Differential Gene Expression in Plants Stressed by the Peroxidizing Herbicide Oxyfluorfen §

Barbara Lederer, Oliver Carsten Knörzer and Peter Böger*

Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, D-78457 Konstanz, Germany. Fax: +49–7531–88 3042.
E-mail: Peter.Boeger@uni-konstanz.de

* Author for correspondence and reprint requests

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The response of plants to the peroxidizing herbicide oxyfluorfen was investigated. The action of this *p*-nitrodiphenyl ether is based on inhibition of plastidic protoporphyrinogen oxidase, which leads to accumulation of protoporphyrin IX in the cytosol yielding reactive oxygen species by light activation. The induction of activities of antioxidative enzymes was followed in *Nicotiana tabacum* plants, var. BelW3. Glutathione reductase activity was elevated by 75% compared to control, monodehydroascorbate reductase by 65% and glutathione *S*-transferase by 110%. The mRNA of ascorbate peroxidase and catalase isoform 2 was induced, the catalase isoform 1 was reduced. These findings were confirmed and supported by measuring enzymatic activity changes in photoheterotrophically grown soybean (*Glycine max*) suspension cultures. To find a possible involvement of compounds regulating oxidative stress response, we investigated the influence of salicylic acid and BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methylester), both inducers of pathogen defense, on soybean cell suspension cultures. The specific activities of glutathione reductase, monodehydroascorbate reductase and glutathione *S*-transferase increased strongly, comparable to oxyfluorfen treatment. Both compounds protected the cells against oxyfluorfen-induced lipid peroxidation and alleviated the accumulation of protoporphyrin IX.

Introduction

Oxidative stress is an impact plants have to deal with since they produced the atmospheric oxidizing environment. Reactive oxygen species (ROS) and their destructive activity are inevitable in metabolism (e.g. Mehler reaction, Polle, 1996) and under certain environmental challenges (e.g. ozone, Sandermann, 1996). Nevertheless, they seem to be indispensable in signal transduction (Foyer *et al.*, 1997) or pathogen defense (Mehdy *et al.*, 1996). Due to this dualism, plants had to develop a sophisticated antioxidative system able to deal with both the threat and benefit of ROS.

We are elucidating the antioxidative system and its regulation and focus on the response of plants to peroxidizing herbicides. Their target is a step in chlorophyll synthesis. Oxyfluorfen, provoking oxidative stress, inhibits the enzyme protoporhyrinogen oxidase (E. C. 1.3.3.4), thereby leading to formation of excess protoporphyrinogen. In the cytosol this is subsequently converted to protoporphyrin IX by a resistant endoplasmatic reticulum associated oxidase (Retzlaff and Böger, 1996). Light-activated protoporphyrin IX generates ROS, most probably singlet oxygen and superoxide anions. Resulting effects are lipid peroxidation, oxidative destruction of proteins, pigments and nucleic acids (Böger and Wakabayashi, 1995).

The plastidic compartment has a strong antioxidative power including a set of enzymes and low molecular weight antioxidants. Due to our experimental approach, ROS originate primary in the cytosol and the chloroplast may merely serve as a sink for detoxifying the membrane-permeable

Abbreviations: APX, ascorbate peroxidase; BTH, benzo-(1,2,3)thiadiazole-7-carbothioic acid S-methylester; CAT, catalase; CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-phenyl phosphate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSH, oxidized glutathione; GST, glutathione *S*-transferase; MDAR, monodehydroascorbate reductase; ROS, reactive oxygen species; SA, salicylic acid; SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7.0.

§ This paper is dedicated to Professor Achim Trebst in honor of his 70th birthday.

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H_2O_2. The highly reactive superoxide anion is supposed to dismutate promptly to peroxide, nonenzymatically and enzymatically by superoxide dismutases (SODs; EC 1.15.1.1). For H_2O_2 annihilation, the cytosol as well as the plastid harbour the ascorbate-glutathione cycle with ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and glutathione reductase (GR; EC 1.6.4.2). Reduction of H_2O_2 can also be catalyzed by catalases (CAT; EC 1.11.1.6). CAT-isoforms in tobacco are assigned to different roles: CAT1, probably mitochondrion associated and the most abundant isoform, protects against H_2O_2 evolved by photorespiration. CAT2 is likely involved in oxidative stress, since it is induced by ozone and SO_2 (Willekens *et al.*, 1994a). Further protective enzymes are peroxidases (PODs; EC 1.11.1.7), quenching peroxide by various reductants, and glutathione *S*-transferases (GSTs; EC 2.5.1.18). The latter are instrumental in detoxifying xenobiotics and evidence is increasing that they detoxify metabolites as well, produced through oxidative stress like organic hydroperoxides and unsaturated alkenals (Berhane *et al.*, 1994, Sommer and Böger, 1999).

In oxidative stress, participation of ROS (like H_2O_2) or glutathione (Chen *et al.*, 1996; Foyer *et al.*, 1997) is discussed among others as signalling molecules which mediate the activation of gene expression. Like salicylic acid (SA), both play a role in signal transduction of pathogen defense. We hypothesized, that salicylic acid (SA) or BTH, an artificial functional analogue, may be likewise involved in regulating oxidative stress response. Experiments of this paper should substantiate this assumption.

Materials and Methods

Growth and treatment of plant material

Suspension cultures of soybean (*Glycine max*) were photoheterotrophically grown and treated as described previously (Knörzer *et al.*, 1996). *Nicotiana tabacum* BelW3 (Dr. K.-J. Kunert, Modderfontein, South Africa), seeds were sterilized by sodium hypochlorite (1.3%, 20 min) and germinated on sterile agar (0.9%, pH 5.7), supplemented with minerals according to Murashige and Skoog (1962) in sterile glass jars (500 ml volume). After 6 weeks of growth (corresponding to approximate 7 cm height) in a thermostated chamber (23 °C, 16 h light /8 h dark, 100 $\mu E\ m^{-1}\ s^{-1}$), the plants were subjected to oxyfluorfen by transferring them on herbicide-containing agar and harvested 72 h later.

Enzyme extraction and assays

Using soybean cultures, soluble protein was extracted according to Knörzer *et al.* (1996). Tobacco leaf samples were ground in a mortar with seasand and liquid nitrogen and extracted by pottering them in extraction buffer (50 mM Tris/HCl (tris[hydroxymethyl]aminomethane), pH 7.0, 20% (v/v) glycerol and 0.1% (v/v) Triton, supplemented with 1 mM of ascorbate, EDTA, and reduced glutathione). The crude cell extracts of both soybean and tobacco were centrifuged for 20 min at 26,900×*g*, and the supernatant was stored at −196 °C for later determination of enzyme activities. Protein content was determined according to Bradford (1976) using bovine serum albumin as standard. Enzymatic activities were determined after Knörzer *et al.* (1996).

Native gel staining of ascorbate peroxidase

Protein extracts (total protein content per lane: 30 µg) were separated by electrophoresis in a nondenaturating 10% acrylamide gel preequilibrated with 2 mM ascorbate, and stained according to Mittler and Zilinskas (1993). The acrylamide gels were equilibrated in 50 mM potassium phosphate, pH 7.0, 2 mM ascorbate for 30 min with buffer exchange every ten minutes, then kept for 20 min in a mixture of 50 mM potassium phosphate, pH 7.0, 4 mM ascorbate, 2 mM H_2O_2, subsequently transferred for 1 min in 50 mM potassium phosphate buffer, pH 7.0, and finally stained in a mixture of 50 mM potassium phosphate pH 7.8, 28 mM TEMED (N, N, N', N'-tetramethylethylenediamine) and 2.45 mM nitroblue tetrazolium.

Extraction and labelling of cDNA probes

Plasmids of pAPX, pCAT1 and pCAT2 (all three gifts of Dr. D. Klessig, Rutgers University, Piscataway, USA) were used to prepare homologous probes for the mRNA of ascorbate peroxidase, catalase 1 and 2 by labelling the excised open reading frames with digoxygenin (DIG-High-

Prime System, Boehringer, Mannheim, Germany) following the instruction of the company.

RNA-Extraction and Northern blotting

Approximately 200 mg of ground tobacco leaves were extracted in TriStar (AGS, Heidelberg, Germany) according to the manufacturers' protocol. The amount of extracted total RNA was determined spectrophotometrically and separated by electrophoresis in a denaturating agarose gel (1%, 2 M formaldehyde, in 200 mM 3-morpholinopropanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0), using a concentration of each 8 µg per slot. Subsequently, the RNA was blotted onto a nylon membrane (Hybond+, Amersham, Braunschweig, Germany), using a downward blotting apparatus (TurboBlotter, Schleicher and Schuell, Dassel, Germany) and fixed by baking. Hybridisation and detection were performed according to the 'The DIG System User's Guide to Filter Hybridisation' (Boehringer, Mannheim, Germany) with some modifications: prehybridisation was carried out in a rotary oven at 42 °C for 1 h and followed by hybridisation for 16 h with the digoxigenin labelled cDNA probes (for labelling procedure, see above). Blots were washed twice in 5 × SSC (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% sodium dodecyl sulfate at room temperature for 15 min each and twice in 1 × SSC, 0.2% sodium dodecyl sulfate at 67 °C for 20 min. Detection was carried out using CSPD (Boehringer, Mannheim, Germany) as chemiluminescent substrate. Equal loading of the RNA was proven by staining the blot, i.e. the rRNA, with methylene blue (data not shown).

Statistics

Sample variation is given as standard error of the mean (SE). Values are means of three independent experiments with tobacco and four independent experiments with soybean cultures.

Results

The influence of oxyfluorfen leads to an increase of most of the activities from enzymes participating in the cellular defense (Table I). The activity of glutathione reductase (GR) was increased in tobacco plants subjected to oxyfluorfen with a maximal increase of 75% at 1 µM oxyfluorfen. Another enzyme of the ascorbate-glutathione cycle, monodehydroascorbate reductase (MDAR), was induced as well by oxidative stress. The maximum increment of enzymatic activity is

Table I. Influence of oxyfluorfen on specific activity of enzymes participating in the antioxidative defense, given as specific activity (µmol min^{-1} mg^{-1} protein) and ± SE. Tobacco plants were incubated for 72 h, soybean cultures for 48 h at 24 °C and 100 µE m^{-2} s^{-1} (16 h/8 h, light/dark) with the concentrations of oxyfluorfen indicated.

	Nicotiana tabacum			*Glycine max*		
	Oxyfluorfen [µM]			Oxyfluorfen [µM]		
Enzyme	0	0.5	1	0	0.1	0.5
GR*)	0.28 ± 0.01	0.38 ± 0.02	0.49 ± 0.07	0.19 ± 0.03	0.24 ± 0.05	0.28 ± 0.05
MDAR	0.26 ± 0.01	0.36 ± 0.04	0.42 ± 0.05	0.86 ± 0.15	1.19 ± 0.12	1.26 ± 0.08
GST	0.23 ± 0.08	0.32 ± 0.07	0.48 ± 0.14	0.20 ± 0.03	0.81 ± 0.06	1.16 ± 0.08
CAT	17.2 ± 1.54	15.4 ± 1.20	16.7 ± 1,39	33.8 ± 0.66	51.3 ± 0.12	57.3 ± 0.06

*) See abbreviation list for enzymes.

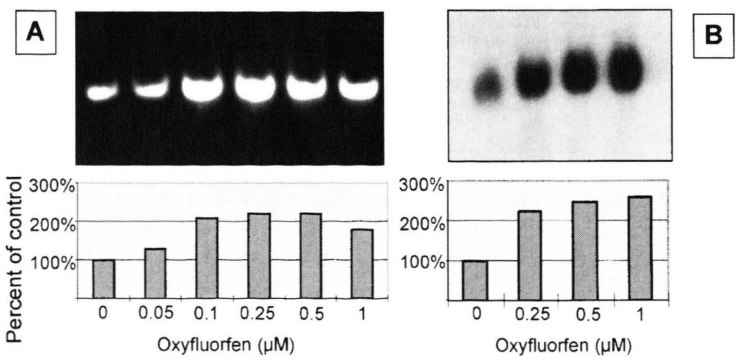

Fig. 1. Enzymatic activity by native gel staining (A) and mRNA content (B) of ascorbate peroxidase (APX) of tobacco plants, incubated for 72 h at 24 °C and 100 µE m^{-2} s^{-1} (16h/8h, light/dark) with the indicated concentrations of oxyfluorfen. Columns represent corresponding densitometric scans.

65% in plants treated with 1 mM oxyfluorfen. In contrast, the activity of dehydroascorbate reductase (DHAR), providing a supplementary pathway of ascorbate regeneration, did not change markedly under these conditions (data not shown). Glutathione *S*-transferase (GST), known to detoxify xenobiotics (Marrs, 1996), raised to maximally 110% under oxyfluorfen influence. The overall activity of the catalases (CAT) did not change after oxyfluorfen treatment. Since these are considered to be most effective in disposal of H_2O_2 at high concentrations in the extraplastidic compartment it has to be investigated further, why no alteration in activity occurs.

Ascorbate peroxidase (APX) was induced under oxyfluorfen influence as well as GR and MDHR (Fig. 1). The enzymatic activity, represented by activity staining of the cytosolic APX, increased with moderate oxyfluorfen concentrations but decreased with higher herbicide doses. The induction of cytosolic APX was markedly due to an elevated amount of mRNA i.e. at the transcriptional level.

Also the CATs were examined for different transcription (Fig. 2). Considering the unaltered enzymatic activity in plants after oxyfluorfen treatment, it had to be ruled out whether this effect is either to due to lack of response of CATs or to different isoforms, yielding in total a constant catalase activity. Checking the mRNA-level the isoforms showed very different responses to oxidative stress. CAT1 mRNA, the isoform which is thought to decompose H_2O_2 resulting from photorespiration, was strongly suppressed. In constrast, CAT2 mRNA, coping with oxidative stress, was induced in the opposite way. Since the mRNA-level represents the CAT-activity (Feierabend, 1996), apparently this shift between the isoforms explains why no overall change in enzymatic activity as a response to oxidative stress was observed.

Salicylic acid (SA), a prominent inductor of defense reactions related to pathogen infection, is considered to be a candidate preventing damage through oxidative stress as well. At the level of enzyme activities soybean suspension cultures showed a similar response to oxyfluorfen treatment as tobacco plants (Table II). With SA present for 48 h, the activity of antioxidative enzymes increased similar to oxyfluorfen treatment. GR

Table II. Influence of salicylic acid and BTH on specific activity of enzymes participating in the antioxidative defense, given as specific activity (μmol min^{-1} mg^{-1} protein) and ± SE. Soybean cultures were incubated for 48 h at 24 °C and 100 μE m^{-2} s^{-1} (16 h/8 h, light/dark) with the indicated concentrations of salicylic acid and BTH, respectively.

	Salicylic acid [mM]			BTH [mM]		
Enzyme	0	0.6	1	0	0.1	0.25
GR	0.13 ± 0.01	0.20 ± 0.02	0.26 ± 0.02	0.19 ± 0.01	0.39 ± 0.03	0.48 ± 0.06
MDAR	0.66 ± 0.06	1.08 ± 0.16	1.33 ± 0.11	0.35 ± 0.04	0.58 ± 0.07	0.67 ± 0.06
GST	0.14 ± 0.02	0.70 ± 0.04	1.05 ± 0.10	0.23 ± 0.05	1.01 ± 0.16	1.15 ± 0.11
CAT	41.3 ± 0.92	68.1 ± 0.12	79.8 ± 4.74	56.1 ± 5.75	50.9 ± 11.2	33.7 ± 11.7

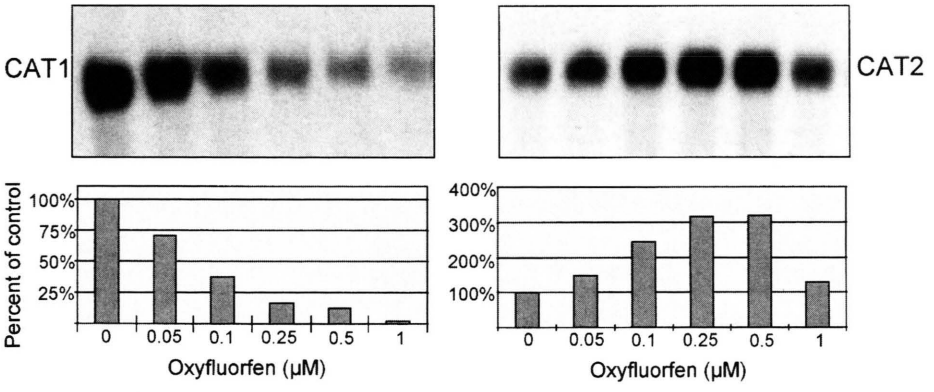

Fig. 2. mRNA content of catalase 1 (CAT1) and catalase 2 (CAT2) of tobacco plants, incubated for 72 h at 24 °C and 100 μE m^{-2} s^{-1} (16h/8h, light/dark) and treated with the indicated concentrations of oxyfluorfen. Columns represent corresponding densitometric scans.

and MDAR, representatives of the ascorbate-glutathione cycle, were induced by factor two by 1 mM SA in a concentration-dependent manner. GST activity raised most, leading to specific activities 8 times higher with preincubation of SA compared to controls (comp. Ulmasov *et al.*, 1995; Xiang *et al.*, 1996). Total CAT activity increased about 2-fold. Treatment with BTH, a functional analogue of SA used in agricultural practice as an activator of the plant defense system (Friedrich *et al.*, 1996; Lawton *et al.*, 1996), results in very similar activation profiles for the enzymes GR, MDHR and GST. CAT activity is reduced somewhat at low levels of BTH, which was not observed with SA.

Eventually we tested whether preincubation with either SA or its analogue BTH could reduce the oxyfluorfen-induced lipid peroxidation. Production of ethane reflects the degree of lipid peroxidation (Böger and Nicolaus, 1993). Preincubation of soybean cultures with SA or BTH strongly decreased oxyfluorfen-induced lipid peroxidation, compared with ethane formation by oxyfluorfen alone (Fig. 3). 200 μM SA lead to complete inhibition of lipid peroxidation, 100 μM was sufficient for this effect with BTH. None of these compounds itself induced ethane formation excluding an activity as prooxidants. The accumulation of protoporphyrin IX was strongly decreased by both compounds.

Discussion

Alterations in the antioxidative system due to the peroxidizing oxyfluorfen reflect the defense against a cytosolic stressor. Several enzymes of the cellular defense are elevated in their activity due to oxyfluorfen, one of them APX. This entry enzyme of the ascorbate-glutathione cycle is risen in a concentration-dependent manner at the transcriptional level due to the peroxidizing effect of oxyfluorfen. However, the enzymatic activity does not parallel this elevation in the higher concentration ranges, producing severe stress. Accordingly, it can be assumed that decrease in enzymatic activity is not due to *translational* effects, since the content of mRNA raises over the whole herbicide concentration range applied. Probably the enzyme is inactivated by the oxidative stress itself or post-transcriptional effects. Mittler *et al.* (1998) reported evidence for the predominant regulation of APX at the transcriptional level which is corroborated by our results.

Two further enzymes, namely MDAR and DHAR, instrumental to regenerate the potent cellular antioxidant ascorbate, show different responses to the treatment with the peroxidizing compound. Whilst MDHR activity increases, that of DHAR does not change markedly. Referring to the actual discussion concerning the role of MDAR and DHAR for regeneration of ascorbate (Foyer and Mullineaux, 1998; Morell *et al.*, 1998), MDAR most likely is predominantly responsible for effective recycling of ascorbate.

GR is a member of the ascorbate-glutathione cycle, protecting the cells from oxidative damage by H_2O_2. Additionally, it keeps the redox balance between the reduced and oxidized form of glutathione, which is considered to be crucial in some regulatory steps (May *et al.*, 1998). Evidence for a significant role of GR in oxidative stress was given by Tanaka *et al.* (1990), who proposed a correlation between oxidative stress sensitivity and reduced activity of GR. Aono *et al.* (1993) demonstrated protection from oxidative stress in GR-overexpressing transgenic plants.

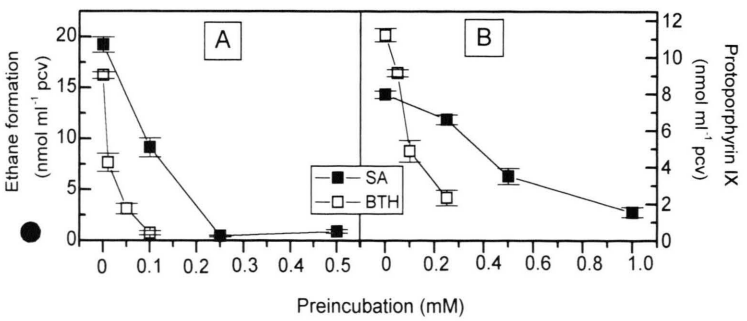

Fig. 3. Decrease of oxyfluorfen-induced lipid peroxidation (A) and protoporphyrin IX accumulation (B) by salicylic acid and BTH. Preincubation with SA and BTH, respectively, for 16 h was followed by application of 0.5 μM oxyfluorfen and a further incubation for 24 h prior to measurement of ethane according to Böger and Nicolaus (1993), respectively extraction of protoporphyrin IX according to Nicolaus *et al.* (1993) pcv, packed cell volume.

Presumably the increase of GST activity under oxyfluorfen stress may have a bearing to a direct detoxification of the compound itself. Despite their well defined activity towards artificial xenobiotics like herbicides as oxyfluorfen, little is known about the GST's 'natural' substrates. Recent research clarified that they may detoxify byproducts of oxidative stress (Berhane *et al.,* 1994; Marrs, 1996), like unsaturated alkenals and hydroperoxides. An induction of GSTs by oxyfluorfen cannot be observed in the dark (our laboratory, unpublished results). This implies that most of the GST activity should be due to ROS. Correspondingly, Mauch and Dudler (1993) observed an induction of the protein amount of GSTs by paraquat and atrazine only in the light, not in the dark. These results indicate the significant role for GSTs in the second line of defense, namely diminishing the toxic consequences of ROS impact.

With CAT a shift in the isoforms rather than a change of overall enzymatic activity occurs. A protective role of CAT has to be assumed since the different compartmentation and function of the isoforms allow for an effective elimination of H_2O_2. The isoform CAT1, likely responsible to detoxify H_2O_2 due to photorespiration, is suppressed under conditions diminishing photorespiration while CAT2, induced by oxidative stress (Willekens *et al.,* 1994b) is strongly induced after oxyfluorfen application. Inhibition of CAT by SA is often discussed, however, inhibition by SA in our enzymatic assay was found negligible. Inhibition of CAT by SA may occur in soybean with concentrations higher than used here (comp. Tenhaken and Rübel, 1997).

Increase of GR, MDAR and GST, resulting from oxyfluorfen treatment, is also observed when SA is applied. These activity changes of antioxidative enzymes are induced by both SA or BTH, suggesting a similar mechanism. Increased radical quenching is supported by the fact, that lipid peroxidation by oxyfluorfen is prevented by a previous SA-treatment.

The observed reduction of the protoporphyrin IX level after preincubation SA with may be caused by activation of GSTs. They may conjugate chlorophyll degradation products, prior to their transport into the vacuole by GS-X pumps (Rea *et al.,* 1998). It has to be pointed out that the effective concentrations of SA and BTH, which lead to reduction of the protoporphyrin IX level, are somewhat higher than those needed to prevent lipid peroxidation measured by ethane formation. Most probably alleviation of lipid peroxidation and suppression of protoporphyrin accumulation are due to two mechanisms, namely degradation of protoporphyrinogen and quenching of ROS and radicals. This is discussed in more detail by Knörzer and Böger (1999).

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