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Original Article

Growth inhibition by selenium is associated with changes in primary metabolism and nutrient levels in Arabidopsis thaliana

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ABSTRACT

Although Selenium (Se) stress is relatively well known for causing growth inhibition, its effects on primary metabolism remain rather unclear. Here, we characterized both the modulation of the expression of specific genes and the metabolic adjustments in *Arabidopsis thaliana* in response to changes in Se level in the soil. Se treatment culminated with strong inhibition of both shoot and root growth. Notably, growth inhibition in Se-treated plants was associated with an incomplete mobilization of starch during the night. Minor changes in amino acids levels were observed in shoots and roots of plants treated with Se whereas the pool size of tricarboxylic acid (TCA) cycle intermediates in root was not altered in response to Se. By contrast, decreased levels of organic acids involved in the first part of the TCA cycle were observed in shoots of Se-treated plants. Furthermore, decreased expression levels of expansins and endotransglucosylases/endohydrolases (XHTs) genes were observed after Se treatment, coupled with a significant decrease in the levels of essential elements. Collectively, our results revealed an exquisite interaction between energy metabolism and Se-mediated control of growth in *Arabidopsis thaliana* to coordinate cell wall extension, starch turnover and the levels of a few essential nutrients.

Key-words: carbon metabolism; cell wall; plant growth; photosynthesis; starch turnover; TCA cycle.

INTRODUCTION

Selenium (Se) has been established as an essential micronutrient for many organisms including mammals, bacteria and some green algae (Zhong and Holmgren 2000, Sun *et al*. 2014, Nancharaiah and Lens 2015). By contrast, there is no evidence that vascular plants require Se for survival; although in some cases, it may promote plant growth (Pilon-Smits *et al*. 2009, Nawaz *et al*. 2015). Accumulation of Se in the environment has been reported to be a cause of toxicity, particularly in aquatic organisms (Hamilton 2004), livestock (Davis *et al*. 2012) and humans (Misra *et al*. 2015). As a first step towards finding solutions for both high levels of Se in the environment, better understanding on the impacts of Se on primary metabolism in higher plants is likely crucial.

Se is part of the VI-A chalcogen group of elements, with the most common valence states being +4 and +6, with Se occurring as Se^{2-} (selenide), Se^{0} (elemental selenium), $Se_2O_3^{-2}$ (thioselenate), SeO_3^{-2} (selenite) and SeO_4^{2-} (selenate), respectively. Notably, the root-to-shoot translocation of Se depends on the species available, selenate being much more easily transported than selenite or organic Se (Li *et al*. 2008, Wang *et al*. 2015). Thus, plants primarily take-up Se as either selenate or selenite, which is further metabolized, via the sulphur (S) assimilation pathway, resulting in the production of selenocysteine, selenomethionine and other Se isologues of various S metabolites (Shibagaki *et al*. 2002, Schiavon *et al*. 2015). Remarkably, the non-specific incorporation of selenoamino acids into proteins may lead to toxicity because of impaired protein function (Terry *et al*. 2000). Furthermore, in plants Se toxicity can also be associated with the formation of reactive oxygen species and oxidative stress (Tamaoki *et al*. 2008, Van Hoewyk 2013, Lehotai *et al*. 2016). Although the physiological effects of Se stress in plants have been relatively well characterized and include leaf chlorosis and reductions in total plant biomass and on photosynthetic efficiency (Terry *et al*. 2000), the impacts of Se on plant carbon status have received little attention to date.

It has recently been demonstrated that selenite induced the reconfiguration of primary metabolism to overcome the consequences of mitochondrial oxidative stress in roots of *Brassica napus* (Dimkovikj and Van Hoewyk 2014). Accordingly, glucose and amino acids levels were increased by Se, while the levels of many tricarboxylic acid (TCA) cycle metabolites were decreased (Dimkovikj and Van Hoewyk 2014). In fact, the functional significance of Se in modulating stress responses is widely accepted based upon a range of experiments indicating its roles in plants (Feng *et al*. 2013, Saidi *et al*. 2014). Although these studies have clearly advanced our understanding of the toxic effects of Se in a specific organ, they provided limited information concerning the general role of Se on the regulation of plant metabolism and growth. Thus, important questions about how central carbon metabolism and plant growth are coordinated with the availability of Se still await clarification. Growth and development of sink organs are

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known to be under the control of carbon availability (Smith and Stitt 2007). Thus, the amount of carbon available for growth is the result of an adequate balance between net photosynthesis, the accumulation of starch and various carbon-containing metabolites during the day light period and their remobilization at night to sink organs (Sulpice *et al.* 2014). Under sink-limited conditions, where the supply of carbon exceeds its use for growth, starch is not completely degraded during the night (Stitt and Zeeman 2012), and growth inhibition is usually observed.

Here, we hypothesized that the growth inhibition observed in plants growing under high Se levels is associated with changes in primary carbon metabolism. We demonstrated that the reduced growth rate of Se-treated plants is likely dependent on starch degradation during the night. Furthermore, in Se-treated plants, we observed changes in the levels of TCA cycle intermediates and in sugars including sucrose, glucose and fructose, as well as a downregulation of genes associated with cell wall metabolism and with altered levels of a few essential nutrients. The results obtained are discussed in the context of current models of growth regulation and metabolic acclimation.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia 0 (Col-0) wild-type seeds were sown on standard greenhouse soil (Stender AG, Schermbeck, Germany) in plastic pots with 0.1 L capacity. Trays containing the pots were placed under a 12/12 h day/night cycle (22/16 °C) with 60/75% relative humidity and 180 μmol $m^{-2}s^{-1}$ light intensity.

Fourteen days after sowing, plants growing singly in pots were watered with 10 mL of deionized water (control) or sodium selenate solution (250 μM). Arabidopsis plants used in the assays were placed in trays together in a random arrangement with 35 pots per tray.

Measurements of photosynthetic parameters

Gas-exchange measurements were performed with an open-flow infrared gas exchange analyser system (Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA) with a portable photosynthesis system to fit whole-plant cuvette. Light was supplied from a series of light-emitting diodes located above the cuvette, providing an irradiance of 300 μmol $m^{-2}s^{-1}$. The reference CO_2 concentration was set at 400 μmol CO_2 mol^{-1} air. Dark respiration was measured on whole rosette using the same protocol after at least 1 h during the dark period. All measurements were performed at 25 °C, and vapour pressure deficit was maintained at 2.0±0.2 KPa while the amount of blue light was set to 10% of photon flux density to optimize stomatal aperture. The ratio of F_v to F_m, which corresponds to the potential quantum yield of the photochemical reactions of PSII and represents a measure of the photochemical efficiency, was measured after dark adaptation of leaves for 30 min using a leaf chamber fluorometer (Model 6400-40, Li-Cor). The determination of the photosynthetic parameters was performed in plants of 35 d after sowing.

Metabolite analysis and enzymatic assays

Whole rosettes were removed with scissors and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Root systems were collected as described by Pfeifer *et al.* (2015) with few modifications. Root systems were thoroughly washed out of the soil using sieves with a mesh size of 1 mm. After washing, root systems were blotted between two sheets of tissue paper to remove excess water and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. The usage of a commercial substrate for Arabidopsis growth allowed us to recover quantitative root material with no significant losses. Thirty plants (six independent samples containing five whole rosettes or five root systems per samples) were harvested 35 d after sowing (21 d after onset of Se treatment). Chlorophyll, sucrose, starch, total protein, total amino acid and nitrate contents were determined as described by Cross *et al.* (2006). NAD and NADP pool sizes and reduction state were measured in acid and alkaline extracts according to the method described by Queval and Noctor (2007). The assays involved the phenazine methosulfate-catalysed reduction of dichlorophenolindophenol in the presence of ethanol and alcohol dehydrogenase (for NAD^+ and NADH) or Glc-6-P and Glc-6-P dehydrogenase (for $NADP^+$ and NADPH).

Metabolite profiling was performed using a gas chromatography-mass spectrometry (GC-MS) system. The extraction, derivatization, standard addition and sample injection were performed exactly as described previously (Lisec *et al.* 2006). Metabolites were identified in comparison to database entries of authentic standard (Kopka *et al.* 2005). The full dataset from the metabolite profiling study is available as Table S1.

Nitrate reductase (NR), sucrose phosphate synthase (SPS), transketolase (TK) and adenosine 5′-diphosphate-glucose pyrophosphorylase (AGPase) were extracted and measured as described previously by Gibon *et al.* (2004). The Rubisco (ribulose 1°5-bisphosphate carboxylase/oxygenase) assay conditions were based on Sulpice *et al.* (2010)

qRT-PCR analysis

Total RNA was isolated using the Trizol® reagent (Ambion, Life Technology) following the company's manual. The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured before and after DNase I digestion using a spectrophotometer. DNAse-treated RNA (2 μg) was used for cDNA synthesis using Superscript™ III reverse transcriptase (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions. Absence of genomic DNA contamination in the RNA samples and RNA integrity were analysed as described by Piques *et al.* (2009). Real-time PCR reactions were performed in a 384-well microtitre plate with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems Applera, Darmstadt, Germany), using Power SYBR Green PCR Master Mix according to

Piques *et al.* (2009). The primers used here were designed using the QUANTPRIME software (Arvidsson *et al.* 2008) and are described in the Table S2. The transcription abundance was calculated by the standard curves of each selected gene and normalized using the constitutively expressed genes *polyubiquitin 10* (AT4G05320) and *S16* (AT2G09990), a 40S ribosomal protein. Data analyses were performed as described by Caldana *et al.* (2007). Four biological replicates were processed for each experimental condition.

Tissue nutrient analysis

Leaf samples were dried at 65 °C for at least 2 d, and reduced to powder (using a mill CIENLAB CE-430; 8 blades, 1,725 r.p.m., 20 mesh size). To quantify the levels of P, K, Ca, Mg, S, Cu, Fe, Mn and Zn, the samples were submitted to a nitric-perchloric digestion (65% and 70%) followed by analysis using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 3000XL). Selenate levels were extracted in 100 mM HCl during 30 min at 70 °C and determined as selenium by atomic absorption spectrophotometry coupled to a graphite oven.

Statistical analyses

All experiments were designed in a completely randomized distribution. Analysis of variance ($P<0.05$) was carried out to determine effects of treatments. Differences among means ($P<0.05$) displayed in figures and tables were examined by the Student's *t*-test. All the statistical analyses were performed using SPSS 8.0 for Windows statistical software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Clear reductions of rosette growth were observed in *Arabidopsis* plants treated with Se and grown side by side with controls (Fig. 1a). In the presence of Se Arabidopsis plants showed a 50% reduction in shoot and root dry weight, as well as total plant dry weight, compared with control plants (Fig. 1b–d). Furthermore, Se treatment reduced the final rosette area by 51% and caused 17% inhibition on the relative growth rate (RGR) across the entire period of measurement (15–35 d) in comparison with control plants (Fig. 1e,f). Notably, shoot-to-root ratio, total number of rosette leaves, specific leaf area (SLA) and water content of the rosette were not significantly affected by Se treatment (Fig. 1g–j).

To further investigate the functional link between Se and growth impairment, we next performed an extensive metabolic characterization in both shoot and root tissues. In control plants, starch was almost exhausted, with only 9% of the levels observed at the end of the day remaining at dawn (Fig. 2a). By contrast, in presence of high levels of Se starch was not completely exhausted, with 38% remaining at the end of the night. Given that the starch turnover was altered by Se treatment, we further determined the sugar levels in both shoot and roots. The levels of sucrose and hexoses in shoots of plants treated with Se were similar to that of control plants at the end of the light period but strongly decreased at the end of the night period (Fig. 2b–d). This is presumably associated to the limited starch degradation not keeping up with the sugars demand for growth. In keeping with this observation, treatment of plants with Se resulted in lower levels of sucrose, glucose and fructose in roots at the end of both night and day (Fig. 2a,b). In addition, neither the rate of net photosynthesis (Fig. 3a) nor the dark respiration (Fig. 3b) were significantly affected by Se. The maximum photochemical efficiency of PSII [maximum variable fluorescence/maximum yield fluorescence (*Fv*/*fm*)] was also not affected by the presence of Se (Fig. 3c). Additionally, no differences were observed in the total chlorophyll (Fig. 3d) and pyridine nucleotide [NAD(P)H] levels between treatments (Fig. 3d–h). Although nitrate content increased in both shoots and roots after Se treatment (Fig. 4a), the activity of nitrate reductase (NR) was unaffected in both tissues following Se treatment in comparison with control plants (Fig. 4b). By contrast to the situation observed for nitrate, the levels of total amino acids (Fig. 4c) and protein (Fig. 4d) in shoots and roots of Se-treated plants were similar to those of control plants (Fig. 4b,c).

We next extended this study to the major pathways of primary metabolism by using an established GC-MS protocol for metabolite profiling (Fernie *et al.* 2004). These studies revealed that among the 38 successfully annotated compounds related to primary metabolism, considerable changes occurred in the levels of a wide range of organic acids, amino acids and sugars (Fig. 5 and Table S1). The levels of sucrose, glucose and fructose were unaltered in shoots of plants treated with Se, but Se led to a decrease of sucrose and hexoses levels in roots (Fig. 5). There were no differences in hexose-phosphates pools in plants treated with Se. Furthermore, there were no changes in the levels of 3-P-glycerate and pyruvate in presence of Se. The analysis of amino acid revealed reductions in the levels of asparagine and glutamate in both shoots and roots of plants grown in soil supplemented with Se and an increase in GABA content (Fig. 5). In addition, glutamine levels were decreased by Se treatment in shoots with no changes in roots in comparison to control plants. The enhancement of Se availability caused a decrease in threonine and tryptophan in shoots but not in roots of Arabidopsis. Decreases in isoleucine and lysine levels were only observed in roots of plants treated with Se. Notably, alanine, valine, arginine, aspartate, serine, cysteine, glycine, methionine, ornithine, phenylalanine and tyrosine levels were unaltered by Se treatment. Our results also demonstrated a decrease in putrescine (49%) and spermidine (42%) level in roots of plants treated with Se. On the other hand, putrescine and spermidine remained at the same level in shoots of Se-treated plants as in the control. A significant increase in homoserine level was only observed in roots of Se-treated plants, while the levels of homoserine remained at the same level in the shoot (Fig. 5). Shoots of Se-treated plants were characterized by increased levels of proline. By contrast, there were no significant differences in proline in roots of plants treated with Se. The levels of three TCA cycle intermediates, namely, aconitate, isocitrate and α-Ketoglutarate were consistently reduced in shoots of Arabidopsis, with no changes in roots of Se-treated plants. In addition, the levels of citrate,

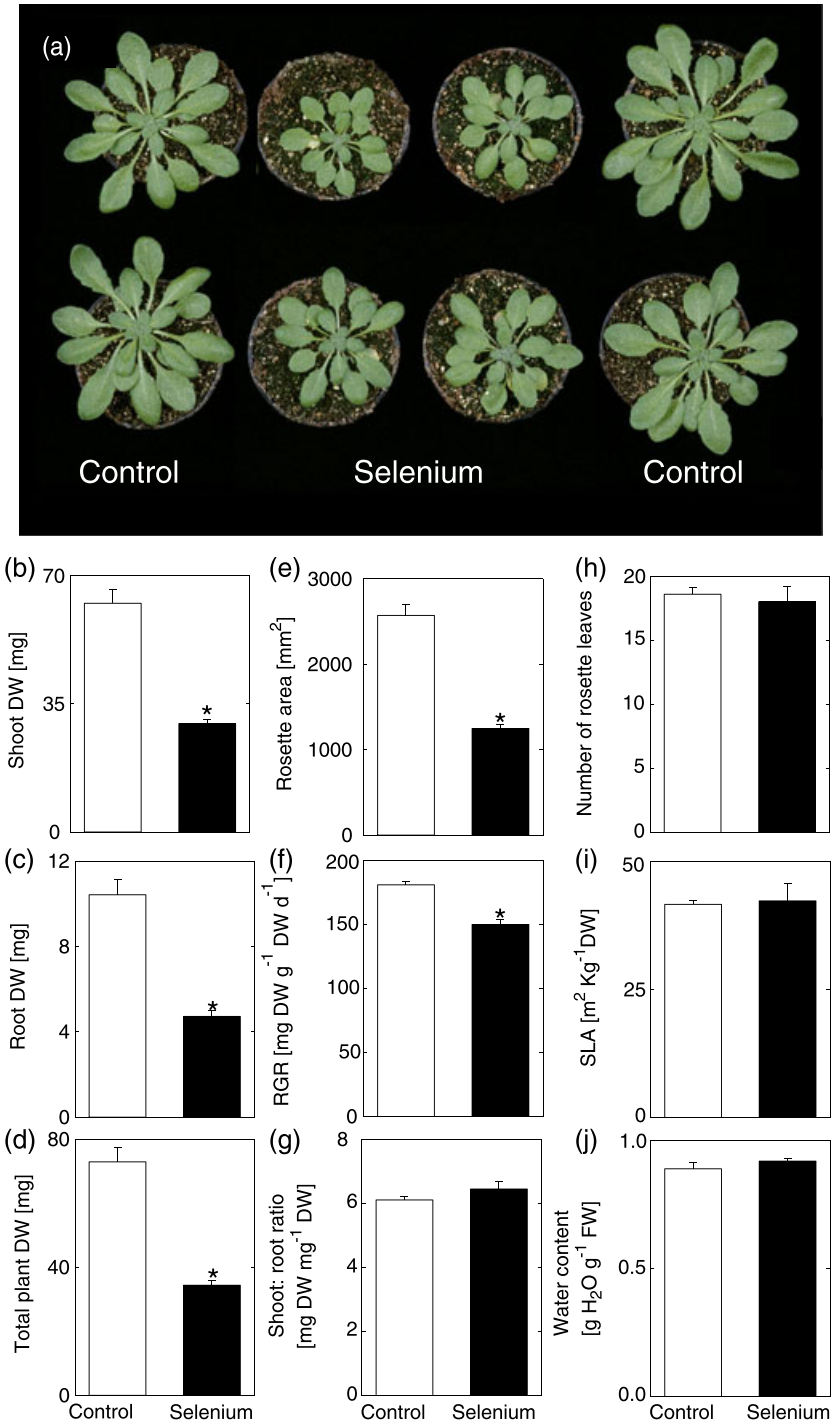

Figure 1. Phenotypic changes of Arabidopsis plants caused by treatment with Se. (a) Photography were taken of 35-day-old plants treated with water (control) or sodium selenate (Se). (b) Rosette dry weight. (c) Root dry weight. (d) Total plant dry weight. (e) Rosette area. (f) Relative growth rate. (g) Shoot-to-root ratio. (h) Number of rosette leaves. (i) Specific leaf area. (j) Water content. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P<0.05$). Values are means±SE of 15 individual determination.

succinate, fumarate and malate in both shoots and roots of plants treated with Se were similar to those observed in control plants.

We next investigated whether the growth and metabolic perturbation here observed could be affecting the expression of several genes related to cell wall synthesis, respiratory metabolism and anti-oxidative system. Interestingly, a remarkable decrease in the expression of genes associated with cell wall such as *expansins* (*EXPL1*, *EXPA1* and *EXPA8*) and *xyloglucan endotransglucosylases/endohydrolases* (*XTHs*) was

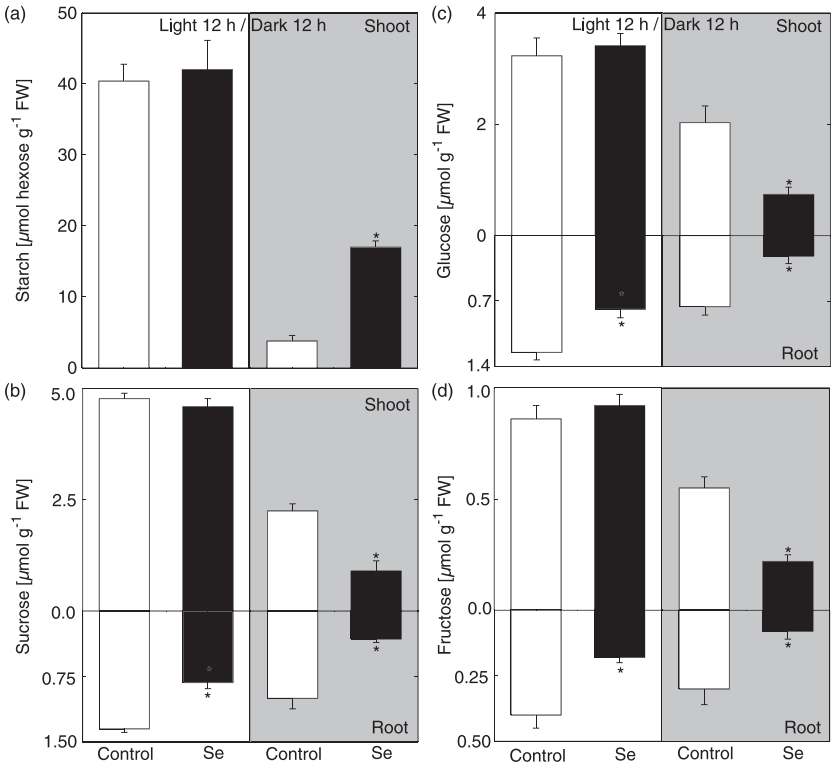

Figure 2. Starch and sugars are affected in plants treated with Se. Starch and sugars were measured in shoots (above sectors) and roots (below sectors) at the end of the light period (white sectors) and the end of the dark period (grey sectors) in material harvested from plants treated with water (control) or sodium selenate (Se). Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P < 0.05$). Values are means±SE of six replicates (each replicate is a pool of five plants).

observed in shoot of plants treated with Se (Fig. 6). By contrast, the transcript levels of *catalase 2* (*CAT2*), *glycolate oxidase 1* (*GOX1*), *serine glyoxylate aminotransferase* (*AGT1*), *serine hydorxymethyltransferase1* and *2* (*SHM1*, *SHM2*), *glycerate kinase* (*GLYK*), *serine descarboxylase* (*SDC*), *glycine descarboxylase H-protein* (*GDCH*), *peroxissomal NAD-malate dehydrogenase 1* (*MDH1*), *hydroxypyruvate reductase* (*HPR1*) and *glyoxylate/hydroxypyruvate reductase A* (*HPR2*) were not affected by the presence of Se (Fig. 6), suggesting that the growth impairment observed here are not mediated by an alteration in the expression levels of such metabolic pathways. To enhance our understanding of the changes in growth and metabolites in plants exposed to Se, we next analysed the expression of genes that encode enzymes of primary metabolism as well as the maximal activities of Rubisco, SPS, TK and AGPase at the end of the light and at the night. The activities of the Rubisco, SPS and TK in plants treated with Se were similar to control plants at the end of the night and the end of the day (Fig. 7a–c). On the other hand, Se treatment led to a significant increase of AGPase activity at the end of the night but not at the end of the day (Fig. 7d). The transcript levels of *rubisco activase* (RCA), *aspartate aminotransferase 1* (*ASP1*) and *fumarate hydratase 1* (*FUM1*) were not affected in plants treated with Se relative to control plants at the end of the night and the end of the day (Fig. 7e). *Glutamate dehydrogenase 1* (*GDH1*) and *glutamine synthetase 2* (*GS2*) showed a significant down-regulation upon Se treatment at the end of the day, whereas *GDH1* and *GS2* expression were not significantly affected at the end of the night. Furthermore, Se treatment led to an increase in the expression of *sucrose phosphate synthase A1* (*SPSA1*) and *ADP glucose pyrophosphorylase small subunit 1* (*APS1*) (Fig. 7e).

In order to further evaluate to what extent the effects of Se on plant growth could be associated with changes in the levels of other nutrients, we next studied whether high levels of Se altered mineral nutrient concentration in both shoots and roots. Se promoted decreases in P (39%) and Ca (38%) levels in shoots while significant increases in S (31%) levels in plants treated with Se as compared with control plants were observed (Table 1). Similarly to the situation observed in shoots, the levels of P and Ca were reduced by 33% and 28% in roots of plants treated with Se, respectively. Furthermore, enhanced levels of S (42%) in roots of plants treated with Se as compared with control plants were observed. Notably, no changes in K and Mg levels in both shoots and roots of Se-treated plants in comparison with control plants was observed. By analysing the levels of micronutrients, it was observed that Zn levels were decreased in both shoots (33%) and roots (40%) of plants treated with Se (Table 1). Conversely, Cu, Mn and Fe levels remained unchanged in Se-treated plants as in the control counterpart. In the present experiments, sodium selenate application resulted in an efficient Se accumulation in both shoots and roots of Arabidopsis plants (Table 1).

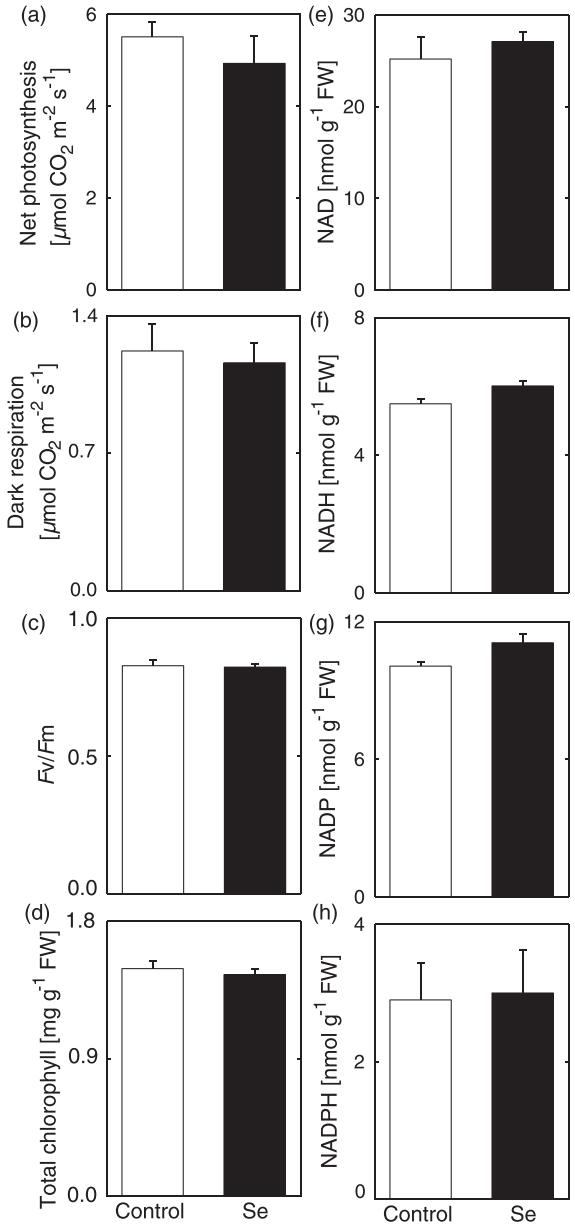

Figure 3. Physiological impacts observed in plants treated with Se. (a) Rate of photosynthesis. (b) Dark respiration. (c) F_v/F_m. (d) Total chlorophyll. (e–h) NAD(P)H levels. Levels of chlorophyll and NAD (P)H were performed using rosettes harvested at the end of the light period from plants treated with water (control) or sodium selenate (Se). Values are means±SE of six replicates (each replicate is a pool of five plants).

DISCUSSION

Plant growth is highly dependent on carbon availability (Smith and Stitt 2007, Pantin *et al.* 2011). Starch synthesized and accumulated during the light period must be remobilized to allow continuous export of sucrose and growth during the night (Gibon *et al.* 2009, Pilkington *et al.* 2015). Collectively, this provides a highly efficient manner for the allocation of photosynthates to maximize vegetative growth (Stitt and Zeeman 2012, Sulpice *et al.* 2014). Our results demonstrated that Se-treated plants retained considerable levels of starch at the end of the night indicating that they were sink-limited (Fig. 2). High levels of free hexoses in leaves of Se-treated plants at the end of the day may also suggest the occurrence of sink-limitation. We cannot formally exclude the possibility that the reduced root growth observed in Se-treated plants might be associated with a lower carbohydrate demand and thus leading to impairment in starch degradation. However, it seems tempting to speculate that the changes in starch metabolism might explain, at least partially, the severe decrease in root growth in Se-treated plants once root growth is regulated to gauge the overall rate of growth to the carbon supply (Yazdanbakhsh *et al.* 2011). In plants treated with Se, there was also no relation between the amplitudes of the changes of SPS transcript and enzyme activity (Fig. 7); this fact apart, several explanations are currently accepted for this difference (discussed in details in Fernie and Stitt 2012, Baerenfaller *et al.* 2012). At the end of the light period, transcription levels of the AGPase small subunit (*APS1*) gene increased, but enzyme activity was not affected in plants treated with Se. It is tempting to speculate that at high concentrations of Se, there is substantial adjustment of the starch turnover that may reflect an altered diel pattern of growth. While the exact nature of the interaction between Se levels, the circadian clock and primary metabolism, and indeed the precise biological function of Se itself in the regulation of metabolism could not be fully elucidated here, they remain as fundamental questions that should be addressed in future studies.

The maximal rate of net photosynthesis and activity of Rubisco as well as TK remained unaltered in Se-treated plants as compared with control (Figs 3 & 7). Se treatment also resulted in a similar activation of *RCA* gene expression (Fig. 7). Furthermore, the photochemical efficiency (F_v/F_m) was also not affected by Se treatment, and there was no change in SLA and shoot:root ratio in plants treated with Se compared with control ones. Taken together, our observations indicate that photosynthetic carbon assimilation was not affected in Se-treated plants. However, it is the developmental programme itself that determines how efficiently carbon is converted into biomass (Sulpice *et al.* 2010). In this context, respiratory carbon is likely an important component in the whole-plant carbon balance (Pyl *et al.* 2012). Dark respiration, photosynthesis and pyridine nucleotide [NAD(P)H] levels were similar in plants treated with Se as compared with controls (Fig. 3). Altogether, these results indicate that the reduced final rosette area and plant biomass were not a consequence of variation in photosynthesis or respiration rates per se. Although Se treatment led to an inhibition of shoot and root growth, the shoot-to-root ratio resembled that of control plants. These data could reflect an unaltered carbohydrate status of Se-treated plants as compared with control plants. However, given the high level of starch in the shoot of plants treated with Se, it probably led to decreased levels of sugars in the root. Collectively, our results are suggestive that Se level orchestrates carbon allocation and growth.

Se increased the amino acids levels in *Brassica napus*, while TCA intermediates were decreased in roots exposed to Se (Dimkovikj and Van Hoewyk 2014). Minor changes in amino

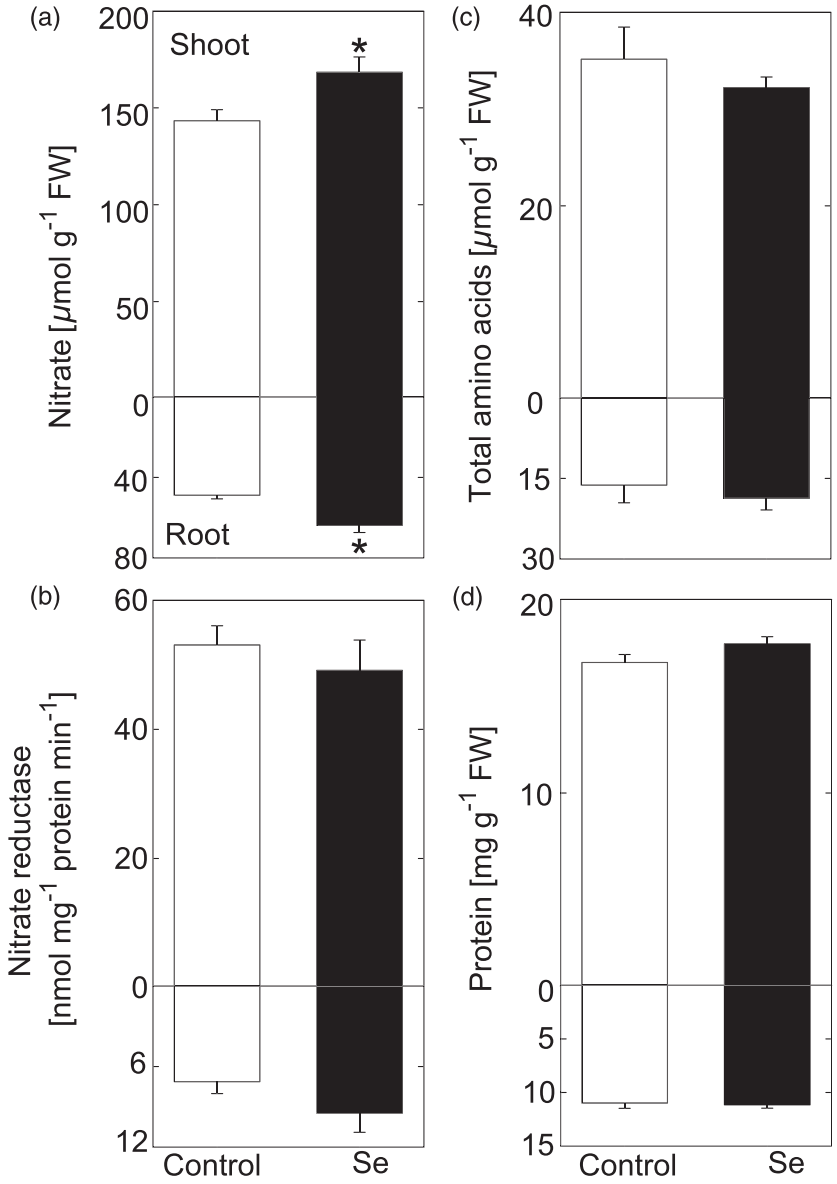

Figure 4. Levels of nitrate, total amino acids, protein and NR activity were analysed in shoot (above sectors) and root (below sectors) of plants treated with Se. (a) Nitrate. (b) Total amino acids. (c) Protein. (d) Nitrate reductase activity. Measurements were performed using rosettes harvested at the end of the light period from plants treated with water (control) or sodium selenate (Se). Values are means±SE of six replicates (each replicate is a pool of five plants).

acids levels in plants treated with Se were observed here (Fig. 4). In both shoot and root of Se-treated plants, the levels of amino acids derived from glycolytic intermediates (e.g. serine, glycine, cysteine, alanine, valine, phenylalanine and tyrosine) were similar to those of control plants. Se did not influence the pool size of TCA intermediates in root of Arabidopsis. By contrast to the situation observed in roots, Se treatment led to decreases in the levels of organic acids (except for citrate) involved in the first part of the TCA cycle, while succinate and other intermediates of the second part of the TCA cycle remained at the same level as in shoots of the control plants. Mitochondrial respiration is divided in three main pathways (glycolysis, the TCA cycle and the mitochondrial electron transport chain) and several unique features of respiratory metabolism are present in plants (Araújo *et al.* 2012). Thus, it is reasonable to assume that an exquisite regulation of plant respiration occurs in response to the presence of high levels of Se. Recent evidence shows that in leaves, the TCA cycle can be more responsible for the production of 2-oxoglutarate for amino acid production than to completely oxidize pyruvate (Sweetlove *et al.* 2010). It seems possible therefore that in response to Se plant respiration is adjusted with the occurrence of non-cyclic modes to allow enhanced amino acid biosynthesis. Remarkably, non-cyclic modes of the TCA cycle in response to several stresses have been previously observed (for a review see Sweetlove *et al.*

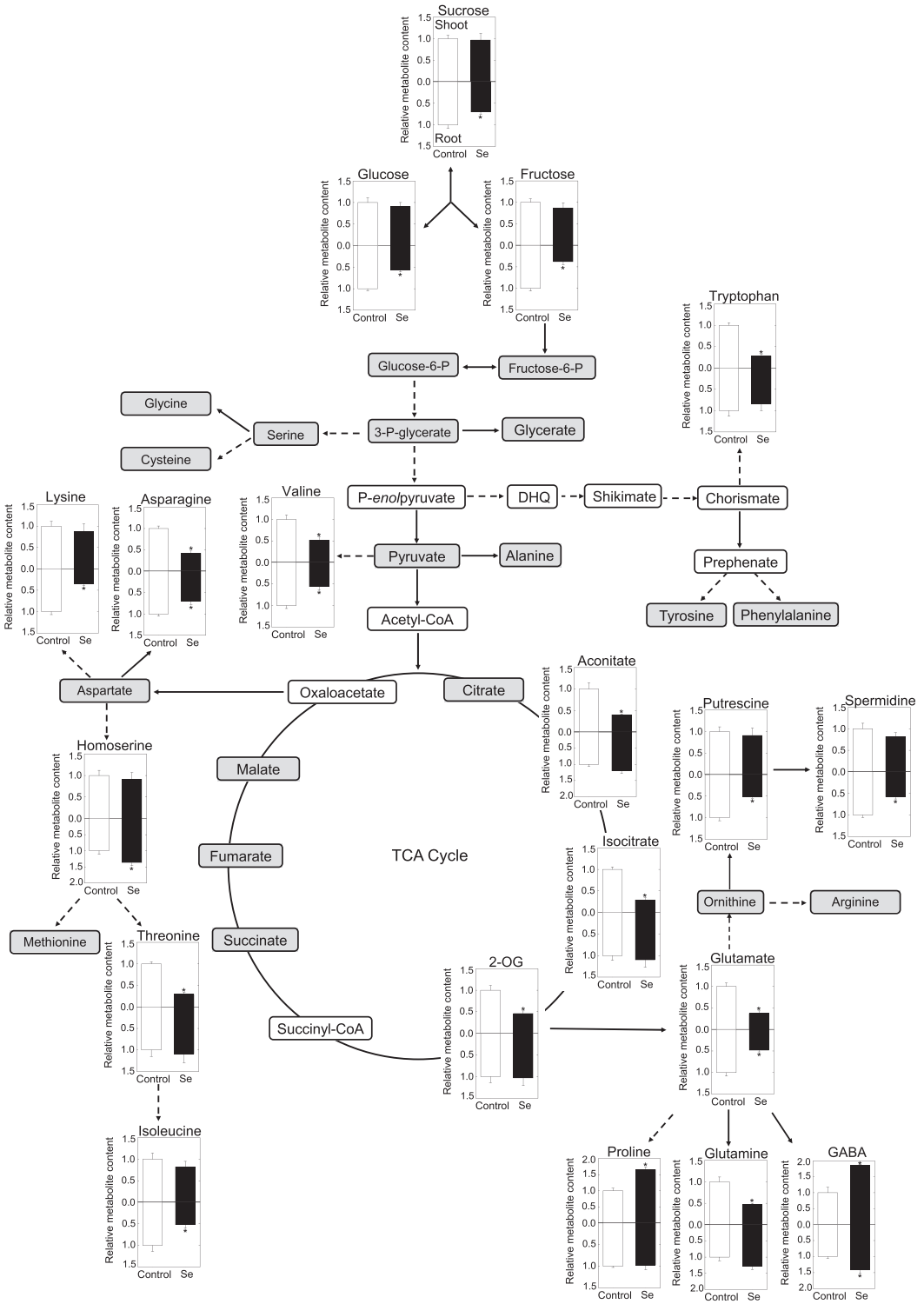

Figure 5. Changes in metabolite profiles in shoot and root of plants treated with Se. Metabolites without a significant difference between treatments are indicated by a grey square. Metabolites outside grey squares indicate that they were not measured. Continuous arrows indicate a one-step reaction, and broken arrows indicate a series of biochemical reactions. Values are presented as means of six replicates (each replicate is a pool of five plants) ± SE. Data are normalized with respect to mean response calculated for the control treatment in each organ. Asterisks indicate values determined by the Student's *t*-test to be significantly different from control ($P < 0.05$). DHQ, 3-dehydroquinate. Measurements were performed using rosettes (above sectors) and roots (below sectors) harvested at the end of the light period from plants treated with water (control) or sodium selenate (Se). The full dataset from the metabolite profiling study is available as Table S1.

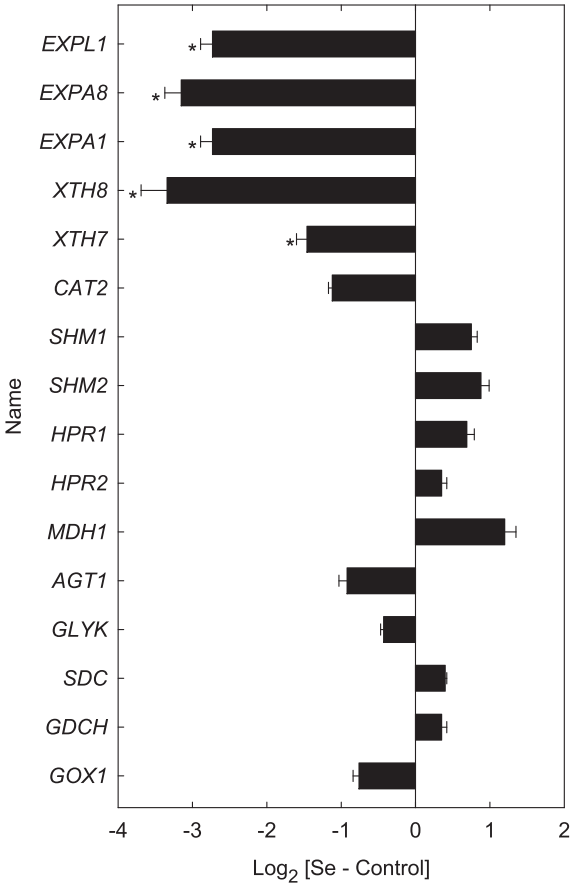

Figure 6. Changes in gene expression in shoots of Arabidopsis plants treated with Se. Data are normalized with respect to mean response obtained for the control treatment. Primer sequences are given in Table S2. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P < 0.05$). Values are means ± SE of four replicates.

2010). The existence of a functional but incomplete TCA cycle in microbial systems in response to aluminum toxicity has been also previously observed (Singh *et al.* 2009, Lemire *et al.* 2010). This elegant metabolic balance allows the accumulation of specific and key metabolites and enables to survive and adapt to hostile environment such as the ones conferred by the presence of Se.

The non-proteinogenic amino acid GABA constitutes a reserve of carbon and nitrogen for amino acid metabolism and TCA activity, particularly in response to biotic and abiotic stress (Nunes-Nesi *et al.* 2010, Renault *et al.* 2013). Moreover, the GABA shunt provides an alternative route of succinate production to that supplied via the operation of the TCA cycle in the mitochondrion (Studart-Guimarães *et al.* 2007, Michaeli *et al.* 2011). The increased GABA levels in Se-treated plants (Fig. 4) could be derived from the direct decarboxylation of glutamate. Aspartate and arginine were not affected by the Se treatment. Furthermore, Se treatment did not significantly affect the expression level of *ASP1* gene as compared with rosette of control plants (Fig. 7). However, other amino acids (e.g. glutamate, glutamine and asparagine) were reduced in shoot and root of plants treated with Se, suggesting a significant alteration of the nitrogen metabolism following Se treatment. Interestingly, *GDH1* and *GS2* genes showed a significant downregulation in rosette of plants treated with Se at the end of the day, but not at the end of the night period (Fig. 7). Thus, although an increased supply of Se leads to increased levels of nitrate in both shoots and roots, total amino acids, protein and nitrate reductase activity remained at the same levels as in the control plants (Fig. 4). This metabolic feature reveals that Arabidopsis responds adaptively to high Se by decreasing the rate of growth, while maintaining the overall protein content, and maintaining or even decreasing the levels of individual amino acids. It is highly possible that in order to keep a similar protein content a higher turnover of non-specific selenoproteins is required in presence of Se. Support to this assumption also comes from recent work in different organisms including human cells (Wallenberg *et al.* 2014), chlamydomonas (Vallentine *et al.* 2014) and *Brassica napus* (Dimkovikj *et al.* 2015) that in presence of Se display increased levels of ubiquitinated proteins as well as proteasome activity. Notably the construction of protein pools is much more energetically expensive as compared with carbohydrate biosynthesis (Pal *et al.* 2013, Ishihara *et al.* 2015). Alternatively, given that ATP is required for the stability and integrity of the 26S proteasome in Arabidopsis (Yang *et al.* 2004), it is possible that the alterations in mitochondrial metabolism took place to support higher ATP demand in presence of Se. Furthermore, despite the absence of changes in dark respiration observed here (Fig. 3b), it should be mentioned that total respiration in *Brassica napus* roots was also increased following Se treatment (Dimkovikj and Van Hoewyk 2014). In summary, it seems reasonable to assume that in order to cope with high levels of Se an extensive metabolic reprogramming occurs, including changes in construction costs, maintenance and growth respiration in both shoots and roots of Arabidopsis, in order to support ATP production and the elimination of malformed proteins at the expense of reduced growth.

It has previously been documented that Se at high concentrations can alter levels of several essential nutrients in wheat plants (*Triticum aestivum*) (Guerrero *et al.* 2014). In the present experiments, Se-treated plants showed an increase in the level of S (Table 1). Furthermore, the levels of important S-containing compounds, such as methionine and cysteine, were not affected by Se treatment (Fig. 5). These results suggest a synergistic instead of an antagonistic relationship between both elements. It was also found that Se treatment triggers a decrease P, Ca and Zn levels, suggesting a modified relationship between the levels of certain nutrients, metabolism and growth. In agreement with this model, plants treated with Se showed a strong decrease in the rate of leaf expansion and biomass accumulation. Expansins (Goh *et al.* 2012) and XTHs (Van Sandt *et al.* 2007) are the best characterized protein classes known to drive cell expansion. Treatment with Se led to a decrease in the expression of these genes, indicating that the effect of Se on plant growth might also be dependent of changes in the transcription of genes associated with cell wall. Indeed, Arabidopsis plants treated with Se showed

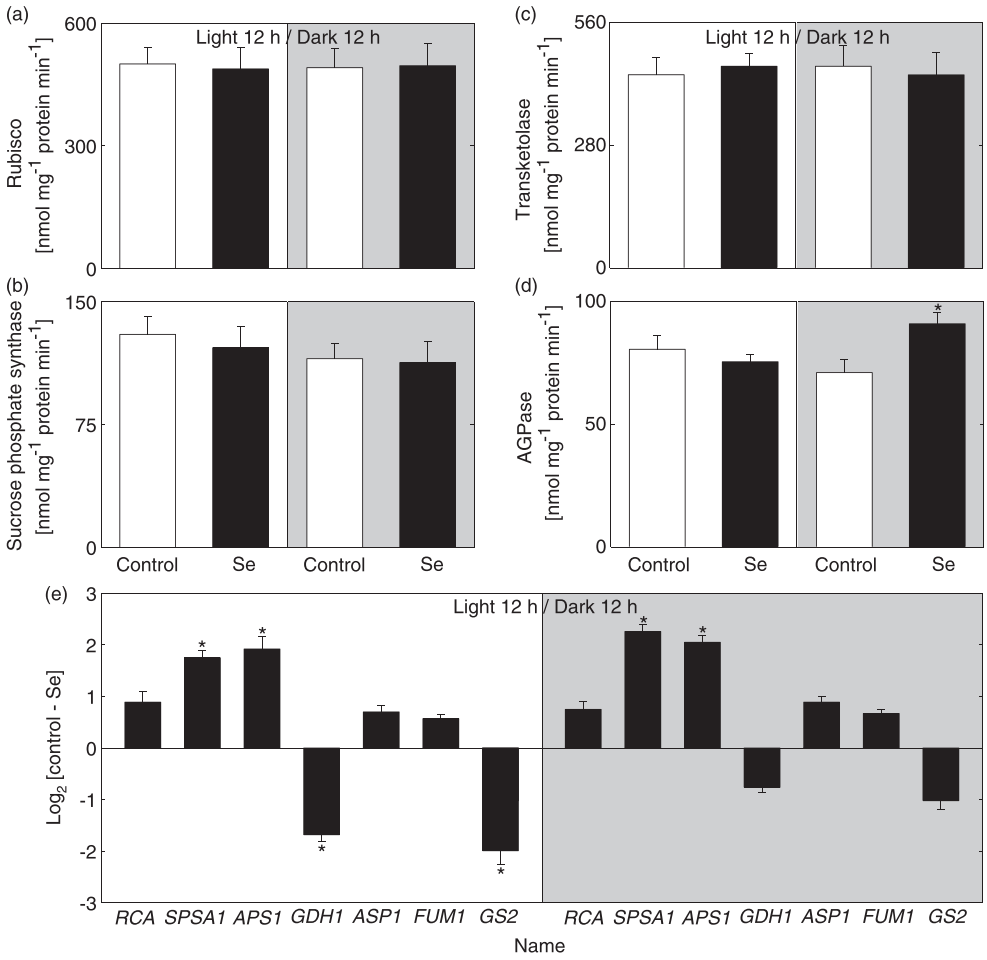

Figure 7. Enzyme activities and expression of primary metabolism-related genes in shoots of plants treated with Se. (a) Rubisco activity. (b) SPS activity. (c) TK activity. (d) AGPase activity. (e) Changes in gene expression. Measurements were performed using whole rosettes harvested at the end of the light (white sectors) and the end of the dark period (grey sectors) from plants treated with water (control) or sodium selenate (Se). For enzyme activities, values are means±SE of six replicates (each replicate is a pool of five plants). For gene expression, values are means±SE of four replicates (each replicate is a pool of five plants). Primer sequences are given in Table S2. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P<0.05$).

Table 1. Levels of macronutrient, micronutrient and selenium in shoot and root of Arabidopsis plants.

	Shoot		Root	
Nutrient	Control	Selenium	Control	Selenium
Macronutrient [mgg^{-1} DW]				
P	1.8±0.2	**1.1±0.1**	2.4±0.3	**1.6±0.2**
K	23.8±1.6	25.0±2.2	15.3±1.6	13.7±1.9
Ca	15.4±0.9	**9.6±1.1**	20.8±1.9	**15.0±1.4**
Mg	2.3±0.2	2.8±0.1	1.9±0.2	2.3±0.3
S	2.7±0.4	**3.9±0.2**	1.1±0.2	**1.9±0.1**
Micronutrient [μgg^{-1} DW]				
Cu	18.1±1.4	16.5±2.0	13.4±0.7	11.5±1.0
Fe	23.0±1.5	26.1±2.8	36.2±4.5	34.3±3.9
Zn	9.4±0.6	**6.3±0.8**	5.7±0.4	**3.4±0.3**
Mn	249.3±19.1	240.0±20.3	221.0±28.4	230.6±26.0
Selenium [μgg^{-1} DW]				
Se	0.8±0.3	**243.8±31.2**	1.4±0.6	**304.5±25.7**

Measurements were performed using whole rosettes and roots harvested at the end of the light period from plants treated with water (control) or sodium selenate (Se). Values in boldface were determined by Student's *t*-test to be significantly different from the control ($P<0.05$). Values are means±SE of six replicates (each replicate is a pool of five plants). DW, dry weight.

downregulation of genes associated with cell cycle and cell wall metabolism (Van Hoewyk *et al*. 2008). These observations are consistent with the growing conception that slow growth in plants contributes to the conservation of carbon resources, ensuring better stress acclimation (Hummel *et al*. 2010).

CONCLUSIONS

Our results demonstrated that Arabidopsis is able to adjust to high levels of Se by an inhibition of both growth and starch degradation. This discovery sheds new light on the modulation between the acquisition and storage of carbon and its usage for plant growth following Se stress. Several lines of evidence also demonstrated that following high levels of Se *Arabidopsis thaliana* necessitate a reconfiguration of primary metabolism to maintain ATP production required most likely for the high turnover of mal formed selenoproteins, which are energetically costly. Further work with specific metabolite flux analyses using available labelled substrates are required to fully understand this intriguing metabolic feature in response to Se. It remains to be explored how exactly starch turnover is regulated and integrated with central metabolism in response to Se.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supporting Information Table S1. Relative metabolite content of plants treated with water 2 (control) or sodium selenite (Se). Values set in bold type were determined by the Student's t-3 test to be significantly different from the control ($P < 0.05$). Data were normalized to the 4 mean response calculated for the control plants in each organ. Values are presented as means 5 of six replicate (each replicate is a pool of five plants) + SE.
Supporting Information Table S2. Primers used in the RT-PCR analyses performed in this study.