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Effect of Cu content on the activity of Cu/ZnSOD1 in the Arabidopsis SUMO E3 ligase *siz1* mutant

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In a previous study, we found copper (Cu) accumulated to a higher level in the aerial parts of soil-grown plants of the SUMO E3 ligase *siz1* mutant than in those of the wild-type. Here, we found that all superoxide dismutase (SOD) isoforms, such as FeSOD, MnSOD and different types of Cu/ZnSOD, were more active in the *siz1* mutant than in the wild type under normal growth conditions. We further examined the expression and enzymatic activity of Cu/ZnSOD1 (CSD1) in shoots of the *siz1* mutant under excess Cu. Shoot CSD1 protein level and activity were reduced in *siz1* with excess Cu but induced in the wild type. SIZ1-dependent SUMOylation may be involved in maintaining CSD1 protein stability or repelling a feedback regulation under Cu stress.

Introduction

The reversible conjugation of the small ubiquitin-like modifier (SUMO) to protein substrates occurs as a post-translational regulatory process in eukaryotic organisms. The components of SUMO conjugation and deconjugation systems are observed in many plant species, including algae, dicots and monocots.[1-3] SUMOylation is involved in many plant physiological processes, such as controlling cell growth and development, embryogenesis and flowering time regulation.[4] It is also involved in actions of both biotic and abiotic stresses, including phosphate starvation responses, salicylic acid-dependent pathogen defense, freezing and cold tolerance, basal thermotolerance and drought response.[4] Despite the existence of several SUMO E3 ligases in Arabidopsis,[5,6] many stress-responsive SUMO conjugations are mainly mediated by the SUMO E3 ligase SIZ1.

We previously observed a phenotype of hypersensitivity to excess Cu in the shoot of the *siz1* mutant.[7] Elemental profiling revealed the *siz1* mutant with a high shoot-to-root ratio of Cu concentration. Exogenous Cu treatments can induce the expression and activity of Cu/Zn superoxide dismutase (Cu/ZnSOD).[8] We wondered whether high endogenous Cu accumulation could also induce the expression and activity of Cu/ZnSOD. Arabidopsis has three Cu/ZnSOD isoforms. In this study, we examined the expression and activity of cytosolic Cu/ZnSOD1 (CSD1) in the *siz1* mutant with high endogenous Cu content.

Results and Discussion

At the cellular level, Cu is a redox-active metal able to facilitate the conversion of O_2 and H_2O_2 to •OH via Fenton and Haber-Weiss reactions. The generated reactive oxygen species can directly interact with the antioxidative defense system, disturbing the cellular redox status and causing lipid peroxidation. Because shoots of soil-grown *siz1* mutant plants accumulated more Cu than those of the wild type, we suspected that the high Cu accumulation would also trigger cellular oxidation. Hence, we examined SOD activities in shoots of plants by gel-stain assay. The activities of all 3 SOD isozymes in Arabidopsis (Cu/ZnSOD, FeSOD and MnSOD) were higher in the *siz1* mutants than in the wild type in the control state (**Fig. 1A**), which indicates that the *siz1* mutants were under more stress than was the wild type. The Cu/ZnSOD activity was particularly increased, which suggests an association with the elevated endogenous Cu content in the mutant lines. Interestingly, the activity of Cu/ZnSOD could be further induced by excess Cu in the wild type, whereas corresponding activities in the *siz1* mutants seemed to have reached a plateau or were slightly reduced. However, the activity of FeSOD was reduced after excess Cu treatment in both *siz1* and the wild type, with the activity of MnSOD not changing much after excess Cu treatment.

To examine whether the regulation of *CSD1* occurred at the transcriptional or post-translational level, we examined the mRNA expression of *CSD1* in shoot and root tissues by real-time quantitative RT-PCR. The mRNA level of *CSD1* was higher in the shoots of *siz1* mutant plants than in those of the wild type (**Fig. 1B**), with no difference in root content between mutant and wild-type plants. These results are consistent with the activity data and suggest that the regulation takes place at the transcriptional level and excess Cu induces *CSD*1 expression. Moreover, the protein level of *CSD1* was increased with Cu treatment in the wild type (**Fig. 1C**), which was consistent with the mRNA expression. Interestingly, both the protein level and activity of

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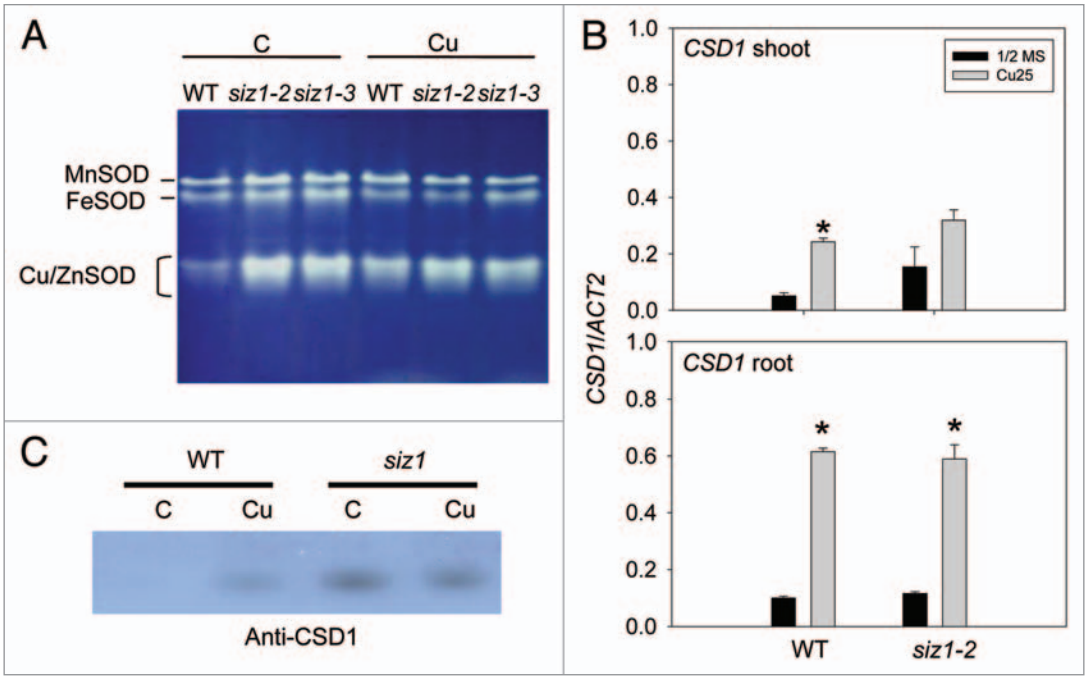

Figure 1. Expression of superoxide dismutase (SOD) enzymes. (A) SOD isozyme activity. Activities of different SOD enzymes in leaves of the wild type (WT) and SUMO E3 ligase mutant *siz1* (*siz1–2* and *siz1–3*) plants grown for 3 weeks and treated with 1/2 MS solution (C) or 1/2 MS + 25 μM $CuSO_4$ (Cu) for 5.5 h; SOD isoforms are indicated. Total proteins (50 μg) were separated on 10% native polyacrylamide gels and stained for total SOD activity. (B) Quantitative RT-PCR analysis of mRNA expression of Cu/ZnSOD1 (CSD1) in shoot and root tissues. Twelve-day-old WT and *siz1–2* plants were treated with 25 μM $CuSO_4$ for 1 d. Y-axis represents expression relative to that of *ACT2*. Means and error bars were calculated from 6 samples of two biological repeats. *p < 0.01 compared with 1/2 MS. (C) Protein gel blot analysis of CSD1 protein level in the wild type and *siz1–2* (*siz1*) with or without Cu treatment.

CSD1 in *siz1* was slightly lower under excess Cu than under control treatment (**Fig. 1A and C**), which implies that SIZ1 may play a role in maintaining the stability of CSD1 at the post-translation level.

Recent reports indicated that ROS could function as a key regulator of the SUMOylation-deSUMOylation equilibrium by influencing the redox states of SUMO cascade enzymes and SUMO protease.[9,10] Whether H_2O_2 levels affect the expression and activity of SODs via SIZ1-dependent SUMOylation as a feedback regulation mechanism remains for further investigation.

In summary, an Arabidopsis Cu/ZnSOD isoform maintained high activity in the shoot of the SUMO E3 ligase mutant *siz1* under normal culture conditions. The high accumulation of endogenous Cu may trigger this antioxidation response. Furthermore, the reduced CSD1 protein accumulation and activity under excess Cu treatment suggests that Cu-induced SUMOylation participates in the control of CSD1 protein stability.

Materials and Methods

Wild-type *Arabidopsis thaliana* (ecotype Columbia) and *siz1* mutant plants were used. Seeds of the T-DNA insertion lines *siz1–2* (SALK_065397) and *siz1–3* (SALK_034008) were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Seeds were surface-sterilized, sowed, treated with Cu, and sampled for quantitative real-time RT-PCR analysis as described in reference 7. Primers for *CSD1* were forward 5'-AGA CCC TGA TGA CCT CGG AAA-3' and reverse 5'-ATG ATG CCG CAA GCA ACA C-3'. The expression of *Actin 2* (*ACT2*) was used as an internal control. Primers for *ACT2* were forward 5'-AGG TCC AGG AAT CGT TCA CAG A-3' and reverse 5'-CCC CAG CTT TTT AAG CCT TTG A-3'.

For SOD activity assay, rosette leaves of 3-week-old plants were cut at the stem-root junction. The detached rosettes were placed for 5.5 h in a Petri dish containing half-strength MS solution with or without 25 μM $CuSO_4$. Treated detached rosettes were ground in liquid nitrogen and resuspended in extraction buffer [50 mM TRIS-HCl, pH 7.6, 330 mM sucrose, 20 mM N-ethylmaleimide and one tablet of protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) per 50 ml]. Samples were centrifuged at 12,000x g for 10 min at 4°C, and protein concentrations were determined by the Bradford method (Bio-Rad). Isolated proteins (50 μg) were separated on 10% native polyacrylamide gels. After electrophoresis, the gels were soaked for 15 min in 15 ml of 0.2% NBT, washed with water, and then soaked in the dark for 25 min in 30 ml phosphate buffered saline containing 0.19% riboflavin and 100 μl TEMED. Bands of SOD activity were observed after exposure to light for 20–30 min at room temperature. Protein gel blot analysis was with anti-CSD1 antibody (dilution 1:10,000). The antibody against CSD1 was prepared using full-length recombinant protein and rabbit immunization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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