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## Chloroplast CuZn-superoxide dismutase and catalase are earliest targets of osmotic stress in lettuce plants.

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### Introduction

Reactive oxygen species (ROS) such as  $O_2^-$  and  $H_2O_2$  are produced as by-products of normal metabolisms e.g. photosynthesis, respiration, lipid catabolism and lignin synthesis. Plant cells scavenge ROS with specific enzymes such as superoxide dismutase (SOD) and catalase that respectively disproportionate  $O_2^-$  and  $H_2O_2$ , and ascorbate peroxidase (APX) that reduces  $H_2O_2$  using ascorbate (AsA). These enzymes and small antioxidant molecules such as glutathione, carotenoids, flavonoids and tocopherols in addition to AsA efficiently protect plant cells from the potential oxidative damages. However, under severe environmental conditions, where ROS-detoxifying enzymes and electron transport systems are impaired, the production of ROS overwhelms the capacity of cells for scavenging them, and oxidative damages to cellular components will develop, leading to cell death.

When plants undergo water deficiency due to drought or high salinity of soil, closure of stomata limits the supply of  $CO_2$  to chloroplasts, causing a lack of electron acceptors. As a result, the electron transport chain is over-reduced and the production of ROS is most likely enhanced. Accumulation of oxidized products of biomolecules under drought stress has been reported (Smiroff, 1993). In this study, we analyzed early biochemical events in lettuce leaves under water stress. We detected an increased ROS production by in vivo ESR at an early stage of water stress, and found that the CuZnSOD in chloroplasts and catalase are earliest and specific targets.

### Materials and Methods

*Plant materials and stress treatment* — Lettuce were cultured hydroponically in 1,000-fold diluted Hyponex (N:P:K = 1:2:1) under continuous light from fluorescent lamps ( $100 \mu E m^{-2} s^{-1}$ ) at 23°C for 3-4 weeks. Osmotic stress was given to roots by transferring the plants to sorbitol solution at various concentrations. Plants were kept illuminated during the stress treatment unless otherwise stated.

*Preparation of leaf extract and thylakoids* — Lettuce leaves were homogenized in a solution containing 50 mM  $KH_2PO_4$ , pH 7.0, and 1 mM EDTA at 4°C, and centrifuged at 10,000 g for 15 min. The supernatant was gel-filtrated with Sephadex G-25 (leaf extract). The pellet was suspended in 50 mM  $KH_2PO_4$ , pH 7.0 (thylakoids). AsA at 1 mM was added in the media for APX.

## Results and Discussion

*Strength of stress made by sorbitol treatment* — When plants were treated with sorbitol at 0.25 M, 0.5 M and 1 M, wilting of leaves started at 4 h, 1 h and 1 h, respectively. When the 0.25 M sorbitol/ 24 h-treated plants were transferred back to distilled water, leaf turgor recovered within 24 h. Hence, the treatment with 0.25 M sorbitol up to 4 h can be regarded as a ‘weak’ stress treatment. For the plants treated with 1 M sorbitol for 4 h, wilted leaves did not recover up to 24 h. Thus, this stress treatment caused an irreversible damage. Sorbitol at 0.25 M induced stomata closure by 50% in 1 h and 100% in 2 h.

*In vivo ESR evidence for an enhanced ROS production in water-stressed leaves* — Leaf sections from variously stress-treated plants were fed with methyl viologen (MV) and free radicals generated in them were determined with ESR. There was not a specific signal detected in darkness, but on illumination the monodehydroascorbate (MDA) radical was observed (Fig. 1, below). The level of photoproduced MDA in the leaves was higher for osmotically stressed plants than that for non-stressed ones. Because MV eliminates the light-dependent reduction of MDA radical to AsA in chloroplasts, level of the light-induced the MDA signal represents the production rate of MDA radical, which in turn represents the production rate of ROS in chloroplasts (Mano et al. 2001). Thus, the photoproduction of ROS in chloroplasts was enhanced at an early stage of water stress.

*Inactivation of SOD and catalase in leaves by osmotic stress* — Weak osmotic stress with 0.25 M sorbitol caused a significant increase in the MDA signal (Fig. 2, below). Corresponding to this increase, SOD activity in leaves was decreased. A fraction (40-50%) of SOD was rapidly inactivated in 1-2 h of treatment, while the rest remained active up to 4 h, suggesting there were at least two SOD populations in leaf cells that had different sensitivities. On the other hand, activities of thylakoid-bound APX, MDA reductase and photosynthetic electron transport rate ( $\text{H}_2\text{O}$  to  $\text{NADP}^+$ ) were not affected by the osmotic stress within 4 h. When sorbitol was given in darkness, SOD activity in leaves was not decreased.

When the plants were treated with sorbitol at higher concentrations, catalase in addition to SOD was inactivated (Fig. 3, below). Catalase is known labile to high temperatures, but in our experimental conditions leaf temperature was kept at  $21.6 \pm 0.3^\circ\text{C}$ . This excludes the heat-inactivation of catalase, but rather suggests a specific inactivation mechanism operating in response to strong osmotic stress.

*Chloroplastic CuZnSOD is specifically inactivated by osmotic stress* — In non-stressed lettuce leaves two major SOD isozymes were identified by polyacrylamide gel electrophoresis/activity staining (Fig. 4, below: lane ‘0’). The lower band corresponded with the stromal isozyme, and is referred to chloroplastic (Cp) SOD. The upper band was of different origin and we tentatively assigned it as a cytosolic isozyme. Both were CuZn-type, as judged by the inhibition by 5 mM KCN (data not shown). Within 2 h of 0.25 M-sorbitol treatment, the Cp-SOD disappeared, while the cytosolic SOD band remained unchanged even 4 h after treatment. This selective disappearance of the Cp-SOD corresponded well with the rapid and partial loss of total leaf SOD (Fig. 2). Thus osmotic stress specifically inactivated Cp-SOD, resulting in a decrease in ROS-scavenging capacity in chloroplasts. Enhanced photoproduction of MDA radical (Figs. 1, 2) could be a consequence of this. The inactivation of Cp-SOD was probably due to a modification of the active site rather than proteolytic degradation because the Cp-SOD protein detected by immunoblotting did not decrease within 4-h treatment (data not shown).

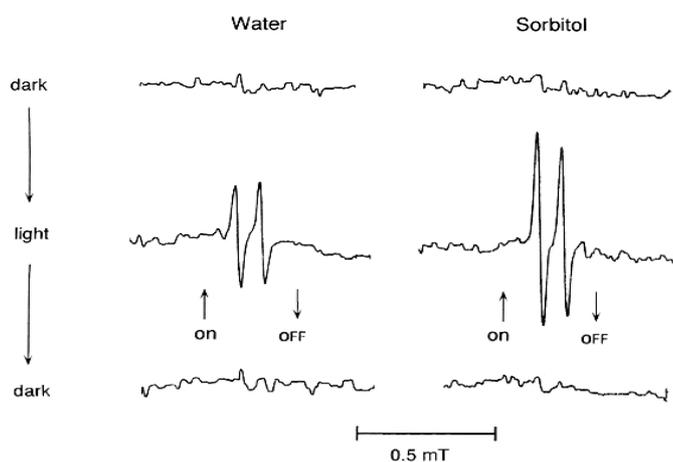
*Concluding remarks* — Cp-SOD and catalase in lettuce leaves were specific primary targets of osmotic stress that was given to the roots. Cp-SOD was sensitive to a weak osmotic stress treatment, while catalase was not inactivated until a strong stress that caused irreversible loss of leaf turgor leaves was given to the plants. Inactivation of these antioxidant enzymes appears to be the initial events that lead to oxidative damages of cells under drought stress.

### Acknowledgement

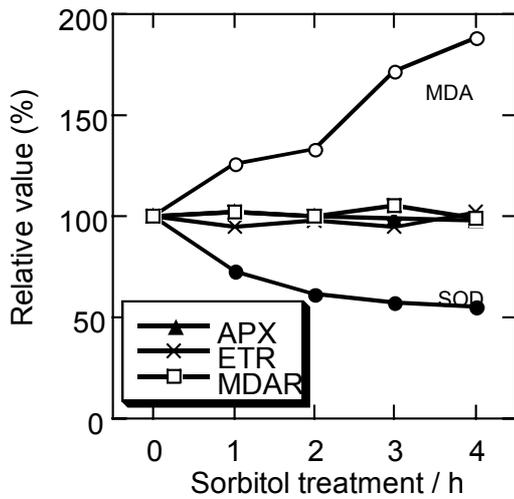
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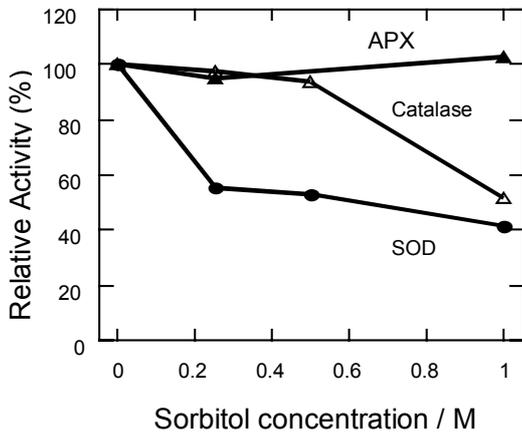
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 Smiroff N (1993) *New Phytol* **125**, 27-58.



**Fig. 1.** ESR spectra of leaf sections. Leaf sections (5 mm × 3 cm) of stress-treated plants were infiltrated with 0.1 mM MV and incubated in darkness for 3 h at 25°C. ESR spectra were recorded in darkness or under illumination ( $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; switched on and off where indicated). The doublet spaced by 0.14 mT represents the MDA radical. In the absence of MV the MDA signal was undetectable both in darkness and under illumination, irrespective of the stress treatment. ESR conditions: microwave frequency, xx GHz; microwave power, xx mW; modulation frequency, 100 kHz; modulation width, xx mT; time constant, xx s; sweep rate, xx mT/min (left to right).



**Fig. 2.** Changes in the photoproduced MDA radical in the MV-fed leaf sections, and in the relevant enzyme activities due to osmotic stress treatment with 0.25 M sorbitol. Leaf extracts and thylakoids were prepared from the stress-treated plants at indicated time. SOD and MDA reductase (MDAR) in leaf extract and APX and electron transport rate ( $H_2O$  to  $NADP^+$ , uncoupled with 0.5  $\mu M$  nigericin; ETR) in thylakoids were assayed, and activities per unit weight of protein (leaf extract) or Chl (thylakoids) were determined.



**Fig. 3.** Sensitivities of antioxidant enzymes in leaves to sorbitol treatment. Activities were determined as in Fig. 2 for the leaves from the plants treated with sorbitol at indicated concentrations for 4 h.



**Fig. 4.** Effects of osmotic stress on the Cp- and cytosolic SODs, as separated by polyacrylamide gel electrophoresis. Stroma fraction (lane 'St') was prepared from chloroplasts isolated from non-stressed. SOD activity was visualized as negative staining with nitrobluetetrazolium.