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Response of an Active-Oxygen Scavenging System to Cadmium in Cadmium-Tolerant Cells of Carrot

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Abstract

Cd-tolerant (CCd1) cells of carrot (*Daucus carota*) were selected from wild (CW) cells under 100 μM Cd. In the CW and CCd1 cells, the maximum activity of superoxide dismutase was found 1 day and 4 days after Cd treatment, respectively. The activity of ascorbate peroxidase in the CW and CCd1 cells decreased by 80-160 and 160 μM Cd, respectively. The activity of glutathione reductase in the CW cells decreased drastically by Cd; whereas, the activity in the CCd1 cells increased. The content of ascorbate in the CW cells decreased remarkably when the Cd treatment was applied, while that in the CCd1 cells also decreased at 4 days but then recovered at 8 days after the Cd treatment. We speculate that Cd tolerance in the CCd1 cells is due to the high activity of glutathione reductase and maintenance of the content of ascorbate.

Abbreviations

APX, ascorbate peroxidase; AsA, ascorbate; CCd1, Cd-tolerant cells of carrot; CW, wild cells of carrot; DHA, dehydroascorbate; GPX, guaiacol peroxidase; GR, glutathione reductase; GSH, gluta thione; PCs, phytochelatins; O_2^-, superoxide radical; SOD, superoxide dismutase.

Introduction

Heavy metals including Cd in excess amounts are toxic to higher plants. In general, phytochelatins (PCs), heavy-metal-chelating compounds, are involved in Cd tolerance of plants (Grill *et al.*, 1985; Zenk, 1996). Recently, Sanita di Toppi and Gabbrielli (1999) mentioned that plants mainly avoid toxicity of Cd by chelation with metallothioneins as well as PCs. They also suggest that other systems such as an antioxidative defense system and an influx or efflux system contribute to Cd detoxification.

It is known that an active oxygen-detoxifying system responds to many stresses including heavy metals (Foyer *et al.*, 1994). Heavy metals such as Cu directly generate active oxygen, but Cd does not (Stohs and Bagchi, 1995). Nevertheless, Cd enhanced activities of the following antioxidative enzymes: superoxide dismutase (SOD) (Dalurzo *et al.*, 1997), catalase (Shaw, 1995), guaiacol peroxidase (GPX) (Dalurzo *et al.*, 1997; Shaw, 1995; Chaoui *et al.*, 1997), and ascorbate peroxidase (APX) (Shaw, 1995; Chaoui *et al.*, 1997). Although the activity of these enzymes was measured in Cd-treated plants, there are no reports on the obvious correlation between enhancement of these enzymes and Cd tolerance.

We have previously shown that Cd-tolerant (CCd1) cells of carrot have a high ability to exhaust Cd, but that under Cd stress, they still contain a high amount of Cd that is toxic to wild (CW) cells (Kim *et al.*, 2000). In the present study, we characterized the response of the active oxygen-scavenging system to Cd stress in the CCd1 and CW cells. Contributions of the system to Cd tolerance are then discussed.

Materials and Methods

Carrots cells and Cd treatment

The CW cells that originated from the hypocotyl of a carrot (*Daucus carota* L. cv. Pikkoro) seedling were subcultured at approximately 3-week intervals on a B5 agar medium containing 1 μM naphthaleneacetic acid, 1 μM benzyladenine, 30 g L^{-1} sucrose, and 0.7% agar. The CCd1 cells were obtained from the CW cells as a result of subculturing on B5 medium containing 100 μM $CdCl_2$ for 3 years. Both cell lines were cultured in darkness at 25 ℃.

These cells were subcultured every week in a Cd-free B5 liquid-medium on a gyratory shaker (100

rpm). An aliquot (1.0 g fresh weight) of the 7-day old cells was inoculated into 20 ml of the B5 liquidmedium containing 0, 40, 80, and 160 μM Cd. A filter-sterilized aqueous solution of $CdCl_2$ was added to the medium. At 1, 2, 4, and 8 days after inoculation cells were collected on filter paper by filtration. Subsequently, the fresh weight of the harvested cells was measured.

Determination of enzyme activities

The cells were homogenized in 5 volumes of 50 mM K-phosphate (pH 7.0) and 1/10 weight of Polyclar-AT, and the homogenate was centrifuged at 15,000 g for 15 min. The supernatant was used for measuring activities of all of the enzymes except the APX (EC 1.11.1.11). For the measurement of APX activity, the cells were homogenized with homogenizing medium, which was composed of 1 mM ascorbate (AsA) and 1 mM EDTA and 50 mM K-phosphate (pH 7.0), and then the supernatant was passed through a gel-filtration column of Sephadex G25 (Amako *et al.*, 1994). All procedures were done at 4 ℃ .

SOD (EC 1.15.1.1) activity in the de-salted supernatant by Sephadex G25 was determined spectrophotometrically by measuring the inhibition of the superoxide radical (O_2^-) dependent reduction of cytochrome c at A_{550}. One unit of SOD activity is defined as the amount of the enzyme that inhibits the reduction of cytochrome c by 50% (McCord and Fridovich, 1969). APX (EC 1.11.1.11) activity was measured according to the method of Amako *et al.* (1994). The activity was determined by following a decrease of AsA in A_{290} (ε=2.8 mM^{-1} cm^{-1}). Catalase (EC 1.11.1.6) activity was assayed in a reaction mixture containing 50 mM K-phosphate (pH 7.0) and 33.5 mM H_2O_2, using an oxygen electrode (Del Rio *et al.*, 1977). The activity of GPX (EC 1.11.1.7) was measured according to the method of Tanaka *et al.* (1985), except that the reaction mixture contained a 100 mM K-phosphate (pH 7.0), 20.1 mM guaiacol, and 12.3 mM H_2O_2. Glutathione reductase (GR; EC 1.6.4.2) activity was monitored at 340 nm as described by Edwards *et al.* (1990). The protein content was determined according to Bradford (1976). All experiments were done in duplicate.

AsA and dehydroascorbate (DHA) contents

AsA and DHA contents were determined according to the method of Takahama and Oniki (1992). AsA content was determined by the measurement of the decrease in the absorbance at 265 nm by ascorbate oxidase (1.25 unit ml^{-1}). The DHA content was determined by measuring an increase of 265 nm absorbance by dithiothreitol.

Results

The effect of Cd on the cell growth of carrot cells

Fig. 1 shows the growth of the CW and CCd1 cells in fresh weight. The doubling time of the CCd1 cells in 0-, 40-, 80-, and 160-μM Cd treatments was 4.7, 4.4, 6.4, and 13 days, respectively. In contrast, the CW cells doubled in 0 and 40 μM Cd treatments at 3.8 and 6.3 days, respectively. However, the CW cells treated with 80-160 μM Cd did not grow. Thus, the CCd1 cells were more tolerant than the CW cells were. The cream color of the CCd1 cells remained in all treatments; however, the CW cells turned brown at more than 80 μM Cd after day 1 of the inoculation (data not shown).

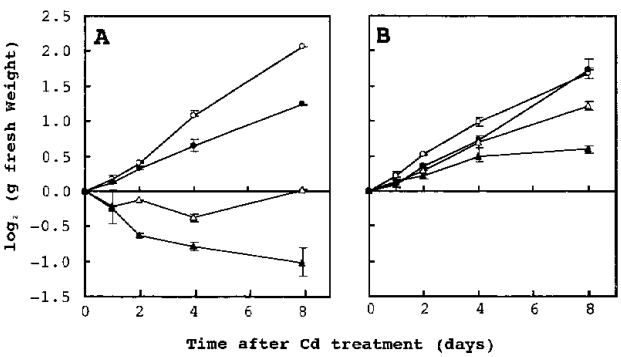

Fig. 1 Effects of Cd on growth in fresh weight of wild (CW, A) and Cd-tolerant (CCd1, B) carrot cells. Cd treatments; 0 (○), 40 (●), 80 (△), and 160 (▲) μM Cd. A bar represents the range between duplications.

The effect of Cd on activities of antioxidative enzymes

After 1 day, the SOD activity in the CW cells increased 5.6-, 2.0-, and 2.3-fold in 40, 80, and 160 μM Cd treatments, respectively, and, later, progressively decreased (**Fig. 2A**). In contrast, the SOD activity in the CCd1 cells peaked 4 days after the Cd treatment (**Fig. 2B**).

The activities of the H_2O_2-scavenging enzymes, catalase and GPX, did not significantly change due to Cd stress in both types of the cells (data not shown). The APX activity in the CW cells decreased by 80- and 160-μM Cd treatments after 4 days (**Fig. 2C**) but the APX activity in CCd1 cells decreased only with the 160-μM Cd treatment (**Fig. 2D**). Just after the Cd treatment, the GR activity in the CW cells decreased remarkably to undetectable levels, and this phenomenon continued during the culture period (**Fig. 2E**). In contrast, the GR activity in the CCd1 cells was enhanced by an increase in the Cd concentration, *e.g.* at 8 days after

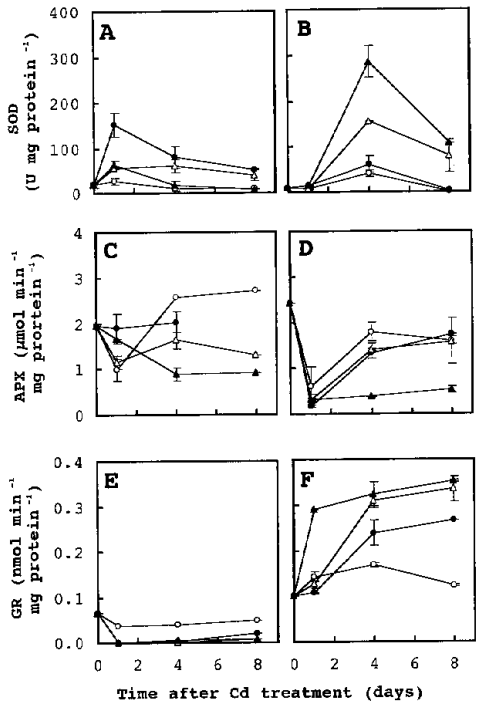

Fig. 2 Effects of Cd on activities of SOD (A, B), APX (C, D), and GR (E, F) in CW (A, C, E) and CCd1 (B, D, F) cells.
For the legend, see Fig. 1

a 160 μM Cd treatment, the activity increased more than 3 fold (**Fig. 2F**).

The effect of Cd on the contents of AsA and DHA

Under Cd-free conditions, both groups of cells contained a similar level of AsA (**Fig. 3**). AsA levels decreased with an increase of Cd concentration at 4 and 8 days of culture in both sets of cells. In the CCd1 cells, the AsA content was partially recovered during the 8-day culture. The AsA/(AsA +DHA) level of the CCd1 cells remained higher than that of the CW cells at all Cd concentrations.

Discussion

It was reported that the Cd-tolerant carrot cells (CCd1) had a stronger capacity of Cd exhaustion than the wild cells (CW) (Kim *et al.*, 2000). In spite of this exhaustion, the CCd1 cells still included a large amount of Cd that exhibited apparent toxicity for the CW cells. For example, the amount of Cd in the CCd1 cells in the 160 μM Cd treatment was higher than that of the CW cells in the 80 μM Cd treatment. Nevertheless, only the CCd1 cells could grow. The quantitative analysis of PCs, which are known to detoxify Cd by chelating in plants (Grill *et al.*, 1985), supported the finding that the PCs synthesis was enhanced by the Cd exposure in both the cell lines, but the CCd1 cells contained lower amounts of PCs than the wild cells (Kim *et al.*,

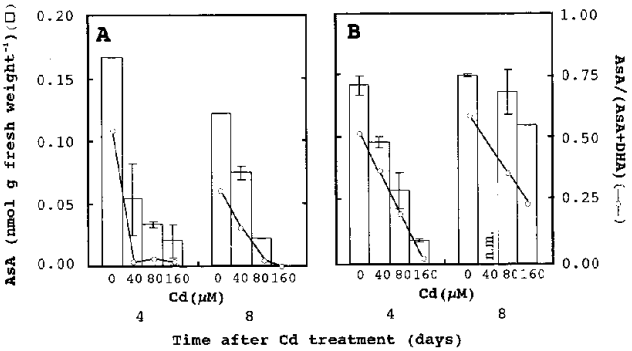

Fig. 3 Effects of Cd on AsA concentration and ratio of AsA/(AsA+DHA) in CW (A) and CCd1 (B) cells.
n.m.; not measured

2000). Therefore, other detoxifying systems should be present in the CCd1 cells. Among them, the most likely mechanism may be an antioxidative defense system because Cd causes oxidative damage as a result of lipid peroxidation and induces activities of antioxidative enzymes in many plants (Sanita di Toppi and Gabbrielli, 1999).

The O_2^- is known to generate by various stresses, and SOD converts O_2^- to H_2O_2 and O_2 to alleviate its toxicity (Scandalios, 1993). The H_2O_2 is further scavenged by catalase, GPX, and the AsA/GSH (glutathione) cycle (Creissen *et al.*, 1994). We measured the activities of these enzymes and compared them in CW cells and CCd1 cells under the Cd stress. The SOD activity of both the cell lines increased remarkably by the Cd treatments at all concentrations tested (**Fig. 2A, B**), whereas the activity peaks appeared in 1 day and 4 days of culture in the CW cells and the CCd1 cells, respectively. This showed that SOD was not a crucial enzyme dominating the high performance of Cd tolerance in the CCd1 cells. In previous studies conflicting results have been obtained on SOD activation by Cd as follows: activation in pea leaves (Dalurzo *et al.*, 1997) and inhibition in mung bean seedlings (Somashekaraiah *et al.*, 1992) and potato tubers (Stroinski and Kozlowska, 1997). From these results it is uncertain whether SOD is always closely associated with Cd tolerance in plant cells.

The activities of H_2O_2-scavenging enzymes, catalase and GPX, and the AsA/GSH cycle enzymes, APX and GR, were analyzed. The dehydroascorbate reductase activity, however, could not be measured because little activity was detected in the homogenates of both the cell lines. Catalase and GPX showed similar activity in both the cells with all treatments (data not shown). The APX activity in the CCd1 cells was not inhibited by the Cd treatment compared to that in the CW cells. However, even when 160 μM Cd was administered to the

medium, respectable activities (ca. 0.5-1 μmol/mg protein) were still retained in both cells (**Fig. 2C, D**). On the other hand, the activity of GR decreased severely after Cd treatment to the CW cells, whereas the activity in the CCd1 cells increased by Cd stress (**Fig. 2E, F**). This phenomenon correlated well with the results of AsA analysis, in which the CCd1 cells showed higher levels in the amount of AsA and the value of AsA/(AsA+DHA) compared with the CW cells under the Cd stress (**Fig. 3**). In addition, we reported that the CW cells had a less reduced form of GSH than the CCd1 cells (Kim *et al.*, 2000). These results suggested that, in the CW cells, the treatment with Cd brought about the deficiency of the AsA/GSH cycle and the H_2O_2-scavenging systems were partially inactivated. In contrast, the CCd1 cells maintained a higher GR activity to keep higher amounts of AsA and GSH than the CW cells. A similar result, namely that the AsA/GSH cycle was activated by Cu in the roots of mung bean, has been reported (Gupta *et al.*, 1999). The activity of the AsA/GSH cycle seemed to participate in the tolerance to heavy metals in plants.

Generally, it is believed that many plants can detoxify Cd by chelating with PCs (Grill *et al.*, 1985). Although Sanita di Toppi and Gabbrielli (1999) suggested that complicated mechanisms for Cd tolerance including not only PC detoxification but also other systems could exist in higher plants there are few examples that showed the presence of the detoxifying mechanisms except for PC by comparison between Cd-sensitive and Cd-tolerant plants. We propose the significance of the AsA/GSH cycle in the Cd tolerance of two carrot cells that show different sensitivities against Cd. Interestingly, the active AsA/GSH cycle of the CCd1 cells under Cd stress did not promote a high level of PCs. This means that the AsA/GSH cycle in the CCd1 cells may act mainly as the H_2O_2-scavenging system rather than to provide GSH to PCs biosynthesis (Noctor and Foyer, 1998).

We found that O_2^-, which was identified by staining of nitro blue tetrazolium, was produced intensively in both the CW and CCd1 cells when they were exposed to Cd stress (data not shown). H_2O_2 generation was also elevated by Cd in *Amaranthus lividus* (Bhattacharjee, 1998) and *Holcus lanatus* (Hendry *et al.*, 1992). Although the mechanism has not been proven, generation of active oxygen by Cd seems to be a ubiquitous phenomenon in plants. To keep a high scavenging capacity of active oxygen, the AsA/GSH cycle appeared to be especially necessary for plant cells to grow under severe Cd stress.

Acknowledgments

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