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Sulfur decreases cadmium translocation and enhances cadmium tolerance by promoting sulfur assimilation and glutathione metabolism in *Brassica chinensis* L.

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ABSTRACT

We investigated the ameliorative role of sulfur (S) in protecting plants against cadmium (Cd) toxicity by using two pakchoi (*Brassica chinensis* L.) cultivars with different Cd tolerance levels. The exposure of pakchoi seedlings to 100 μM Cd inhibited plant growth, increased superoxide content, enhanced membrane lipid peroxidation, and induced Cd accumulation in the roots and shoots. Application of S to Cd-stressed plants alleviated Cd-induced oxidative stress by promoting the capacity of the ascorbate (AsA)–glutathione (GSH) cycle, enhanced S assimilation by increasing the activity of ATP sulfurylase (ATPS) and *o*-acetylserine(thiol)lyase (OASTL), and decreased Cd translocation from the roots to the shoots by enhancing phytochelatins (PCs) biosynthesis. Results suggested that S reversed Cd-induced growth inhibition and oxidative stress by restraining Cd translocation from the roots to the shoots and upregulating S assimilation and GSH metabolism, including the AsA–GSH cycle and PCs synthesis.

1. Introduction

With rapid industrial and agricultural development, large areas of cultivated soils worldwide have been contaminated by cadmium (Cd) because of excessive utilization of chemical fertilizers, wastewater irrigation, and sewage sludge in the past decades (Zhang et al., 2013a; Fan et al., 2010; Liang et al., 2014). Cd, one of the most widespread and toxic heavy metal elements, poses a serious hazard to human health through the food chain (Wang et al., 2013). Cd inhibits physiological processes and normal growth and reduces the biomass and yield of plants (Anjum et al., 2008). These changes have been linked to the overproduction of reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2), resulting in membrane damage and electrolyte leakage (Saidi et al., 2014; Khan et al., 2015).

Glutathione (GSH) is an essential metabolite with multiple functions in plants. As an important non-enzymatic antioxidant in the cellular milieu, GSH can scavenge ROS and alleviate membrane damage through the ascorbate (AsA)–GSH cycle (Yuan et al., 2013; Anjum et al., 2008). GSH also functions as a component of GSH S-transferase (GST)-based detoxification mechanisms (Rausch and Wachter, 2005; Corticeiro et al., 2013; Dixon et al., 2010) and as a substrate for the biosynthesis of phytochelatins (PCs), which are compounds that sequester metals to form a complex to be transported into the vacuole (Zenk, 1996; Lavoie et al., 2009; Zhang et al., 2013b; Bhargava et al., 2005). GSH synthesis, which starts from inorganic sulfate, requires S assimilation and cysteine (Cys) biosynthesis pathways. After uptake of sulfate ions by sulfate transporters, sulfate is activated by ATP via ATP sulfurylase (ATPS). The product, 5′-adenylylsulfate (APS), is reduced to sulfite by APS reductase. Sulfite is then reduced by sulfite reductase to H_2S, which is coupled to *o*-acetylserine(OAS) through *o*-acetylserine (thiol)lyase (OASTL) to form Cys (Rausch and Wachter, 2005). GSH is synthesized from glycine (Gly), glutamate (Glu), and Cys in two ATP-dependent enzymatic reactions (Noctor et al., 2012). γ-Glutamylcysteine synthetase (γ-ECS) initially binds Cys and Glu to form the intermediate γ-glutamylcysteine (γ-EC); GSH is then formed by glutathione synthetase (GS) through the binding of Gly to γ-EC (Nagalakshmi and Prasad, 2001).

Sulfur (S) is an essential macronutrient that plays a vital role in regulating plant growth, development, and responses to biotic and abiotic stresses. Under Cd stress, S improves growth, increases anti-oxidative capacity, and reduces ROS and lipid peroxidation (Liang et al., 2014; Khan et al., 2015; Asgher et al. 2014). Zhang

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et al. (2013b) found that S addition could restrain Cd uptake, increase biomass, and promote synthesis of the non-protein thiol (NPT) pool (including PCs and GSH) in rice to alleviate Cd toxicity. Anjum et al. (2008) presented that high S availability in soil protected mustard from Cd toxicity by improving leaf AsA and GSH. Bashir et al. (2013) reported that S deficiency increased the level of oxidative stress and restricted the GSH biosynthesis pathway in *Arabidopsis thaliana* under Cd stress. Therefore, high S levels could lead to high GSH levels to enhance Cd tolerance in plants. To date, the mechanisms through which enzymes and metabolites involved in GSH metabolism respond to the interactive effects between Cd and S remain poorly understood.

Pakchoi (*Brassica chinensis* L.) is widely cultivated in China because of its high nutritional value, rapid growth, and low production cost (Sun et al., 2010). This vegetable has a relatively high uptake coefficient for heavy metals and has been the subject of research on the environmental risks of heavy metal-polluted soils (Chen et al., 2010). To elucidate the possible mechanisms of S-enhanced resistance and/or tolerance to Cd stress, we analyzed the effects of exogenous S in Cd-stressed pakchoi by determining changes in plant growth, Cd accumulation, oxidative stress, and key enzymes and metabolites involved in GSH metabolism.

2. Materials and methods

2.1. Plant material and growth conditions

Healthy pakchoi seeds (*B. chinensis L.*) from Aikangqing (tolerant to Cd stress) and Qibaoqing (sensitive to Cd stress) cultivars were surface sterilized with 5% sodium hypochlorite for 15 min and germinated on moist filter paper placed in an incubator at 25 °C for 48 h. Uniformly germinated seeds were transferred to a hydroponic growth system with Hoagland nutrient solution. Two-week-old uniform seedlings were transplanted into plastic pots (12 plants per pot) filled with 1L of Hoagland nutrient solution. $CdCl_2 \cdot 2.5H_2O$ or Na_2SO_4 was then added into the solution to prepare the following treatments: (1) Control, 0 mM $CdCl_2 \cdot 2.5H_2O$+0 mM Na_2SO_4; (2) S, 0 mM $CdCl_2 \cdot 2.5H_2O$+4 mM Na_2SO_4; (3) Cd, 0.1 mM $CdCl_2 \cdot 2.5H_2O$+0 mM Na_2SO_4; and (4) Cd+S, 0.1 mM $CdCl_2 \cdot 2.5H_2O$+4 mM Na_2SO_4.The solution was adjusted to pH 6.5 and renewed every 3 d. The seedlings were maintained in a cultivation chamber under controlled conditions, with a light/dark photoperiod of 14/10 h, a photon flux density of 150 μmol/m^2 s at the leaf level, a day/night temperature of 25 °C/18 °C, and a relative humidity of 65 ± 5%. After 3 d of exposure to the treatments, the plants were harvested and washed with 5 mM $CaCl_2$, followed by distilled water. Approximately 0.1 g of the fresh leaf and root samples were frozen in liquid nitrogen and stored at −80 °C until further physiological and biochemical analyses. Meanwhile, the sampled plants were separated into the shoots and roots and dried at 80 °C in an oven to a constant weight. The dried plant tissues were prepared for Cd measurement.

2.2. Cd content

Approximately 0.1 g of dried fine powder from the roots and shoots was digested with a mixture of HNO_3 and $HClO_4$ (v/v=4:1) at 220 °C. Cd content was determined through flame atomic absorbance spectrometry (Hitachi 180-80, Japan).

2.3. Assessment of oxidative damage

Lipid peroxidation was measured by determining the malondialdehyde (MDA) equivalents by using 2-thiobarbituric acid. MDA content was analyzed spectrophotometrically at 450, 532 and 600 nm as described by Lei et al. (2007). Superoxide (O_2^-) content was determined spectrophotometrically at 530 nm by monitoring nitrite formation from hydroxylamine in the presence of O_2^- as described by He et al. (2011).

2.4. AsA and NPT assays

AsA content was estimated as described by Zhang and Kirkham (1996). Total AsA (AsA+DHA) was determined after DHA reduced to AsA with dithiothreitol (DTT), and DHA content was estimated from the difference between the total AsA and AsA. Samples (0.2 g) were extracted in ice-cold 5% (*w*/*v*) meta-phosphoric acid and centrifuged at 22,000 g for 15 min at 4 °C. For total AsA determination, the reaction mixture comprised 0.3 mL of the supernatant, 0.75 mL of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.15 mL of 10 mM DTT. After incubation for 10 min at 25 °C, the solution was added with 0.15 mL of 0.5% N-ethylmaleimide to remove excess DTT. For AsA determination, a similar reaction mixture was used except that 0.3 mL of H_2O was added rather than DTT and N-ethylmaleimide. Color was developed in both reaction mixtures after adding the following reagents: 0.6 mL of 10% TCA, 0.6 mL of 44% *ortho*-phosphoric acid, 0.6 mL of 4% α, α′-dipyridyl in 70% ethanol and 0.3% (*w*/*v*) $FeCl_3$. After vortex mixing, the mixture was incubated at 40 °C for 40 min and absorbance was recorded at 525 nm.

NPT content was measured following the procedure of Devi and Prasad (1998). NPT were extracted by homogenizing 0.2 g of the samples in 2 mL of 5% sulfosalicylic acid and then centrifuged at 10,000 g for 15 min at 4 °C. For NPT determination, the reaction mixture contained 0.2 mL of the supernatant, 2 mL of 0.2 M Tris-HCl (pH 8.2) and 0.15 mL of 10 mM 5,5%-Dithio-bis-(2-nitrobenzoic acid) (DTNB). The mixture was incubated for 20 min, and absorbance was determined at 412 nm.

GSH content was determined according to previously described methods of Nagalakshmi and Prasad (2001) with slight modifications. Samples (0.2 g) were homogenized in 2 mL of 5% sulfosalicylic acid at 4 °C. The homogenate was then centrifuged at 12,000 g for 10 min. Approximately 100 μL of the supernatant was added to 100 μL of 5% sulfosalicylic acid, and the mixture was neutralized by adding 48 μL of 1.84 M triethanolamine. About 50 μL of the sample was used to determine total GSH (GSH+GSSG). Another 50 μL of the sample was pretreated with 50 μL of 2-vinylpyridine for 60 min at 25 °C to mask GSH by derivatization and allow the determination of GSSG alone. Both types of samples were added with 20 μL of 10 mM NADPH, 80 μL of 12.5 mM DTNB, and 706 μL of 50 mM phosphate buffer (pH 7.5) containing 2.5 mM EDTA. Approximately 20 μL of GR (50 U/mL) was then added, and changes in absorbance were monitored at 412 nm. GSH content was estimated from the difference between total GSH (GSH+GSSG) and GSSG.

PCs content was calculated according to the method of Bhargava et al. (2005) by subtracting the content of GSH from that of NPT.

2.5. ATPS, OASTL, γ-ECS, and GST activity assays

ATPS (EC 2.7.7.4) activity was estimated using molybdate-dependent formation of pyrophosphate as described by Lappartient and Touraine (1996). Samples (100 mg) were ground in a mortar at 4 °C with 20 mMTris-HCl (pH 8.0) containing 10 mM EDTA, 2 mM DTT, and 0.01 g/mL polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was used for ATPS assays. The reaction was initiated by adding 0.1 mL of crude extract to 0.5 mL of the reaction mixture containing 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 U/mL of sulfate-free inorganic pyrophosphatase (Sigma) in 80 mM Tris-HC1 buffer (pH 8.0). Another aliquot from the same