An *O*-Acetylserine(thiol)lyase Homolog with L-Cysteine Desulfhydrase Activity Regulates Cysteine Homeostasis in Arabidopsis[1][C][W]

Consolación Álvarez, Leticia Calo, Luis C. Romero, Irene García, and Cecilia Gotor*

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas y Universidad de Sevilla, 41092 Seville, Spain

Cysteine (Cys) occupies a central position in plant metabolism due to its biochemical functions. Arabidopsis (*Arabidopsis thaliana*) cells contain different *O*-acetylserine(thiol)lyase (OASTL) enzymes that catalyze the biosynthesis of Cys. Because they are localized in the cytosol, plastids, and mitochondria, this results in multiple subcellular Cys pools. Much progress has been made on the most abundant OASTL enzymes; however, information on the less abundant OASTL-like proteins has been scarce. To unequivocally establish the enzymatic reaction catalyzed by the minor cytosolic OASTL isoform CS-LIKE (for Cys synthase-like; At5g28030), we expressed this enzyme in bacteria and characterized the purified recombinant protein. Our results demonstrate that CS-LIKE catalyzes the desulfuration of L-Cys to sulfide plus ammonia and pyruvate. Thus, CS-LIKE is a novel L-Cys desulfhydrase (EC 4.4.1.1), and we propose to designate it DES1. The impact and functionality of DES1 in Cys metabolism was revealed by the phenotype of the T-DNA insertion mutants *des1-1* and *des1-2*. Mutation of the *DES1* gene leads to premature leaf senescence, as demonstrated by the increased expression of senescence-associated genes and transcription factors. Also, the absence of DES1 significantly reduces the total Cys desulfuration activity in leaves, and there is a concomitant increase in the total Cys content. As a consequence, the expression levels of sulfur-responsive genes are deregulated, and the mutant plants show enhanced antioxidant defenses and tolerance to conditions that promote oxidative stress. Our results suggest that DES1 from Arabidopsis is an L-Cys desulfhydrase involved in maintaining Cys homeostasis, mainly at late developmental stages or under environmental perturbations.

Sulfur is a macronutrient that is essential for plant growth and development. The most abundant form of sulfur in nature and the source of sulfur for plants is sulfate; this form is reduced and assimilated into Cys. In addition to its role as an amino acid in proteins, Cys functions as a precursor for a huge number of essential biomolecules, such as vitamins and cofactors (Droux, 2004; Wirtz and Droux, 2005), antioxidants like glutathione, which is regarded as the major determinant of cellular redox homeostasis (Meyer and Hell, 2005; Mullineaux and Rausch, 2005), and many defense compounds (Rausch and Wachter, 2005). All of these biomolecules contain sulfur moieties that act as functional groups and are derived from Cys. The biosynthesis of Cys is accomplished through two sequential reactions catalyzed by the enzymes Ser acetyltransferase (SAT; EC 2.3.1.30), which synthesizes the intermediary product *O*-acetylserine (OAS), and *O*-acetylserine(thiol)lyase (OASTL; EC 2.5.1.47), which combines sulfide with OAS to produce Cys. Together, these two enzymes form the heterooligomeric Cys synthase complex, which was first described in bacteria and has since been extensively studied in plants (Droux et al., 1998; Wirtz and Hell, 2006). Plant cells contain different SAT and OASTL enzymes localized in the cytosol, plastids, and mitochondria, resulting in a complex variety of isoforms and different subcellular Cys pools. Arabidopsis (*Arabidopsis thaliana*) is the best investigated plant system; its genome encodes five different *SAT* (Howarth et al., 2003) and eight *OASTL* (Wirtz et al., 2004) genes.

The OASTL enzymes belong to the superfamily of β-substituting Ala synthases (Hatzfeld et al., 2000; Supplemental Table S1). The proteins encoded by *OAS-A1* (At4g14880), *OAS-B* (At2g43750), and *OAS-C* (At3g59760) are considered authentic OASTLs that are located in the cytosol, plastids, and mitochondria of Arabidopsis cells, respectively (Wirtz et al., 2004). A property that defines an authentic OASTL is the ability to interact with SAT, and this can be demonstrated using different approaches (Bogdanova and Hell,

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* Corresponding author; e-mail gotor@ibvf.csic.es.

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1997; Droux et al., 1998; Wirtz et al., 2001; Bonner et al., 2005; Heeg et al., 2008). The OASTL family also includes another isoform located in the mitochondria encoded by *ATCYS-C1* (At3g61440), which actually functions as a β-cyanoalanine synthase (CAS; EC 4.4.1.9; Hatzfeld et al., 2000; Yamaguchi et al., 2000). According to the Genevestigator microarray database (www.genevestigator.com) and numerous studies from a large number of laboratories, the three OASTL isoforms OAS-A1, OAS-B, and OAS-C and the CAS isoform ATCYSC1 are the most highly expressed in Arabidopsis cells. The remaining OASTL-like proteins located in the cytosol (encoded by *ATCYS-D1* [At3g04940], *ATCYS-D2* [At5g28020], and *CS-LIKE* [At5g28030]) and in the plastid (encoded by *CS26* [At3g03630]) are expressed at much lower levels and have not been characterized enzymatically. Their functions in plant sulfur metabolism are currently unknown. In particular, the CS-LIKE protein was identified by sequence homology upon the completion of the sequencing of the Arabidopsis genome. Because of its cytosolic localization, it is thought to have an auxiliary function with respect to the major cytosolic isoform OAS-A1.

While Cys and glutathione are usually considered to be components of cellular antioxidant systems, emerging evidence points to the existence of reactive sulfur species. Thiols are easily oxidized to species with sulfur in higher oxidation states. These species subsequently inhibit enzymes; therefore, they can be considered to be a new class of oxidative stress agents (Giles and Jacob, 2002). Furthermore, thiol groups can generate reactive oxygen species (ROS) through the Fenton reaction catalyzed by iron. Evidence has shown that free Cys, but not glutathione, exacerbates the prooxidant properties of ferrous iron (Vanlerberghe et al., 2002; Jacob et al., 2003; Park and Imlay, 2003). Therefore, under nonstress conditions, Cys levels should remain low; these levels have been calculated to be at least 10 times lower than those of glutathione, which ranges in the millimolar order (Meyer et al., 2001; Krueger et al., 2009).

Several candidate Cys-degrading enzymes have been reported to exist in different plant species (Papenbrock et al., 2007). One class of these enzymes is the L-Cys desulfhydrases (DESs; EC 4.4.1.1), which catalyze the formation of sulfide, ammonia, and pyruvate in a stoichiometric ratio of 1:1:1 and require pyridoxal phosphate (PLP) as a cofactor. Such activity has been found in plants; however, the corresponding genes and pure proteins have remained unisolated (Schmidt, 2005). In addition to DESs, D-Cys desulfhydrases (EC 4.4.1.15) that specifically use D-Cys as their substrate and are completely distinct from their L-counterparts have been shown to exist in several plant species (Rennenberg et al., 1987). In Arabidopsis, two genes have been identified, At1g48420 and At3g26115, that encode proteins possessing this activity (Papenbrock et al., 2007). Another Cys desulfuration reaction catalyzed by the L-Cys desulfurases (EC 2.8.1.7) occurs in iron-sulfur cluster biosynthesis and involves the formation of L-Ala and elemental sulfur or H_2S from Cys, also with PLP as a cofactor. These proteins are now known as NifS-like proteins (Schmidt, 2005), and they likely provide sulfur for the formation of biotin and Fe-S cluster assembly for mitochondrial and cytosolic proteins and for thiamine synthesis in the chloroplast (Van Hoewyk et al., 2008).

In this work, our aim was to characterize in detail the protein named CS-LIKE. We expressed this protein in bacteria and found that CS-LIKE is a DES enzyme (EC 4.4.1.1). We confirmed this assertion by characterizing T-DNA-tagged mutants, which were found to have increased levels of Cys and glutathione that allow the mutants to tolerate oxidative stress conditions.

RESULTS

Expression and Functional Characterization of the CS-LIKE Recombinant Protein

With the aim of identifying the enzymatic reaction that is catalyzed by the minor cytosolic OASTL isoform in Arabidopsis, CS-LIKE, we expressed the corresponding full-length cDNA (*At5g28030*) in *Escherichia coli* as a 6× His N-terminally tagged recombinant protein. The recombinant protein was purified to apparent homogeneity by affinity chromatography using nickel-nitrilotriacetic acid agarose (Ni-NTA) resin under nondenaturing conditions to preserve the enzymatic activity. We selected the elution fractions containing the unique protein band of estimated size as visualized by SDS-PAGE (Supplemental Fig. S1) and discarded the elution fractions containing any other protein bands to be sure that the enzymatic reaction we were measuring was due to the recombinant CS-LIKE. We were able to recover 0.16 mg of purified protein per 100 mL of *E. coli* culture with a yield of 55% (Table I).

Different enzymatic assays were carried out with the bacterial crude extracts and purified recombinant protein. While we were unable to detect β-cyanoalanine synthase activity, we measured both OASTL and DES activities, both in bacterial extracts and with the purified recombinant CS-LIKE. After the purification process, the specific DES activity of the protein increased 4.5-fold and the specific OASTL activity diminished 7.3-fold (Table I). These results indicate that CS-LIKE protein predominantly catalyzes the degradation of Cys rather than its synthesis and that the OASTL reaction is a side reaction of the DES reaction. We also measured the D-Cys desulfhydrase activity of the recombinant protein and determined a specific activity of 4.7 units mg^{-1}, which is about 10-fold lower than the measured DES activity.

The DES activity was quantified as the release of sulfide from L-Cys as described previously (Riemenschneider et al., 2005a); however, it has been

Table I. *Purification of CS-LIKE expressed in E. coli*

The gene encoding CS-LIKE was cloned in the pDEST17 vector using the Gateway technology. The protein was expressed in *E. coli* and purified using the Ni-NTA purification system as described in "Materials and Methods." DES and OASTL activities were measured as described.

Purification Step	Protein	Specific Activity		Total Activity		Purification Factor		Yield	
		DES	OASTL	DES	OASTL	DES	OASTL	DES	OASTL
	mg	*units mg*$^{-1}$		*units*				*%*	
Crude extract	1.29	10.0	8.8	13.0	11.3	–	–	–	–
Ni-NTA chromatography	0.16	44.5	1.2	7.1	0.2	4.5	0.1	54.6	1.7

reported that the degradation of L-Cys can release, besides sulfide, L-Ala or pyruvate plus ammonium (Rennenberg et al., 1987; Zheng et al., 1993). In characterizing the products of the enzymatic reaction catalyzed by CS-LIKE, we failed to detect L-Ala by HPLC, but we easily detected pyruvate and ammonium. The formation of pyruvate and ammonium in the reaction mixture containing L-Cys and recombinant CS-LIKE protein was detected as consumption of NADH by reactions catalyzed by lactate dehydrogenase and Glu dehydrogenase, respectively, as described in "Materials and Methods." In 1 mL of reaction mixture containing 8 μg of the recombinant protein in the presence of a saturating concentration of Cys, we detected the formation of 96.44 nmol of ammonium and 104.17 nmol of pyruvate. Furthermore, in the same reaction mixture, we colorimetrically quantified the release of 105.18 nmol of sulfide. Thus, we conclude that CS-LIKE catalyzes the desulfuration of L-Cys to equimolar amounts of pyruvate plus NH_3 and SH_2 and that CS-LIKE is a DES.

The conclusion that CS-LIKE is a DES is supported by the alignment of CS-LIKE protein with the major OASTL isoforms (Supplemental Fig. S2). Residues 74 to 78 of OAS-A1, with the sequence TSGNT, form a loop that is involved in sulfur incorporation into Cys (Bonner et al., 2005). This loop is highly conserved across the major OASTL isoforms, but in CS-LIKE, Gly replaces Ser-75. Furthermore, CS-LIKE has nonconservative amino acid changes in the β8A-β9A loop, which is highly conserved in true OASTL enzymes and has been shown to be important for the interaction with SAT (Bonner et al., 2005). In contrast, all of the residues that are responsible for PLP anchoring are conserved in CS-LIKE, suggesting that the desulfuration of L-Cys is dependent on PLP. We have observed that 100 μM aminooxyacetate, a known inhibitor of PLP-dependent enzymes, causes an 80% reduction in DES enzyme activity of recombinant CS-LIKE. This result demonstrates that PLP is a required cofactor for this activity, as has been reported previously for other plant systems (Rennenberg et al., 1987).

We characterized the DES and OASTL reactions catalyzed by purified recombinant CS-LIKE by performing substrate saturation experiments (Supplemental Fig. S3). The K_m value for L-Cys in the DES reaction is 13-fold lower than that for OAS in the OASTL reaction (Table II), indicating a much higher affinity of CS-LIKE for L-Cys as a substrate. Although V_{max} is higher for the Cys synthesis reaction, the catalytic efficiencies (k_{cat}/K_m) of CS-LIKE for both substrates, L-Cys and OAS, are in the same order of magnitude. These results further reinforce the idea that CS-LIKE protein functions as a DES with a side OASTL reaction.

Based on the experimental data described above, we propose to rename Arabidopsis CS-LIKE protein DES1, and we will use this nomenclature for the rest of this article.

Identification and Characterization of Arabidopsis *des1* Mutants

With the aim of determining the function of the DES1 protein in Arabidopsis, T-DNA insertion mutants from the existing collections were screened. The SALK_103855 and RATM13-2715-1_G mutants, designated *des1-1* and *des1-2*, were selected for further

Table II. *Catalytic properties of the CS-LIKE recombinant protein for the DES and OASTL enzymatic reactions*

The substrate saturation experiments were performed with CS-LIKE recombinant protein and Cys as a substrate for the DES reaction; OAS and H_2S were used as cosubstrates for the OASTL reaction. The kinetic constants were calculated from the Hanes plots of substrate concentration versus substrate concentration/initial velocity.

DES Reaction			OASTL Reaction			
K_{mcys}	V_{max}	k_{cat}/K_{mcys}	K_{mOAS}	K_{mH2S}	V_{max}	k_{cat}/K_{mOAS}
mM	*μmol min^{-1} mg^{-1}*	*mM^{-1}min^{-1}*	*mM*	*mM*	*μmol min^{-1} mg^{-1}*	*mM^{-1}min^{-1}*
0.4 ± 0.1	0.04 ± 0.003	3.5	5.2 ± 0.6	0.25 ± 0.01	1.8 ± 0.04	5.9

analysis (Supplemental Fig. S4). Reverse transcription (RT)-PCR analysis was conducted on the homozygous mutant plants using specific primers, and no *DES1* transcript was detected. This result suggests that both *des1-1* and *des1-2* contain knockout mutations. A Southern-blot analysis was also performed to determine the number of T-DNA insertions. A single unique T-DNA insertion site was confirmed to exist in the *des1-1* genome with the appearance of a single hybridization band (Supplemental Fig. S4).

We screened the *des1-1* and *des1-2* mutant plants for potential growth defects and found no significant phenotypic differences from wild-type plants under normal growth conditions, either in soil or on solid Murashige and Skoog (MS) medium in petri dishes. It was apparent, however, that these mutants flowered before wild-type plants. It is well established that late-flowering plants form more leaves (Koornneef et al., 1991). Therefore, we determined the number of rosette leaves of the mutant and wild-type plants at flowering time after being grown under long-day conditions. The mutants produced 14.0 ± 1.5 rosette leaves, while the wild-type plants produced 17.5 ± 1.8 rosette leaves, under our experimental conditions. This result suggests that the *des1* mutants have an early-flowering phenotype. Based on this phenotype, we propose that the mutation in the *DES1* gene leads to premature leaf senescence. Many aspects of the regulatory network controlling leaf senescence in Arabidopsis have been identified, including the increased expression of senescence-associated genes and transcription factors (Lim et al., 2007). To confirm that *des1* mutants initiate leaf senescence earlier, we performed real-time quantitative (q) RT-PCR for the senescence-associated genes *SEN1* and *SAG21* and for *NAP*, which is a member of the NAC transcription factor gene family. We observed an induction of the expression levels of these senescence gene markers in leaves of *des1-1* and *des1-2* plants when compared with their respective wild-type plants at the same developmental stage (Fig. 1), thus confirming the onset of leaf senescence in these mutant plants.

Biochemical characterization of both *des1-1* and *des1-2* plants revealed that the total DES activity was reduced by 20% to 25% in both mutants relative to their respective wild types (Table III). Interestingly, the reduction in total DES activity was the same regardless of the growth stage of the plant, as much in mature plants as in seedlings. This result further confirms that the DES1 protein is a DES enzyme. This finding also suggests that cytosolic DES1 contributes considerably to the total L-Cys desulfuration of the plant. Accordingly, the total amounts of Cys in the *des1-1* and *des1-2* mutants were about 20% and 25% higher, respectively, than those found in their respective wild-type ecotypes, both in mature plants and seedlings. In addition, the glutathione content was also significantly higher (Table III).

We also performed qRT-PCR on the OASTL gene family in the *des1-1* mutant and wild-type plants. We

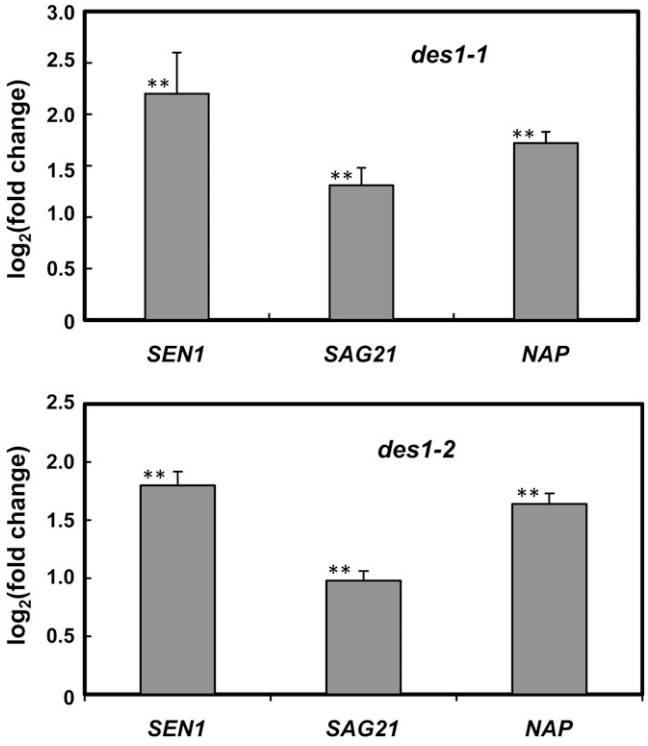


Figure 1. Relative expression levels of senescence-associated genes in *des1* mutant plants. Real-time RT-PCR analysis of the expression of *SEN1* (*At4g35770*), *SAG21* (*At4g02380*), and *NAP* (*At1g69490*) genes was performed in leaves from Col-0 and No-0 wild-type and *des1-1* and *des1-2* mutant plants grown for 5 weeks under control conditions. The transcript levels were normalized to the internal control, the constitutive *UBQ10* gene. Data shown are means + SD of three independent analyses made from RNA obtained from different plants grown in different pots at the same time, and they represent the transcript level of each gene in the mutant plants relative to the transcript level in the corresponding wild-type ecotype. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$.

observed opposing regulatory patterns in the transcript levels of the different gene members: a slight repression of the most abundant *OAS-A1* and *OAS-B* genes and a significant induction of the three β-cyanoalanine synthase-like-encoding genes (Fig. 2). The induction of *CYS-C1*, *CYS-D1*, and *CYS-D2* could be an indication that these enzymes possess similar functions to DES1. To check this hypothesis, we measured the total DES activity in their respective previously described deficient mutants (Watanabe et al., 2008a). None of the mutants showed a reduction in DES activity relative to wild-type plants, as was shown for the two *des1* mutants.

Many studies on the regulation of the sulfate assimilatory pathway have shown that there is negative regulation by reduced sulfur-containing compounds and positive regulation by OAS (Hawkesford, 2000; Hawkesford and De Kok, 2006; Kopriva, 2006). Some components of the pathway have been shown to be specifically sensitive to this regulation at the transcript level (Hirai et al., 2003; Nikiforova et al., 2003). Thus, a

Table III. *Enzyme activity and thiol content*

DES enzyme activity and total levels of Cys and glutathione were measured in the leaves of Col-0 and No-0 wild-type and *des1* mutant plants grown for 5 weeks in soil under control conditions and in whole 2-week-old seedlings grown on MS solid medium. Values are means ± SD of four independent experiments made from different plants and seedlings grown in different pots and plates, respectively. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$

Plant Line	DES Activity	Total Cys	Total Glutathione
	nmol min^{-1} mg^{-1}	*nmol g^{-1} fresh wt*	
Leaves from 5-week-old plants grown in soil			
Col-0	12.4 ± 0.8	29.8 ± 2.3	302.8 ± 21.8
des1-1	10.1 ± 0.7**	36.1 ± 4.6**	345.2 ± 12.3**
No-0	15.5 ± 0.9	29.0 ± 3.4	322.2 ± 11.3
des1-2	13.1 ± 0.8**	36.2 ± 7.3**	390.0 ± 16.2**
Whole 2-week-old seedlings grown on MS medium			
Col-0	6.9 ± 0.05	47.7 ± 4.1	730.7 ± 51.2
des1-1	5.9 ± 0.1**	58.0 ± 3.8**	867.2 ± 10.2**
No-0	7.15 ± 0.28	50.3 ± 2.4	606.6 ± 14.6
des1-2	6.2 ± 0.4**	62.9 ± 2.8**	693.3 ± 13.7**

lower intracellular content of sulfate, Cys, and glutathione is concomitant with increasing transporter activity predominantly at the level of the mRNA (Buchner et al., 2004). To demonstrate that the 20% to 25% increase in the Cys level found in the *des1* mutants is significant, and therefore to demonstrate the importance of DES1 in plant metabolism, we studied the regulation of sulfate transporter genes. Both ecotype Columbia (Col-0) wild-type and *des1-1* plants were grown simultaneously in soil irrigated with sufficient-sulfate solution (described as control conditions) and low-sulfate solution. Then, we determined the levels of different sulfate transporter genes by qRT-PCR in leaves of both genotypes grown in the two different sulfate conditions. The expression of all of the analyzed transporters responded very differently to sulfur nutrition in *des1-1* mutant plants than in wild-type plants (Fig. 3). In Col-0 plants, a significant increase in expression of the tested sulfate transporters was observed in low-sulfate compared with control conditions, as reported previously (Takahashi et al., 2000; Yoshimoto et al., 2002; Buchner et al., 2004; Kataoka et al., 2004). However, in *des1-1* plants under sulfur limitation, the *SULTR2.1* and *SULTR3.1* transporters were down-regulated, *SULTR2.2* expression was unaffected, and *SULTR1.2* was induced, but to a significantly lower extent than in the wild type.

5′-Adenylylsulfate (APS) reductase is also susceptible to negative control by thiols and positive regulation by sulfur limitation at the mRNA level (Vauclare et al., 2002). Accordingly, we extended the qRT-PCR analysis in Col-0 wild-type and *des1-1* mutant plants

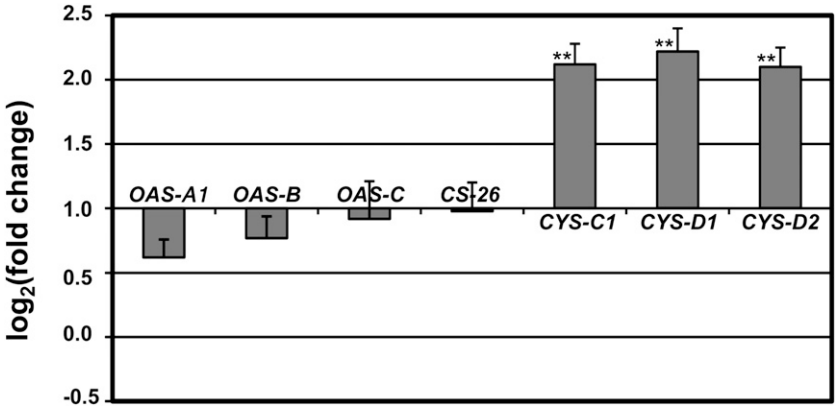


Figure 2. Relative expression levels of the OASTL gene family in the *des1-1* mutant plants. Real-time RT-PCR analysis of the expression of the *OAS-A1* (*At4g14880*), *OAS-B* (*At2g43750*), *OAS-C* (*At3g59760*), *CS-26* (*At3g03630*), *CYS-C1* (*At3g61440*), *CYS-D1* (*At3g04940*), and *CYS-D2* (*At5g28020*) genes was performed in leaves from the wild-type Col-0 and *des1-1* mutant plants grown for 5 weeks under control conditions. The transcript levels were normalized to the constitutive *UBQ10* gene. Data shown are means + SD of three independent analyses made from RNA obtained from different plants grown in different pots at the same time, and they represent the transcript level of each gene in the mutant plants relative to the transcript level in the wild-type plants. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$.

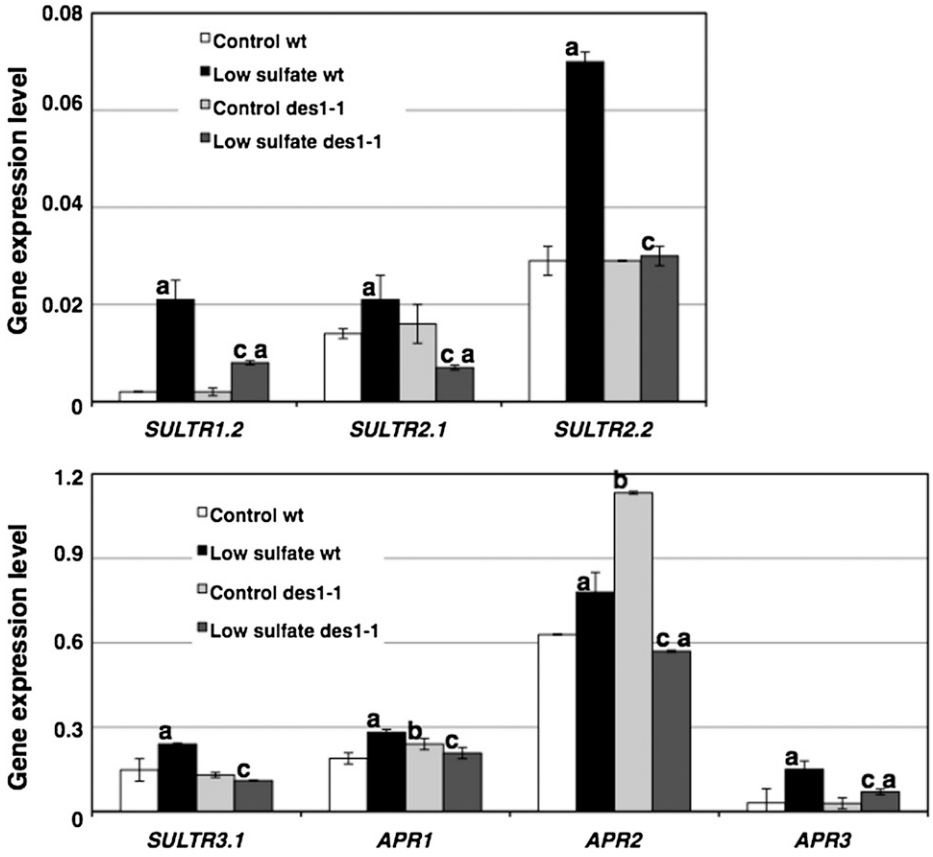


Figure 3. Sulfate regulation of the expression levels of sulfur-responsive genes in the Col-0 wild-type (wt) and *des1-1* mutant plants. Real-time RT-PCR analysis of expression of the *SULTR1.2* (*At1g78000*), *SULTR2.1* (*At5g10180*), *SULTR2.2* (*At1g77990*), *SULTR3.1* (*At3g51895*), *APR1* (*At4g04610*), *APR2* (*At1g62180*), and *APR3* (*At4g21990*) genes was performed in leaves from the Col-0 wild-type and *des1-1* mutant plants grown in soil and irrigated with Hoagland medium (control conditions) or deionized water (low-sulfate conditions). The transcript levels were normalized using the constitutive *UBQ10* gene as an internal control. Data shown are means ± SD of three independent analyses made from RNA obtained from different plants grown in different pots at the same time and are divided into two different graphs due to the different scales. ANOVA was performed using the software OriginPro 7.5. Significant differences between each plant line grown under low-sulfate and control conditions are indicated by the letter a ($P < 0.05$). Significant differences between *des1-1* and wild-type plants grown under control conditions are indicated by the letter b ($P < 0.05$). Significant differences between *des1-1* and wild-type plants grown under low-sulfate conditions are indicated by the letter c ($P < 0.05$).

grown in the two different sulfate concentrations to the three members of the APS reductase gene family from Arabidopsis (Fig. 3). Under conditions of sufficient sulfate, *APR1* and *APR2* expression levels were increased in the *des1-1* mutant with respect to the wild type. Moreover, we observed opposite responses in the wild type and the mutant in the regulation of the expression levels of *APR1* and *APR2*. Both transcripts were up-regulated in the wild type and down-regulated in the *des1-1* mutant under low-sulfate conditions. *APR3* gene expression was induced in both genotypes, but considerably less so in the mutant.

Tolerance of *des1* Mutants to Abiotic Stress

We showed previously that depleting the cytosolic levels of Cys alters the antioxidative capacity of the cytosol in Arabidopsis; as a consequence, there is an increased sensitivity to cadmium (Cd; Lopez-Martin et al., 2008a). We next wanted to study how an increase in the levels of cytosolic Cys, as in the *des1* mutants, would affect Cd sensitivity. We performed a Cd tolerance test comparing the *des1-1* and *des1-2* mutant plants with their respective wild-type ecotypes. Seeds were germinated on solid MS medium containing two concentrations of $CdCl_2$ and allowed to grow for 14 d. On 175 μM Cd, a high proportion of the Col-0 and ecotype Nossen (No-0) wild-type seeds failed to germinate; those that germinated developed chlorotic leaves. In contrast, seeds of both mutants were able to germinate on this Cd concentration and produced green leaves. At 250 μM, the highest tested $CdCl_2$ concentration, the difference between the wild-type ecotypes and their respective mutants was much more remarkable (Fig. 4A). We quantified the tolerance as the ratio between the total number of live seedlings

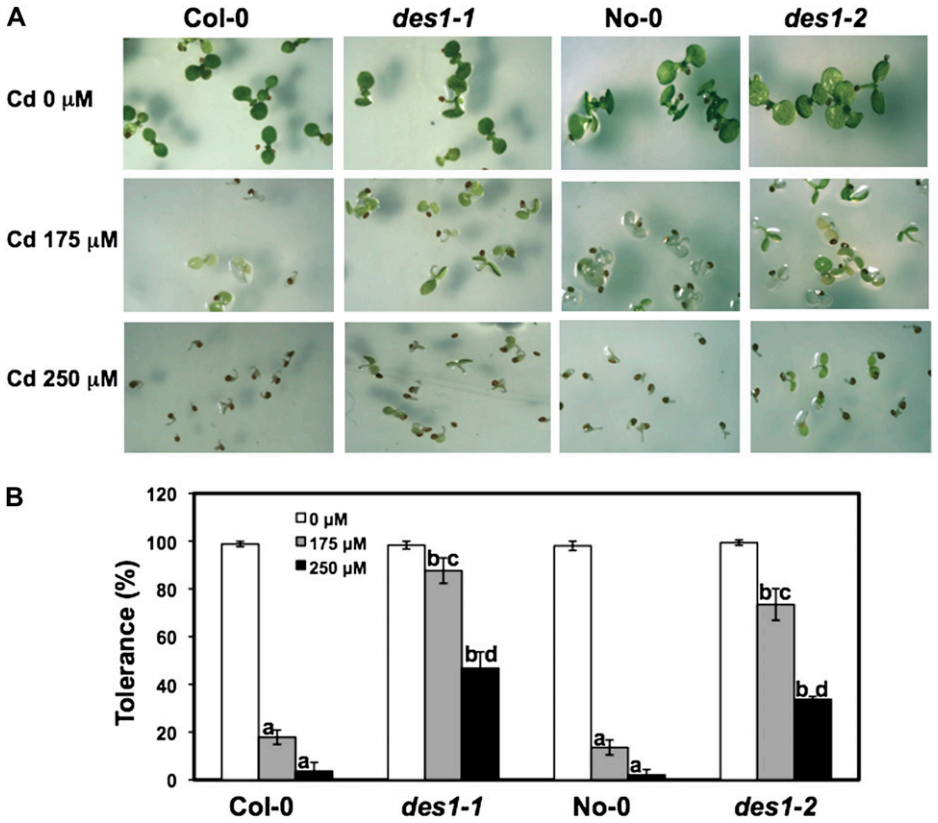


Figure 4. Sensitivity of the *des1-1* and *des1-2* mutant plants to Cd stress. A, Col-0 and No-0 wild-type and mutant seeds were germinated on solid MS medium containing varying concentrations of $CdCl_2$ as stated, and photographs were taken after 14 d of growth. B, The tolerance to Cd was calculated as the ratio of the total number of live seedlings to the total number of sown seeds after 14 d of growth in the presence of different concentrations of Cd. Data shown are means ± SD of three independent analyses made in separate batches at different times. ANOVA was performed using the software OriginPro 7.5. Significant differences between the wild-type plants grown in the presence and absence of Cd are indicated by the letter a ($P < 0.05$). Significant differences between the mutant plants grown in the presence and absence of Cd are indicated by the letter b ($P < 0.05$). Significant differences between the mutant plants and their respective wild-type plants grown in 175 μM Cd are indicated by the letter c ($P < 0.05$). Significant differences between the mutant plants and their respective wild-type plants grown in 250 μM Cd are indicated by the letter d ($P < 0.05$). [See online article for color version of this figure.]

and the total number of sown seeds after 14 d of growth. With this metric, the Col-0 and No-0 wild types showed 18% and 14% tolerance, respectively, to 175 μM Cd and the *des1-1* and *des1-2* mutants showed 88% and 75% tolerance, respectively. At 250 μM Cd, we observed only about 4% and 2% tolerance in the wild-type ecotypes; in contrast, the tolerance of both mutant strains was between 47% and 34% (Fig. 4B). These data show that the *des1* mutants have an increased tolerance to Cd.

A large number of reports have shown that Cd treatment generates oxidative stress in different plant systems (Sandalio et al., 2001; Schutzendubel et al., 2001; Romero-Puertas et al., 2007). We wanted to know if the enhanced Cd tolerance in the *des1* mutants would correlate with enhanced antioxidative defenses. Imaging of ROS in vivo in plant tissues by confocal laser microscopy is a very useful technique (Sandalio et al., 2008); we specifically stained for hydrogen peroxide (H_2O_2) in roots of *des1-1* mutant seedlings after a short treatment with 250 μM $CdCl_2$ and compared the staining with Col-0 wild-type roots (Fig. 5A). In wild-type roots, we observed an increase in fluorescence emission resulting from the oxidation of the nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to the highly fluorescent product; this signal reflects significant production of H_2O_2. In contrast, roots of the *des1-1* mutant had a much lower fluorescence emission than wild-type roots. In green tissues, chlorophyll autofluorescence interfered with the H_2O_2 detection. The significant decrease in H_2O_2 production observed in the *des1-1* mutant roots compared with the wild type upon Cd treatment was further confirmed in whole seedlings by spectrofluorimetric quantification (Fig. 5B). The concentrations of H_2O_2 in Col-0 and *des1-1* seedlings were identical in the absence of Cd. In contrast, ROS increased 64% more in the Col-0 seedlings after Cd treatment but remained almost unchanged (only 7% more) in the *des1-1* seedlings.

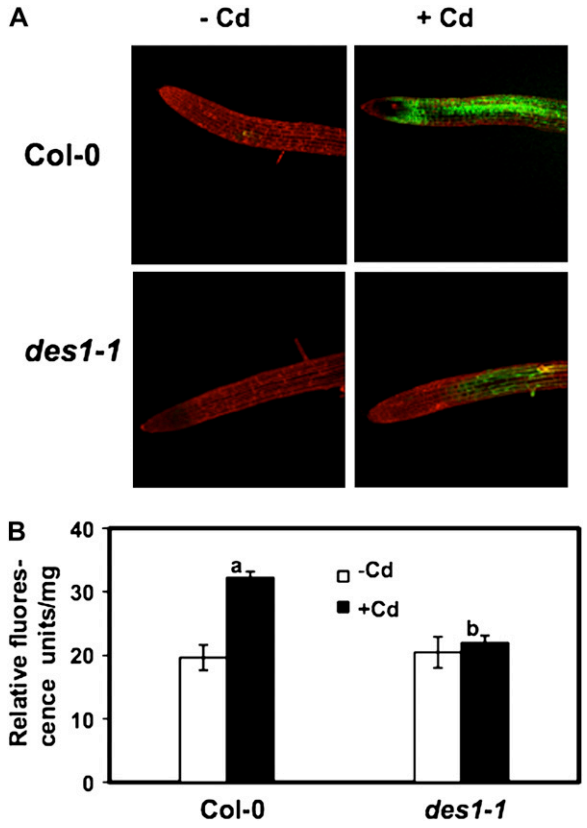


Figure 5. Fluorescence microscopic detection of H_2O_2 in root tissues and quantification in whole seedlings after Cd treatment. A, Roots from 5-d-old wild-type Col-0 and *des1-1* mutant plants grown on solid MS medium were soaked for 10 min in the absence or presence of 250 μM $CdCl_2$, rinsed with water, and then loaded with H_2DCFDA for 5 min in the presence of propidium iodide to visualize cell walls (pseudocolored in red) by confocal microscopy. H_2O_2 is pseudocolored in green. The experiments were repeated using roots from plants grown on different plates at least five times with similar results. B, Whole 2-week-old wild-type Col-0 and *des1-1* seedlings grown on solid MS medium were subjected to the same Cd treatment, then harvested and ground, and H_2O_2 was quantified by H_2DCFDA fluorescence as described in "Materials and Methods." Data shown are means ± SD of three independent analyses made in seedlings grown on different plates. ANOVA was performed using the software OriginPro 7.5. Significant differences between Cd-treated and untreated wild-type plants are indicated by the letter a ($P < 0.05$). Significant differences between Cd-treated *des1-1* mutant and Cd-treated wild-type plants are indicated by the letter b ($P < 0.05$).

We further tested the tolerance of *des1-1* and *des1-2* mutant plants by exogenously applying H_2O_2 to the growth medium. At concentrations of 4 mM and lower, no phenotypic differences could be observed between the wild-type ecotypes and their respective mutant seeds after 14 d of growth. In the presence of 6 mM H_2O_2, however, seeds of both mutants germinated and produced green leaves better than the Col-0 and No-0 wild types; the seedlings remained alive but with a significantly reduced size compared with seedlings grown in the absence of H_2O_2 (Fig. 6A). By quantifying the tolerance to H_2O_2, we found that in medium containing 6 mM H_2O_2 the *des1-1* and *des1-2* seedlings had 71% and 84% tolerance, respectively, whereas the Col-0 and No-0 wild types had only 13% and 39% tolerance, respectively (Fig. 6B).

We also examined the activities of antioxidant enzymes in the leaves. We found that these enzymes were significantly induced in both *des1-1* and *des1-2* mutant plants as compared with wild-type plants; this induction is likely responsible for the antioxidant protection against the abiotic stresses and the significant decrease in H_2O_2 production described above. We visualized one isoform of ascorbate peroxidase (APX) activity by nondenaturing PAGE. The APX activity was shown to qualitatively increase in gel band intensity; we calculated a roughly 2-fold increase in APX activity over wild-type levels in both *des1* mutants. In general, different peroxidases are known to use different substrates to metabolize H_2O_2. When guaiacol was used as a substrate, peroxidase activities were also found to be 2-fold enhanced in the both *des1* mutant plants (Fig. 7).

DISCUSSION

Cys occupies a central position in plant primary and secondary metabolism due to its biochemical functions. Cys residues are essential for protein structure and many protein functions, including catalytic activities, binding capacities, and redox modulations. In addition to its role in proteins, Cys is the precursor molecule of many essential biomolecules and defense molecules that are involved in plant protection (Rausch and Wachter, 2005; Wirtz and Droux, 2005). These diverse functions rely on the very high reactivity of the thiol moiety, but because of this reactivity, Cys can be a very toxic molecule when present above a certain concentration threshold (Park and Imlay, 2003). Thus, it is essential to maintain Cys homeostasis.

In recent years, the enzymes involved in Cys biosynthesis have been heavily studied in plants. Very recently, much progress has been made in understanding the OASTL enzymes from Arabidopsis, with the focus being mainly on the functional characterization of the most abundant enzymes (Heeg et al., 2008; Lopez-Martin et al., 2008a; Watanabe et al., 2008a). Less is known about the low abundance OASTL-like proteins; these proteins have been suggested to play an auxiliary role in Cys biosynthesis under particular conditions. Our data have demonstrated, however, that the minor cytosolic OASTL protein CS-LIKE catalyzes the enzymatic desulfuration of L-Cys to sulfide, ammonia, and pyruvate. This conclusion is mainly inferred from the affinity of CS-LIKE for L-Cys as a substrate, which was found to be more than 10 times higher than that for OAS. The three major OASTLs from Arabidopsis have K_m values for OAS that are at least five times lower than what we report for CS-LIKE (Jost et al., 2000). Also, their K_m values for sulfide are 2 orders of magnitude lower than that determined for

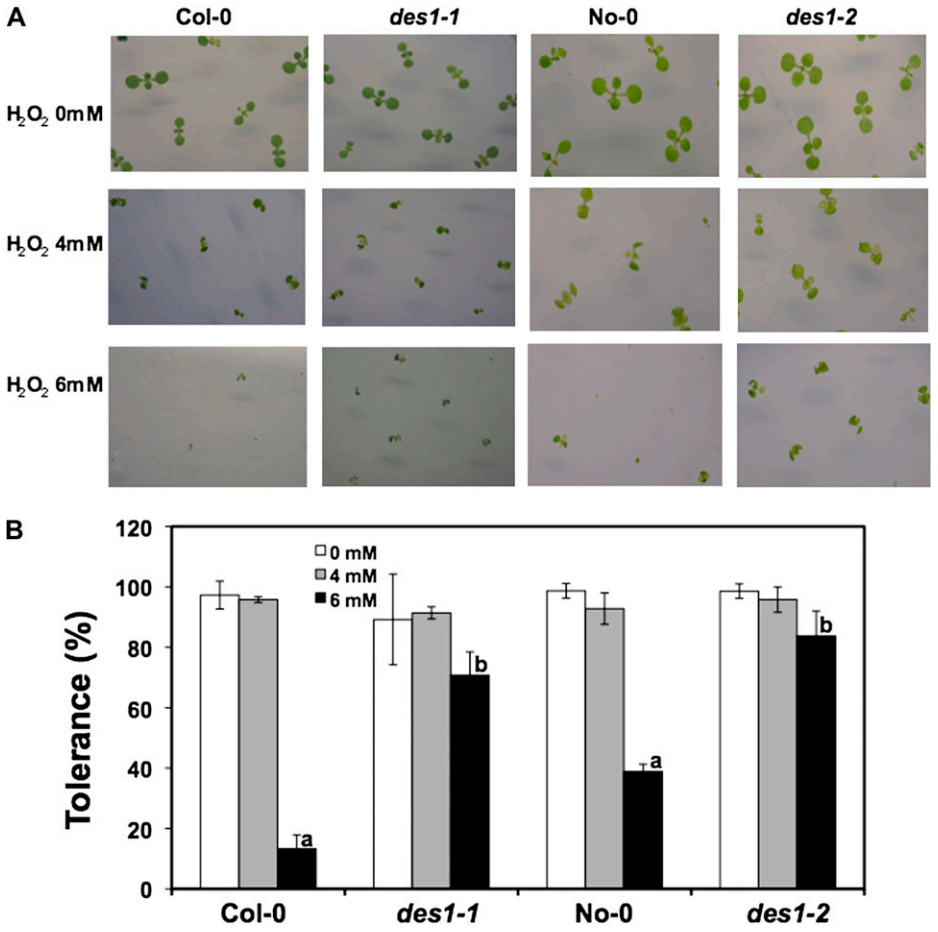
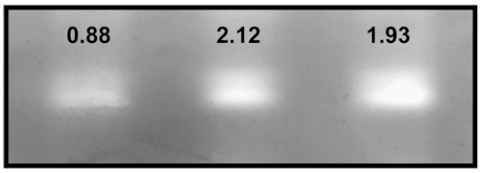


Figure 6. Sensitivity of the *des1-1* and *des1-2* mutant plants to oxidative stress. A, Col-0 and No-0 wild-type and mutant seeds were germinated on solid MS medium containing varying concentrations of H_2O_2 as stated, and photographs were taken after 10 d of growth. B, The tolerance to H_2O_2 was calculated as the ratio of the total number of live seedlings to the total number of sown seeds after 10 d of growth in the presence of different concentrations of H_2O_2. Data shown are means ± SD of three independent analyses made in separate batches at different times. ANOVA was performed using the software OriginPro 7.5. Significant differences between wild-type plants grown in 6 mM H_2O_2 and in the absence of H_2O_2 are indicated with the letter a ($P < 0.05$). Significant differences between the mutant plants and their respective wild-type plants grown in 6 mM H_2O_2 are indicated with the letter b ($P < 0.05$). [See online article for color version of this figure.]

recombinant CS-LIKE (Wirtz et al., 2004). It seems apparent, therefore, from our studies on the recombinant protein that CS-LIKE is not a true OASTL isoform, as has been suggested previously. One main characteristic of the OASTL proteins is the ability to interact with SAT, as has been described in different plant species (Droux et al., 1992, 1998; Ruffet et al., 1994; Bogdanova and Hell, 1997; Wirtz et al., 2001; Berkowitz et al., 2002; Wirtz and Hell, 2006, 2007). The highly conserved β8A-β9A surface loop has been proposed to be the site of interaction with SAT (Bonner et al., 2005). The sequence alignment of CS-LIKE with the cytosolic OASTL shows several amino acid differences in this loop, reinforcing our conclusion that CS-LIKE is not a true OASTL enzyme. Its inability to interact with SAT, apparently due to its structural features, was experimentally demonstrated when Arabidopsis OASTL proteins were purified on SAT immobilized resin and then separated by two-dimensional PAGE and identified by matrix-assisted laser-desorption ionization time of flight mass spectrometry (Heeg et al., 2008). Therefore, our enzymatic characterization of recombinant CS-LIKE protein, together with an analysis of its structural features and experimental data from other investigators, suggest that CS-LIKE is a novel DES located in the cytoplasm. We propose to designate it as DES1. Interestingly, the existence of enzymes with H_2S-releasing activity that are localized in the cytosol and distinct from OASTL proteins was suggested previously during the characterization of RNA interference-transgenic potato (*Solanum tuberosum*) plants with reduced OASTL (Riemenschneider et al., 2005b).

The function of the DES1 enzyme and its impact on plant metabolism are further revealed by the phenotype of the null mutants. In both mutant alleles, the absence of DES1 significantly reduces the total Cys desulfhydrase activity both in the leaves of mature plants and in seedlings, and this results in a concomitant increase in the total Cys content. As a consequence, the expression levels of genes in the sulfate assimilation pathway, which are susceptible to sulfur availability, are deregulated. We clearly observed differential responses of the different sulfate transporter and APS reductase genes to sulfur limitation conditions in the *des1-1* mutant compared with wild-type plants. These results demonstrated that mutation of DES1 has a significant effect on the two main regulatory points of the sulfate assimilatory pathway (Hawkesford, 2000; Hawkesford and De Kok, 2006; Kopriva, 2006), highlighting the importance of this enzyme.

A Relative quantification of APX activity in gel



Col-0 *des1-1* *des1-2*

B

Plant line	GPX activity (U/mg)
Col-0	0.098 ± 0.021
des1-1	0.192 ± 0.028 (196%)**
No-0	0.121 ± 0.009
des1-2	0.209 ± 0.021 (173%)**

Figure 7. APX and guaiacol peroxidase (GPX) activity levels in the *des1-1* and *des1-2* mutant plants. A, In-gel activity of APX in leaf extracts. Leaf protein extracts (30 μg) were loaded in parallel onto two nondenaturing polyacrylamide gels; after electrophoresis, one gel was stained to develop the APX activity and the other was stained with Coomassie Brilliant Blue as described in "Materials and Methods." The Coomassie Brilliant Blue-stained gel is shown in Supplemental Figure S5. The quantification of each APX activity band relative to the Coomassie Brilliant Blue-stained band was calculated with Quantity One software. The experiment was repeated at least three times with similar results. B, Guaiacol peroxidase activity determined in leaf extracts from Col-0 and No-0 wild-type and mutant plants as described in "Materials and Methods." Values are means ± SD from three independent experiments made using leaf extracts obtained from different plants grown in different pots. Percentages with regard to the respective wild type are given in parentheses. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$.

In addition, the *des1-1* and *des1-2* mutant plants show enhanced antioxidant defenses, reflected in a significant decrease in ROS production and an ability to tolerate conditions of oxidative stress. This apparent contradiction, an increase in the concentration of a reducing agent triggering antioxidant defenses, can be explained by considering that intracellular Cys reduces ferric iron with exceptional speed and promotes oxidative damage through the Fenton reaction (Park and Imlay, 2003). Thus, although it is normally viewed as a reducing agent, Cys can induce the expression of antioxidant enzymes; for instance, cytosolic copper/zinc superoxide dismutase is induced by glutathione, Cys, and dithiothreitol (Herouart et al., 1993). Furthermore, transgenic tobacco (*Nicotiana tabacum*) plants that express the wheat (*Triticum aestivum*) OASTL gene *cys1* have higher intracellular levels of Cys and glutathione and also display enhanced ROS-scavenging systems, such as elevated copper/zinc superoxide dismutase transcript levels and superoxide dismutase activities, and a greater tolerance to methyl viologen-induced photooxidative damage (Youssefian et al., 2001). Overexpression of the *E. coli* SAT gene *cysE* in tobacco also increases the levels of Cys and concomitantly increases the resistance to oxidative stress generated by exogenous H_2O_2. Plants with the bacterial SAT targeted to the cytosol are more resistant than plants with the protein targeted to the chloroplasts (Blaszczyk et al., 1999). Also, we have demonstrated that knockout *oas-a1* mutants that are deficient in the most abundant form of cytosolic OASTL show phenotypic characteristics that are apparently opposite those of the *des1* mutants (Lopez-Martin et al., 2008a). These data from the *oas-a1* mutants, together with our data here on the *des1* mutants, support our claim that Cys in the cytosol is an important determinant of antioxidative capacity in Arabidopsis (Lopez-Martin et al., 2008b).

We propose that DES1 contributes to the maintenance of Cys homeostasis under certain conditions or growth stages. The Genevestigator data show that the highest expression of *DES1* is observed in the senescent growth stages, suggesting a requirement for DES1 at a late developmental stage, when it probably plays a role in the mobilization of sulfur. Accordingly, it has been determined previously in Arabidopsis plants that the OASTL activity decreases with increasing age, whereas the DES activity increases in older plants (Burandt et al., 2001). Leaf senescence is associated with an intense proteolytic activity that produces the release of Cys, and Cys desulfuration must be an essential way to avoid the accumulation of toxic levels of Cys in the cell. This is in agreement with the observed phenotype of premature leaf senescence in the *des1* mutants as a consequence of their inability to desulfurate Cys, and it is confirmed by our studies of the expression of senescence-associated genes. Also, our analysis of the expression levels of the different members of the OASTL gene family showed a significant induction of *CYS-C1*, *CYS-D1*, and *CYS-D2* expression, which could be reasonable for CYS-C1 because this β-cyanoalanine synthase is involved in the detoxification of cyanide and cyanide is coproduced during ethylene synthesis in senescent plants (Lim et al., 2007).

Therefore, Cys homeostasis should be precisely maintained in the cytosol, which is the compartment where Cys biosynthesis mainly occurs. This conclusion is confirmed by the repression in the *des1* mutants of the major OASTL enzymes that catalyze the synthesis of Cys, which can be explained as a strategy to avoid the accumulation of Cys. We have previously demonstrated that the cytosolic OASTL isoform OAS-A1 is the major contributor to Cys biosynthesis (Lopez-Martin et al., 2008a). Independent investigations on the Arabidopsis OASTL and SAT gene families have also demonstrated that the cytosol is the major site of Cys synthesis (Haas et al., 2008; Heeg et al., 2008; Watanabe et al., 2008a, 2008b). Cys concentrations in the cytosol are estimated to be over 300 μM, whereas the other cell compartments contain below 10 μM Cys (Krueger et al., 2009). Thus, Cys synthesis and degradation in the cytosol should be coordinated through OAS-A1 and DES1 activities throughout the life cycle of the plant.

In conclusion, although we cannot rule out a role in a secondary metabolite biosynthetic pathway, we suggest that DES1 (previously known as CS-LIKE) of Arabidopsis is a DES involved in precisely maintaining Cys homeostasis in the cytosol at some specific developmental stage or under environmental perturbations.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Treatments

Arabidopsis (*Arabidopsis thaliana*) wild-type ecotypes Col-0 and No-0 and the SALK_03855 and RIKEN RATM13-27151_G mutants were used in this work (Alonso et al., 2003; Ito et al., 2005). The plants were grown either on solid MS medium in petri dishes or in soil. Plants grown in soil were irrigated with Hoagland II medium (Jones, 1982) containing 0.75 mM sulfate, and these conditions were referred to as control conditions; alternatively, they were irrigated with deionized water, referred to as low-sulfate conditions. Plants were grown under a photoperiod of 16 h of white light (120 μE m^{-2} s^{-1}) at 20°C and 8 h of dark at 18°C.

Cd or H_2O_2 stress was imposed on plants growing on solid medium with the addition of $CdCl_2$ or H_2O_2 to the medium at the indicated concentrations. The tolerance to the chemical was calculated as the ratio between the total number of live seedlings and the total number of sown seeds after growth in the presence of different concentrations of the chemical.

Expression of CS-LIKE in *Escherichia coli* Using Gateway Technology

To construct the expression clone, initially the *CS-LIKE* (*At5g28030*) cDNA was directionally cloned into the pENTR/D-TOPO vector for entry into the Gateway System (Invitrogen). Total RNA was extracted from Arabidopsis wild-type leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Subsequently, a 972-bp sequence encoding the full-length CS-LIKE protein was amplified by PCR using the primers GW-CSL-F and GW-CSL-R (Supplemental Table S2) and the proofreading Platinum Pfx DNA polymerase (Invitrogen). PCR conditions were as follows: a denaturation cycle of 2 min at 94°C, followed by 30 amplification cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The amplified cDNA was then ligated into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning Kit (Invitrogen) following the manufacturer's instructions. Positive clones were identified by PCR and chosen for plasmid DNA isolation. The *CS-LIKE* cDNA was then cloned into the expression vector pDEST17 using the *E. coli* Expression Systems with Gateway Technology (Invitrogen), which allowed us to generate a fusion protein with an N-terminal 6× His tag confirmed by sequencing, and its expression was inducible by L-Ara in BL21-AI *E. coli* cells.

Purification of Recombinant CS-LIKE Protein

The 6× His-tagged recombinant protein was isolated from 100 mL of BL21-AI *E. coli* cells cultured at 28°C to an optical density at 600 nm (OD_{600}) of 0.4 and then induced with 0.1% L-Ara overnight at 22°C. The purification was performed under nondenaturing conditions by affinity to nickel resin using the Ni-NTA Purification System (Invitrogen) according to the manufacturer's instructions. The purification process was visualized by SDS-PAGE using 12% (w/v) polyacrylamide gels and Coomassie Brilliant Blue staining.

Determination of Enzyme Activities

Plant leaf material was ground in 50 mM phosphate buffer (pH 7.5), 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride using a mortar and pestle with liquid nitrogen. After centrifugation at 15,000*g* for 15 min at 4°C, the resulting supernatant was used as plant soluble extract for OASTL activity measurements. Plant leaf material ground in 20 mM Tris-HCl (pH 8) as described above was used for plant soluble extract for DES activity measurements. The total amount of protein in the extracts was determined by the Bradford (1976) method.

OASTL activity was measured using the method described previously (Barroso et al., 1995) in soluble plant, soluble bacterial, or purified protein extracts.

DES activity was measured by the release of sulfide from L-Cys as described previously (Riemenschneider et al., 2005a). The assay contained in a total volume of 1 mL: 1 mM dithiothreitol, 1 mM L-Cys, 100 mM Tris-HCl, pH 8.0, and enzyme extract. The reaction was initiated by the addition of L-Cys. After incubation for 15 min at 37°C, the reaction was terminated with the addition of 100 μL of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 μL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm, and the enzyme activity was calculated using the extinction coefficient of 15×10^6 cm^2 mol^{-1} (Papenbrock and Schmidt, 2000). D-Cys desulfhydrase activity was determined in the same way, but D-Cys was used instead of L-Cys (Riemenschneider et al., 2005c).

The substrate saturation experiments were performed with CS-LIKE recombinant protein and Cys as the substrate for the DES reaction by varying the Cys concentration from 0.03 to 2 mM. OAS and H_2S were cosubstrates for the OASTL reaction; the OAS concentration was varied from 0.05 to 12 mM, and the H_2S concentration was varied from 10 to 1,000 μM. Data from at least four replicate experiments were pooled and analyzed. The kinetic parameters for the DES and OASTL reactions were determined from a Hanes plot of s/vi against s, where s is the substrate concentration and vi is the initial velocity.

CAS activity was measured by the release of sulfide from L-Cys in the presence of cyanide following the method and extinction coefficient described earlier (Meyer et al., 2003).

Glu dehydrogenase activity was assayed by following the oxidation of NADH at 340 nm as described previously (Glevarec et al., 2004). The assay contained in a total volume of 850 μL: 120 mM Tris-HCl, pH 9, 12 mM 2-oxoglutarate, 0.25 mM NADH, and 500 μL of the DES reaction. The reaction was initiated by the addition of 6 units of bovine liver Glu dehydrogenase (Sigma), and the OD_{340} (extinction coefficient of 6.2 mM^{-1} cm^{-1}) was recorded for a period of 15 min.

Lactate dehydrogenase activity was assayed using the spectrophotometric method of following the oxidation of NADH at 340 nm (Riemenschneider et al., 2005c). The reaction was performed in 1 mL containing 50 mM potassium phosphate, pH 8.0, 0.23 mM NADH, and 500 μL of the DES reaction. The reaction was initiated by the addition of 6 units of rabbit muscle lactate dehydrogenase (Sigma), and the OD_{340} was recorded for a period of 15 min.

APX activity was detected on native PAGE gels according to the method described earlier (Mittler and Zilinskas, 1993). Equal amounts of soluble protein (30 μg) were separated on a discontinuous polyacrylamide gel under nondenaturing conditions at 4°C as described earlier (Laemmli, 1970); specific activity staining followed. The stacking and separating gels contained 4% and 10% polyacrylamide, respectively. The carrier buffer contained 2 mM ascorbate, and a prerun of 30 min was performed to let ascorbate enter the gel before the samples were loaded. After protein separation, the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min, followed by 20 min of incubation in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2. The gel was then washed with the buffer for 1 min and visualized by immersion in 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM tetramethyl ethylene diamine and 2.5 mM nitroblue tetrazolium. The APX activity appeared as an achromatic band on a purple-blue background. An identical gel was run in parallel and stained with Coomassie Brilliant Blue to detect all protein bands. The resulting two gels were scanned, and the APX activity band was quantified relative to the Coomassie Brilliant Blue-stained band using the Quantity One software (Bio-Rad).

Guaiacol peroxidase activity was assayed colorimetrically with guaiacol as a substrate. The reaction mixture contained 50 mM phosphate buffer (pH 6.1), 0.25 mM H_2O_2, 6.25 mM guaiacol, and enzyme extract. The linear increase in absorption at 470 nm, due to the formation of tetraguaiacol, was followed for 2 min (extinction coefficient of 26.6 mM^{-1} cm^{-1}).

Mutant Isolation by PCR Screening

To identify individuals that were homozygous for the insertion, genomic DNA was extracted from either 30 mg mL^{-1} kanamycin-resistant (SALK mutant) or 25 mg mL^{-1} hygromycin-resistant (RIKEN mutant) seedlings and

subjected to PCR genotyping using the following primer pairs (Supplemental Table S2): LBb1/GW-CSL-R, GW-CSL-F/GW-CSL-R, and GW-CSL-F/LBb1 for the SALK mutant and DS3-4/GW-CSL-R and GW-CSL-F/GW-CSL-R for the RIKEN mutant. PCR conditions were as described previously (Lopez-Martin et al., 2008a).

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from Arabidopsis leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). An aliquot of the cDNA was amplified in subsequent PCRs using the following primers: GW-CSL-F and GW-CSL-R for the *DES1* (*At5g28030*) gene and UBQ10F and UBQ10R for the constitutive *UBQ10* gene, which was used as a control (Supplemental Table S2). PCR conditions were as described above.

DNA Isolation and Southern-Blot Analysis

Total DNA was isolated from Arabidopsis leaves and digested with *Xba*I, a restriction enzyme that does not cut inside the *DES1* gene, following a previously described method (Lopez-Martin et al., 2008a).

Real-Time RT-PCR

qRT-PCR was used to analyze the expression of senescence-associated genes, the OASTL gene family, and sulfur-responsive genes in the *des1-1* mutant plants. First-strand cDNA was synthesized as described above. Gene-specific primers for each gene were designed using the Vector NTI Advance 10 software (Invitrogen; Supplemental Table S2). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad); the signals were detected on an iCYCLER (Bio-Rad) according to the manufacturer's instructions. The cycling profile consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A melt curve from 60°C to 90°C was run following the PCR cycling. The expression levels of the genes of interest were normalized to that of the constitutive *UBQ10* gene by subtracting the cycle threshold (CT) value of *UBQ10* from the CT value of the gene (ΔCT). The fold change was calculated as $2^{-(\Delta CT\ mutant\ -\ \Delta CT\ wild\ type)}$. The results shown are means ± SD of at least three independent RNA samples.

Quantification of Thiol Compounds

To quantify the total Cys and glutathione contents, thiols were extracted, reduced with $NaBH_4$, and quantified by reverse-phase HPLC after derivatization with monobromobimane (Molecular Probes) as described previously (Dominguez-Solis et al., 2001).

Detection and Quantification of H_2O_2

For the fluorimetric detection of H_2O_2, roots from 1-week old seedlings grown on vertical MS plates were treated with 250 μM $CdCl_2$ for 5 min, rinsed with water, and then incubated for 5 min with 10 mM H_2DCFDA (Molecular Probes) in the presence of 10 mM propidium iodide to visualize the cell walls. The samples were observed using a TCS SP2 spectral confocal microscope (Leica Microsystems) with the following settings: excitation, 488 nm; emission, 500 to 550 nm for fluorescein detection and 600 to 650 nm for propidium detection.

The quantification of H_2O_2 was performed following the protocol described previously (Joo et al., 2005). Basically, frozen plant tissue was hand ground in liquid nitrogen, and the powder was weighed and immediately taken up in 10 mM Tris-HCl buffer, pH 7.5. The extract was centrifuged twice at 15,000*g* for 10 min. We performed each measurement on two equal aliquots, to one of which we added 100 mM ascorbate, and they were allowed to react for 15 min. Then ROS levels were assayed by adding H_2DCFDA in dimethyl sulfoxide to both aliquots to a final concentration of 25 μM and incubating at 30°C for 30 min. Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation/emission wavelengths set to 485 and 525 nm, respectively. We then subtracted the ascorbate-insensitive background from each experimental value. Total protein was quantified using the method of Bradford (1976). The average fluorescence value obtained from three successive measurements was divided by the protein content and expressed as relative fluorescence units per milligram of protein.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Purification of the CS-LIKE recombinant protein as shown by SDS-PAGE.

Supplemental Figure S2. Amino acid alignment of CS-LIKE (CSL) and the major OASTL isoforms in Arabidopsis: cytosolic OAS-A1, plastid OAS-B, and mitochondrial OAS-C.

Supplemental Figure S3. Michaelis-Menten plots for DES and OASTL reactions with the substrates L-Cys and OAS, respectively.

Supplemental Figure S4. Intron-exon organization of the *DES1* gene (*At5g28030*) in the *des1* mutants, and RT-PCR and Southern-blot analyses.

Supplemental Figure S5. Nondenaturing polyacrylamide gel stained with Coomassie Brilliant Blue in parallel with the gel shown in Figure 7A as a control for protein loading.

Supplemental Table S1. Arabidopsis OASTL gene family.

Supplemental Table S2. Sequences of oligonucleotides used in this work.

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