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Research article

Superoxide generated from the glutathione-mediated reduction of selenite damages the iron-sulfur cluster of chloroplastic ferredoxin

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ABSTRACT

Selenium assimilation in plants is facilitated by several enzymes that participate in the transport and assimilation of sulfate. Manipulation of genes that function in sulfur metabolism dramatically affects selenium toxicity and accumulation. However, it has been proposed that selenite is not reduced by sulfite reductase. Instead, selenite can be non-enzymatically reduced by glutathione, generating selenodiglutathione and superoxide. The damaging effects of superoxide on iron-sulfur clusters in cytosolic and mitochondrial proteins are well known. However, it is unknown if superoxide damages chloroplastic iron-sulfur proteins. The goals of this study were twofold: to determine whether decreased activity of sulfite reductase impacts selenium tolerance in Arabidopsis, and to determine if superoxide generated from the glutathione-mediated reduction of selenite damages the iron-sulfur cluster of ferredoxin. Our data demonstrate that knockdown of sulfite reductase in Arabidopsis does not affect selenite tolerance or selenium accumulation. Additionally, we provide *in vitro* evidence that the non-enzymatic reduction of selenite damages the iron-sulfur cluster of ferredoxin, a plastidial protein that is an essential component of the photosynthetic light reactions. Damage to ferredoxin's iron-sulfur cluster was associated with formation of apo-ferredoxin and impaired activity. We conclude that if superoxide damages iron-sulfur clusters of ferredoxin *in planta*, then it might contribute to photosynthetic impairment often associated with abiotic stress, including toxic levels of selenium.

1. Introduction

Plants can accumulate and metabolize selenium (Se), but do not require it as a trace element, in contrast to some algae, many bacteria, and animals (Pilon-Smits and Quinn, 2010). Selenium metabolism in plants has nutritional significance for consumers, including humans, whose worldwide Se requirement is provided primarily through a plant-based diet (Rayman, 2000). Se is chemically similar to sulfur (S), and the uptake and reduction of inorganic Se is mediated by transporters and enzymes involved in the S assimilation pathway, as reviewed elsewhere (Zhu et al., 2009).

Arabidopsis plants with mutations in sulfate transporters and sulfur assimilatory enzymes have played a significant role in our understanding of pathways involved in Se uptake and metabolism. For example, mutation in the sulfate transporter (*SULTR1;2*) increased tolerance to selenate 6-fold compared to wildtype (WT) plants, which was explained by restricting the amount of selenate transported into roots (El Kassis et al., 2007). Once selenate is transported into plant cells, its reduction likely occurs predominantly in plastids where it is metabolized by S assimilatory enzymes. The rate-limiting step of selenate reduction is controlled by ATP-sulfurylase, which hydrolyzes ATP and couples AMP to sulfate, and presumably to selenate as well (Dilworth and Bandurski, 1977). Overexpression of *ATPS1* increased the reduction of selenate almost

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3-fold, resulting in increased selenate tolerance and Se accumulation (Pilon-Smits et al., 1999; Sors et al., 2005). Furthermore, mutation of *APR2*, the enzyme that catalyzes the formation of sulfite from phosphorylated sulfate, decreased selenate tolerance 2.5-fold, and coincided with increased levels of selenate and total Se compared to WT plants (Grant et al., 2011). In summary, altered expression of genes participating in sulfate uptake and reduction results in concomitant changes in Se tolerance and accumulation.

Sulfite reductase (SiR)-the enzyme that catalyzes the reaction of sulfite to sulfide-is a rate-limiting enzyme in the sulfate assimilation pathway (Khan et al., 2010) and is capable of alleviating sulfite toxicity (Yarmolinsky et al., 2013). It is not known whether altered SiR expression affects Se tolerance and accumulation in plants. While enzymes involved in sulfur metabolism are generally assumed to metabolize Se analogs, SiR may not be important for selenite reduction and detoxification. In a study that compared the incorporation of radioactive sulfite and selenite into cysteine and selenocysteine, respectively, two pieces of evidence emerged suggesting that sulfite and selenite reduction occur *via* two independent pathways (Ng and Anderson, 1979). Cyanide, a potent SiR inhibitor, blocked sulfite's incorporation into cysteine, but had no effect on the incorporation of selenite into selenocysteine. This suggests that plant SiR is not a selenite reductase, as similarly reported for *Escherichia coli* SiR (Turner et al., 1998). Secondly, the *in-vitro* incorporation of selenite into selenocysteine was dependent upon the reductant glutathione (GSH).

Because of these findings, it has been proposed that selenite reduction in plants and other organisms is mainly non-enzymatic and mediated by GSH (Ng and Anderson, 1979; Kessi and Hanselmann, 2004). Indeed, depletion of internal GSH in *Rhodobacter rubrum* and *Rhodobacter capsulatus* decreased selenite reduction (Kessi, 2006). *In-vitro* evidence demonstrated that non-enzymatic reduction of selenite by GSH rapidly yields selenodiglutathione and superoxide (Seko et al., 1989; Kessi and Hanselmann, 2004; Chen et al., 2007). Additionally, *in vivo* studies using human cells (Wallenberg et al., 2010), Arabidopsis (Grant et al., 2011), and the green alga *Chlamydomonas reinhardti* (Vallentine et al., 2014) also demonstrate that selenite treatment produces toxic levels of superoxide. Superoxide is a reactive oxygen species (ROS) that can induce oxidative stress by oxidizing and impairing protein structure. For example, iron-sulfur (Fe–S) clusters present as cofactors in various Fe–S proteins (Balk and Pilon, 2011) are sensitive to superoxide, as reviewed elsewhere (Py et al., 2011). In both plants and animals, ROS can damage the Fe–S cluster of aconitase, an important participant of the tricarboxylic acid cycle (Verniquet et al., 1991; Gardner and Fridovich, 1991). It was recently demonstrated that selenite-induced superoxide decreased aconitase activity and levels of tricarboxylic acid cycle metabolites in *Brassica napus* (Dimkovikj and Van Hoewyk, 2014). Additionally, the Fe–S cluster in isopropylmalate dehydratase, an Fe–S protein involved in branched-chain amino acid metabolism, is also sensitive to copper-induced oxidative stress (Macomber and Imlay, 2009).

The effects of superoxide on a chloroplastic Fe–S protein have not been examined. Some chloroplastic Fe–S proteins mediate electron transport during the light reactions (Balk and Pilon, 2011). Fe–S clusters found in the cytochrome b_6f complex, photosystem I, and ferredoxin (Fd) are vital for plant development and photosynthesis (Van Hoewyk et al., 2007). Fd mediates the reduction of $NADP^+$ to NADPH. Knockout of isoform Fd2 resulted in growth impairment and ROS accumulation (Voss et al., 2008).

Numerous abiotic stressors result in superoxide accumulation (Mittler, 2002). For example, inorganic Se induces ROS accumulation and has been reported to decrease efficiency of photosystem II in Arabidopsis (Grant et al., 2011), wheat (Łabanowska et al., 2012) and *Stanleya albescens* (Freeman et al., 2010). The exact cellular mechanism is not clear, but if superoxide also damages the chloroplastic Fe–S clusters, this would likely diminish photosynthetic capacity. We reasoned that since (*i*) selenate is likely reduced to selenite in chloroplasts and if (*ii*) the non-enzymatic reduction of selenite yields superoxide, then it would potentially damage chloroplastic proteins that contain an Fe–S cluster. To test this hypothesis, the integrity of the Fe–S cluster of Fd was analyzed in a reaction containing selenite and GSH. We additionally examined if Arabidopsis plants with a mutation in SiR had altered selenite tolerance and selenium accumulation.

2. Methods

2.1. Plant selenium toxicity and elemental analysis

Seeds of SiR mutants plants used in this study were obtained as SIR KD1T seeds (Yarmolinsky et al., 2013) and *sir1-1* seeds (Khan et al., 2010), and separately compared to Columbia ecotype in the same genetic background. Plants were grown in a growth chamber (150 μE, 16 h light/8 h dark cycle, 24 C) on agar plates containing 1% sucrose and 0.5 strength Murashige and Skoog media consisting of 2.5 mM KNO_3, 25 μM FeNa-EDTA, 1 mM KH_2PO_4, 1 mMKH_2PO_4, 1 mM $Ca(NO_3)_2$.4H2O, 35 μM H_3BO_3, 7 μM $MnCl_2$, 0.25 μM $CuSO_4$, 2 mM $ZnSO_4$, and 0.4 μM $Na_2MoO_4 \cdot 2H_2O$ (Murashige and Skoog, 1962). To test the effect of selenite, agar media plates contained either 0 or 15 μM sodium selenite, a concentration known to induce toxicity (Tamaoki et al., 2008). To confirm selenite toxicity at the above-mentioned concentration, plants ($n = 20–30$) were grown with and without selenite. Selenite tolerance was determined by measuring root length in the WT and mutant plants after 14 days of growth on vertical plates. To determine elemental content, plants were grown for 21 d on horizontal agar plates; this longer time point was selected in order to obtain enough for elemental analysis. Four biological replicates per treatment consisting of several pooled plants each were rinsed and dried, and then acid digested prior to ICP-OES analysis. Elemental analysis was carried out as described before (Van Hoewyk et al., 2007; Pilon Smits et al., 1999). This included appropriate quality assurance and control, by means of National Institute of Standards and Technology standards for all elements, and a quality control checked every 15 samples. All statistical analyses (ANOVA and Student's t tests) were performed using the Kaleida-graph software package (Synergy Software).

2.2. Plant stress-induced phenotype measurements in sir1-1 plants

Total GSH content, including the pool of reduced and oxidized glutathione (GSSG), was estimated spectrophotometrically at A_{412} using Ellman's Reagent, as previously described (Grant et al., 2011). Reduced GSH was estimated as the difference between total GSH and GSSG in five individual plants. The ratio of variable and maximal Chlorophyll fluorescence (F_V/F_M), which represents the maximum photochemical efficiency of photosystem II, was measured in 25–30 dark-adapted plants using a hand-held chlorophyll fluorimeter (Photon System Instruments; Bratislava, Czech Republic) and calculated as described previously (Maxwell and Johnson, 2000).

2.3. Selenite and superoxide measurements

Ten micrograms of Fd, purified as previously described (Pilon et al., 1992; Ye et al., 2005), was incubated in a 1 mL volume containing 50 mM tricine/KOH buffer (pH 7.6), +/− 1 mM GSH, and+/− 0.1 mM sodium selenite, as described in Table 2. After a 2 h incubation at room temperature, the sample was centrifuged and the