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H_2O_2 GENERATION AND THE INFLUENCE OF ANTIOXIDANTS DURING THE 2,3,5-TRIIODOBENZOIC ACID-MEDIATED INDUCTION OF GLUTATHIONE S-TRANSFERASE IN SOYBEAN

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Key Word Index—*Glycine max*; Fabaceae; glutathione S-transferase; oxidative burst; 2,3,5-triiodobenzoic acid (TIBA).

Abstract—The induction of glutathione S-transferase (GST, EC 2.5.1.18) with 2,3,5-triiodobenzoic acid (TIBA) in soybean (*Glycine max*) hypocotyls was diminished by the natural antioxidants ascorbate and glutathione but not with 1,4-dithio-threitol (DTT). DTT, 1,2-benzenediol and 1,4-benzenediol induced GST activity in soybean hypocotyls. Furthermore, in the presence of luminol TIBA-treated soybean suspension cells exhibited strong biphasic chemiluminescence. The induced chemiluminescence was inhibited by added catalase (EC 1.11.1.6) and superoxide dismutase (EC 1.15.1.1) but not by the peroxidase inhibitor azide. These findings indicate that as a response to TIBA-treatment, soybean cells produce H_2O_2 and O^{2-} without involvement of peroxidase (EC 1.11.1.7). The time course of active oxygen species production was nearly identical to that described for cells treated with an incompatible pathogen.

INTRODUCTION

In the last decade, it has become increasingly evident that active oxygen species (AOS), namely H_2O_2, O^{2-}, etc., function as cellular signals for the activation of antioxidative enzymes, in particular glutathione S-transferase (GST, EC. 2.5.1.18) [1–4]. This family of enzymes catalyzes the conjugation of glutathione to electrophilic molecules [5]. Some GSTs additionally exhibit glutathione peroxidase activity toward fatty acid hydroperoxides [6]. Both activities of GSTs may protect cells from oxidative injury caused by the products of an oxidative burst [4]. Subcellular regulatory units responding to H_2O_2 have been described for mammalian cells (the antioxidant responsive element, ARE, [1]) or have been proposed for plant cells [7]. Increased levels of H_2O_2 have been shown to induce GST in soybean cells [4]. In addition to H_2O_2, GSTs of both plants and animals are induced by a wide range of different compounds. For mammalian GSTs, evidence suggests that an oxidative signal released in various manners (e.g. the production or release of AOS by (i) reversible redox cycles of quinones, (ii) cytochrome P-450 monoxygenase-dependent metabolism, (iii) inhibition of AOS detoxifying enzymes, and (iv) disturbance of the mitochondrial electron transport system) by various compounds, might be a common step in the GST induction pathway [3]. In plants, this hypothesis remains to be proven.

We have previously shown that intra- and extracellular GSTs from soybean exhibiting high glutathione peroxidase activity toward fatty acid hydroperoxides are induced by oxidatively active compounds and various other chemicals [8, 9]. The induction level was particularly high after treatment with the electrophilic compound 2,3,5-triiodobenzoic acid (TIBA). We examined further whether an oxidative signal, namely the production or release of AOS, is part of the GST-induction pathway and whether the induction is suppressed by antioxidants.

In this communication we report on the diminution of TIBA-mediated GST-induction with antioxidants in soybean hypocotyls as well as rapid TIBA-induced H_2O_2 production in soybean cell suspensions.

RESULTS AND DISCUSSION

Earlier findings indicated a potential role for oxidants, in particular H_2O_2, in the induction pathway for soybean GST [4, 9]. In this context antioxidants would play an opposite role. It was therefore interesting to see whether antioxidants would suppress or diminish induction of GST.

The measurement of antioxidant contents revealed

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Table 1. Contents of total and reduced ascorbate and homoglutathione/glutathione of soybean hypocotyls. Soybean seedlings were either untreated or incubated in 10 mM Na phosphate buffer for 24 h without (control) or with antioxidants. Prior to incubation the roots were dissected

Treatment	Total	Reduced
Ascorbate content		(μmol/g fr.wt.)
None	1.6±0.3	1.48±0.26 (92%)
Control	6.56±3.57	1.17±0.48 (18%)
10 mM Ascorbate	110±11.1	90±10 (82%)
Homoglutathione/glutathione content		(nmol/g fr.wt.)
None	190±28	175±25 (92%)
Control	280±39	264±35 (94%)
10 mM Glutathione	1843±86	1752±76 (95%)

Average values ± SD, $n = 3$.

a moderate increase of the ascorbate and GSH contents in hypocotyls of untreated soybean seedlings (Table 1). Furthermore, an oxidative effect with respect to ascorbate but not to GSH was measurable, suggesting a distinct control of the redox states of both antioxidants. These effects were due to the dissection of roots prior to incubation, a treatment that was necessary to ensure an effective uptake of xenobiotic compounds and antioxidants. Only a modest increase of the basal GST-activity level (Table 2) was ensued from the incubation conditions. With respect to a potential control of GST by oxidants, this increase of GST activity may reflect the observed enrichment of oxidized ascorbate.

However, after incubation with 10 mM ascorbate or GSH, both physiological antioxidants were

Table 2. Effect of antioxidants on TIBA-induced GST activity in soybean hypocotyls. Incubation conditions had a modest effect on GST activity basal level (control: incubation for 24 h in 10 mM Na phosphate buffer, pH 7.3, 0.1% DMSO, prior to incubation the roots were dissected)

Treatment	GST activity (nkat mg^{-1} protein)
None	0.23±0.10
Control	0.40±0.08
Ascorbate (10 mM)	0.37±0.06
GSH (10 mM)	0.37±0.08
DTT (10 mM)	1.00±0.13*
1,2-Benzenediol (1 mM)	1.72±0.25*
1,4-Benzenediol (1 mM)	0.57±0.03*
TIBA (100 μM)	2.86±0.82†
TIBA+Ascorbate	1.36±0.12‡
TIBA+GSH	1.22±0.15‡
TIBA+DTT	2.83±0.08

* $p < 0.05$ as vs control, $n = 3$.
† $p < 0.05$, as vs control, $n = 23$.
‡ $p < 0.05$ as vs TIBA treatment, $n = 3$.
Average values ± SD.

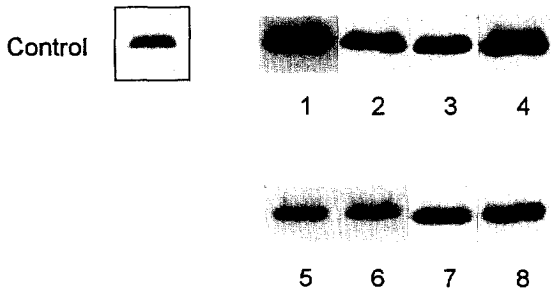

Fig. 1. Induction of soybean GST with TIBA and its reversal with glutathione (GSH) or ascorbate. Immunoblot analysis of soybean GST. Proteins were electrophoretically separated and transferred onto a nitrocellulose membrane. The blot was probed with a rabbit antiserum to maize GST, and developed with horseradish peroxidase-conjugated anti-rabbit IgG and a chemiluminescence-based detection system. Concentrations were as follows: TIBA: 100 μM; glutathione, ascorbate and 1,4-dithiothreitol (DTT): 10 mM; 1,2-benzenediol: 1 mM. Lane 1, TIBA; lane 2, TIBA+GSH; lane 3, TIBA+ascorbate; lane 4, TIBA+DTT; Lane 5, GSH; lane 6, ascorbate; lane 7, DTT; lane 8, 1,2-benzenediol.

accumulated in soybean hypocotyls to 82% and 95%, respectively, in reduced form—and their levels increased up to 16-fold and 6.5-fold respectively (Table 1). While the high content of ascorbate and GSH had no effect on the basal GST activity and protein content compared to the control (Table 2 and Fig. 1), they both diminished the induced activity level. This reduction was accompanied by a reduced GST protein contents as indicated by immunoblotting (Fig. 1), suggesting that both antioxidants interfere with the GST-induction pathway and thereby diminish the induction level.

A contrary effect was observed with the non-physiological antioxidants DTT, 1,2-benzenediol and 1,4-benzenediol. While these agents induced GST activity, DTT did not diminish the TIBA-induced GST activity level and protein content (Table 2 and Fig. 1).

10 mM GSH diminished also the induction of soybean GST-activity by other xenobiotics (Table 3;

Table 3. Effect of 10 mM glutathione (GSH) on the induction of soybean GST activity with 100 μM 2,4-D and PBA (2-(1-pyrenoic-) benzoic acid). Seeds were treated as described in Table 2

Treatment	GST activity (nkat mg^{-1} protein)
Control	0.40±0.08
2,4-D	1.65±0.19
2,4-D+GSH	1.11±0.08*
PBA	1.36±0.11
PBA+GSA	0.66±0.13*

* $p < 0.05$ as vs 2,4-D/PBA treatment, $n = 3$.
Average values ± SD.

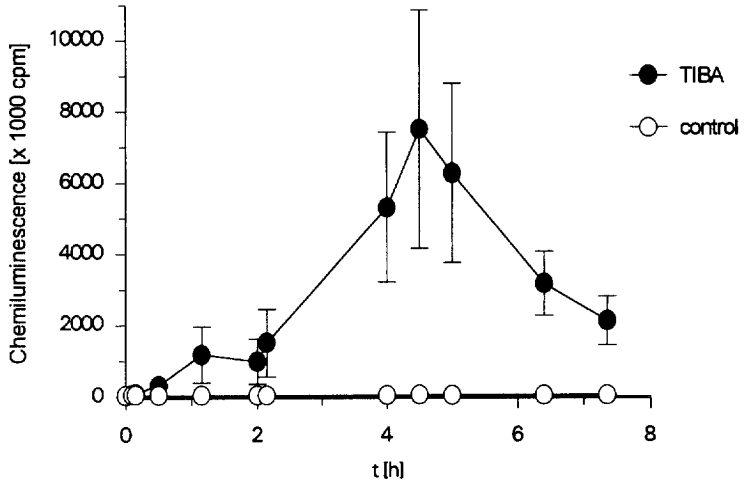

Fig. 2. Chemiluminescence of soybean cells treated with 100 μM TIBA. ○—○, treated cells; ●—●, control cells. Cells were cultured in continuous light for four days after subculturing. TIBA was added to a final concentration of 100 μM and 0.1% DMSO. Control: 0.1% DMSO. pH in both treated and control cells was 7.0 (initiation of the experiment) and 7.1–7.2 (end of experiment). Mean values and standard deviation of three independent experiments are shown.

2,4-dichlorophenoxyacetic acid or 2-(1-pyrenoic-) benzoic acid).

One main difference between physiological and non-physiological antioxidants is that the former are held in reduced state by a series of enzymatic reactions (e.g. the reactions of glutathione reductase, EC 1.6.4.2; dehydroascorbate reductase, EC 1.8.5.1; and NADPH generating processes) while the latter are not. In particular, for both benzenediols it was shown that they can be metabolized to quinoid structures and act as oxidants via reversible redox cycles [1]. Thus, DTT, 1,2-benzenediol and 1,4-benzenediol may act as oxidants.

Together with the earlier findings that H_2O_2 and other oxidants induced soybean GST, these results were further hints of an oxidant controlled GST induction pathway in soybean.

We investigated further whether the induction of GST was accompanied by the release or production of AOS. Because the measurement of oxidants in whole plant is hardly possible, in particular if a time course is required, we employed soybean cells for the measurement of oxidants. GST in these cells has been previously shown to be induced by TIBA, 2,4-D, PBA and various other agents [8].

AOS were detected by measuring the chemiluminescence in the presence of luminol (CL), which has been shown to be an appropriate method [10–12]. CL started to increase markedly 3 min after exposure to 100 μM TIBA. While the control cells showed only very low background luminescence, TIBA-treatment led to a 25–50-fold increase during the first hour. After a plateau, lasting about 1 h, a further increase in CL was observed with a maximal level around 4–5 h after treatment (increase 200–300-fold). Figure 2 shows the results of three representative experiments. Obviously there was an effect of light on the extent but not on the time course of chemiluminescence. Normally cells were cultured in the dark and only for experiments were they subcultured and grown for 4 days in continuous light. An additional 7 days in continuous light before subculturing doubled the maximal measured CL (increase 350–600-fold). The observed inhibitory effects of added superoxide dismutase and catalase (Table 4) suggest the generation of both hydrogen peroxide and O^{2-} after TIBA treatment. Obviously CL depends essentially on both AOS, because enzymatic removal of one reduced CL to much less than 50% (about 1/20, see Table 4).

Since as much as 10 mM of the peroxidase inhibitor azide had no effect on CL, the observed CL seems rather unlikely to be a peroxidase-catalyzed reaction.

The rapid production and accumulation of AOS, commonly called the oxidative burst, has been shown to occur in various plant/cell culture systems after treatment with fungal pathogens or elicitors [13–15]. In particular, the production of hydrogen peroxide (H_2O_2) and superoxide radical anion (O^{2-}) has been observed. It has been proposed that AOS are produced by an activated NADPH-oxidase complex residing in the plasma membrane similar to that of mammalian neutrophils [7, 14, 15].

Table 4. Effect of putative inhibitors on TIBA-induced chemiluminescence in the presence of luminol ($n = 3$)

Treatment	Chemiluminescence (%)
TIBA	100
TIBA + catalase	6 ± 6
TIBA + SOD	8 ± 6
TIBA + azide (10 mM)	105 ± 12

Recently, the oxidative burst has been seen as a signal for the induction of protective, antioxidant enzymes like GST and glutathione peroxidase (Gpx, EC 1.11.1.9) [4, 16]. These enzymes would then protect cells from oxidative injury by the products of the oxidative burst.

The time course of fungal pathogen-derived AOS production has been described with a maximum of AOS release around 1–2 h after treatment for both compatible and incompatible pathogens, and with a second maximum around 5–6 h only after treatment with an incompatible pathogen [7, 14].

The TIBA-produced oxidative burst closely resembles that triggered by an incompatible pathogen with respect to time course and formation of both AOS, namely H_2O_2 and O^{2-}. This indicates that TIBA may interfere somewhat with the activation pathway of the NADPH-oxidase complex. Further studies are required to establish where TIBA acts.

We have previously shown that both TIBA and H_2O_2 induced intra- and extra-cellular GSTs in soybean [8, 9]. The presently observed TIBA-stimulated production of H_2O_2 may indicate that GST induction by TIBA is mediated, at least in part, by an increased H_2O_2 level. Further, weaker, GST activating, xenobiotic compounds like 2,4-dichlorophenoxy acetic acid and 2,4-dichloro-benzoic acid also had a lower stimulating effect on CL (concentration: 100 μM; data not shown). These findings corroborate the above mentioned thesis that H_2O_2 might be a physiological signal in plants which stimulates antioxidative enzymes, thereby protecting cells from oxidative injury by the products of an oxidative burst [4].

In addition, chemical compounds like TIBA will be useful tools to study AOS release in plants. In particular, a potential activation of the NADPH-oxidase complex would be of great interest. Since the oxidative burst is part of the self-defense mechanism in plants against pathogens, it will be interesting to investigate if compounds exhibiting the observed oxidative potency also induce the plant's pathogen defense mechanisms.

EXPERIMENTAL

Cells

Soybean cells (*Glycine max* L. cv Harosoy 63) were cultured and maintained as described in Ref. [17]. For experiments, cells were grown in continuous light at a quantum flux density of 445 μmol m^{-2} s^{-1} (Eye NH220 FLX and Eye Multi Metal E-40 MF 400-LE/BUH, Japan). Four-day-old cells with a cell density of 0.16 ± 0.03 ml cells per ml medium, were used.

Incubation conditions

The incubation mixtures in CL experiments contained 0.1% dimethylsulfoxide in a total volume of 44 ml with or without (control) 100 μM TIBA.

Chemiluminescence measurement

Chemiluminescence was measured as described in Ref. [12] using a Beckman LS 1801 scintillation counter in the single photon mode. The effect of putative inhibitors on chemiluminescence was determined 5 h after incubation with TIBA. Superoxide dismutase and catalase were purchased from Boehringer Mannheim, Germany (SOD 400 U/test, from bovine erythrocytes; catalase, from bovine liver, 400 U/test).

Soybean seeds (*Glycine max* L. cv Asgrow) were sown as described in [8], except that after dissection of roots seeds were incubated in 10 mM Na phosphate buffer, pH 7.3 for 24 h. Then the hypocotyls were dissected and samples for GST activity determination were prepared as follows: 2 g hypocotyl were homogenized with an Ultra Turrax Homogenisator in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.5 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT, then filtered through two layers of Miracloth and centrifuged at 6000*g* for 10 min. The supernatant was centrifuged again for 1.5 h at 105,000*g*. This supernatant was used directly for activity assays and for immunoblot analysis. Immunoblotting and electrophoresis were carried out as described in Ref. [8].

Ascorbate contents were determined according to Ref. [18]. Glutathione contents were determined with the "catalytic assay" according to Ref. [19] and corrected for homoglutathione contents according to Ref. [20]. Oxidized glutathione was detected after derivatization of reduced glutathione with 4-vinylpyridine. GST activity was detected according to Ref. [5].

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