DOI: 10.1007/s10535-014-0412-y BIOLOGIA PLANTARUM 58 (4): 773-777, 2014

BRIEF COMMUNICATION

Effect of cadmium on antioxidative enzymes, glutathione content, and glutathionylation in tall fescue

D. DEL BUONO[1]*, T. MIMMO[2], R. TERZANO[3], N. TOMASI[4], and S. CESCO[2]

Dipartimento di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi di Perugia, I-06121 Perugia, Italia[1]
Facoltà di Scienze e Tecnologie, Libera Università di Bolzano, I-39100 Bolzano, Italia[2]
Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli studi di Bari "Aldo Moro", I-70126 Bari, Italia[3]
Dipartimento di Scienze Agrarie e Ambientali, Università di Udine, I-33100 Udine, Italia[4]

Abstract

The aim of this work was to assess the effect of different Cd^{2+}concentrations on some antioxidant enzymes in *Festuca arundinacea.* Increased activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione S-transferase, and glutathione reductase were ascertained in response to low Cd^{2+} concentrations (0 - 20 μM), whereas the enzyme activities were less increased or decreased at a higher Cd^{2+} dosage (50 μM) and a longer exposure. The content of reduced glutathione (GSH) decreased significantly with increasing Cd^{2+} concentrations, whereas the content of oxidized glutathione (GSSG) increased proportionally to the amount of Cd^{2+} applied. Further experiments, performed by incubating the enzyme extracts with oxidized glutathione, evidenced that the addition of GSSG to the incubation mixtures caused significant decreases of some enzymatic activities. Finally, the effect of glutathione S-transferase, *Fa*GST I, extracted from fescue seedlings and purified till homogeneity, on these enzyme activities was investigated. It was found that *Fa*GST I enhanced the decreased enzymatic activities caused by GSSG.

Additional key words: ascorbate peroxidase, dehydroascorbate reductase, *Festuca arundinacea,* glutathione peroxidase, glutathione reductase, glutathione S-transferase, monodehydroascorbate reductase, oxidative stress.

Cadmium is a toxic metal and occurs widely in nature in small amounts of about 0.2 μg g^{-1}(soil), nonetheless, fertilization and industrial activities have led to Cd^{2+} dispersion in the environment. Most plant species are sensitive to Cd^{2+} which causes changes in photosynthesis, plant growth, uptake, and distribution of nutrients (Rizzardo *et al*. 2012). In addition, Cd^{2+} causes an oxidative stress (Fornazier *et al.* 2002, Wang *et al.* 2011, Tian *et al.* 2012). To counteract the oxidative stress, plants possess antioxidant enzymes; among them ascorbate peroxidase (APX; EC 1.11.1.11), glutathione peroxidase (GPOX; EC 1.11.1.9), dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and glutathione reductase (GR; EC 1.6.4.2) are particularly important (Panda *et al.* 2003, Del Buono *et al*. 2011). In addition, plants possess glutathione S-transferases (GSTs; EC 2.5.1.18), enzymes involved in facing the oxidative stress and in inactivating a variety of toxic compounds (Del Buono and Ioli 2011).

In general, oxidative modifications of sensitive proteins, as those having oxidable cysteine (Cys) residues, can cause losses or changes in their activity (Dalle-Donne *et al*. 2007). In order to prevent their oxidation, cells contain high amounts of GSH, the main cellular non-protein thiol, which operates for the inactivation of oxidants and toxic compounds. GSH is transformed into its disulfide oxidized form GSSG

Submitted 4 October 2013, *last revision* 22 January 2014, *accepted* 6 February 2014.
Abbreviations: APX - ascorbate peroxidase; DHAR - dehydroascorbate reductase; GPOX - glutathione peroxidase; GR - glutathione reductase; GRX - disulphide oxidoreductases glutaredoxin; GSH - reduced glutathione; GSSG - oxidized glutathione; GST - glutathione S-transferase; MDHAR - monodehydroascorbate reductase.
Acknowledgements: This work has been financially supported by the Italian MIUR (FIRB-Programma" Futuro in Ricerca" 2012 - RBFR127WJ9).
* Corresponding author; fax: (+39) 075 585 6239; e-mail: delbuono@unipg.it

(Zagorchev *et al.* 2013).

Recently, the idea that GSSG has also some important protective and regulatory functions is emerging. In particular, GSSG is very reactive towards Cys sulfhydryl groups of proteins, and its reaction with them, named S-glutathionylation, produces conjugates linked by a mixed disulfide bridge GS-S-protein (PSSG) (Bradford and Aruni 2012). Thus, S-glutathionylation is a reversible modification and it protects thiols against irreversible oxidation and also alter, either positively or negatively, the activity of many proteins (Del Buono *et al*. 2009, Gao *et al*. 2009). The GS-S-protein bonds can be broken by de-glutathionylation which occurs *via* direct thiol/ disulfide exchange with GSH. This reverse reaction starts when the redox status of the cells has been restored and it is catalyzed by the disulphide oxidoreductases glutaredoxins (GRXs) (Rouhier *et al*. 2008, Gao *et al*. 2009). Though some experiments evidenced that the de-glutathionylation of glutathionylated proteins can be catalyzed by glutaredoxin (Gao *et al*. 2009, Pastore and Piemonte 2012), only few studies performed on mammals suggested a catalytic involvement of stress and drug-inducible GSTs in promoting S-glutathionylation (Manevich and Fischer 2005, Townsend *et al*. 2009). For mammals it has been found that GSTs can mediate the forward reaction of the post-translational process of glutathionylation (Tew 2007, 2008). In particular, it has been shown that GSTs promote the glutathionylation of 1-*cis*-peroxiredoxin by determining its loss of peroxidase activity (Tew 2007). Also, mouse embryo fibroblast cells deficient in glutathione S-transferase P 1-1 (GSTP1-1) and/or GSTP2-2 show reduced capacity for S-glutathionylation confirming that GSTs are responsible for post-translational S-glutathionylation (Townsend 2006).

This research aimed at investigating the effect of Cd^{2+} on a widespread grass, *Festuca arundinacea*, which shows tolerance to some herbicides and heavy metals and that can be found in polluted habitats (Borin *et al*. 2005). In particular, the study aimed at assessing: *1*) the effects of different Cd^{2+} concentrations on APX, GPOX, DHAR, MDHAR, GR, and GST; *2*) the effects of Cd^{2+} on the content of GSH and GSSG; *3*) the effects of GSSG on the activity of the above mentioned enzymes by *in vitro* assays; and *4*) the catalytic role of a GST extracted from shoots of fescue on S-glutathionylation of the enzymes studied.

Seeds of tall fescue (*Festuca arundinacea* Schreb. hybrid Villageoise) were germinated in plastic pots (0.01 m^2) containing quartz sand in the dark at a temperature of 16 °C and a relative humidity of 80 %. After two days, the seedlings were grown under a 12-h photoperiod, an irradiance of 200 μmol m^{-2} s^{-1}, day/night temperatures of 23/21 °C, and a relative humidity of 80 %. When the seedlings were 12-d-old, the pots were divided into five groups: one group was left as control, and to the others 100 cm^3 of solutions containing 5, 10, 20, or 50 μM $CdCl_2$ were added. These concentrations were chosen because they are close to those found in heavy metal-polluted soils and close to the toxicity range for plants, which is 5 - 30 μg g^{-1}(soil) (Steinkellner *et al.* 1998).

For preparation of enzymes, samples of shoots (2.0 g of leaves and stems) were ground to a fine powder and then suspended in a buffer (1:5, m/v) composed of 100 mM Tris-HCl (pH 7.5), 2 mM Na_2-EDTA, 1 mM dithiothreitol, and 1.5 % (m/v) polyvinylpolypyrrolidone. After filtration, the homogenate was centrifuged at 10 000 *g* and 4 °C for 20 min and the supernatant was used for determinations of APX, GPOX, GR, and GST activities (Liu *et al.* 2004, for detail see Del Buono *et al.* 2011). For preparations of DHAR and MDHAR, the powder was suspended in a buffer (1:5, m/v) composed of 100 mM phosphate buffer (pH 7.0), 2 mM ascorbic acid, 1 mM Na_2-EDTA, 2.5 % (v/v) glycerol, and 2 % (m/v) polyvinylpolypyrrolidone. After filtration, the suspension was centrifuged at 10 000 *g* and 4 °C for 20 min and the activities of DHAR and MDHAR were measured (Hodges *et al*. 1997, Del Buono *et al.* 2011). For determination of GSH and GSSG content, shoots (0.5 g) were homogenized in 3.0 cm^3 of 5 % (m/v) sulfosalicylic acid and then centrifuged at 10 000 *g* and 4 °C for 15 min. The supernatant was used for glutathione measurements (Anderson 1985). *Fa*GST I, the most stress-inducible GST in fescue, was extracted and purified from shoots according to a previously published procedure (Del Buono *et al*. 2007).

To test the effect of GSSG+/-*Fa*GST I on APX, GPOX, GR, DHAR, MDHAR, and GST, enzymes preparations, obtained from untreated shoots as described above, were incubated at 35 °C with 5 mM GSSG or with 5 mM GSSG + 0.1 U of *Fa*GST I. Other samples were maintained in the same conditions of incubation but in absence of GSSG or *Fa*GST I, and then they were left as controls. The enzyme activities were then monitored after 1 and 3 h.

All analyses were carried out in triplicate and a statistical analysis was performed using the analysis of variance (*ANOVA*). Significant differences between values were determined at $P \leq 0.05$, according to the Fisher's LSD test.

The activities of APX, DHAR, MDHAR, GR, and GST were significantly increased by Cd^{2+} proportionally to the dosage applied (Table 1). However, the highest dosage (50 μM) was less effective in inducing the enzymes or it even decreased their activities depending on the length of the treatment. The GPOX activity was generally depressed by Cd^{2+} (Table 1).

Since targets of ROS in the cells are compounds sensitive to oxidation and among them sulfhydryl-containing molecules are particularly important, the effects of Cd^{2+} on the content of GSH and GSSG have been investigated. Cd^{2+} exerted a concentration-dependent effect on the GSH content. It was significantly decreased by 20 and 50 μM Cd^{2+}. On the other hand, 5 and 10 μM Cd^{2+} did not influence the GSH content (Table 1). In contrast, the content of GSSG increased proportionally to the concentrations of Cd^{2+} applied (Table 1). The increase of the GSSG content and the

Table 1. The activities [nmol mg^{-1}(protein) s^{-1}] of APX, GPOX, DHAR, MDHAR, GR, and GST and the content [nmol g^{-1}(f.m.)] of GSH and GSSG in the shoots of *Festuca arundinacea* at 24, 48, and 72 h after the treatments with 0, 5, 10, 20, and 50 μM Cd^{2+}. For each line, means followed by different letters are significantly different at $P \leq 0.05$, $n = 3$.

Parameter	Exposure [h]	0 μM Cd^{2+}	5 μM Cd^{2+}	10 μM Cd^{2+}	20 μM Cd^{2+}	50 μM Cd^{2+}
APX	24	10.20 a	14.40 b	15.70 b	29.40 c	33.50 d
	48	10.20 a	21.50 b	25.00 c	28.30 d	25.30 c
	72	10.90 a	21.80 b	22.50 b	24.50 c	15.80 d
GPOX	24	0.31 a	0.28 a	0.23 b	0.20 c	0.20 c
	48	0.30 a	0.25 b	0.23 b	0.25 b	0.20 c
	72	0.31 a	0.32 a	0.31 a	0.32 a	0.13 b
DHAR	24	2.40 a	2.69 b	2.72 b	2.90 c	3.45 d
	48	2.26 a	2.74 b	2.96 c	2.98 c	2.90 c
	72	2.36 a	2.61 b	2.67 b	2.86 c	2.00 d
MDHAR	24	0.06 a	0.08 b	0.08 b	0.10 c	0.08 b
	48	0.07 a	0.07 a	0.09 b	0.09 b	0.08 a
	72	0.07 a	0.08 a	0.08 b	0.08 b	0.08 b
GR	24	0.36 a	0.32 a	0.35 a	0.55 b	0.48 c
	48	0.38 a	0.41 a	0.45 b	0.65 c	0.48 b
	72	0.45 a	0.46 a	0.60 b	0.75 c	0.28 d
GST	24	1.01 a	1.20 b	1.57 c	2.25 d	1.36 e
	48	1.24 a	1.23 a	1.45 b	2.12 c	1.74 d
	72	1.61 a	1.56 a	1.90 b	2.33 c	2.01 b
GSH	72	245.00 a	238.00 a	240.00 a	225.00 b	195.00 c
GSSG	72	12.50 a	13.50 a	21.00 b	24.00 c	27.50 d

Table 2. Enzymatic extracts obtained from fescue shoots were incubated with GSSG or GSSG + *Fa*GST I for 3 h and the activities [nmol mg^{-1}(protein) s^{-1}] of APX, GPOX, DHAR, MDHAR, GR, and GST were measured after 1 and 3 h of incubation. For each line, means followed by different letters are significantly different from the relative control at $P \leq 0.05$, $n = 3$.

Parameter	Exposure 1 h			Exposure 3 h		
	control	GSSG	GSSG + *Fa*GST I	control	GSSG	GSSG + *Fa*GST I
APX	10.20 a	9.60 a	5.60 b	5.20 a	4.70 a	3.20 b
GPOX	0.29 a	0.27 a	0.30 a	0.28 a	0.24 a	0.13 b
DHAR	2.20 a	2.15 a	2.21 a	1.90 a	1.95 a	1.89 a
MDHAR	0.06 a	0.06 a	0.06 a	0.05 a	0.05 a	0.05 a
GR	0.36 a	0.04 b	0.04 b	0.10 a	0.01 b	0.01 b
GST	1.42 a	1.04 b	0.80 c	0.90 a	0.82 b	0.74 c

reduction of that of GSH indicate that the antioxidant defenses required considerable amounts of GSH to remove the oxidative stress generated by Cd^{2+}. The total amount of glutathione, expressed as GSH+GSSG, decreased significantly with the increasing Cd^{2+} concentration, and it indicates that Cd^{2+} exerted interferences on GSH biosynthesis or on sulphur assimilation in general.

Given that in response to increasing Cd^{2+} concentrations GSSG accumulated and the activities of some enzymes declined with treatment duration, further experiments were done 72 h after the treatment in order to study their relation. As already discussed earlier, recent studies demonstrated that some enzymes under oxidative perturbations, when GSSG accumulates, can lose activity for S-glutathionylation (Townsend *et al*. 2009). Therefore, the effect of GSSG on the activity of APX, GPOX, DHAR, MDHAR, GR, and GST was investigated. In addition, in order to ascertain the involvement of GST on S-glutathionylation, the effect of *Fa*GST I, GST extracted from the fescue shoots and purified till homogeneity, was evaluated by incubating GSSG + *Fa*GST I with the enzymatic preparations (Table 2). Despite that all the samples, controls included, showed losses in the enzyme activities after 3 h of incubation, the residual activities permitted to investigate the effect due to GSSG+/-*Fa*GST I on the enzymes studied. Regarding the results of the incubations with GSSG alone, the experiments evidence that oxidized glutathione strongly decreased the activities of GR and GST (Table 2). Therefore, these results indicate that both GST and GR are regulated by GSSG (Townsend *et al*. 2009). In contrast, the other tested enzymes were unaffected by the incubations with GSSG.

On the other hand, when the enzymatic preparations were incubated with GSSG + *Fa*GST I, significant decreases in the enzyme activities were ascertained after 1 and 3 h of incubation for APX and GST with respect to

the control samples and those incubated with GSSG alone. In addition to these, GPOX also showed significant decreases of activity at 3 h of incubations with GSSG + *Fa*GST I. DHAR, and MDHAR were unaffected by GSSG+/-*Fa*GST I (Table 2).

This experiment highlights some important regulatory functions of *Fa*GST I that enhanced the S-glutathionylation of APX, GPOX, and GST. Further, it evidences that *Fa*GST I could regulate the activity of the studied enzymes by potentiating the reactivity of GSSG towards these proteins. In addition, the experiments also evidence that *Fa*GST I decreased the total GST activity (Table 2); this is in agreement with other literature reports which indicated that GSTs themselves are redox-regulated by S-glutathionylation (Townsend *et al*. 2009).

In conclusion, our findings highlight that in plants under the oxidative stress induced by Cd^{2+}, the regulation by glutathionylation is a pivotal post-translational regulatory mechanism of certain enzymes. Moreover, the demonstrated decrease in the enzyme activities catalyzed by *Fa*GST I is the first evidence that the regulation *via* S-glutathionylation, well known in mammal cells, occurs also in plants. Whilst the catalytic detoxification properties of GST isozymes have been a primary research focus for the last decades, our results emphasize another biological importance. In fact, GSTs are linked to the forward S-glutathionylation reaction, and for this, they take an active part in a regulatory mechanism which governs and regulates the activity and functions of other proteins. Such findings extend the role of GSH, GSSG, and GST further than to the sole detoxification reactions.

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