 - 0.910)	3.89E-08	0.895 (0.859 - 0.965)	1.04E-04	1.008 (0.868 - 1.135)	8.67E-01
894041H01	0.925 (0.868 - 1.052)	1.31E-02	0.966 (0.911 - 1.041)	6.51E-02	1.027 (0.830 - 1.282)	6.45E-01
894017C09	1.176 (0.945 - 1.423)	2.31E-02	0.834 (0.745 - 0.924)	5.93E-04	0.879 (0.823 - 0.957)	1.20E-03
894100F04	0.963 (0.850 - 1.034)	2.27E-01	0.922 (0.846 - 0.991)	4.99E-03	0.953 (0.803 - 1.157)	3.66E-01
894083B07	0.979 (0.867 - 1.102)	4.17E-01	0.925 (0.765 - 1.180)	1.88E-01	1.071 (0.760 - 2.530)	6.87E-01
963047E03	1.034 (0.950 - 1.115)	1.27E-01	0.942 (0.799 - 1.048)	1.08E-01	1.081 (0.896 - 1.351)	2.50E-01
963041E04	1.031 (0.878 - 1.361)	5.94E-01	0.910 (0.762 - 0.989)	1.25E-02	0.936 (0.704 - 1.119)	2.74E-01
963053C08	1.026 (0.910 - 1.285)	5.49E-01	0.905 (0.844 - 0.983)	2.34E-03	0.608 (0.158 - 0.983)	6.14E-02
894004A09	1.425 (1.015 - 1.601)	1.44E-03	0.854 (0.793 - 0.908)	9.13E-06	0.952 (0.905 - 0.990)	5.68E-03
894019B05	1.116 (1.029 - 1.194)	1.25E-03	0.861 (0.730 - 1.059)	1.31E-02	0.822 (0.629 - 0.943)	1.18E-02
894068A11	0.967 (0.926 - 0.998)	2.05E-02	0.726 (0.625 - 0.839)	2.02E-04	0.767 (0.329 - 1.063)	8.33E-02
894069B11	0.919 (0.872 - 0.966)	6.57E-04	0.791 (0.653 - 1.291)	1.52E-02	1.019 (0.756 - 1.365)	8.36E-01
894006E05	1.185 (0.922 - 1.365)	8.47E-03	0.788 (0.736 - 0.867)	6.37E-06	0.846 (0.659 - 1.006)	4.91E-02
894069E01	0.899 (0.812 - 0.946)	1.23E-03	0.723 (0.662 - 0.797)	1.46E-06	0.687 (0.605 - 0.838)	1.51E-04
894002C07	1.072 (0.834 - 1.219)	2.07E-01	0.842 (0.749 - 0.963)	1.63E-03	0.828 (0.731 - 0.994)	1.70E-03

Table III: Fold induction of the 14 selected genes present in figure 4 of chapter 2 by a dark-light shift, 2 mM H_2O_2, 5 μM menadione,0.1 mM t-BOOH, 5 μM NR or 1 μM RB analyzed by real-time RT-PCR

Figure 4:

Clone ID	d-l shift 20 min	d-l shift 60min	d-l shift 120 min	H_2O_2 20 min	H_2O_2 60 min	H_2O_2 120 min	Menadione 20 min	Menadione 60 min	Menadione 120 min
894016F07	0.6	1.0	0.5	1.4	0.9	2.0	1.2	1.3	0.3
963041H11	1.8	0.4	0.1	1.7	1.9	3.1	1.4	2.2	1.3
894028F11	0.6	0.2	0.1	1.0	1.4	1.6	1.8	4.6	1.4
963046E04	2.0	2.8	0.8	0.7	2.9	6.3	2.0	6.4	5.0
894024F09	2.6	11.1	0.9	1.2	2.6	1.6	2.5	1.9	2.8
894008C03	1.9	7.5	0.6	2.8	5.5	14.8	7.2	3.9	0.3
894100A09	1.4	1.1	0.8	2.4	10.0	10.8	2.2	5.0	0.7
963032A08	2.4	3.4	0.2	1.8	4.7	30.3	3.8	10.9	0.7
894082A11	1.0	0.8	0.4	3.9	19.3	121.4	179.4	70.0	3.7
963036A07	0.9	2.3	0.9	1.0	2.4	3.8	1.5	2.5	1.2
894077F06	0.9	2.0	0.9	1.1	2.5	2.6	3.7	3.0	2.4
894096D05	0.7	3.5	0.9	2.0	3.2	8.9	10.9	4.0	1.8
894006G02	0.9	3.4	0.8	1.2	3.6	5.2	10.2	4.7	1.3
894009B03	2.8	7.0	1.3	1.8	4.4	7.4	18.8	9.2	1.1
963068F01	1.7	6.2	0.6	11.8	18.7	96.6	5.4	27.8	5.1
894096A07	3.5	9.1	0.9	3.6	16.9	53.1	11.6	27.6	3.6
963050F02	1.1	4.3	0.2	2.4	9.6	543.7	15.6	8.7	0.6
894022H10	2.3	1.1	0.5	2.0	8.8	34.2	27.3	4.6	1.8

Clone ID	t-BOOH 20 min	t-BOOH 60 min	t-BOOH 120 min	NR light 20 min	NR light 60 min	NR light 120 min	RB light 20 min	RB light 60 min	RB light 120 min
894016F07	1.1	1.6	7.1	1.8	1.4	1.9	0.9	1.0	1.1
963041H11	0.9	1.6	3.9	1.5	4.8	12.3	1.3	1.5	2.3
894028F11	5.5	3.7	7.8	8.2	24.3	35.9	0.9	2.3	4.2
963046E04	1.5	4.7	12.4	25.0	78.9	92.5	10.3	47.4	27.7
894024F09	1.4	1.0	4.6	1.9	10.5	10.0	0.9	4.8	1.7
894008C03	3.1	8.4	4.9	8.0	6.5	3.3	1.9	2.6	2.4
894100A09	1.6	1.8	2.8	3.6	8.5	15.0	1.5	3.5	1.6
963032A08	10.1	32.3	8.7	8.5	17.6	42.3	0.8	1.6	1.3
894082A11	8.0	49.7	49.6	38.4	210.2	806.3	0.9	8.2	11.4
963036A07	1.0	2.5	5.7	1.3	5.0	13.5	1.1	2.6	2.1
894077F06	1.5	4.0	5.2	1.4	4.9	12.6	1.3	2.6	2.7
894096D05	1.3	5.0	7.1	0.8	7.8	18.4	0.8	2.5	2.3
894006G02	1.7	7.2	7.1	0.9	6.7	12.5	0.9	2.2	2.0
894009B03	2.5	9.4	7.1	1.8	18.1	22.6	0.6	1.6	1.4
963068F01	2.2	31.1	91.8	8.4	16.1	155.1	0.8	3.7	2.7
894096A07	5.2	35.3	28.9	4.0	20.7	43.5	1.4	4.0	3.3
963050F02	4.4	26.7	53.1	74.2	504.8	664.7	0.7	6.8	2.7
894022H10	2.1	4.7	12.3	3.0	40.2	115.4	0.8	1.6	3.3

CURRICULUM VITAE

Beat Fischer

02.02.1974	Born in Muri (AG), Switzerland
1981-1986	Primary school in Merenschwand (AG)
1986-1990	Secondary school in Muri (AG)
1990-1994	Gymnasium for mathematics and science at the Kontonsschule Wohlen (AG) with graduation diploma (Matura Typus C)
1994-2000	Undergraduate studies in the faculty of Biochemistry at the University of Fribourg and the faculty of Biology II at the University of Basel with specialization in molecular- and microbiology
2000-2004	Doctoral studies in the Department of Environmental Microbiology and Molecular Ecotoxicology (MIX) at the Swiss Federal Institute for Environmental Science and Technology (EAWAG) in Dübendorf (ZH)