Isolation and Characterization of an Arabidopsis Mutant That Overaccumulates O-Acetyl-L-Ser

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O-Acetyl-L-Ser (OAS) is a positive regulator for the expression of sulfur (S) deficiency-inducible genes. In this study, through the isolation and analysis of Arabidopsis mutants exhibiting altered expression of S-responsive genes, we identified a thiol reductase as a regulator of the OAS levels. Ethyl methanesulfonate-mutagenized M2 seeds of transgenic Arabidopsis NOB7 carrying a chimeric S-responsive promoter driving the green fluorescent protein gene were screened for mutants with altered levels of green fluorescence compared to parental NOB7 line. One of the lines exhibited elevated levels of green fluorescence and mRNA accumulation of several endogenous S-responsive genes and carried a single recessive mutation responsible for the phenotype. OAS concentration in the rosette leaves of the mutant was about five times higher than that of wild-type plants. Based upon the high OAS levels, the mutant was named osh1-1 (OAS high accumulation). The OSH1 locus was mapped to a 30-kb region in chromosome V. DNA sequence analysis revealed no base change in this region; however, a demethylated C residue was found in the first exon of At5g01580. At5g01580 mRNA accumulation was higher in the 30-kb region in chromosome V. The 30-kb region in chromosome V. DNA sequence analysis revealed no base change in this region; however, a demethylated C residue was found in the first exon of At5g01580. At5g01580 mRNA accumulation was higher in osh1-1 than in wild type, while transcript levels of other genes in the mapped region were not significantly altered in osh1-1. A line of transgenic plants overexpressing At5g01580 had elevated levels of endogenous S-responsive genes. These results suggest that elevated expression of At5g01580 is the cause of osh1 phenotype. Based on sequence similarity to animal thiol reductases, At5g01580 was tested for and exhibited thiol reductase activity. Possible roles of a thiol reductase in OAS metabolism are discussed.

Sulfur (S) is one of the essential elements of higher plants and is an important constituent of proteins, lipids, secondary metabolites, and coenzymes. In proteins, S participates in the reversible formation of disulfide bonds. The importance of S can be further evidenced by the integral role of iron-S proteins in electron transfer in the chloroplast (for review, see Marschner, 1995). In addition, glutathione (GSH) has roles as a signaling molecule, is involved in the cell cycle, serves as a source of reducing equivalents in the antioxidative pathway and, by formation of phytochelatins, is involved in the detoxification of heavy metals (for review, see May et al., 1998; Cobbett and Goldsborough, 2002).

The S metabolic pathway in plants was recently completely defined, and most of the genes encoding enzymes in this pathway have been cloned. Some of enzymes involved in the pathway were activated under S deficiency, and this corresponded to increases in mRNA accumulation (for review, see Leustek et al., 2000; Saito, 2000).

In addition to levels of available S, nitrogen (N) nutrition also regulates S metabolism. Activities of ATP sulfurylase (ATPS) and adenosine 5’-phosphosulfate reductase (APR) were decreased under nitrate deficiency and increased by application of NH$_4$ to the medium in *N. tabacum* cells (Brunold, 1993). Accumulation of mRNAs of APR genes was also increased by application of nitrate or NH$_4$ in Arabidopsis (Koprivova et al., 2000). O-Acetyl-L-Ser (OAS) is synthesized from Ser and, along with sulfide ion, serves as a cosubstrate for the production of Cys at the merging step of S and N metabolisms. Evidence shows that OAS acts as a signal molecule regulating S metabolism in response to S and N availability. OAS concentration is drastically increased by sulfate deficiency or nitrate application and is positively correlated with the ratio of nitrate to sulfate in the medium (Kim et al., 1999). OAS-supplemented medium enhanced both the activity of APR and the assimilation of sulfate to Cys in *L. minor* (Neuenschwander et al., 1991). Accumulation of mRNAs for a sulfate transporter in *Hordeum vulgare* and for APR in Arabidopsis was increased by OAS application (Smith et al., 1997; Koprivova et al., 2000).

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1 *This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 12138201 to S.N. and T.F.), by a 21st COE project (to T.F.), and by Research Fellowships of Japan Society for the Promotion of Science (grant no. 9846 to N.O.).

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[w]The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.047068.
Moreover, analysis of global transcriptome analysis suggests that OAS is a general regulator for the S deficiency-regulated gene expression in Arabidopsis (Hirai et al., 2003). GSH, a tripeptide of γ-Glu-Cys-Gly, is another molecule regulating S metabolism. GSH is the major form of available S within the plant (May et al., 1998). In split root experiments using *Brassica napus*, ATPS activity and sulfate uptake rate were elevated in sulfate-fed roots when the other one-half of the roots was starved for sulfate. This elevated rate was negatively correlated with GSH concentration in phloem sap (Lappartient and Touraine, 1996). Accumulation of ATPS and sulfate transporter mRNAs was decreased by application of GSH or Cys to the medium. However, when buthionine sulfoximine, an inhibitor of GSH synthesis from Cys, was applied simultaneously with Cys to Arabidopsis, reduction of the levels of ATPS and sulfate transporter mRNAs was not observed (Lappartient et al., 1999). The level of APR mRNAs was also decreased by GSH but not by simultaneous application of Cys and buthionine sulfoximine in Arabidopsis (Vaucellare et al., 2002). These results suggest that GSH acts as a signal molecule transmitting S status over a long distance to regulate gene expression.

Aside from the signal metabolites OAS and GSH, little is known about regulation of S metabolism. It is of crucial importance to identify regulatory proteins, if any, involved in regulation of genes for S metabolism. It is also important to reveal the mechanisms of signal molecule homeostasis and of regulation of S metabolism by these signals. To this end, we generated transgenic Arabidopsis plants in which regulation of gene expression by S nutrition was able to be visualized (Ohkama et al., 2002). We used the gene encoding the β-subunit of β-conglycinin, one of the major seed storage proteins of soybean (Shortwell and Larkins, 1989), as a model for S-responsive gene. Regulation of β-subunit gene expression in seeds by S has been reported in transgenic *Petunia hybrida* and Arabidopsis (Fujiiwara et al., 1992; Naito et al., 1994). When the β-subunit gene promoter was fused with a β-glucuronidase (GUS) reporter gene and introduced in Arabidopsis, the GUS activity was increased by sulfate deficiency, demonstrating that S regulation of this gene was at the level of transcription (Hirai et al., 1995).

Deletion analysis of the β-subunit gene promoter defined the S-responsive region to within 235 bp (βSR; S-responsive region; Awazuhara et al., 2002a). In transgenic Arabidopsis carrying three copies of βSR inserted in tandem into the cauliflower mosaic virus (CaMV) 35S RNA promoter, termed P35S:βSRX3, and fused with the GUS gene, GUS activity was observed not only in seeds but also in nonseed tissues including leaves and was increased by sulfate deficiency (Awazuhara et al., 2002a), suggesting that the mechanisms of S regulation of the β-subunit gene expression were common among the tissues examined. It was also found that the β-subunit gene promoter activity was up-regulated by application of OAS (Kim et al., 1999) and accumulation of β-subunit protein was decreased by GSH (Awazuhara et al., 2002b). Additionally, activity of βSR was also shown to be up-regulated by OAS and down-regulated by GSH (Y. Sogawa, N. Ohkama-Ohtsu, and T. Fujiwara, unpublished data). As OAS and GSH regulate the β-subunit gene in a similar manner for genes involved in S metabolism, it is likely that the β-subunit gene and other S-responsive genes are regulated by S nutrition via similar mechanisms.

To gain more insight into the regulation of S metabolism, we exploited a genetic strategy to isolate mutants exhibiting altered response to S nutrition. Response of βSR to S nutrition was visualized using green fluorescent protein (GFP) gene fusion in transgenic Arabidopsis line NOB7 (Ohkama et al., 2002). In this study, we report a mutant with an elevated level of GFP expression using NOB7 as a parental line. This mutant showed high levels of expression for S-responsive genes and elevated level of OAS and was therefore designated as OAS high accumulation (*osh1-1*). We also demonstrate that the causal gene for the phenotype of *osh1-1* is a homolog of thiol reductase from animals.

### RESULTS

#### Isolation of an S-Response Mutant, *osh1-1*

Transgenic Arabidopsis NOB7 plants carry GFP reporter gene under the control of the 35S:βSRX3 chimeric promoter, and expression from the S-respon-

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**Table 1. Segregation of *osh1-1* phenotype in F2 progenies**

Relative GFP fluorescent intensities in shoots of 14-d-old plants were quantified and normalized with the average of wild-type plants as in Figure 1B. As all GFP fluorescence values in homozygous *osh1-1* lines tested were above 1.25 and those in wild-type plants were below 1.25, plants with GFP fluorescence value higher and lower than 1.25 were counted as *osh1-1* and wild type, respectively.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Generation</th>
<th>No. of Plants Tested</th>
<th>Phenotype</th>
<th>Chi-Square (^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type × <em>osh1-1</em>(^{a})</td>
<td>F2</td>
<td>125</td>
<td>95</td>
<td>0.67(^{c})</td>
</tr>
<tr>
<td><em>osh1-1</em> × wild type(^{a})</td>
<td>F2</td>
<td>125</td>
<td>96</td>
<td>0.22(^{c})</td>
</tr>
</tbody>
</table>

\(^{a}\)Female × male. \(^{b}\)Chi-square values calculated based on expected ratio of 3:1 segregation. \(^{c}\)Not significant (\(P > 0.05\)) by Student’s t test.
sive promoter region of the soybean β-conglycinin β-subunit gene (βBbr) was visualized with GFP (Ohka-
ma et al., 2002). Twenty thousand M2 seeds originated
from 10,000 M1 plants were sown on plates with 1.5 mm sulfate (referred to as +S) and another 20,000
seeds were sown on plates with 0.0015 mm sulfate (re-
ferred to as −S). Fourteen days after germination, GFP
fluorescence in leaves was examined using a quantita-
tive fluorescent system. Plants showing altered GFP
fluorescence compared to wild-type plants (nonmuta-
genized NOB7 plants) were selected as putative mu-
tants and transferred to soil. M3 seeds were retested
for inheritance of the higher GFP fluorescence. From
six putative mutants, osh1-1, which exhibited higher
GFP fluorescence than wild type irrespective of sulfate
condition, was chosen for further analysis.

In backcrossing experiments with NOB7, the
phenotype of all (40/40) F1 plants was wild type when
osh1-1 was used as female. When osh1-1 was used as
a male, 37 out of 40 F1 plants exhibited wild-type
phenotype. The reason why the other three plants
were scored as mutants is likely to be due to variation
in GFP fluorescence. The phenotype of F2 progeny
segregated in a 3:1 ratio (wild type:mutant), indicating
that the phenotype is caused by a single nuclear locus
(Table I). These findings indicated that the high GFP
fluorescence phenotype of osh1-1 was caused by a sin-
gle recessive mutation.

osh1-1 Shows Higher Expression of S-Responsive Genes

osh1-1 was backcrossed three times with the wild-
type transgenic NOB7 line, and a line segregating with
the osh1-1 phenotype was established. Wild-type and
osh1-1 plants were grown on +S agar plates for 10 d
and then transferred to fresh +S or −S agar plates and
grown for an additional 4 d. As shown in Figure 1, A
and B, GFP fluorescence of osh1-1 was higher than that
of wild-type plants under both +S and −S conditions.
osh1-1 plants were smaller than wild type with both
shoot fresh weight (FW) and root length about one-
half as those of wild type (Fig. 1C).

In addition to an increased GFP fluorescence driven
by the 35S::βBbr×3 promoter, expression of endogenous
S-responsive genes was also higher in osh1-1 (Fig. 2).
Relative mRNA accumulations of sulfate transporter
(Sultr) 2;2 and APR 1 to ubiquitin5 in leaves were
determined using real-time PCR analysis. Sultr2;2 and
APR1 are involved in sulfate transport and assimila-
tion, respectively, and mRNA levels are reported to be
increased by sulfate deficiency (Setya et al., 1996;
Takahashi et al., 1997, 2000). In agreement with these
previous reports, mRNA levels of Sultr2;2 and APR1
were increased by S deficiency in wild-type plants
(Fig. 2). In osh1-1, accumulation of mRNA for Sultr2;2
and APR1 was higher both under +S and −S con-
ditions (Fig. 2), indicating that osh1-1 is a mutant with
constitutive high expression of S-responsive genes. As
similar trends were also observed when β-tubulin was
used as an internal standard (data not shown), it was
unlikely that increase in mRNA levels of Sultr2;2 and
APR1 was due to decrease in those of internal controls
in osh1-1.

Figure 1. GFP expression and appearance of osh1-1. A, A false image representing GFP fluorescence (left) and photos taken under white light (right) of wild-type NOB7 and osh1-1 mutant plants. Plants were grown vertically on agar plates containing 1.5 mm sulfate. After 10 d of germination, plants were transferred to fresh agar plate containing 1.5 (+S) or 0.0015 (−S) mm sulfate and grown for an additional 4 d. Green fluorescence against red chlorophyll autofluorescence signals was visualized using a quantitative fluorescent imaging system. Bars = 1 cm. B, Quantification of relative GFP fluorescent intensity. Plants were grown as in A. GFP fluorescent intensity in leaves of wild type (white bars) and osh1-1 (black bars) was determined. Intensity of chlorophyll autofluorescence was also determined and relative fluo-
rescent intensity was calculated. Values are normalized with the means
of wild-type plants (+S). Means and SD of 10 (+S) and 5 (−S) plants are shown. C, Fresh weight of shoots and root length of plants grown as in A. Means and SD of 8 plants are shown. Asterisks in B and C indicate significant difference between wild-type and osh1-1 plants (P < 0.05, Student’s t test). Relative GFP fluorescence intensity was significantly different between the +S and −S conditions in the osh1-1 plants (P < 0.05, Student’s t test).
osh1-1 Has Higher OAS, Ser, and Thr Concentrations

Concentration of OAS, a positive regulatory metabolite for S-responsive genes (Kim et al., 1999; Koprivova et al., 2000), was determined in leaves of osh1-1 and wild-type plants. Consistent with the report by Kim et al. (1999), the concentration of OAS was increased by −S condition (Table II). In osh1-1, OAS concentration was about 3- and 5-fold higher under +S and −S conditions, respectively (Table II). Concentrations of GSH, which is considered to be a negative regulatory metabolite for S-responsive genes (Lappartient et al., 1999), and γ-glutamyl-Cys (γ-EC), a precursor of GSH, were not significantly different between osh1-1 and wild type. Concentrations of other amino acids were also determined in leaves of osh1-1 and wild type (Table II). Under +S condition, concentrations of Ser and Thr were significantly increased in osh1-1 by approximately 60% compared to wild type. Under the −S condition, in addition to Ser and Thr, concentrations of Asp, Asn+Gln, and Gly were also significantly higher in osh1-1.

Ser Application to Plants Elevates OAS and Thr Levels

As Ser is a precursor of OAS, it was possible that an elevated level of Ser in osh1-1 was a factor in the increase of OAS concentration. To test this hypothesis, we measured OAS concentration in leaves of plants applied with Ser. Wild-type NOB7 plants grown on normal (+S) agar plates for 10 d were transferred to fresh agar plates supplemented with 10, 30, or 100 mM of Ser. Four days following transfer, Ser and OAS concentrations in leaves were determined. Concentration of Ser increased more than 50-fold when Ser was applied at above 10 mM (data not shown). OAS concentration was about 7-, 11-, and 19-fold higher when Ser was applied at 10, 30, and 100 mM, respectively (Fig. 3A), showing the dependence of OAS concentration on the availability of Ser. As the Thr level was increased in osh1-1 under +S condition (Table II), we tested the possibility that an increase in Thr concentration was caused by an increase in Ser concentration. As shown in Figure 3B, Thr level in

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**Table II. Concentrations of OAS, GSH, γ-EC and amino acids in osh1-1 and wild-type plants grown under +S and −S conditions**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>osh1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+S</td>
<td>−S</td>
</tr>
<tr>
<td>OAS</td>
<td>0.9 ± 0.2</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>GSH</td>
<td>109 ± 4</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>γ-EC</td>
<td>8.3 ± 1.6</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Asp</td>
<td>1,376 ± 158</td>
<td>1,218 ± 171</td>
</tr>
<tr>
<td>Thr</td>
<td>428 ± 71</td>
<td>378 ± 50</td>
</tr>
<tr>
<td>Ser</td>
<td>843 ± 199</td>
<td>673 ± 113</td>
</tr>
<tr>
<td>Asn + Glu</td>
<td>1,557 ± 207</td>
<td>1,359 ± 223</td>
</tr>
<tr>
<td>Gln</td>
<td>3,115 ± 704</td>
<td>3,020 ± 142</td>
</tr>
<tr>
<td>Pro</td>
<td>155 ± 22</td>
<td>200 ± 48</td>
</tr>
<tr>
<td>Gly</td>
<td>224 ± 40</td>
<td>152 ± 23</td>
</tr>
<tr>
<td>Ala</td>
<td>449 ± 37</td>
<td>563 ± 30</td>
</tr>
<tr>
<td>Cys</td>
<td>11.7 ± 0.6</td>
<td>9.9 ± 0.8</td>
</tr>
<tr>
<td>Ile</td>
<td>43 ± 14</td>
<td>40 ± 9</td>
</tr>
<tr>
<td>Leu</td>
<td>52 ± 7</td>
<td>52 ± 5</td>
</tr>
<tr>
<td>Tyr</td>
<td>16 ± 7</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Phe</td>
<td>29 ± 12</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>His</td>
<td>6 ± 3</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

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leaves was increased by Ser application. Level of GFP fluorescence in leaves of wild-type NOB7 plants applied with Ser was also determined (Fig. 3C). GFP fluorescence was significantly increased when Ser was applied at above 30 mM. Application of 100 mM mannitol did not affect levels of GFP fluorescence, indicating that elevation of GFP fluorescence by Ser was not due to an increase in osmotic pressure. In NOC2 plants carrying GFP gene driven under an authentic CaMV 35S promoter (Ohkama et al., 2002), GFP fluorescence in shoots of NOB7 and NOC2 plants were determined as in Figure 1B. Values are normalized with the means without Ser. Asterisks indicate significant difference from values without Ser \( (P < 0.05, \text{ Student's t-test}) \).

Positional Identification of OSH1

To identify the gene responsible for the mutant phenotype, the osh1 locus was genetically mapped. The mutant (Columbia ecotype [Col-0] accession) was crossed with Landsberg erecta ecotype accession. The F1 plants were selfed and homozygous osh1 plants were selected from the segregating F2 population based on strong GFP fluorescence. After a rough mapping, osh1 was mapped to the top region of Chromosome V. Several new markers were developed based on the genomic sequences of bacterial artificial chromosome (BAC) clones in this region (Supplemental Tables I and II, available at www.plantphysiol.org). Fine mapping using these new markers delimited OSH1 to an approximately 30-kb region of the BAC clone F7A7 between the markers F7A7.2 and F7A7.3 (Fig. 4A). Genomic sequences of wild type and osh1-1 were compared in this region, but no difference was observed.

As we were confident of our mapping data, we examined the possibility that epigenetic differences such as a difference in methylation may be present in this region. To test this, isoschizomeric restriction enzymes HpaII and MspI were used. When the second C of the recognition sequence, CCGG, is methylated, DNA is not cleaved with HpaII while cleaved with MspI. Four overlapping probes covering the mapped region (Fig. 4B) were used to detect the difference in digestion pattern between wild type and osh1-1 by Southern hybridization. When cleaved with HpaII, a 2.3-kb band corresponding to 221,355 to 223,688-bp region of chromosome V was observed when probe “a” was used with genomic DNA from osh1-1, but not with that from wild type. No other difference was observed in the mapped region. The 2.3-kb band was observed with DNA from both genotypes when cleaved with MspI (data not shown). To confirm the result of Southern hybridization, HpaII- or MspI-digested DNA was amplified with two primer sets covering each end of the 2.3-kb fragment (Fig. 4C). With the primers covering the left end, HpaII-digested DNA from both genotypes was not amplified, indicating that DNA was cleaved with HpaII in both genotypes. With primers covering the right end, HpaII-digested DNA from wild type was amplified but not efficiently amplified when DNA from osh1-1 was used. Essentially no amplification was observed with MspI-digested DNA for both ends in both genotypes. These results suggested that the second C of the recognition sequence at 223,689 of chromosome V was methylated in wild type but demethylated in osh1-1.

To directly determine the methylation status, we applied the bisulfite sequencing method (Jacobsen et al., 2000) to 223,234 to 224,340-bp region of chromosome V. Bisulfite sequencing of wild-type DNA detected five hypermethylated C residues that are clustered in a 72-bp region, of which the one at 223,689 was demethylated in osh1-1 (Fig. 4, E and F). No other hypermethylation was observed in either genotype in the 1.1-kb genome DNA region analyzed. The three recombinants identified between the F7A7.1 and F7A7.2 markers (Fig. 4A) also carried the demethylation of C at 223,689, supporting that the phenotype of osh1-1 was caused by this demethylation.

Expression of the Demethylated At5g01580 Was Elevated in osh1-1

The C residue at 223,689 of chromosome V is located in the predicted first exon of At5g01580 based on the Arabidopsis Information Resource database (http://www.arabidopsis.org/; Fig. 4, D and E). By using the database information, we isolated cDNA for At5g01580. Sequence of the cDNA was consistent with the exon/intron structure of the translated region in the database. To examine effect of demethylation on...
Figure 4. Positional cloning of the OSH1. A, Physical map of a region of chromosome V where OSH1 was mapped. BAC clones, markers used for fine mapping using 3,600 chromatids, and the number of recombinants detected are indicated. OSH1 was mapped between the markers F7A7.2 and F7A7.3. B, Four overlapping probes (a–d) used for Southern hybridization to detect methylation of HpaII site. The thin vertical lines indicate the positions of CCGG sequence. The thick horizontal bar represents the 2.3-kb HpaII fragment (221,355–223,688 bp of chromosome V) that was detected in osh1-1 but not in wild type. Asterisk indicates the HpaII site that was methylated in wild type but was demethylated in osh1-1 (see below). C, Detection of difference in methylation status by PCR. Genomic DNA from wild type and osh1-1 was cleaved with MspI or HpaII and amplified with two primer sets covering each end of the 2.3-kb HpaII fragment in B. Results of three replicates with independent DNA extraction are shown. Intact DNA (uncut) was also amplified. D, Predicted genes within the mapped region and their orientations. E, Detection
mRNA accumulation, real-time PCR analysis was carried out. Relative abundance of At5g01580 mRNAs to ubiquitin5 was about 2-fold higher (P < 0.02 by Student’s t test) in osh1-1 than in wild-type NOB7 both under +S and −S conditions (Fig. 5A). Accumulation of At5g01580 mRNAs was not increased by −S in both wild type and osh1-1 (Fig. 5A). Similar trends were also observed when β-tubulin was used as an internal standard (data not shown). Relative transcript accumulations of 11 other genes in the mapped region, from At5g01550 to At5g01660 (Fig. 4D), were also examined and no significant difference was observed between wild type and osh1-1 (Table III). These results suggest that At5g01580 is the causal gene for the phenotype of osh1-1.

For further confirmation, a transgenic Arabidopsis line carrying At5g01580 open reading frame (ORF) driven under the CaMV 35S RNA promoter (35S::At5g01580) was obtained. In this line, relative mRNA accumulation of At5g01580 increased about 2,000-fold compared to that in the vector control line (Table IV). Relative mRNA levels of both of APR1 and Sultr2;2 were severalfold higher than those in the vector control line under the +S condition (Table IV). Under the −S condition, accumulation of APR1 and Sultr2;2 mRNAs were not significantly different between the 35S::At5g01580 line and the vector control line, but it tend to be higher in the 35S::At5g01580 line. Although it is absolutely necessary to obtain additional independent transgenic lines to confirm that overexpression of At5g01580 causes elevated accumulation of OAS and mRNAs of APR1 and Sultr2;2, the phenotype of the single transgenic line obtained thus far strongly supports that At5g01580 is the causal gene for the phenotype of osh1-1.

As no expressed sequence tag corresponding to At5g01580 was found in GenBank database, expression of At5g01580 was considered to be very low. In fact, the mRNA level of At5g01580 was about 10²- and 10³-fold lower than those of ubiquitin5 and β-tubulin, respectively, judging from the amplification curve in real-time PCR analysis (data not shown).

To test tissue-specific expression, relative mRNA accumulation of At5g01580 was examined in various tissues by real-time PCR analysis (Fig. 5B). In 2-week-old plants, the levels of At5g01580 mRNAs were about 7-fold higher in roots than in rosette leaves. In rosette leaves, there was no major difference in the mRNA accumulation between 2-week-old and 5-week-old plants. In 5-week-old plants, accumulation of At5g01580 mRNAs in stems and flowers were about 17- and 4-fold higher than those in rosette leaves, respectively.

At5g01580 Is Homologous to Lysosomal Thiol Reductase from Animals

The predicted protein sequence of At5g01580 (233 amino acids; GenBank accession no. NP_195778) contained the domain of γ-interferon-inducible lysosomal thiol reductase (GILT; Inter-Pro accession no. IPR004911). BLAST searches against the GenBank protein database revealed that the predicted protein

![Figure 5](https://www.plantphysiol.org/)

Figure 5. A, Accumulation of At5g01580 mRNAs in osh1-1. Plants were grown as in Figure 1A. Total RNA extracted from shoots was reverse transcribed and subjected to real-time PCR analysis to monitor amplification of At5g01580 cDNAs in wild type (white bars) and osh1-1 (black bars). Accumulation of these mRNAs relative to that of ubiquitin5 was determined. Values are normalized with the means of wild-type plants (+S). Means and SD of seven plants are shown. Asterisks indicate significant difference between wild type and osh1-1 plants (P < 0.05, Student’s t test). B, Accumulation of At5g01580 mRNAs in various tissues of nontransgenic Col-0 plants. Total RNA was extracted from rosette leaves (Lf) and roots (Rt) of 2-week-old plants (2w), rosette leaves, stems (St), flowers (Fl), and siliques (Sq) of 5-week-old plants (5w). Accumulation of the mRNAs relative to that of ubiquitin5 was determined as in A, and values were normalized with the means of 2-week-old rosette leaves. Means and SD of three plants are shown.
Table III. Relative mRNA accumulations of genes within the mapped region in shoots of osh1-1

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Relative mRNA Accumulation (osh1-1/wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g01550</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>At5g01560</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>At5g01570</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>At5g01580</td>
<td>2.1 ± 1.4*</td>
</tr>
<tr>
<td>At5g01590</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>At5g01600</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>At5g01610</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>At5g01620</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>At5g01630</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>At5g01640</td>
<td>1.9 ± 1.4</td>
</tr>
<tr>
<td>At5g01650</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>At5g01660</td>
<td>2.1 ± 1.8</td>
</tr>
</tbody>
</table>

sequence of At5g01580 shares relatively high identities with lysosomal thiol reductase from Mus musculus (GenBank accession no. NP_075552; 34%) and Homo sapiens (human; GenBank accession no. P13284; 33%), or with lysosomal thiol reductase-like protein from Rattus norvegicus (GenBank accession no. XP_214298; 35%), Drosophila melanogaster (GenBank accession no. NP_651166; 35%), and Caenorhabditis elegans (GenBank accession no. T21922; 31%). BLAST searches against the GenBank translated nucleotide sequence database revealed five other Arabidopsis genes with relatively high identity to the predicted protein sequence of At5g01580: At1g07080 (53% identity), At4g12890 (42%), At4g12900 (39%), At4g12960 (39%), and At4g12870 (39%). The latter four genes are clustered on chromosome IV. The same BLAST search revealed two Oryza sativa cDNA clones (GenBank accession nos. AK106050 and AK071633) with 47% and 46% identities, respectively. These O. sativa clones were considered to contain full-length ORF. As shown in the phylogenetic tree, At5g01580, At1g07080, and the two O. sativa genes were categorized in the same subgroup (Fig. 6A).

GILT of human was shown to reduce disulfide bonds optimally at acidic conditions (Arunachalam et al. 2000). This protein contains 261 amino acid residues comprised of a 37 aa-long signal peptide and a 22- amino acid-long proform. Both N- and C-termini of the proform are cleaved to generate the mature form of GILT. The proform was shown to localize to the secretory pathway, while the mature form was localized to lysosomes and endosomes. GILT in animals is considered to function in reducing disulfide bonds of antigens in antigen presenting cells, which facilitates subsequent proteolysis (Maric et al., 2001).

Figure 6B shows comparison of the predicted amino acid sequences of At5g01580 and the GILT of human. The predicted sequence of At5g01580 shared relatively high identities and similarities with the human GILT not only for the mature protein region but also for the signal peptide and the C-terminal propeptide regions of human GILT. TargetP (Emanuelsson et al., 2000; http://www.cbs.dtu.dk/services/TargetP/) and PSORT (Nakai and Kanehisa, 1992; http://psort.nibb.ac.jp/form.html) suggest that the At5g01580 protein is a secretory protein, and iPSORT (Bannai et al., 2002; http://hypothesiscreator.net/iPSORT/) suggests that the N-terminal 30 amino acid is the signal peptide of the At5g01580 protein.

Table IV. Relative mRNA accumulations of At5g01580, APR1 and Sultr2;2 in a line of transgenic Arabidopsis carrying 35S::At5g01580

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>Control</th>
<th>35S::At5g01580</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>+S</td>
<td>−S</td>
</tr>
<tr>
<td>Relative mRNA accumulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At5g01580</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>APR1</td>
<td>1.0 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Sultr2;2</td>
<td>1.0 ± 0.3</td>
<td>3.6 ± 1.3</td>
</tr>
</tbody>
</table>

Recombinant At5g01580 Protein Has Thiol Reductase Activity at Neutral pH

As the predicted protein sequence of the At5g01580 shared relatively high identities with GILT from ani-
mals, we examined whether At5g01580 protein has thiol reductase activity. Escherichia coli-expressed At5g01580 protein was reacted with cy3-conjugated F(ab')2 fragment of IgG as a substrate, and reduction of F(ab')2 into H' and L chains via Fab' was analyzed by nonreducing SDS-PAGE followed by detection of cy3 fluorescence. As shown in Figure 7, when thioredoxin (positive control) or At5g01580 protein was used, the amounts of F(ab')2 were decreased and signals corresponding to H' and L chains increased at pH 5.0 to 7.0. On the other hand, when lysozyme (negative control) was used, only weak signals of H' and L chains were observed. As pointed out by Arunachalam et al. (2000), these weak signals are likely to be due to reduction of F(ab')2 caused by dithiothreitol contained in the reaction mixture, whose effect is more evident at neutral pH. In contrast to the GILT of human, which has the highest activity at acidic conditions (Arunachalam et al., 2000), At5g01580 protein did not reduce F(ab')2 at pH 4.0.

At5g01580 Protein Is Localized to Endoplasmic Reticulum

To study subcellular localization in living cells, GFP was fused in-frame to the C terminus of At5g01580 protein and placed under the control of a CaMV 35S promoter. The construct was expressed transiently in epidermal cells of Arabidopsis leaves by microprojectile bombardment. As a control, free GFP was also expressed. The GFP fluorescence was analyzed under a confocal laser scanning microscope. As shown in Figure 8A, free GFP predominantly localized in the nucleus and around the plasma membrane, whereas the GFP-tagged At5g01580 localized in the endoplasmic reticulum (ER)-like reticulate structures in addition to the nucleus and around the plasma membrane (Fig. 8A). Transient expression of these constructs in tobacco Bright Yellow-2 cells showed essentially the same pattern of GFP fluorescence to that in Arabidopsis epidermal cells (Fig. 8B). These results suggested that At5g01580 protein was transported through ER.

DISCUSSION

In this study, an Arabidopsis mutant, osh1-1, was isolated exhibiting elevated expression of a heterologous S-responsive promoter, P35S:Pβx3. We showed that the expression of endogenous S-Responsive genes was also up-regulated in osh1-1. During the course of this study, a mutant having a lesion in the sulfate transporter 1,2 gene (Shibagaki et al., 2002; Yoshimoto et al., 2002) was also isolated (N. Ohkama-Ohtsu, Y. Ide, and T. Fujiwara, unpublished data), which supports the validity of our screening strategy. To our
knowledge, this is the first identification of mutants that over-accumulate OAS and of a key gene responsible for S-deficiency regulated gene expression in higher plants.

In osh1-1, Ser and OAS concentrations were elevated (Table II). As Ser is a precursor of OAS, we considered that increase of Ser content is the primary cause for the overaccumulation of OAS. In fact, OAS concentration in leaves was elevated by application of Ser to the culture medium (Fig. 3A). OAS is a metabolite at the marginal step of S and N assimilations and its concentration responds to sulfate and nitrate concentrations in the culture medium (Kim et al., 1999). Our results suggest that OAS concentration is regulated not only by sulfate and nitrate in the culture medium but also by Ser content in tissue. In support of this notion, concentrations of sulfate and nitrate were not significantly changed in osh1-1 (data not shown). GFP fluorescence in NOB7 was increased by application of Ser at above 30 mM (Fig. 3C), while it was increased by applying OAS at 3 mM (Y. Sogawa, N. Ohkama-Ohtsu, and T. Fujiwara, unpublished data). From these results we may consider that effect of Ser on expression of S-responsive genes was through the changes in OAS concentrations. OAS is synthesized from Ser by Ser acetyltransferase (SAT). In Arabidopsis, three isoforms present in chloroplasts, mitochondria, and cytosol are reported (Noji et al., 1998). Among the isoforms, the cytosolic SAT is strongly feedback-inhibited by Cys, whereas the other isoforms are not (Noji et al., 1998). It has been demonstrated that Ser accumulates in siliques of Arabidopsis exposed to sulfur deficiency. However, its increment is at maximum 2-fold, while OAS concentration changes as much as 5-fold (Kim et al., 1999). These suggest that changes in Ser concentration have a strong impact on OAS concentration, supporting our present findings. These also suggest the importance of SAT in regulating OAS concentrations, although it is not clear which isoform of SAT is responsible for the accumulation of OAS.

After the fine mapping and sequencing, it was found that no base change was present in the mapped region of osh1-1 genome, suggesting that the genetic lesion in the osh1-1 genome is epigenetic. The osh1-1 mutant was isolated from ethyl methanesulfonate (EMS) mutagenized M2 population. It is not clear how EMS treatment caused epigenetic changes in At5g01580 in the mutant, but our results suggest that EMS can cause epigenetic changes. The facts that the phenotype of the osh1-1 mutant is stable for at least four generations and that the osh1-1 mutation was unambiguously mapped to about the 30-kb region suggest that the epigenetic lesion of the osh1-1 mutant is stable. Epigenetic changes are in many cases variable, but examples of stable epigenetic changes are also reported. For example, several epigenetic mutations induced by ddm1 mutations in Arabidopsis are stably inherited in the succeeding generations (Kakutani et al., 1999).

We therefore examined epigenetic changes in the mapped region. Southern-blot analysis using the methylation-sensitive restriction enzyme revealed a hypomethylation(s) in a CCGG sequence in the ORF of At5g01580 in the osh1-1 genome as compared to the wild-type genome. Bisulfite sequencing analysis revealed a small cluster of methylated C residues, all in dinucleotides CG (CpG), within the At5g01580 ORF, and one of them is specifically demethylated in osh1-1 (Fig. 4). Methylation of C residue in CpG is a common
feature in C methylation. Based on these findings, we thought it possible that expression of At5g01580 and/or other genes located very close to At5g01580 is altered in the mutant. Accumulation of transcripts for all genes located in the mapped region was determined and At5g01580 was found to be the only gene whose transcript accumulation was significantly increased in osh1-1 (Fig. 5; Table III). Based on the mapping data, we confirmed that the osh1-1 mutation resides among the 12 genes in the mapped region and epigenetic mutations lead to changes in phenotype through changes in gene expression. Thus, it is most likely that osh1 phenotype was due to increase in expression of At5g01580, which is likely to be caused by demethylation of the C residue at 223689 bp of chromosome V.

For further confirmation, we obtained a line of transgenic Arabidopsis plants overexpressing At5g01580 and found that accumulation of APR1 and Sultr2;2 transcripts increased in rosette leaves under +S condition (Table IV). We acknowledge that results from a single line of transgenic plants cannot be used to draw conclusions; however, the phenotype of this transgenic line supports At5g01580 being the causal gene of osh1-1 mutant. It is clearly necessary to obtain additional independent transgenic lines and to determine OAS levels in these transgenic lines before drawing a final conclusion.

DNA methylation has long been a factor affecting transcription. A number of examples have been reported in which transcriptional gene silencing is associated with DNA methylation in the promoter region of a gene (Morel et al., 2000). In the case of At5g01580, however, methylation was found in the ORF and there was essentially no methylation in the promoter region. It has been reported that posttranscriptional gene silencing is associated with DNA methylation in its coding region (English et al., 1996; Morel et al., 2000). Although the cause-effect relationship is unclear in these cases, a report by Hisano et al. (2003) is worth mentioning. Through the analysis of the mouse Tact1/Act17b gene, they found that a CpG island present in the ORF was demethylated in cells expressing the gene, while it is methylated in cells that do not express the Tact1/Act17b gene. With the use of transfection assays, they demonstrated that methylation of the CpG island in the ORF represses its mRNA accumulation in somatic cells, and demethylation is necessary for the gene expression. Although its mechanism has to be elucidated, it is in fact possible that the methylation status of a peculiar C residue in the At5g01580 ORF is a key determinant for its expression.

The predicted amino acid sequence of At5g01580 has a high homology with animal GILT (Fig. 6), a lysosomal thiol reductase. We demonstrated that the E. coli-expressed recombinant At5g01580 protein had thiol reductase activity under neutral conditions (Fig. 7). Reverse formation of disulfide bond is known to be one of mechanisms regulating activity of enzymes. Thioredoxin was shown to regulate formation of disulfide bond of enzymes involved in carbon metabolism (Ruelland and Miginiac-Maslow, 1999). Similarly, At5g01580 may be involved in the regulation of Ser concentration. Identification of the target molecule(s) for At5g01580 will give further insights into this question. A possible candidate target molecule is APR. It is reported that APR is activated by oxidation of two hydrogen sulfides of Cys in the enzyme into a disulfide bond by oxidized glutathione and reversibly inactivated by reduced thioredoxin (Bick et al., 2001). At5g01580 may replace thioredoxin to reduce APR activity, leading to decrease of sulfide levels and subsequently caused relative increase of OAS levels.

Transient expression analysis of GFP-tagged At5g01580 revealed that the protein is located in the ER-like structure (Fig. 8), suggesting that At5g01580 is transported through ER. This is in agreement with the computer predictions of subcellular localization. It is possible that At5g01580 is transported to an organelle whose pH is neutral. It is worth noting that among the several possible mechanisms for the increase in Ser concentrations in osh1-1, change in photorespiration (Douce et al., 2001) is likely to be a major contributor. Photorespiration is a process in which three organelles, namely chloroplasts, mitochondria, and peroxisomes, are involved and is a major process of Ser production in plants (Somerville and Ogren, 1981). In osh1-1 mutant, it is possible that photorespiration is activated and resulted in elevated accumulation of Ser concentrations. This may explain why amino acids other than Ser were also elevated in osh1-1 (Table II). Accumulation of At5g01580 mRNA in wild type was not strongly regulated by S deficiency and expressed both in roots and aerial portions of plants. It is likely that At5g01580 is an important protein required in the plant body for the homeostasis of OAS concentration irrespective of S nutrition status. The findings that OAS levels and accumulation of APR1 and Sultr2;2 mRNAs in the osh1-1 mutant are all elevated both in +S and −S conditions (Table II; Fig. 2) support this view.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

In the experiments described here, wild type refers to the unmutagenized transgenic line of Arabidopsis L. Heynh. ecotype Col-0. NOB7 (Ohkama et al., 2002). NOB7 carries a chimeric P35S-βαx3 promoter fused to the GFP ORF. P35S-βαx3 promoter is comprised of a backbone of CaMV 35S RNA promoter with an insertion of three tandem repeats of S-responsive elements (βα) corresponding to the −307 to −73-bp region from the promoter of the β-subunit of β-conglycinin (Awa-zuhara et al., 2002a). NOC2 line of transgenic Arabidopsis carries the GFP ORF under control of the CaMV 35S RNA promoter (Ohkama et al., 2002).

Surface sterilized seeds were sown on hydroponic culture medium containing 1.5 mM sulfate (+S condition; Hirai et al., 1995) containing 1% (w/v) Suc and solidified with either 1% agarose (LC3; Takara, Kyoto) or 0.8% agar (Wako Pure Chemicals, Osaka). For −S condition (0.0015 mM sulfate), MgSO4 was replaced with equal molars of MgCl2 (Hirai et al., 1995). For agar plates, agar was washed with 6 L of deionized water before use to eliminate sulfate. Immediately after sowing, seeds were vernalized for 3 d at 4°C in the dark. Plants were grown at 22°C under a 16-h light/8-h dark cycle. Ten days after germination, plants were transferred to fresh agar plates with +S or −S.
condition and grown for an additional 4 d. For growing the plants further, plants were transplanted to soil and watered with hydroponic culture medium (Fujisawa et al., 1992).

Mutagenesis and Mutant Isolation

EMS-mutagenized M2 seeds of NOB7 were surface sterilized and sown either on + S or − S plates. After vernalization for 3 d at 4°C in dark, M2 plants were grown for 14 d before determination of GFP fluorescence. Those M2 plants exhibiting altered GFP fluorescence compared to NOB7 were chosen and transferred to soil. M3 seeds were retested for inheritance of the observed phenotype.

Positional Cloning

For mapping, mutants (Col-0 ecotype) were crossed with Ler plants. Fourteen-day-old F2 seedlings that displayed the mutant phenotype were chosen based on GFP fluorescence. DNA was extracted from shoots of these plants and analyzed for cosegregation with respect to single sequence length polymorphism markers (Bell and Ecker, 1994; Lukowitz et al., 2000). To identify the mutated gene, new single sequence length polymorphism markers (Supplemental Table I) and cleaved amplified polymorphic sequences markers (Supplemental Table II) were generated based on Cereon Arabidopsis Polymorphism Collection (http://www.arabidopsis.org/Cereon/index.html).

Sequence Analysis

DNA sequence of the mapped region was determined as described (Baird et al., 2000). The sequence was amplified PCR by Hitachi (Saitama, Japan), Genesit-Win software (version 6; Software Development, Tokyo) was used for general sequence analysis. The BLAST search program (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) was used for searching known protein domains or signatures in the InterPro database (Mulder et al., 2003; http://www.ebi.ac.uk/interpro/index.html) was used for searching known protein domains or signatures in the InterPro database (Apweiler et al., 2001). Sequences were aligned using CLUSTALW (Higgins et al., 1996) at DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). Phylogenetic tree was constructed using DendroMaker for Macintosh version 4.1 software (http://www.cib.nig.ac.jp/dda/timanish/dendromaker/home.html).

Quantitation of GFP Fluorescence and Concentrations of Anions, Thiols, OAS, and Amino Acids

GFP fluorescence was determined as described in Niwa et al. (1999). Concentrations of anions and thiols were determined as described (Kuzuhara et al., 2000; Ohkama et al., 2002). Extraction and analysis of OAS was performed as described by Kuzuhara et al. (1996) between the Sall and Nol sites. The construct of At5g01580 ORF under the CaMV 35S RNA promoter from pNO5 was then subcloned into the binary T-D plasmid vector pTF336 (Awazuhara et al., 2002a) to generate pNO6. Arabidopsis Col-0 was transformed with pNO6 or pTF336 by Agrobacterium tumefaciens-mediated floral dip method (Clough and Bent, 1998). T2 seeds were sown on + S agar plate containing kanamycin (50 μg ml−1) and grown for 7 d. Kanamycin resistant plants were then transferred to + S agar plates without kanamycin. Total RNA was extracted from shoots 4 d after the transfer, reverse-transcribed, and subjected to real-time PCR analysis.

Microprojectile Bombardment and Confocal Laser Scanning Microscopy

To construct pNO7 that carries GFP-tagged At5g01580 ORF driven under the CaMV 35S RNA promoter (35S::At5g01580), reverse-transcribed RNA was amplified using two sets of primers: At5g01580 1F (5′-ACGCGTCGACATGGCATCATATCAGAGGCTTTG-3′) and At5g01580 1R (5′-CTTCTCTGTGCTTCTGAAC-3′), and At5g01580 2F (5′-TAAATCTCCTCATCCATCGG-3′), and At5g01580 2R (5′-TACGCCATGGAACACACCCACCATGCACTGATCGG-3′). The two amplified DNA fragments were subjected to a second PCR with primers At5g01580 1F and At5g01580 2R. The amplified DNA was cloned into 35S::sGFP(S65T) plasmid (Chiu et al., 1996) between the Sall and Nol sites. The construct of At5g01580 ORF under the CaMV 35S RNA promoter from pNO6 was then subcloned into the HindIII-EcoRI sites of the binary Ti-plasmid vector pTF336 (Awazuhara et al., 2002a) to generate pNO6. Arabidopsis Col-0 was transformed with pNO6 or pTF336 by Agrobacterium tumefaciens-mediated floral dip method (Clough and Bent, 1998). T2 seeds were sown on + S agar plate containing kanamycin (50 μg ml−1) and grown for 7 d. Kanamycin resistant plants were then transferred to + S agar plates without kanamycin. Total RNA was extracted from shoots 4 d after the transfer, reverse-transcribed, and subjected to real-time PCR analysis.

Bisulfite Sequencing

XhoI-digested genomic DNA isolated from wild-type and oshl-1 plants was treated with sodium bisulfite and amplified by PCR as described (Jacobsen et al., 2000). Primers 5′-TGTGATGGCTATTTGATATATATAAA-3′ and 5′-CCCTCTCTTCGTGAATCCCA-3′ and 5′-TGGGATCC(T/C)AAATGATGATATAAG-3′ and 5′-ACTTCCTCACTCTTTCGAC-3′ were used for amplifying the top strand of chromosome V for 222,319 to 223,873 and 222,664 to 224,608 bp, respectively. Amplified DNA was directly sequenced. In this method, unmethylated C residue is converted to uridine, while methylated C remains unchanged. Peak heights of C and T signals for each C position were determined and normalized to that of average value in each sequence.

Real-Time PCR Analysis

Real-time PCR analysis was carried out as described (Goto and Naito, 2002) using ubiquitin5 (GenBank accession no. AY084978), GUS-b-tubulin (GenBank accession no. AY059075), and actin8 (GenBank accession no. U42007) as internal controls. The forward and reverse primers used are listed in Supplemental Table III. For calculation of relative values of transcript accumulations, serial dilutions of a batch of first-strand cDNA stock were used throughout the experiment.

Complementation Analysis Using Transgenic Arabidopsis Overexpressing At5g01580

To construct pNOS that carries At5g01580 ORF driven under the CaMV 35S RNA promoter (35S::At5g01580), reverse-transcribed RNA was amplified using two sets of primers: At5g01580 1F (5′-ACGCGTCGACATGGCATCATATCAGAGGCTTTG-3′) and At5g01580 1R (5′-CTTCTCTGTGCTTCTGAAC-3′), and At5g01580 2F (5′-TAAATCTCCTCATCCATCGG-3′), and At5g01580 2R (5′-TACGCCATGGAACACACCCACCATGCACTGATCGG-3′). The two amplified DNA fragments were subjected to a second PCR with primers At5g01580 1F and At5g01580 2R. The amplified DNA was cloned into 35S::sGFP(S65T) plasmid (Chiu et al., 1996) between the Sall and Nol sites. The construct of At5g01580 ORF under the CaMV 35S RNA promoter from pNOS was then subcloned into the HindIII-EcoRI sites of the binary Ti-plasmid vector pTFS36 (Awazuhara et al., 2002a) to generate pNOS. Arabidopsis Col-0 was transformed with pNOS or pTF336 by Agrobacterium tumefaciens-mediated floral dip method (Clough and Bent, 1998). T2 seeds were sown on + S agar plate containing kanamycin (50 μg ml−1) and grown for 7 d. Kanamycin resistant plants were then transferred to + S agar plates without kanamycin. Total RNA was extracted from shoots 4 d after the transfer, reverse-transcribed, and subjected to real-time PCR analysis.
In Vitro Assay for Thiol Reductase Activity

To construct the plasmid pNO9 for production of recombinant At5g01580 protein, the At5g01580 ORF was amplified as in pNO7 except that At5g01580 3R primer (5‘-CCGCTCCAGGATGGAAATCAGACAAAATCCGG-3’) was used in place of At5g01580 2R. The At5g01580 ORF in the amplified fragment is devoid of the termination codon and is flanked by SalI and XhoI sites. The DNA was cloned into pIVEX2.3-MCS in R50 500 E. coli circular template kit (Roche Diagnostics, Mannheim, Germany) between SalI and XhoI sites to generate the At5g01580 ORF tagged with six His codons at the C terminus. His-tagged At5g01580 protein was expressed in the RTS 500 instruments (Roche Diagnostics) and purified using Ni-NTA agarose (Qiagen, Valencia, CA) as recommended by the manufacturers.

In vitro assay for thiol reductase activity was performed based on the method of Arunachalam et al. (2000) with modifications. Cy3-conjugated affinity-purified sheep anti-mouse F(ab)2 fragment (Sigma-Aldrich, St. Louis) was used as a substrate. F(ab)2 was denatured by boiling in 0.2% SDS and diluted in 0.1% Triton X-100. Recombinant At5g01580 protein or E. coli thioredoxin (Wako Pure Chemicals, Osaka) or egg white lysozyme (Wako Pure Chemicals) was preactivated with dithiothreitol (final concentration, 25 μM) for 10 min at 37°C, and added to the reaction mixture (20 μL) at 300 nM. Each reaction contained 4 ng of cy3-conjugated F(ab)2. After 15 h incubation at 37°C, the reaction was terminated by the addition of iodoacetamide (Sigma-Aldrich) to 5 mM and the samples were analyzed by nonreducing SDS-PAGE. Fluorescence from cy3 was scanned using Typhoon8600 (Amersham Pharmacia Biotech) with an excitation at 532 nm and an emission at 565 to 595 nm.

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center (Columbus, OH) for a BAC clone F7A7. We are grateful to Kumi Fujiwara for general assistance, Miwa Hashimoto, Mao Sugawara, Reiko Mishina, Yuko Kawara, and Kayoko Aizawa for excellent technical assistance, and to Dr. Annita G. Peterson for careful reading of the manuscript. We used the Radiobiology Laboratory of the Graduate School of Agriculture, Hokkaido University.

Received June 1, 2004; returned for revision July 26, 2004; accepted July 26, 2004.

LITERATURE CITED


