Antioxidant Responses of Rice Seedlings to Sulfur Deficiency

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ABSTRACT

In this study, we investigated the antioxidant responses of rice seedlings to sulfur (S) deficiency. Sulfur deficiency resulted in a reduction of shoot growth but not root growth. Accumulation of 
\[ \text{H}_2\text{O}_2 \]
 and malondialdehyde in leaves was induced by S deficiency. The contents of cysteine, glutathione (GSH) and the ratio of GSH/oxidized glutathione, and the activities of ascorbate peroxidase, glutathione reductase, and catalase were lower in S-deficient leaves compared to control leaves. However, the content of ascorbate (AsA) and the ratio of AsA/dehydroascorbate were not affected and superoxide dismutase was increased by S deficiency. Our results demonstrate that S deficiency altered changes in antioxidant status and induced oxidative stress in rice seedlings.

Key words: Antioxidant response, Oxidative stress, Sulfur deficiency, Rice.

INTRODUCTION

Formation of reactive oxygen species (ROS), such as \( \text{O}_2^- \), hydroxyl radical, and \( \text{H}_2\text{O}_2 \), is intrinsic to metabolism of aerobic organisms. Plants have evolved a complex antioxidant network to maintain ROS at a steady-state level when they are subjected to hazardous environmental conditions (Grat
că et al. 2005). When the redox cellular status is compromised, antioxidants such as ascorbate (AsA) and reduced glutathione (GSH), and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) help to scavenge excessive ROS (Foyer and Noctor 2011, Noctor et al. 2012, Gest et al. 2013). An imbalance between ROS production and scavenging leads to cellular damage.

Sulfur (S) is an essential nutrient required for all living organisms. The need of higher plants for S has long been recognized. S is required for protein synthesis. In addition, it is an integral constituent of the amino acids such as cysteine (Cys) and methionine and a variety of cellular constituents such as GSH (Rausch and Wachter 2005). Thus, S plays an important role in plant
growth and in the regulation of plant development. In recent years, S deficiency has become an increasing problem for agriculture. The occurrence of S deficiency is mainly due to the significant decreases in atmospheric inputs of S in industrialized countries, or an imbalance of S in fertilizers in relation to the supply of other major nutrients (Zhao et al. 1999).

Sulfur is taken up by plants in its inorganic sulfate form, followed by reduction into sulfide and incorporation into Cys. Cys is further converted to GSH. The decrease in GSH content under S-deficient conditions has been reported previously (Lappartient and Touraine 1996, Hirai et al. 2003, Nikiforova et al. 2003, Kandlbinder et al. 2004, Kopriva and Rennenberg 2004). Sulfur deficiency is reported to decrease the synthesis and activity of the enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco), CO₂ assimilation rate, and chlorophyll content (Xu et al. 1996, Hawkesford 2000, Kandlbinder et al. 2004, Tewari et al. 2004). As a result, S deficiency could lead to enhanced H₂O₂ production in plants. Indeed, increase in H₂O₂ production has been demonstrated in S-deficient Arabidopsis roots (Schachtman and Shin 2007), maize leaves (Tewari et al. 2004) and mulberry leaves (Tewari et al. 2010). The changes in the activities of antioxidant enzymes in response to S deficiency have been studied in Arabidopsis (Kandlbinder et al. 2004), maize (Tewari et al. 2004) and mulberry (Tewari et al. 2010). However, little has been known about the changes in antioxidant responses of rice seedlings to S deficiency. The present study was undertaken with the objective to examine the effect of S deficiency on antioxidant responses in leaves of rice seedlings.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter papers at 37°C in the dark. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a beaker containing half-strength Kimura B nutrient solution with sufficient S supply (control) or deficient S supply (–S). Nutrient solution for the control contains the following macro- and micro-elements: 182.3 μM (NH₄)₂SO₄, 91.6 μM KNO₃, 273.9 μM MgSO₄·7 H₂O, 91.1 μM KH₂PO₄, 182.5 μM Ca(NO₃)₂, 30.6 μM Fe-citrate, 0.25 μM H₂BO₃, 0.2 μM MnSO₄·H₂O, 0.2 μM ZnSO₄·7 H₂O, 0.05 μM CuSO₄·5H₂O and 0.07 μM H₂MoO₄ (Kimura 1931). Sulfur deficiency in the nutrient solution was ensured by replacing sulfate salts with respective chlorides to ensure adequate supply of N, Mg, Mn, Zn, and Cu. The nutrient solutions (pH 4.7) were replaced every 3 d. The concentration of chlorine in S-deficient nutrient solution did not affect the metabolism of rice seedlings. Kimura B nutrient solution contains the desired nutrients for growing rice plants. Since young rice seedlings were used for the present study, the nutrient solution contained no silicon, although silicon is essential for growth of sturdy rice plants in the field.

The hydroponically cultivated seedlings were grown in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30/25°C day/night and 90% relative humidity. When the third leaves of control or –S seedlings were fully grown (12 d after sowing), the second leaves of control and –S seedlings were excised to determine the contents of chlorophyll, H₂O₂, malondialdehyde (MDA), Cys, and antioxidants (AsA and GSH), and the activities of antioxidant enzymes (SOD, APX, GR, and CAT).

**Growth Response**

At the end of treatment, the seedlings were divided into their separate parts (shoots, adventitious roots, and primary roots). The length of the shoots and primary roots and the fresh weight (FW) and dry weight (DW) of shoots and roots (adventitious roots plus primary roots) were measured. For DW determination, the shoots and roots were dried at 65°C for 48 h, a time when DW was constant.

**Determination of H₂O₂, Chlorophyll, and MDA**

The H₂O₂ content was measured colorimetrically and was extracted with sodium phosphate buffer (50 mM, pH 6.8) containing 1 mM hydroxylamine, a catalase inhibitor (Jana and Choudhuri 1981). The reaction mixture consisted
of 3 mL of leaf extract supernatant and 1 mL reagent [0.1% (v/v) TiCl$_4$ in 20% (v/v) H$_2$SO$_4$]. The blank probe consisted of 50 mM phosphate buffer in the absence of leaf extract. The absorbance was measured at 410 nm. The amount of H$_2$O$_2$ was calculated by using a standard curve prepared with known concentrations of H$_2$O$_2$.

The chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. Malondialdehyde, routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined by the thiobarbituric acid reaction as described by Heath and Packer (1968).

**Determination of AsA, Dehydroascorbate (DHA), GSH, Oxidized glutathione (GSSG), and Cys**

Ascorbate and DHA contents in 5% (w/v) trichloroacetic acid were determined as described by Law et al. (1983). The assay is based on the reduction of Fe$^{3+}$ to Fe$^{2+}$ by AsA. The Fe$^{2+}$ then forms complexes with bipyridyl, giving a pink color that absorbs at 525 nm. GSH and GSSG contents in 3% sulfosalicylic acid extract were determined by the method of Smith (1985). The content of GSH was spectrophotometrically determined with an enzyme-recycling assay at 412 nm. The assay is based on sequential oxidation of GSH by 5, 5-dithiobis-(2-nitrobenzoic acid) and reduction by NADPH in the presence of known amount of GR. The procedure to determine Cys content was previously described (Gaitonde 1967).

**Enzyme Extraction and Assays**

Leaf samples were excised and immediately used for enzyme extraction. All operations were carried out at 4°C. For extraction of enzymes, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. For analysis of APX activity, 2 mM AsA was added to the extraction buffer. The homogenate was centrifuged at 12,000 x g. SOD activity was determined according to Paolletti et al. (1986). The reaction mixture (2.73 mL) contained 100 mM triethanolamine-diethanolamine buffer (pH 7.4), 7.5 mM NADH, EDTA/MnCl$_2$ (100 mM/50 mM, pH 7.4), 10 mM 2-mercaptoethanol, and enzyme extract (0.2 mL). The reaction was started by the addition of NADH. The reaction was allowed to proceed for 10 min. The absorbance was measured at 340 nm. One unit of SOD was defined as the amount of enzyme that inhibits by 50% the rate of NADH oxidation observed in blank sample. CAT activity was assayed according to Kato and Shimizu (1987). The decrease in H$_2$O$_2$ was followed as the decline in the absorbance at 240 nm, and the activity was calculated using the extinction coefficient (40 mM$^{-1}$cm$^{-1}$ at 240 nm) for H$_2$O$_2$. One unit of CAT was defined as the amount of enzyme which degraded 1 µmol H$_2$O$_2$ per min. APX activity was determined according to Nakano and Asada (1981). The decrease in AsA concentration was followed as a decline in the absorbance at 290 nm and activity was calculated using the extinction coefficient (2.8 mM$^{-1}$cm$^{-1}$ at 290 nm) for AsA. One unit of activity for APX was defined as the amount of enzyme that degraded 1 µmol of AsA per min. GR activity was determined by the method of Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreased 1 optical density min$^{-1}$ at 340 nm. Enzyme activities were expressed on a milligram protein basis. For protein determination, enzyme extracts were used for determination of protein by the method of Bradford (1976).

**Statistical Analysis**

Statistical differences between measurements ($n = 4$ or 40) for different treatments were analyzed following Student’s $t$-test or Duncan’s multiple range test. A $P<0.05$ was considered statistically significant.

**RESULTS**

**Effect of S Deficiency on Growth Response**

Effect of S deficiency on growth response was shown in Table 1. Shoot length but not root length was significantly reduced by S deficiency. S deficiency also resulted in a decline in the FW and DW of shoots. However, the FW and DW of roots were not affected by S deficiency.

**Effect of S Deficiency on Chlorophyll, Cys, H$_2$O$_2$, and MDA**

Deficiency of S significantly decreased the contents of chlorophyll and Cys in the second leaves (Table 2). In the present study, H$_2$O$_2$ production and MDA, an indicator of lipid
peroxidation, were also measured. We observed that S deficiency increased H$_2$O$_2$ and MDA contents in the second leaves of rice seedlings (Table 2).

**Effect of S Deficiency on Antioxidant Status**

When rice seedlings were grown under conditions of S deficiency for 12 d, it was observed that S deficiency had no effect on AsA, DHA, and the AsA/DHA in the second leaves of seedlings (Table 2). In contrast, S deficiency resulted in a reduction of GSH content and the GSH/GSSG ratio in the second leaves of seedlings (Table 2). However, S deficiency increased GSSG content (Table 2). As to the effect of S deficiency on the activities of antioxidant enzymes such as SOD, APX, GR, and CAT, it was observed that S

**Table 1.** Effect of sulfur (S) deficiency on the length, fresh weight (FW), and dry weight (DW) and concentrations of S in roots and shoots of rice seedlings. Rice seedlings were grown under S-sufficient (control) and -deficient (-S) conditions for 12 d. Data are means ± SE (n = 40). *P < 0.05 compared with control.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Control</th>
<th>-S</th>
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</thead>
<tbody>
<tr>
<td>Shoot length (cm)</td>
<td>14.0 ± 0.2</td>
<td>12.6 ± 0.2*</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>4.7 ± 0.1</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>Shoot FW (mg seedling$^{-1}$)</td>
<td>65.8 ± 1.9</td>
<td>43.5 ± 1.9*</td>
</tr>
<tr>
<td>Root FW (mg seedling$^{-1}$)</td>
<td>49.5 ± 1.5</td>
<td>49.7 ± 2.7</td>
</tr>
<tr>
<td>Shoot DW (mg seedling$^{-1}$)</td>
<td>15.3 ± 0.5</td>
<td>13.3 ± 0.3*</td>
</tr>
<tr>
<td>Root DW (mg seedling$^{-1}$)</td>
<td>6.9 ± 0.2</td>
<td>6.9 ± 0.2</td>
</tr>
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</table>

**Table 2.** Effect of sulfur (S) deficiency on the contents of chlorophyll, H$_2$O$_2$, Cys, MDA, and antioxidants (AsA, DHA, GSH and GSSG), the ratios of AsA/DHA and GSH/GSSG, and the activities of antioxidant enzymes (SOD,GR,APX,CAT) in the second leaves of rice seedlings. Rice seedlings were grown under S-sufficient (control) and -deficient (-S) conditions for 12 d. The second leaves were then used to determine antioxidants and antioxidant enzymes. Data are means ± SE (n = 4). *P < 0.05 compared with control. Cys: cysteine; MDA: malondialdehyde; AsA: ascorbate; DHA: dehydroascorbate; GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; GR: glutathione reductase; APX: ascorbate peroxidase; and CAT: catalase.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>-S</th>
</tr>
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<tbody>
<tr>
<td>Chl (mg g$^{-1}$FW)</td>
<td>2.60 ± 0.06</td>
<td>2.00 ± 0.07*</td>
</tr>
<tr>
<td>Cys (μmol g$^{-1}$FW)</td>
<td>0.42 ± 0.01</td>
<td>0.26 ± 0.01*</td>
</tr>
<tr>
<td>H$_2$O$_2$ (μmol g$^{-1}$FW)</td>
<td>32.50 ± 0.70</td>
<td>37.30 ± 0.20*</td>
</tr>
<tr>
<td>MDA (nmol g$^{-1}$FW)</td>
<td>36.30 ± 3.50</td>
<td>46.20 ± 4.30*</td>
</tr>
<tr>
<td>AsA (μmol g$^{-1}$FW)</td>
<td>3.06 ± 0.20</td>
<td>3.20 ± 0.10</td>
</tr>
<tr>
<td>DHA (μmol g$^{-1}$FW)</td>
<td>0.58 ± 0.10</td>
<td>0.66 ± 0.10</td>
</tr>
<tr>
<td>AsA/DHA ratio</td>
<td>5.90 ± 0.50</td>
<td>4.80 ± 0.50</td>
</tr>
<tr>
<td>GSH (nmol g$^{-1}$FW)</td>
<td>65.60 ± 5.20</td>
<td>37.80 ± 6.30*</td>
</tr>
<tr>
<td>GSSG (nmol g$^{-1}$FW)</td>
<td>20.90 ± 2.70</td>
<td>31.30 ± 3.40*</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td>3.10 ± 0.20</td>
<td>1.20 ± 0.40*</td>
</tr>
<tr>
<td>SOD (units mg$^{-1}$protein)</td>
<td>1.60 ± 0.06</td>
<td>2.50 ± 0.12*</td>
</tr>
<tr>
<td>APX (unit mg$^{-1}$protein)</td>
<td>1.50 ± 0.12</td>
<td>1.00 ± 0.09*</td>
</tr>
<tr>
<td>GR (unit mg$^{-1}$protein)</td>
<td>0.12 ± 0.01</td>
<td>0.08 ± 0.01*</td>
</tr>
<tr>
<td>CAT (unit mg$^{-1}$protein)</td>
<td>0.17 ± 0.01</td>
<td>0.13 ± 0.01*</td>
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</table>
deficiency increased SOD activity, but decreased APX, GR, and CAT activities (Table 2).

**DISCUSSION**

In this study, rice seedlings grown under S-sufficient and -deficient conditions for 12 d, a time when the third leaves of both S-deficient and -sufficient rice seedlings were fully grown. Sulfur deficiency alone resulted in a reduction of shoot growth but not root growth (Table 1). This is consistent with the general notion that the growth of shoots is more affected by S deficiency than root growth (Tewari *et al.* 2010). In the previous work, we also observed that nitrogen deficiency resulted in a decline in shoot growth but not root growth (Lin *et al.* 2011). It appears that the effect of S deficiency on growth response in rice seedlings resembles that of nitrogen deficiency. Plant exposure to nutritional deficiencies commonly results in a decrease in shoot biomass production and in the partitioning of biomass in favor of the roots, so that shoot to root ratio is significantly decreased (Tewari *et al.* 2007). Apparently, altered growth pattern has a specific role in the adaptation of plants to nutrient limitation to improve nutrient uptake.

When rice seedlings were grown under S-deficient conditions, chlorosis or decrease in chlorophyll was first observed in the second leaves (Table 2). For this reason, the second leaves of rice seedlings were used to perform all measurements except growth response. In this study, all the measurements were expressed on FW or protein basis. Slight change of FW or protein content in the second leaves of the seedlings in response to S deficiency was observed (data not shown). Thus, the data of all the measurements reported here are unlikely due to the change in FW or protein content.

Cysteine and GSH are S-containing compounds. S deficiency decreased the contents of Cys and GSH in the second leaves (Table 2). It appears that analyses of Cys and GSH are accurate tool to diagnose S deficiency. S deficiency decreased Cys and GSH contents has also been reported in Arabidopsis (Hirai *et al.* 2003, Nikiforova *et al.* 2003, Kandlbinder *et al.* 2004), mulberry (Tewari *et al.* 2010), and canola (Lappartient and Touraine 1996).

An increase in \( \text{H}_2\text{O}_2 \) production has been reported upon exposure of plants to S-deficient conditions (Schachtman and Shin 2007, Tewari *et al.* 2010). In this study, we also observed an increase in \( \text{H}_2\text{O}_2 \) content in the second leaves (Table 2). SOD catalyzes the dismutation of two molecules of superoxide into oxygen an \( \text{H}_2\text{O}_2 \). APX and CAT are responsible for scavenging or eliminating \( \text{H}_2\text{O}_2 \). We observed that in S-deficient leaves SOD activity was greater while APX and CAT activities were lower than their respective control leaves (Table 2). This may result in a higher \( \text{H}_2\text{O}_2 \) content in S-deficient leaves. Decreased activity of CAT has also been demonstrated in S-deficient mulberry plants (Tewari *et al.* 2010). In contrast, S deficiency has been found to increase the activity of APX in Arabidopsis (Kandlbinder *et al.* 2004), maize (Tewari *et al.* 2004) and mulberry (Tewari *et al.* 2010). A role for plasma membrane NADPH oxidase in the production of the \( \text{H}_2\text{O}_2 \) has been a recent focus in ROS signaling research (Sagi and Flurher 2006). Whether S deficiency causes \( \text{H}_2\text{O}_2 \) production via an increase in NADPH oxidase remains to be investigated.

Exposure to S deficiency has been linked to a production of \( \text{H}_2\text{O}_2 \) (Tewari *et al.* 2004, 2010, Schachtman and Shin 2007), thereby imposing a cellular oxidative challenge. Lipid peroxidation, clear marker of ROS-induced oxidative damage, was shown to occur under S deficiency (Tewari *et al.* 2004, 2010). However, in a recent review, Chen and Niki (2011) showed that lipid peroxidation products may also induce adaptive response and increase tolerance to forthcoming oxidative stress by upregulating defense capacity. In the current study, it seemed that the involvement of oxidative stress endorsed increased lipid peroxidation level in leaves of rice seedlings exposed to S deficiency.

Taken together, our results demonstrate that deficiency of S altered changes in antioxidant status and induced oxidative stress in leaves of rice seedlings.

**ACKNOWLEDGMENTS**

This work was supported by a research grant from the National Science Council of the Republic of China (NSC 100-2313-B002-002).
REFERENCES


