Running head:

OASTLs confer Se resistance and degrade L-SeCys in *Arabidopsis*

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Title:

Impairment in O-acetylserine-(thiol) lyase A and B, but not C, confers higher selenate sensitivity and uncovers role for A, B and C as L-Cys and L-SeCys desulfhydrases in *Arabidopsis*

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Summary:

The cytosolic OASTLA and chloroplastic OASTLB have significantly higher desulfhydrase activity rates than the cytosolic DES1 and are able to degrade L-Cys and L-SeCys to sulfide and selenide, respectively in *Arabidopsis*.

Footnotes:

List of author contributions:

A.K. designed and performed experiments and analyzed the data. A.B. involved in SO kinetic assay and selenate detection. A.S. involved in RNA extraction. D.O. performed Western-blot, S.S and Z.N. participated in native SDS-PAGE assays. M.S. conceived the original idea, designed the research plan, and supervised the research work; the article was jointly written by A.K. and M.S.

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ABSTRACT

The role of the cytosolic O-acetylserine-(thiol) lyase A (OASTLA), chloroplastic OASTLB and mitochondrion OASTLC in plant resistance/sensitivity to selenate was studied in *Arabidopsis* plants. Impairment in OASTLA and B resulted in reduced biomass, chlorophyll and soluble protein levels compared with impaired OASTL C and Wild-Type treated with selenate. The lower organic-Se and protein-Se levels followed by decreased organic-S, S in proteins and total glutathione in *oastlA* and *oastlB* compared to Wild-Type and *oastlC* are indicative that Se accumulation is not the main cause for the stress symptoms, but rather the interference of Se with the S-reduction pathway. The increase in sulfite oxidase, adenosine 5′-phosphosulfate reductase, sulfite reductase and OASTL activity levels, followed by enhanced sulfite and sulfide, indicate a futile anabolic S-starvation response to selenate-induced organic-S catabolism in *oastlA* and *oastlB* compared to Wild-Type and *oastlC*.

Additionally, the catabolic pathway of L-cysteine degradation was enhanced by selenate, and similar to L-cysteine producing activity, *oastlA* and *B* exhibited a significant decrease in L-cysteine desulfhydrase (DES) activity, compared with WT, indicating a major role of OASTLs in L-cysteine degradation. This notion was further evidenced by sulfide dependent DES in-gel activity, immunoblotting, immunoprecipitation with specific antibodies and identification of unique peptides in activity bands generated by OASTLA, B and C. Similar responses of the OASTLs in Seleno-Cysteine degradation was demonstrated in selenate stressed plants. Notably, no L-cysteine and L-Seleno-Cysteine DES activity bands but those related to OASTLs were evident. These results indicate the significance of OASTLs in degrading L-cysteine and L-SelenoCysteine in *Arabidopsis*.

Keywords: Sulfur and selenium metabolism**,** L-Cys desulfhydrase, OASTLs, SeCys desulfhydrase, *Arabidopsis*

INTRODUCTION

The amino acid selenocysteine, a component of selenoproteins in humans, contains Se (Papp et al., 2007). Plants serve as a source of Se for mammalians; yet Se is not essential for plants. The chemical similarity of Se to S, enables plants to readily uptake Se via root sulfate transporters (Pilon-Smits and Quinn, 2010; Sors et al., 2005) and incorporate it into organic Se compounds via the S assimilation pathway components (Grant et al., 2011). The pathway (see Supplemental Fig. S1) is initiated with the adenylation of sulfate/selenate by ATP sulfurylase (ATPS, EC 2.7.7.4) to generate adenosine 5′-phosphosulfate (APS)/adenosine 5′-phosphoselenate (APSe). APS/APSe is then reduced to sulfite/selenite by the plastidic APS reductase (APR, EC 1.8.99.2) (Mroczek-Zdyrska and Wójcik, 2012; Schiavon et al., 2015; White, 2015). The toxic sulfite/selenite is further reduced to sulfide by the chloroplast-localized sulfite reductase (SiR, EC 1.8.7.1) (Khan et al., 2010; Yarmolinsky et al., 2013), whereas selenite can be reduced enzymatically to selenide by SiR and non-enzymatically by reduced glutathione [GSH (Fisher et al., 2016; Seko et al., 1989; White, 2015)]. Further, the generated sulfide/selenide together with O-acetylserine (OAS), the latter catalyzed by serine acetyltransferase (SAT, EC 2.3.1.30), are incorporated into cysteine (Cys) or selenocysteine (SeCys) in a reaction catalyzed by the O-acetylserine-(thiol) lyase (OASTL, EC 2.5.1.47) in cytosol (OASTLA), plastid (OASTLB), and mitochondria (OASTLC) [(Pilon-Smits and Womg, 2012; White, 2015; Wirtz and Hell, 2006) (see in Supplemental Fig. S1)]. The replacement of Cys by SeCys in proteins is thought to be a cause for selenate toxicity in *Arabidopsis* (Sabbagh and Van Hoewyk, 2012; Van Hoewyk, 2018).

Three major Se toxicity mechanisms were recently concluded in plants: competition between Se and S in primary and secondary metabolism, incorporation of SeCys and SeMet into proteins, and disruption caused by oxidative stress of metabolism and cellular structures (Lima et al., 2018; White, 2018). Amongst others, the role of Cys catabolic enzymes in L-SeCys degradation was not shown. Moreover, while the cytosolic L-cysteine desulfhydrase1 (DES1, EC 4.4.1.1) was shown to catalyze the degradation of cysteine to sulfide, pyruvate and ammonia (Alvarez et al., 2012b; Gotor et al., 2013; Romero et al., 2013), DES1 or other L-Cys catabolic enzymes were not shown to increase L-Cys and/or L-SeCys degradation in response to selenate.

In the current study we show that *oastlA and oastlB Atabidopsis* mutants are more sensitive to the presence of selenate in the growth medium than *oastlC* and WT. The

105 significantly lower selenium level in the protein fraction and biomass of *oastlA* and *oastlB*
106 compared to WT and *oastlC* mutant led us to explore the interference of selenate in the sulfate
107 reduction pathway as the cause for the lower growth rate in the two mutants. The results
108 demonstrate that in the absence of active OASTLA and OASTLB, the selenate interference in
109 the S assimilation pathway leads to a decrease in organic S, S in protein fraction and total
110 glutathione and has a more negative effect on the resulting biomass accumulation than the
111 expected positive effect of lower selenium content in the organic fraction. Increase in the activity
112 of core enzymes of the sulfate reduction pathway, the APR and SiR, followed by enhanced
113 sulfite and sulfide levels uncovers a futile anabolic S-starvation response to the selenate induced
114 organic-S catabolism in *oastlA* and *oastlB* compared to WT and *oastlC.* A major role for the
115 OASTL proteins in acting as L-Cys and L-SeCys desulfhydrases in control and Selenate stressed
116 plants was demonstrated as well.
117
118 **RESULTS**
119 ***oastlA* and *oastlB* mutants are more sensitive to selenate than *WT* and *oastlC***
120 Replacement of cysteine by selenocysteine in proteins is considered to be toxic to plants
121 (Lima et al., 2018; White, 2018); whereas minimizing the incorporation of selenocysteine into
122 proteins has been shown to be an effective strategy to increase Se tolerance in plants (Van
123 Hoewyk et al., 2005). *Arabidopsis* mutants impaired in the OASTLs: the cytosolic (*oastlA*),
124 plastidal (*oastlB*) and mitochondrial (*oastlC*), were employed to examine whether these
125 impairments will reduce selenium in the protein fraction and thus enhance plant tolerance to
126 selenium toxicity.

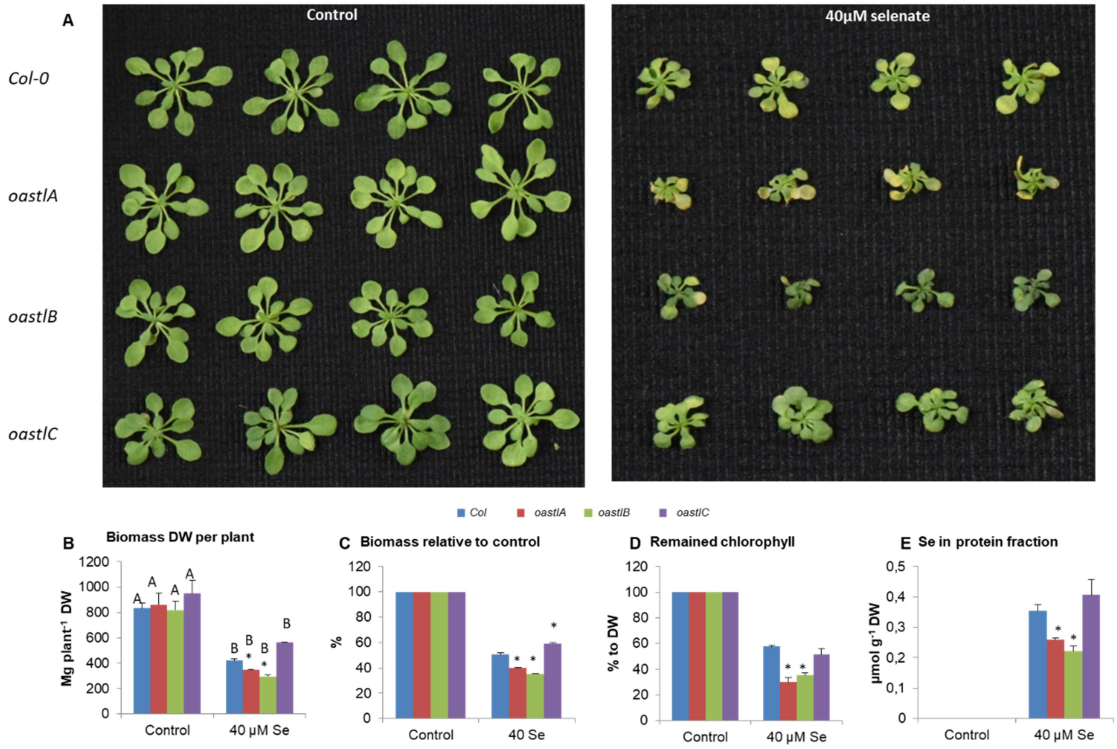

Figure 1. The effect of 40 µM selenate treatment on growth parameters and Se in protein fraction in WT, *oastlA, oastlB* and *oastlC KO* mutants of *Arabidopsis thaliana*. A. Phenotype of plants grown for 14 days under control (left panel) and 40µM selenate treated (right panel) conditions. The weight of plant biomass accumulation (B) and relative weight to their own controls (C) supplemented with or without selenate for 14 days. The values are means ± SE (n = 15). Remaining chlorophyll level (D) and Se in protein fraction (E). The values are means ± SE (n = 4). Values denoted with different letters are significantly different according to the T- test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different uppercase letters indicate significant differences between control and Se treatment of each genotype. Asterisks indicate significant differences between *WT* and *oas-tlA, oas-tlB, oastlC* KO plants subjected to the same treatment.

The mutation of the various OASTL mutant plants was verified by immunoblot analysis, using an OASTL C polyclonal antiserum which also cross-reacts with OASTL A and OASTL B (kindly provided by Prof. Dr. S. Kopriva, University of Cologne, Köln). The three bands detected in WT fractionated proteins corresponded to OASTL A, B, and C from bottom to top respectively, whereas the lower, middle or upper bands were missing in the fractionated proteins of *oastlA, oastlB* or *oastlC* mutants respectively (Supplemental Fig. S2A).

oastlA, oastlB or *oastlC* mutants grown with *WT* plants on 0.5 MS agar medium in the presence and absence (control) of 40 µM selenate for 2 weeks exhibited similar growth as *WT* plant in the absence of selenate and showed a significant reduction in growth rate and chlorophyll level in response to selenate. Yet, the reduction in biomass and chlorophyll content

148 shown in *oastlA* and *oastlB* mutants was significantly higher than in *WT* and *oastlC* (Fig. 1A-D),
149 in spite of the lower Se level in the protein fraction of the *oastlA* and *oastlB* (Fig. 1E).
150 Since at high levels selenate and Se can act as pro-oxidants and cause oxidative stress
151 (Grant et al., 2011; Mroczek-Zdyrska and Wójcik, 2012), Se and selenate levels were determined
152 in the leaves. Yet, no higher total Se and selenate were detected in *oastlA* and *oastlB* as
153 compared to *WT* and *oastlC* (Supplemental Fig. S2B and C) which could be attributed to the
154 decreased biomass accumulation and higher chlorophyll degradation rates in *oastlA* and *oastlB*
155 mutants. These results indicate that the levels of Se in the protein fraction, total Se and selenate
156 in leaf tissue are not the only cause for the higher sensitivity to Selenate noticed in *oastlA* and
157 *oastlB* compared to WT and *oastlC* mutant.
158

159 **The interference in sulfate assimilation by selenate is stronger in *oastA* and *B* than in WT**
160 **and *oastlC* mutant**

161 Since higher sensitivity in the *oastlA* and *oastlB* mutants was not related to Se content in
162 the leaves, the sulfate reduction pathway was further studied, estimating the level of selenate
163 interference. Cysteine biosynthesis, the last step of the sulfate reduction pathway catalyzed by
164 OASTLs was studied in the three mutants in comparison to WT. *OASTLA, OASTLB* or *OASTLC*
165 transcripts were not expressed in *oastlA, oastlB* or *oastlC* mutants respectively, indicating the
166 complete knockout of these genes (Supplemental Fig. S2D-F). Importantly, under control
167 conditions *OASTLB* transcript was enhanced in *AtoastA* and *C* mutants, *OASTLA* in *AtoastB*,
168 whereas *OASTC* was enhanced in *AtoastB,* indicating a level of complementation to compensate
169 for the mutation among the various transcripts. The presence of selenate in the growth medium
170 resulted in a significant enhancement of the transcripts, exhibiting the increase of the three
171 transcripts in *WT*, as well as *OASTLA* and *B* transcripts in *oastlC*, whereas in *oastlA* the transcript
172 of *OASTLB* and in *oastlB OASTLA,* both were significantly increased (Supplemental Fig. S2D-
173 F). Similarly, the absence of either OASTLA or C in *oastlA* or *oastlC* mutants respectively,
174 resulted in the enhancement of the two other functioning OASTLs in each of the mutants as
175 compared to the proteins expression in WT leaves grown under unstressed conditions, whereas in
176 *oastlB* only OASTLA exhibited such response (Supplemental Fig. S2A). Supplementation of
177 selenate to the growth medium resulted in increased expression of OASTLB and C proteins in

178 WT leaves, and the enhancement of OASTLB in *oastlA*, OASTLA in *oastlB* and OASTLB in
179 *AtoastlC* as compared to their control unstressed plants (Supplemental Fig. S2A).
180 In-spite of the enhanced protein expression of OASTLB and C in the absence of selenate
181 and of OASTLB in the presence of selenate, the impairment in OASTLA resulted in a strongly
182 decreased cysteine production activity of OASTL in *AtoastlA* mutant, exhibiting a reduction in
183 activity by 87% and 72% as compared to WT under control and selenate treated conditions,
184 respectively. The *AtoastlB* mutant showed a significant decline of 28% in both conditions, while
185 in *oastlC* OASTL activity was similar to the activity in WT plants. Interestingly, the activity in
186 all the examined genotypes was significantly increased with selenate treatment, likely as the
187 result of the enhanced expression of at least one of the active OASTL proteins (Fig. 2A). The
188 comparable activity rates to WT found in *oastlC* mutant might be the result of the enhanced
189 expression of the two major proteins, the OASTLA and B as compared to WT in control as well
190 as under stressed conditions (Supplemental Fig. S2A). Taken together, the enhanced OASTL
191 activity in leaves of selenate stressed WT and the OASTL mutants may indicate S-limitation type
192 response shown before in *Arabidopsis* leaves in response to limited S supply (Barraso et al.,
193 1995; Hesse et al., 1999).
194 To examine this assumption the Cys, total glutathione and total organic S levels, as well
195 as the S level in protein fraction were detected. No differences were noticed in cysteine level
196 between WT and mutants in control and selenate treated plants, whereas in selenate treated
197 plants a higher cysteine level was evident with WT and *oastlC* compared to these plants grown
198 under control conditions (Fig. 2B). Determination of total glutathione revealed a decrease only in
199 *oastlC* leaves whereas the other mutants exhibited comparable levels to WT leaves in plants
200 grown in control conditions. In contrast, *oastlA* and *oastB* showed decreased total glutathione
201 level compared to WT and *oastlC* leaves in plants exposed to selenate. Significantly, all
202 genotypes exposed to selenate exhibited significantly lower glutathione levels than control plants
203 (Fig. 2C). Determination of the organic S level revealed a significant reduction in all the
204 genotypes at 40 μM selenate compared to untreated controls. Under the selenate stress, *oastlA*
205 and *oastlB* exhibited a significant decrease in organic S as compared to WT and *oastlC* (Fig.2D).
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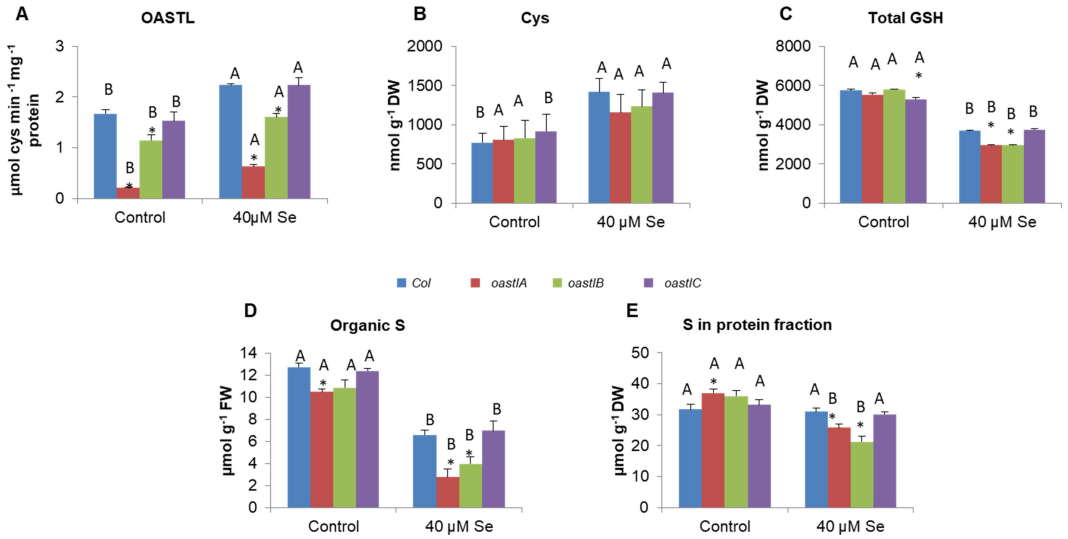

Figure 2. Cys biosynthesis and S containing compounds in WT, *oastlA, oastlB* and *oastlC* KO mutants supplemented with or without selenate for 14 days. The effect of 40 µM selenate treatment on OAS-TL activity (A), Cysteine level (B), Total glutathione (C), Total organic S (D) and S in protein fraction (E). The values are means ± SE (n = 4). Values denoted with different letters are significantly different according to T-test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different uppercase letters indicate significant differences between control and Se treatment of each genotype. Asterisks indicate significant differences between WT and the KO plants *oastlA, oastlB and oastlC* subjected to the same treatment.

The level of S in the protein fraction, under control conditions, was higher in *oastlA* than in WT, whereas in the other mutants it was similar to *WT*. Selenate supplementation resulted in a significantly lower S content in the protein fraction in *oastlA* and *oastlB* as compared to their controls and selenate treated WT and *oastlC* (Fig.2E). Taken together, the lower total glutathione, organic S and S in protein fraction in the leaf tissue of mutants impaired in OASTLA or B proteins fed with selenate (Fig. 2C, D and E), indicates possible interference in the S reduction pathway by selenate, resulting in retarded plant growth as compared to WT and *oastlC* mutant.

The presence of selenate in the growth medium induces catabolic processes in *Arabidopsis*

Cysteine can be found in plants as free cysteine, being among the amino acids that build proteins, as well as an intermediate of Met biosynthesis, GSH and many other thiol species (Hesse and Hoefgen, 2003; Ravanel et al., 1998). Under the long day (14/10 light/dark) growth conditions free cysteine levels in leaves of plants growing in plates containing 0.5 MS medium,

234 were similar in the mutants compared to WT either in control or in selenate treated plants (Fig.
235 2B). Considering the lowest cysteine generating activity level of OASTL was evident in *oastlA*
236 and *B* (Fig. 2B), this result indicates a possible catabolic protein degrading activity which can act
237 as an additional source of free L-cysteine in *oastlA* and *B*. In support of this notion is the
238 significantly lower soluble protein content evident in *oastlA* and *B* compared to WT and *oastlC*
239 mutant grown with selenate in the growth medium (Fig. 3A).

240 Recently, a lower biomass accumulation rate, the result of reduced organic S content was
241 shown to be the consequence of higher L-cysteine degradation by desulfhydrase (DES) activity
242 of OASTL in the perennial halophyte *Sarcocornia*, but not in the annual halophyte *Salicornia*,
243 both fed with high sulfate (Kurmanbayeva et al., 2017). To examine whether OASTLs play a
244 role in cysteine degradation also in non-halophyte plants, we studied first the expression of the
245 cytosolic *DES1* claimed previously to play an important role in cytosolic L-Cys degradation
246 (Álvarez et al., 2010). Unlike the *OASTLs* whose transcript levels were enhanced in WT plants in
247 response to selenate (Supplemental Fig. S2 D-F), a significant decrease in *DES1* transcript
248 abundance was evident in WT, *oastlB* and *C,* whereas in *oastlA* mutant a non-significant
249 decrease was noticed. Under control and selenate stress conditions, *DES1* transcript abundance
250 was the highest in *oastlB* (Fig. 3B).

251 Unlike the *DES1* transcript abundance in *WT* and *OASTLs* mutants, but similar to the L-
252 Cys generation activities of the OASTLs (Fig. 2A), the total L-Cys DES kinetic activity was
253 enhanced in all genotypes grown with selenate compared to unstressed control plants (Fig. 3C).
254 DES activity rate was significantly lower in *oastlA* and *oastlB* mutants compared to *WT* grown
255 under unstressed conditions, while when exposed to selenate *oastlA* exhibited a significant lower
256 activity rate than *WT* and the other genotypes (Fig.3C). These results indicate a possible role of
257 these OASTL isoforms in L-Cys degradation and not only in L-Cys biosynthesis (Figs.2A, 3C).
258

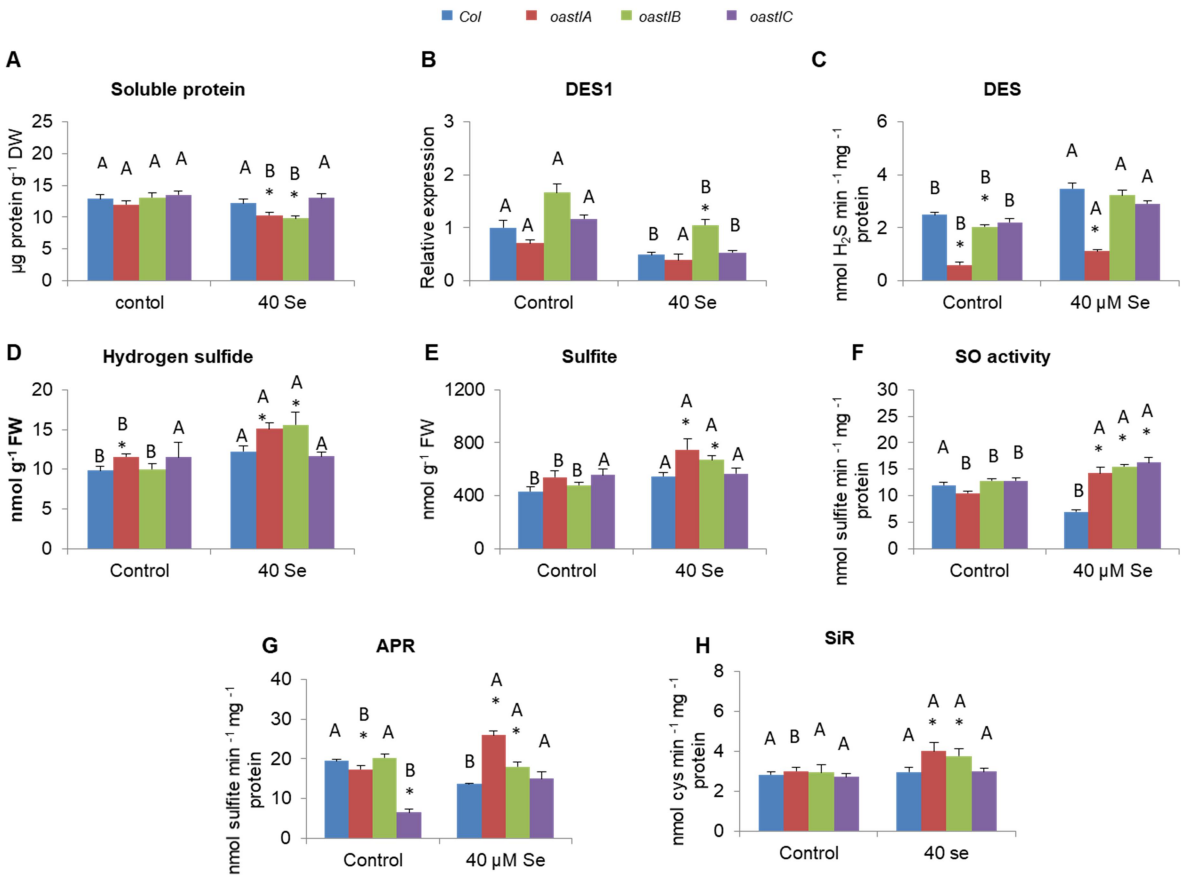

Figure 3. Soluble protein level and S metabolism components in WT, *oastlA, oastlB* and *oastlC* KO mutants supplemented with or without selenate for 14 days. The effect of 40 µM selenate treatment on soluble protein content (A), transcript expression of DES1 (B), L-Cysteine desulfhydrase (DES) activity (C), sulfide (D) and sulfite levels (E), Sulfite oxidase (SO) activity (F), Adenosine 5′-phosphosulfate reductase (APR) activity (G) and Sulfite reductase (SiR) activity (H). Transcript level of *DES1* was detected by quantitative reverse transcription-PCR using *ACTIN2* as the housekeeping transcript for normalization. The relative expression in the various genotypes was analyzed using the normalized WT control as reference. The values are means ± SE (n = 4). Values denoted with different letters are significantly different according to T-test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different uppercase letters indicate significant differences between control and Se treatment of each genotype. Asterisks indicate significant differences between WT and *oastlA, oastlB, oastlC* plants subjected to the same treatment.

Notably, in spite of the lower or similar DES activity rate, *oastlA* and *oastlB* mutants exhibited higher sensitivity to selenate stress, accumulating lower biomass. This can be attributed to the lowest capacity of L-Cys biosynthesis by OASTL in *oastlA* and *oastlB* mutants grown

278 with selenate that resulted in low levels of organic sulfur, total glutathione as well as low S in the
279 protein fraction (Fig. 2A,C,D,E). This could result in S-starvation type responses such as the
280 higher sulfide level evident in these mutants compared to WT and *oastlC* when stressed by
281 selenate, exhibiting, excluding *oastlC,* higher sulfide than the control unstressed plants, (Fig.
282 3D). The higher sulfite level detected in these mutants compared to WT and *oastlC* grown with
283 selenate (Fig. 3E), shown before when detected on fresh weight basis to be toxic in *Arabidopsis*
284 and tomato plants (Brychkova et al., 2012; Brychkova et al., 2013; Yarmolinsky et al., 2014), is
285 likely another S-starvation type response and an additional cause for the mutants' sensitivity to
286 selenate. Interestingly, while under control unstressed conditions SO activity was similar in
287 mutants and in WT treated with selenate, the mutants exhibited a drastically higher SO activity
288 rate than WT (Fig. 3F). This enhanced SO activity in the mutants compared to WT is a typical
289 response of SO to sulfite increase as was shown before (Brychkova et al., 2012; 2013;
290 Yarmolinsky et al., 2013; 2014). The selenate induced sulfite increase in *oastlA* and *oastlB*
291 mutants is most likely the result of the increased APR activity rate in these mutants compared to
292 WT and *oastlC* grown with selenate (Fig. 3G). The increase in APR activity rate in *oastlA* and
293 *oastlB* leaves is an expected response to S-starvation (Lee et al., 2011 and references therein), as
294 featured in the current study by lower glutathione, organic S and S in the protein fraction of these
295 mutants compared to WT and *oastlC* (Fig. 2C,D,E). Further reinforcement for this notion is the
296 significant increase in SiR activity resulting in the enhanced sulfide in *oastlA* and *oastlB* treated
297 with selenate, whereas no differences between the genotypes were noticed in the unstressed
298 plants (Fig. 3H, D). Taken together, the increase in SO, APR and SiR activity, followed by the
299 enhanced sulfite and sulfide levels (Fig. 3D-H), indicates a futile anabolic S-starvation response
300 to the selenate induced organic-S catabolism (Fig. 2C, D, E) in *oastlA* and *oastlB* compared to
301 Wild-Type and *oastlC*.
302
303 **OAS-TLs degrades L-Cys and L-SeCys to H_2S and H_2Se respectively**
304 The decrease in L-Cys desulfydrase kinetic activity in the *oastlA* and *oastlB* mutants
305 compared to WT (Fig. 3C) raised the possibility that OASTLs may act as L-Cys and L-SeCys
306 desulfhydrases. This was examined by employing crude protein extracted from leaves of 14 day
307 old selenate stressed and unstressed WT, *oastlA*, *B* and *C* plants, fractionated by native SDS-
308 PAGE (Sagi and Fluhr, 2001; Srivastava et al., 2017; Yesbergenova et al., 2005) to distinguish

309 between the activity bands of the various OASTLs. The gels were then subjected to DES activity
310 reaction solution containing L-Cys or L-SeCys as a substrate under reducing conditions,
311 generated in the presence of β-Mercaptoethanol. The reaction of lead (as lead acetate) with
312 sulfide or selenide (the consequence of desulfhydration activity that degrades L-Cys or L-SeCys,
313 respectively), results in a brown-dark precipitate exhibiting the activity band at the position of
314 the fractionated active enzyme. When the substrates (L-Cys or L-SeCys) or reducing conditions
315 (β-Mercaptoethanol) were omitted from the reaction solution, the activity bands, the precipitated
316 product of the generated lead sulfide or lead selenide, were not seen (Supplemental Fig. S3A). In
317 contrast, when the substrate was present under reducing condition, three distinguishable activity
318 bands were present in the location of the fractionated WT proteins (Fig.4A). Significantly, the
319 fractionated proteins extracted from *oastlA*, *oastlB* and *oastlC* mutants exhibited the absence of
320 the lowest, middle and most upper activity band respectively, compared to WT, with L-Cys as
321 the substrate (Fig.4A). When L-SeCys was present in the reaction solution as the substrate, the
322 two lowest activity bands with the same mobility as with the L-Cys desulfhydrase activity were
323 present in the fractionated WT proteins, whereas the fractionated proteins of *oastlA* and *oastlB*
324 mutants were missing the lowest and middle bands, respectively (Fig. 4B). The absence of the
325 most upper activity band of OASTLC in WT and *oastlA*, *oastlB* mutants, is likely the result of
326 the relatively low L-SeCys desulfhydrase activity rate by OASTLC, being below the detection
327 level when employing L-SeCys as a substrate. Importantly, the presence of selenate in the
328 growth medium resulted in the general enhancement of L-Cys and L-SeCys desulfhydrase
329 activities of OAS-TL enzymes compared to the control conditions (Fig.4A and B). These results
330 indicate that OASTLA, OASTLB and OASTLC play a role not only in Cys/SeCys biosynthesis,
331 but also in degrading L-Cys and L-SeCys.

332 Interestingly, while the homozygous *Atdes1* KO T-DNA mutant SALK_103855 was
333 shown to express ca 45 to 60% of *DES1* (AT5G28030) compared to WT root transcript (see
334 Figure 7C in Mei et al., 2019), the homozygous KO mutant SALK_103855 exhibited almost the
335 absence and ca 29% transcript expression compared to WT employing oligonucleotides upstream
336 and downstream along the transcript length, respectively. SALK_205358, shown before to be a
337 *des1* null mutant (Jin et al., 2018) exhibited ca 40% decrease in *DES1* compared to the
338 expression in WT leaves, employing the oligonucleotides described above (Supplemental Fig.
339 S3B and E). Yet, in-spite of the transcript decrease the *Atdes1* KO (SALK_205358) exhibited

340 kinetic activities of cysteine generation by OASTLs and total desulfhydration activity in control
341 and selenate stressed plants comparable to WT (Fig. S3C and D, respectively).
342 Since SALK_103855, KO mutant of *Atdes1* grown on MS medium was also
343 demonstrated to be responsible for only a minor portion of the Cys-desulfudrase activity
344 compared to WT [14% of the total activity in leaves of plants grown in plates containing agar
345 with MS medium (Alvarez et al., 2010)], an in-gel desulfydrase activity was employed to further
346 examine the significance of DES1 and the OASTLs in desulfhydrase activity in Arabidopsis
347 plants. NATIVE-SDS was employed to fractionate the proteins (Sagi and Fluhr, 2001;
348 Yesbergenova et al., 2005) for desulfhydrase in-gel activities in WT and homozygous
349 SALK_205358 *Atdes1* mutant employing L-Cys or L-SeCys as substrates. The existence of
350 OASTLA, OASTLB and OASTLC activity bands with relative intensity in *Atdes1* mutant as
351 with WT was evident (Supplemental Fig. S3E). We further examined L-Cys desulfhydrase
352 activity in SALK_103855 leaves and further noticed similar activity bands in WT and the *Atdes1*
353 homozygous mutant (Supplemental Fig. S3F, left insert). These results demonstrate that the
354 OASTL enzymes may play a significant role in degrading L-cys and L-SeCys to sulfide and
355 selenide respectively in Arabidopsis leaves.
356 To further support the identification of the enzymes which generated the activity bands, a
357 Western blot analysis employing the lower middle and upper sliced activity bands in WT (Fig.
358 4A and B) was performed after the fractionation of the proteins in SDS-PAGE, using OASTL C
359 antibody which cross reacts also with OAS-TL A and B proteins (kindly provided by Prof. R.
360 Hell, Heidelberg University, Germany). Different mobility of the single bands was demonstrated
361 (Fig.4C left side), supporting the notion of OASTL A, B and C in L-Cys and OASTL A, B
362 isoforms playing a role in L-SeCys degradation (Fig.4C right side). Additional support for this
363 notion was achieved by immunoprecipitation analysis. The proteins that generated L-Cys and
364 SeCys DES activity bands were fully pulled down after the proteins were immunoprecipitated by
365 *Arabidopsis* OAS-TL B specific antibody (kindly provided by Prof. Dr. S. Kopriva, University
366 of Cologne, Germany) that also cross reacts with OASTLA and C isoforms (Fig. 4D left and
367 right side respectively).
368

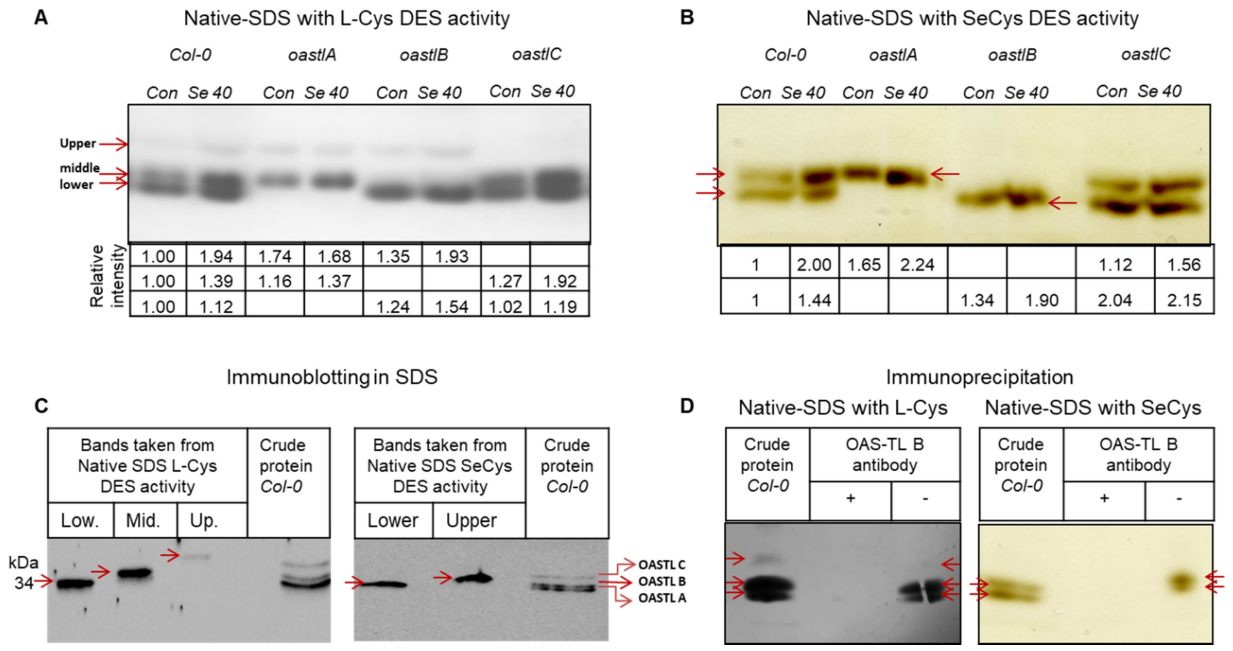

Figure 4. NATIVE-SDS on L-Cys and SeCys desulfhydrase activities in WT, oastlA, oastlB and oastlC KO mutants supplemented with or without selenate for 14 days. Effect of 40 µM selenate treatment on L-Cysteine (A) and SeCys (B) degradation activities. C. Immunodetection of the sliced WT activity bands from 4A are in left insert and the sliced WT activity bands sliced from 4B are in the right insert. D. Desulfhydrase activity of WT crude protein extract without immunoprecipitation (left lane) with immunoprecipitation in the presence (+) or absence (-) of specific antibodies. L-cys and L-Secys desulfhydrase activities are left and right inserts respectively. Red arrows indicate activity band.

Finally, the identification of tripsinized unique peptides in the three sliced activity bands after the fractionation of WT crude protein extract (Fig.4A and B) was performed. The lowest sliced activity bands of L-Cys and L-SeCys desulfhydrase activities revealed 23 and 20 unique peptides overlapping 66% and 65% of the Arabidopsis OAS-TL A protein sequence, respectively (Supplemental Table S1A-C). Together with the OAS-TL A peptides, a lower amount of 14 and 9 peptides of OASTLB were identified in the lowest sliced L-Cys and SeCys desulfhydrase activity bands, respectively (Supplemental Table S1A-C). In the middle activity band, sliced from L-Cys and SeCys desulfhydrase activity, 23 and 18 unique peptides were identified showing 58% and 60.5% overlap of OAS-TL B protein sequence, respectively (Supplemental Table S1A-C). Additionally, 18 and 15 peptides of OASTLA proteins in the L-Cys and SeCys desulfhydrase activity bands and 6 unique peptides of OASTLC protein were identified in L-Cys desulfhydrase activity. The upper band sliced from L-Cys desulfhydrase activity revealed 13 specific unique peptides with 45% coverage of OASTLC protein. Six and 11 unique peptides of OASTLA and B proteins respectively were identified as well (Supplemental Table S1A-C). The

391 lack of a full separation between the 3 OASTLs activity bands is likely the result of the relatively
392 high identity between the three proteins [ca 70 to 80% identity (Supplemental Fig.S4)] that
393 prevented the clear complete separation in-spite of the 16 cm fractionation length by SDS-
394 PAGE. Yet, based on the identification by the use of the three mutants exposed to in-gel activity,
395 immunoblot and immunoprecipitation by the specific antibodies employed (Fig. 4), as well as the
396 majority of the overlapping unique peptides (Supplemental Table S1), we can conclude that
397 OASTLA, B and C are localized to the highest, second-highest and the lowest activity bands,
398 respectively of L-Cys and SeCys desulfhydrase activity.

399 Significantly, only two unique peptides of DES1 were identified in the lower and middle
400 bands and none in the most upper band of L-Cys desulfhydrase activity bands (Supplemental
401 Table S1). Considering that the number of theoretic tryptic peptides in DES1 is similar to that of
402 OASTLs (estimated by the use of Expasy: https://web.expasy.org/peptide_mass/), these results
403 indicate that DES1 is a less abundant protein or it undergoes ionization. Yet, since the semi
404 quantitation was made by calculating the peak area of each peptide, whereas to overcome
405 differential ionization, the area of the protein was calculated as the average of the three most
406 intense peptides from each protein; the data indicate that OASTL A, B and C are more abundant
407 than DES1 protein (Supplemental Table S1D).
408 The less abundant DES1 protein in the activity bands does not necessarily indicate
409 decreased L-Cys and L-SeCys DES activity by the WT DES1 compared to the OASTLs. Yet,
410 firstly, the kinetic activity revealed a major significant decrease of 80 and 65% in the Cys-
411 desulfhydrase activity of the cytosolic *oastlA* leaves in plants grown in control or selenate
412 stressed plants, respectively compared to WT (Fig.3C). Additionally, activity bands, additive to
413 OASTL, A, B and C, that could be attributed to DES1, were not noticed, neither above, nor
414 below and none were noticed at the position of the migrated OASTL A, B and C when the
415 activity bands were absent in the related impaired mutants under control and selenate stressed
416 conditions (Fig. 4A and B). Taken together these results (Supplemental Table S1A-D, Figs 3C,
417 4A, B) strongly support the notion that OAS-TL A, B and C proteins play a major role in L-Cys
418 and L-SeCys degradation in *Arabidopsis*. In the presence of selenate, plants impaired in OASTL
419 A and B exhibit higher catabolic processing rates of chlorophyll and protein degradation, the
420 result of enhanced sulfur metabolism interference in the mutants compared with WT.
421

422 **DISCUSSION**

423 **Impairment in OASTLA and OASTLB leads to higher sensitivity to selenate**

424 Non-specific incorporation of seleno-amino acids SeCys and SeMet into proteins instead
425 of cysteine and methionine is thought to be a main cause for Se induced toxicity in many plants
426 (Sors et al., 2005; Terry et al., 2000). Yet, lower Se in protein fraction was evident in *apr2* KO
427 mutants exhibiting stronger sensitivity than WT to the Se treatment (Grant et al., 2011).
428 Similarly, determination of the total Se and Se content in protein fraction revealed a significantly
429 lower rate in *oastlA* and *oastlB* mutants, compared with WT and *oastlC* (Fig. 1E and
430 Supplemental Fig. S2B). This indicates that the accumulated Se in *oastlA* and *oastlB* mutant
431 proteins is not the main cause for their stronger sensitivity to Se compared with WT.

432 The uptake and assimilation of Se has similar effects on gene expression as with the case
433 of S-starvation, resulting in greater S uptake and assimilation rates by plants [(White, 2018) and
434 references therein]. Plants exposed to high Se level exhibited symptoms such as growth
435 inhibition and chlorosis (Grant et al., 2011; Sabbagh and Van Hoewyk, 2012; Van Hoewyk,
436 2018). Interestingly, the overexpression of OASTL in Arabidopsis and tobacco increased
437 tolerance to the toxicity of Cd stress by increasing thiol availability (Domínguez-Solís et al.,
438 2004). Among the three *Arabidopsis* OASTL mutants grown for 2 weeks with 40µM selenate, a
439 decrease in biomass accumulation and chlorophyll content compared to WT was noticed in
440 *oastlA* and *oastlB* but not in *oastlC* mutant (Fig.1). This is likely since the cytosolic and
441 chloroplastic OASTL A and B respectively, exhibit significantly higher activity rates compared
442 with the mitochondrion localized OASTL C, the latter being responsible for only 5-12% of
443 OASTL activity in WT plants (Heeg et al., 2008; Kuske et al., 1996). The results demonstrate
444 that dissimilar to WT and impairment in OASTLC, in the absence of active OASTLA and
445 OASTLB, the selenate interference in S assimilation pathway leads to a stronger decrease in
446 organic S, S in protein fraction and total glutathione, and has a more negative effect on the
447 remaining chlorophyll level and the resulting biomass accumulation than the expected positive
448 effect of lower selenium content in the organic fraction (Figs 1 and 2).

449 The free cysteine levels were similar in WT and the mutants exposed to selenate
450 (Fig.2B), whereas the Cys generation activity rate differed as shown here in response to selenate
451 (Fig.2A). This was also shown previously in an O-acetylserine (thiol) lyase reduced isoform by
452 RNA interference in potato, as well as in *Arabidopsis* KO mutants (Heeg et al., 2008;

453 Riemenschneider et al., 2005), indicating the feasibility of an additional Cys source affecting free
454 Cys level. The source can be protein degradation, indicated by the lower soluble protein level in
455 *oastlA* and *oastlB* KO mutants compared to WT and *oastlC* treated with selenate (Fig. 3A). The
456 protein degradation is the result of selenate induced stress, being stronger in *oastlA* and *B* as
457 indicated by the lower remaining chlorophyll (Fig. 1D), organic-S, and S in protein fraction (Fig.
458 2D and E).

459 Sulfite is a strong nucleophile that should be tightly regulated to avoid its deleterious
460 reaction with a wide variety of cellular components (Brychkova et al., 2007; Lang et al., 2007;
461 Brychkova et al., 2013; Yarmolinsky et al., 2013; Brychkova et al., 2015). The increased sulfite
462 level in *oastlA* and *oastlB* mutants compared to WT (Fig. 3D) is a deleterious effect of a futile S-
463 starvation response, the result of the low organic sulfur, total glutathione as well as low S in the
464 protein fraction (Fig. 2A,C,D,E) in *oastlA* and *oastlB* mutants treated with selenate. APR activity
465 increase in *oastlA* and *oastlB* leaves that caused the enhanced sulfite level in these 2 mutants is a
466 response to S-starvation (Lee et al., 2011) and the followed sulfide level increase, the result of
467 higher SiR activity in these 2 mutants, further supports the notion of selenate induced S-
468 starvation. The futileness of such response is firstly because of the waste of reducing agents and
469 ATP (see in Supplementary Fig. S1) in the process ending with excess sulfide, the result of the
470 inability of *oastlA* and *oastlB* plants to assimilate sulfide to L-Cys generation. Additionally, the
471 sulfite level increase, shown before to induce oxidative stress (Brychkova et al., 2012;
472 Yarmolinsky et al., 2014), indeed led to oxidative stress (Supplementary Fig. S5) that had an
473 additive deleterious effect on the growth of *oastlA* and *oastlB* mutant plants. The oxidative stress
474 could further induce APR to generate more sulfite as demonstrated before (Bick et al., 2001;
475 Koprivova et al., 2008). Notably, the levels of hydrogen peroxide (H_2O_2), the carbonyl toxic
476 malondialdehyde (MDA), the product of lipid peroxidation by prolonged oxidative stress (Tian
477 et al., 2017) and the antioxidant anthocyanins (Gould et al., 2002; Xu et al., 2017), were
478 enhanced (on FW, and except H_2O_2, on soluble protein basis as well) in the selenate treated
479 plants compared to control plants. MDA and anthocyanin levels were significantly higher in
480 *oastlA* and *oastlB* compared to the selenate treated WT and *oastlC* mutant, whereas, H_2O_2 was
481 significantly higher in the 3 mutants compared to WT treated with selenate. The enhancement in
482 H_2O_2, MDA and anthocyanins levels in *oastlA* and *oastlB* mutants (Supplemental Fig. S5) is
483 indicative of the higher oxidative stress level in these plants as shown before (Gould et al., 2002;

484 Srivastava et al., 2017; Xu et al., 2017; Yarmolinsky et al., 2014). Taken together the results
485 indicate that the decrease in *oastlA* and *oastlB* biomass accumulation is likely the result of
486 selenate induced S-starvation responses causing waste of reducing agents and ATP, as well as
487 sulfite induced oxidative stress.
488

489 **OASTL A and B act as major L-Cys and L-SeCys desulfhydrases in *Arabidopsis***

490 The similar free cysteine level in WT and *oastlA* and *B* mutants, compared with the lower
491 cysteine generating activity rate of OASTL and the lower soluble protein level evident in the
492 mutants exposed to selenate (Figs 2A, B, Supplemental S3A), led us to examine a role for
493 OASTL proteins in degrading L-Cys and SeCys to sulfide and selenide respectively.
494 Additionally, albeit in a small rate comparing to the biosynthesis by OASTLs, the degrading
495 activity of the active enzymes is likely beneficial to plants, decreasing the levels of L-SeCys
496 (compare the rate in Fig. 2A to the rate in Fig.3C) and its incorporation into proteins.

497 L-Cys desulfhydrase activity in WT showed 3 activity bands, where the upper band intensity
498 was weaker than that of the other two bands. L-SeCys desulfhydrase exhibited only 2 activity
499 bands, with the absence of the upper band likely the result of detection limits (Fig. 4A and B).
500 Importantly, fractionated proteins extracted from *oastlA* and *oastlB* KO mutants exhibited in-gel
501 activity absent of the lower and middle activity band, respectively with both activity types; the L-
502 Cys and SeCys DES, whereas the most upper band was seen only when L-Cys was used as the
503 substrate and only in WT, *oastlA* and *oastlB* but not in *oastlC* (Fig. 4A and B). These results
504 indicate that the activity bands with the highest, second-highest and the lowest mobility are of
505 OASTLA, B and C, respectively. Additionally, the results show that the three OASTLs have the
506 capacity of L-SeCys and L-Cys DES activities. This was further supported by the use of specific
507 antibodies to identify the sliced activity bands by Western blot after further SDS fractionation
508 (Fig. 4C), as well as by immunoprecipitation assay (Fig. 4D). Finally it was supported by the
509 identification of the tripsinized unique peptides (Supplemental Table S1) in the three sliced WT
510 activity bands (Fig.4A and B).

511 Importantly, DES1 was shown to degrade L-Cys, estimated by the use of the *des1* null mutants
512 to be responsible for up to 14% of the total L-Cys desulfhydrase activity compared to WT plants
513 grown in plated on MS medium (Álvarez et al., 2010). This suggests the existence of additional
514 source/s that can contribute to the majority (ca 86%) of H_2S production by degrading L-Cys.

515 Notably, the impairment of *oastlA* and *oastlB* mutants led to a significantly decreased sulfide
516 production activity rate by L-Cys desulfhydrase [ca 76% and 18% reduction respectively
517 (Fig3C)] compared to WT, indicating the important role of the OASTLs in degrading L-Cys.
518 Intriguingly, only very few unique peptides of DES1 were identified in the major activity bands,
519 the highest and second-highest mobility bands, of L-Cys desulfhydrase activity and none in the
520 SeCys desulfhydrase activity bands, indicating that the OASTLs are more abundant proteins than
521 DES1 (Supplemental Table S1). Taking together i) the lower abundancy of DES1 compared to
522 the OASTLs and ii) the significant decrease in the Cys-desulfhydrase kinetic activity of the
523 *oastlA* leaves in plants grown in control or selenate stressed plants compared to WT (Fig.3C) as
524 well as iii) the absence of activity bands, additive to OASTL A, B and C activity bands that
525 could be attributed to DES1 in control and selenate stressed condition (Fig. 4A and B), the result
526 strongly support the notion that OASTL A, B and C play a major role in L-Cys and L-SeCys
527 degradation in *Arabidopsis*.
528
529 **CONCLUSIONS**

530 Using *Arabidopsis* O-acetylserine-(thiol) lyases mutants, impaired in the biosynthesis of cysteine
531 (Cys) from the substrates sulfide and O-acetylserine, it was shown that the absence of active
532 OASTLA and OASTLB, confers higher reduced growth, lower remaining chlorophyll, lower
533 sulfur in protein fraction and lower total glutathione than in selenate stressed WT and *oastlC*
534 mutant as the result of the interference of selenate with the sulfate reduction pathway.
535 Additionally it was shown that the absence of active OASTLA or OASTLB confers higher
536 sensitivity to selenate compared to WT or the absence of active OASTLC. Further, it was shown
537 here that the cytosolic OASTLA, chloroplat localized OASTLB and the mitochondrion localized
538 OASTLC has an important role in desulfhydrase activity degrading not only L-Cys but also
539 SeCys to sulfide and selenide, respectively.
540
541 **MATERIALS AND METHODS**

542 **Plant Material, Growth Conditions and Selenium treatment**
543 *Arabidopsis thaliana* var. Columbia wild type (*WT*), *oastlA (Salk_072213), oastlB (Salk*
544 *_021183), oastlC (Salk_000860) and des1* (SALK_205358) lines were used for Selenium

545 treatment. SALK_103855 (*des1*) was employed for in-gel DES activity and transcript
546 expression. All the mutants were derived from the Columbia (*Col*) ecotype. Experiments were
547 carried out in the growth room at Sde Boqer Campus, Ben-Gurion University of the Negev,
548 under 14 h light/10 h darkness, 22˚C and 75–85% relative humidity under photosynthetically
549 active radiation of 150 µmol m^{-2} sec^{-1} as described in Brychkova et al., 2007.
550 Seeds were germinated and grown in standard 90 mm Petri dishes on solid ½ MS
551 medium for 9 days (2 days in dark and 7 days in growth room). The seedlings were transferred to
552 large Petri dishes of 155x30 mm diameter and height respectively, supplied with ½ MS medium
553 (Murashige and Skoog, 1962) supplemented with or without 40µM of Na_2SeO_4. All treatments
554 were performed in six replicates. The weight of shoot biomass accumulation was determined 14
555 days after the treatment and results were expressed as average plant growth rate in mg $plant^{-15}$
556
557 **RNA Isolation, cDNA Preparation, and transcript analyses by Real-time PCR**
558 To quantify the transcripts using quantitative reverse transcriptase-polymerase chain reaction
559 (qRT-PCR), total RNA was prepared using the Aurum™ total RNA Mini Kit (Bio-Rad,
560 Hercules, CA) according to the manufacturer's instructions. The cDNA was prepared in a 10 µl
561 volume containing 350 ng of plant total RNA that was reverse-transcribed with an iScript™
562 cDNA Synthesis Kit using modified MMLV-derived reverse transcriptase (Bio-Rad), a blend of
563 oligo-d(T) and random hexamer primers, according to the manufacturer's instructions. The
564 generated cDNA was diluted 10 times and the quantitative analysis of transcripts was performed
565 employing the sets of primers from both sites of insertions shown in Supplemental Table S2 as
566 previously described (Brychkova et al., 2007). The transcripts level detected by Real-time PCR
567 were normalized using *ACTIN2* (At3g18780) and *ELONGATION FACTOR1-α* (At5g60390) and
568 compared to WT grown under the control condition. The data are presented as relative
569 expression (means ± SE, $n = 4$). All PCR fragments were sequenced for verification.
570
571 **Chlorophyll, MDA, H_2O_2 and Anthocyanin determination**
572 For detection of remaining content of chlorophyll (%) the leaf discs (7 mm diameter)
573 were immersed in 90% ethanol and incubated at 4°C for 2 d in the dark. Absorbance of the
574 extracted chlorophyll was measured at 652 nm, and total chlorophyll was estimated (Ritchie,
575 2006). MDA levels were determined as described by Srivastava et.al., 2017.

576 For detecting H_2O_2, frozen leaves were extracted in 50 mM P buffer (pH 7.5) at a ratio of
577 1:8 (w/v) and centrifuged (Eppendorf 5417R) twice at 18,000*g* for 20 min. The reaction mixture
578 for detecting H_2O_2 consisted of 0.85 mm 4-aminoantipyrine, 3.4 mm 3,5-dichloro-2-
579 hydroxobenzene sulfonate, 4.5 U mL^{-1} HRP in 2 mL of 50 mM P buffer (pH 7.5) in the presence
580 or absence of 2 mM tungstic acid and 100 μM DPI as described in Yesbergenova et al. 2005.
581 Absorbance was measured after 5 min at 515 nm as described above.
582 The anthocyanin content was determined based on a modification of protocols described
583 by Laby et al. (2000) and Kant et al. (2006). Approximately 100 mg of fresh plants grown in ½
584 MS medium were crushed in 600 μL methanol acidified with 1% HCl. The extract was
585 centrifuged for 10 sec at 4000g. Five hundred μL of double distilled water were added to the
586 collected sand, mixture was gently vortexed and then 700 μL chloroform was added and mixed
587 for 20 sec followed by centrifugation at 4000 g for 2 min. The total anthocyanin in the aqueous
588 phase was determined by detecting the optical density (OD) at A530 and A657 nm. The amount
589 of anthocyanin was calculated by subtracting the A657 from the A530 (Laby et al., 2000).
590
591 **Enzyme activity measurements**
592 Protein extraction, desalting, concentration determination and kinetic assays for APR, SO, SiR,
593 OAS-TL and DES activities were assayed and expressed as described before; OAS-TL and SiR
594 activity in nmol Cys mg^{-1} protein min^{-1}, APR and SO in nmol sulfite mg^{-1} protein min^{-1} and
595 DES activity in nmol H_2S mg^{-1} protein min^{-1} (Brychkova et al., 2012a; Brychkova et al., 2012b;
596 Kurmanbayeva et.al., 2017). In brief, APR activity employing APS as substrate was detected
597 using the sulfite-specific fuchsine colorimetric detection method. SO activity was measured as
598 the disappearance of sulfite. The desalted protein extracts were treated with 1 mM tungstate for
599 30 min at 4°C to inhibit SO activity. SiR activity was estimated by the coupled SiR/OASTL
600 assay with the addition of NADPH and tungstic acid. The resultant generated Cys was detected
601 as described before (Gaitonde, 1967; Brychkova et al., 2012b). OAS-TL activity was initiated by
602 incubating 75 μL Reaction mixture (RM) containing 25 mM OAS, 1 mM DTT, 1.42 mM
603 Pyridoxal-phosphate (PLP), 60 mM Na_2S and 200 mM P-buffer (pH 7.5) with 25 μL of protein
604 extract for 10 min at 30°C. Generated Cysteine was detected at 560 nm by a spectrophotometer
605 (Gaitonde, 1967).

606 DES activity was detected as H_2S formation in the presence of L-cysteine. The assay
607 solution contained 0.1 M Tris-HCl, pH 9.0, 2.5 mM dithiothreitol, 0.8 mM L-cysteine and 10 μg
608 of desalted protein in a total volume of 0.2 mL. After incubation at 37 °C for 30 min, H_2S was
609 detected according to Bloem et al., 2004 with 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 20 mM
610 N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of
611 methylene blue was measured at 670 nm. Na_2S*9H_2O was used as a standard.
612
613 **Protein extraction, fractionation for in gel DES activity**
614 Whole protein from Arabidopsis seedlings was extracted as described by Sagi and Fluhr,
615 2001. Concentrations of total soluble protein in the resulting supernatant were determined
616 according to Bradford, 1976. Native-SDS PAGE was carried out as follows: samples were
617 subjected to a Bio-Rad Protean II xi slab cell (Bio-Rad, Richmond, CA, USA), with a
618 discontinuous buffer system (Laemmli, 1970) in 7.5% (w/v) polyacrylamide separating gels and
619 4% (w/v) stacking gels. Native-SDS PAGE was carried out using 1.5 mm thick slabs loaded with
620 200 μg of proteins. Regeneration of the active proteins after native-SDS PAGE was carried out
621 by removal of the SDS as described by Sagi and Fluhr, 2001 with slight modification: by shaking
622 the gel for 1 h in 10 mM Tris-HCl buffer (pH 7.8) solution (65 ml buffer per ml of gel)
623 containing 2 mM EDTA.
624 DES activity was detected using a modification of an in-gel visualization protocol for
625 H_2S (Manchenko, 2002). Lead acetate was employed to detect the generated H_2S and possibly
626 H_2Se, producing dark brown lead sulfide/selenide bands. The reaction solution contained 0.15 M
627 Tris–HCl, pH 8.5, 1 mM Dithiothreitol (DTT), 50 mM β-mercaptoethanol, 5 mM L-cysteine or
628 2.5 mM SelenoCystine, 0.1 mM pyridoxal 5-phosphate (PLP), 0.4 mM lead acetate. The reaction
629 was stopped by immersion of the gel in double-distilled water.
630
631 **Western Blot and Immunoprecipitation Analysis**
632 Western blot proteins were separated by SDS–PAGE carried out in 12.5%
633 polyacrylamide gels and were blotted to polyvinylidene difluoride membranes (Immun-Blot
634 membranes, Bio-Rad). The blotted proteins were subjected to immuno-detection with antibodies
635 raised against Arabidopsis chloroplastic OAS-TL C (diluted1:1000) (kindly provided by Prof.

636 R.Hell, University of Heidelberg, Heidelberg) and followed by secondary antibodies as described
637 by Heeg et al., 2008.
638 For the immunoprecipitation assay 200 µg of protein from *WT* were incubated with 80 µl
639 of the OAS-TL B (diluted1:5000) (kindly provided by Prof. Dr. S. Kopriva, University of
640 Cologne, Köln) antibodies in Tris-buffer saline (TBS) for 30 min at room temperature and then
641 kept at 4°C for overnight. Protein extract that had not been mixed with antibody was employed
642 as control. The mixture and the control solutions were incubated with 120 µl of Protein G
643 Agarose at 4°C for 2h with continuous shaking and then centrifuged at 10,000g for 5 min,
644 followed by removal of supernatant for analysis by the Native-SDS DES assay.
645
646 **Determination of selenium and sulfur-containing metabolites**
647 To determine the total selenium content, 100 mg of the dried and powdered leaves were
648 placed in glass tubes, digested with 70% $HN0_3$, heated at $220^{O}C$ for 4 hours and quantified by
649 inductively coupled plasma emission spectrometry (ICP-AES) (Kalra, 1997).
650 Se in protein fraction: 400 g fresh weight was extracted in 2 mL buffer containing 100
651 mM NaCl, 50 mM Tris HCl (pH 7.5), 0.5% (v/v) Triton X-100, 1 mM DTT, and 0.1 mM PMSF.
652 The homogenate was cleared by centrifugation (7,500g for 10 min). A small sample was taken
653 for protein determination, and the volume of the extract was measured. The proteins in the
654 extract were precipitated by adding TCA to a final concentration of 15% (w/v). The mixture was
655 incubated on ice for 30 min and then centrifuged for 20 min at 7,000g at 4°C. The pellet was
656 washed with ice-cold acetone, dried, and dissolved in 1 mL of concentrated nitric acid. After acid
657 digestion, the Se was determined by ICP (Pilon-Smits et al., 1999).
658 Selenate detection was done according to (Schiavon et al., 2012) with slight
659 modifications. The frozen tissues (200 mg) were ground in liquid nitrogen, and then 5 mL of
660 distilled water was added. The obtained extracts were filtered (0.22µm, Millipore) and analyzed
661 for sulfate and selenate concentrations by Ion chromatography using a Dionex IonPac AS19
662 4x250mm column.
663 Organic S and Se were calculated by subtracting the sum of sulfate/selenate, sulfite and
664 sulfide from the total sulfur/selenium content.
665 Determination of sulfite, free Cys and total glutathione was performed as described
666 (Brychkova et al., 2013; Yarmolinsky et al., 2014). Sulfide was extracted (1:4, w/v) in 0.1 M

667 Tris–HCl, pH 8.0 buffer in the presence of 0.5 M sodium sulfate to minimize sulfite self-
668 oxidation to sulfate. After centrifugation at 1800 g for 15 min, the supernatant was further de-
669 proteinized employing Sephadex G-25 Column (Pd-10, GE Healthcare) loaded with the same
670 extraction solution. Immediately after the separation through the column 600 µl of 2% cadmium
671 acetate were added to the 600 µl of the sample and kept on ice. The levels of sulfide were
672 detected by 40 µl NN-Dimethyl-1,4-phenylen-diammonium-dichloride dissolved in 7.2 N HCl
673 and 4 µl FeCl3 dissolved in 1.2 N HCl from 240 µl of preincubated sample with 2% cadmium
674 acetate (Siegel, 1965). Sulfide was detected at 625 nm.
675
676 **Protein sequencing**
677 To identify the proteins participating in the cysteine desulfhydrase activity, the activity bands
678 from the in-gel activity of DES were sliced from the native gel, and fractionated with 12.5%
679 SDS-PAGE (Fig. 4). Thereafter the proteins were stained by Coomassie Brilliant Blue, and the
680 stained bands were excised from the gel, trypsinized and the resulting peptides were separated by
681 HPLC and analyzed by LC-MS/MS on Q-Exactive (Thermo) at The Smoler Protein Research
682 Center (Technion University, Haifa, Israel).
683 All the identified peptides were filtered with high confidence, top rank, mass accuracy, and a
684 minimum of 2 peptides. High confidence peptides passed the 1% FDR threshold. (*FDR =false
685 discovery rate, is the estimated fraction of false positives in a list of peptides). Semi quantitation
686 was done by calculating the peak area of each peptide. The area of the protein is the average of
687 the three most intense peptides from each protein. Analysis of peptide sequences was performed
688 by employing Proteome Discoverer™ Software ver. 1.4.1.14 (Thermo Fisher Scientific Inc.
689 https://www.thermofisher.com/order/catalog/product/ IQLAAEGABSFAKJMAUH).
690
691 **Accession Numbers**
692 Sequence data for this article can be found in the Arabidopsis Genome Initiative or
693 GenBank/EMBL databases under the following accession numbers: At4g14880 *(OASTL A*),
694 At2g43750 (*OASTL B*), At3g59760 (*OASTL C*), AT5G28030 (*DES1*), AT3G18780 (*ACTIN 2*),
695 AT5G60390 (*EF 1-α*)

696

697 **Supplemental Figures**

698 **Supplemental Figure S1.** Schematic model of Sulfur (S) and Selenium (Se) metabolism in Arabidopsis
699 plants [after White (2015)].

700 **Supplemental Figure S2.** OASTLs protein and transcript expressions and Se containing compounds in
701 WT, *oastlA, oastlB* and *oastlC KO* mutants supplemented with or without selenate for 14 days.

702 **Supplemental Figure S3.** S/Se metabolism activities and DES1 transcript in WT and *Atdes1* KO mutants
703 supplemented with or without selenate for 14 days.

704 **Supplemental Figure S4.** Multiple sequence alignment of OASTL A, B and C proteins of in *Arabidopsis*
705 *thaliana*.

706 **Supplemental Figure S5.** H_2O_2 MDA and anthocyanin level in WT, *oastlA, oastlB* and *oastlC* KO
707 mutants supplemented with or without selenate for 14 days.

708 **Supplemental Tables**

709 **Supplemental Table S1.** Identified and overlapped unique peptides from L-Cys and L-SeCys
710 desulfhydrase activity. **Supplemental Table S2.** List of gene primers used for quantitative real-time PCR.

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713 Heidelberg, Heidelberg) for providing OAS-TL A and B antibodies.

714

715 **FIGURE LEGENDS**

716 **Figure 1.** The effect of 40 µM selenate treatment on growth parameters and Se in protein fraction in WT,
717 *oastlA, oastlB* and *oastlC KO* mutants of *Arabidopsis thaliana*. A. Phenotype of plants grown for 14 days
718 under control (left panel) and 40µM selenate treated (right panel) conditions. The weight of plant biomass
719 accumulation (B) and relative weight to their own controls (C) supplemented with or without selenate for
720 14 days. The values are means ± SE (n = 15). Remaining chlorophyll level (D) and Se in protein fraction
721 (E). The values are means ± SE (n = 4). Values denoted with different letters are significantly different
722 according to the T- test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different uppercase

723 letters indicate significant differences between control and Se treatment of each genotype. Asterisks
724 indicate significant differences between *WT* and *oas-tlA, oas-tlB, oastlC* KO plants subjected to the same
725 treatment.

726 **Figure 2.** Cys biosynthesis and S containing compounds in WT, *oastlA, oastlB* and *oastlC* KO mutants
727 supplemented with or without selenate for 14 days. The effect of 40 µM selenate treatment on OAS-TL
728 activity (A), Cysteine level (B), Total glutathione (C), Total organic S (D) and S in protein fraction (E).
729 The values are means ± SE (n = 4). Values denoted with different letters are significantly different
730 according to T-test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different uppercase
731 letters indicate significant differences between control and Se treatment of each genotype. Asterisks
732 indicate significant differences between WT and the KO plants *oastlA, oastlB and oastlC* subjected to the
733 same treatment.

734 **Figure 3.** Soluble protein level and S metabolism components in WT, *oastlA, oastlB* and *oastlC*
735 KO mutants supplemented with or without selenate for 14 days. The effect of 40 µM selenate
736 treatment on soluble protein content (A), transcript expression of DES1 (B), L-Cysteine
737 desulfhydrase (DES) activity (C), sulfide (D) and sulfite levels (E), Sulfite oxidase (SO) activity
738 (F), Adenosine 5′-phosphosulfate reductase (APR) activity (G) and Sulfite reductase (SiR)
739 activity (H). Transcript level of DES1 was detected by quantitative reverse transcription-PCR
740 using *ACTIN2* as the housekeeping transcript for normalization. The relative expression in the
741 various genotypes was analyzed using the normalized WT control as reference. The values are
742 means ± SE (n = 4). Values denoted with different letters are significantly different according to
743 T-test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different uppercase letters
744 indicate significant differences between control and Se treatment of each genotype. Asterisks
745 indicate significant differences between WT and *oastlA, oastlB, oastlC* plants subjected to the
746 same treatment.

747 **Figure 4.** NATIVE-SDS on L-Cys and SeCys desulfhydrase activities in WT, oastlA, oastlB and oastlC
748 KO mutants supplemented with or without selenate for 14 days. Effect of 40 µM selenate treatment on L-
749 Cysteine (A) and SeCys (B) degradation activities. C. Immunodetection of the sliced WT activity bands
750 from 4A are in left insert and the sliced WT activity bands sliced from 4B are in the right insert. D.
751 Desulfhydrase activity of WT crude protein extract without immunoprecipitation (left lane) with
752 immunoprecipitation in the presence (+) or absence (-) of specific antibodies. L-cys and L-Secys
753 desulfhydrase activities are left and right inserts respectively. Red arrows indicate activity band.

754

755 **Supplemental Figures**

756 **Supplemental Figure S1.** Schematic model of Sulfur (S) and Selenium (Se) metabolism in Arabidopsis
757 plants [after White (2015)]. ATP sufurylase (ATPS) catalyzes the adenylation of sulfate/selenate to
758 adenosine 5′-phosphosulfate (APS)/adenosine phosphoselenate (APSe) using ATP as an electron donor.
759 Then, APS/APSe is reduced by the plastidic enzyme APS reductase (APR) to sulfite/selenite in the
760 presence of two molecules of reduced glutathione, which acts as an electron donor. The generated
761 sulfite/selenite can be reduced to sulfide/selenide by the Sulfite Reductase (SiR) employing 3 molecules
762 of reduced ferredoxin. Alternatively, selenite can also be reduced non-enzymatically by glutathione
763 (GSH) to selenide. The sulfide/selenide together with O-acetyl-L-Serine (OAS) are the substrates for
764 Cysteine (Cys)/Selenocysteine (SeCys) biosynthesis catalyzed by O-acetylserine-(thiol) lyase (OAS-TL).

765 **Supplemental Figure S2.** OASTLs protein and transcripts expression, as well as Se containing
766 compounds in *WT, AtoastlA, AtoastlB* and *AtoastlC* KO mutants supplemented with or without selenate
767 for 14 days. Effect of 40µM Selenate treatment on OASTLA, OASTLB and OASTLC level in WT and
768 the various mutants analysed by Western blot employing specific antibody (A), total Se (B), selenate (C)
769 and transcripts analyses of *OASTLA* (D), *OASTLB* (E) and *OASTLC* (F). Transcript levels of *OASTLA,*
770 *OASTLB* and *OASTLC* were detected in the various genotypes by quantitative reverse transcription-PCR
771 using *ACTIN2* as housekeeping transcript for normalization. The relative expression of each normalized
772 *OASTL* in the various genotypes was analyzed using WT control as the reference. The values are means ±
773 SE (n = 4). Values denoted with different letters are significantly different according to T-test analyses, P
774 < 0.05 (JMP 8.0 software, http://www.jmp.com/). Different uppercase letters indicate significant
775 differences between control and Se treatment of each genotype. Asterisks indicate significant differences
776 between WT and *oas-tlA, oastlB, oastlC* KO plants subjected to the same treatment. The specific antibody
777 kindly provided by Prof. Dr. S. Kopriva, University of Cologne, Germany.

778

779 **Supplemental Figure S3.** S/Se metabolism activities and DES1 transcript in WT and *Atdes1* KO mutants
780 supplemented with or without selenate for 14 days. L-Cys/SeCys NATIVE-SDS degradation activities in
781 the presence and absence of L-Cys/SeCys or BME (A), *DES1* transcripts expression with primers
782 constructed upstream and downstream along the transcript length (B), OAS-TL (C) and DES kinetic
783 activities (D), as well as, L-Cysteine (left side) and SeCys (right side) in-gel desulfhydrase activities in
784 WT and *Atdes1* SALK_205358 leaves (E). DES1 transcript expression and in-gel L-Cysteine
785 desulfhydrase activity in WT and *Atdes1* SALK_103855 (F). Transcript level of *Des1* was detected by
786 quantitative reverse transcription-PCR using *ACTIN2* as the housekeeping transcript for normalization.

787 The relative expression in the *Atdes1* with the 2 coupled primers was analyzed using the normalized WT
788 control as reference. In-gel L-Cysteine desulfhydrase activities were employed after protein fractionation
789 in SDS gel performed in Protean® II xi (www.bio-rad.com) in A and E and mini Protean3 (www.bio-
790 rad.com) in F. The values are means ± SE (n = 4). Values denoted with different letters are significantly
791 different according to T-test analyses, $P < 0.05$ (JMP 8.0 software, http://www.jmp.com/). Different
792 uppercase letters indicate significant differences between control and Se treatment of each genotype.
793 Different lower-case letters indicate differences between genotypes within the same primer. Asterisks
794 indicate significant differences between WT and *des1* KO plants subjected to the same treatment
795
796 **Supplemental Figure S4.** Multiple sequence alignment of OASTL A, B and C proteins in *Arabidopsis*
797 *thaliana*. The percentage of sequence identity between the proteins is presented below.

798 **Supplemental Figure S5.** Hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and anthocyanin level in
799 WT, *AtoastlA, AtoastlB* and *AtoastlC* KO mutants supplemented with or without selenate for 14 days. The
800 effect of 40µM Selenate treatment on H_2O_2 (A), MDA (B) and anthocyanin (C) levels calculated on FW
801 (left) and soluble protein basis (right). The values are means ± SE (n = 4). Values denoted with different
802 letters are significantly different according to T-test analyses, $P < 0.05$ (JMP 8.0 software,
803 http://www.jmp.com/). Different uppercase letters indicate significant differences between control and Se
804 treatment of each genotype.
805

806 **Supplemental Tables**

807 **Supplemental Table S1.** Identified and overlapped unique peptides from L-Cys and L-SeCys
808 desulfhydrase activity. **A**. List of the number coverage percentage **B**. Identified and overlapped unique
809 peptides from L-Cys desulfhydrase activity. **C**. Identified and overlapped unique peptides from L-SeCys
810 desulfhydrase activity. D. Abundance of OASTL A, B and C as compared to DES1 protein at the lower,
811 middle and upper activity bands. The semi quantitation was made by calculating the peak area of
812 each peptide, whereas to overcome differential ionization, the area of the protein was calculated
813 as the average of three most intense peptides from each protein. The calculated area of the DES
814 protein is marked in red. The activity bands (as shown in Fig. 4A and B) were sliced and fractionated in
815 12.5 % SDS-PAGE. Further the bands were digested by trypsin and analyzed by LC-MS/MS as described
816 in Materials and Methods. Blue color – overlapped areas. The presented data are representative of 3
817 independent experiments with similar results. *(see below).
818

819 **Supplemental Table S2.** List of gene primers used for quantitative real-time PCR.

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