Successful Fertilization Requires the Presence of at Least One Major *O*-Acetylserine(thiol)lyase for Cysteine Synthesis in Pollen of Arabidopsis[1][C][W][OPEN]

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The synthesis of cysteine (Cys) is a master control switch of plant primary metabolism that coordinates the flux of sulfur with carbon and nitrogen metabolism. In Arabidopsis (*Arabidopsis thaliana*), nine genes encode for *O*-acetylserine(thiol)lyase (OAS-TL)-like proteins, of which the major isoforms, OAS-TL A, OAS-TL B, and OAS-TL C, catalyze the formation of Cys by combining *O*-acetylserine and sulfide in the cytosol, the plastids, and the mitochondria, respectively. So far, the significance of individual OAS-TL-like enzymes is unresolved. Generation of all major OAS-TL double loss-of-function mutants in combination with radiolabeled tracer studies revealed that subcellular localization of OAS-TL proteins is more important for efficient Cys synthesis than total cellular OAS-TL activity in leaves. The absence of *oastl* triple embryos after targeted crosses indicated the exclusiveness of Cys synthesis by the three major OAS-TLs and ruled out alternative sulfur fixation by other OAS-TL-like proteins. Analyses of *oastlABC* pollen demonstrated that the presence of at least one functional OAS-TL isoform is essential for the proper function of the male gametophyte, although the synthesis of histidine, lysine, and tryptophan is dispensable in pollen. Comparisons of *oastlABC* pollen derived from genetically different parent plant combinations allowed us to separate distinct functions of Cys and glutathione in pollen and revealed an additional role of glutathione for pollen germination. In contrast, female gametogenesis was not affected by the absence of major OAS-TLs, indicating significant transport of Cys into the developing ovule from the mother plant.

Sulfur assimilation in plants is hallmarked by two reaction sequences, namely sulfate reduction and Cys synthesis. The sulfate reduction pathway consists of three steps and produces sulfide from sulfate, which is available in the soil and transported into the roots by specific transporters (Takahashi et al., 2011). Sulfide is subsequently incorporated into the amino acid *O*-acetylserine (OAS) by *O*-acetylserine(thiol)lyase (OAS-TL; EC 2.5.1.47) to produce Cys (Hell and Wirtz, 2011). Cys then serves as the sulfur source for all organic metabolites containing reduced sulfur in plants, including proteins, cofactors, and secondary metabolites. The tripeptide glutathione (GSH) is one of the most important Cys-derived metabolites, since it has an important function in redox homeostasis and the control of development (Meyer and Rausch, 2008). Impaired GSH synthesis negatively affects growth of the shoot and root system of Arabidopsis (*Arabidopsis thaliana*; Vernoux et al., 2000; Xiang et al., 2001), and loss-of-function mutants for the first enzyme (GSH1, Glu-Cys ligase; EC 6.3.2.2) or the second enzyme (GSH2, glutathione synthase; EC 6.3.2.3) of the two-step pathway leading to GSH formation show an embryo- and seedling-lethal phenotype, respectively (Cairns et al., 2006; Pasternak et al., 2008).

Cys synthesis by OAS-TL constitutes the direct link between carbon and nitrogen (OAS) as well as sulfur (sulfide) metabolism and, therefore, can be designated as one of the central reactions in plant primary metabolism. The genome of the model plant Arabidopsis encodes nine OAS-TL-like enzymes: OAS-TL A1 (At4g14880), OAS-TL B (At2g43750), and OAS-TL C (At3g59760) are the major isoforms and are localized in the cytosol, plastids, and mitochondria, respectively (Jost et al., 2000). *OAS-TL A2* (At3g22460) encodes a truncated and nonfunctional protein (Jost et al., 2000). In the following, therefore, OAS-TL A1 is referred to as OAS-TL A. CYS D1 (At3g04940) and CYS D2 (At5g28020) show OAS-TL activity in vitro (Yamaguchi et al., 2000). Whether they contribute to net Cys

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synthesis in vivo is unknown (Heeg et al., 2008). *CS26* (At3g03630) encodes a plastidic *S*-sulfocysteine synthase, which prefers thiosulfate instead of sulfide as substrate and produces *S*-sulfocysteine (Bermúdez et al., 2010). Whether thiosulfate is taken up from the soil or formed within the plant is unclear, but its presence in Arabidopsis was demonstrated (Tsakraklides et al., 2002). However, the synthesis of *S*-sulfocysteine from thiosulfate potentially constitutes an alternative sulfur fixation pathway. So far, CS26 was shown to be important for the regulation of redox homeostasis in plastids under certain stress conditions (Bermúdez et al., 2010). DES1 (At5g28030; formerly known as CS-LIKE) is a Cys desulfhydrase (EC 4.4.1.15) that releases sulfide in the cytosol (Alvarez et al., 2010). As a Cys-consuming enzyme, it contributes to Cys homeostasis, especially in late vegetative development and under certain stress conditions (Alvarez et al., 2010, 2012). *CYS C1* (At3g61440), finally, encodes a mitochondrial β-cyanoalanine synthase (EC 4.4.1.9), which detoxifies cyanide by incorporation into Cys (Yamaguchi et al., 2000; Watanabe et al., 2008a; García et al., 2010). The major isoforms OAS-TL A, OAS-TL B, and OAS-TL C as well as CYS D1 and CYS D2 can interact with serine acetyltransferase (SAT; EC 2.3.1.30) in the cysteine synthase complex (CSC; Heeg et al., 2008). Although SAT acetylates Ser at the hydroxyl group to form OAS, the direct substrate of OAS-TL, formation of the CSC has no substrate-channeling function but contributes to the demand-driven regulation of Cys synthesis (Hell and Wirtz, 2011).

The subcellular compartmentation of Cys precursor formation is a remarkable feature of Cys synthesis in higher plants that implies a high degree of regulation between the participating compartments: while sulfate is exclusively reduced to sulfide in plastids (Takahashi et al., 2011), the synthesis of OAS and the incorporation of sulfide take place in all three compartments where SAT and OAS-TL are present, namely in the cytosol, plastids, and mitochondria. Reverse genetics approaches proved a certain redundancy between the different SAT and OAS-TL isoforms, which demonstrates that sulfide, OAS, and Cys can be exchanged between these compartments (Haas et al., 2008; Heeg et al., 2008; Watanabe et al., 2008a, 2008b). Indeed, sulfide can easily diffuse through membranes (Mathai et al., 2009), but OAS and Cys need to be actively transported. However, the identity of these transporters is unknown. Although sulfide, OAS, and Cys can pass the mitochondrial membrane (Wirtz et al., 2012), the loss-of-function mutant for mitochondrial OAS-TL C is the only single *oastl* knockout mutant that displays a significant growth phenotype (Heeg et al., 2008). This result was astonishing, since OAS-TL C contributes only 5% to extractable foliar OAS-TL activity (Heeg et al., 2008). The retarded growth of the *oastlC* mutant, however, cannot be explained by the lack of sulfide detoxification in mitochondria by OAS-TL C, due to an alternative detoxification mechanism for sulfide in mitochondria (Birke et al., 2012). These data question the total redundancy between the different OAS-TL isoforms and suggest specific functions in the different subcellular compartments.

Despite its central position in the primary metabolism of higher plants, fundamental questions about Cys synthesis are still unanswered. First, the contribution of OAS-TL-like proteins, especially CYS D1, CYS D2, and CS26, to the fixation of sulfur in planta is unknown. Second, the significance of Cys synthesis by the major OAS-TL proteins in the different subcellular compartments during sporophyte and gametophyte development is unclear. In this study, we addressed these questions using a reverse genetics approach. We were able to prove that fixation of sulfur is carried out exclusively by the major OAS-TL isoforms OAS-TL A, OAS-TL B, and OAS-TL C and elucidated specific functions for OAS-TL A in the cytosol and OAS-TL C in mitochondria of leaf cells. Furthermore, we demonstrate that Cys can be supplied by the mother plant for the development of female gametophytes lacking OAS-TL activity. In contrast, the presence of at least one functional OAS-TL isoform is essential in the male gametophyte.

RESULTS

Generation and Characterization of *oastl* Double Mutants

Loss of a single OAS-TL isoform in the Arabidopsis transfer DNA (T-DNA) insertion mutants *oastlA*, *oastlB*, and *oastlC* did not cause lethality of the plants (Heeg et al., 2008; Watanabe et al., 2008a), which indicates redundancy between the different isoforms. However, the *oastlC* mutant displayed a minor but significant growth retardation (Heeg et al., 2008), suggesting a specific function of OAS-TL C in mitochondria. To further analyze the relevance of OAS-TL activities in different subcellular compartments, the *oastl* single mutants were crossed to generate the double mutants *oastlBC* $A^{+/+}$, *oastlAC* $B^{+/+}$, and *oastlAB* $C^{+/+}$, leaving only one major OAS-TL in the cytosol, in the plastids, and in the mitochondria, respectively.

Immunological detection confirmed the absence of the respective OAS-TL isoforms in all *oastl* double mutants compared with the wild type (Fig. 1A). However, extractable OAS-TL activity was not altered in the *oastlBC* $A^{+/+}$ mutant compared with the wild type (Fig. 1B). In contrast, OAS-TL activities were decreased to 30% and 3% in the *oastlAC* $B^{+/+}$ and *oastlAB* $C^{+/+}$ mutants, respectively (Fig. 1B). In none of the *oastl* double mutants was the abundance of the remaining OAS-TL isoform altered, as observed by relative quantification of the immunological signals (Fig. 1A). The rosette morphology of the *oastl* double mutants was comparable to that of the wild type (Fig. 1C). However, *oastlAC* $B^{+/+}$ and *oastlAB* $C^{+/+}$ mutants were smaller in size, which was reflected by a decreased shoot biomass, whereas the *oastlBC* $A^{+/+}$ mutant did not show any difference from the wild type (Fig. 1D). The fresh weight of 6- and 8-week-old *oastlAC* $B^{+/+}$

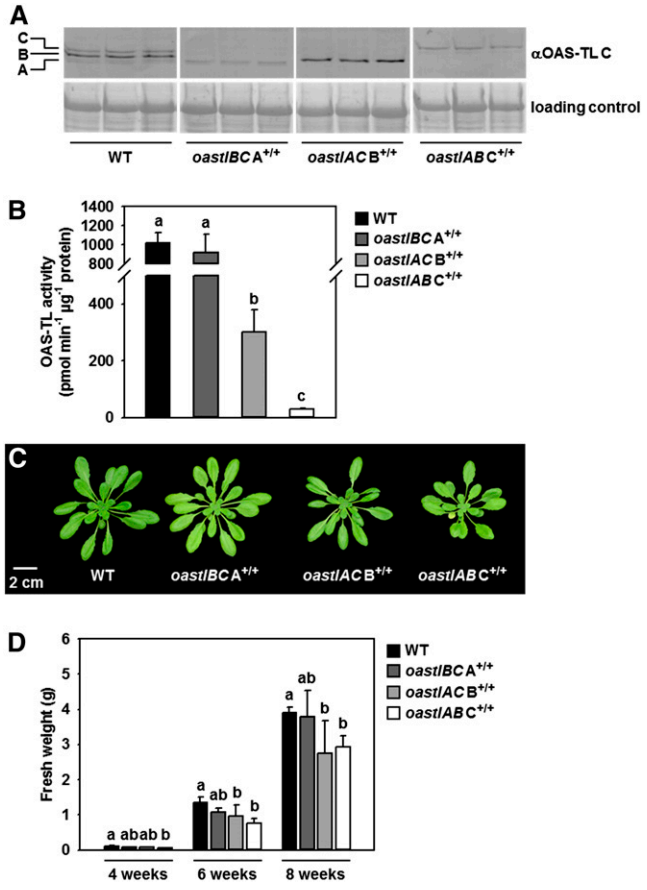

Figure 1. Characterization of *oastl* double mutants. A, Soluble leaf proteins (30 μg) extracted from 8-week-old plants grown on soil ($n = 3$) were used for immunological detection of OAS-TL proteins using an antiserum recognizing the major OAS-TL isoforms (Heeg et al., 2008). Ponceau S-stained Rubisco (large subunit) was used as a loading control. B, Total OAS-TL activity was determined for leaves from plants described in A ($n = 5$). C, Phenotypes of the wild type (WT) and the *oastl* double mutants *oastlBC* $A^{+/+}$, *oastlAC* $B^{+/+}$, and *oastlAB* $C^{+/+}$ grown on soil under short-day conditions for 6 weeks. D, Fresh weight of the wild type and the *oastl* double mutants described in A was determined after 4, 6, and 8 weeks ($n = 5$). The phenotypes (C) and fresh weights (D) of the wild type and the *oastlAB* $C^{+/+}$ mutant were already published by Heeg et al. (2008). Mean values ± SD are shown. Statistical differences are indicated by different letters (Holm-Sidak one-way ANOVA, $P \leq 0.05$). [See online article for color version of this figure.]

mutants was decreased by 30% compared with the wild type, whereas no significant difference was observed for 4-week-old plants. In contrast, the shoot biomass of 4-week-old *oastlAB* $C^{+/+}$ mutants was already decreased by 45%, and the fresh weight did not reach more than 74% after 8 weeks compared with the wild type.

To determine the relevance of OAS-TL proteins in different subcellular compartments for Cys synthesis in planta, the flux of sulfur and the carbon/nitrogen-containing backbone, OAS, into thiols was investigated in leaves of the *oastl* double mutants. As an indicator for the reduction of sulfur in planta, incorporation of sulfur into thiols was determined by feeding of leaves with ^{35}S-labeled sulfate (Fig. 2). Incorporation of ^{35}S into Cys within 45 min was 40% lower in the *oastlAC* $B^{+/+}$ mutant compared with the wild type (Fig. 2A), and the incorporation rate into GSH was only 50% in both mutants lacking the cytosolic OAS-TL (*oastlAC* $B^{+/+}$ and *oastlAB* $C^{+/+}$; Fig. 2B). In contrast, the flux of ^{35}S into thiols was not affected in the *oastlBC* $A^{+/+}$ mutant compared with the wild type. Determination of steady-state levels of thiols, however, revealed a 1.6-fold increase for Cys in the *oastlBC* $A^{+/+}$ mutant (Fig. 3A). Additionally, γ-glutamylcysteine, the intermediate metabolite in GSH synthesis, accumulated more than 3-fold in the *oastlBC* $A^{+/+}$ mutant (Fig. 3B), whereas the glutathione concentration was not altered compared with the wild type (Fig. 3C). Despite the decreased incorporation of ^{35}S into thiols in the *oastlAC* $B^{+/+}$ mutant (Fig. 2), the Cys steady-state level remained unchanged (Fig. 3A). However, glutathione concentration was decreased to 70% compared with the wild type (Fig. 3C). Neither the concentration of Cys nor glutathione was altered in the *oastlAB* $C^{+/+}$ mutant (Fig. 3, A and C).

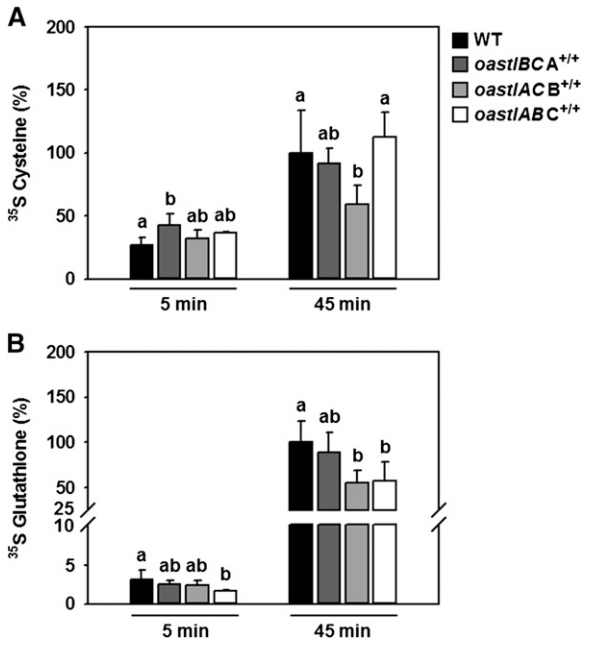

Figure 2. Incorporation of radiolabeled sulfur into thiols in the *oastl* double mutants. Leaf pieces of hydroponically grown wild-type plants (WT) and *oastl* double mutants *oastlBC* $A^{+/+}$, *oastlAC* $B^{+/+}$, and *oastlAB* $C^{+/+}$ ($n = 4$–9) were incubated in one-half-strength Hoagland solution supplemented with 125 nM $^{35}SO_4^{2-}$. Samples were taken after 5 and 45 min. Thiols were separated by HPLC, and the incorporation of ^{35}S into Cys (A) and glutathione (B) was quantified by liquid scintillation counting of the respective fractions. Data for the wild type and the *oastlAB* $C^{+/+}$ mutant were already published by Heeg et al. (2008). Mean values ± SD are shown. Statistical differences are indicated by different letters (Holm-Sidak one-way ANOVA, $P \leq 0.05$).

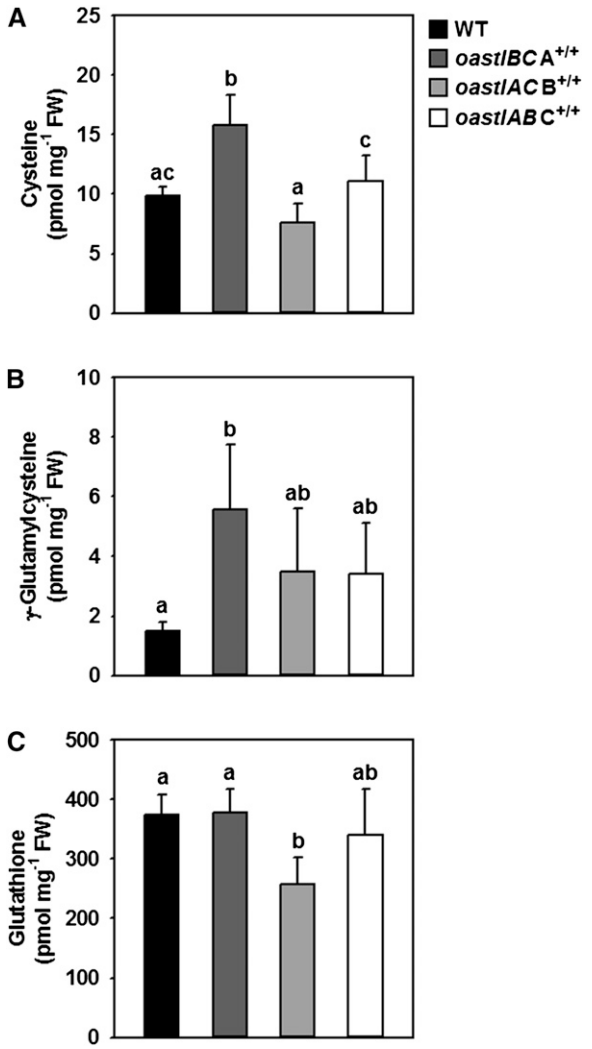

Figure 3. Thiol steady-state levels in *oastl* double mutants. Thiols were extracted from leaves of 8-week-old wild-type plants (WT) and *oastlBC* $A^{+/+}$, *oastlAC* $B^{+/+}$, and *oastlAB* $C^{+/+}$ double mutants grown on soil. The steady-state levels of Cys (A), γ-glutamylcysteine (B), and glutathione (C) were quantified by HPLC upon selective labeling with the fluorescent dye monobromobimane ($n = 5$). Mean values ± SD are shown. Statistical differences are indicated by different letters (Holm-Sidak one-way ANOVA, $P \leq 0.05$). FW, Fresh weight.

In planta OAS synthesis and flux of the carbon backbone into thiols were investigated upon feeding of leaves with ^{3}H-labeled Ser (Fig. 4). The incorporation rate of [^{3}H]Ser into Cys (Fig. 4A) and GSH (Fig. 4B) was about 50% lower in the *oastlAC* $B^{+/+}$ mutant compared with the wild type. The same trend, although not statistically significant, was observed for the *oastlBC* $A^{+/+}$ mutant (−20%; Fig. 4, A and B). In contrast, the incorporation rate was not altered in the *oastlAB* $C^{+/+}$ mutant, which possesses functional OAS-TL C and thus CSC in mitochondria (Fig. 4, A and B). In agreement with the lowered incorporation from Ser into Cys, the OAS steady-state level was decreased to 30% and 24% compared with the wild type in the *oastlBC* $A^{+/+}$ and *oastlAC* $B^{+/+}$ mutants, respectively (Fig. 4C). In the *oastlAB* $C^{+/+}$ mutant, the OAS steady-state level increased by a factor of 1.7 compared with the wild type (Fig. 4C). The observed changes of flux and OAS steady-state levels did not correlate with a change in in vitro SAT activity (Fig. 4D), determined in the presence of excess substrates. However, it was already shown that in vivo SAT activity is regulated by reversible posttranslational modulation in the CSC rather than by adjustment of SAT amount (for review, see Hell and Wirtz, 2011).

Taken together, one can conclude from these results that (1) the incorporation of sulfur into thiols but not thiol steady-state levels is decreased in mutants lacking cytosolic OAS-TL A; (2) lack of mitochondrial OAS-TL C diminishes the incorporation of OAS into thiols without affecting thiol steady-state levels; and (3) only the absence of both OAS-TL A and OAS-TL C results in a decreased incorporation of sulfur and the carbon/nitrogen backbone into thiols, which also causes lower thiol steady-state levels.

Generation of the *oastl* Triple Collection

The presence of eight OAS-TL-like proteins and the viability of the *oastl* single and double mutants prompted us to question the exclusiveness of Cys synthesis by the three major isoforms in Arabidopsis. For that reason, the *oastlBC* $A^{+/+}$ mutant was crossed with the *oastlAB* $C^{+/+}$ and *oastlAC* $B^{+/+}$ mutants to generate an *oastlABC* triple mutant. Successful crossing was confirmed by genotyping the T1 generation by PCR. In the T2 generation, a total of nine plants could be identified with only one functional allele of *OAS-TL A*, *OAS-TL B*, or *OAS-TL C* left, whereas the presence of the T-DNA insertion could be confirmed in the remaining five *OAS-TL* alleles (Fig. 5A). The mutants were named *oastlBC* $A^{+/-}$, *oastlAC* $B^{+/-}$, and *oastlAB* $C^{+/-}$, respectively, and all three mutants together are referred to as the *oastl* triple collection. The offspring of the *oastl* triple collection grown on soil were screened for homozygous *oastlABC* triple mutants, but neither in the T3 nor the T4 generation could such a mutant be identified ($n = 508$). Furthermore, the segregation of *oastlBC* $A^{+/-}$ and *oastlAB* $C^{+/-}$ was analyzed upon germination on solid growth medium (Table I). The germination rate of the seeds from both mutants (97% and 92%, respectively) was similar to the germination rate of wild-type seeds (93%). Determination of the seedling genotypes by PCR revealed no *oastlABC* triple mutant ($n = 309$ and 132, respectively). In both cases, approximately half of the seedlings (57% and 65%, respectively) were homozygous for the respective wild-type allele, whereas the remaining seedlings were heterozygous (Table I). This segregation pattern can only be explained by a gamete-lethal phenotype of the *oastlABC* triple mutant.

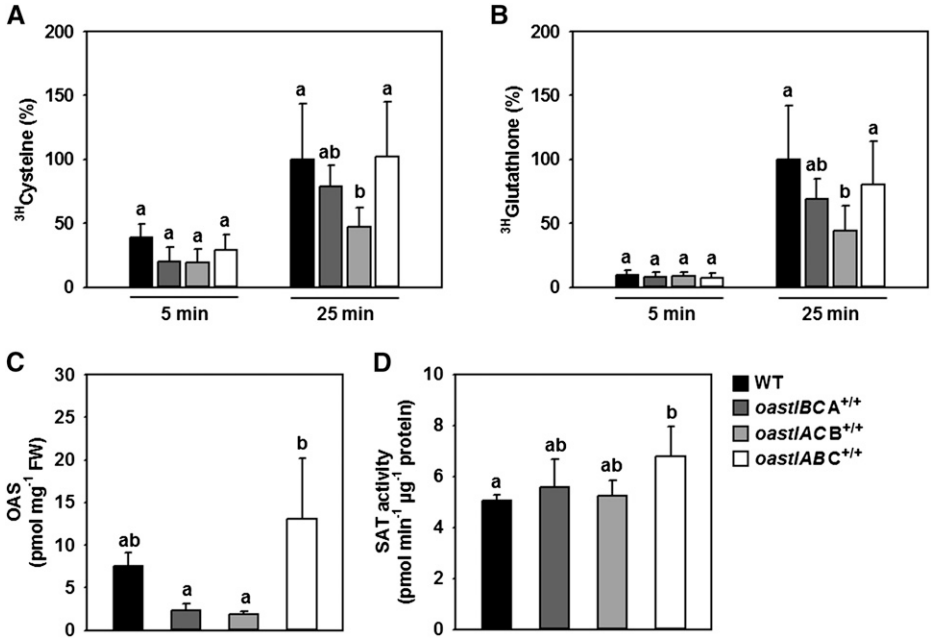

Figure 4. Incorporation of ^{3}H-labeled Ser into thiols in the *oastl* double mutants and quantification of OAS steady-state levels and SAT activity. A and B, Leaf pieces of hydroponically grown wild-type plants (WT) and the *oastl* double mutants *oastlBC* A$^{+/+}$, *oastlAC* B$^{+/+}$, and *oastlAB* C$^{+/+}$ (n = 5–11) were incubated in one-half-strength Hoagland solution supplemented with 2.5 µM ^{3}H-labeled Ser for 5 and 25 min. Thiols were extracted and selectively labeled with the fluorescent dye monobromobimane. The resulting derivatives were separated by HPLC, and incorporation of ^{3}H into Cys (A) and glutathione (B) was quantified by liquid scintillation counting of the respective fractions. For easier comparison of the genotypes, the incorporation of ^{3}H after 25 min into the respective thiol (Cys or glutathione) of the wild type was set at 100%. C, Foliar OAS steady-state levels of 8-week-old wild-type plants and *oastl* double mutants grown on soil was determined upon selective labeling with Accq-Tag (n = 5). D, Total extractable SAT activity was determined for the plants described in C (n = 5). Mean values ± SD are shown. Statistical differences are indicated by different letters (Holm-Sidak one-way ANOVA, $P \leq 0.05$). FW, Fresh weight.

Phenotype of the *oastl* Mutants in the Generative Phase of the Life Cycle

The gamete-lethal phenotype of the *oastlABC* triple mutant suggested a significant function of Cys synthesis by the major OAS-TLs in the generative phase of the life cycle. For that reason, flower and seed development were analyzed in the *oastl* double mutants and plants of the *oastl* triple collection. No significant difference could be observed regarding flowering time and flower morphology for any of the investigated mutants compared with the wild type. However, the stem was shorter in the *oastlAC* B$^{+/+}$ (77%) and *oastlAC* B$^{+/-}$ mutants (67%) compared with the wild type (Fig. 5B; Supplemental Fig. S1), and the length of the siliques was decreased to 49% and 42%, respectively (Fig. 5, B and C). In addition, the number of fully developed seeds per silique was decreased by more than 85% in these mutants, resulting in almost no seed yield (Fig. 5D). In the *oastlAB* C$^{+/-}$ mutant, the silique length was also slightly decreased by 13% (Fig. 5C), which correlated with a lower number of fully developed seeds (38% of the wild type; Fig. 5D). In the remaining mutants, silique length and the number of fully developed seeds were comparable to the wild type (Fig. 5). No significant differences could be observed regarding the number of ovules and aborted seeds per silique in any of the investigated mutants compared with the wild type (Supplemental Fig. S2, A and B), demonstrating that a substantial number of ovules in the siliques of the *oastlAC* B$^{+/+}$, *oastlAC* B$^{+/-}$, and *oastlAB* C$^{+/-}$ mutants were unfertilized (Supplemental Fig. S2C). The absence of aborted seeds excluded embryo lethality caused by the *oastlABC* triple mutation, which is in agreement with the observed germination rate (Table I).

The observed silique phenotype suggested a defect in development and/or functionality of the female and/or male gametophyte in the *oastlAC* B$^{+/+}$, *oastlAC* B$^{+/-}$, and *oastlAB* C$^{+/-}$ mutants. To further localize the defect, mutants from the *oastl* triple collection and the wild type were reciprocally crossed and the silique phenotype was analyzed. Additionally, all genotypes were crossed with themselves as reference. When pollen from a wild-type plant was transferred to pistils from another wild-type plant, the length of the silique reached approximately 1 cm (Fig. 6A), and 40% of the ovules were fertilized and developed to mature seeds (Fig. 6B). This result represented a technical control, illustrating the difference between the efficiency of self-pollination (Fig. 5) and manual crosses (Fig. 6). When the *oastlBC* A$^{+/-}$ mutant and the wild type were used for the crosses, silique length (Fig. 6A) and the

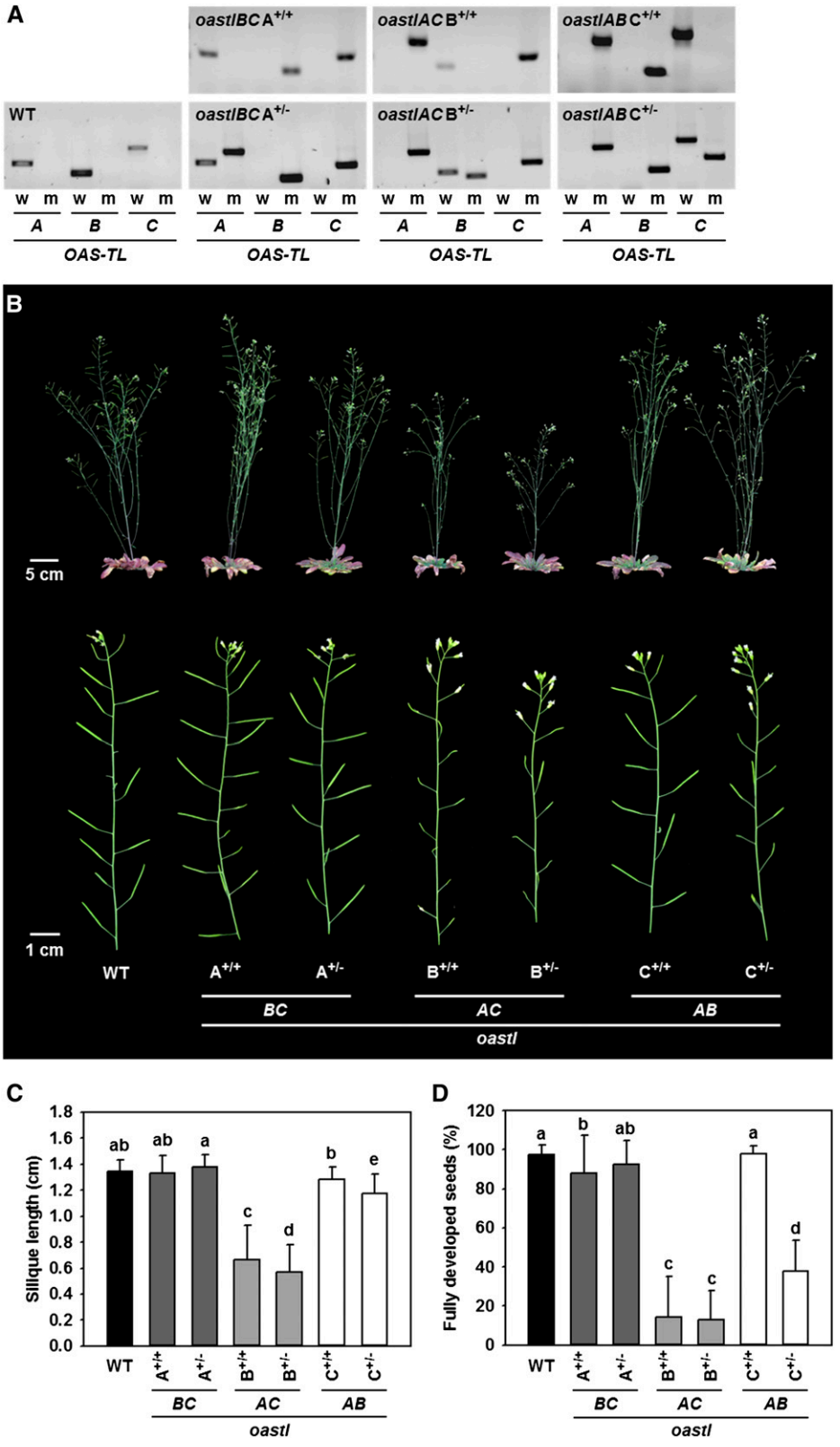

Figure 5. Phenotypes of *oastl* mutants in the generative phase of the life cycle. A, The genotypes of the wild type (WT), the *oastl* double mutants, and the mutants from the *oastl* triple collection were confirmed by PCR using gene- and T-DNA-specific primers for *OAS-TL A*, *OAS-TL B*, and *OAS-TL C*. m, Mutant (T-DNA) allele; w, wild-type allele. B, The phenotypes of whole plants and the upper part of the primary stem were documented approximately 3 weeks after bolting. C, Lengths of siliques that were harvested 5 to 10 cm underneath the top of the primary inflorescence stem of plants shown in B ($n = 30–50$). D, Percentages of fully developed seeds within the siliques described in C. Mean values ± SD are shown. Statistical differences are indicated by different letters (Holm-Sidak one-way ANOVA, $P \leq 0.05$). [See online article for color version of this figure.]

number of fully developed seeds (Fig. 6B) were always comparable to the wild-type reference, irrespective of the parents' genotype. In contrast to this, silique length and the number of fully developed seeds were significantly decreased when pollen from the *oastlAC* $B^{+/-}$ and *oastlAB* $C^{+/-}$ mutants were transferred to pistils

Table I. *Segregation analysis of oastlBC $A^{+/-}$ and oastlAB $C^{+/-}$ mutants*

Seeds from the wild type and the *oastl* double mutants *oastlBC* $A^{+/-}$ and *oastlAB* $C^{+/-}$ were germinated on solid growth medium, the genotype of the germinated seedlings was determined by PCR, and relative frequency is given in percentage. The germination rate was determined to exclude any embryo- or early seedling-lethal phenotypes of the *oastl* triple mutant.

Plant	No. of Seeds/Seedlings	Homozygous for the Wild-Type Allele	Homozygous for the Mutant Allele	Heterozygous	Germination Rate
Wild type	198	100%	0%	0%	93%
oastlBC $A^{+/-}$	309	57%	0%	42%	97%
oastlAB $C^{+/-}$	132	65%	0%	35%	92%

from these mutants or the wild type. On the other hand, when the *oastlAC* $B^{+/-}$ and *oastlAB* $C^{+/-}$ mutants were crossed with pollen from the wild type, silique length and the number of fully developed seeds were comparable to the wild-type reference. The same observation held true when the *oastlAC* $B^{+/+}$ double mutant was used for similar crosses (Supplemental Fig. S3). These results pointed to a defect in pollen and not ovule development and/or functionality in the *oastlAC* $B^{+/+}$, *oastlAC* $B^{+/-}$, and *oastlAB* $C^{+/-}$ mutants. In agreement, in vitro OAS-TL activity was high in pollen of wild-type plants, suggesting an important function of Cys in the pollen (Supplemental Fig. S4).

Analysis of Pollen Viability and Functionality in *oastl* Mutants

Alexander viability staining of the pollen from the *oastl* double mutants and the mutants from the *oastl* triple collection did not reveal differences compared with pollen from the wild type (Supplemental Fig. S5). However, this staining method only provides information about the presence of cytoplasm and does not allow any conclusion regarding the functionality of the pollen (Nepi and Franchi, 2000). Therefore, in vitro germination of the pollen was investigated (Supplemental Fig. S6). The germination rate for pollen from the wild type under the conditions used in this work constantly reached 40% in two independent experiments with pollen from nine flowers of three plants (Fig. 7). Pollen from *oastlAC* $B^{+/+}$ and *oastlAC* $B^{+/-}$ plants germinated with a significantly lower rate than pollen from the wild type (8.5% and 12.3%, respectively; Fig. 7). The same trend was observed for pollen from *oastlAB* $C^{+/-}$, but this was not significant. Interestingly, pollen of the other mutants (i.e. those having at least one functional *OAS-TL A* allele including *oastlBC* $A^{+/-}$) germinated like pollen from the wild type. Since 50% of pollen from *oastlAB* $C^{+/-}$, *oastlAC* $B^{+/-}$, and *oastlBC* $A^{+/-}$ mother plants are isogenic and have no functional major OAS-TL left, these germination phenotypes are not explainable by the genetic equipment of the pollen alone. Furthermore, pollen from the *oastlAC* $B^{+/+}$ double mutant, which contains a functional OAS-TL B, were as affected as pollen from the *oastlAC* $B^{+/-}$ mutant, pointing again toward an impact of the genetic

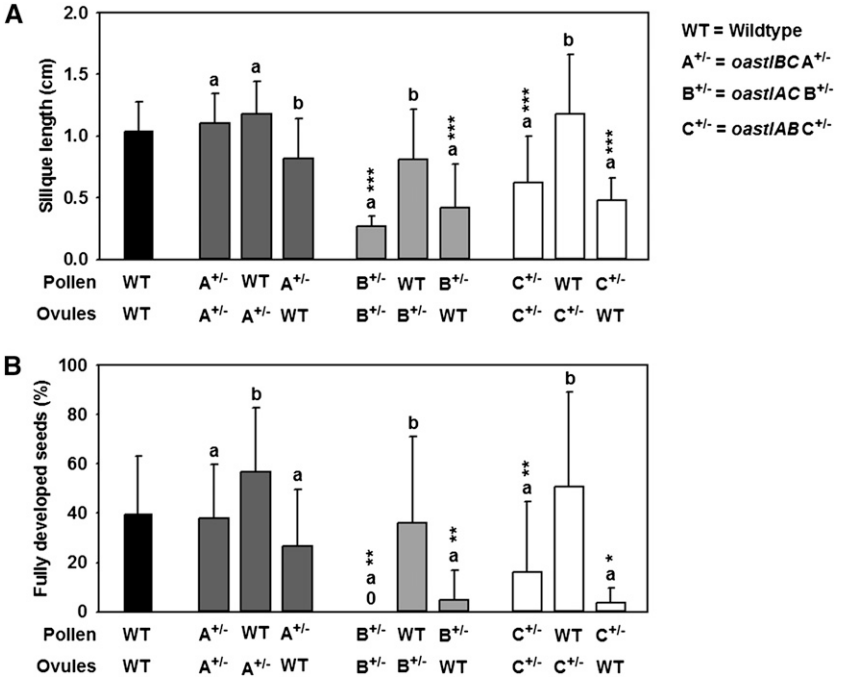

Figure 6. Reciprocal crosses with wild-type plants (WT) and the mutants from the *oastl* triple collection. A, Wild-type plants and mutants from the *oastl* triple collection were crossed by hand 2 to 3 weeks after bolting, and the lengths of the formed siliques were determined (n = 5–30). B, Percentages of fully developed seeds in the siliques described in A. Mean values ± SD are shown. Statistical differences were determined by Holm-Sidak one-way ANOVA ($P \leq 0.05$). Statistical differences within one group are indicated by different letters, whereas differences compared with the wild-type reference are indicated by asterisks (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

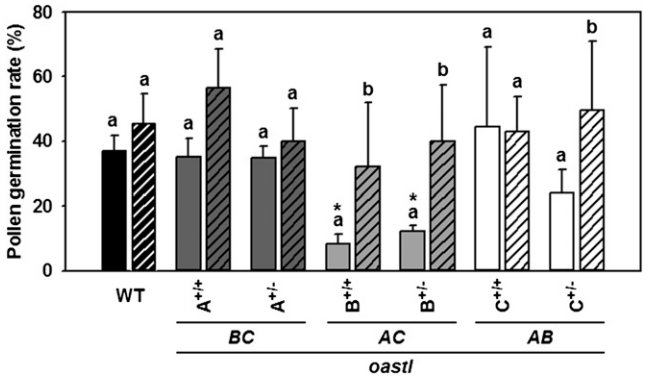

Figure 7. In vitro germination rate of pollen from the *oastl* mutants. Pollen of five to 10 flowers from wild-type plants (WT), *oastl* double mutants, and mutants from the *oastl* triple collection grown on soil ($n = 5$) were transferred to germination medium supplemented without (plain bars) and with 1 mM glutathione (striped bars). Germination was analyzed 26 to 28 h after transfer of the pollen to germination medium ($n = 50–150$). Mean values ± SD are shown. Statistical differences were determined by Holm-Sidak one-way ANOVA ($P \leq 0.05$). Statistical differences between the means of one genotype are indicated by different letters, whereas differences compared with the wild-type reference are indicated by asterisks ($*P \leq 0.05$).

background of the mother plant from which the pollen derived. The pollen germination defect coincided with decreased steady-state levels of glutathione in vegetative tissue of the mother plants (Supplemental Fig. S7). Previous studies had revealed a beneficial impact of GSH on pollen germination in the *pad2-1* mutant background, having only 20% of wild-type GSH synthesis capacity in leaves left (Zechmann et al., 2011). Indeed, application of GSH restored the germination rates of pollen from all *oastl* mutants to the wild-type level (Fig. 7; Supplemental Fig. S6). The germination rate of pollen from the wild type was not significantly affected by this treatment.

Verification of a Gene Dose Effect in the *oastl* Mutants

The *oastlAB* $C^{+/-}$ mutant showed differences regarding the silique phenotype and pollen functionality compared with the corresponding double mutant *oastlAB* $C^{+/+}$ (Figs. 5 and 7). This observation could be the result of a gene dose effect caused by the additional T-DNA insertion in one of the *OAS-TL C* alleles. Immunological detection of OAS-TL proteins indeed revealed a 50% decrease in the amount of OAS-TL C protein in leaves of *oastlAB* $C^{+/-}$ compared with *oastlAB* $C^{+/+}$ (Fig. 8A). As a consequence, extractable OAS-TL activity was further decreased by 40% in the *oastlAB* $C^{+/-}$ mutant compared with the double mutant *oastlAB* $C^{+/+}$ (Fig. 8B). The gene dose effect on protein and activity levels was also observable for the *oastlBC* $A^{+/-}$ and *oastlAC* $B^{+/-}$ mutants compared with the corresponding double mutants (Fig. 8). However, in contrast to the *oastlAB* $C^{+/-}$ mutant, this gene dose effect did not become apparent in phenotypical differences (Figs. 5 and 7).

Contribution of Additional OAS-TL-Like Proteins to Cys Synthesis

Gamete lethality of the *oastlABC* triple mutation proved that, at least in pollen, Cys is exclusively provided by OAS-TL, and loss of OAS-TL A, OAS-TL B, and OAS-TL C together cannot be compensated by the remaining OAS-TL-like proteins. However, it cannot be ruled out that, during vegetative growth, one of the OAS-TL-like proteins might provide sufficient Cys for the survival of the plant. For that reason, transcript amounts of the *OAS-TL-like* genes in leaves were determined by quantitative real-time (qRT)-PCR in the *oastlAC* $B^{+/-}$ mutant, which showed the strongest phenotype of all investigated mutants compared with the wild type. The transcript amounts of *CYS D1* and *CYS D2* were not up-regulated in the mutant compared with the wild type (Supplemental Fig. S8), suggesting that CYS D1 and CYS D2 did not compensate for the loss of the major OAS-TLs. This is in agreement with their, from a biochemical point of view, inefficiency for Cys synthesis, although OAS-TL activity has been shown for both enzymes in vitro (Yamaguchi et al., 2000). The amount of *CS26* and *DES1* transcript was also not changed, whereas the amount of *CYS C1* transcript was increased by a factor of 1.5 compared with the wild type (Supplemental Fig. S8). However, CYS C1 has been shown to catalyze the in vivo detoxification of cyanide and not the synthesis of Cys (Hatzfeld et al., 2000; Bonner et al., 2005; for review, see Heeg et al., 2008). A compensatory contribution of OAS-TL-like proteins in vegetative organs, therefore, seems very unlikely.

DISCUSSION

Subcellular Localization of OAS-TL Proteins Is More Important for Efficient Cys Synthesis Than Total Cellular OAS-TL Activity

For a long time, the presence of OAS-TL in the cytosol, plastids, and mitochondria of Arabidopsis cells was attributed to the necessity of Cys production for protein synthesis in these compartments (Lunn et al., 1990). The viability of Arabidopsis mutants lacking OAS-TL activity in one of the three compartments, however, proved a certain redundancy between the OAS-TL isoforms (Heeg et al., 2008; Watanabe et al., 2008a). On the other hand, this study revealed significant differences in the growth of the *oastl* double mutants, suggesting specific functions of the individual isoforms. Specifically, the double mutants lacking cytosolic OAS-TL A were retarded in growth, which can be explained by a decreased incorporation of sulfur into thiols in these mutants. Hence, a predominant role of OAS-TL A regarding total net Cys synthesis can

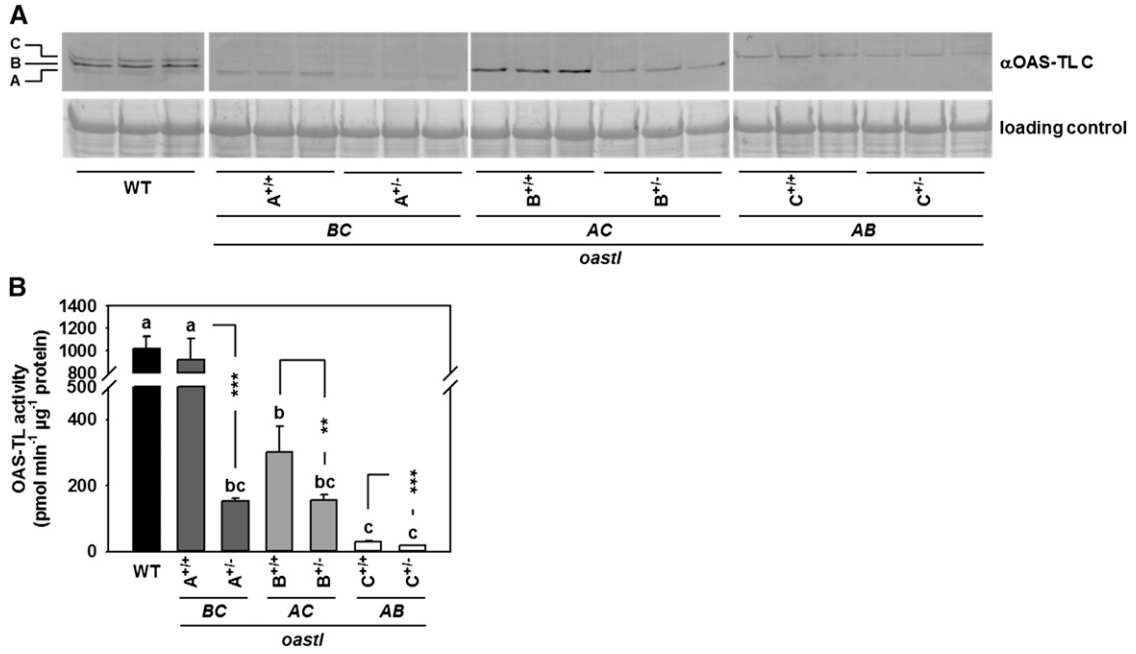

Figure 8. Immunological detection of OAS-TL proteins and OAS-TL activity in the *oastl* mutants. A, Soluble leaf proteins (30 μg) extracted from 8-week-old plants grown on soil ($n = 3$) were used for immunological detection of OAS-TL proteins using an antiserum recognizing the major OAS-TL isoforms (Heeg et al., 2008). Ponceau S-stained Rubisco (large subunit) was used as a loading control. B, Total OAS-TL activity was determined for leaves from the plants described in A ($n = 5$). Mean values ± SD are shown. Statistical differences between all means are indicated by different letters (Holm-Sidak one-way ANOVA, $P \leq 0.05$). Additionally, Student's *t* test was used to determine statistical differences between the mutants of the *oastl* triple collection and the corresponding double mutant, and these differences are indicated by asterisks (**$P \leq 0.01$, ***$P \leq 0.001$). WT, Wild type.

be concluded, which is in agreement with previous observations (Heeg et al., 2008) and with the relevance of OAS-TL A for those stress responses of the plant that depend on Cys synthesis (e.g. cadmium stress response; Dominguez-Solís et al., 2001).

On the other hand, steady-state levels and flux of the carbon backbone OAS into thiols was decreased in mutants lacking mitochondrial OAS-TL C, namely *oastlBC* $A^{+/+}$ and *oastlAC* $B^{+/+}$. This supported the hypothesis that OAS-TL C is important for the regulation of OAS synthesis by mitochondrial SAT3 and, in consequence, also Cys synthesis (Haas et al., 2008; Heeg et al., 2008; Watanabe et al., 2008b). Indeed, the export of OAS from mitochondria was shown to be dependent on SAT3 and OAS-TL C, demonstrating that the formation of the mitochondrial CSC significantly contributes to the regulation of net OAS synthesis of the cell (Wirtz et al., 2012). Astonishingly, no specific function could be identified for OAS-TL B so far, although OAS-TL B contributed 30% to total OAS-TL activity and is localized in the plastids, the compartment of sulfide formation (Hell and Wirtz, 2011). However, it cannot be ruled out that OAS-TL B plays an important role under certain stress conditions, presumably those predominantly affecting the chloroplast (e.g. high light). Nevertheless, despite its almost negligible contribution to total cellular OAS-TL activity (5% or less), mitochondrial OAS-TL C is far more important than plastidic OAS-TL B for total Cys synthesis under nonstress conditions. Consequently, the *oastlAC* $B^{+/+}$ mutant, having 30% of total cellular OAS-TL activity left, was the most affected mutant with respect to flux as well as metabolite steady-state levels compared with the wild type. The *oastlAB* $C^{+/+}$ mutant, which lacked more than 95% of extractable OAS-TL activity, was less affected compared with the *oastlAC* $B^{+/+}$ mutant. Hence, the presence of OAS-TL in a specific compartment is more important than its contribution to total cellular OAS-TL activity. This can be explained by the specific functions of OAS-TL A and OAS-TL C regarding the incorporation of sulfide into OAS and the regulation of net OAS synthesis, respectively. It is indeed reasonable to assume that decreased OAS synthesis contributes to the development of the growth phenotype of the *oastlC* single mutant (Heeg et al., 2008). However, it cannot be excluded that alterations of downstream reactions contribute to the *oastlC* phenotype. A prime candidate for such a pleiotropic contribution is Cys-dependent detoxification of reactive oxygen species in mitochondria. However, several lines of evidence demonstrate the presence of GSH and the glutathione reductase system in mitochondria, which play a more important role for the detoxification of reactive oxygen species in other subcellular compartments than Cys (Noctor et al., 2011).

Cys Is Exclusively Provided by the Major OAS-TLs and Is the Only Entry Point of Sulfur into Organic Metabolism

The gamete lethality of the *oastlABC* triple mutant demonstrated that Cys synthesis by OAS-TL A, OAS-TL B, or OAS-TL C is essential for the reproduction of Arabidopsis. In agreement, the complete lack of sulfide production by sulfite reductase (SiR; EC 1.8.7.1) in the *sir1-2* mutant is seedling lethal (Khan et al., 2010). We conclude from these results that the incorporation of thiosulfate into *S*-sulfocysteine by CS26 (Bermúdez et al., 2010) is not an alternative sulfur fixation pathway. The function of CS26, therefore, seems to be restricted to specialized plastidic processes like light-dependent redox control (Bermúdez et al., 2010). Consequently, OAS-TL A, OAS-TL B, and OAS-TL C represent the only enzymes that provide sufficient Cys for the survival of Arabidopsis.

Cys Synthesis by the Female Gametophyte Is Dispensable

In principle, gamete lethality caused by the lack of Cys synthesis might affect the female, male, or both gametophytes. However, the haploid *oastlABC* triple mutant ovules are functional, since they could be fertilized by wild-type pollen in reciprocal crossing experiments, narrowing down gamete lethality to the male gametophyte. Since Cys is essential for translation and important for protein structure and function, it is required during the development of the female gametophyte. Therefore, Cys supply by the mother plant is the most likely explanation for the functionality of the *oastlABC* triple mutant ovules. The funiculus indeed provides a physical connection between the maternal tissue and the ovules, and several amino acid transporters are expressed in funiculi and ovules of Arabidopsis (for review, see Tegeder and Rentsch, 2010). A significant contribution of amino acid transport to the development and function of the female gametophyte becomes evident by the successful female gametogenesis of His, Lys, or Pro auxotrophic mutants (Song et al., 2004; Hudson et al., 2006; Muralla et al., 2007; Funck et al., 2012). However, the embryo-lethal phenotype of mutants lacking His or Lys synthesis demonstrates that the developing sporophyte later relies on the synthesis of these amino acids (Song et al., 2004; Hudson et al., 2006; Muralla et al., 2007). The sources and requirements for amino acids during reproductive stages appear to be quite different, since the lack of Trp synthesis causes a female-specific defect in the transmission of the mutant phenotype, demonstrating that the synthesis of Trp by the female gametophyte itself is essential (Niyogi et al., 1993).

The Presence of at Least One Functional OAS-TL Isoform in the Male Gametophyte Is Essential for the Proper Function of Pollen

In contrast to the female gametophyte, the male gametophyte forms an independent unit that is released from the anthers of the sporophyte in the form of the pollen grains. Nevertheless, the expression of several amino acid transporters in the tapetum, the pollen, and the pollen-transmitting track of the pistil suggests the transport of amino acids from the sporophyte to the male gametophyte when the pollen is associated with sporophytic tissues (for review, see Tegeder and Rentsch, 2010). In agreement with this, the lack of His, Lys, or Trp synthesis within the pollen does not affect pollen functionality (Niyogi et al., 1993; Song et al., 2004; Hudson et al., 2006; Muralla et al., 2007). In surprising contrast, Cys cannot be supplied by the mother plant in sufficient amounts to fulfill male gametophyte function and needs to be synthesized within the pollen itself, which was demonstrated by the dysfunctionality of the *oastlABC* triple mutant pollen. In agreement, in vitro OAS-TL activity was surprisingly high in germinating pollen of the wild type, even exceeding the activity in rosette leaves, pointing to a high demand of Cys synthesis in this tissue.

However, the dysfunctionality of the *oastlABC* triple mutant pollen generated by different genotypes of the *oastl* triple collection became apparent at different developmental stages of the male gametophyte: *oastlABC* triple mutant pollen derived from mother plants without a functional *OAS-TL A* allele (*oastlAC* $B^{+/-}$ and *oastlAB* $C^{+/-}$) showed a strongly reduced germination rate compared with pollen from wild-type plants. In contrast, *oastlABC* triple mutant pollen derived from a mother plant with a functional *OAS-TL A* allele (*oastlBC* $A^{+/-}$) germinated but failed to fertilize the female gametophyte, pointing to an essential function of Cys during elongated pollen tube growth and/or fertilization. The latter can be explained by high-energy-consuming processes during pollen tube growth that rely on energy production by the mitochondrial respiratory chain (De Paepe et al., 1993). The essential complexes I, II, and III of the respiratory chain contain significant amounts of iron-sulfur (Fe-S) clusters (Vigani et al., 2009), which are built from Cys independently in plastids and mitochondria (Van Hoewyk et al., 2007). In contrast to translation, Fe-S cluster synthesis consumes Cys irreversibly, since Cys is degraded during the formation of the Fe-S cluster. Consequently, pollen tube growth requires the degradation of significant amounts of Cys to allow the recycling of Fe-S cluster-containing enzymes. Additionally, the absence of OAS-TL activity in the *oastlABC* triple mutant pollen may cause high sulfide levels by functional sulfate reduction in pollen. Elevated sulfide levels can interfere with efficient mitochondrial respiration, since complex IV of the mitochondrial respiratory chain is strongly inhibited by sulfide (50% inhibition of initial activity < 10 nM; Birke et al., 2012). Thus, impaired mitochondrial respiration provides a reasonable explanation for the dysfunctionality of the *oastlABC* triple mutant pollen generated by the *oastlBC* $A^{+/-}$ mutant.

In contrast to the *oastlABC* triple mutant pollen derived from the *oastlBC* $A^{+/-}$ mutant, germination

of the *oastlABC* triple mutant pollen derived from mother plants without a functional *OAS-TL A* allele (*oastlAC* $B^{+/-}$ and *oastlAB* $C^{+/-}$) was strongly reduced. Thus, the germination rate of the *oastlABC* triple mutant pollen depends on the presence of OAS-TL A activity in the mother plant, which determines the incorporation of sulfur into Cys, the limiting precursor of GSH synthesis. The latter led us to suggest that the germination defect of pollen from the *oastlAC* $B^{+/-}$ and *oastlAB* $C^{+/-}$ mutants was caused by decreased GSH supply from the mother plant to the pollen. Indeed, germination of the *oastlABC* triple mutant pollen derived from both mutants could be restored by GSH application. These findings are corroborated by the prominent role of GSH for optimal germination, as shown by chemical inhibition of GSH synthesis in wild-type pollen (Zechmann et al., 2011). Furthermore, pollen of the homozygous *pad2-1* mutant, which has a significantly decreased GSH synthesis capacity (Parisy et al., 2007), also displays a germination defect that can be restored by GSH application (Zechmann et al., 2011). Surprisingly, *gsh1*$^{-}$ and *gsh2*$^{-}$ pollen from heterozygous *gsh1*$^{+/-}$ and *gsh2*$^{+/-}$ mutants can germinate and contribute to fertilization and the generation of homozygous mutant embryos, although these pollen completely lack GSH synthesis (Cairns et al., 2006; Pasternak et al., 2008). The major difference between *pad2-1* and *gsh1*$^{-}$ or *gsh2*$^{-}$ pollen is the amount of GSH that can be supplied by the mother plant to these pollen. While the homozygous *pad2-1* mother plant has only 20% of the wild-type glutathione level in vegetative tissues left (Parisy et al., 2007), the *gsh1*$^{-}$ and *gsh2*$^{-}$ pollen derive from heterozygous plants with unaffected glutathione levels. The seemingly contradictory result for germination rates between *pad2-1* (having decreased GSH synthesis capacity) and *gsh1*$^{-}$ or *gsh2*$^{-}$ pollen (lacking GSH synthesis) becomes explainable if the mother plant preloads the pollen with significant amounts of GSH.

GSH preloading by the mother plant would also explain why pollen generated by the *oastlBC* $A^{+/-}$ mutant was able to germinate, although this segregating pollen population also comprises the *oastlABC* triple mutant pollen. The presence of one functional *OAS-TL A* allele in the *oastlBC* $A^{+/-}$ mother plant was obviously sufficient to generate enough Cys for adequate GSH synthesis and preloading of the pollen with GSH. However, GSH synthesis in the pollen contributes to establishing sufficient GSH levels for successful pollen germination, as shown by the decreased germination rate of pollen derived from the *oastlAC* $B^{+/+}$ double mutant in comparison with the wild type and the *oastlAB* $C^{+/+}$ double mutant. The comparison with the latter corroborates the minor contribution of OAS-TL B to net Cys synthesis in the leaves when compared with OAS-TL C.

The prominent role of GSH for pollen germination could be explained by the manifold functions of glutathione in the water stress response (for review, see Chan et al., 2013), since desiccation of the pollen grain during male gametogenesis is crucial for the maximum maintenance of pollen viability and function (Twell, 2004). In this respect, it is noteworthy that Pro, another key player of the water stress response (Yoshiba et al., 1997), is the only amino acid known so far that needs to be synthesized within the pollen in addition to Cys (Funck et al., 2012). Thus, only amino acids with specialized functions, like Cys and Pro, have been shown so far to be essential for male gametophyte function, while the synthesis of other proteinogenic amino acids, like His, Lys, and Trp, is dispensable in pollen (Niyogi et al., 1993; Song et al., 2004; Hudson et al., 2006; Muralla et al., 2007).

CONCLUSION

Subcellular compartmentation of OAS-TL activity contributes to the complex regulation of net cellular Cys synthesis and adjusts fluxes of sulfur and carbon into thiols. In this respect, the presence of OAS-TL C in the mitochondria is crucial for the regulation of carbon flux, as the regulation of OAS synthesis by CSC formation is primarily carried out in this subcellular compartment. However, the bulk of plastid-originated sulfide is incorporated into Cys in the cytosol by OAS-TL A. For that reason, mitochondrial OAS-TL C and cytosolic OAS-TL A are more important for net Cys synthesis than plastidic OAS-TL B, at least under nonstressed conditions, irrespective of their relative contributions to total OAS-TL activity. However, a partial redundancy between the three isoforms ensures the survival of the plant, in case one or two subcellular compartments lack OAS-TL activity. Analysis of triple *oastl* loss-of-function pollen demonstrates that at least one functional OAS-TL isoform is required in pollen during elongated tube growth and/or fertilization. In contrast, glutathione, which constitutes the storage compound for Cys in vegetative tissues, is supplied to the pollen by the mother plant in significant amounts to promote pollen germination. Surprisingly, *oastlABC* triple mutant ovules are viable and can attract pollen for successful fertilization, which strongly indicates a significant transport of Cys or another reduced sulfur-containing compound from the maternal tissue to the ovule.

MATERIALS AND METHODS

Plants

All experiments were performed using Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia. Nontransgenic plants are referred to as the wild type. Transgenic plants used in this study were generated using the T-DNA insertion mutants described by Heeg et al. (2008).

Plant Growth Conditions

Plants were grown on soil under short-day conditions (day/night cycle of 8.5/15.5 h, 50%–60% humidity, 70–100 μE m^{-2} s^{-1}, day/night temperature of 22°C/18°C) as described by Heeg et al. (2008). To induce bolting, 8-week-old plants were transferred to long-day conditions (day/night cycle of 14/10 h).

For determination of incorporation rates, plants were grown hydroponically as described by Heeg et al. (2008). For segregation analysis, seeds were surface sterilized with 70% (v/v) ethanol for 5 min, washed with sterile water, and plated on Arabidopsis growth medium (Haughn and Somerville, 1986) or one-half-strength Murashige and Skoog medium (Murashige and Skoog salt including vitamins [Duchefa Biochemie] and 1% [w/v] Suc, pH 5.8), both including 0.8% (w/v) microagar (Duchefa Biochemie). Plates were kept in the dark at 4°C for two nights, and seedlings were subsequently grown under short-day conditions (day/night cycle of 8/16 h, 70–100 μE m^{-2} s^{-1}, day/night temperature of 22°C/18°C) in a growth cabinet (Percival) in the horizontal position.

Crossing of Plants and Analysis of Siliques

To generate the *oastl* double mutants, the respective single mutants were crossed, and the genotype of the F1 and F2 generations was determined by PCR. To generate the *oastl* triple collection, the *oastl* double mutants were crossed, and the genotype of the filial generations was determined by PCR. To detect a female or male gamete-lethal phenotype, reciprocal crosses with the *oastl* triple collection and the wild type were performed 2 to 3 weeks after bolting using pollen from flowers that opened that day and pistils from still-closed flowers. The silique phenotype was analyzed in ripe siliques shortly before abscission. At this stage, embryos with normal and abnormal development can be distinguished from unfertilized ovules (Meinke, 1994). To describe the silique phenotype, the length of the silique as well as the number of unfertilized ovules, aborted seeds, and fully developed seeds per silique were determined. Classification of the seed phenotype was thereby done according to Meinke (1994): small, white, fist-shaped structures were classified as unfertilized ovules; brown, small, and sometimes shriveled structures as aborted seeds; and whitish/greenish, shiny seeds as fully developed seeds. Silique preparation was performed using fine forceps (Dumont 3-4) and a binocular microscope (MZFLIII; Leica). For phenotypical characterization of the *oastl* triple collection, only siliques 5 to 10 cm underneath the top of the primary stem were used for analysis to ensure comparable developmental stages.

Pollen Analysis

Pollen analysis was performed 4 to 6 weeks after bolting using five to 10 flowers from five plants of each genotype. For pollen preparation, stamens from flowers that opened on that day were removed from the rest of the flower using fine forceps (Dumont 3-4).

To test the viability of the pollen, whole stamens were placed on a microscope slide and covered with Alexander staining solution (Alexander, 1969). The staining of the pollen was analyzed after heating followed by incubation at room temperature for 24 h using an inverse epifluorescence microscope (DM IRB attached to a DFC320 camera; Leica).

To determine the in vitro germination rate, pollen were transferred on a microscope slide covered with germination medium with or without 1 mM glutathione (Zechmann et al., 2011). The microscope slides were placed on a net positioned in a box filled with water to keep humidity high. Germination was monitored after incubation for 26 to 28 h at 20°C in the dark. Micrographs were taken of several randomly chosen areas using the epifluorescence microscope and camera mentioned above, and the amount of germinated pollen (defined as the appearance of a pollen tube, irrespective of the tube length) was determined.

For the quantification of OAS-TL activity in germinated pollen, pollen was released by vigorously vortexing a total of 280 flowers for 2 min in 14 pools, each supplemented with 1 mL of germination medium (19.8% [w/v] Suc, 0.05% [w/v] lactalbumin hydrolysate, 10 mM myoinositol, 5 mM MES, 5 mM $CaCl_2$, 1.5 mM H_3BO_3, 1 mM KCl, and 0.8 mM $MgSO_4$, pH 5.8). After the removal of flowers and tissue parts using forceps, pollen was harvested at 5,000*g* for 6 min and pollen from seven tubes was pooled. After another centrifugation step, pollen was resuspended in 750 μL of germination medium and transferred to a petri dish. The sealed petri dish was incubated overnight at 20°C to 25°C in the dark. Germinated pollen was harvested by centrifugation and resuspended in 130 μL of protein extraction buffer (see below). After sonication for 3 min in ice water and centrifugation for 2 min at 25,000*g*, the supernatant was used for the determination of protein concentration and OAS-TL activity.

Determination of the Genotype by PCR

Genomic DNA was isolated according to Edwards et al. (1991) with minor modifications. The DNA was dissolved in 50 μL of sterile water, and 1 μL was used for PCR. To isolate genomic DNA from 2- to 3-week-old seedlings grown on a plate, the protocol was adjusted to 100 μL of extraction buffer and DNA was dissolved in 30 μL of sterile water. For standard PCR using *Taq* DNA polymerase (New England Biolabs), the same primers as described by Heeg et al. (2008) were used, with the exception that the improved T-DNA-specific LBb1.3 primer (5′-ATTTTGCCGATTTCGGAAC-3′) was used instead of LB_SALK.

Determination of Enzyme Activities, Immunological Detection, and Purification of OAS-TL Proteins

A total of 50 mg of leaf and stem material from 8-week-old soil-grown plants was ground to a fine powder in the presence of liquid nitrogen. Soluble proteins were extracted using 300 μL of extraction buffer (50 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% [v/v] glycerol, 10 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) at room temperature and vigorous mixing for 15 min. After centrifugation at 25,000*g* for 10 min at 4°C, the supernatant was applied to a NAP5 column (GE Healthcare) that was equilibrated in resuspension buffer (50 mM HEPES-KOH, pH 7.4, and 1 mM EDTA). Proteins were eluted using 1 mL of resuspension buffer, and protein concentrations as well as enzymatic activities were determined as described by Heeg et al. (2008). Two micrograms (the wild type, *oastlBC* $A^{+/+}$, and *oastlAC* $B^{+/+}$) or 20 μg (other genotypes) of soluble leaf and stem protein were used for the determination of OAS-TL activity, and 100 μg (all genotypes) was used for the determination of SAT activity. OAS-TL activity in flowers and germinated pollen (see above) was determined in four technical replicates using 2 and 0.1 μg of soluble proteins extracted from a pool of 140 and 280 flowers from approximately 20 plants, respectively. A total of 30 μg of the extracted leaf proteins was separated by discontinuous SDS-PAGE and blotted on a nitrocellulose membrane using a Mini-Protean II system (Bio-Rad). Immunological detection of OAS-TL proteins using anti-OAS-TL C was done as described by Heeg et al. (2008). Proteins of the OAS-TL family were isolated from leaves of 9-week-old plants grown on soil, separated by two-dimensional PAGE, and identified by mass spectrometry as described by Heeg et al. (2008).

Determination of Metabolites and Incorporation Rates Using $^{35}SO_4^{2-}$ or ^{3}H-Labeled Ser

Hydrophilic metabolites were extracted from 50 mg of leaf material from 8-week-old soil-grown plants as described by Wirtz and Hell (2003). Derivatization, separation, and quantification of thiols and OAS were performed as described by Heeg et al. (2008). Incorporation rates of ^{35}S and ^3H-labeled Ser into thiols in leaf pieces were determined as described by Heeg et al. (2008) and Haas et al. (2008), respectively.

qRT-PCR

Expression of the genes encoding OAS-TL-like proteins was determined in rosette leaves of 8-week-old plants grown on soil. Total RNA was extracted from 50 mg of ground leaf material using the peqGOLD Total RNA Kit (Peqlab). On-column DNase treatment was done using the peqGOLD DNase I Digest Kit (Peqlab). Total RNA (2.5 μg) was reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific) and oligo(dT) primers. qRT-PCR was performed in a Rotor-Gene Q cycler (Qiagen) using the Rotor-Gene SYBR Green PCR kit (Qiagen) with the primers given in Supplemental Table S1.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers OAS-TL A1 (At4g14880), OAS-TL A2 (At3g22460), OAS-TL B (At2g43750), OAS-TL C (At3g59760), CYS C1 (At3g61440), CYS D1 (At3g04940), CYS D2 (At5g28020), CS26 (At3g03630) and DES1 (At5g28030).

Supplemental Data

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

Supplemental Figure S1. Stem length of *oastl* mutants.

Supplemental Figure S2. Number of ovules, aborted seeds, and unfertilized ovules within siliques of *oastl* mutants.

Supplemental Figure S3. Reciprocal crosses with wild-type plants and the *oastlAC* $B^{+/+}$ double mutant.

Supplemental Figure S4. OAS-TL activity in different tissues of wild-type plants.

Supplemental Figure S5. Alexander viability staining of pollen from the *oastl* mutants.

Supplemental Figure S6. In vitro germination of pollen from the *oastl* mutants.

Supplemental Figure S7. Glutathione steady-state levels in *oastl* double mutants and the mutants of the *oastl* triple collection.

Supplemental Figure S8. Relative transcript levels of *OAS-TL-like* genes in the wild type and the *oastlAC* $B^{+/-}$ mutant.

Supplemental Table S1. Nucleotide sequences of primers used for qRT-PCR.

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LITERATURE CITED

Alexander MP (1969) Differential staining of aborted and nonaborted pollen. Stain Technol **44:** 117–122

Alvarez C, Calo L, Romero LC, García I, Gotor C (2010) An *O*-acetylserine (thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiol **152:** 656–669

Alvarez C, García I, Moreno I, Pérez-Pérez ME, Crespo JL, Romero LC, Gotor C (2012) Cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile in *Arabidopsis*. Plant Cell **24:** 4621–4634

Bermúdez MA, Páez-Ochoa MA, Gotor C, Romero LC (2010) *Arabidopsis S*-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control. Plant Cell **22:** 403–416

Birke H, Haas FH, De Kok LJ, Balk J, Wirtz M, Hell R (2012) Cysteine biosynthesis, in concert with a novel mechanism, contributes to sulfide detoxification in mitochondria of *Arabidopsis thaliana*. Biochem J **445:** 275–283

Bonner ER, Cahoon RE, Knapke SM, Jez JM (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

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. Plant Physiol **141:** 446–455

Chan KX, Wirtz M, Phua SY, Estavillo GM, Pogson BJ (2013) Balancing metabolites in drought: the sulfur assimilation conundrum. Trends Plant Sci **18:** 18–29

De Paepe R, Forchioni A, Chétrit P, Vedel F (1993) Specific mitochondrial proteins in pollen: presence of an additional ATP synthase beta subunit. Proc Natl Acad Sci USA **90:** 5934–5938

Dominguez-Solís JR, Gutierrez-Alcalá G, Vega JM, Romero LC, 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

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19:** 1349

Funck D, Winter G, Baumgarten L, Forlani G (2012) Requirement of proline synthesis during Arabidopsis reproductive development. BMC Plant Biol **12:** 191–202

García I, Castellano JM, Vioque B, Solano R, Gotor C, Romero LC (2010) Mitochondrial β-cyanoalanine synthase is essential for root hair formation in *Arabidopsis thaliana*. Plant Cell **22:** 3268–3279

Haas FH, Heeg C, Queiroz R, Bauer A, Wirtz M, Hell R (2008) Mitochondrial serine acetyltransferase functions as a pacemaker of cysteine synthesis in plant cells. Plant Physiol **148:** 1055–1067

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

Haughn GW, Somerville C (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet **204:** 430–434

Heeg C, Kruse C, Jost R, Gutensohn M, Ruppert T, Wirtz M, 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

Hell R, Wirtz M (2011) Molecular biology, biochemistry and cellular physiology of cysteine metabolism in *Arabidopsis thaliana*. The Arabidopsis Book **9:** e0154, doi/10.1199/tab.0154

Hudson AO, Singh BK, Leustek T, Gilvarg C (2006) An LL-diaminopimelate aminotransferase defines a novel variant of the lysine biosynthesis pathway in plants. Plant Physiol **140:** 292–301

Jost R, Berkowitz O, Wirtz M, Hopkins L, Hawkesford MJ, Hell R (2000) Genomic and functional characterization of the *oas* gene family encoding *O*-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. Gene **253:** 237–247

Khan MS, Haas FH, Samami AA, Gholami AM, Bauer A, Fellenberg K, Reichelt M, Hänsch R, Mendel RR, Meyer AJ, et al (2010) Sulfite reductase defines a newly discovered bottleneck for assimilatory sulfate reduction and is essential for growth and development in *Arabidopsis thaliana*. Plant Cell **22:** 1216–1231

Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP-sulfurylase and *O*-acetylserine(thiol)lyase in spinach leaves. Plant Physiol **94:** 1345–1352

Mathai JC, Missner A, Kügler P, Saparov SM, Zeidel ML, Lee JK, Pohl P (2009) No facilitator required for membrane transport of hydrogen sulfide. Proc Natl Acad Sci USA **106:** 16633–16638

Meinke DW (1994) Seed development in *Arabidopsis thaliana*. *In* EM Meyerowitz, CR Somerville, eds, Arabidopsis, Vol 27. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 253–295

Meyer AJ, Rausch T (2008) Biosynthesis, compartmentation and cellular functions of glutathione in plant cells. *In* R Hell, C Dahl, DB Knaff, T Leustek, eds, Sulfur Metabolism in Phototrophic Organisms, Vol 27. Springer, Dordrecht, The Netherlands, pp 161–184

Muralla R, Sweeney C, Stepansky A, Leustek T, Meinke D (2007) Genetic dissection of histidine biosynthesis in Arabidopsis. Plant Physiol **144:** 890–903

Nepi M, Franchi GG (2000) Cytochemistry of mature angiosperm pollen. Plant Syst Evol **222:** 45–62

Niyogi KK, Last RL, Fink GR, Keith B (1993) Suppressors of *trp1* fluorescence identify a new *Arabidopsis* gene, *TRP4*, encoding the anthranilate synthase β subunit. Plant Cell **5:** 1011–1027

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011) Glutathione. The Arabidopsis Book **9:** e0142, doi/10.1199/tab.0142

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F (2007) Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. Plant J **49:** 159–172

Pasternak M, Lim B, Wirtz M, Hell R, Cobbett CS, Meyer AJ (2008) Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. Plant J **53:** 999–1012

Song JT, Lu H, Greenberg JT (2004) Divergent roles in *Arabidopsis thaliana* development and defense of two homologous genes, *ABERRANT GROWTH AND DEATH2* and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1*, encoding novel aminotransferases. Plant Cell **16:** 353–366

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol **62:** 157–184

Tegeder M, Rentsch D (2010) Uptake and partitioning of amino acids and peptides. Mol Plant **3:** 997–1011

Tsakraklides G, Martin M, Chalam R, Tarczynski MC, Schmidt A, Leustek T (2002) Sulfate reduction is increased in transgenic *Arabidopsis thaliana* expressing 5′-adenylylsulfate reductase from *Pseudomonas aeruginosa*. Plant J **32:** 879–889

Twell D (2004) Pollen developmental biology. *In* SD O'Neill, JA Roberts, eds, Annual Plant Reviews: Plant Reproduction, Vol 6. Wiley-Blackwell, Chichester, UK, pp 86–153

Van Hoewyk D, Abdel-Ghany SE, Cohu CM, Herbert SK, Kugrens P, Pilon M, Pilon-Smits EA (2007) Chloroplast iron-sulfur cluster protein maturation requires the essential cysteine desulfurase CpNifS. Proc Natl Acad Sci USA **104:** 5686–5691

Vernoux T, Wilson RC, Seeley KA, Reichheld JP, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inzé D, et al (2000) The *ROOT MERISTEMLESS1/CADMIUM SENSITIVE2* gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell **12:** 97–110

Vigani G, Maffi D, Zocchi G (2009) Iron availability affects the function of mitochondria in cucumber roots. New Phytol **182:** 127–136

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

Watanabe M, Mochida K, Kato T, Tabata S, Yoshimoto N, Noji M, 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

Wirtz M, Beard KF, Lee CP, Boltz A, Schwarzländer M, Fuchs C, Meyer AJ, Heeg C, Sweetlove LJ, Ratcliffe RG, et al (2012) Mitochondrial cysteine synthase complex regulates O-acetylserine biosynthesis in plants. J Biol Chem **287:** 27941–27947

Wirtz M, Hell R (2003) Production of cysteine for bacterial and plant biotechnology: application of cysteine feedback-insensitive isoforms of serine acetyltransferase. Amino Acids **24:** 195–203

Xiang C, Werner BL, Christensen EM, Oliver DJ (2001) The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. Plant Physiol **126:** 564–574

Yamaguchi Y, Nakamura T, Kusano T, Sano H (2000) Three Arabidopsis genes encoding proteins with differential activities for cysteine synthase and β-cyanoalanine synthase. Plant Cell Physiol **41:** 465–476

Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol **38:** 1095–1102

Zechmann B, Koffler BE, Russell SD (2011) Glutathione synthesis is essential for pollen germination in vitro. BMC Plant Biol **11:** 54–64