Involvement of CDSP 32, a drought-induced thioredoxin, in the response to oxidative stress in potato plants

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Abstract In animal cells, yeast and bacteria, thioredoxins are known to participate in the response to oxidative stress. We recently identified a novel type of plant thioredoxin named CDSP 32 for chloroplastic drought-induced stress protein of 32 kDa. In the present work, we measured comparable increases in the glutathione oxidation ratio and in the level of chlorophyll thermoluminescence, a specific marker for thylakoid lipid peroxidation in *Solanum tuberosum* plants subjected to drought or oxidative treatments (photooxidative stress, gamma irradiation and methyl viologen spraying). Further, substantial accumulations of CDSP 32 mRNA and protein were revealed upon oxidative treatments. These data show for the first time in plants the induction of a thioredoxin by oxidative stress. We conclude that CDSP 32 may preserve chloroplastic structures against oxidative injury upon drought.

Key words: Chloroplast; Drought; Oxidative stress; Thioredoxin; *Solanum tuberosum*

1. Introduction

Plants are frequently exposed to environmental constraints resulting in metabolism alterations and decreased growth. Drought is known to induce, through stomatal closure, a reduced CO_2 availability at the rubisco site [1]. Consecutively, an excess of absorbed light energy occurs and provokes a deviation of reducing power to oxygen and an increased production of active oxygen species (AOS) [2]. These molecules may lead to damage such as lipid peroxidation [3] and protein oxidation [4]. However, a remarkable tolerance of the photosynthetic apparatus to dehydration has been reported [5,6]. To limit AOS formation, plants have evolved an array of protection mechanisms, such as heat dissipation of excess excitation energy through carotenoids and photorespiration [1]. Further, enhanced activities of chloroplastic Cu/Zn superoxide dismutase [7] and hydrogen peroxide scavenging system [8] have been reported upon water deficit.

Thioredoxins are small oxidoreductases functioning as hydrogen donor for target proteins [9] and their involvement in the response to oxidative stress is well-documented in bacteria, yeast and animal cells. Thus, *Escherichia coli* and yeast thioredoxin-deficient mutants are more sensitive to H_2O_2 [10,11] and *E. coli* thioredoxin has been shown to enhance recovery of human cells after H_2O_2 exposure [12]. In contrast, the participation of plant thioredoxins in the response to oxidative stress remains still largely unknown. We recently identified a novel type of thioredoxin, termed CDSP 32 for chloroplastic drought-induced stress protein of 32 kDa, highly synthesized upon severe water deficit in *Solanum tuberosum* plants [13,14]. The mature protein was found to contain 243 residues and two domains homologous to thioredoxin proteins. While the C-terminal half part of CDSP 32 displayed typical features of thioredoxins, i.e. most residues recognized as important for structure and activity in the *E. coli* protein, particularly a CGPC motif corresponding to the active site, the N-terminal half part was also found to contain most residues well-conserved in thioredoxins, except a potential active site [14]. Further, based on reduction assay of insulin disulfide bridges, the recombinant C-terminal part of CDSP 32 was shown to possess thioredoxin activity [14].

Here, we investigated the occurrence of oxidative stress within chloroplast upon the drought conditions known to enhance CDSP 32 synthesis and we analyzed *CDSP 32* expression upon oxidative treatments.

2. Materials and methods

2.1. Plant material, growth conditions and experimental treatments

Drought and oxidative treatments were applied on 3-weeks-old *S. tuberosum* L, cv Haig, plants grown on compost in a phytotron [13]. Water deficit was applied by withholding watering for 10 days. Leaf relative water content, RWC, was determined as described by Pruvot et al. [13]. High light treatment (1200 μmol photons m^{-2} s^{-1}) under low temperature (8°C) was performed in a phytotron for 3 to 6 days. Gamma irradiation (100 Grays) was delivered using a source of cobalt 60 (0.659 Grays min^{-1}). Methyl viologen (Sigma, St. Louis, USA) treatment was performed by spraying 10 ml per plant of a 10 μM solution in 0.25% Tween 20. After irradiation or spraying, plants were grown under 300 μmol photons m^{-2} s^{-1} for 6 h to 3 days.

2.2. Glutathione analysis

Leaf samples (0.5 g) were immediately frozen in liquid nitrogen, blended in 5% sulfosalicylic acid and centrifuged (20 000 × *g*, 4°C, 15 min). The supernatant was filtered through 0.45 μm microfilters. Total glutathione (GSH+GSSG) was determined using the modified GSSG reductase recycling procedure described by Anderson et al. [15]. GSSG was determined using the same procedure, after removal of GSH from the sample with 2-vinylpyridine. The reaction was monitored at 412 nm, and the rate of increase in absorbance was compared to specific standards for GSH and GSSG (0–1 nmol equivalent GSH).

2.3. Chlorophyll thermoluminescence measurements

Excited forms of lipid peroxides are able to transfer their energy to chlorophyll. Heating a leaf sample allows desexcitation of chlorophyll by photon emission. This process, termed chlorophyll thermoluminescence, is used to estimate the level of lipid peroxidation within chloro-

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Abbreviations: CDSP, chloroplastic drought-induced stress protein; GSH and GSSG, reduced and oxidized glutathione, respectively; MV, methyl viologen; RWC, relative water content; TL, chorophyll thermoluminescence

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plast [16]. Measurements were performed on 1 cm diameter leaf discs [17]. The TL signal was detected with a photomultiplier Hamamatsu R376 on a sample heated from 25 to 150°C (3°C s^{-1}). Temperature was controlled using a thermocouple placed between the sample and the heating element.

2.4. Extraction of leaf proteins, electrophoresis and Western analysis

After extraction [18], leaf soluble protein content was determined using a modified Lowry method (Sigma). Proteins (20 μg per lane) were separated by SDS–PAGE [19] using 12% (wt/v) acrylamide gels and electroblotted onto 0.45 μm nitrocellulose (Schleicher and Schuell, Dassel, Germany). Western blot analysis was performed using a serum raised against CDSP 32 diluted 1:1500 [14].

2.5. RNA isolation and Northern blot analysis

Total leaf RNAs were prepared and separated in formaldehyde gels [14]. Gels were stained with ethidium bromide to ensure equal loading (20 μg per lane). After blotting to Biodyne B (Pall Gelman Sciences, Ann Arbor, USA), hybridization to a random primed CDSP 32 cDNA probe labelled with [α-^{32}P]dATP (3000 Ci $mmol^{-1}$, Amersham-Pharmacia, Rainham, UK) was carried out in 7% PEG-8000, 10% SDS and 100 μg ml^{-1} salmon sperm DNA at 65°C for 16 h. Membranes were washed in 2×SSC, 0.1% SDS at 25, 35 and 45°C successively (20 min each) and exposed to an autoradiography film at −80°C. Band intensities were analyzed using the logiciel Gene Tools for Syngene (Synoptics ltd. 1998, Cambridge, UK).

3. Results

Potato plants were subjected to different treatments, i.e. (i) progressive drought down to a leaf RWC around 70%; (ii) high light/low temperature (1200 μmol photons m^{-2} s^{-1}/8°C), conditions resulting in an excess of excitation energy and in enhanced formation of AOS in chloroplast [1]; (iii) gamma irradiation (100 Grays), the capture of ionizing radiations by water molecules provoking the formation of hydroxyl radicals and hydrogen peroxide [20]; (iv) spraying with a 10 μM solution of methyl viologen, an electron acceptor at the PSI site that reduces O_2 to superoxide [21]. We determined the intensity of oxidative stress conditions within chloroplast by measuring on one hand, the GSH oxidation status, a large amount of cell glutathione (35 to 60%) being found in chloroplast [22,23], and on the other hand, the chlorophyll thermoluminescence signal, a specific marker for peroxidation of thylakoid lipids.

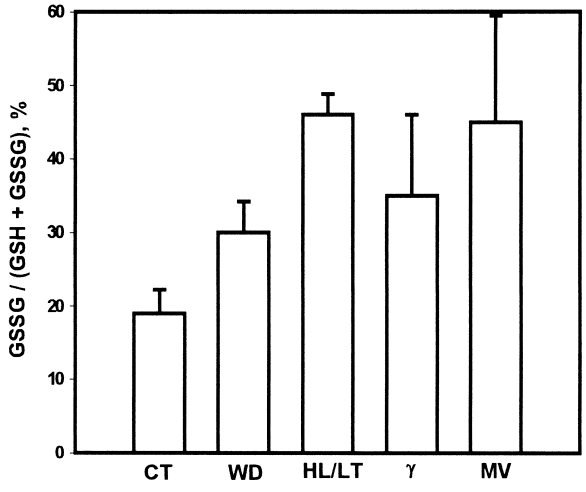

Fig. 1. Percentage of oxidized glutathione in leaf potato plants subjected to drought and oxidative treatments. Percentage of oxidized to total glutathione is reported as GSH equivalents. CT, control plants; WD, drought-stressed plants (70% RWC); HL/LT, plants subjected to high light (1200 μmol photons m^{-2} s^{-1})/low temperature (8°C) for 6 days; γ, irradiated plants (100 Grays) 3 days after treatment; MV, plants sprayed with 10 μM methyl viologen, 3 days after treatment. Values are means ± S.E.M. of four independent samples.

3.1. GSH oxidation status in potato plants subjected to water deficit or oxidative treatments

We measured the amounts of total glutathione (GSH+GSSG) and oxidized glutathione (GSSG) in leaves and the oxidation ratio was calculated as the percentage of oxidized to total glutathione as GSH equivalents. In control well-watered plants, around 18% of the glutathione pool was found oxidized (Fig. 1). In plants subjected to water deficit (leaf RWC around 70%), the ratio of oxidized glutathione increased to 30%. In agreement with these data, Sgherri and Navari-Izzo [24] reported an increase from 12 to 28% of the GSSG/(GSH+GSSG) ratio in sunflower seedlings droughted from 98% to 78% RWC. In plants subjected to oxidative treatments, the ratio was 35% 3 days after irradiation and around 45% 3 days after methyl viologen spraying and after 6 days of growth under high light/low temperature (Fig. 1). Thus, the increase in GSH oxidation ratio occurring upon drought appears in an extent comparable to those observed upon oxidative treatments.

3.2. Chlorophyll thermoluminescence in potato plants subjected to water deficit or oxidative treatments

The level of lipid peroxidation in thylakoids was estimated using chlorophyll thermoluminescence (TL) measurements. Intensity of photon emission by chlorophylls at 90°C has been correlated with the level of lipid peroxidation as measured by thio-barbituric acid reactive substances or malondialdehyde contents in tobacco leaves [16]. Typical TL patterns of potato leaf discs are shown in Fig. 2A. Samples from stressed plants exhibited a peak of TL at around 90°C, absent in controls. Using this method on drought-stressed plants, we observed a twice higher TL value at 90°C than in well-watered plants (Fig. 2B). This result, indicating the occurrence of lipid peroxidation in chloroplast, is consistent with reports showing an increased malondialdehyde content, a product of lipid peroxidation, in leaves of wheat [25] and pea [26] upon mild water deficit. In plants either subjected to high light/low temperature for 6 days, or irradiated (3 days after treatment), the increase in TL at 90°C was similar to that observed in droughted plants. In methyl viologen-treated plants, a twice higher TL signal was noticed 6 h after spraying (data not shown) and the signal was 8-fold higher after 3 days, but there was a large variability among samples, probably related to damage heterogeneity on leaves (Fig. 2B).

3.3. Expression of CDSP 32 in response to oxidative treatments

The levels of CDSP 32 transcript and protein were analyzed in potato plants exposed to high light/low temperature conditions for 3 to 6 days. After 3 days, plants exhibited some leaf chlorosis and anthocyanin accumulation, and small necrosis spots appeared after 6 days (data not shown). Fig. 3A, C shows an increase in the transcript level from 3 days of treatment, its abundance being 10-fold higher than control after 6 days. The protein amount was 4-fold higher after 3 days, and remained at high levels for 6 days (Fig. 3B, C).

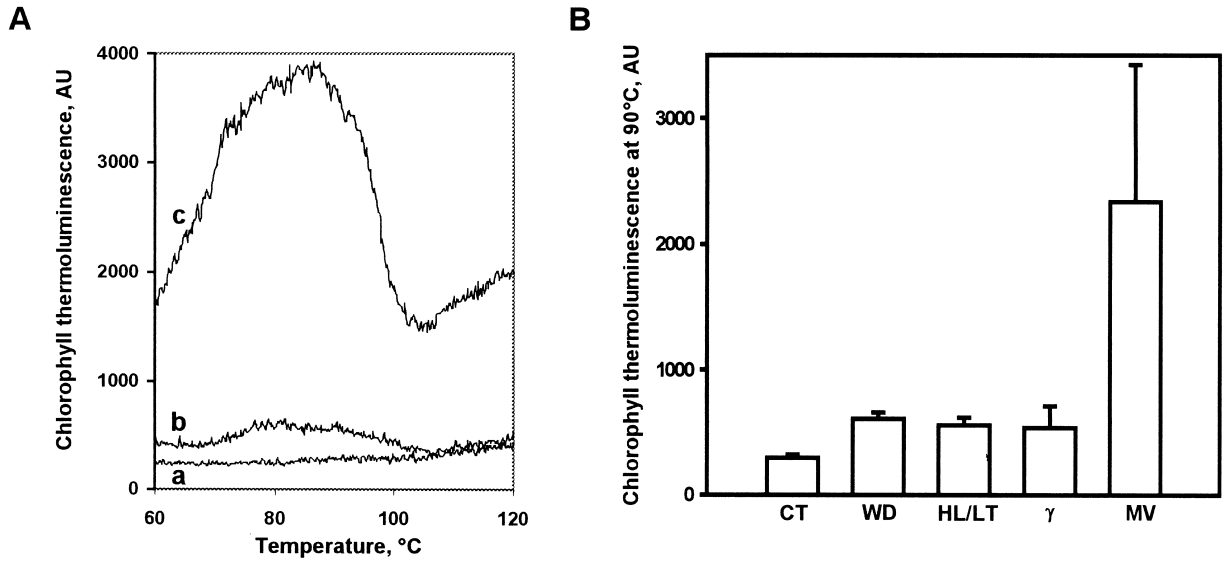

Fig. 2. Leaf chlorophyll thermoluminescence in leaf potato plants subjected to drought and oxidative treatments. A: Representative chlorophyll thermoluminescence signals from potato leaf discs between 60 and 120°C. a, controls; b, droughted plants (70% RWC); c, plants treated with 10 μM methyl viologen and grown in the phytotron for 3 days. B: Level of chlorophyll thermoluminescence signal at 90°C. CT, controls; WD, drought-stressed plants (70% RWC); HL/LT, plants subjected to high light (1200 μmol photons m^{-2} s^{-1}) and low temperature (8°C) for 6 days; γ, irradiated plants (100 Grays), 3 days after treatment; MV, plants sprayed with 10 μM methyl viologen, 3 days after treatment. Values are means ± S.E.M. of four independent samples.

We then analyzed *CDSP 32* expression in plants subjected to gamma irradiation (100 Grays) and transferred in a phytotron for 3 days. An accumulation of anthocyanins was observed in leaves a few hours after irradiation, and small necrosis spots appeared after 3 days (data not shown). A noticeable increase in the transcript level was observed from 6 h to 3 days, the transcript abundance being 4- to 5-fold higher than in control (Fig. 3D, F). The CDSP 32 protein amount was found to increase 6 h after irradiation and to reach a maximal abundance (7.5-fold the control level) after 2 days (Fig. 3E, F).

Finally, *CDSP 32* expression was analyzed in plants sprayed with 10 μM methyl viologen. Leaves rapidly exhibited bleaching spots (1 day after treatment), their size increasing until 3 days (data not shown). The CDSP 32 transcript abundance dramatically increased (100-fold compared to control) 6 h after treatment, and then substantially decreased (Fig. 3G, I). In comparison, a much lower increase in the protein level was observed, with a 3-fold higher abundance after 2 days (Fig. 3H, I). Altogether, Northern and Western analyses demonstrate that *CDSP 32*, previously identified as drought-in-

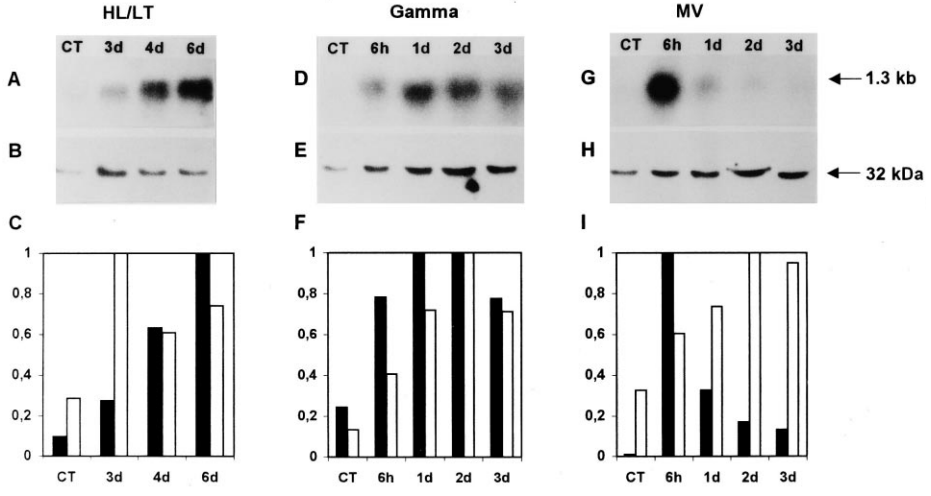

Fig. 3. Expression of *CDSP 32* gene in potato plants subjected to oxidative treatments. A, D, G: RNA blot analysis. Leaf total RNAs (20 μg per lane) were loaded and hybridized with a CDSP 32 probe. B, E, H: Immunoblot analysis of leaf soluble proteins using a serum against CDSP 32. C, F, I: Relative abundances of CDSP 32 mRNA (■) and protein (□). A, B, C: Plants (HL/LT) exposed to high light (1200 μmol photons m^{-2} s^{-1})/low temperature (8°C) for 3 days (3d), 4 days (4d), or 6 days (6d). D, E, F: γ irradiated plants (100 Grays) grown in the phytotron for 6 h (6h), 1 day (1d), 2 days (2d) or 3 days (3d). G, H, I: Plants sprayed with 10 μM methyl viologen (MV) grown in the phytotron for 6 h (6h), 1 day (1d), 2 days (2d) or 3 days (3d). CT, controls.

duced, is also substantially induced in whole potato plants subjected to oxidative stress conditions.

4. Discussion

We report, for the first time in plants, the induction by oxidative stress of a thioredoxin, CDSP 32. In plants, three main types of thioredoxins have been identified, each consisting of a multigene family. Thioredoxin h is located in the cytosol, while thioredoxins f and m are chloroplastic [27]. Until now, the main known function of plant thioredoxins is the regulation of chloroplastic enzyme activities by controlling their redox state. Very recently, Lemaire et al. [28] have shown in *Chlamydomonas reinhardtii* that thioredoxin h and m genes were regulated by light and circadian clock. Otherwise, very little is known about the expression of plant thioredoxin genes, particularly upon environmental constraints. In bacteria, yeast and mammals, thioredoxins play an essential role in the response to oxidative stress. Takemoto et al. [10] have shown that an *E. coli* thioredoxin-deficient mutant was more sensitive to H_2O_2 than the wild-type strain. In yeast, deletion of both thioredoxin genes led to pleiotropic effects including H_2O_2 hypersensibility [11]. Low levels of recombinant *E. coli* thioredoxin significantly increased the ability of human lens epithelial cells to recover from exposure to H_2O_2 [12] and bovine liver thioredoxin has been reported to regenerate in vitro the activity of proteins inactivated by oxidative stress in human endothelial cells [29]. Moreover, thioredoxin-dependent peroxide reductases, proteins able to reduce H_2O_2 and alkyl hydroperoxides using thioredoxin as an electron donor, have been identified in various organisms ranging from prokaryotes to mammals [30,31]. In contrast, no study has shown any direct involvement of plant thioredoxins in the response to oxidative stress. Recently, Mouaheb et al. [32] reported that *Arabidopsis thaliana* thioredoxins h_3 and h_4 were able to complement the phenotype of H_2O_2 hypersensibility in a yeast thioredoxin mutant. Note also that a thioredoxin-dependent peroxide reductase has been identified in plants. The protein, homologous to bacterial alkyl hydroperoxide reductase, is located in chloroplast and, using a transgenic approach, has been shown to exert a protective function in photosynthesis [33].

In the water stress conditions inducing *CDSP 32* expression, we measured a significant increase in the GSSG/(GSH+GSSG) and we observed a twice higher TL signal, indicating the occurrence of lipid peroxidation within chloroplast. Thus, our experiments show that water deficit results in substantial changes in the chloroplastic redox state leading to oxidative damage. Further, these modifications are in an extent comparable to those measured in plants subjected to severe oxidative treatments where *CDSP 32* expression is also highly enhanced. We conclude that the induction of CDSP 32 by drought very likely results from changes in the chloroplast redox state and we propose that the protein participates in the response to oxidative stress within chloroplast upon water deficit. CDSP 32 may either regenerate proteins inactivated by redox change or supply electrons to a thioredoxin-dependent protein involved in scavenging of peroxides. Further insight about the CDSP 32 function should be gained by the analysis of transgenic plants or mutants upon environmental stress.

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