Antioxidative Responses of Tobacco Expressing a Bacterial Glutathione Reductase

Barbara Lederer and Peter Böger*

Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, D-78457 Konstanz, Germany. Fax: +49-7531-883042. E-mail: peter.boeger@uni-konstanz.de

* Author for correspondence and reprint requests

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Reports on stress response of tobacco expressing a bacterial glutathione reductase (GR) do not agree. To clarify this situation we investigated several parameters using the tobacco BelW3 line and its transformant BelW3*gor* expressing an *E. coli* GR.
This alteration in the activity of GR led to an ambiguous modification of the antioxidative system. In contrast to the wild type, the transgenic tobacco suffered lipid peroxidation under moderate light intensities, while it was found to be more resistant towards oxidative stress induced by paraquat or hydrogen peroxide. Transcript levels for violaxanthin deepoxidase and cytosolic Cu-Zn-superoxide dismutase were strongly reduced in BelW3*gor* plants as compared to BelW3.

Key words: Glutathione Reductase, Transgenic, Oxidative Stress

Introduction

Enzymatic protection against reactive oxygen species (ROS) deals with the disposal of superoxide anions by superoxide dismutases and the decomposition of hydrogen peroxide by catalases or peroxidases. Regeneration of resulting oxidized intermediates is achieved by the enzymes of the ascorbate-glutathione cycle, and by low molecular weight antioxidants (see Inzé and van Montagu, 1995). The defense of plants against biotic stress involves generation of ROS as well. Thus, the antioxidative network must be adjusted to various initiating events. Especially the chloroplast provides numerous protection devices like carotenoids, or ascorbic acid and glutathione, which are regenerated by enzymatic reactions.

Glutathione reductase (GR) is thought to be a bottleneck in the antioxidative cascade of plants, since it is present in lowest amounts compared to other enzymes of the defense system against free radical attack (see Polle, 2001). A promising candidate to test this assumption is the tobacco cultivar BelW3. Its ozone sensitivity was explained by diminished activity of GR, accounting for only half the activity of ozone-tolerant cultivars (Tanaka *et al.*, 1990). Subsequently, increase of GR activity by genetic means was performed by several laboratories. In some cases, transformation slightly reduced the susceptibility towards oxidative stress. Several authors reported resistance against paraquat but no resistance against ozone despite of overexpression of GR (Aono *et al.*, 1991; Foyer *et al.*, 1991; Aono *et al.*, 1993; Creissen *et al.*, 1996).

The findings of these authors are inconsistent as far as strengthening of stress resistance is concerned. Accordingly, our studies resumed this line of research by extending the number of physiological parameters investigated like ion leakage, peroxide formation and degradation, peroxidative ethane evolution, decay of the D1 protein and messenger RNA level using low and moderate light conditions.

Abbreviations: APX, ascorbate peroxidase (EC 1.11.1.11); bhy, β-carotene hydroxylase; CAT, catalase (EC 1.11.1.6); chlFeSOD, plastidic Fe-superoxide dismutase; cytCuZnSOD, cytosolic Cu-Zn-superoxide dismutase; GOR, bacterial glutathione reductase; GPX, glutathione peroxidase (EC 1.11.1.9); GR, glutathione reductase (EC 1.6.4.2); GSH, reduced glutathione; ROS, reactive oxygen species; SOD, superoxide dismutase (EC 1.15.1.1); vde, violaxanthin deepoxidase. Paraquat is 1,1′-dimethyl-4,4′-bipyridylium dichloride.

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Materials and Methods

Plant material and growth conditions

Tobacco (*Nicotiana tabacum* cv. BelW3 and BelW3*gor*) was provided by K.-J. Kunert [see Greer and Perham (1986) for the *gor*-gene from *E. coli* and Tyystjärvi *et al.* (1999) for constructs]. Plants were grown for two months in a thermostated chamber (24 °C, 16 h light, 8 h darkness) under sterile conditions in glass containers on Murashige and Skoog basal salt medium (pH 5.7) supplemented with 0.9% (w/v) agar. Light intensities were either 50–100 μEinstein (E) m^{-2} s^{-1} (low light) or 400–500 μE m^{-2} s^{-1} (moderate light) and were provided by Osram Universal White L65W/25S and Osram Natura De Luxe L58W/76. Plants reached an approx. height of 10 cm. For paraquat treatment intact plants were submersed in appropriate micromolar solutions under conditions as indicated in the legends of Figs. 3 and 4. Leaf disks were excised with a cork borer (7 mm diameter) and incubated in bi-distilled water under shaking in gas-tight vials for one day at 24 °C and 400–500 μE m^{-2} s^{-1} provided by Osram Concentra 230V R95 150W lamps. In general, samples were harvested always at the same time of day to avoid effects due to diurnal alterations.

Determination of oxidative and antioxidative parameters

Extraction of chlorophyll with methanol and spectroscopic determination followed the method of Böger (1964). – Measurement of reduced and oxidized ascorbic acid was carried out as described in Knörzer *et al.* (1996).

Changes in conductivity were quantified with a conductivity meter (WTW LF 530, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim) measuring the bathing medium of the leaf disks, with the value for the first 15-min incubation time subtracted to adjust the exudation due to cutting out the leaf disks.

Hydrogen peroxide was determined *ex planta* in the medium of the leaf disks, essentially according to Schwacke and Hager (1992). Calibration of the luminometer (1250, LKB Wallac, Freiburg) was carried out with commercial hydrogen peroxide solution and measurement of the resulting luminol chemiluminescence.

Ethane and ethylene were determined by gas chromatography [headspace DANI HS 86.50; gas chromatograph Perkin Elmer F 22; column alumina F1; see Knörzer *et al.* (1999) for details].

For protein extraction, plant material was harvested and immediately frozen in liquid nitrogen. Extraction of total soluble protein was carried out by an ice-cold mixture of 50 mM tris-(hydroxymethyl)-aminomethane (Tris)/HCl, pH 7; 20% (v/v) glycerol; 1 mM ascorbic acid; 1 mM dithiothreitol; 1 mM EDTA; 1 mM GSH; 1% (v/v) Triton 100 and 1% polyvinylpyrrolidone 10000. The crude protein extract was separated from cell debris by centrifugation at 26000 × *g*, 4 °C. Glutathione reductase activity was measured by a spectroscopic assay according to Halliwell and Foyer (1978). To determine the amount of D1 protein, protein extracts were separated electrophoretically by SDS-PAGE and transferred onto membranes. Immunological detection (see Durner *et al.*, 1993) was performed with an antibody (Herrmann *et al.*, 1985), using alkaline phosphatase as secondary antibody and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as color substrates. Equal loading was checked by staining the membranes with Ponceau red.

RNA extraction and detection (Northern blot)

Plant material was immediately frozen in liquid nitrogen and pulverized. Total RNA was extracted with TriStarTM according to the manufacturer's recommendations (AGS, Heidelberg). Detection of mRNA was performed using the products and protocols of the non-radioactive Dig system from Roche (Mannheim). Hybridization was done at 42 °C in high SDS concentration hybridization buffer [7% SDS; 50% formamide; 5 × SSC (salt sodium citrate); 2% blocking reagent; 50 mM sodium phosphate; pH 7.0; 0.1% N-lauroylsarcosine], followed by washing at room temperature in 0.5 × SSC (75 mM NaCl; 7.5 mM sodium citrate; pH 7) and at 65 °C in 0.1 × SSC. Gene probes for GOR were provided by K.-J. Kunert; for CAT1, 2, PR1a and APX by D. Klessig; for CAT3, chlFeSOD (= SOD2), cytCuZnSOD (= SOD3), GPX by D. Inzé; and probes for vde and bhy by S. Römer.

All experiments were repeated at least three times, standard deviations are given in table and figures when appropriate. Figs. 2–4 show a typical finding. All blots for each mRNA shown (= two boxes in a row) were produced simultaneously and detected on one membrane.

Results

Enhanced light stress in BelW3gor plants

The degree of damage in wild-type (BelW3) and transgenic tobacco (BelW3*gor*) with overexpression of a bacterial glutathione reductase, grown either under low or moderate light, was determined after incubation at moderate light for one day.

Transgenic plants showed a higher sensitivity towards light induced peroxidative damage (Table I). Ethane emission and ion leakage, indicators of lipid peroxidation and subsequent membrane damage, were about twice as high in the transgenic plant than in the wild-type. When both tobacco lines were grown under low light, ethylene emission, hydrogen peroxide exudation, chlorophyll content and the chlorophyll a/b ratio did not differ significantly. However, when grown under moderate light, the transformant had a reduced content of chlorophyll, a lower chlorophyll a/b ratio, and markedly reduced fresh weight compared to the wild-type.

Less stress damage in BelW3gor after paraquat and hydrogen peroxide treatment

Paraquat treatment increased ethane emission and exudation of hydrogen peroxide in wild-type plants adapted to low light (Fig. 1). Both parameters were elevated only slightly in the transgenic line compared to the untreated control. Paraquat-induced lipid peroxidation was less when the plant lines were grown under moderate light, shown by smaller increases of ethane and hydrogen peroxide formation.

Table I. Light-induced oxidative damage and glutathione-reductase activity in BelW3 and transgenic BelW3*gor* tobacco plants.
Leaf disks of two-month old plants adapted to low light (upper panel) or moderate light (lower panel) were kept for further 24 h under moderate light. Thereafter, emission of ethane and ethylene, ion leakage (conductivity), hydrogen peroxide exudation, chlorophyll content and fresh weight, respectively, were determined. The activity of glutathione reductase in two-month old plants was measured prior to the 24-h incubation under moderate light.

Plants grown under low light ($50-100\ \mu E\ m^{-2}\ s^{-1}$)	BelW3	BelW3*gor*	Change in BelW3*gor* vs. BelW3
Ethane [pmol/vial][a]	51 ± 11	104 ± 13	203%
Ethylene [pmol/vial][a]	700 ± 90	500 ± 136	71%
Conductivity [mS/vial][b]	3 ± 1	6 ± 1	182%
H_2O_2 [pmol/vial][b]	47 ± 4	54 ± 3	115%
Chlorophyll [μg/vial][c]	90 ± 12	85 ± 15	94%
Chl a/b ratio[c]	3.0 ± 0.2	3.0 ± 0.2	99%
Fresh weight [mg/vial][c]	91 ± 9	104 ± 5	114%
Plants grown under moderate light ($400-500\ \mu E\ m^2\ s^{-1}$)			
Ethane [pmol/vial][a]	76 ± 12	200 ± 32	263%
Ethylene [pmol/vial][a]	845 ± 94	796 ± 161	94%
Conductivity [mS/vial][b]	17 ± 4	30 ± 1	173%
H_2O_2 [pmol/vial] [b]	25 ± 4	24 ± 0	98%
Chlorophyll [μg/vial][c]	37 ± 9	30 ± 3	81%
Chl a/b ratio[c]	3.1 ± 0.1	2.2 ± 0.1	70%
Fresh weight [mg/vial][c]	109 ± 13	67 ± 0	61%
Glutathione reductase activity [μmol min^{-1} mg^{-1} protein]	0.38 ± 0.02	0.84 ± 0.05	221%

[a] A vial included a gas volume of 5.4 ml.
[b] A vial included 5 ml of water.
[c] For measurement 15 leaf disks (7 mm diameter) were placed into one vial.

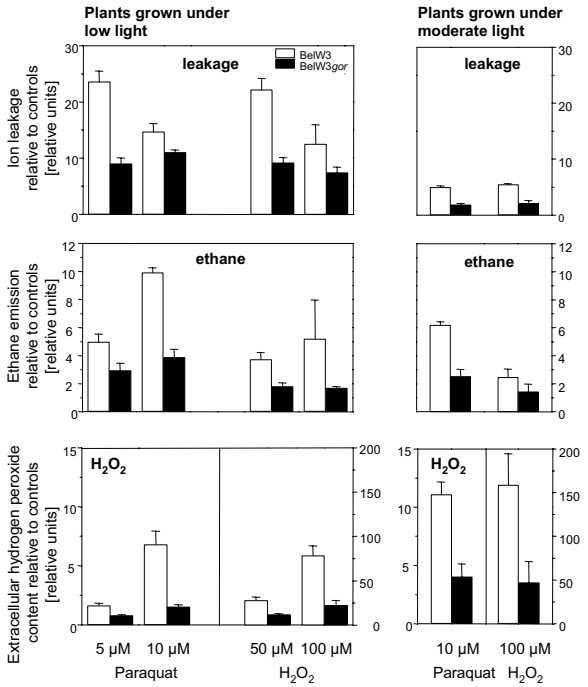

Fig. 1. Oxidative damage in BelW3 and BelW3*gor* tobacco plants. Leaf disks of plants grown under low or moderate light were treated for one day with paraquat (5 and 10 μM) or hydrogen peroxide (50 and 100 μM) and thereafter ion leakage, ethane emission and hydrogen peroxide content were determined. Numerals at the ordinate show relative units of alterations as compared to the corresponding control which is set to (1). Absolute values of the controls are given in Table I.

Exogenous application of hydrogen peroxide resulted in enhanced ethane emission and ion leakage, being less pronounced in transgenic than in wild-type plants. Moreover, the transgenic plants revealed a greater capacity to decompose hydrogen peroxide. As checked by control experiments, chemical quenching was not responsible for peroxide degradation.

In comparison to the wild-type, the constitutive activity of glutathione reductase in the transgenic BelW3*gor*-line was 2.2-fold higher (see Table I, bottom row), but the levels of reduced and oxidized glutathione were comparable in both plant lines (not documented). After paraquat treatment intact wild-type and transformant plants showed an slight increase (about 20%) of ascorbic acid and glutathione. Paraquat up to 5 μM did not alter the redox state of both low-molecular weight antioxidants with no significant difference between the plant lines (not documented).

Different expression of genes for antioxidative enzymes in BelW3, BelW3gor and Samsun

To characterize the sensitivity of BelW3*gor* plants to light stress compared to BelW3 plants in more detail, differences in the expression levels of genes for antioxidative enzymes were analyzed. Additionally, the tobacco cultivar Samsun was included.

Plants grown under moderate light conditions had less D1 protein than plants grown under low light (Fig. 2). The expression levels of genes for the antioxidative enzymes APX, CAT1, CAT2 and chlFeSOD were higher under moderate light. In all

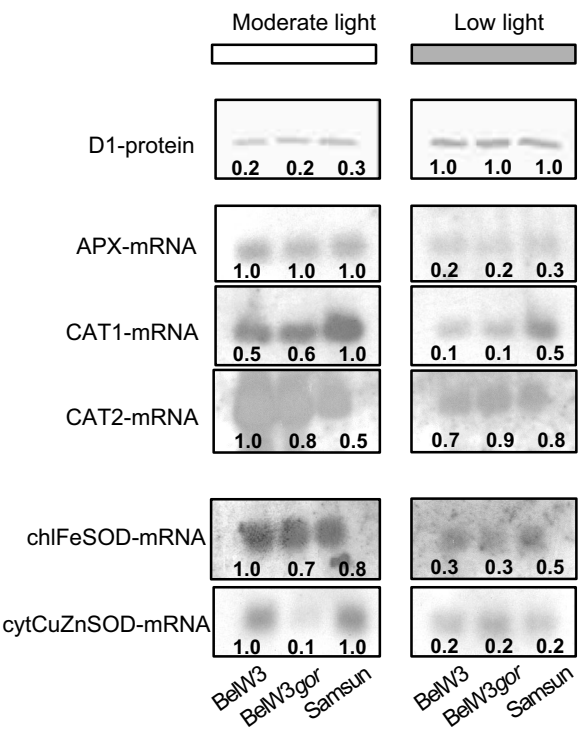

Fig. 2. Impact of different light intensities on transcription of antioxidative enzymes in BelW3, BelW3*gor* and tobacco cultivar Samsun. Amount of D1 protein and transcript levels of cytosolic ascorbate peroxidase (APX), catalases (CAT1, CAT2) and superoxide dismutases (plastidic Fe-superoxide dismutase [chlFeSOD], cytosolic Cu-Zn-superoxide dismutase [cytCuZnSOD]) were determined in two-month old plants grown under moderate or low light. The numerals in the boxes indicate the relative staining intensity of the bands. The strongest one is set to (1).

three tobacco lines, transcript levels for cytosolic APX and plastidic chlFeSOD were changed in the same manner by increased light intensity. In contrast, the transcript pattern of the genes coding for CAT1 and CAT2 differed in Samsun and the two BelW3 lines. When grown in low or moderate light, the CAT1 mRNA level was found to be markedly lower in BelW3 compared to Samsun. In all three lines, however, in moderate light the CAT1 level was higher than in low light. Under low light conditions, CAT2 mRNA was transcribed in all three lines in similar amounts. In moderate light, however, the expression level of CAT2 mRNA was considerably higher in BelW3 than in Samsun.

BelW3 and BelW3*gor* plants grown under low or moderate light showed no differences in the amount of APX, CAT1, CAT2 or chlFeSOD mRNA. The expression of the gene coding for cytCuZnSOD was likewise similar in BelW3 and BelW3*gor* grown under low light, but under moderate light conditions it was increased only in BelW3.

D1 protein and transcript levels of antioxidative enzymes

Degradation of the D1 protein by paraquat indicates a damaged photosynthetic machinery. In wild-type plants, the D1 protein was degraded in the presence of paraquat in a concentration-dependent manner (Fig. 3). In contrast, the D1 degradation was stalled in the glutathione reductase expressing line.

mRNA levels for the hydrogen-peroxide removing enzymes (catalases CAT1, CAT2 and CAT3, ascorbate peroxidase APX and glutathione peroxidase GPX) were not considerably altered, neither in the wild-type nor in the transgenic line. In contrast, the pathogenesis-related protein PR1a, a gene for stress response, was induced in a concentration-dependent manner in BelW3 and in BelW3*gor* plants (not documented). The genes for enzymes detoxifying superoxide anions, chlFeSOD (located in the chloroplast) and cytCuZnSOD (present in the cytosol) were not activated by treatment with paraquat either (Fig. 4). This was observed for both plant lines, even though the transcript level of the cytCuZnSOD was lower in BelW3*gor* than in the wild-type BelW3. The transcript level of the xanthophyll-cycle gene violaxanthin deepoxidase (vde) was much lower in the transgenic line than in the wild-type. Another carotenoid biosynthesis gene, β-carotene hydroxylase (bhy), was equally expressed in both tobacco lines and remained unaltered after paraquat treatment.

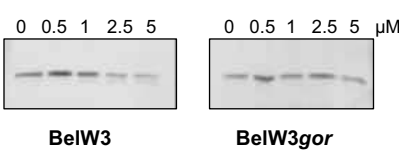

Fig. 3. Alteration of the D1 protein content of BelW3 and BelW3*gor* plants after paraquat treatment. Two-month old intact plants grown under moderate light were treated for three days with 0, 0.5, 1, 2.5 and 5 μM paraquat, respectively. The D1 protein was detected immunologically.

Discussion

Compared to the tobacco cultivar Samsun, the BelW3 plant lines had considerably less CAT1 mRNA. This CAT isoform accounts for approx. 80% of the total catalase activity in tobacco plants (Chamnongpol *et al.*, 1996). The particular importance of CAT1 for stress resistance is evident in CAT1 antisense plants (Willekens *et al.*, 1997), which show a significantly higher sensitivity towards light stress and ozone. Lack of CAT1 in

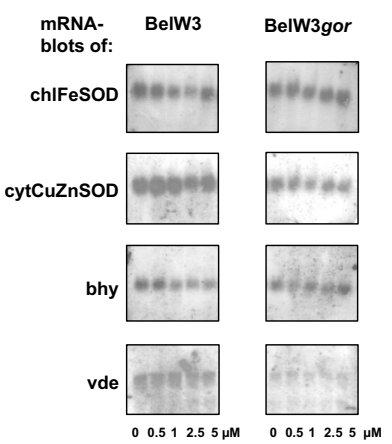

Fig. 4. Impact of paraquat on transcription of antioxidative enzymes in BelW3 and BelW3*gor* plants. Two-month old intact plants grown under moderate light were treated with 0, 0.5, 1, 2.5 and 5 μM paraquat, respectively. Three days later transcript levels were determined using specific gene probes for superoxide dismutases (plastidic Fe-superoxide dismutase [chlFeSOD] and cytosolic Cu-Zn-superoxide dismutase [cytCuZnSOD]), β-carotene hydroxylase (bhy) and violaxanthin deepoxidase (vde).

BelW3 plants seems to provoke a compensatory induction of CAT2 mRNA when the light intensity is increased. Also CAT2 plays a role in the defense against oxidative stress (Willekens *et al.*, 1995) and therefore could counteract the accumulation of hydrogen peroxide under light stress.

Transformation of BelW3 with the glutathione reductase from *E. coli* caused a stronger light sensitivity. The level of violaxanthin deepoxidase mRNA was lower in transgenic plants than in the wild-type BelW3, whereas the transcript level of β-carotene hydroxylase was similar in both BelW3 lines. The elevated sensitivity towards excess light of BelW3*gor* plants compared to the wild-type BelW3 did not impede its tolerance against other forms of stress. Superoxide anions, generated primarily in the chloroplast by paraquat, provoked less stress-induced damage in the transgenic line expressing the bacterial glutathione reductase (GOR) in the cytosol. In addition, BelW3*gor* had a greater capacity to detoxify hydrogen peroxide. Degradation of hydrogen peroxide was neither influenced by alterations of APX mRNA and CAT mRNA levels nor by changes in the pattern of anionic peroxidases, as determined by activity-staining of isoforms (data not shown).

Since glutathione reductase cannot react with hydrogen peroxide, the peroxide degradation is an indirect effect resulting from the expression of foreign glutathione reductase. We conclude that the elevated glutathione reductase activity fortifies the ascorbate-glutathione cycle. The initiating steps of this cycle can proceed either enzymatically or non-enzymatically. The final re-reduction of glutathione can only be mediated by glutathione reductase, which is the rate-limiting enzyme of the ascorbate-glutathione cycle. This could explain the greater capacity of BelW3*gor* to detoxify hydrogen peroxide.

In accordance with our findings, it was reported that hydrogen peroxide accumulation in catalase-inhibited plants was prevented by an elevation of glutathione reductase activity (Streb and Feierabend, 1996). Photooxidative stress or paraquat treatment can lead to a compensatory increase of glutathione reductase in the cytosol, since plastidic glutathione reductase is easily inactivated by an oxidative impact (Casano *et al.*, 1999). Likewise, studies on stress resistance of tobacco lines over-expressing ascorbate peroxidase emphasize the significance of the cytosolic ascorbate glutathione cycle (Pitcher *et al.*, 1994). In addition, the importance of cytosolic regeneration of the oxidized form of glutathione was reported in case the plastidic capacity is overtaxed (Foyer *et al.*, 2001). Accordingly, protection against oxidative stress achieved by plastidic and cytosolic interaction can explain the observed protection of the D1 protein in the transgenic BelW3*gor* plants.

The mRNA level of cytCuZnSOD was markedly lower in BelW3*gor* plants than in BelW3 under moderate light, but it was similar under low light conditions. A relationship between SOD and GR or GSH, respectively, has been reported. An increased oxidation state of GSH repressed cytCuZnSOD in *Pinus* (Wingsle and Karpinski, 1996). A significant reduction in total SOD activity was found with a *Nicotiana tabacum* strain which expressed bacterial glutathione reductase in the cytosol (Aono *et al.*, 1995). In our study, only the cytCuZnSOD mRNA transcription was repressed, but not that of the chlFeSOD.

In conclusion, expression of the bacterial glutathione reductase is accompanied by concomitant reduction of the transcript levels of cytosolic cytCuZnSOD and plastidic violaxanthin deepoxidase. Although the capacity to reduce glutathione is increased in BelW3*gor* plants, its capacity to detoxify cytosolic superoxide anions by cytCuZnSOD is lower. Since violaxanthin deepoxidase is the predominant protective enzyme in xanthophyll-mediated energy dissipation, reduced transcript levels impair the protection of the photosynthetic machinery and lead to enhanced light sensitivity. This explains why a transgenic plant producing additional glutathione reductase is more sensitive to certain forms of oxidative stress than the wild-type. Our study gives evidence for the effects of an imbalanced antioxidative network as a result of genetic manipulation of one compound.

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