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Arabidopsis S-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control

Authors:

Maria Angeles Bermúdez, Maria Angeles Páez-Ochoa, Cecilia Gotor and Luis C. Romero

Authors` affiliations:

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas, Avda. Américo Vespucio, 49, 41092 Sevilla, Spain.

Running title:

Chloroplastic S-sulfocysteine synthase

Corresponding author: Luis C. Romero

e-mail: lromero@ibvf.csic.es; Telephone: + 34-954489516. fax: + 34-954460065

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Synopsis.
S-sulfocysteine synthase is a newly discovered enzymatic activity located in chloroplasts of *Arabidopsis thaliana.* It plays an important role in chloroplast function and is essential for light-dependent redox regulation within the chloroplast. The loss of this activity resulted in accumulation of reactive oxygen species and dramatic changes in phenotype that were dependent on the light regime.

ABSTRACT

In bacteria, the biosynthesis of cysteine is accomplished by two enzymes that are encoded by the *cysK* and *cysM* genes. CysM is also able to use thiosulfate as a substrate to produce S-sulfocysteine. In plant cells, the biosynthesis of cysteine occurs in the cytosol, mitochondria and chloroplasts. Chloroplasts contain two O-acetylserine(thiol)lyase homologs, which are encoded by the *OAS-B* and *CS26* genes in *Arabidopsis thaliana*. An *in vitro* enzymatic analysis of the recombinant CS26 protein demonstrated that this isoform possesses S-sulfocysteine synthase activity and lacks O-acetylserine(thiol)lyase activity. *In vivo* functional analysis of this enzyme in knockout mutants demonstrated that mutation of *CS26* suppressed the S-sulfocysteine synthase activity that was detected in wild type; furthermore, the *cs26* mutants exhibited a reduction in size and showed paleness, but penetrance of the growth phenotype depended on the light regime. The *cs26* mutant plants also had reductions in chlorophyll content and photosynthetic activity (neither of which were observed in *oas-b* mutants), as well as elevated glutathione levels. However, *cs26* leaves were not able to properly detoxify reactive oxygen species, which accumulated to high levels under long-day growth conditions. The transcriptional profile of the *cs26* mutant revealed that the mutation had a pleiotropic effect on many cellular and metabolic processes. Our findings reveal that S-sulfocysteine and the activity of S-sulfocysteine synthase play important roles in chloroplast function and are essential for light-dependent redox regulation within the chloroplast.

INTRODUCTION

The biosynthesis of cysteine (Cys) represents the final step of the assimilation of inorganic sulfate in bacteria, archaea and plants. The biosynthesis of Cys is accomplished by the sequential actions of two enzymes. The first enzyme is serine acetyltransferase (SAT), which synthesizes the intermediate O-acetylserine (OAS) from acetyl-CoA and serine; the second enzyme is O-acetylserine(thiol)lyase (OASTL), which incorporates the sulfide (derived from the assimilatory reduction of sulfate) into OAS to produce Cys. In *Escherichia coli* and *Salmonella typhimurium*, the SAT enzyme is encoded by the *cysE* gene, whereas the two OASTL isoforms are encoded by the *cysK* and *cysM* genes (Hulanicka et al., 1986; Denk and Bock, 1987; Byrne et al., 1988). Although the enzyme encoded by *cysK* only catalyzes the incorporation of sulfide, the enzyme encoded by *cysM* is also able to catalyze the incorporation of thiosulfate into OAS to form the thioester S-sulfocysteine (S-Cys) (Hulanicka et al., 1979; Nakamura et al., 1984), which is then converted into Cys and sulfate to serve as a sulfur source.

In plant cells, such as *Arabidopsis thaliana*, the biosynthesis of cysteine can occur in three different cellular compartments: the cytosol, the chloroplast and the mitochondria. The biosynthetic enzymes are encoded by members of a large multigene family and are represented by five different SAT isoforms (Howarth et al., 2003) and nine OASTL isoforms (Wirtz et al., 2004) (Figure 1). At the transcriptional level, the most abundant *OASTL* transcripts are those representing the cytosolic OAS-A1, the plastidial OAS-B and the mitochondrial OAS-C isoforms. However, one gene, *OAS-A2*, does not produce a functional protein due to an in-frame stop codon and an unspliced intron. Yet, analysis of null alleles of *oas-A1* and *oas-B* indicate that the major cytosolic and plastidial enzymes are dispensable for growth under normal conditions, although together they constitute 95% of the total OASTL activity (Heeg et al., 2008; Watanabe et al., 2008a). The double mutant, *oas-A1oas-B*, as well as the null allele of *oas-C,* exhibits a slight growth retardation phenotype, revealing that Cys and sulfide are exchanged between the cytosol and the organelles (Heeg et al., 2008).

Although the major plant OASTL isoforms are functionally redundant under normal

growth conditions and because Cys can be translocated from the organelles to the cytosol (and vice versa), the cytosolic Cys pool seems to be critical for maintaining the redox capacity of the cytosol during environmental and intracellular variation (Lopez-Martin et al., 2008). Furthermore, the major cytosolic OASTL isoform, OAS-A1, is essential for heavy metal tolerance: over-expression is sufficient to confer tolerance to elevated cadmium concentrations (Dominguez-Solis et al., 2001; Dominguez-Solis et al., 2004; Lopez-Martin et al., 2008). Recently, the minor cytosolic OASTL isoform, CS-LIKE has been described to be an L-cysteine desulfhydrase enzyme involved in maintaining cysteine homeostasis, mainly at late developmental stages or under environmental perturbations (Alvarez et al., 2009).

Another OASTL homolog, CYS-C1, exhibits OASTL activity but is in fact a β-cyanoalanine synthase enzyme that uses Cys to detoxify cyanide within the mitochondria (Hatzfeld et al., 2000).

The remaining genes, *CS26*, *CYS-D1* and *CYS-D*, are each transcribed at very low levels, and their functional roles have not yet been explored. Protein sequence analysis predicts that CS26 is localized in the chloroplast; in addition, the protein lacks the ß8a-ß9a loop necessary for interaction with SAT (Bonner et al., 2005). Proteomic analysis of chloroplast fractions by LTQ-Orbitrap mass spectrometry confirmed the localization of CS26 in the chloroplast; in addition, the small number of CS26 tryptic peptides identified confirms the low abundance of this protein (Zybailov et al., 2008).

We have functionally characterized the minor chloroplastic isoform, CS26, by purifying a recombinant form of the protein from *E. coli* and analyzing its enzymatic properties. The functional role of this enzyme *in planta* was analyzed in null mutant alleles in *Arabidopsis* by comparing different single mutants of both chloroplastic proteins, CS26 and OAS-B.

RESULTS

Characterization of the CS26 recombinant protein

The deduced amino acid sequences of the homologous proteins OAS-B and CS26

were aligned with the most abundant cytosolic protein, OAS-A1 (Supplemental Figure 1 online). The comparison revealed that CS26 contains an extension C-terminal to the chloroplast transit peptide compared to OAS-B. The N-terminal chloroplast transit peptide (cTP) of the native protein was predicted by the TargetP program, and a protein lacking the 94 N-terminal amino acids was considered to be the mature CS26 protein. We cloned the coding region of the *Arabidopsis* CS26 gene corresponding to the mature protein into the Gateway destination vector pDEST™17 for high-level expression of the recombinant protein in *E. coli*. To facilitate purification of the recombinant protein, expression from the pDEST™17 vector generates a N-terminal 6xHis-tagged protein. The recombinant protein expressed in bacteria was then purified using a Ni-NTA agarose column under native conditions (Supplemental Figure 2 online). Approximately 0.12 mg of purified recombinant protein was isolated from 100 mL of bacterial liquid culture with a yield of 56% (Table 1). The identity of the protein was confirmed by excising the protein band from an SDS-PAGE gel and submitting it to MALDI-TOF mass spectrometry.

Enzymatic determinations of the O-acetylserine(thiol)lyase (OASTL), S-sulfocysteine synthase (SSCS), ß-cyanoalanine synthase (CAS) and L-cysteine desulfhydrase (LCD) activities were measured using the bacterial crude extract following arabinose induction and the purified recombinant His-CS26 protein. Although we were unable to detect OASTL, CAS or LCD activities from the purified recombinant His-CS26, the protein did exhibit S-sulfocysteine synthase activity, as measured by thiosulfate consumption in the enzymatic reaction mixture (Table 1).

The reaction product formed by the recombinant His-CS26 enzyme in the presence of thiosulfate and O-acetylserine was identified by mass spectrometry after HPLC separation. The main product formed in the enzymatic reaction was a molecule with an m/z of 201.9 ± 0.1, whose mass spectrum precisely matched the theoretical spectrum of S-Cys and the spectra obtained from standard S-Cys samples (Figure 2). The small peaks at m/z 202.9 and 203.9 also corresponded to S-Cys, but with different isotopic distributions. The abundance of these peaks also matched the expected isotopic distribution for a molecule with the molecular formula $C_3H_7NO_5S_2$ (Figure 2A). Although the mass spectra of the reaction products exactly matched the spectra of theoretical and standard samples, the major peak was isolated and fragmented in the ion trap. The major peak detected after fragmentation

(MS^2) corresponded to an ion with an m/z of 120.0 ± 0.1, resulting from a loss of a SO_3 fragment, forming a Cys radical (Figure 3). Further fragmentation of the ion with an m/z of 120.0 resulted in the formation of two ions with m/z values of 92.2 and 74.2. The first ion corresponded to a loss of the CO, and the second peak corresponded to a loss of $CO + H_2O$, forming the immonium ion of the parental m/z 120.0 radical.

The kinetic properties of the recombinant protein were determined by an Eadie-Hofstee plot. The estimated Km values for the substrates O-acetylserine and thiosulfate were 0.46 mM and 0.93 mM, respectively. The maximum level of activity was observed in the pH range of 8-9 and at an optimum temperature of 30°C. Amino-oxyacetate (AOA; 10 mM), an inhibitor of pyridoxal-5′-phosphate (PLP)-dependent enzymes such as S-sulfocysteine synthase, inhibited the enzyme by 76%.

Sequence comparison of the chloroplastic enzymes OAS-B and CS26 with the bacterial *cysK* and *cysM* (encoding OASTL and SSCS, respectively) revealed a 16 amino acid deletion in the CS26 protein between the ß8A and ß9A domains, relative to the OASTL enzymes. This deletion was also present in the S-sulfocysteine synthase enzymes encoded by the bacterial *cysM* genes. This region is involved in the active site of the nucleophile (sulfide) in the OASTL enzymes or thiosulfate in the SSCS enzymes (Figure 4).

Identification and biochemical characterization of the *Arabidopsis cs26* and *oas-b* mutants

To determine the specific functions of the CS26 S-sulfocysteine synthase in the chloroplast of *A. thaliana*, we screened various T-DNA insertion mutants from available collections. For comparison, two T-DNA insertion mutants of the chloroplastic *OAS-B* gene were also analyzed. Two independent alleles were characterized for each gene: two mutants contained T-DNA insertions in the first exon of the *CS26* gene; and two mutants contained T-DNA insertions in the second exon and the first intron of the *OAS-B* gene, respectively (Supplemental Figure 3 online). Reverse transcription (RT)-PCR analysis of the homozygous mutant plants using gene-specific primers revealed no detectable transcripts, suggesting that the four alleles represent knockout events. Southern blot analysis was also performed to determine the number of T-DNA insertions; unique T-DNA insertion sites

were confirmed by the appearance of a single hybridized band in the genomes of the SALK_034133 and GABI_684B07 mutants, which were chosen for further analysis. The two mutants are hereafter referred to as *cs26* and *oas-b*, respectively.

Biochemical characterization of the *cs26* mutant plants revealed that the mutation had no significant effect on the total OASTL enzyme activity in leaves when compared to wild type plants, which was expected from the lack of OASTL activity exhibited by the recombinant protein. Whereas the level of SSCS activity in crude extracts from the wild type leaves was low, we were unable to detect any SSCS activity in the *cs26* mutant (Table 2). Comparison of the total leaf concentrations of Cys and glutathione revealed that although the amount of Cys remained unchanged, the total glutathione content was significantly elevated. In contrast, the *oas-b* mutant plants displayed a 20% decrease in the total OASTL enzyme activity, which did not correlate with a reduction in the total Cys or glutathione levels in leaves (Table 2). In addition, a significant increase in SSCS activity was detected in the *oas-b* mutant.

The *cs26* but not the *oas-b* mutant plants exhibited phenotypic differences that depended on light conditions

When phenotypic traits of the different plants were compared, *oas-b* mutant plants showed no phenotypic differences from the wild type under long-day conditions; however, *cs-26* mutant plants were significantly smaller and had pale green leaves (Figure 5A). Mutant plants containing the other *cs26* allele showed a similar phenotype as the one selected (Supplemental Figure 4 online). The fresh weight of *cs26* plants was, on average, only 24% of the fresh weight of wild type plants. To verify that the observed phenotype of the *cs26* mutant plants was indeed due to disruption of the *CS26* gene, the mutant was complemented using the full-length cDNA fragment (Supplemental Figure 3 online). The complemented *cs26*::P35S-CS26 line, which expressed the *CS26* gene at levels comparable to wild type, rescued the phenotype of mutant plants grown under long-day conditions (Figure 5A). The complemented line showed higher SSCS activity (37.4 ± 1.51 mU mg^{-1}) than the wild type line.

Interestingly, the phenotypic characteristics of the *cs26* mutant plants were dependent on the light regime; they exhibited a more severe reduction in size and increased paleness

when grown under continuous light (Figure 5B). In contrast, under short-day conditions, the *cs26* mutant plants were phenotypically indistinguishable from the wild type plants (Figure 5A). The mutant lines growing on MS medium containing sucrose also looked like wild type plants.

The phenotype of the *cs26* mutant suggested a possible defect in photosynthesis under long-day conditions. To investigate this possibility, we measured pigment contents and photosynthetic activity (Figure 6). A pigment analysis revealed reductions of 34% and 38% in *Chl a* and *b,* respectively, based on fresh weight; there was also a 25% reduction in carotenoid content in the *cs26* mutant plants relative to wild type plants. However, no significant differences were detected in the *oas-b* mutant plants (Figure 6A). In contrast, when *cs26* mutant plants were grown under short-day conditions, the levels of photosynthetic pigments were very similar to wild type. Although we have presented only the data for one of the alleles for each mutant, our results were consistently similar (within experimental error) for the other alleles. A defect in photosynthesis in the *cs26* mutant plants grown in long-day conditions was also observed when the rate of photosynthesis was assessed by oxygen evolution. The mutant showed a severe reduction (60%) in photosynthetic activity when compared to wild type, whereas the photosynthetic activity of the *oas-b* mutant was similar to that of the wild type (Figure 6B). The differences in oxygen evolution disappeared in when the plants were grown with short-day conditions (Figure 6C).

Mutation of the *CS26* gene results in an increased production of reactive oxygen species during a long diurnal cycle

The defect in photosynthesis observed in the *cs26* mutant plants under long-day growth conditions correlated with an increase in the production of reactive oxygen species (ROS), such as the superoxide radical anion and hydrogen peroxide (Figure 7). Superoxide production was visualized by nitroblue tetrazolium (NBT) staining of leaves excised from plants grown under long-day or short-day conditions. The dark blue stain, due to superoxide production, was clearly observed across the surface of the leaves excised from *cs26* mutant plants with long-day growth conditions when compared to the wild type plants. The production of H_2O_2, detected by the histochemical diaminobenzidine (DAB) method, was

clearly observed in leaves of the mutant plants under long-day growth conditions as a brown staining that was homogeneously distributed throughout the leaves. ROS accumulation was not detected in either the mutant or the wild type under short-day conditions.

S-sulfocysteine synthase activity in chloroplasts

To confirm the chloroplastic localization of the SSCS enzyme, we obtained chloroplast preparations from wild type and *oas-b* and *cs26* mutant plants. The SSCS activity in the wild type plants was present in the chloroplast preparations with a specific activity of 84.7 ± 2.6 mU per mg, a 10-fold enrichment compared to the whole-leaf crude extract, indicating that the preferential localization of this enzyme is in the chloroplast (Figure 8). As demonstrated in the whole-leaf extract, we did not detect SSCS activity in chloroplasts that were isolated from the *cs26* mutant. The SSCS activity was also easily detected in chloroplasts from the *oas-b* mutant, which exhibited a slight increase in activity relative to wild type.

Transcriptional profile of the *cs26* mutant line

Using the Affymetrix ATH1 GeneChips, we performed a comparative transcriptomic analysis on leaves of the *cs26* and wild type plants (Figure 9A). Total RNA was extracted from the leaves of 3-week-old plants grown under identical long-day conditions on soil (three biological replicates for each genotype), and these samples were used to prepare complementary RNA, which was then hybridized to the chips (raw data, Supplemental Dataset 1; Gene Expression Omnibus repository GSE19241).

The normalized data from the replicates showed differential expression of 743 genes in the *cs26* mutant in long-day conditions, with 372 genes down-regulated and 371 genes up-regulated by more than twofold, with a False Discovery Rate (FDR) of < 0.05 and an intensity signal restriction of lgSignal >7 (Figure 9B).

The 743 altered genes were classified into 11 functional groups based on the Gene Ontology categorization from The *Arabidopsis* Information Resource (TAIR; http://www.*Arabidopsis*.org). The most abundant groups corresponded to proteins that respond to abiotic and biotic stimuli (Figure 9C). This group included *COR15A, COR15B,*

COR78 and *COR414-TM,* the four most highly repressed genes in the *cs26* mutant, which are involved in cold acclimation. On the other hand, the most induced genes were the oxidoreductase 2OG-Fe(II) oxygenase, *CATION EXCHANGER 3 (CAX3)*, *PATHOGENESIS-RELATED GENE 1* (*PR-1*) and other genes mostly related to biotic stress responses. In general, the transcriptional profile of the *cs26* mutant indicates that the mutation has a pleiotropic effect on many cellular and metabolic processes.

In order to isolate the effect produced by the mutation from side effects produced by the long-day-dependent stress, we also compared the transcript profiles of the *cs26* and wild type plants using the leaves of 5-week-old plants grown under identical short-day conditions on soil, where ROS accumulation was not detected (Figure 7). The normalized data from the replicates showed differential expression of 251 genes in the *cs26* mutant, with 172 genes down-regulated and 79 genes up-regulated by more than twofold, with an FDR of < 0.05 and an intensity signal restriction of lgSignal >7 (raw data, Supplemental Dataset 2; Gene Expression Omnibus repository GSE19241). The Venn diagram of short- and long-day transcript profiles show 112 common genes; 9 of them were up-regulated and 6 down-regulated in both profiles (Figure 9B).

The nine up regulated genes in both profiles matches the most highly induced genes under short-day growth conditions. In this condition, many of the induced genes were encoded by the chloroplast genome, such as *RBCL* (large subunit of RUBISCO, AtCg00490), *ACCD* (beta subunit of the Acetyl-CoA carboxylase complex, AtCg00500), *RPL20* (chloroplast ribosomal protein L20, AtCg00660), *RPS15* (chloroplast ribosomal protein S15, AtCg01120) and *PSAI* (the subunit I of photosystem I, AtCg00510). Several genes that respond to stress signals, such as the *PLANT-DEFENSIN 1.2A* and *1.2B* (At5g44420 and At2g26020), are also significantly induced in the mutant line under both short- and long-day conditions. Interestingly, many of the genes highly induced in the mutant under long-day growth conditions were the most repressed under short-day growth conditions, such as *PR1*, the oxidoreductase 2OG-Fe(II) oxygenase and *CAX3*.

A selection of sixteen genes, up- and down-regulated, from the microarray experiments were analyzed by quantitative Real-Time-PCR to validate the data. All the selected genes without exceptions showed the same pattern of regulation as quantified in the microarray and by qRT-PCR (Supplemental Table 1 online).

DISCUSSION

Recent reports from several laboratories have demonstrated that the major O-acetylserine(thiol)lyase isoforms within the cytosol (OAS-A1), the chloroplast (OAS-B) and the mitochondria (OAS-C) are redundant enzymes, because sulfide, OAS and Cys exchange between the cytosol and the organelles. Therefore, the main contribution of the mitochondrial enzyme is related to the synthesis of OAS, and the cytosol is the main compartment that contributes to the biosynthesis of Cys (Heeg et al., 2008; Lopez-Martin et al., 2008; Watanabe et al., 2008a; Watanabe et al., 2008b; Krueger et al., 2009). If cysteine can be easily translocated between subcellular compartments, as the experimental data suggests, why are so many isoforms with the same enzymatic activity required in plant cells?

The biosynthesis of Cys in bacteria is accomplished by isoenzymes A (encoded by *cysK*) and B (encoded by *cysM*); the latter is also able to incorporate thiosulfate to produce S-Cys. S-Cys biosynthesis has been also reported in several purple photosynthetic bacterial strains, such as *Rhodospirillum tenue, Rhodopseudomonas gelatinosa, Chromatium vinosum* and *Thiocapsa pfennigii,* among others (Hensel and Trüper, 1976). However, this activity has not been previously reported in plants or eukaryotic microorganisms, nor has the existence of the metabolite S-sulfocysteine been reported. Currently, the one exception that has been reported is the cysteine synthase enzyme from *Trichomonas vaginalis,* which is an anaerobic protozoan parasite of humans; this enzyme is able to use sulfide or thiosulfate as a substrate to produce Cys or S-Cys (Westrop et al., 2006). This protozoan lacks glutathione and therefore uses Cys as the major cellular reducing agent.

Our work clearly demonstrates that the minor chloroplastic O-acetylserine(thiol)lyase isoform encoded by the *CS26* gene from *Arabidopsis* is in fact a S-sulfocysteine synthase enzyme. This conclusion is based on the fact that the recombinant protein had no detectable OASTL activity but catalyzed the formation of S-Cys, which was identified by mass spectrometry. The SSCS activity observed in the recombinant protein

was not due to the deletion of the N-terminal signal peptide or the addition of the His-tag, because the null allele mutants of this gene lost SSCS activity when compared to that of the wild type. Furthermore, the complemented *cs26* null allele recovered SSCS activity. Therefore, the enzymatic SSCS activity of the CS26 protein is not an artifact of the recombinant protein. In addition, a biochemical comparison of the *oas-b* and *cs26* null mutants demonstrated that the *cs26* mutation had no influence on the level of OASTL activity, whereas the *oas-b* mutant had significantly less OASTL activity, similar to the values previously reported by other authors (Watanabe et al., 2008a). The loss of CS26 function resulted in dramatic changes in phenotype and ROS accumulation, which were dependent on the light regime. Although other studies have not described this anomalous phenotype in *cs26* null mutants, they routinely cultured plants on germination media containing 1% sucrose (Watanabe et al., 2008a).

A further argument for the function of CS26 is that comparison of the protein sequences of CS26 and OAS-B and homologous proteins from bacteria (encoded by *cysK* and *cysM*) shows that CS26 is more similar to the CysM enzyme. The CS26 protein has a 16 amino acid deletion between the ß8A and ß9A domains, which are necessary for interaction with SAT (Bonner et al., 2005). This deletion is also present in the isoform encoded by *cysM* from *E. coli,* which is unable to interact with SAT (Claus et al., 2005; Zhao et al., 2006). The inability to bind SAT has also been suggested for the CS26 protein from *Arabidopsis*, because this protein was not retained on a SAT affinity column (Heeg et al., 2008).

The CS26 protein is predicted to be localized within the chloroplast based on its N-terminal signal peptide. A proteomic analysis of chloroplast fractions by LTQ-Orbitrap mass spectrometry confirmed the localization of CS26 to the chloroplast; additionally, the small number of tryptic peptides identified confirms the low abundance of this protein (Zybailov et al., 2008). Our analysis of SSCS activity in an enriched chloroplast preparation from the wild type and mutant lines supports the subcellular localization of CS26. The presence of sulfocysteine synthase activity in the chloroplast could represent residual activity due to an endosymbiotic origin. The role of *cysM* in bacteria has not been clearly determined; however, this isoenzyme is required for efficient Cys biosynthesis during anaerobic growth (Filutowicz et al., 1982). Although S-Cys can be degraded to generate

Cys, the phenotype observed in the null alleles under long-day growth conditions and comparison of that phenotype with the phenotype of the *oas-b* mutant suggests that S-Cys must play an important role in the chloroplast and also that this function does not rely on the capacity to synthesize Cys.

The transcriptional profile of the *cs26* mutant under short-day growth conditions showed that the mutation produces significant changes in the transcript levels of nuclear- and chloroplast-encoded genes, suggesting that S-Cys must have an important role in chloroplast function. The increased number of transcripts that changed under long-day growth conditions could be due to a synergistic effect of the oxidative stress that the mutant is exposed to during the prolonged period of light. Many of these genes are involved in biotic stress responses and correlate with previously published observations about the chloroplast signaling crosstalk between light acclimation and immunity in *Arabidopsis* (Muhlenbock et al., 2008).

Although we are not able to predict the molecular target of S-sulfocysteine, a recent publication has identified *CS26* as one of the target genes of the Long-Term Acclimation (LTR) signaling pathway—genes regulated to compensate for the lack of LTR signaling (Pesaresi et al., 2009). This conclusion is supported by the identification of commonly regulated genes in the *stn7-1, psad1-1* and *psae1-3* mutants, impaired in different components necessary for state transitions. The *psad1-1* and *psae1-3* mutants lack of PSI subunit D1 and E1, respectively. The *stn7-1* mutant lacks the thylakoid protein kinase STN7 and shows drastically reduced phosphorylation of the light-harvesting system of PSII (LHCII) and markedly reduced state transitions. Either short-term and long-term light quality acclimation in plants requires a severe photosystem stoichiometry adjustment that is regulated by the plastoquinone (PQ) redox state and the STN7 kinase (Kanervo et al., 2005; Dietzel et al., 2008). STN7, and its ortholog STT7 in *Chlamydomonas*, contains a transmembrane region that separates its catalytic kinase domain on the stromal side from its N-terminal end in the thylakoid lumen with two conserved cysteines that are critical for its activity. A disulfide bridge between these two cysteines is required for kinase activity but how the redox states of these two cysteines are regulated in the lumen remains an open question (Lemeille et al., 2009). The protein CS26 contains a signal peptide longer than its counterpart OAS-B and computer predictions suggest that this protein is located in the

lumen (The Plant Proteome Database, http://ppdb.tc.cornell.edu/(Zybailov et al., 2008)). S-Cys ($RSSO_3^-$) may act chemically as an oxidant molecule by reacting with reduced thiols (R-SH) according to the following reaction: $RSSO_3^- + RSH \leftrightarrows RSSR + HSO_3^-$. Although the equilibrium of this reaction is shifted to the left in normal conditions, when RSH is in greater abundance than RSSR, the equilibrium shifts to the right side (Neta and Huie, 1985). Therefore, S-Cys in the lumen may act by regulating the oxidation of the thiol residues of STN7, therefore affecting light acclimation.

Although the hypothesis suggested above requires a thorough investigation, which is already underway, the work presented here demonstrates for the first time the presence of S-sulfocysteine synthase activity in plants and the importance of the metabolite S-sulfocysteine in the redox regulation of the chloroplast in certain light conditions. Even under short-day conditions where ROS accumulation was not observed, the absence of S-Cys and the activity of S-Cys synthase could trigger several signaling events involved in many biological processes related to photosynthesis maintenance and stress responses.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (accession Col-0) and the SALK_02183, SALK_034133, GABI_376E08 and GABI_684B07 mutants used in this work were provided by the NASC (European *Arabidopsis* Stock Centre). The plants were grown in soil supplemented with Hoagland media or in solid Murashige & Skoog media in Petri dishes supplemented with 30 mg mL^{-1} kanamycin (for the SALK mutants) or 7.5 mg mL^{-1} sulfadiazine (for the GABI mutants). Plants were grown under a long-day photoperiod of 16 h of white light at 20ºC/8 h in the dark at 18ºC, under a short-day photoperiod of 8 h of white light at 20ºC/16 h in the dark at 18ºC or under continuous white light. The light intensity ranged from 140 – 160 E m^{-2} s-^{1}.

To generate the *cs26* complementation lines, the 1215-bp cDNA fragment containing the full-length coding sequence of *cs26* was obtained by RT-PCR amplification

that was extended from the CACC at the 5'-end using the proofreading Platinum Pfx enzyme (Invitrogen). The fragment was first cloned into the pENTR/D-TOPO vector (Invitrogen) and then transferred into the vector pMDC32 (Curtis and Grossniklaus, 2003) using the Gateway technology (Invitrogen) according to manufacturer's instructions. The final construct was introduced by electroporation into *Agrobacterium tumefaciens*, which was then introduced into the *cs26* null plants using the floral dipping method (Clough and Bent, 1998).

Bacterial expression and purification of the recombinant protein

The deduced amino acid sequences of the homologous proteins OAS-B and CS26 were aligned with the sequence of the most abundant cytosolic protein OAS-A1. The comparison revealed that CS26 contains an extension at the C-terminus of the chloroplast transit peptide compared to OAS-B. The TargetP program was used to predict the N-terminal chloroplast transit peptide (cTP) of the native protein; a protein with the 94 N-terminal amino acids deleted was considered to be the mature CS26 protein. We cloned the mature coding region of the *Arabidopsis* CS26 gene into the Gateway destination vector pDEST™17 for high-level expression of the recombinant protein in *E. coli*. The pDEST™17 vector generated a N-terminal 6xHis-tagged protein in order to facilitate purification of the recombinant protein.

Chemically competent *E. coli* BL21-A1 cells (Invitrogen) were transformed with the pDEST™17 vector (Invitrogen) containing the coding region of the *CS26* gene with the N-terminal 94-amino acid deletion and selected on LB agar medium containing 100 µg mL^{-1} ampicillin. Single colonies were picked and inoculated into 5 mL LB medium containing 100 µg mL^{-1} ampicillin and incubated a 37°C until the bacterial culture reached an optical density (OD_{600}) between 0.6 and 1.0. This culture was used to inoculate a fresh 100 mL LB/ampicillin culture and incubated at 28°C until the OD_{600} reached 0.4. At this point, the cells were induced with 0.2% (w/v) arabinose and incubated overnight at 28°C. Bacteria were harvested by centrifugation and resuspended in 8 mL of 20 mM Tris-HCl buffer, pH 8.0. The protein crude extract was obtained after sonication of the bacterial sample and centrifugation of the lysate for 10 min at 4°C to pellet the cellular debris.

The recombinant protein was purified in a Ni-NTA agarose column (Invitrogen) under native conditions. The bacterial crude extract (approx. 8 mL) was added to a 10 mL column containing 1.5 mL of Ni-NTA resin and incubated with gentle agitation for 1 h. The resin was settled by centrifugation (800 x g), and the supernatant was removed by aspiration. The resin was washed four times, each time with 8 mL of wash buffer (50 mM NaH$_2$PO$_4$, pH 8.0; 100 mM NaCl; 20 mM imidazole); the protein was finally eluted with 8 mL of elution buffer (50 mM NaH$_2$PO$_4$, pH 8.0; 100 mM NaCl; 250 mM imidazole). One milliliter fractions were collected and analyzed by SDS-PAGE.

RNA isolation, RT-PCR analysis and identification of T-DNA insertion lines

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 PCR reactions using the following primers: F-26, 5'-CGTCTCCTTCGCTCCGTCTTCTTCCTCAGT-3' and R-26, 5'-ATATCTCATCTCTTGGACTTCTCTGTTG-3' for the CS26 gene; F-B, 5'-CATGGCGGCGACATCTTCCTCT-3' and R-B 5'-AAGCTCGGGCTGCATTTGC-3' for the OAS-B gene; and UBQ10F, 5'-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3' and UBQ10R, 5'-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3' for the constitutive *UBQ10* gene, which was used as a control. PCR conditions were as follows: a denaturation cycle of 2 min at 94ºC, 35 amplification cycles of 1 min at 94ºC, 1 min at 60ºC and 1 min at 72ºC and an extension cycle of 5 min at 72ºC.

To identify individual plants with homozygous T-DNA insertions, genomic DNA was extracted from the kanamycin-resistant seedlings of the SALK mutants or from sulfadiazine-resistant seedlings of the GABI mutants. DNA was subjected to PCR genotyping using the following primers: F-26, R26, LBb1, 5'-GCGTGGACCGCTTGCTGCAACT-3' and LBb2, 5'-CCCATTTGGACGTGAATGTAGACAC-3' for the different *cs26* alleles; F-B, R-B, LBb1 and LBb2 for the different *oas-b* alleles. PCR conditions were as follows: a denaturation cycle of 2 min at 94ºC, 35 amplification cycles of 1 min at 94ºC, 1 min at 60ºC and 1 min at 72ºC and an extension cycle of 5 min at 72ºC.

Microarray hybridization and data analysis

Total RNA was isolated with the Trizol reagent (Invitrogen) followed by cleaning with the RNeasy Plant Mini Kit (Qiagen); this preparation was used to synthesize biotinylated cDNA for hybridization to *Arabidopsis* ATH1 arrays (Affymetrix), using the 3′ Amplification One-Cycle Target Labeling Kit. Briefly, 4 mg of RNA was reverse transcribed to produce first strand cDNA using a $(dT)_{24}$ primer with a T7 RNA polymerase promoter site added to the 3' end. After second strand synthesis, *in vitro* transcription was performed using T7 RNA polymerase and biotinylated nucleotides to produce biotin-labeled complementary RNA (cRNA). The cRNA preparations (15 μg) were fragmented into 35 to 200 bp fragments at 95°C for 35 min. The fragmented cRNA was hybridized to the *Arabidopsis* ATH1 microarrays at 45°C for 16 h. Each microarray was washed and stained in the Affymetrix Fluidics Station 400 following standard protocols. Microarrays were scanned using an Affymetrix GeneChip® Scanner 3000.

Microarray analysis was performed using the affylmGUI R package (Wettenhall et al., 2006). The Robust Multi-array Analysis (RMA) algorithm was used for background correction, normalization and summarization of expression levels (Irizarry et al., 2003). Differential expression analysis was performed using Bayes t-statistics using the linear models for microarray data (Limma)*,* which is included in the affylmGUI package. *P*-values were corrected for multiple-testing using Benjamini-Hochberg's method (FDR, False Discovery Rate) (Benjamini and Hochberg, 1995; Reiner et al., 2003). A cutoff value of twofold, FDR value of less than 0.05 and lgSignal >7 were adopted to discriminate the genes with altered expression.

Quantitative real-time reverse transcriptase-PCR (qRT-PCR) was used to validate microarray data. Gene-specific primers were designed using the Vector NTI Advance 11 software (Invitrogen). Real-time PCR reaction was performed using iQ™ SYBR Green Supermix (Bio-Rad), and 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, 45 cycles of 95°C for 15 s and 60°C for 1 min. A melt curve from 60 to 90°C was run following the PCR cycling. The expression level of the genes of interest was normalized to that of the constitutive *UBQ10* gene by subtracting the CT value of *UBQ10* from the CT

value of the gene (ΔC_T). Fold change was calculated as $2^{-(\Delta C_T \text{ mutant} - \Delta C_T \text{ wild type})}$. The results shown are mean values ± SD. Three biological replicates were performed, and each reaction was run in triplicate.

Assay of enzymatic activities

OASTL activity was measured in plant and bacterial crude extracts using a previously described method (Barroso et al., 1995). ß-Cyanoalanine synthase activity was measured by the release of sulfide from L-Cys and cyanide as previously described (Papenbrock and Schmidt, 2000; Meyer et al., 2003). L-Cys desulfhydrase activity was also measured by the release of sulfide from Cys as previously described (Riemenschneider et al., 2005). S-Sulfocysteine synthase activity was measured by incubation of a mixture consisting of 250 µmol Tris-HCl, pH 8.0, 15 µmol $Na_2S_2O_3$, 60 µmol O-acetylserine and the enzyme solution in a final volume of 1.5 mL. After preincubation for 30 s at 30°C, the reaction was initiated by the addition of O-acetylserine and then incubated at 30°C for 30 min. The reaction was stopped by transferring 0.2 mL of the reaction mixture into 0.2 mL of ethanol, followed by the addition of 2 mL of water. One milliliter of the diluted sample was used for colorimetric determination of thiosulfate, as previously described (Sorbo, 1957). One unit of enzyme activity was defined as the amount that catalyzed the consumption of 1 µmol of thiosulfate per min. The total amounts of protein were determined by the Bradford method (Bradford, 1976).

Analysis by HPLC-ESI/MS

Analyses were carried out with a linear ion trap mass spectrometer equipped with an electrospray ionization source (ESI) (Bruker Daltonics) coupled to a liquid chromatograph (Ultimate 3000, Dionex). The analyses were performed by injecting 5 µL aliquots of a standard solution or sample extract onto a reverse phase column (Acclaim 120 Å C18, 2.1 x 100 mm, 3 µm particle size) fitted with a precolumn (Acclaim 120 Å C18, 2.1 x 10 mm, 3 µm particle size). The temperature of the column was fixed at 25ºC and the sample was eluted at a flow rate of 0.25 mL min^{-1} with an elution gradient composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). We used the following gradient profile: a linear gradient from 0 to 10% B (0-6 min), from 10 to 50% B

(6-7 min), 50% B (7-10 min) and a linear gradient from 50 to 0% B (10-15 min). Samples were detected in positive ion mode with ESI parameter values of 40 V for the nebulizer, 9 L min^{-1} of drying gas and 365°C for the drying gas temperature. The MS signal was optimized by direct injection of a standard solution (in 0.1% formic acid) of the metabolite. The spectra were acquired in the mass/charge ratio (m/z) range of 50-700. The system was controlled with the software package HyStar (version 3.2, Bruker Daltonics) and the data were processed with the Data Analysis software (version 3.2, Bruker Daltonics).

Isolation of chloroplasts

Enriched chloroplast preparations from *Arabidopsis* were isolated from 40 g of leaves according to previous work (Kieselbach et al., 1998). The leaves were homogenized with a mixer in 170 mL of a solution containing 20 mM Tricine-NaOH (pH 8.4), 300 mM sorbitol, 10 mM EDTA, 10 mM KCl, 0.25% BSA (w / v), 4.5 mM sodium ascorbate and 5 mM cysteine. Homogenates were then filtered through 4 layers of nylon mesh (20 μm) and centrifuged for 1 min at 1000xg. The pellet was resuspended with a brush in a solution containing 20 mM HEPES-NaOH (pH 7.8), 300 mM sorbitol, 5 mM MgCl$_2$, 2.5 mM EDTA, 10 mM KCl, centrifuged again and the pellet resuspended in 6 mL of the above solution. This provides 60-70% intact chloroplasts estimated by microscopic observation, containing 50-60 mg of chlorophyll. For analysis by LC / MS, chloroplasts were concentrated in 1.5 mL of 0.1 N HCl and 0.1% formic acid, and passed through a 0.22 μm filter.

Quantification of Thiol Compounds, Photosynthetic Pigments and Photosynthetic Oxygen Evolution

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 previously described (Dominguez-Solis et al., 2001). Photosynthetic pigments were extracted from 0.1 g (fresh weight) of leaf tissue and used for the estimation of Chl a, Chl b and total carotenoids according to previous work (Lichtenthaler, 1987).

Photosynthetic oxygen evolution from leaves was measured with a Clark-type 2 electrode (Hansatech). Leaves from 4-week-old plants grown under long-day conditions or from 5-week-old plants grown under short-day conditions were excised, washed with distilled water and cut into 2 mm diameter disks. The samples were then immediately transferred into assay buffer (50 mM HEPES/NaOH, 0.5 mM $CaSO_4$, pH 7.2) and vacuum infiltrated for 15 min. Thirty leaf disks for each measurement were placed in 2 mL of buffer in the oxygen electrode cuvette that was maintained at 25ºC; argon gas was then bubbled into the buffer to remove dissolved O_2. The leaf disks were preilluminated for 3 min and photosynthesis was initiated by addition of 50 μL of 0.42 M $NaHCO_3$. The increase in the O_2 level in the buffer was measured for 10-12 min under 500 μmol m^{-2} s^{-1} of incident light.

Detection of Reactive Oxygen Species

For the detection of the superoxide radical anion, leaves were immersed in a solution of 0.5 mg mL^{-1} NBT (nitroblue tetrazolium, Sigma-Aldrich) in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl and 0.05 M $MgCl_2$ for 2 h at room temperature in the dark and then photographed. For the detection of H_2O_2, leaves were immersed in 1 mg mL^{-1} DAB (diaminobenzidine, Sigma-Aldrich), fixed with a solution of 3:1:1 (v/v/v) ethanol:lactic acid:glycerol and photographed.

Accession numbers

Sequence and microarray data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers:

CS26 (At3g03630) , *OAS-B* (At2g43750), *OAS-A1* (At4g14880), *OAS-A2* (At3g22460), *OAS-C* (At3g59760), *CYS-C1* (At3g61440), *CYS-D1* (At3g04940), *CYS-D2* (At5g28020), *CS-LIKE/DES1* (At5g28030), .

CS26 T-DNA mutants: SALK_034133 and GABI_376E08

OAS-B T-DNA mutants: GABI_684B07 and SALK_02183

Microarray Gene Expression Omnibus (GEO) accession number GSE19241.

Supplemental data online

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

Supplemental Figure 1. Alignment of the cytosolic OAS-A1 and plastid CS26 and OAS-B deduced amino acid sequences.

Supplemental Figure 2. SDS-PAGE analysis of the His-CS26 recombinant protein.

Supplemental Figure 3. Characterization of the *cs26* and *oas-b* mutant lines.

Supplemental Figure 4. Phenotypes of the null alleles from mutants *cs26*.

Supplemental Table 1. Validation of microarray data.

Supplemental Dataset 1. List of differentially regulated genes in leaves of the *cs26* mutant grown with long-day conditions compared to wild type.

Supplemental Dataset 2. List of differentially regulated genes in leaves of the *cs26* mutant grown with short-day conditions compared to wild type

ACKNOWLEDGMENTS

FIGURE LEGENDS

Figure 1. Cysteine biosynthesis network and subcellular localization of SAT and OASTL enzymes in *Arabidopsis thaliana* cells. The biosynthesis of cysteine can occur in any of three different cellular compartments: the cytosol, the chloroplast and the mitochondria. The biosynthetic enzymes are represented by five different SAT isoforms and eight functional OASTL isoforms.

Figure 2. ESI/MS mass spectra of S-Cys in positive ionization mode.
(A) The theoretical mass spectrum and the molecular structure of S-Cys.
(B) The mass spectrum of a standard solution of S-Cys.
(C) The mass spectrum of the enzymatic reaction products produced by recombinant His-CS26 using thiosulfate and O-acetylserine as substrates

Figure 3. Fragmentation pattern of S-Cys samples by ESI/MS in positive ionization mode.
(A) Mass spectrum of the S-Cys ion $[M+H]^+$ isolated in the ion trap.
(B) MS^2 fragmentation pattern of the parental ion at m/z 201.9.
(C) MS^3 fragmentation pattern of the parental ion at m/z 120.0. The arrow indicates the fragmented ion.
(D) The bottom panel shows the details of the ionization and fragmentation of S-Cys samples and identification of the detected ions.

Figure 4. Partial alignment of the chloroplastic OAS-B and CS26 deduced amino acid sequences. The alignment was created with the Vector NTI software to compare the proteins encoded by the *cysK* and *cysM* genes from *Escherichia coli* and *Salmonella typhimurium*. The blue arrow indicates the ß-sheet domains deduced from the crystal structure of the cytosolic OAS-A1 from *Arabidopsis thaliana* (Bonner et al., 2005).

Figure 5. Phenotypes of the null mutants *cs26* and *oas-b* and the complemented line *cs26*::P35S-CS26.

(A) Plants grown on soil under long-day (LD) or short-day (SD) conditions for 3 and 4 weeks, respectively.

(B) Plants grown on soil under continuous light for 4 weeks.

Figure 6. Pigment contents and photosynthetic activity in leaves of the null mutants *cs26* and *oas-b*.

(A) Chlorophylls a and b and carotenoid contents were measured in acetone extracts of leaves of wild type and mutant plants grown under long-day conditions for 4 weeks. Values are means $\pm$ SD of three independent determinations. Asterisks (*) indicate statistical significance of $P < 0.01$.

(B) Photosynthetic oxygen evolution measured with an oxygen electrode from disks excised from leaves of wild type and mutant plants grown under long-day conditions for 4 weeks. A representative experiment is shown.

(C) Photosynthetic oxygen evolution measured with an oxygen electrode from disks excised from leaves of wild type and mutant plants grown under short-day conditions for 5 weeks. A representative experiment is shown.

Figure 7. **Detection of accumulation of reactive oxygen species in the leaves of the null mutant *cs26*.**

(A) and **(B)** Histochemical detection of the superoxide radical anion by staining with nitroblue tetrazolium chloride (NBT) in the wild type **(A)** and *cs26* mutant **(B)** in leaves from 3-week-old plants grown in long-day conditions.

(C) and **(D)** Histochemical detection of H_2O_2 by staining with 3,3'-diaminobenzidine (DAB) in the wild type **(C)** and *cs26* mutant **(D)** leaves from 3-week-old plants growing in long-day conditions.

(E) and **(F)** Detection of the superoxide radical anion in the wild type **(E)** and *cs26* mutant **(F)** leaves from 5-week-old plants grown in short-day conditions.

(G) and **(H)** Detection of H_2O_2 in the wild type **(G)** and *cs26* mutant **(H)** leaves from 5-week-old plants grown in short-day conditions. The experiments were repeated at least five times with similar results.

Figure 8. S-sulfocysteine synthase (SSCS) activities in whole-leaf and chloroplast extracts. SSCS activity was measured in whole-leaf (white columns) and chloroplast extracts (black columns) from the wild type and the *oas-b* and *cs26* mutant lines. Values are means ± SD of three independent determinations.

Figure 9. Transcriptional profile of the cs26 mutant grown under long- and short-day photoperiod.

(A) Phenotypes of *cs26* and wild type plants grown with long-day (LD) and short-day (SD) conditions.

(B) Venn diagram showing the number of genes that changed in expression in the *cs26* mutant compared to wt plants in long-day (yellow circle) and short-day (blue circle) conditions. The intersection (green area) shows the number of genes that are common in both profiles. Blue arrows show the number of genes that are up-regulated. Red arrows show the number of genes that are down regulated.

(C) Gene Ontology categorization of biological processes of the genes that have modified expression in long-day (yellow bars) and short-day (blue bars) growth conditions.

Table 1. Purification of His-CS26 expressed in *E. coli.*

The gene encoding CS26 was cloned into the pDEST^{TM}17 vector using the Gateway technology, and the protein expressed in *E. coli* was purified using the Ni-NTA purification system as described in the Materials and Methods. S-sulfocysteine synthase (SSCS) and O-acetylserine(thiol)lyase (OASTL) activities were measured as described. nd, not detectable.

Purification step	Protein (mg)	Specific activity (U mg^{-1})		Total activity (U)		Purification Factor		Yield (%)	
		SSCS	OASTL	SSCS	OASTL	SSCS	OASTL	SSCS	OASTL
Crude extract	4.00	0.60	0.32	2.39	1.25	-	-	-	-
Ni-NTA agarose chromatography	0.12	11.46	nd	1.38	nd	19.1	nd	57.6	nd

Table 2. Enzyme activity levels and thiol contents in leaves.

Enzyme activities and quantification of thiol compounds were determined in leaves of wild type and the *cs26* and *oas-b* mutant plants grown on soil under long-day conditions. Values are means $\pm$ SD (n = 4). Asterisks (*) indicate statistical significance of *P* < 0.01. nd, not detectable.

Plant line	OASTL activity (U/mg prot)	SSCS activity (mU/mg prot)	Total Cysteine content (nmol/g FW)	Total Glutathione content (nmol/g FW)
Wild type	3.65 ± 0.08	20.7 ± 2.1	16.6 ± 2.0	256.7 ± 17
cs26	3.36 ± 0.04	nd	17.9 ± 1.6	338.0 ± 21 [*]
oas-b	2.92 ± 0.05 [*]	31.7 ± 3.8 [*]	16.3 ± 1.8	269.16 ± 15

REFERENCES

Alvarez, C., Calo, L., Romero, L.C., Garcia, I., and Gotor, C. (2009). An O-Acetylserine(thiol)lyase Homolog with L-Cysteine Desulfhydrase Activity Regulates Cysteine Homeostasis in *Arabidopsis thaliana*. Plant Physiol.**,** pp.109.147975.

Barroso, C., Vega, J.M., and Gotor, C. (1995). A new member of the cytosolic O-acetylserine(thiol)lyase gene family in Arabidopsis *thaliana*. FEBS Let. **363:** 1-5.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. J R Statist Soc B **57:** 289-300.

Bonner, E.R., Cahoon, R.E., Knapke, S.M., and Jez, J.M. (2005). Molecular basis of cysteine biosynthesis in plants - Structural and functional analysis of O-acetylserine sulfhydrylase from *Arabidopsis thaliana*. J. Biol. Chem. **280:** 38803-38813.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254.

Byrne, C.R., Monroe, R.S., Ward, K.A., and Kredich, N.M. (1988). DNA-Sequences of the cysK regions of *Salmonella typhimurium* and *Escherichia coli* and linkage of the cysK regions to ptsH. J. Bacteriol. **170:** 3150-3157.

Claus, M.T., Zocher, G.E., Maier, T.H.P., and Schulz, G.E. (2005). Structure of the O-acetylserine sulfhydrylase isoenzyme CysM from *Escherichia coli*. Biochemistry **44:** 8620-8626.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735-743.

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. **133:** 462-469.

Denk, D., and Bock, A. (1987). L-Cysteine biosynthesis in *Escherichia coli* - Nucleotide-sequence and expression of the serine acetyltransferase (cysE) gene from the wild-type and a cysteine-excreting mutant. J. Gen. Microbiol. **133:** 515-525.

Dietzel, L., Brautigam, K., and Pfannschmidt, T. (2008). Photosynthetic acclimation: state transitions and adjustment of photosystem stoichiometry--functional relationships between short-term and long-term light quality acclimation in plants. FEBS J. **275:** 1080-1088.

Dominguez-Solis, J.R., Gutierrez-Alcala, G., Vega, J.M., Romero, L.C., and Gotor, C. (2001). The cytosolic O-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. J. Biol. Chem. **276:** 9297-9302.

Dominguez-Solis, J.R., Lopez-Martin, M.C., Ager, F.J., Ynsa, M.D., Romero, L.C., and Gotor, C. (2004). Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*. Plant Biotech. J. **2:** 469-476.

Filutowicz, M., Wiater, A., and Hulanicka, D. (1982). Delayed inducibility of sulphite reductase in cysM mutants of *Salmonella typhimurium* under anaerobic conditions. J. Gen. Microbiol. **128:** 1791-1794.

Hatzfeld, Y., Maruyama, A., Schmidt, A., Noji, M., Ishizawa, K., and Saito, K. (2000). beta-cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and *Arabidopsis*. Plant Physiol. **123:** 1163-1171.

Heeg, C., Kruse, C., Jost, R., Gutensohn, M., Ruppert, T., Wirtz, M., and Hell, R. (2008). Analysis of the *Arabidopsis* O-Acetylserine(thiol)lyase Gene Family Demonstrates Compartment-Specific Differences in the Regulation of Cysteine Synthesis. Plant Cell **20:** 168-185.

Hensel, G., and Trüper, H.G. (1976). Cysteine and S-sulfocysteine biosynthesis in phototrophic bacteria. Arch. Microbiol. **109:** 101-103.

Howarth, J.R., Dominguez-Solis, J.R., Gutierrez-Alcala, G., Wray, J.L., Romero, L.C., and Gotor, C. (2003). The serine acetyltransferase gene family in *Arabidopsis thaliana* and the regulation of its expression by cadmium. Plant Mol. Biol. **51:** 589-598.

Hulanicka, M.D., Hallquist, S.G., Kredich, N.M., and Mojica, T. (1979). Regulation of O-acetylserine sulfhydrylase-B by L-cysteine in *Salmonella typhimurium*. J. Bacteriol. **140:** 141-146.

Hulanicka, M.D., Garrett, C., Jaguraburdzy, G., and Kredich, N.M. (1986). Cloning and characterization of the cysAMK region of *Salmonella typhimurium.* J. Bacteriol. **168:** 322-327.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:** 249-264.

Kanervo, E., Suorsa, M., and Aro, E.M. (2005). Functional flexibility and acclimation of the thylakoid membrane. Photochem. Photobiol. Sci. **4:** 1072-1080.

Kieselbach, T., Hagman, Andersson, B., and Schroder, W.P. (1998). The thylakoid lumen of chloroplasts. Isolation and characterization. J. Biol. Chem. **273:** 6710-6716.

Krueger, S., Niehl, A., Lopez-Martín, M.C., Steinhauser, D., Donath, A., Hildebrandt, T., Romero, L.C., Hoefgen, R., Gotor, C., and Hesse, H. (2009). Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in *Arabidopsis*. Plant Cell cell Environ. **32:** 349-367.

Lemeille, S., Willig, A., Depege-Fargeix, N., Delessert, C., Bassi, R., and Rochaix, J.D. (2009). Analysis of the chloroplast protein kinase Stt7 during state transitions. PLoS Biol. **7:** e45.

Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids - Pigments of photosynthetic biomembranes. Methods Enzymol. **148:** 350-382.

Lopez-Martin, M.C., Becana, M., Romero, L.C., and Gotor, C. (2008). Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in *Arabidopsis*. Plant Physiol. **147:** 562-572.

Meyer, T., Burow, M., Bauer, M., and Papenbrock, J. (2003). *Arabidopsis* sulfurtransferases: investigation of their function during senescence and in cyanide detoxification. Planta **217:** 1-10.

Muhlenbock, P., Szechynska-Hebda, M., Plaszczyca, M., Baudo, M., Mullineaux, P.M., Parker, J.E., Karpinska, B., and Karpinski, S. (2008). Chloroplast Signaling and LESION SIMULATING DISEASE1 Regulate Crosstalk between Light Acclimation and Immunity in *Arabidopsis*. Plant Cell **20:** 2339-2356.

Nakamura, T., Iwahashi, H., and Eguchi, Y. (1984). Enzymatic proof for the identity of the S-sulfocysteine synthase and cysteine synthase-B of *Salmonella typhimurium*. J. Bacteriol. **158:** 1122-1127.

Neta, P., and Huie, R.E. (1985). Free-radical chemistry of sulfite. Environ. Health Perspect. **64:** 209-217.

Papenbrock, J., and Schmidt, A. (2000). Characterization of two sulfurtransferase isozymes from *Arabidopsis thaliana*. Eur. J. Biochem. **267:** 5571-5579.

Pesaresi, P., Hertle, A., Pribil, M., Kleine, T., Wagner, R., Strissel, H., Ihnatowicz, A., Bonardi, V., Scharfenberg, M., Schneider, A., Pfannschmidt, T., and Leister, D. (2009). *Arabidopsis* STN7 kinase provides a link between short- and long-term photosynthetic acclimation. Plant Cell **21:** 2402-2423.

Reiner, A., Yekutieli, D., and Benjamini, Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics **19:** 368-375.

Riemenschneider, A., Riedel, K., Hoefgen, R., Papenbrock, J., and Hesse, H. (2005). Impact of reduced O-acetylserine(thiol)lyase isoform contents on potato plant metabolism. Plant Physiol. **137:** 892-900.

Sorbo, B. (1957). A colorimetric method for the determination of thiosulfate. Biochim. Biophys. Acta **23:** 412-416.

Watanabe, M., Kusano, M., Oikawa, A., Fukushima, A., Noji, M., and Saito, K. (2008a). Physiological roles of the beta-substituted alanine synthase gene family in *Arabidopsis*. Plant Physiol. **146:** 310-320.

Watanabe, M., Mochida, K., Kato, T., Tabata, S., Yoshimoto, N., Noji, M., and Saito, K. (2008b). Comparative Genomics and Reverse Genetics Analysis Reveal Indispensable Functions of the Serine Acetyltransferase Gene Family in *Arabidopsis*. Plant Cell **20:** 2484-2496.

Westrop, G.D., Goodall, G., Mottram, J.C., and Coombs, G.H. (2006). Cysteine Biosynthesis in *Trichomonas vaginalis* Involves Cysteine Synthase Utilizing O-Phosphoserine. J. Biol. Chem. **281:** 25062-25075.

Wettenhall, J.M., Simpson, K.M., Satterley, K., and Smyth, G.K. (2006). affylmGUI: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics **22:** 897-899.

Wirtz, M., Droux, M., and Hell, R. (2004). O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in *Arabidopsis thaliana*. J. Exp. Bot. **55:** 1785-1798.

Zhao, C., Kumada, Y., Imanaka, H., Imamura, K., and Nakanishi, K. (2006). Cloning, overexpression, purification, and characterization of O-acetylserine sulfhydrylase-B from *Escherichia coli*. Prot. Expr. Purif. **47:** 607-613.

Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., and van Wijk, K.J. (2008). Sorting Signals, N-Terminal Modifications and Abundance of the Chloroplast Proteome. PLoS ONE **3:** e1994.