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Short Communication

Independent roles of glutathione and *O*-acetyl-L-serine in regulation of sulfur-responsive gene expression in *Arabidopsis thaliana*

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Abstract We investigated the effects of a four-day treatment with 0.3 mM glutathione (GSH) and 10 mM *O*-acetyl-L-serine (OAS), negative and positive regulators of sulfur-responsive gene expression, respectively, alone or in combination, on the sulfur-responsive gene expression in ten-day-old *Arabidopsis thaliana* plants. We determined the relationship between the concentrations of GSH, OAS and sulfate, and expression of sulfur-responsive genes in rosette leaves. The concentrations of GSH and OAS correlated negatively and positively with expression of genes, respectively, suggesting that GSH and OAS independently affect expression of sulfur-responsive genes.

Key words: *Arabidopsis thaliana*, β subunit of β-conglycinin, glutathione, *O*-acetyl-L-serine, sulfur deficiency.

Plants take up sulfate from soil, and reduce it for biosynthesis of sulfur-containing organic compounds (Figure 1). Plants also respond to sulfur-deficiency by activating sulfate uptake and the assimilation pathway (for reviews see Saito 2000; Leustek et al. 2000). For example, under sulfur deficiency, mRNAs for sulfur transporters and sulfur assimilation enzymes accumulate at high levels (Takahashi et al. 1997).

Glutathione (GSH) and *O*-acetyl-L-serine (OAS) were reported to affect sulfur-responsive gene expression. Exogenous application of GSH downregulated expression of a sulfur transporter gene *Sultr2;2*, and a gene encoding ATP sulfurylase (Lappartient et al. 1999). Exogenous application of OAS resulted in upregulation of expression of genes encoding adenosine 5′-phosphosulfate reductase, chloroplastic and cytosolic cysteine synthase and serine acetyltransferase (Koprivova et al. 2000).

Although these studies suggested that GSH and OAS are key metabolites in the regulation of gene expression, the reciprocal relationship of the roles of GSH and OAS in the regulation of sulfur-responsive gene expression has not been examined. In this study, we examined the relationship between GSH and OAS in the regulation of sulfur-responsive gene expression.

We used a transgenic *A. thaliana line*, NOB7 (Ohkama et al. 2002), which carries the green fluorescence protein (GFP) open reading frame driven by a chimeric promoter comprised of a cauliflower mosaic virus 35S RNA promoter and a trimer of the β_{SR} fragment. β_{SR} is a 235 bp promoter region of the β-conglycinin β subunit gene, a sulfur deficiency-upregulated soybean seed storage protein gene, and this region was sufficient for induction by a sulfur-deficient condition (Awazuhara et al. 2002). β_{SR} is activated by sulfur-deficiency, and NOB7 plants accumulate GFP at a high level under a sulfur-deficient condition (Ohkama et al. 2002).

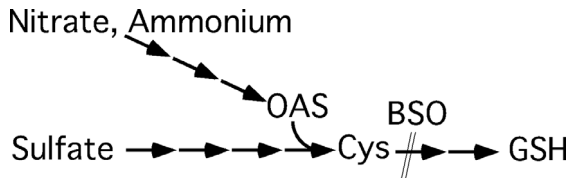

Figure 1. A simple scheme of sulfur assimilation pathway from sulfate to glutathione. Sulfate in the environment is taken up and reduced to form cysteine (Cys). *O*-acetyl-L-serine (OAS) is synthesized from the N assimilation pathway and conjugate with reduced sulfur to form Cys. Glutathione (GSH) is synthesized from Cys and the synthesis is inhibited by buthionine sulfoximine (BSO). OAS and GSH have been shown to affect expression of sulfur-responsive genes.

Abbreviations: BSO, buthionine sulfoximine; β_{SR}, sulfur-responsive 235 bp promoter region of the β subunit gene of β-conglycinin; GFP, green fluorescence protein; GSH, OAS, *O*-acetyl-L-serine; $[OAS]_{leaves}$, concentration of sulfate in rosette leaves.

We examined the effects of exogenous application of GSH and OAS on the expression of sulfur-responsive genes or elements, β_{SR}, *Sultr2;2*, *APR1*, and *SAT1* in NOB7 plants. There was no significant difference in expression patterns of *Sultr2;2*, *APR1*, and *SAT1* between the NOB7 plants and Col-0 wild-type plants in any of the following experiments (data not shown). Plants were initially grown on agarose plates containing 1,500 μM sulfate for ten days. Then they were cultured for four days on fresh agarose plates containing 1,500 μM or 1.5 μM sulfate with or without 0.3 mM GSH, 10 mM OAS or 1 mM BSO alone or in combination (Table 1). The media used in the present study were as described previously (Hirai et al. 1995). OAS, GSH and BSO were filter-sterilized and added after autoclaving the media to avoid possible degradation during autoclaving. To avoid any changes in pH in the media by addition of these reagents, we dissolved OAS, GSH and BSO in a small volume of media and readjusted the pH prior to filter sterilization.

After the treatment, we collected the rosette leaves of the plants and determined the concentrations of GSH, OAS and sulfate, referred to hereafter as $[GSH]_{leaves}$, $[OAS]_{leaves}$, and $[sulfate]_{leaves}$, respectively (Table 2), and levels of GFP fluorescence, and accumulation of mRNA for *Sultr2;2*, *APR1*, and *SAT1* (Table 3). For the quantification of GFP, we used relative GFP fluorescence (Table 3), which represents the intensity of fluorescence corresponding to GFP in NOB7 plants relative to chlorophyll autofluorescence as described by Niwa et al. (1999). The relative levels of *Sultr2;2*, *APR1* or *SAT1* transcript accumulation to that of β-tubulin transcript were caluculated (Table 3).

Table 1. List of treatments.

Treatments Sulfate (μM)	GSH	OAS	BSO	Abbreviations
1500	−	−	−	C
1500	−	−	+	CB
1500	+	−	−	CG
1500	−	+	−	CO
1500	−	+	+	COB
1.5	−	−	−	ΔS
1.5	+	−	−	ΔSG
1.5	−	+	−	ΔSO

GSH, OAS and BSO were applied at the initial concentrations of 0.3, 10, and 1 mM, respectively. In the following tables and text, the abbreviations shown here will be used to describe each treatment.

Treatment with GSH under a sulfur-deficient condition increased the $[GSH]_{leaves}$ but not $[OAS]_{leaves}$ or $[sulfate]_{leaves}$ (Table 2, compare ΔS and ΔSG). Under sulfur deficiency, plants treated with GSH accumulated less GFP, and mRNAs for *Sultr2;2*, *APR1* and *SAT1* than those not treated with GSH (Table 3, compare ΔS and ΔSG). Buthionine sulfoximine (BSO), an inhibitor of GSH biosynthesis lowered $[GSH]_{leaves}$ but not $[OAS]_{leaves}$ or $[sulfate]_{leaves}$ (Table 2, compare C and CB). BSO increased the GFP fluorescence, accumulation of mRNA for *Sultr2;2*, *APR1*, and *SAT* (Table 3, compare C and CB). In summary, in plants under sulfur deficiency, the increase in $[GSH]_{leaves}$ correlated with repression of the expression of sulfur-responsive genes including β_{SR}, suggesting that under a limited sulfur supply, $[GSH]_{leaves}$ is a key determinant for the downregulation of the sulfur-responsive genes.

Although application of GSH in the presence of 1.5 mM sulfate increased $[GSH]_{leaves}$ (Table 2, compare C and CG), it had no significant effect on the expression of sulfur-responsive genes (Table 3, compare C and CG), or $[OAS]_{leaves}$, or $[sulfate]_{leaves}$ (Table 2, compare C and CG). These results suggest that the negative effect of GSH on the expression of sulfur-responsive genes was not evident in plants grown with normal levels of sulfate. In these plants, $[GSH]_{leaves}$ is typically higher than 180 nmol·gFW^{-1}.

Treatment with OAS in the presence of 1.5 mM sulfate increased $[OAS]_{leaves}$ (Table 2, compare C and CO) and levels of GFP and mRNAs corresponding to *Sultr2;2* and *APR1* (Table 3, compare C and CO). OAS had no effect on $[sulfate]_{leaves}$, but increased $[GSH]_{leaves}$ (Table 2, compare C and CO). In summary, $[OAS]_{leaves}$ increases

Table 2. Concentrations of GSH, OAS, and sulfate in rosette leaves of *A. thaliana*.

Treatment	$[GSH]_{leaves}$ (nmol·gFW^{-1})	$[OAS]_{leaves}$ (nmol·gFW^{-1})	$[sulfate]_{leaves}$ (μmol·gFW^{-1})
C	181±25a	3.7±0.9a	11.4±1.8a
CB	15±4b	2.2±0.8a	11.0±0.8a
CG	361±120c	3.7±2.0a	11.1±3.2a
CO	388±13c	15.0±3.5bc	10.2±0.9a
COB	71±26d	11.6±1.7b	10.2±0.4a
ΔS	59±26d	8.3±2.5b	2.6±0.6b
ΔSG	167±45a	10.1±0.2b	3.3±1.0b
ΔSO	81±23d	16.7±1.5c	2.5±3.3b

Plants were treated with BSO, GSH or OAS alone or in combination as shown in Table 1. The concentrations of GSH and sulfate were determined as described by Ohkama et al. (2000). The concentration of OAS was determined as described by Kim et al. (1999). Means±SD of five replications are shown. Different letters indicate significant difference at $P<0.05$ estimated by Student's *t*-test.

Table 3. Relative GFP fluorescence and relative mRNA accumulation in rosette leaves of *A. thaliana*.

Treatment	Relative GFP fluorescence	Relative mRNA accumulation		
		Sultr2;2	*APR1*	*SAT1*
C	1.00±0.15a	1.00±0.17a	1.00±0.23a	1.00±0.01a
CB	3.54±0.30b	1.51±0.22b	1.91±0.90b	2.64±0.44b
CG	1.01±0.31a	0.92±0.16a	1.01±0.18a	1.19±0.20a
CO	2.13±0.13c	1.53±0.25b	1.77±0.26b	1.36±0.20c
COB	4.00±0.08d	1.69±0.19b	2.52±0.27c	1.85±0.24d
ΔS	4.66±0.15e	2.10±0.17c	1.94±0.14b	1.24±0.16c
ΔSG	3.02±0.58b	1.31±0.09b	1.24±0.30a	0.80±0.08a
ΔSO	4.06±0.41bde	2.42±0.50c	1.81±0.20b	1.51±0.31cd

Plants were treated with BSO, GSH or OAS alone or in combination as shown in Table 1. Relative GFP fluorescence represents intensity of fluorescence corresponding to GFP fluorescence in NOB7 plants relative to chlorophyll autofluorescence as described by Niwa et al. (1999). Relative mRNA accumulation represents mRNA accumulation of *Sultr2;2*, *APR1* or *SAT1* transcripts relative to *β-tublin* transcripts in each sample. Total RNA was prepared using an RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After treatment with DNaseI (Invitrogen, Carlsbad, CA, U.S.A), reverse transcription was performed using SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen) and Oligo(dT)12–18 Primer (Invitrogen). SmartCycler™ (Cepheid, Sunnyvale, CA, U.S.A) was used for amplification. The pairs of oligonucleotide primers used for amplification were; 5′-GCTCGCTAATCCTACCTTTGG-3′ and 5′-AGCCTTGGGAATGGGATAAG-3′ for *β-tublin*; 5′-CGACATGTCTTGCGTGATGGGCG-3′ and 5′-GCTCGCTTCAATTTGTGAAGTACCCT-3′ for *Sultr2;2*; 5′-CGACATGTCTTGCGTGATGGGCG-3′ and 5′-TCCTCAATCTCAACCACATCAAC-3′ for *APR1*; 5′-CTTTCGCCGCGATATTCAT-3′ and 5′-CCGACCCTGATCCAATCTTA-3′ for *SAT1*. Means±SD of three replications are shown. Different letters indicate significant difference at $P<0.05$ estimated by Student's t-test.

with the increase in the expression of GFP, *Sultr2;2*, *APR1* (Table 3), suggesting that $[OAS]_{leaves}$ is a key determinant for gene expression in plants grown in the presence of 1.5 mM sulfate.

Under sulfur deficiency, $[OAS]_{leaves}$ and expression of sulfur-responsive genes were high (Tables 2 and 3, compare C and ΔS). Treatment with OAS increased $[OAS]_{leaves}$ under sulfur deficiency (Table 2, compare ΔS and ΔSO), but not the expression of sulfur-responsive genes (Table 3, compare ΔS and ΔSO). $[GSH]_{leaves}$ and $[sulfate]_{leaves}$ were not significantly affected by OAS (Table 2, compare ΔS and ΔSO). In summary, the positive effect of OAS on expression of sulfur-responsive genes was not evident when $[OAS]_{leaves}$ was higher than 8.3 nmol·g fresh weight^{-1}, suggesting that $[OAS]_{leaves}$ is not a key determinant for the gene expression in plants with high $[OAS]_{leaves}$.

Application of neither OAS nor BSO significantly affected $[sulfate]_{leaves}$ (Table 2). Application of OAS and that of OAS+BSO increased $[OAS]_{leaves}$ within a similar range (Table 2, CO and COB, above 8.3 nmol·g fresh weight^{-1}). $[OAS]_{leaves}$ was low both in plants treated with GSH or BSO (Table 2, CG and CB, about 4 nmol·g fresh weight^{-1}). $[GSH]_{leaves}$ was about five-fold higher in the plants treated with OAS than in those treated with OAS and BSO (Table 2, compare CO and COB). Similarly, $[GSH]_{leaves}$ was about 24-fold higher in the plants treated with GSH than in those treated with BSO (Table 3; compare CG and CB). Expression of sulfur-responsive genes was lower in plants treated with OAS than in those treated with BSO (Table 3, compare CO and CB), and lower in plants treated with GSH than in those treated with BSO (Table 3, compare CG and CB). These results suggest that the negative effect of GSH is evident irrespective of $[OAS]_{leaves}$.

Figure 2 illustrates the correlation between GSH and gene expression levels. When $[OAS]_{leaves}$ is low (Figure 2, closed circles), the increase in $[GSH]_{leaves}$ from low (about 60 nmol·g fresh weight^{-1}) to high (more than 180 nmol·g fresh weight^{-1}) downregulates expression of sulfur-responsive genes, and the negative effect of GSH is not evident when $[GSH]_{plants}$ is higher than 180 nmol·g fresh weight^{-1}.

Expression of the sulfur-responsive genes, namely β_{SR}, *Sultr2;2*, and *APR1*, was higher in plants with a high $[OAS]_{leaves}$ (Figure 2, open circles) than in plants with a low $[OAS]_{leaves}$ even at a similar level of $[GSH]_{leaves}$ (Figure 2). These results suggest that the positive effect of OAS on expression of β_{SR}, *Sultr2;2*, and *APR* is evident irrespective of $[GSH]_{leaves}$, although this was not evident in *SAT1* (Figure 2). Taken together our results suggest that GSH and OAS affect the expression of sulfur-responsive genes independently within the ranges of $[GSH]_{leaves}$ and $[OAS]_{leaves}$ examined.

In the data analysis shown in Figure 2, we disregarded $[sulfate]_{leaves}$. Exogenous application of OAS without alteration of sulfate concentration in the media has been reported to increase the expression of the β-conglycinin β subunit gene (Kim et al. 1999), suggesting that sulfate does not directly regulate the expression of the β subunit gene. It is reasonable to assume a similar response in NOB7 plants carrying a portion of the β subunit gene promoter as a sulfur regulatory element.

GSH and OAS have been reported to regulate the expression of sulfur-responsive genes, but to our knowledge, the relationship between GSH and OAS has

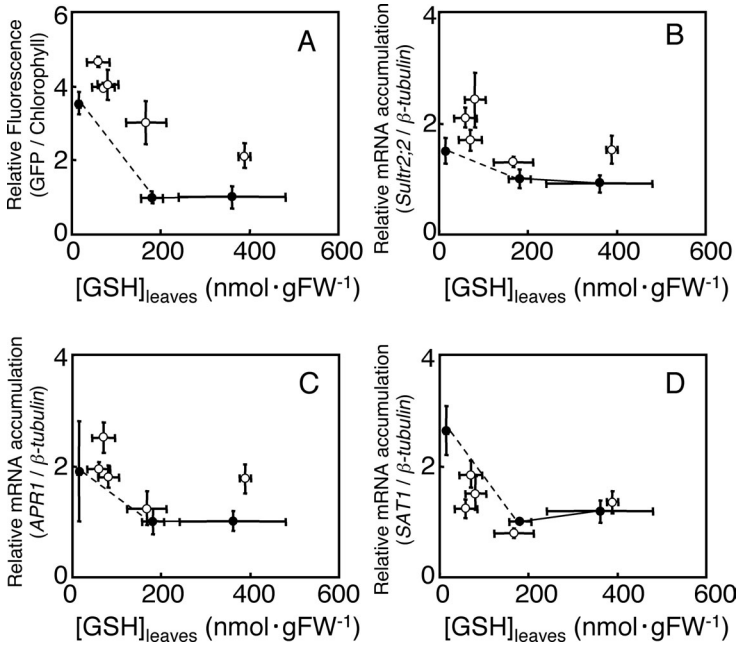

Figure 2. Relationship between concentrations of GSH and expression of sulfur-responsive genes, β_{SR} (A), *Sultr2;2* (B), *APR1* (C) and *SAT1* (D) in rosette leaves of *Arabidopsis thaliana*. The plants were treated with sulfur deficiency, OAS, GSH and BSO alone or in combination as shown in Table 1. The data presented in Tables 2 and 3 are plotted. Closed circle points represent data points with low concentrations (less than 5 nmol·gFW^{-1}) of OAS in leaves, and open circle points represents data with high concentrations (more than 5 nmol·gFW^{-1}) of OAS in leaves.

not been investigated. Our results show the presence of upper concentration thresholds for the negative effect of GSH and the positive effect of OAS in the regulation of sulfur-responsive gene expression. More importantly, we also demonstrated that the expression of sulfur-responsive genes is determined by GSH and OAS and the combination of the negative effect of GSH and the positive effect of OAS.

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