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Adenosine 5′-phosphosulfate reductase (APR2) mutation in *Arabidopsis* implicates glutathione deficiency in selenate toxicity

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APR2 is the dominant APR (adenosine 5′-phosphosulfate reductase) in the model plant *Arabidopsis thaliana*, and converts activated sulfate to sulfite, a key reaction in the sulfate reduction pathway. To determine whether APR2 has a role in selenium tolerance and metabolism, a mutant *Arabidopsis* line (*apr2-1*) was studied. *apr2-1* plants had decreased selenate tolerance and photosynthetic efficiency. Sulfur metabolism was perturbed in *apr2-1* plants grown on selenate, as observed by an increase in total sulfur and sulfate, and a 2-fold decrease in glutathione concentration. The altered sulfur metabolism in *apr2-1* grown on selenate did not reflect typical sulfate starvation, as cysteine and methionine levels were increased. Knockout of APR2 also increased the accumulation of total selenium and selenate. However, the accumulation of selenite and selenium incorporation in protein was lower in *apr2-1* mutants. Decreased incorporation of selenium in protein is typically associated with increased selenium tolerance in plants. However, because the *apr2-1* mutant exhibited decreased tolerance to selenate, we propose that selenium toxicity can also be caused by selenate's disruption of glutathione biosynthesis leading to enhanced levels of damaging ROS (reactive oxygen species).

Key words: buthionine sulfoximine, reactive oxygen species, selenium tolerance, selenoprotein, sulfur, vascular plant.

INTRODUCTION

Unlike some bacteria and animals, vascular plants have not been shown to require selenium as an essential trace element. However, plants can still accumulate selenium in their tissues due to non-specific uptake and assimilation. Selenate and sulfate have long been known to compete for transport in plants [1], and most selenate uptake into *Arabidopsis thaliana* (thale cress) roots is probably mediated by the high-affinity sulfate transporter SULTR1;2 [2]. In fact, due to the chemical similarity between sulfur and selenium, nearly all plant enzymes participating in essential sulfur metabolism are thought to be able to use selenium analogues as substrates [3]. Replacement of sulfur by selenium can be detrimental; this is particularly true when cysteine is replaced by selenocysteine in proteins [4]. Therefore selenium incorporation into plant proteins is considered toxic, and preventing such misincorporation has been shown to be a successful approach to enhance plant selenium tolerance [5,6].

Interest in plant selenium metabolism and selenium accumulation stems from two areas of research. First, selenium can accumulate to potentially toxic levels in the environment, both due to natural and anthropogenic causes, and can pose threats to aquatic organisms [7], livestock [8] and humans [9]. Plants can be used to extract excess selenium from soil and water, alleviating public health concerns. Therefore plants with enhanced selenium tolerance and accumulation may potentially be used for the phytoremediation of selenium [10] in polluted terrestrial or aquatic ecosystems. Secondly, humans require selenium as an essential trace element for at least 25 selenoproteins [11], and plants are the primary source of essential selenium in the human diet. Although nutritional selenium deficiency in humans is associated with infertility and death [12], there is conflicting evidence on the protective benefits of a selenium-enriched diet [13]. For example, elevated levels of selenium in humans are associated with a decreased risk of bladder cancer [14], but an increased risk of Type 2 diabetes [15]. Thus crops with the ability to fortify the human diet with selenium might be desirable, particularly in areas where selenium is naturally present at low levels in soil, such as the U.K. [16], Scandinavia [17] and New Zealand [18].

The challenges of enhancing selenium phytoremediation and dietary biofortification can be addressed by better understanding genetic factors that mediate selenium accumulation, metabolism and tolerance in plants. Enhanced selenium tolerance and accumulation can be achieved via genetic manipulation. For example, a broccoli methyltransferase involved in ubiquinone synthesis was recently overexpressed in *Arabidopsis*, which increased selenium volatilization and tolerance, but decreased selenium accumulation; what makes the work by Zhou et al. [19] intriguing is that ubiquinone has a well-known biochemical role in mitochondrial respiration. More typically, enhanced selenium tolerance and accumulation is achieved by manipulating enzymes involved in sulfur metabolism. For instance, increased selenium tolerance was observed in *Arabidopsis* plants with a knockout of SULTR1;2; the enhanced selenium tolerance was explained by the increased sulfate/selenium ratio [2]. Barberon et al. [20] observed further selenium tolerance and decreased accumulation in the *sultr1;1-sultr1;2* double mutant. However, selenium tolerance and accumulation are not always inversely correlated. For example, overexpression of ATP sulfurylase (the first enzyme in the sulfate reduction pathways) enhanced both selenium tolerance and accumulation in *Brassica juncea* [21]. Additionally, overexpression of CpNifS, a chloroplastic protein with cysteine desulfurase and selenocysteine lyase activity, enhanced selenium tolerance and accumulation in *Arabidopsis*;

Abbreviations used: APR, adenosine 5′-phosphosulfate reductase; BSO, buthionine sulfoximine; Col, Columbia; MS, Murashige and Skoog; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; SiR, sulfite reductase; SULTR, sulfate transporter; T-DNA, transferred DNA.

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the enhanced tolerance to selenium was explained by a decrease of selenium incorporation in proteins [6].

As already stated, it is well established that some enzymes participating in sulfur metabolism also mediate selenium uptake and reduction (for a review, see [22]). The key enzyme in the sulfur reduction pathway is APR (adenosine 5′-phosphosulfate reductase), which reduces activated sulfate to sulfite [23], although a role for sulfite reductase has also recently been proposed [24]. All three isoenzymes of APR are localized to plastids and are negatively regulated by cysteine as well as by glutathione [25], the dominant form of non-protein thiols in plants. Overexpression of a *Pseudomonas* APR in *Arabidopsis* [26] and corn [27] decreased the concentration of sulfate and increased the concentration of reduced sulfur compounds. However, these plants also exhibited chlorosis, suggesting that APR activity needs to be strictly regulated.

Recently it was revealed that APR2 is the major isoenzyme that catalyses sulfate reduction in *Arabidopsis* plants, as knockout of APR2 reduced total APR activity by approx. 80 % [28]. The decreased APR activity in plants resulted in increased sulfate accumulation. The viability of *apr2-1* plants suggests that the activity of APR1 and APR3 is sufficient for plant growth and development. APR1 and APR3 appear to complement APR2 in *Arabidopsis*, at least under controlled environmental conditions. However, there is evidence to suggest that the three isoenzymes of APR are not functionally redundant [29]. For example, transcripts of the three APR isoforms are differentially regulated in response to light [30], nitrogen [31], salt and H_2O_2 [32]. Furthermore, all three isoenzymes of APR are differentially activated by a family of MYB transcription factors involved in glucosinolate biosynthesis [33], which is induced by biotic stress. Therefore it is likely that the APR isoenzymes have unique roles during development and stress.

A role for APR in selenate tolerance is suggested by results from several earlier studies. Transcripts of all three APR genes were up-regulated in root, but not shoot, tissue when *Arabidopsis* plants were grown for 10 days on selenate [34] and selenite [35]. Furthermore, overexpression of the *Pseudomonas* APR in *Arabidopsis* enhanced plant tolerance to selenate; these transgenic plants had a decrease in selenium accumulation, which was accompanied by an overall larger pool of reduced selenium [36]. In the present study, we directly investigate the role of APR2 in selenium tolerance and accumulation using *apr2-1* mutants with a complete knockout of APR2 [28]. The data presented show that disruption of APR2 decreases selenium tolerance, and suggest that APR2 is a key enzyme mediating selenium accumulation and metabolism in *Arabidopsis*. Furthermore, the present study sheds new light on the mechanisms of selenium toxicity in plants.

EXPERIMENTAL

Plant material and growing conditions

The APR2 knockout plants used in the present study were initially described by Loudet et al. [28]. Briefly, *apr2-1* seeds were generated by the GABI-KAT programme [37]. The T-DNA (transferred DNA) insert in the coding region of *APR2* (At1g62180) was confirmed using primers that flanked the T-DNA insert (5′-TGAGGTTCAAGCTTTAGTGAGGA-3′, 5′-TATGGATGTTCCGGTGAATGCATT-3′) and a primer that hybridized to the T-DNA insert (5′-ATATTGACCATCATACTCATTGC-3′). *apr2-1* seeds were deemed homozygous by PCR analysis (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/438/bj4380325add.htm), and all of the progeny grew on the selective herbicide sulfadiazine. These homozygous knockouts were compared with their wild-type background, Col (Columbia). Plants were grown in a growth chamber (150 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} PAR, 16 h light/8 h dark cycle, 24 °C) on agar plates containing half-strength MS (Murashige and Skoog) medium + 1 % sucrose with or without 40 μM sodium selenate (Na_2SeO_4).

Selenium and sulfur metabolism, and quantification of metabolites

Selenate tolerance was determined by measuring the total plant biomass and root length of seedlings ($n = 30$) grown for 14 days on vertical plates with or without 40 μM selenate as described [21]. Total plant biomass was additionally measured at day 14. Tolerance to 15 μM sodium selenite and 80 mM NaCl was also measured, as determined by measuring root length of plants after 14 days of growth.

Total sulfur and selenium accumulation was assayed by growing seedlings for 21 days on horizontal agar plates containing half-strength MS with or without 20 μM selenate. A lower selenate concentration was used to collect enough biomass in the selenate-sensitive *apr2-1* plants; unless otherwise described, all subsequent studies employed 20 μM selenate. Shoots were harvested, separated, washed to remove any external selenium and dried for 72 h at 70 °C. Five separately pooled shoot samples were then acid digested and analysed by means of ICP (inductively coupled plasma)-atomic emission spectrometry as described [21]. Incorporation of selenium in protein was determined from five separately pooled samples each containing seedlings grown for 21 days on agar medium supplied with 20 μM selenate as described previously [6]. Levels of selenate and selenite were measured by digesting fresh plant material ($n = 3$ independently pooled samples containing 65–75 seedlings) with a heated solution of tetramethylammonium. Samples were then analysed at Brooks Rand Labs using a Hamilton PRPX-100 method via HPLC-ICP-DRC (dynamic reaction cell)-MS, as similarly described elsewhere [38].

Levels of amino acids, sulfate, sulfite and glutathione were measured in shoots from plants grown for 21 days with or without selenate. For the estimation of free amino acids, plant samples were precipitated with 5 % sulfosalicylic acid, and the supernatant was measured on a Hitachi L-8900 amino acid analyser according to the manufacturer's instructions.

Sulfate levels were estimated spectrophotometrically by measuring the amount of barium sulfate, which formed when sulfate was allowed to react with barium chloride [39]. Two leaves (20–25 mg) from the same plant were ground in liquid nitrogen, and extracted in 200 μl of distilled H_2O ($n = 5$ biological replicates). The extract was passed through a 0.22 μM filter before adding 100 μl of 20 % trichloroacetic acid and incubating for 40 min at 70 °C. Samples were then centrifuged, and 200 μl of supernatant was added to a 200 μl solution containing 0.7 % barium chloride and 10 % poly(ethylene glycol)-3350. This mixture was incubated at 25 °C for 10 min before the A (absorbance) was measured at 600 nm. Sulfite was measured spectrophotometrically using a kit according to the manufacturer's instructions (R-BioPharm); briefly, sulfite was indirectly estimated based on the amount of sulfate produced by sulfite oxidase. Total glutathione content, including the pool of reduced and oxidized GSH, was estimated spectrophotometrically using Ellman's Reagent, as previously described [34]. The amount of reduced glutathione was estimated by the difference between total glutathione from oxidized glutathione.

Photosynthetic and stress-induced measurements

Chlorophyll and anthocyanin were measured in wild-type and mutant plants grown for 2 weeks with and without

40 μM selenate. Chlorophyll content was measured as described previously [40]. Anthocyanin content was estimated spectrophotometrically at A_{530} after subtracting for non-specific background at 657 nm, as described previously [41]. For measurements related to photosynthetic efficiency, plants were grown for 10 days without selenate before being transferred to medium with or without 40 μM selenate for an additional 14 days. This was done to ensure equal biomass and development of full leaves prior to the onset of selenate stress. Maximum photochemical efficiency of photosystem II (F_v/F_m) in dark-adapted plants was determined by using a hand-held chlorophyll fluorimeter (Photon System Instruments) and calculated as described previously [42]. Photosynthetic electron transport rate was measured in dark-adapted plants at varying light intensities (e.g. 20, 50, 100, 300 and 500 μEinsteins).

For the visual estimation of selenate-induced formation of ROS (reactive oxygen species) formation, plants were grown for 10 days on control medium to ensure equal biomass before being transferred to 40 μM selenate for an additional 14 days. Accumulation of superoxide was detected with Nitro Blue Tetrazolium as described in [43]. Estimation of lipid peroxidation was determined by the formation of malondialdehyde; this procedure was based on modifications of a TBARS (thiobarbituric acid-reacting substance) assay described previously [44]. Briefly, 100 mg of shoots was ground in liquid nitrogen and suspended in PBS containing 0.1 mM PMSF and 10 % trichloroacetic acid. A 1:1 volume of supernatant and 0.8 % thiobarbituric acid was vortex mixed and heated at 100 °C for 45 min. The formation of malondialdehyde was determined spectrophotometrically at A_{535}, after correcting for non-specific background absorbance (A_{600}).

RT–PCR (reverse transcription–PCR), immunoblotting and statistics

For the estimation of transcript abundance, 30–40 plants were grown for 14 days on vertical plates with or without 20 μM selenate. Root and shoot material (100 mg) were separated and processed from three separately pooled samples; mRNA from each biological replicate was extracted using an RNeasy Plant Mini Kit (Qiagen) and converted into cDNA by SuperScript® reverse transcriptase (Invitrogen), and subjected to RT–PCR analysis as described previously [45]. Gene-specific primers were designed for APR1 (At4g04610), APR1f (5′-CATTGGAGCCAAAAGTTTCGCA-3′) and APR1r (5′-CGCCATTGCATTTAGTGGTGCAGA-3′); APR3 (At4g21990), APR3f (5′-CCTTCTTCAGATCTCAAAGTAAC-3′) and APR3r (5′-GCTATTGCCTTTAGTGGAGCTGAA-3′); SiR (sulfite reductase) (AT5G04590), SiRf (5′-CCAAACTGCAATGGCTTGCC-3′) and SiRr (5′-CAGTTTCTCGAATCCCATGC-3′); SULTR1;1 (AT4G08620), SULTR1;1f (5′-GCCATCACAATCGCTCTCCAA-3′) and SULTRr (5′-TTGCCAATTCCACCCATGC-3′); SULTR1;2 (AT1G78000), SULTR1;2f (5′-GGATCCAGAGATGGCTACATGA-3′) and SULTR1;2r (5′-TCGATGTCCGTAACAGGTGAC-3′); actin2 as a control (AT3G18780), ActinF (5′-TGCAGGAGATGATGCTCCCAG-3′) and ActinR (5′-ATCCAGCACAATACCGGTTGTA-3′). The PCR reactions were still in exponential phase once they were stopped (cycle 17 for actin; cycle 21 for APR1, SULTR1;1 and SULTR1;2; cycle 24 for APR3 and SiR). Transcript levels were estimated based on PCR band intensities, which were quantified using ImageJ64 imaging software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Immunoblotting was used to estimate APR and SiR polypeptide levels in Col and *apr2-1* grown with or without selenate. Shoot protein (20 μg) was separated by SDS/PAGE and transferred to nitrocellulose by electroblotting. APR and SiR polypeptides were detected using antibodies that have been described previously [31,46]. Briefly, protein gels were analysed with APR antisera against recombinant *Arabidopsis* APR2 and SiR antisera against Maize SiR. Protein gels were allowed to then react against a secondary antibody against rabbits. Immunoreactive proteins were detected using alkaline phosphatase. Antisera against APR and SiR polypeptides produced bands of 50 and 71 kDa respectively, which are in agreement with their estimated sizes.

All statistical analyses (ANOVA, Student's *t* tests) were performed using the Kleida-graph software package (Synergy Software).

RESULTS

Arabidopsis plants of ecotype Col with a T-DNA insert in the APR2-coding region failed to produce a measurable *APR2* transcript (Supplementary Figure S1). The *apr2-1* plants were compared with wild-type Col for their ability to grow with or without selenate, the most abundant bioavailable form of selenium in oxic soils. On control medium the Col and mutant *apr2-1* plants were indistinguishable from each other in their growth and development, and there was no difference in biomass and root growth. The tolerance to selenate, as judged from biomass and root length, was reduced in *apr2-1* plants by 3.2-fold and 2.7-fold respectively relative to Col control plants (Figure 1A).

The effect of loss of APR2 function was specific to selenate because treatment with 15 μM selenite had the same effect on the wild-type and mutant (Figure 1B). To determine whether knockout of APR2 in *Arabidopsis* also impairs its tolerance to another form of abiotic stress, Col and *apr2-1* plants were grown on 80 mM NaCl. Although this stress restricted root length, there was no significant difference ($P > 0.05$) between the wild-type and mutant (Figure 1B). Thus knockout of APR2 specifically impairs tolerance to selenate.

To establish whether there are physiological differences between Col and *apr2-1* plants when grown on selenate, pigments and photosynthetic parameters were measured. After 2 weeks of growth on medium with 40 μM selenate, there was a decrease in chlorophyll concentration in *apr2-1* compared with Col; there was, however, no difference in chlorophyll content between the two genotypes when grown without selenate (Figure 2A). Anthocyanin, a pigment that typically accumulates during stress, accumulated in both Col and *apr2-1* when grown on selenate compared with control medium. Although anthocyanin did not accumulate differentially in mutant and wild-type in the absence of selenium, its levels were 2-fold higher in *apr2-1* compared with Col when grown on selenium (Figure 2B). To gain more insight into the effects of selenium in APR2-impaired plants, photosynthetic performance of Col and *apr2-1* was measured using a chlorophyll fluorimeter. In order to ensure equal biomass and development of full leaves prior to measurements, plants were grown without selenate for 10 days before being transferred to medium with and without 40 μM selenate for an additional 2 weeks. The F_v/F_m ratio, which is indicative of the maximum photochemical efficiency [42], was lower in *apr2-1* than in Col, when treated both with and without selenium (Figure 2c). Impaired photosynthetic efficiency in *apr2-1* was further reflected by a lower photosynthetic electron transport rate of Photosystem II; the reduced electron transport rate in *apr2-1* was exacerbated when grown on selenium (Figure 2D).

To determine how the decreased selenate tolerance in *apr2-1* is associated with total selenium and sulfur accumulation, *apr2-1* and Col plants were grown on selenate for 3 weeks

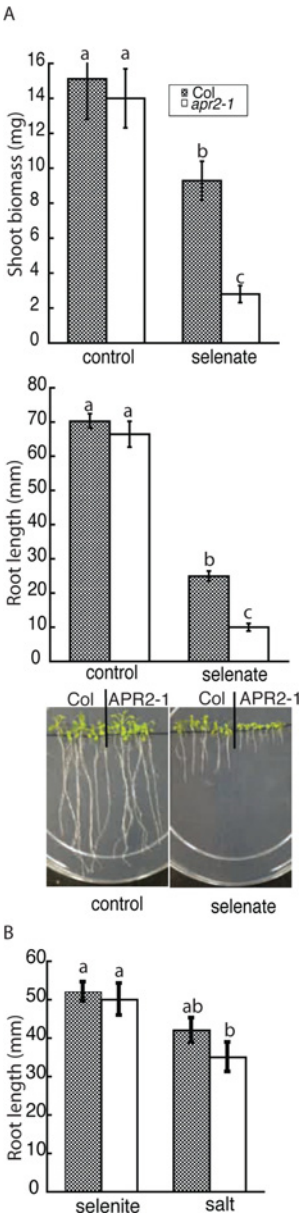

Figure 1 ***apr2-1*** **is sensitive to selenate, but not selenite or salt**

(**A**) Selenate tolerance as determined by measuring the root length and biomass of Col ecotype and *ap2-1* (Col background) grown for 14 days on 0.5 × MS medium (control) and medium supplemented with 40 μM selenate. (**B**) Root length of Col and *apr2-1* on 15 μM selenite and 80 mM NaCl. Results shown are the mean ($n = 30$ seedlings) and S.E.M., and are representative of two different experimental replicates. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$).

and tissue selenium and sulfur levels were compared. Shoot concentration of total selenium was nearly 2-fold higher in *apr2-1* compared with Col (Figure 3A). Total sulfur concentration was also enhanced in the mutant plants, when grown both with and without selenate (Figure 3B). A decrease in APR activity has previously been reported to increase levels of sulfate [28]. To determine if disruption of *APR2* in *Arabidopsis* similarly affects the accumulation of inorganic selenium species, concentrations of selenate and selenite were estimated. *apr2-1* plants accumulated more selenate and less selenite compared with Col when grown on selenium (Figures 4A and 4B). A similar pattern appears for sulfate, i.e. sulfate accumulates more in *apr2-1* than in Col, when grown both with and without selenium (Figure 4C). However, as barium chloride reacts with both sulfate and selenate, it should be noted that levels of sulfate may be overestimated by 2–4 % in plants grown on selenate. Sulfite is the product of APR activity, and represents the first form of reduced sulfur in the plant cell. *apr2-1* mutants accumulated nearly the same amount of sulfite compared with Col when grown with or without selenium (Figure 4D). In the presence of selenate, the overall trend is that knockout of APR2 decreases accumulation of selenite and sulfite, resulting in an increase in the pool of the sulfate and selenate, a possible reactant for the APR2 enzyme. Notably, when grown on selenate, the ratio of sulfate/sulfite is much higher than that of selenate/selenite in both Col and *apr2-1* (Figure 4E).

As shown above, *apr2-1* plants accumulate more total sulfur than the wild-type when grown on selenate. To determine if this increase in total sulfur corresponds to an equal increase in organic sulfur compounds, cysteine, methionine, and glutathione were measured. There was no difference in levels of cysteine and methionine between Col and APR2 knockouts on control medium. Surprisingly, both cysteine and methionine accumulated to higher levels in *apr2-1* compared with Col grown on selenate (Figures 5A and 5B). In fact, total accumulation of all amino acids in *apr2-1* was 1.3- and 2.3-fold higher compared with Col on control and selenate medium respectively (Figure 5C). Accumulation of total amino acids in *apr2-1* is mostly attributed to increased levels of glutamine, glutamic acid and asparagine, and is associated with a higher concentration of ammonia in these plants (Supplementary Table S1 available at http://www.BiochemJ.org/bj/438/bj4380325add.htm).

Glutathione represents the majority of non-protein thiols stored in plants. Reduced and oxidized levels of glutathione were measured in Col and *apr2-1* and compared with *cad2-1*, a mutant (Col ecotype) in glutamate–cysteine ligase with impaired glutathione synthesis [47]. On control medium, WT and *apr2-1* had nearly the same levels of glutathione, yet glutathione concentration in both accessions was 2-fold higher compared with *cad2-1*.

Selenate had the effect of decreasing total and reduced glutathione in all three plant lines. Although the amount of reduced glutathione was nearly the same in Col and *apr2-1* on control medium, when grown on 20 and 40 μM selenate, levels of reduced glutathione in *apr2-1* declined 2- and 3-fold respectively compared with Col (Figures 6B and 6C). The lower levels of glutathione in *apr2-1* plants could help explain the phenotypes of these plants on selenate. We were therefore interested in analysing and comparing the phenotypes of *apr2-1* plants with those of *cad2-1* plants and of plants treated with 200 μM BSO (buthionine sulfoximine), a known inhibitor of glutamate–cysteine ligase. BSO decreased glutathione levels in Col and *apr2-1* relative to plants grown on the same selenate concentration without BSO (Figure 6D). Selenate tolerance in the three accessions was measured on 20 and 40 μM selenate as well as on 20 μM selenate plus BSO. Compared with wild-type, *cad2-1* was most sensitive to selenate, followed by *apr2-1* (Figure 6E). Among the three lines, a positive correlation exists between selenate tolerance and glutathione, particularly glutathione (Figure 6F). Furthermore, selenate tolerance is also positively correlated with the glutathione redox ratio.

To gain more insight into the consequence of elevated selenate in *apr2-1*, the effects of selenate and glutathione on each other were further examined using an *in vitro* approach. When selenate and glutathione were mixed in an aqueous solution, measured levels of selenate decreased compared with a negative control lacking glutathione (Supplementary Figure S2 available at http://www.BiochemJ.org/bj/438/bj4380325add.htm). However,

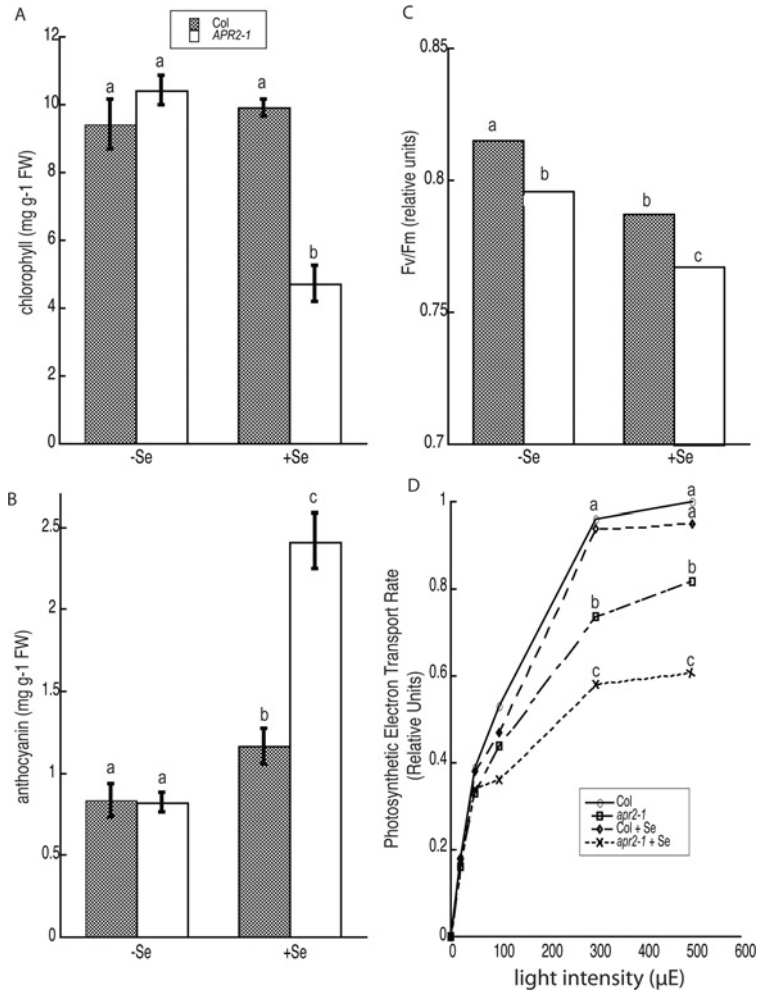

Figure 2 Influence of selenate treatment on photosynthetic parameters of *apr2-1*

Measurement of (**A**) chlorophyll and (**B**) anthocyanin in Col and *apr2-1* grown for 14 days on 0.5 × MS medium (control) and medium supplemented with 40 μM selenate. Results shown are the mean ($n = 5$ independently pooled samples of 3–4 seedlings) and S.E.M. (**C**) F_v/F_m ratio, which represents the maximum photochemical efficiency, and (**D**) photosynthetic electron transport rate were measured in five different Col and *apr2-1* plants. Values are relative to the highest rate of electron transport in Col plants grown without selenate. Plants were grown for 10 days on control media and then for an additional 14 days on medium with or without 40 μM selenate. Results shown are the mean and S.E.M., and are representative of an experimental replicate; different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$). Note that standard error bars were too small to be plotted in (**C**, **D**).

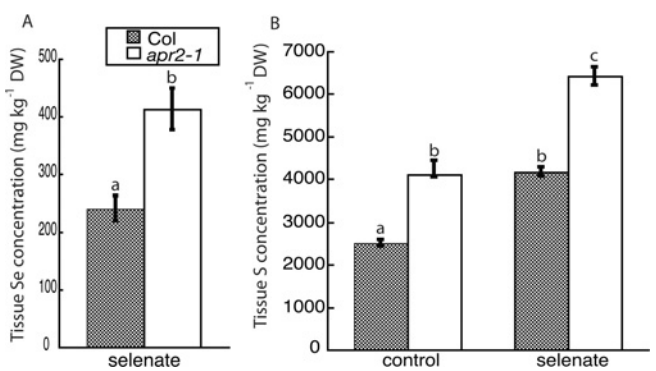

Figure 3 Accumulation of total selenium and sulfur

Shoot concentrations of (**A**) selenium and (**B**) sulfur in Col and *apr2-1* plants grown on medium with and without 20 μM selenate for 21 days. Results shown are the mean ($n = 5$ independently pooled samples of roughly 20 seedlings) and S.E.M. Note that selenium is absent in plants grown without selenate. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$).

the glutathione-mediated decrease in selenate was lower compared with the enzymatic reduction of selenate via ATP sulfurylase. Furthermore, the presence of selenate in an aqueous solution mixed with glutathione had the effect of increasing levels of oxidized glutathione.

Selenium toxicity is thought to occur when selenocysteine replaces cysteine in proteins. In order to explore how selenium contributes to *apr2-1's* sensitivity to selenate, the amount of selenium in protein was measured in wild-type and *apr2-1* plants after growing on 20 μM selenate for 3 weeks. Although the sulfur content in protein was the same among the two plant types, the selenium content in protein was lower in *apr2-1* compared with Col (Figure 7).

Selenium was reported to induce the formation of ROS in plants [19,48], thereby causing oxidative stress. To further probe the mechanisms of elevated selenate toxicity in *apr2-1*, the formation of superoxide was estimated in wild-type and mutant plants grown on 40 μM selenate. The *apr2-1* plants, which were shown earlier to accumulate more selenate, had a substantially higher amount of superoxide compared with Col throughout the entire shoot system

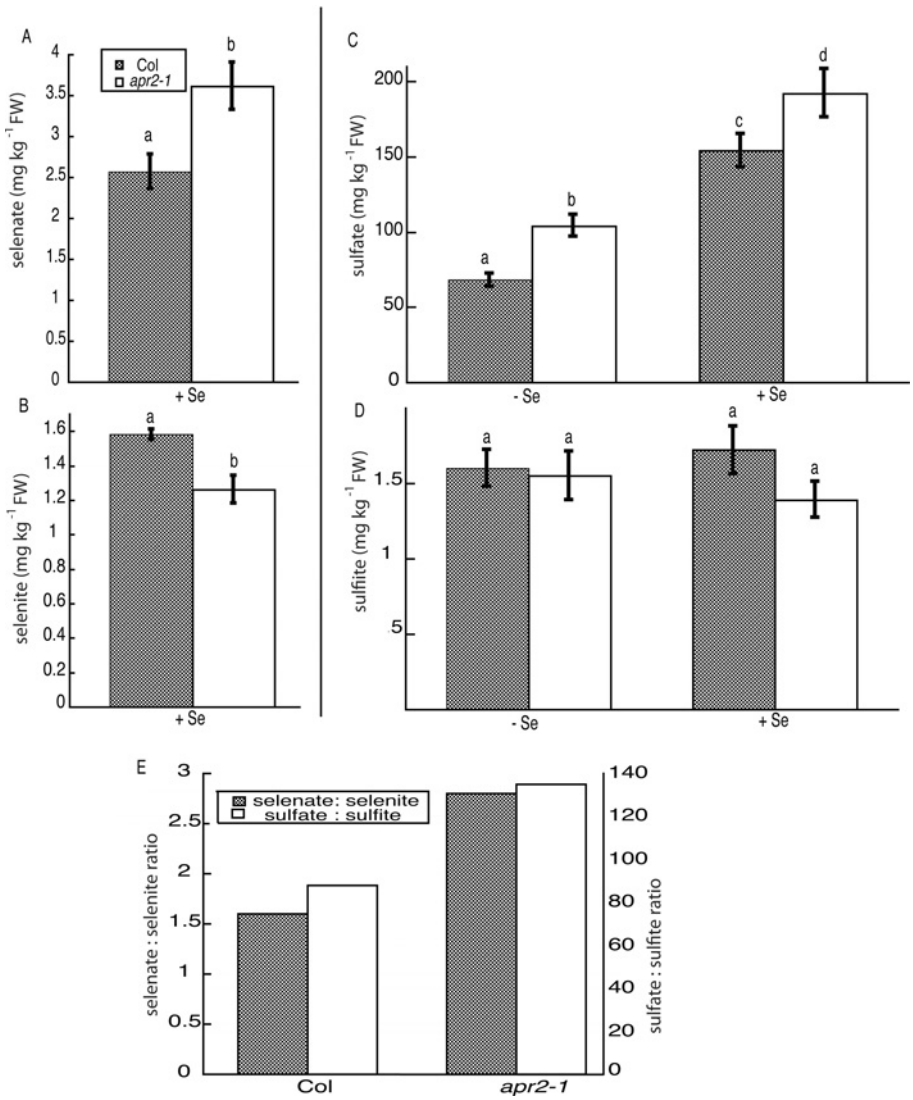

Figure 4 Accumulation of inorganic sulfur and selenium metabolites

Shoot concentrations of inorganic (**A**) selenate and (**B**) selenite were measured along with (**C**) sulfate and (**D**) sulfite in Col and *apr2-1* plants grown on medium with and without 20 μM selenate for 21 days. Results shown are the mean ($n = 3$ independently pooled samples of 65–75 seedlings for selenate and selenite measurements; $n = 5$ independently pooled samples of 20 seedling for sulfite measurements; for sulfate measurements, 2–3 leaves from the same plant were measured in five different individuals) and S.E.M. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$). (**E**) The ratio of selenate to selenite (left *y*-axis) and sulfate to sulfite (right *y*-axis) in Col and *apr2-1* was estimated by using the mean values of data from plants grown on selenate (**A**–**D**).

(Figure 8). To investigate whether the increased superoxide levels corresponded with an increase in lipid peroxidation, the latter was estimated by measuring the formation of malondialdehyde. Despite selenate's (and selenite's) reported capacity to induce superoxide accumulation, there was no difference between Col and *apr2-1* in the amount of lipid peroxidation among treatments (Supplementary Figure S3 available at http://www.BiochemJ.org/bj/438/bj4380325add.htm).

Plants with a full knockout of APR2 are viable when grown on soil, clearly suggesting that APR1 and APR3 provide sufficient activity to complete normal plant development and reproduction. Abundance of root and shoot transcripts regulating sulfur metabolism was analysed to determine whether there is differential expression between Col and *apr2-1* when grown with or without selenate. Transcripts measured included APR1 and 3, SiR and the two high-affinity root sulfate transporters SULTR1;1 and SULTR1;2. In the absence of selenium, there was no difference in the abundance in transcripts between wild-type and the mutant in either the root or shoot material. However, when grown on selenate, SULTR1;1 accumulated nearly 1.5-fold in the roots of *apr2-1* compared with Col (Supplementary Figure S4 available at http://www.BiochemJ.org/bj/438/bj4380325add.htm). SiR transcript levels were somewhat lower in the roots and higher in the shoots of *apr2-1* relative to Col, but not significantly ($P > 0.05$). In the shoots, APR1 transcript levels increased 2-fold in *apr2-1* compared with Col.

Given that APR can be regulated post-transcriptionally [32], the abundance of APR polypeptides was estimated in the shoots of plants using an APR antibody that cross-reacts with all APR isoenzymes. As expected, APR levels decreased in *apr2-1* plants grown with or without selenate (Figure 9). In both Col and *apr2-1*, APR levels decreased when grown on selenate compared with control medium. The decreased levels of APR1 and APR3 in *apr2-1* on selenate compared with control medium are in contrast

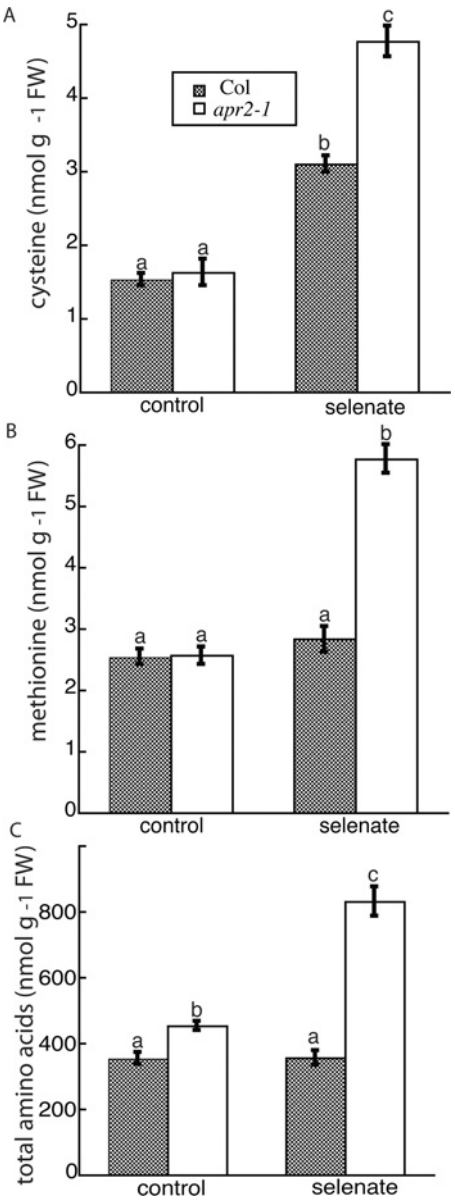

Figure 5 Cysteine, methionine and total amino acid measurements

Shoot concentration of (**A**) cysteine, (**B**) methionine and (**C**) total amino acids in Col and *apr2-1* plants grown on control and 20 μM selenate for 21 days. Results shown are the mean ($n = 3$ independently pooled samples of 30–40 plants) and S.E.M. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$).

to the overall increased expression of the transcripts that encode these polypeptides when *apr2-1* plants were grown on selenate. Lastly, levels of SiR polypeptides remained nearly unchanged in the shoots of *apr2-1* grown with or without selenate compared with Col.

DISCUSSION

APR reduces activated sulfate to sulfite, which is viewed as a key step in sulfur reduction and assimilation in plants [23]. Knockout of *APR2* in *Arabidopsis* severely decreased selenate tolerance, but not selenite and salt tolerance, compared with the Col wild-type. Furthermore, *apr2-1* plants had increased levels of selenate, but decreased levels of selenite, compared with Col. The present study represents the first line of evidence that a plant APR protein is specifically involved in selenate tolerance and selenium metabolism.

Selenate induces a sulfur starvation response in plants, which causes an increase in the expression of sulfate transporters and leads to accumulation of sulfate [34]. Given that *apr2-1* plants already accumulate more sulfate [28], the enhanced accumulation of total sulfur in *apr2-1* plants grown on selenate is probably due to the increase in sulfate levels, as decreased concentrations of glutathione, the dominant form of the non-protein thiol pool, were observed. Similarly, an increased accumulation of selenium in *apr2-1* is probably due to the observed increase in selenate. This result can be explained by the increased expression of the sulfate transporter SULTR1;1 in *apr2-1* (Supplementary Figure S4), which probably mediates selenate uptake in addition to sulfate [2,20].

APR activity was recently shown to be partially uncoupled with cysteine accumulation [28]. However, given the decrease in glutathione in *apr2-1* plants when treated with selenate, it was surprising that concentrations of cysteine and methionine were higher in *apr2-1* compared with Col. An increase in cysteine is also observed in *cad2-1* [47], and cysteine might similarly accumulate in *apr2-1* on selenate as glutathione synthesis decreases. Additionally, during typical sulfate starvation, concentrations of cysteine and methionine decrease. Therefore the observed alteration in sulfur-containing amino acids in *apr2-1* does not parallel sulfate deficiency, which is in agreement with the observation that sulfate accumulates in these plants. Although a majority of other amino acids remained unchanged between Col and *apr2-1* on selenate, levels of glutamine, glutamate and asparagine all doubled in *apr2-1* compared with Col on selenate. Glutamine is the first amino acid formed during nitrogen assimilation from ammonia; glutamine is a precursor of asparagine, another amino acid involved in nitrogen transport. Sulfur and nitrogen metabolism are closely co-regulated. Both glutamine and asparagine are known to accumulate during sulfur starvation in plants, which may help prevent the accumulation of toxic ammonia as the nitrogen/sulfur ratio increases and amino acid metabolism is altered [49,50]. Indeed, accumulation of glutamine and asparagine during cultivation in the presence of selenate was correlated with a 3-fold increase in the concentration of ammonia in *apr2-1* compared with Col (Supplementary Table S1). Altogether, the altered amino acid metabolism of selenate-treated *apr2-1* is reminiscent and remarkably similar to phenotypes observed for a knockdown of SiR when grown on control medium: both mutants accumulate sulfate, ammonia and total amino acids including cysteine and methionine. Although glutathione levels were not altered in the SiR mutant, incorporation of labelled sulfate (^{35}S) into cysteine and glutathione were greatly reduced [24].

The increase in selenate and total selenium in the *apr2-1* plants did not correspond to an increase in the amount of selenium in protein. These observations would support the hypothesis that APR2 has a role in selenate reduction, which would probably limit the assimilation of selenate to selenocysteine. What makes the decreased selenium incorporation in protein in the *apr2-1* plants noteworthy is the amount of evidence [6,51,52] and support for the idea [5,35] that selenium toxicity in non-hyperaccumulating plants can be mitigated by lowering the incorporation of selenium in protein. If selenium toxicity in *Arabidopsis* could only simply be explained by the amount of selenium in protein, *apr2-1* would be expected to be more tolerant to selenate than Col. In the case of the *apr2-1* plants, selenium toxicity and the amount of selenium

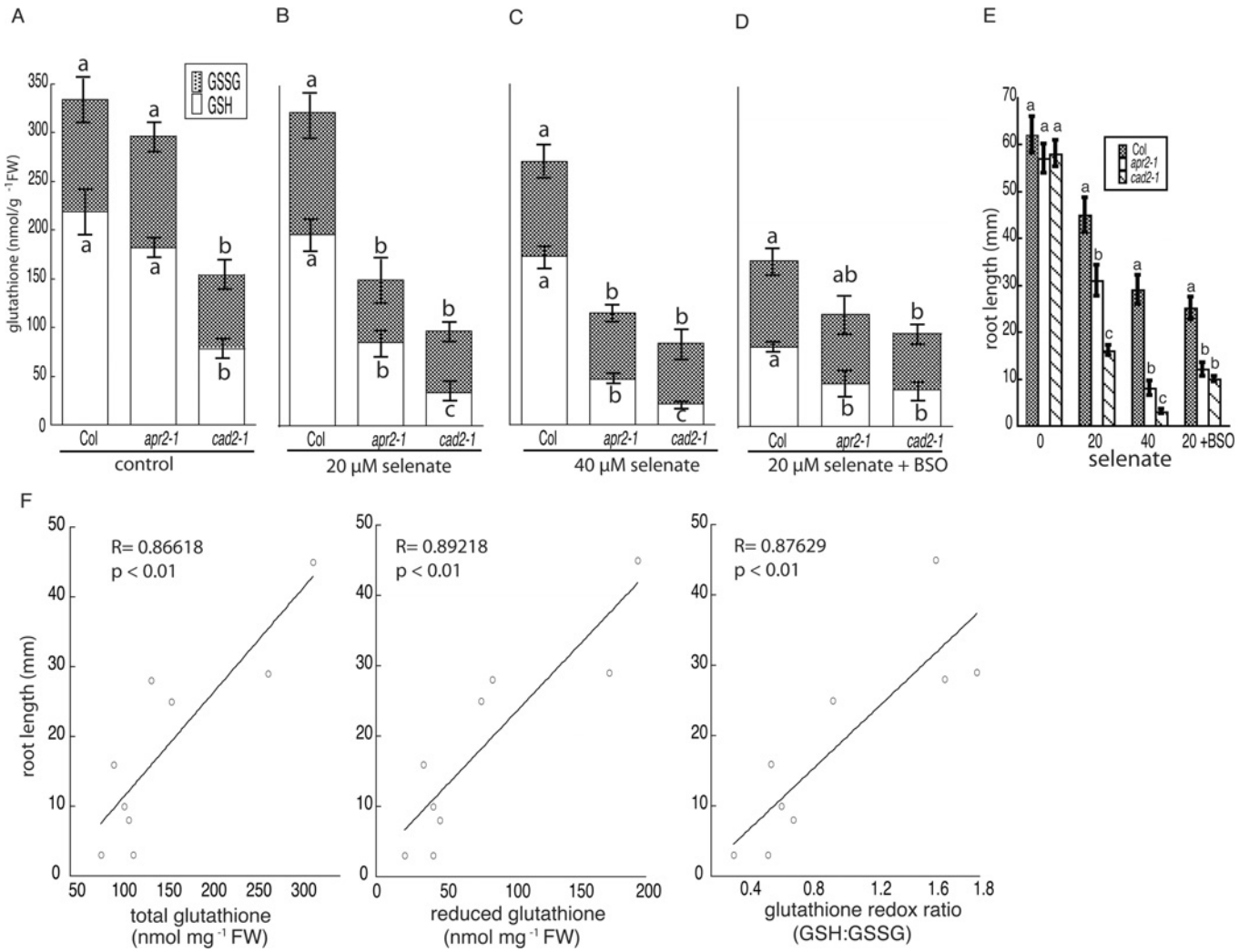

Figure 6 Accumulation of glutathione

Shoot concentrations of reduced (light boxes) and oxidized (dark boxes) glutathione were measured in Col, *apr2-1*, and *cad2-1* plants grown without selenate (**A**), 20 μM selenate (**B**), 40 μM selenate (**C**) and 20 μM selenate supplemented with 200 μM of BSO for 14 days. Reduced glutathione was estimated by the difference of total glutathione and oxidized glutathione, which was extracted using 2-vinylpyridine. (**E**) Root length was measured (prior to glutathione measurements) in the plants grown on the three different conditions containing selenate. Shown are the mean ($n = 30$ for root length and five independently pooled samples of 5–15 plants for sulfur metabolites) and S.E.M. Different letters above/below bars denote a significant difference between plants among each treatment ($P < 0.05$). (**F**) A correlation between mean glutathione (**B**–**D**) and root length (**E**) for each of the three lines grown on media containing 20 μM selenate ($\pm$200 μM BSO) and 40 μM selenate.

in protein appears to be uncoupled, indicating that an additional factor(s) is causing the selenate-induced stress.

A notable change in *apr2-1* plants was the decrease in glutathione, especially when treated with selenate. Glutathione accumulation in *Arabidopsis*, also previously shown to be uncoupled with APR activity [28,32], is known to decrease on exposure to selenate [34]. To further evaluate the role of glutathione in selenate toxicity, levels of reduced and oxidized glutathione in *apr2-1* were compared with wild-type and *cad2-1* plants with impaired glutathione biosynthesis. *cad2-1* mutants are particularly sensitive to selenate, but not selenite, suggesting that glutathione appears to be specifically important for selenate tolerance [53]. We sought to determine whether there is a relationship between selenate tolerance and the broad range of glutathione levels observed in the three different lines grown. The results (Figure 6F) indicate that selenate tolerance correlates very strongly with reduced glutathione, and to a slightly lesser extent total glutathione and the redox ratio of glutathione. Reduced levels of GSH were recently shown to perturb auxin accumulation and transport in *Arabidopsis* roots, and had the effect of greatly decreasing root length, even without selenate [54]. In view of this observation, it is possible that selenate tolerance as determined by root length is indirectly attributable to selenate and a more direct consequence of glutathione depletion in *apr2-1*.

In an attempt to increase both glutathione concentration and selenate tolerance, *apr2-1* plants grown on 40 μM selenate were supplemented with 50 μM sulfite. This treatment indeed enhanced both glutathione concentration and selenate tolerance compared with growth on 40 μM selenate, but did not completely restore it to the levels observed in wild-type plants (Supplementary Figure S5 available at http://www.BiochemJ.org/bj/438/bj4380325add.htm). This may be because *apr2-1* still accumulated more selenate compared with wild-type, straining the glutathione status in the mutant.

To gain more insight into how glutathione and selenate interact *in vitro*, selenate was mixed with either glutathione or

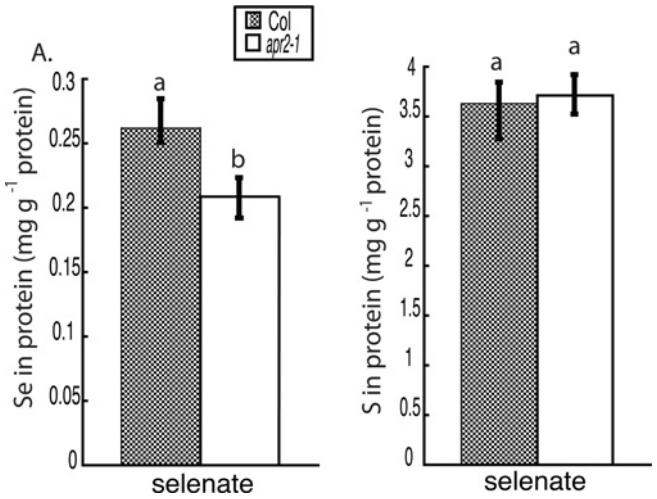

Figure 7 Sulfur and selenium in protein

The amount of (**A**) sulfur and (**B**) selenium in protein from plants grown for 21 days with and without 20 μM selenate. Results shown are the mean ($n = 5$ independently pooled samples of 20–25 seedlings) and S.E.M. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$).

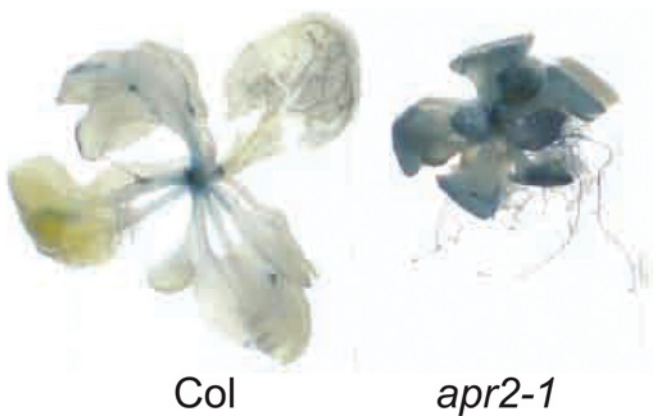

Figure 8 Influence of selenate treatment on superoxide formation in *apr2-1*

The amount of selenate-induced superoxide formation visualized by *in situ* Nitro Blue Tetrazolium staining and in Col and *apr2-1*. Plants were grown for 10 days without selenate, followed by 14 days on 40 μM selenate for the detection of superoxide. The image of superoxide staining is representative of two additional experiments.

ATP-sulfurylase, which served as a positive control. Our results (Supplementary Figure S2) suggest that selenate directly interacts with glutathione, as measurable selenate declined. Furthermore, selenate appears to affect glutathione redox status in an *in vitro* assay; levels of oxidized glutathione increased as the selenate concentration also increased. This *in vitro* result coincides with a similar observation in *apr2-1* plants, i.e. elevated selenate accumulation and a higher proportion of oxidized glutathione. How glutathione interacts with selenate *in planta* is not known, but it seems clear that it has more of a role in mitigating selenate rather than selenite stress. It is tempting to hypothesize that the increased pool of selenate in *apr2-1* directly impairs the cellular glutathione status, and perhaps most probably in root cells that are a suggested target of selenate toxicity [2]. Is it also possible that glutathione is involved in conjugating or sequestering selenate into a benign form in plants? Nonetheless, our overall results suggest that selenate tolerance is impaired by APR2 disruption and that this strongly correlates with reduced glutathione levels.

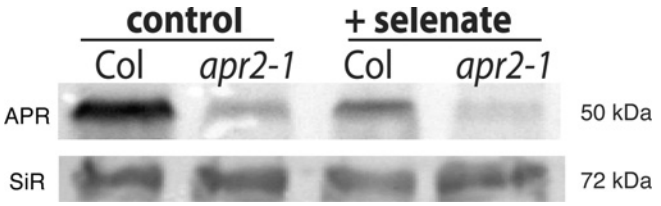

Figure 9 Effect of selenate treatment on levels of immunoreactive APR and SiR polypeptides

Immunoblot of APR and SiR in shoot extracts. Col and *apr2-1* plants were grown for 10 days on 20 μM selenate. Twenty micrograms of total protein were loaded per lane. The immunoblot is representative of two additional experiments.

Selenium induces the formation of ROS in plants [19,48]. The *apr2-1* plants, which accumulate more selenate, had a substantially higher amount of superoxide compared with Col throughout the entire shoot system. The increased superoxide in *apr2-1* could possibly be attributable directly to the increased selenate and/or the decreased glutathione concentration.

It is generally assumed that proteins involved in sulfate uptake and sulfur assimilation can also transport and metabolize selenate and selenium analogues. For example, yeast ATP sulfurylase, which is upstream of APR, also acts on selenate, which probably forms adenosine phosphoselenate. However, to our knowledge, attempts to isolate and confirm the identity of adenosine phosphoselenate have not been successful due to the product's instability [55]. Downstream of APR, selenite can be reduced non-enzymatically via glutathione to elemental selenium *in vitro* [55] and in *Escherichia coli* [56]; so far it has not been reported whether selenite reduction in plants is mediated non-enzymatically or via SiR.

This leaves the question as to whether or not APR is involved in the reduction of adenosine phosphoselenate to selenite. The lack of available adenosine phosphoselenate limits the ability to draw conclusions on the role of APR2 in selenate reduction. Nonetheless, *apr2-1* accumulated more selenate and less selenite compared with wild-type plants, which is in agreement with a possible role for APR2 in selenate reduction. Intriguingly, the overall ratio of sulfate to sulfite was much higher than the ratio of selenate to selenite in both Col and *apr2-1*. If the concentration of sulfate is 80–100-fold higher than that of selenate, why are levels of selenite and sulfite so similar? One possible explanation is that APR proteins have a greater activity on adenosine phosphoselenate than on adenosine phosphosulfate. It is not uncommon for enzymes to exhibit a higher activity for the selenium analogue than the sulfur analogue. For example, CpNifS has a greater activity on selenocysteine than cysteine [57]. Alternatively, the relative levels of selenite and sulfite could be explained in *apr2-1* given two separate possible scenarios: (i) SiR has reduced activity on selenite compared with sulfite; (ii) *apr2-1* plants might bottleneck selenite relative to sulfite if high concentrations of reduced glutathione can non-enzymatically mediate the conversion of selenite, as also demonstrated *in vitro* [55].

In the present study, we demonstrate that selenate toxicity is increased in APR2-impaired plants. Furthermore, it appears that selenate toxicity is not solely due to the non-specific incorporation of selenium in protein, but that selenate accumulation may also contribute to toxicity. Selenate may generate ROS, either directly or indirectly by lowering glutathione levels. Future strategies to enhance selenate tolerance in plants may focus on pathways involved in glutathione accumulation. Furthermore, future research may direct efforts to biochemically demonstrate

whether APR and SiR participate in selenium assimilation, similar to ATP sulfurylase, in order to arrive at a fuller understanding of the biochemical mechanisms of selenium metabolism in plants.

AUTHUR CONTRIBUTION

Doug Van Hoewyk designed the study. Kevron Grant, Nicole Carey, Miguel Mendoza and Doug Van Hoewyk performed all of the experiments, except for the amino acid analysis performed by John Schulze and the estimation of total sulfur and selenium performed by Elizabeth Pilon-Smits and Marinus Pilon. Doug Van Hoewyk analysed all of the data, created all of the Figures and wrote the paper. Elizabeth Pilon-Smits and Marinus Pilon also assisted in writing and preparing the paper.

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SUPPLEMENTARY ONLINE DATA

Adenosine 5′-phosphosulfate reductase (APR2) mutation in *Arabidopsis* implicates glutathione deficiency in selenate toxicity

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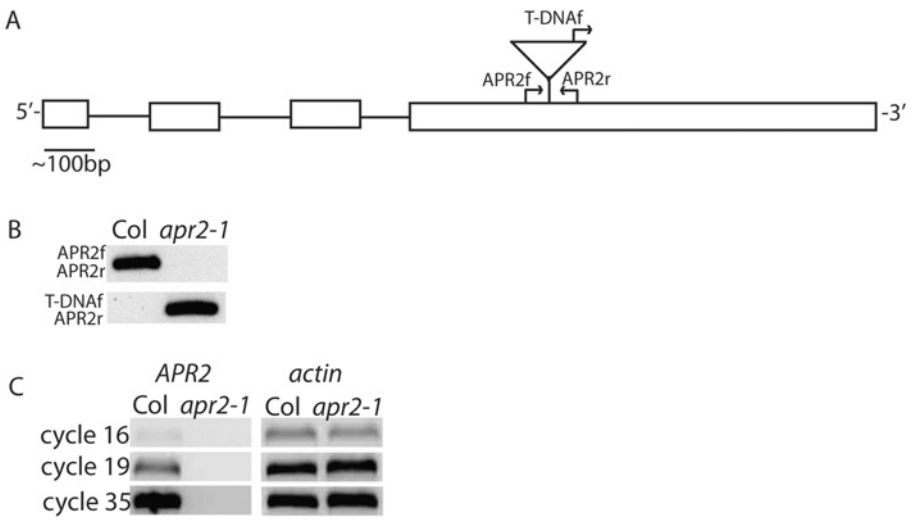

Figure S1 T-DNA location in *apr2-1* plants

(**A**) Map of the T-DNA location into the coding region of the *APR2* (**B**). PCR was used to confirm the T-DNA insert using a primer that hybridized to the T-DNA (T-DNAf, 5′-ATATTGACCATCATACTCATTGC-3′) and primers that hybridized to regions flanking the T-DNA insert (ARP2f, 5′-TGAGGTTCAAGCTTTAGTGAGGA-3′; APR2r, 5′-TATGGATGTTCCGGTGAATGCATT-3′). Introns and exons are represented by lines and boxes respectively. (**C**) RT–PCR analysis of *APR2* (5′-CGCTCCTTGGTGCCCTTTCT-3′, 5′-AGCCCGTGGAGCTCTTTTCG-3′) and *actin* (5′-TGCAGGAGATGATGCTCCCAG-3′; 5′-ATCCAGCACAATACCGGTTGTA-3′) transcripts. Reactions were stopped at cycles 16, 19 and 35.

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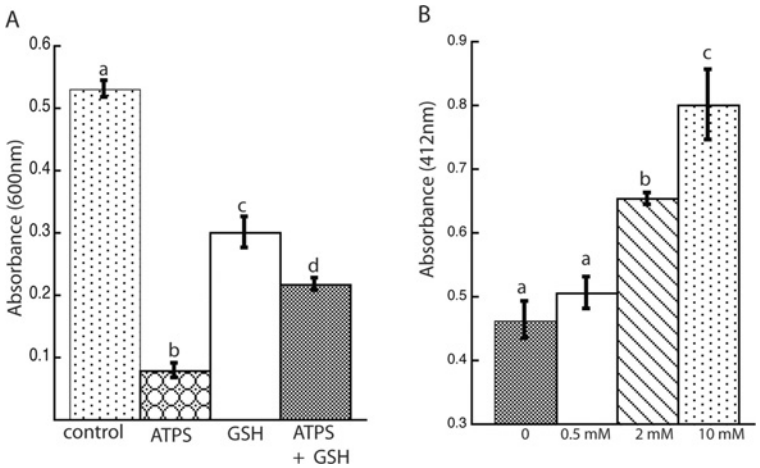

Figure S2 ***In vitro*** **assay on glutathione and selenate mixed in an aqueous solution**

(**A**) A solution containing 1 mM sodium-selenate, 0.1 mM ATP, 1 mM $MgCl_2$ and 10 mM P-K buffer were mixed in a tube and were allowed to react for 5 h with or without 1 unit of ATPS (ATP sulfurylase, 1 mM glutathione or both ATPS and glutathione. Remaining selenate was measured at $A_{600\,nm}$ after reacting with barium chloride. (**B**) A solution containing 1 mM glutathione was allowed to react for 5 h with 0, 0.2, 1 and 10 mM sodium selenate. Oxidized glutathione was extracted and its concentration estimated using Ellman's reagent at an A of 412 nm. Shown are the mean ($n = 5$ replicates) and S.E.M. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$). Results are representative of two additional experiments.

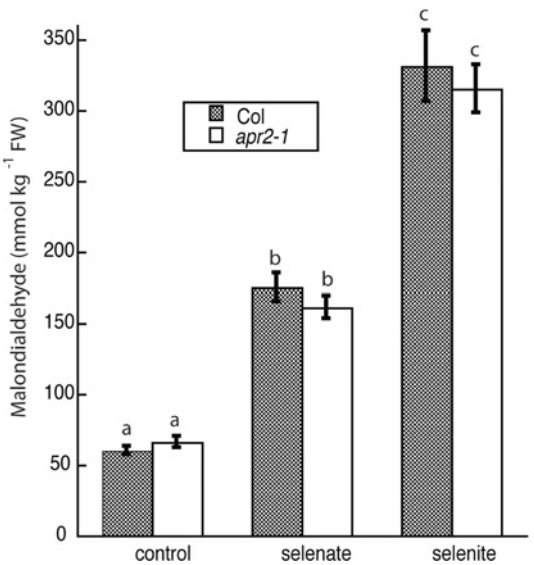

Figure S3 **Influence of selenate treatment on lipid peroxidation in** ***apr2-1***

The amount of selenate-induced malondialdehyde in Col and *apr2-1*. Plants were grown for 10 days without selenate, followed by 14 days on control medium, 40 μM selenate and 15 μM selenite. Shown are the mean ($n = 5$ pooled samples of 5 seedlings) and S.E.M, and are similar to a repeated experiment. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$).

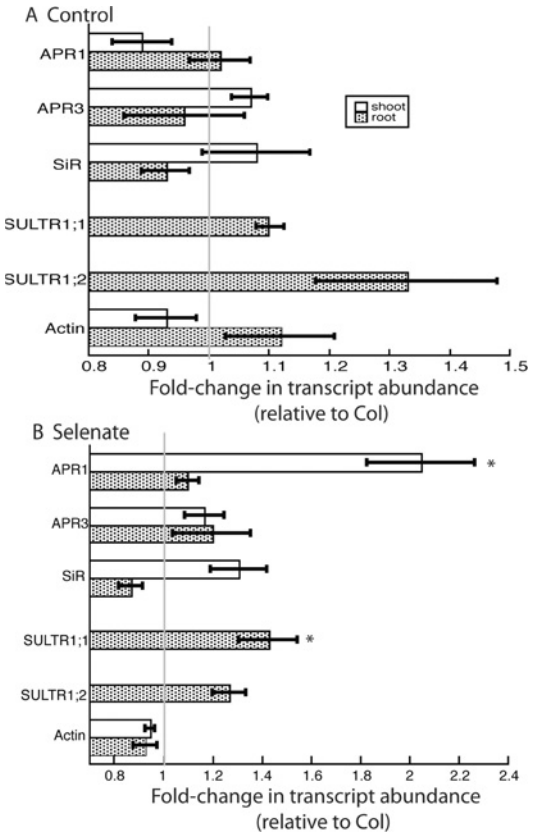

Figure S4 Differential expression of sulfur-related transcripts

The transcript fold-change of sulfur-metabolism genes in the roots and leaves of *apr2-1* plants grown on control (**A**) and selenate (**B**). mRNA was extracted from the roots and leaves of Col and *apr2-1* plants grown for 10 days on 20 μM selenate ($n = 3$ independently pooled samples), and reverse transcribed to make cDNA. Relative mRNA levels were determined by semi-quantitative RT–PCR. Shown are values of transcript abundance in *apr2-1* relative to Col, and are representative of two additional experiments each containing one biological replicate. Results represent the mean intensity and S.E.M. of PCR bands as quantified by Image J64. Asterisks denote a significant difference ($P < 0.05$) of band intensity in *apr2-1* compared with Col. Levels of SULTR1;1 and SULTR1;2 were not measured in the shoot.

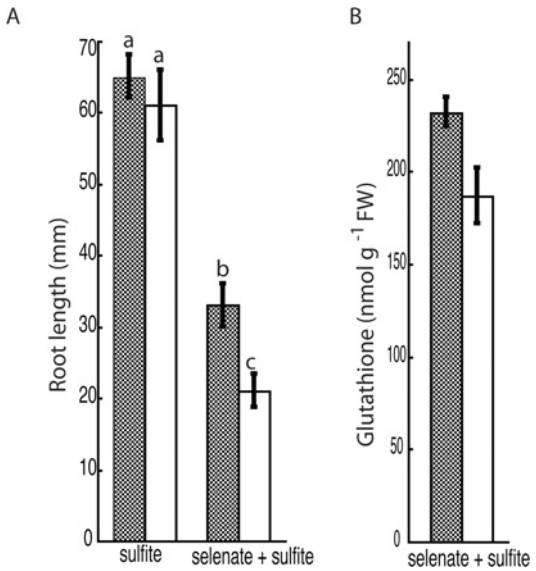

Figure S5 Accumulation of glutathione in plants supplemented with sulfite

Root lengths (**A**) were measured in Col and *apr2-1* plants grown on 50 μM sulfite with or without 40 μM selenate. (**B**) Total glutathione concentrations were subsequently measured. Shown are the mean ($n = 30$ for root length and $n = 5$ for glutathione) and S.E.M. Different letters above bars denote a significant difference between plants among each treatment ($P < 0.05$).

Table S1 Amino acid analysis

Amino acids and ammonia content (nmol/g of fresh weight) in the shoot material of plants grown on control and selenate for 21 days. Shown are the mean ($n = 3$ independently pooled samples) and S.E.M. Values in bold represent a significant difference ($P < 0.05$).

	Control		Selenate	
	Col	*apr2-1*	Col	*apr2-1*
Cys	1.53 (0.08)	1.62 (0.18)	3.1 (0.11)	**4.76 (0.19)**
Met	2.54 (0.12)	2.56 (0.14)	2.83 (0.11)	**5.77 (0.21)**
Asn	36.4 (1.6)	**41.7 (1.7)**	31.84 (0.13)	**95.6 (5.2)**
Gln	144.6 (8.7)	**189 (4.3)**	186.4 (5.3)	**554.8 (34.4)**
Glu	45.5 (2.1)	**57.8 (3.6)**	32.9 (2.1)	**63.25 (2.2)**
Ala	7.7 (2.1)	7.3 (5.4)	5.0 (0.11)	4.9 (0.41)
Asp	15.4 (1.5)	19.2 (2.62)	10.8 (0.44)	12.6 (0.68)
Arg	40.3 (2.6)	40.9 (1.7)	29.8 (1.7)	26.7 (0.6)
Gly	15.7 (1.4)	**24.6 (1.9)**	15.4 (1.4)	**11.0 (0.8)**
His	3.35 (0.32)	4.2 (0.21)	3.5 (0.11)	**5.55 (0.32)**
Ile	0.63 (0.04)	0.73 (0.13)	0.94 (0.03)	0.94 (0.04)
Leu	0.7 (0.08)	0.81 (0.11)	.86 (0.08)	1.15 (0.03)
Lys	2.2 (0.2)	2.8 (0.3)	1.94 (0.08)	1.95 (0.14)
Phe	1.25 (0.21)	1.1 (0.06)	1.8 (0.2)	1.31 (0.11)
Pro	6.01 (2.4)	**17.5 (1.7)**	3.56 (0.44)	**10.3 (0.56)**
Ser	21.6 (1.1)	**29.1 (1.1)**	17.4 (2.1)	20.6 (1.0)
Thr	7.43 (0.45)	9.04 (0.63)	5.91 (0.21)	6.7 (0.33)
Tyr	0.75(0.09)	0.62 (0.04)	0.49 (1.1)	0.56 (0.16)
Val	1.32 (0.08)	1.62 (0.21)	1.81 (0.06)	2.13 (0.15)
NH_3	38.0 (1.4)	**57.2 (4.2)**	45.5 (3.3)	**135.8 (3.8)**

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