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Variation in Sulfur and Selenium Accumulation Is Controlled by Naturally Occurring Isoforms of the Key Sulfur Assimilation Enzyme ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE2 across the Arabidopsis Species Range^{1[W][OPEN]}

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Natural variation allows the investigation of both the fundamental functions of genes and their role in local adaptation. As one of the essential macronutrients, sulfur is vital for plant growth and development and also for crop yield and quality. Selenium and sulfur are assimilated by the same process, and although plants do not require selenium, plant-based selenium is an important source of this essential element for animals. Here, we report the use of linkage mapping in synthetic F2 populations and complementation to investigate the genetic architecture of variation in total leaf sulfur and selenium concentrations in a diverse set of Arabidopsis (*Arabidopsis thaliana*) accessions. We identify in accessions collected from Sweden and the Czech Republic two variants of the enzyme ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE2 (APR2) with strongly diminished catalytic capacity. APR2 is a key enzyme in both sulfate and selenate reduction, and its reduced activity in the loss-of-function allele *apr2-1* and the two Arabidopsis accessions Hodonín and Shahdara leads to a lowering of sulfur flux from sulfate into the reduced sulfur compounds, cysteine and glutathione, and into proteins, concomitant with an increase in the accumulation of sulfate in leaves. We conclude from our observation, and the previously identified weak allele of APR2 from the Shahdara accession collected in Tajikistan, that the catalytic capacity of APR2 varies by 4 orders of magnitude across the Arabidopsis species range, driving significant differences in sulfur and selenium metabolism. The selective benefit, if any, of this large variation remains to be explored.

Sulfur is one of the essential mineral nutrients for all organisms and is required for the biosynthesis of

sulfur-containing amino acids, lipids, vitamins, and secondary metabolites, the catalytic and regulatory activity of enzymes, and the stabilization of protein structures. However, animals can only utilize sulfur that has been incorporated into biomolecules primarily originating from plants. Sufficient sulfur fertilization is also important to maximize yields of various crops, including rapeseed (*Brassica napus*) and wheat (*Triticum aestivum*; Bloem et al., 2004; Dubousset et al., 2010; Steinfurth et al., 2012). Interestingly, sulfur fertilization can also be important for food quality, with well-fertilized wheat crops producing flour with improved bread-making qualities (Shahsavani and Gholami, 2008).

Over the last 50 years, the levels of sulfur in soils have been impacted significantly by the release of sulfur dioxide into the atmosphere as a result of the combustion of fossil fuels (Menz and Seip, 2004). In the atmosphere, sulfur dioxide is oxidized to sulfuric acid and thereafter deposited on soils as acid rain. Regulations controlling the release of sulfur dioxide from power stations introduced in the 1970s have caused significant declines in sulfate deposition due to this acid rain (Menz and Seip, 2004). These reductions in sulfate deposition are

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significant enough to have increased the need for sulfur fertilization of agricultural crops such as rapeseed and wheat (Dubousset et al., 2010; Steinfurth et al., 2012). The essentiality of sulfur for plants and the relatively recent major fluctuations in soil sulfate deposition make the study of natural variation in sulfur homeostasis by plants attractive. Such studies not only have the potential to identify new molecular mechanisms involved in sulfur homeostasis but also could provide a platform for probing at the genetic level interactions between natural plant populations and a changing soil environment.

The sulfur analog selenium is also widely distributed in soil and is incorporated into biomolecules in plants in place of sulfur via sulfur assimilatory processes. Although selenium is not required by plants, it is essential for animals, and plant-based selenium is the primary source of this important nutrient for humans. In animals, selenium plays a vital role in numerous different selenoproteins involved in redox reactions, selenium storage, and hormone biosynthesis (Underwood, 1981; Rayman, 2012; Roman et al., 2014). In these proteins, selenium is incorporated as seleno-Cys. Unlike plants where selenium is incorporated into biomolecules nonspecifically as a sulfur analog, seleno-Cys in animals is biosynthesized by the action of a specific seleno-Cys synthase that converts Ser-tRNA to seleno-Cys-tRNA (Roman et al., 2014). To help prevent the negative health effects of selenium deficiency in the diet, fortification of various foods with selenium is already practiced, and biofortification of crops such as wheat, through the addition of selenium to fertilizers, is being proposed (Lyons et al., 2003). Moreover, there is a growing body of studies suggesting that supraoptimal dietary levels of selenium in the form of seleno-Met and seleno-methylseleno-Cys may be helpful in preventing certain cancers, although the efficacy of these supplements is still debated (Rayman, 2012; Steinfurth et al., 2012). Therefore, the identification of genes that control variation in the uptake and metabolism of sulfur and selenium in plants is not only an essential task for understanding the molecular mechanisms of plant nutrition but also important for crop yield, food quality, and human health.

Plants mainly take up sulfur from the soil in the form of sulfate. After uptake of sulfate via the sulfate transporters *SULTR1;1* and *SULTR1;2* in the root (Yoshimoto et al., 2007; Barberon et al., 2008), sulfate needs to be reduced to sulfide before it can be incorporated into Cys, the first sulfur-containing compound in the assimilatory process (for review, see Takahashi et al., 2011). Sulfate is first activated through adenylation by ATP SULFURYLASE (ATPS) to form adenosine 5'-phosphosulfate (APS) in both plastids and the cytosol (Rotte and Leustek, 2000). After activation, sulfate as APS is reduced to sulfite by APS REDUCTASE (APR) using reduced glutathione (GSH) as the electron donor (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Alternatively, APS can be phosphorylated by APS kinase to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which acts as the sulfate donor for sulfotransferases to incorporate sulfate

directly into saccharides and secondary metabolites such as glucosinolates (Mugford et al., 2009). Sulfite produced by APR is further reduced to sulfide by sulfite reductase (Khan et al., 2010). In the final step, sulfide is combined with *O*-acetyl-Ser to form Cys, catalyzed by *O*-acetyl-Ser (thiol)lyase (Wirtz and Hell, 2006). In plants, selenium is taken up as selenate via sulfate transporters (Shibagaki et al., 2002). After uptake, it is thought that selenate is reduced to selenite via the action of ATP sulfurylase and APS reductase (Shaw and Anderson, 1972; Pilon-Smiths et al., 1999; Sors et al., 2005a, 2005b). Selenite is most likely nonenzymatically reduced to selenide and combined with *O*-acetyl-Ser to form seleno-Cys catalyzed by *O*-acetyl-Ser(thiol)lyase (Ng and Anderson, 1978).

Sulfate uptake and reduction represent the two control points for sulfur homeostasis (Kopriva et al., 2009). Based on sequence similarity, there are 14 genes annotated as sulfate transporters in the *Arabidopsis thaliana* genome, and at least six of them have evidence supporting their functions (Kopriva et al., 2009; Takahashi, 2010). Among these, *SULTR1;1* and *SULTR1;2* encode high-affinity sulfate transporters in the root responsible for sulfate uptake from the soil solution (Rouached et al., 2008; Takahashi, 2010). Genes encoding both ATPS and APR also form gene families in *Arabidopsis*, with ATPS being encoded by four genes and APR by three (Kopriva et al., 2009). ATPS enzymes localize to both the cytosol and plastids, whereas APR enzymes localize solely to the plastids (Lunn et al., 1990; Rotte and Leustek, 2000). ATPS1 and APR2 are responsible for the majority of ATP sulfurylase and APS reductase activity of seedlings.

In a quantitative trait locus (QTL) analysis of sulfate content in *Arabidopsis* leaves using recombinant inbred lines generated by crossing Bayreuth (Bay-0) and Shahdara (Sha), *APR2* was identified as a locus controlling variation in sulfate accumulation between the Bay-0 and Sha accessions (Loudet et al., 2007). The causal quantitative trait nucleotide in the Sha allele of *APR2* results in a substitution of Ala-399 to Glu-399. The substitution localizes in the thioredoxin active site, which reduces the affinity of *APR2* for GSH and results in a loss of 99.8% of enzyme activity. The loss-of-function Sha *APR2* allele leads to a significant decrease in sulfate reduction and a concomitant increase in leaf sulfate accumulation. In the same analysis, a second QTL for leaf sulfate accumulation was described (Loudet et al., 2007). This second QTL was recently established to be driven by an *ATPS1* expression-level polymorphism between Bay-0 and Sha, with Bay-0 showing reduced expression of *ATPS1* compared with Sha (Koprivova et al., 2013).

Both Loudet et al. (2007) and Koprivova et al. (2013) focused their studies on the genetic architecture of natural variation for leaf sulfate using a single recombinant population created by crossing the Bay-0 and Sha accessions. This recombinant inbred population has proved a powerful tool for the identification of novel alleles controlling several different traits (Loudet et al., 2008; Jiménez-Gómez et al., 2010; Jasinski et al., 2012; Pineau et al., 2012; Anwer et al., 2014). However, because this set

of recombinant inbred lines is composed of genotypes from only two accessions, its value is limited when trying to understand allelic diversity across the *Arabidopsis* species as a whole. To address this limitation, we performed experiments using a set of 349 *Arabidopsis* accessions collected from across the species range. These 349 accessions were selected from a worldwide collection of 5,810 accessions sampled to minimize redundancy and close family relatedness (Baxter et al., 2010; Platt et al., 2010). To allow us to further probe *Arabidopsis* species-wide allelic diversity, we also utilized the complete genome sequences of 855 *Arabidopsis* accessions from the 1,001 Genomes Project (<http://signal.salk.edu/atg1001/index.php>).

Since sulfate uptake and accumulation is only one step in the complex process of sulfur assimilation in plants, uncovering the genetic architecture of sulfate accumulation, as performed by Loudet et al. (2007) and Koprivova et al. (2013), could potentially overlook genetic variation in other important aspects of sulfur metabolism. To enable us to identify natural genetic variation in sulfur homeostasis beyond just sulfate accumulation, we chose to quantify total leaf sulfur. This potentially allowed us to capture variation in the accumulation of both sulfate and other important sulfur-containing metabolites such as GSH and glucosinolates.

Furthermore, to test genetically the long-held assumption that selenium in plants is metabolized as a sulfur analog, we also phenotyped the same set of plants for total leaf selenium. Through a combination of high-throughput elemental analysis, linkage mapping, genetic and transgenic complementation, reciprocal grafting, and protein haplotype analysis, we have established that the natural variation in both leaf sulfur and selenium content in *Arabidopsis* is controlled by several rare APR2 variants across the whole of the *Arabidopsis* species.

RESULTS

Natural Variation in Leaf Sulfur and Selenium Content in a Worldwide Sample of *Arabidopsis* Accessions

To examine the natural variation in total leaf sulfur and selenium content in *Arabidopsis*, we employed a population of 349 accessions collected across the species range. These accessions were grown in an artificial soil in a controlled environment with short days to limit flowering. After 5 weeks of growth, leaves were harvested and analyzed for multiple elements by inductively coupled plasma mass spectrometry (ICP-MS). We observed large variations in both total leaf sulfur and selenium concentrations across the 349 accessions analyzed, with total leaf sulfur ranging from 3,000 to 19,000 mg kg⁻¹ dry weight (Fig. 1A) and total leaf selenium ranging from 6 to 26 mg kg⁻¹ dry weight (Fig. 1B).

Genome-wide association (GWA) mapping is a powerful method for testing the phenotypic effect of multiple alleles across the genome and is successful for the identification of relatively strong-effect alleles at relatively high frequency in the population. For example, this

approach has been used successfully in *Arabidopsis* to identify variation at the sodium transporter *High-affinity Potassium Transporter1* and the zinc transport *Heavy Metal Adenosine Triphosphatase3* as controlling a significant proportion of the population-wide diversity in the leaf accumulation capacity of sodium and cadmium, respectively (Baxter et al., 2010; Chao et al., 2012). Surprisingly, no peaks of single-nucleotide polymorphisms (SNPs) significantly associated with variation in the concentration of total leaf sulfur or selenium were identified using GWA mapping. Indeed, only a single SNP (chromosome 5 position 10832256), located in AT5G28820, a gene of no previously known function, exceeded our experiment-wide significance threshold ($P < 2.3 \times 10^{-7}$) for total leaf sulfur (Supplemental Fig. S1), and no SNP with significant associations were identified for total leaf selenium accumulation (Supplemental Fig. S2). However, by calculating broad sense heritability, which allows an estimation of the proportion of the phenotypic variance accounted for by genetics, we found that most of the variation in total leaf sulfur and selenium in the set of 349 *Arabidopsis* accessions can be accounted for by genotype (heritability = 0.75 for sulfur and 0.68 for selenium). Similar levels of heritability have been observed previously for sulfur accumulation in leaves in a smaller population of 96 *Arabidopsis* accessions (Baxter et al., 2012). The high heritability of these traits and the inability of GWA to identify SNPs responsible for variation in total leaf sulfur and selenium concentration are the expectations for traits controlled by many alleles of small effect or the segregation of very rare alleles of large effect.

Identification of the Causal Locus for Variation in Total Leaf Sulfur and Selenium of *Arabidopsis*

Compared with GWA mapping, linkage mapping in a synthetic F2 population is more powerful for the identification of weak or rare alleles responsible for natural variation due to the high representation of alleles in a biparental cross. However, to take advantage of this power, it is important to select parents that contain the genetic variation of interest. To achieve this, we selected the accession with the highest total leaf sulfur and selenium content, an accession collected in the Czech Republic near Hodonín (Hod). This accession was crossed with the Columbia-0 (Col-0) accession, which has total leaf sulfur and selenium concentrations close to the population median (Fig. 1). Leaves of F1 hybrid plants were analyzed and found to have the same total leaf sulfur (Fig. 2A) and selenium (Fig. 2C) concentrations as the Col-0 parent, indicating that the locus (loci) controlling these two phenotypes are dominant in Col-0. To further confirm this, we grew 328 F2 individuals from this cross and analyzed leaves for total sulfur and selenium. We observed a total leaf sulfur content in F2 plants consistent with a single recessive locus driving elevated total leaf sulfur in Hod, with 72 plants showing a total leaf sulfur phenotype similar to Hod, 196 similar to Col-0, and 60 plants showing a

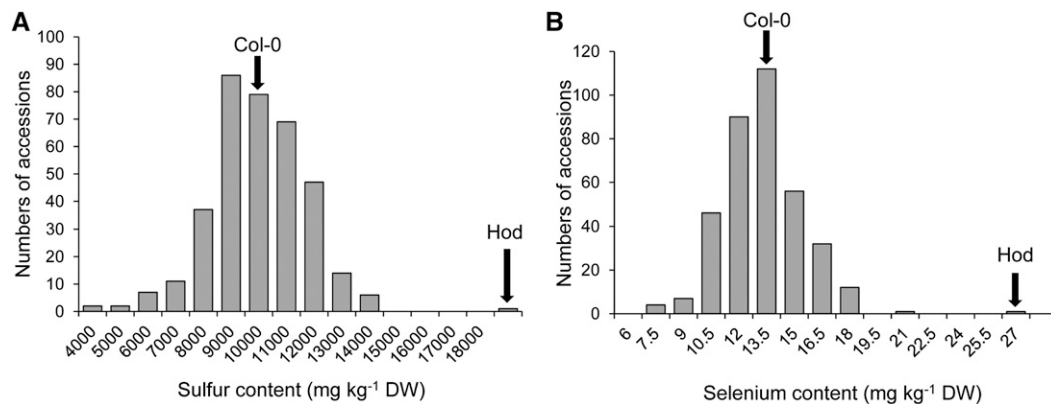


Figure 1. Natural variation in total sulfur (A) and selenium (B) concentrations in leaves of 349 *Arabidopsis* accessions grown together in a controlled environment. Arrows indicate leaf sulfur or selenium concentrations of Col-0 and Hod. All leaf sulfur and selenium data are accessible using the digital object identifier 10.4231/T9H41PBV (see <http://dx.doi.org/>). DW, Dry weight.

phenotype intermediate between the two parents (Fig. 2B). A similar distribution was observed for total leaf selenium in the same population (Fig. 2D). The correlation coefficient between the total leaf concentrations of sulfur and selenium in this F2 population was high ($r^2 = 0.78$; Fig. 3), suggesting that the two phenotypes are controlled by the same locus. We also observed a correlation ($r^2 = 0.51$) between the concentrations of total leaf sulfur and selenium in the 349 *Arabidopsis* accessions from which Hod was chosen.

To map the causal locus driving both elevated total leaf sulfur and selenium in Hod, we performed extreme array mapping (XAM), in which we combined bulk segregant analysis with SNP microarray genotyping (Becker et al., 2011). From the 328 phenotyped Col-0 \times Hod F2 plants, we pooled separately 57 individuals with extreme high total leaf sulfur concentration and 61 individuals with extreme low total leaf sulfur concentration. Genomic DNA was extracted from the two tissue pools, labeled, and hybridized separately to the Affymetrix SNP tiling array Atsnptile 1. The allele frequency differences between the two pools for all the polymorphic probes were assessed (Becker et al., 2011). Based on these allele frequency differences, we mapped the causal locus for high total leaf sulfur to the long arm of chromosome 1, with the depletion in Col-0 genotypes peaking at 23 Mb (Fig. 4A). The single strong XAM peak further supports the prediction of a single recessive locus controlling the Hod high total leaf sulfur and selenium phenotype.

To narrow the mapping interval of the high total leaf sulfur and selenium locus, we genotyped 328 F2 individuals from the same population used for the XAM with six cleaved-amplified polymorphic sequence (CAPS) or simple sequence length polymorphism PCR-based markers spanning the identified 20- to 24-Mb interval on chromosome 1 (Fig. 4B). Based on the total leaf sulfur and selenium phenotype of the F2 individuals or their F3 progeny, we identified 27 recombination events between the causal locus and the tested markers. Among

these 27 recombinants, the number of crossover events between the causal locus and the markers CF20M, CF22FMA, CF225HK, CF23M, CF235HK, and CF24MA were 19, nine, two, zero, five, and eight, respectively. Based on this result, the mapping interval for the causal polymorphism was narrowed to a 1-Mb interval between markers CF225HK and CF235HK (Fig. 4B). To further narrow this mapping interval, five more polymorphic markers between the markers CF225HK and CF235HK were developed and used to genotype an enlarged mapping population of 1,084 F2 individuals. Eighteen individuals with crossovers between the two markers were identified (Fig. 4C). We genotyped all of these recombinants at five newly developed markers polymorphic between Col-0 and Hod and phenotyped all plants in the F2 and/or F3 generation. Based on the association of genotype and phenotype, the causal polymorphism was mapped to a 101-kb interval between markers CF229954 and CF23055 (Fig. 4C). This interval contains 30 genes, including the *APR2* gene encoding APS reductase (Fig. 4D). As *APR2* is known to have a key role in sulfur assimilation (Kopriva et al., 2009) and has been shown to play an important role in regulating sulfate content in *Arabidopsis* (Loudet et al., 2007), we chose it as a strong candidate for the causal gene of the high total leaf sulfur and selenium phenotype of Hod.

***APR2* Is the Causal Locus Underlying Natural Variation in Total Leaf Sulfur and Selenium in the Hod Accession**

To test for false negatives in the GWA analysis, SNPs linked to *APR2* were reanalyzed. Seven SNPs within the *APR2* gene were tested, and one exhibited a nominal *P* value of 0.0012 for total leaf selenium, but no significant variation in total leaf sulfur was mapped to the *APR2* locus. Selecting a 100-kb window surrounding *APR2* identified 247 previously genotyped SNPs. This increased SNP number still did not detect any associations between genotypes linked to *APR2* and either total leaf sulfur or

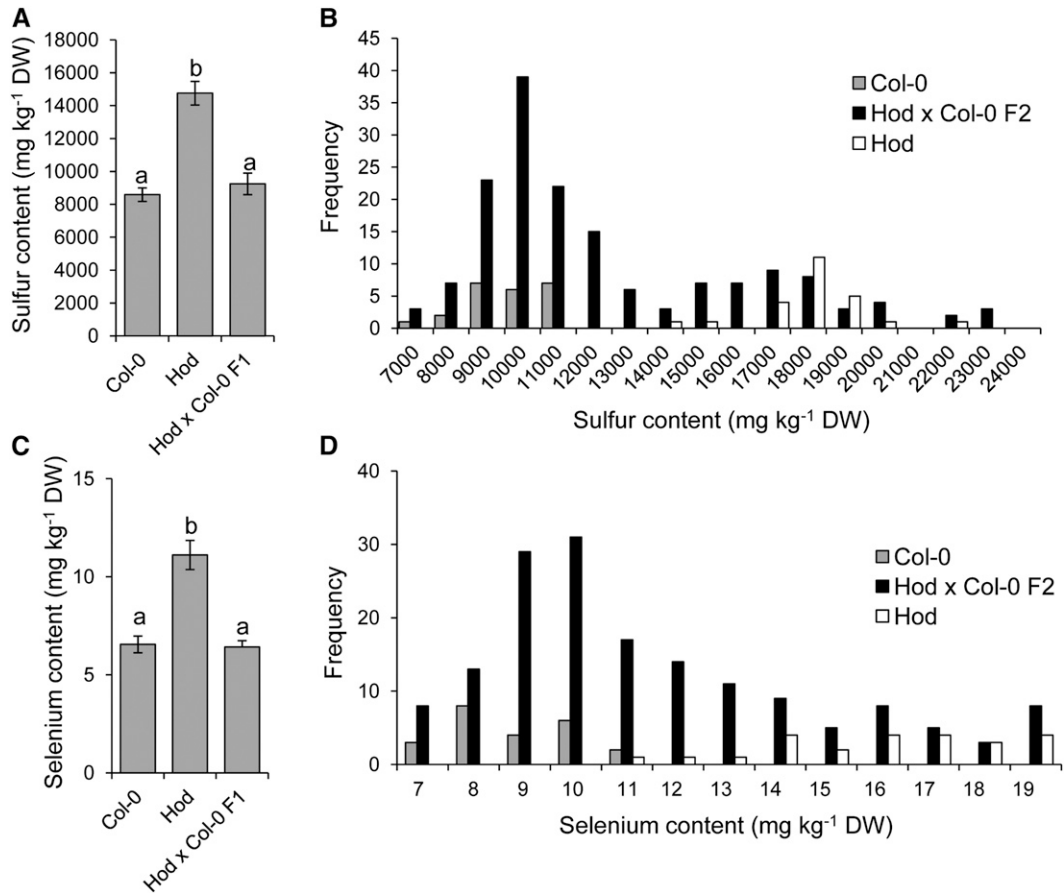


Figure 2. High leaf sulfur and selenium in the Arabidopsis Hod accession is recessive. A and C, Total leaf sulfur (A) and selenium (C) concentrations in Hod, Col-0, and their F1 progeny. Data represent means \pm SE ($n = 12$ independent plants per genotype). B and D, Frequency distribution of total leaf sulfur (B) and selenium (D) concentrations in Col-0, Hod, and F2 progeny from a Col-0 \times Hod cross. Letters above bars indicate significant groups using a one-way ANOVA Tukey's honestly significant difference test using a 95% confidence interval. Data are accessible using the digital object identifier 10.4231/T96Q1V5R (see <http://dx.doi.org/>). DW, Dry weight.

selenium accumulation that exceeded the now approximately 1,000-fold reduced multiple test correction of $P < 2.202 \times 10^{-4}$ as compared with the genome-wide analysis (Supplemental Table S1). The mapping of the trait to this location in the line cross, but failure to find

an association, is consistent with the presence of a rare allele of *APR2* with a strong effect on total leaf sulfur and selenium accumulation in the Hod accession.

To further examine if *APR2* is the causal locus, we phenotyped three independent transfer DNA (T-DNA)

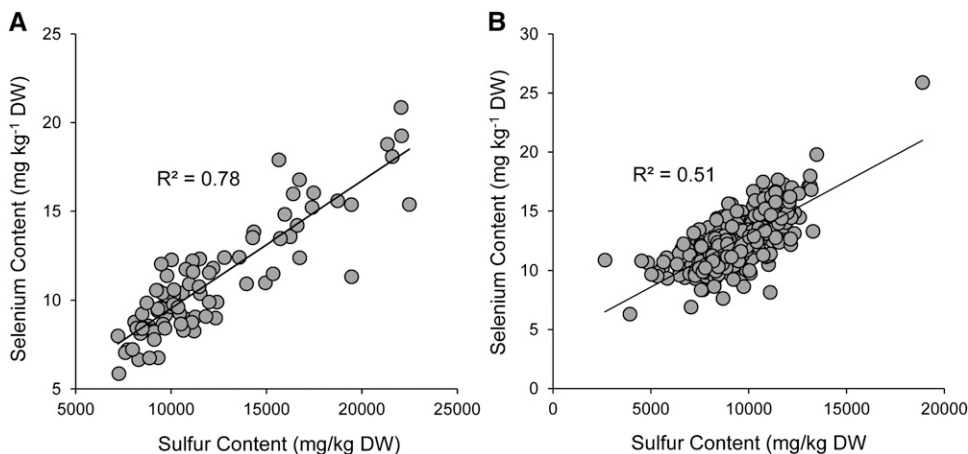
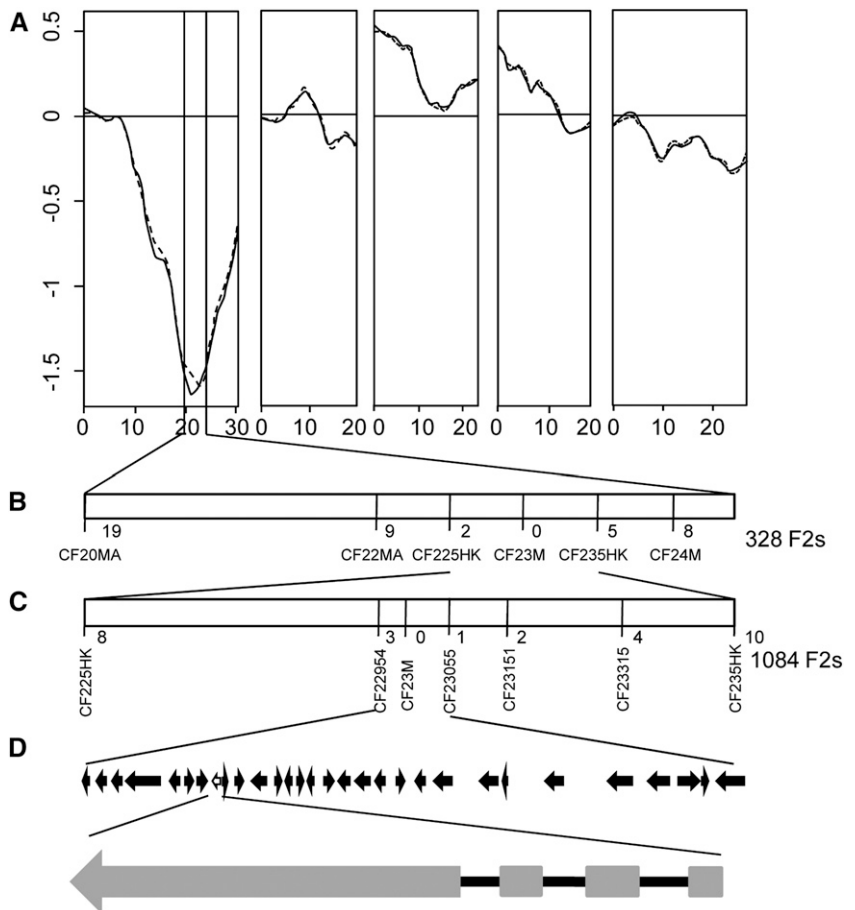


Figure 3. Correlation between the concentrations of total sulfur and selenium in leaves from F2 plants of a Hod \times Col-0 cross (A) and leaves of the 349 Arabidopsis accessions Hod was selected from (B). Selenium and sulfur data for A are accessible using the digital object identifier 10.4231/T96Q1V5R and data for B using the digital object identifier 10.4231/T9H41PBV (see <http://dx.doi.org/>). DW, Dry weight.

Figure 4. Map-based cloning of the high leaf sulfur locus in the *Arabidopsis* Hod accession. A, DNA microarray-based bulk segregant analysis of the leaf sulfur concentration trait using leaf phenotyped F2 progeny from the cross Hod × Col-0. Lines represent allele frequency differences between high and low total leaf sulfur pools of F2 plants at SNPs that are polymorphic between Hod and Col-0. Solid lines indicate sense strand probes, and dashed lines indicate antisense strand probes. B, PCR-based genotyping of 328 F2 plants (Col-0 × Hod) narrowed the causal gene to between CAPS makers CF225HK and CF235HK. C, Fine-mapping using 1,084 F2 plants (Col-0 × Hod) narrowed the causal gene to a 101-kb region between CAPS makers CF22945 and CF23055. Numbers under the horizontal lines in B and C represent the number of recombinants between the indicated marker and the causal gene. D, Candidate causal genes in the mapped region and the gene structure of *APR2* (bottom). Gray bars indicate exons, and black lines indicate introns.



insertional alleles of *APR2* with T-DNA inserts in the fourth exon (*apr2-1*; GABI_108G02) and 169 bp (*apr2-2*; SALK_119683) and 116 bp (*apr2-3*; SALK_035546) upstream of the *APR2* translational start site. Analysis of total leaf sulfur and selenium of the T-DNA insertional alleles revealed that *apr2-1* phenocopied Hod (Fig. 5A), while the *apr2-2* and *apr2-3* alleles with T-DNA insertions in the *APR2* promoter showed a similar but weaker phenotype compared with Hod (Fig. 5, A and C). These results support the hypothesis that polymorphism(s) at *APR2* are responsible for the high total leaf sulfur and selenium phenotype in Hod. To validate this hypothesis, genetic complementation was performed by crossing the three *apr2* alleles with Hod. The F1 hybrid plants from these crosses all exhibited the same high total leaf sulfur and selenium concentration as the *apr2* parents, establishing that an unknown natural polymorphism(s) in *APR2* leads to the high total leaf sulfur (Fig. 5A) and selenium (Fig. 5C) in Hod.

To reinforce this conclusion, transgenic complementation was performed by introducing an *APR2* genomic fragment from Col-0 (including a 1.5-kb promoter region, the gene body, and an 896-bp downstream sequence) into Hod. Transgenic lines in the T2 generation were grown, and total leaf sulfur and selenium were analyzed. T2 plants were also tested for GUS activity as

a reporter of the transformation vector by histochemical staining to confirm that an individual T2 plant contained the transgenic fragment. Individuals without the transgenic fragment were removed from further analysis. Using this approach, all seven independent Hod lines transformed with *APR2*^{Col-0} showed significantly reduced total leaf sulfur and selenium, indistinguishable from Col-0 (Fig. 5, B and D). The successful complementation of the elevated total leaf sulfur and selenium phenotype of Hod by a genomic DNA fragment containing *APR2*^{Col-0} establishes beyond doubt that *APR2* is the causal locus driving natural variation in both total leaf sulfur and selenium between Col-0 and Hod.

The High Total Leaf Sulfur Phenotype in Hod Is Driven Primarily by the Shoot

It has been reported that *APR2* is expressed both in shoot and root, and this was confirmed here using quantitative real-time (qRT)-PCR (Fig. 6). Expression of *APR2* in *apr2-1* is completely lost and partially lost in *apr2-2* and *apr2-3*, respectively, confirming *apr2-1* as a loss-of-function allele, as determined previously (Loudet et al., 2007), whereas *apr2-2* and *apr2-3* are only partial loss-of-function alleles. Interestingly, even though

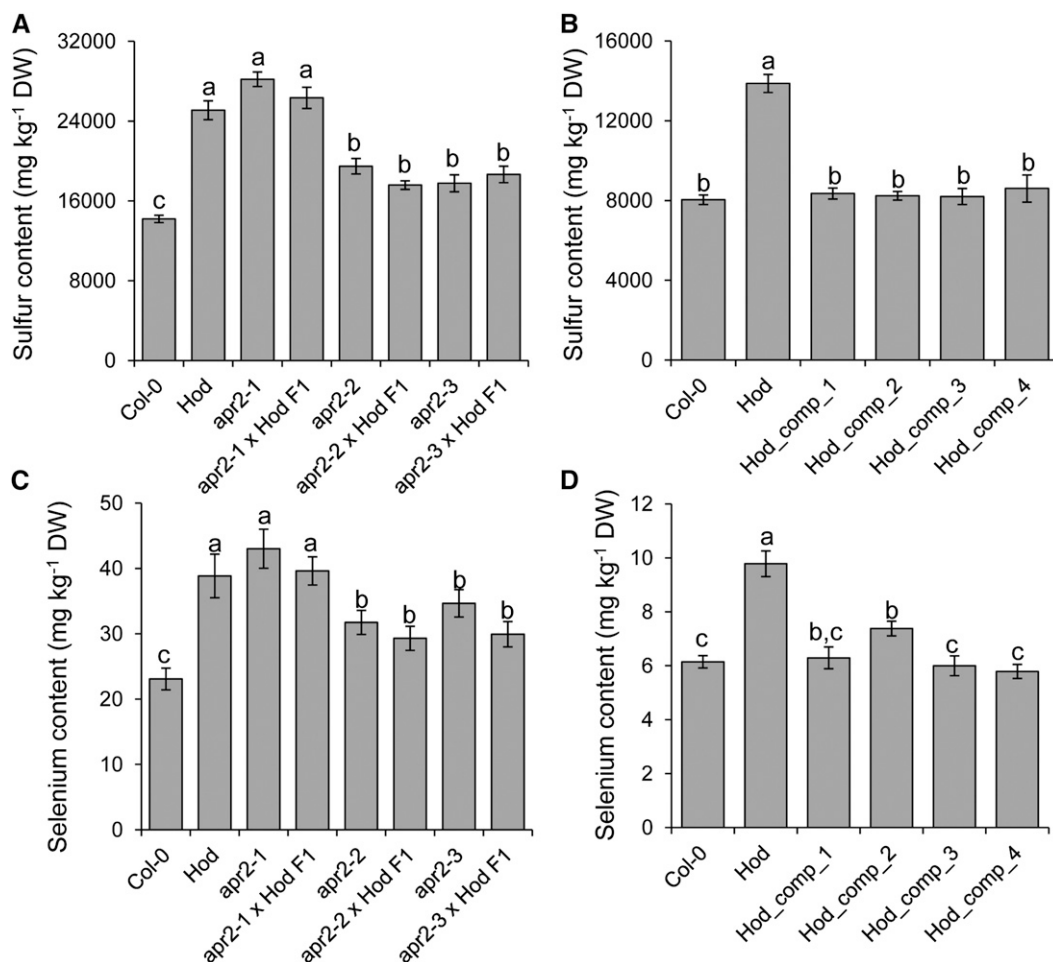


Figure 5. Complementation of the high total leaf sulfur and selenium of the Arabidopsis Hod accession. A and C, Total leaf sulfur (A) and selenium (C) of Col-0, Hod, *apr2-1*, and their F1 progeny. B and D, Total leaf sulfur (B) and selenium (D) concentrations of Hod transformed with Col-0 *APR2* genomic DNA. Data represent means \pm SE ($n = 12$ independent plants in A and C and $n = 4$ –12 plants in B and D). Letters indicate significant groups using a one-way ANOVA Tukey's honestly significant difference test using a 95% confidence interval. All data are accessible using the digital object identifier 10.4231/T9BG2KW2 (see <http://dx.doi.org/>). DW, Dry weight.

APR2^{Hod} is a loss-of-function allele relative to *APR2^{Col-0}* (Fig. 5, A and C), the expression of *APR2* in Hod is essentially the same as in Col-0. This suggests that the loss of function of *APR2^{Hod}* is not due to an expression-level polymorphism and is more likely caused by a polymorphism in the *APR2* gene body. This is similar to the weak allele of *APR2* identified previously in the Sha accession (Loudet et al., 2007). Given that *APR2* is expressed in both root and shoot, to determine which tissue controls the *APR2*-dependent variation in total leaf sulfur between Hod and Col-0, it was necessary to perform a reciprocal grafting experiment (Fig. 7). This experiment established that elevated leaf sulfur in Hod is primarily driven by the shoot. Total leaf sulfur in self-grafted and nongrafted Hod plants was found to be indistinguishable from grafted plants with a Hod shoot and Col-0 root (Fig. 7). However, grafted plants with a Col-0 shoot and Hod root were indistinguishable from nongrafted Col-0 and only slightly higher than self-grafted Col-0 plants.

An Amino Acid Substitution in Hod Leads to Loss of Function of *APR2*

Consistent with our genetic analysis establishing that *APR2^{Hod}* is hypofunctional compared with *APR2^{Col-0}*, we found that the APS reductase activity in Hod is reduced to the level of the *apr2-1* null allele (Fig. 8A). Furthermore, *APR2^{Hod}* is unable to restore the APR activity of *apr2-1* when both are present in F1 Hod \times *apr2-1* hybrid plants (Fig. 8A). This is in contrast to the *APR2^{Col-0}* allele, which is able to fully restore the APR activity of *APR2^{Hod}* in hybrid Hod \times Col-0 F1 plants (Fig. 8A). These observations are fully consistent with *APR2^{Hod}* being a recessive loss-of-function allele of *APR2*. In order to identify the polymorphism(s) causing loss of function of *APR2^{Hod}*, we sequenced the *APR2^{Hod}* genomic region covering the gene body, promoter, and 3' terminus. After assembling the sequenced fragments, 116 polymorphic sites between Hod and Col-0 were observed, of which 61 are localized in the promoter, five in the 5'-untranslated region, 12 in

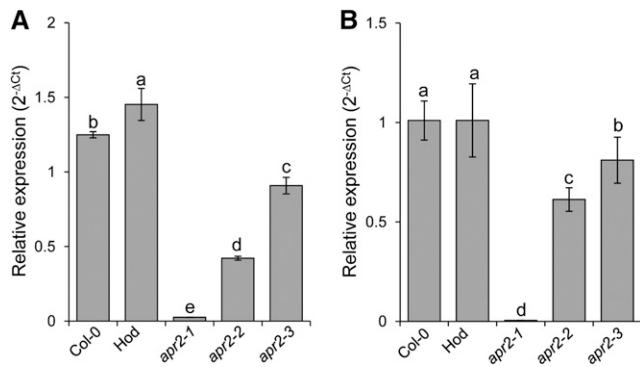


Figure 6. Expression of *APR2* in root (A) and shoot (B) in different accessions and *apr2* alleles. *UBIQUITIN-CONJUGATING ENZYME21* (*At5g25760*) was used as the control gene, and four PCRs were done per replicate using three independent biological samples per genotype, with error bars representing SD.

introns, and 26 in exons. Of those in the promoter, 47 are SNPs and 15 are short insertions/deletions (Supplemental Table S2). Although insertions/deletions in the promoter can drive expression-level polymorphisms, which can form the basis of variation in gene function (Rus et al., 2006; Baxter et al., 2008), this was ruled out as a cause of the loss of function of *APR2^{Hod}* because the expression of *APR2* in Hod is very similar to *APR2* expression in Col-0 (Fig. 6).

Therefore, we focused on the protein-coding portion of *APR2^{Hod}*. Of the polymorphic sites in the *APR2* exons between *APR2^{Hod}* and *APR2^{Col-0}*, 10 are non-synonymous substitutions resulting in amino acid changes (Supplemental Table S2), raising the possibility that these changes may impair the enzymatic activity of the *APR2^{Hod}* protein. To examine this possibility, we purified both recombinant *APR2^{Hod}* and *APR2^{Col-0}* proteins, measured their APR activity in vitro, and derived various kinetic constants for the enzymes for both the APS and GSH substrates. This analysis clearly established that the *APR2^{Hod}* enzyme has extremely impaired catalytic capacity compared with the *APR2^{Col-0}* enzyme for both substrates (Table I), confirming that one or more amino acid substitutions are responsible for the loss of function of *APR2^{Hod}*. We also assayed the activity of purified recombinant *APR2^{Bay-0}* and *APR2^{Sha}* proteins as controls, since kinetic data have previously been published on these enzymes and to allow comparison with the kinetic parameters of the various *APR2* variants (Loudet et al., 2007). The *APR2* protein haplotype in Hod is very similar to the haplotype of the Catania-1 (Ct-1) accession reported previously (Loudet et al., 2007), with the exception of a Gly-216 amino acid residue in the central region of the *APR2^{Col-0}* protein that is changed to an Arg in the *APR2^{Hod}* enzyme. We observed that the concentration of total leaf sulfur and selenium in Ct-1 is similar to that in Col-0 and much lower than in Hod, supporting the conclusion that this Gly/Arg polymorphism is causal for the loss of activity of *APR2^{Hod}*. This Gly-216 is part of the conserved Arg

loop that interacts with the APS substrate (Chartron et al., 2006; Bhawe et al., 2012). Therefore, it is possible that this Gly/Arg polymorphism in the *APR2^{Hod}* protein might alter the binding of APS to the enzyme reducing its activity. However, we observed that the K_m values for APS of *APR2^{Hod}* and *APR2^{Col-0}* are very similar (Table I) and therefore conclude that Gly-216 is perhaps more likely to be involved in the *APR2* catalytic mechanism.

The Frequency of Weak Alleles of *APR2* in the Global Arabidopsis Population

The previously identified weak allele of *APR2* in the Sha accession was unique to Sha within a small but diverse collection of 32 accessions (Loudet et al., 2007), suggesting that it is relatively rare. To perform a more in-depth analysis of the frequency of *APR2* alleles, including the Sha allele and the newly identified Hod allele, we examined the *APR2* protein haplotype in 855 Arabidopsis accessions that have had their whole genomes sequenced as part of the 1,001 Genomes Project (<http://signal.salk.edu/atg1001/index.php>). Neither the Sha nor the Hod *APR2* allele was found to be present in any other accession, establishing that these two hypofunctional alleles are very rare in the global Arabidopsis population. Finding two extremely rare independent hypofunctional *APR2* protein haplotypes inspired us to search for additional rare alleles. We selected LAC-5, Tammisari-2 (Tamm-2), Oystese-0 (Oy-0), and Glueckingen (Gu-1) as candidates for containing hypofunctional *APR2* alleles, as these are the four accessions with the highest total leaf sulfur concentrations from the 349 accessions originally screened (excluding Hod). Furthermore, we analyzed *APR2* sequences of the 855 resequenced Arabidopsis accessions to look for

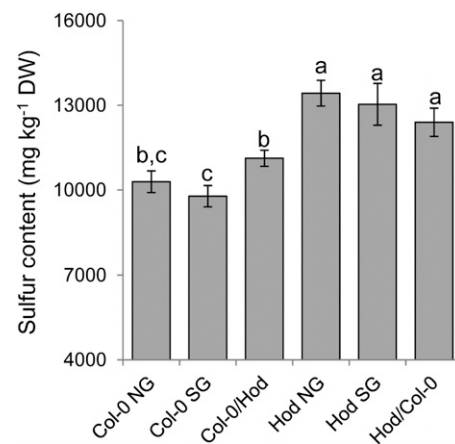


Figure 7. Reciprocal grafting establishes that shoots are primarily responsible for the high total leaf sulfur concentration in the Arabidopsis Hod accession. DW, Dry weight; NG, nongrafted plants; SG, self-grafted plants; Col-0/Hod, Col-0 shoot grafted onto a Hod root; Hod/Col-0, Hod shoot grafted onto a Col-0 root. Data represent means \pm SE ($n = 11-14$). Letters above each bar indicate statistically significant groups using a one-way ANOVA with groupings by Tukey's honestly significant difference test using a 95% confidence interval.

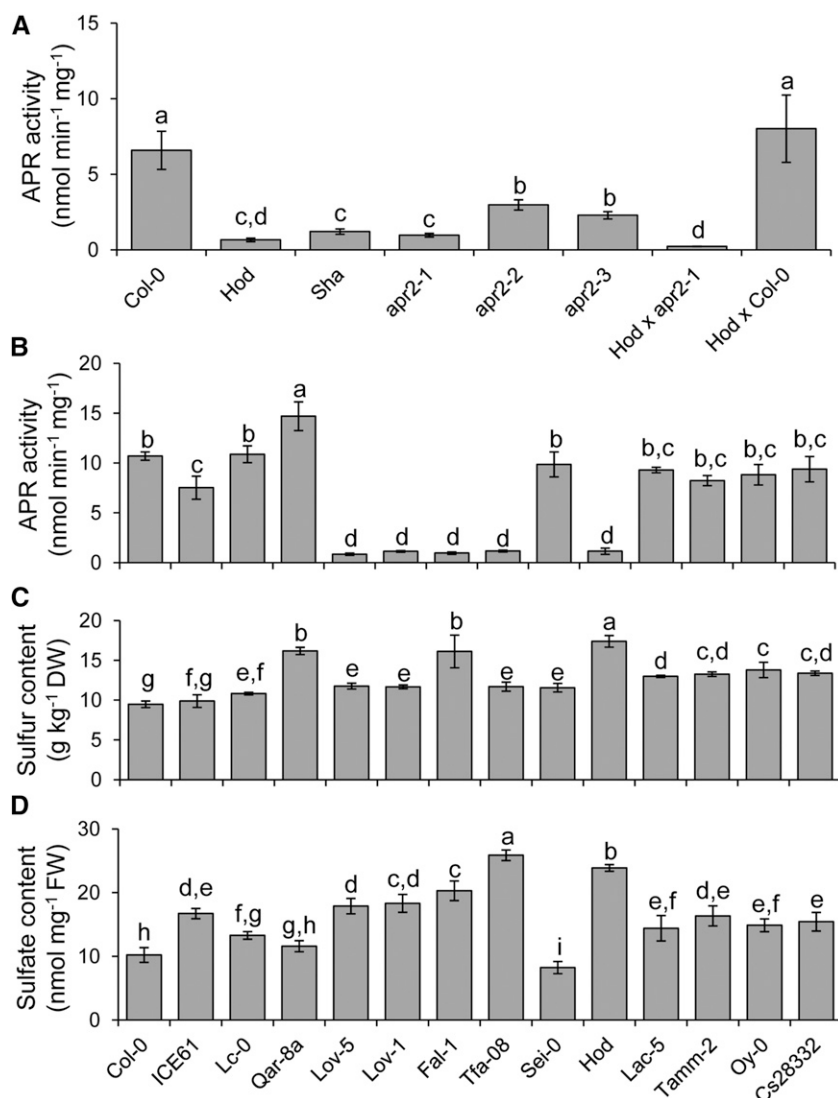


Figure 8. APR activity (A and B), total leaf sulfur concentration (C), and leaf sulfate concentration (D) in different natural accessions, *apr2* alleles, and F1 hybrids. Data represent means \pm SE ($n = 3$). Letters above each bar indicate statistically significant groups using a one-way ANOVA with groupings by Tukey's honestly significant difference test using a 95% confidence interval. DW, Dry weight; FW, fresh weight.

nonsynonymous substitutions in the *APR2* coding region. Eight accessions with amino acid changes in conserved regions of *APR2* were selected, making a total of 12 additional accessions for which APS reductase activity was measured in cell-free extracts (Fig. 8B). Compared with Col-0, five of the tested accessions were found to have significantly reduced APS reductase activity, whereas one accession had significant increased activity (Fig. 8B). Accessions with significantly reduced

APR activity are Lovvik-1 (Lov-1), Lov-5, Faberget-Lower-1 (Fal-1), Tfa-08, and Stepnoje-1 (ICE61). Among them, the first four accessions are all from western Sweden and share the same *APR2* protein haplotype (Table II). This western Swedish haplotype has an amino acid change of Phe-265 to Ser-265 in the α 8-helix (Chartron et al., 2006; Stevenson et al., 2013) of the PAPS reductase domain of the *APR2* enzyme (Table II), and the total APR activity is reduced to similar levels as in

Table I. Enzyme kinetics constants of different *APR2* variants

K_{cat} , the catalytic number, is equal to $V_{max}/[E]$.

Accession	APS				GSH			
	V_{max}	K_m	K_{cat}	K_{cat}/K_m	V_{max}	K_m	K_{cat}	K_{cat}/K_m
	units mg^{-1}	mM	s^{-1}	$1/(M s^{-1})$	units mg^{-1}	mM	s^{-1}	$1/(M s^{-1})$
Col-0	58.8	0.02	49	2,450,000	62.5	13.5	52.1	3,900
Bay-0	50	0.014	41.7	2,980,000	52	12	43.3	3,608
Sha	0.4	0.012	0.33	27,500	0.06	31	0.05	1.6
Hod	0.001	0.0082	0.001	122	0.00144	10.56	0.0012	0.11
Lov-5	0.089	0.258	0.074	287	0.0097	1.168	0.0081	6.92

Hod (Fig. 8B). The accession ICE61 represents a singleton haplotype in the 855 resequenced accessions, which has an amino acid change of Ala-155 to Val-155 in the α 3-helix (Stevenson et al., 2013) of the PAPS reductase domain (Table II). The APS reductase in accession ICE61 is reduced to 70% of that in Col-0 (Fig. 8B).

Analysis of total leaf sulfur contents in these accessions with alternative *APR2* alleles from across the Arabidopsis species revealed a link between the strong reduction of APR activity and high total leaf sulfur content in many accessions (Fig. 8C). However, APR activity is not always correlated with total sulfur content in the leaves, as the reduction of APR activity in ICE61 did not affect total leaf sulfur accumulation and the increased activity in Qartaba (Qar-8a) was accompanied by high total leaf sulfur content. Similarly, the mechanism underlying high total leaf sulfur accumulation in LAC-5, Tamm-2, Oy-0, and Gu-1 is independent from APR, as the high total leaf sulfur phenotype was not linked to a decrease in APR enzyme activity. Since APR2 was previously shown to be responsible for variation in sulfate levels (Loudet et al., 2007), we also determined the foliar contents of this sulfur-containing metabolite. Interestingly, there was a better correlation between APR and leaf sulfate levels, as all accessions with APR activity lower than Col-0 accumulated sulfate (Fig. 8D). There is a good correlation between high leaf sulfate accumulation and high total leaf sulfur content, suggesting that sulfate is the major sulfur pool in Arabidopsis leaves. In some accessions, however, these two traits are uncoupled (e.g. Fal-1 and Tfa-08; Fig. 8, C and D), pointing to variation in the relative size of the leaf sulfate pool.

To further confirm that the western Swedish APR2 variant is hypofunctional, we expressed an *APR2^{Lov-5}* complementary DNA (cDNA) in bacteria as a representative of the western Swedish APR2 protein haplotype. Recombinant *APR2^{Lov-5}* enzyme was purified and assayed for APR activity. This analysis established that the APS reductase activity of *APR2^{Lov-5}* is very low compared with that of *APR2^{Col-0}* (Table I) and suggests that the amino acid change Phe-265/Ser-265 (Table II)

impairs the activity of *APR2^{Lov-5}*. Surprisingly, this Phe-265/Ser-265 polymorphism in *APR2^{Lov-5}* (and in the other western Swedish accessions Lov-1, Tfa-1, and Fal-1) is in the α 8-helix of the PAPS reductase domain, a region that does not have any predicted catalytic function (Chartron et al., 2006). However, this residue is conserved as a Phe or Tyr across all other 855 accessions tested as well as APS and PAPS reductases from other plant and bacterial species, suggesting that it may play an important role, perhaps in maintaining the proper tertiary or quaternary structure of APR2. Indeed, this amino acid substitution significantly affects the binding of both substrates, APS and GSH, pointing to a more global effect on the enzyme structure.

Loss of APR2 Activity Leads to Reduced Sulfur Assimilation and Increased Accumulation of Sulfate

To understand why the reduced function of *APR2* results in high total leaf sulfur, we measured the flux of sulfur from sulfate into the reduced sulfur compounds Cys and GSH, and into proteins, in Arabidopsis lines with hypofunctional *APR2* alleles. We measured this flux as ³⁵S assimilated into reduced sulfur-containing compounds as a percentage of the total [³⁵S]sulfate taken up by the plant. In these same lines, we also measured the sulfate content of the leaves. Accessions and mutants with weak alleles of *APR2*, including Hod, Sha, *apr2-1*, *apr2-2*, and *apr2-3*, have reduced flux of sulfur through the sulfate assimilation pathway compared with Col-0, which possesses a strong *APR2* allele (Fig. 9A). Based on these observations, we hypothesized that sulfate should accumulate in those accessions with a weak *APR2* allele, where sulfate assimilation is reduced. Measurement of leaf sulfate confirmed this hypothesis. Hod, *apr2-1*, and *apr2-2*, all with weak alleles of *APR2*, have increased accumulation of sulfate in leaves compared with Col-0, which possesses a strong allele of *APR2* (Fig. 9B). In the F1 hybrid plants from a Hod × *apr2-1* cross, the *APR2^{Hod}* allele is unable to complement the elevated sulfate accumulation of *apr2-1*, whereas the *APR2^{Col-0}* allele is able to complement (Fig. 9B). These results confirm that elevated

Table II. Rare *APR2* coding haplotypes that have amino acid changes in conserved domains

Accession	Position																				APR Activity <i>mmol min⁻¹ mg⁻¹</i>
	5	16	21	24	40	56	58	65	107	111	155	182	216	242	265	343	349	385	399	427	
Col-0	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	F	N	K	I	A	P	10.7
ICE61	A	S	G	S	T	-	S	T	R	Q	V	G	G	D	F	N	R	I	A	P	7.5
Lc-0	V	S	R	A	N	-	T	T	K	E	A	D	G	D	F	N	R	I	A	P	10.9
Qartaba	V	S	G	S	T	-	S	T	R	Q	A	G	G	G	F	S	R	I	A	P	14.7
Lov-5	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	S	N	R	I	A	P	0.9
Lov-1	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	S	N	R	I	A	P	1.1
Fal-1	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	S	N	R	I	A	P	1.0
Tfa-08	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	S	N	R	I	A	P	1.2
Sei-0	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	F	N	R	I	A	S	9.9
Sha	V	S	G	S	T	-	S	T	R	Q	A	G	G	D	F	N	R	V	E	P	2.1
Hod	V	T	G	S	N	S	T	L	K	E	A	G	R	D	F	N	R	V	A	P	1.2

leaf sulfate in Hod is driven by the reduced function of *APR2*^{Hod}. Taken together, the sulfur flux and sulfate accumulation data strongly support our conclusion that, in the case of Hod, the reduced function of *APR2* activity limits the plant's ability to reduce sulfate, causing sulfate to accumulate in leaf tissue. However, this enhanced sulfate accumulation in Hod does not appear to be driven by constitutively elevated sulfate uptake, as the rate of sulfate uptake in Hod is indistinguishable from that in Col-0 (Supplemental Fig. S3) when measured as total accumulation of ³⁵S after exposure of the plants to [³⁵S] sulfate. In contrast, although Sha has a weak allele of *APR2* and reduced flux through the sulfate assimilation pathway, it does not show higher sulfate concentrations compared with Col-0. This suggests that further levels of regulation on sulfate accumulation exist besides *APR2*, such that high sulfate in Sha, due to weak *APR2* activity, can be suppressed.

DISCUSSION

The wide variation in total leaf sulfur and selenium and its high heritability in the population of 349 accessions we studied suggest that this population is a good resource for investigating the genetic architecture of phenotypic variation in these traits. However, even though we observed high heritability for total leaf sulfur and selenium accumulation, genome-wide and targeted association mapping failed to identify the genetic basis of this heritable variation. This missing heritability is not unusual in a genome-wide association study (GWAS; Brachi et al., 2011), with human height being a classic example. Human height has a heritability of approximately 80% (Visscher 2008). However, a GWAS on 90,000 individuals identified allelic variation that accounted for less than 10% of this heritable variation in height (McEvoy and Visscher, 2009). It was concluded that heritable variation in height is controlled by numerous small-effect alleles. Such an explanation also could cause the failure of our GWAS to identify genetic

variation that explains the heritable variation we observe in total leaf sulfur and selenium concentrations. However, the much smaller population size used in our study compared with the human height study means that we cannot exclude the possibility of nondetection of the phenotypic effects of large-effect rare alleles. Allelic heterogeneity, in which multiple alleles of the same gene are associated with a phenotype, as is the case for variation in leaf sodium governed by *High-affinity Potassium Transporter1* (Segura et al., 2012), also could diminish the sensitivity of GWA mapping using single-locus tests. However, the use of a multilocus test, as used here, did not improve our detection of loci linked to variation in leaf sulfur or selenium.

Investigating the genetic basis of high total leaf sulfur and selenium in accessions in the extreme tail of the phenotypic distribution using synthetic F2 populations, we were able to overcome some of these limitations of association mapping. Creating a synthetic F2 population between Col-0 (with average total leaf sulfur and selenium) and Hod (with high total leaf sulfur and selenium concentrations), and combining its analysis with available sequence data, allowed us to detect three different large-effect rare alleles of *APR2*. These alleles were contained in the Hod accession from the Czech Republic, four accessions from western Sweden, and ICE61 from southwestern Russia. These alleles were most likely not detected using association mapping due to their low frequency in the phenotyped population of 349 accessions studied.

The large-effect nature and low frequency of these new *APR2* alleles in the *Arabidopsis* species-wide collection used in this study are consistent with them being of recent adaptive significance (Brachi et al., 2012; Rockman, 2012). Because of the species-wide nature of the collection of accessions used, locally adaptive alleles would be sampled at a low frequency in the population. Furthermore, large-effect new mutations are theorized to play an important role in bouts of rapid evolution when a genotype suddenly finds itself in an environment to which it is badly adapted (Rockman, 2012). This raises the

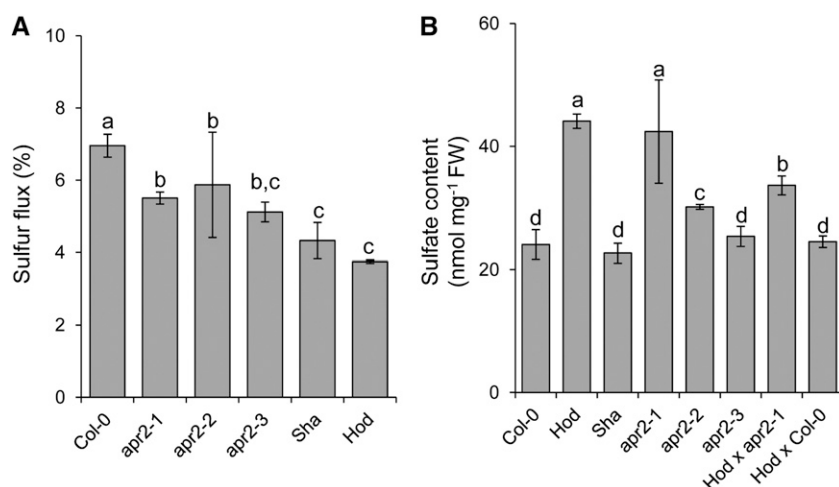


Figure 9. Sulfur flux in whole seedlings (A) and leaf sulfate content (B) in natural accessions, *apr2* alleles, and F1 hybrids. Data represent means \pm SE ($n = 3$). Letters above each bar indicate statistically significant groups using a one-way ANOVA with groupings by Tukey's honestly significant difference test using a 95% confidence interval. Sulfur flux was quantified as the incorporation of [³⁵S] sulfate into thiols and proteins as a percentage of the total [³⁵S] sulfate taken up by the seedlings. FW, Fresh weight.

question, to what mismatched environment might the founders of the accessions containing the reduced-function alleles of *APR2* have been exposed? One interesting possibility is that these reduced-function *APR2* alleles are adaptive to soils with elevated sulfate concentrations, where the capacity to limit the energy-demanding reduction of sulfate may have a fitness advantage. In support of this notion, we note that, at least for the European accessions possessing these weak alleles of *APR2*, they were collected from central Europe and Scandinavia, regions known to have experienced high levels of sulfate deposition due to acid rain in the 1950s, 1960s, and 1970s (Menz and Seip, 2004). Alternatively, the impairment of sulfate reduction rate might be an adaptation to a lower growth rate in adverse conditions to prevent the accumulation of reduced sulfur compounds (e.g. during nitrogen limitation; Koprivova et al., 2000). The identification of four individuals with the same weak allele of *APR2* in a localized area in western Sweden close to the town of Hårnösand is also consistent with this allele being locally adaptive. Although these observations are intriguing, more work is needed to establish if these weak alleles of *APR2* are adaptive, and if so, to what.

The availability of these newly identified natural alleles of *APR2* that encode enzymes varying across a broad range of catalytic capacity provides a unique opportunity to better understand the relationship between structure and function in this important enzyme. This potential is clearly highlighted by the unpredicted behavior of the enzyme encoded by the *APR2^{Hod}* and Swedish alleles. The *APR2^{Hod}* allele encodes an enzyme with a polymorphism that, based on our current knowledge, should be in the APS-binding loop. This polymorphism reduces the activity of the *APR2^{Hod}* enzyme. However, it does not affect the enzyme's binding affinity for APS. The Swedish *APR2* allele contains a polymorphism that produces a nonconservative amino acid change within the PAPS reductase domain of the enzyme. This change reduces the catalytic efficiency of the enzyme by over 1,000-fold, even though, based on our current understanding, this polymorphism should be in a region of secondary structure that has no catalytic activity.

Overall, our data support the model that reduced-function or loss-of-function alleles of *APR2* drive the elevated accumulation of total leaf sulfur (primarily as sulfate) by reducing the flux of sulfur from inorganic sulfate into reduced organic forms (e.g. Cys and GSH). This limitation in the assimilation of sulfur results in the accumulation of the pathway substrate, sulfate. This is consistent with previous studies showing a major role of APR in the control of flux through the sulfate assimilation pathway (Vauclare et al., 2002; Scheerer et al., 2010). It is also possible that the reduction in sulfate assimilation rate may lead to the signaling of sulfur deficiency, giving rise to further elevated uptake and accumulation of sulfate. However, we do not observe an increased sulfate uptake rate in Hod. Additionally, we observe *APR2* mRNA levels in Hod to be similar to those in Col-0 when

growing in sulfate-replete conditions. Since the level of *APR2* mRNA is normally strongly induced by sulfur starvation (Gutierrez-Marcos et al., 1996), this suggests that Hod is not experiencing sulfur deficiency. This indicates that sulfur deficiency signaling in Hod may not play a key role in its elevated sulfate accumulation. Intriguingly, the reciprocal grafting experiments established that reduced *APR2* function solely in the leaves is sufficient to achieve the elevated total leaf sulfur concentrations observed in the Hod accession. Even though *APR2* is known to be active in both roots and leaves, the activity in the leaves is known to be much higher (Koprivova et al., 2000). The fact that sulfur accumulates only in plants with a loss of *APR2* activity in the leaves, therefore, is consistent with our proposed role of the loss of function of *APR2* in high total leaf sulfur in Hod.

It is clear from this and previous studies (Loudet et al., 2007; Koprivova et al., 2013) that limitation of the catalytic activity of the ATP sulfurylase or APS reductase, enzymes that work sequentially to reduce sulfate to sulfite, is a significant source of natural variation in sulfate accumulation in Arabidopsis. Furthermore, for those accessions with high APR activity and high leaf sulfate, we would propose that they have a reduced-function allele of *ATPS1* (Koprivova et al., 2013). However, it is also evident that variation in leaf sulfate accumulation cannot always be explained in this way. The four western Swedish accessions (Lov-1, Lov-5, Fal-1, and Tfa-08) share the same weak allele of *APR2*, and all have similarly low APR activity. However, Tfa-08 accumulates significantly more leaf sulfate than the other three Swedish accessions, suggesting that these accessions contain other loci that limit sulfate accumulation. Making crosses between Tfa-08 and these other lower sulfate-accumulating accessions with weak alleles of *APR2* could provide a way to discover new genes and alleles involved in sulfur homeostasis in plants.

Unlike animals, plants do not have specific mechanisms for incorporating selenium into the amino acids seleno-Cys and seleno-Met. Rather, selenium in plants is thought to be metabolized nonspecifically via the sulfate assimilation pathway (for review, see Sors et al., 2005b). Heterologous and ectopic expression studies in plants of genes encoding ATP sulfurylase and APS reductase singly and together support this conclusion (Pilon-Smits et al., 1999; Sors et al., 2005a), although the role of these genes in tolerance to elevated selenate is less clear (Hatzfeld et al., 1998; Pilon-Smits et al., 1999; Sors et al., 2005a). Our observation that elevated total leaf selenium in Arabidopsis in Hod is caused by a reduction in function of *APR2* provides independent, nonheterologous, genetic evidence supporting these earlier reports that APR activity is required for selenium assimilation in plants. Uncovering this new role for natural genetic variation in *APR2* opens up a new avenue for the exploitation of natural variation in sulfur metabolism for the manipulation of dietary selenium concentrations in crop plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana plants used for elemental analysis by ICP-MS were grown in a controlled common garden with a photoperiod of 10 h of light ($90 \mu\text{mol m}^{-2} \text{s}^{-1}$)/14 h of dark, humidity of 60%, and temperature ranging from 19°C to 22°C. Detailed information for planting and management was the same as described previously (Lahner et al., 2003). Plants used for the measurement of sulfate content, APR activity, and sulfur flux were grown for 2 weeks on vertical plates with Murashige and Skoog medium without Suc supplemented with 0.8% agarose. The plates were placed in a controlled-environment room at 20°C under a 16-h-light/8-h-dark cycle. Plants for expression analysis of APR2 were grown in axenic conditions as described previously (Chao et al., 2012). Briefly, sterilized seeds were sown on one-half-strength Murashige and Skoog medium (Sigma-Aldrich) solidified with 1.2% agar containing Suc. Plates were placed at 4°C for 3 d to achieve optimum germination and then transferred into a growth chamber with a photocycle of 16 h of light ($90 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8 h of dark and temperature of 22°C for another 3 weeks.

Elemental Analysis

Total leaf elemental concentrations were determined as described previously (Lahner et al., 2003). In brief, one to two leaves were harvested from 5-week-old plants with a sharp knife and clean plastic tweezers. Leaves were rinsed three times with ultrapure water (18 M Ω) and placed at the bottom of Pyrex digestion tubes. Samples were subsequently dried at 92°C for 20 h. Dried samples together with analytical blanks and standard reference material (National Institute of Standards and Technology, Standard Reference Material 1574) were digested with 0.7 mL of concentrated nitric acid (OmniTrace; VWR Scientific Products) with gallium added as an internal standard. After completing digestion at 118°C, all tubes were diluted to 6 mL with 18-M Ω water and analyzed by ICP-MS (Elan DRCe; PerkinElmer) for lithium, boron, sodium, magnesium, phosphorus, potassium, calcium, manganese, iron, cobalt, nickel, copper, zinc, arsenic, selenium, molybdenum, and cadmium. The raw data were normalized using a previously described method (Lahner et al., 2003). Unnormalized data have been deposited on the iHUB site (previously known as PiIMS; Baxter et al., 2007) for viewing and download at <http://www.ionomicshub.org>.

Genome-Wide and Targeted Association Mapping

GWA analyses were performed using the MLM package (Segura et al., 2012) and SNP data from the tiling array data from call method 75 (Atwell et al., 2010) comprising just over 214,000 SNPs. Phenotypic data consisting of measurements of sulfur and selenium accumulation were obtained from the iHUB site (<http://www.ionomicshub.org>) as data set doi: 10.4231/T9H41PBV (Nordborg 360 HapMap) and originate from 331 of the 360 accessions selected previously to represent the species-wide diversity of *Arabidopsis* (Baxter et al., 2010; Platt et al., 2010). Leaf tissue was analyzed for sulfur and selenium accumulation by ICP-MS analysis (Lahner et al., 2003). MLM optimal models were achieved at the first iteration, and inclusion of the top SNP from this first iteration reduced the *P* values of the remaining SNPs and also reduced the fit of those at APR2. Thus, controlling for the genetic effects of the top-associated SNP did not improve the fit of SNP segregation to the phenotypic data. The optimal outputs, therefore, are similar to what would be obtained by ordinary mixed-model association mapping. For targeted, candidate gene, association analysis, SNP data were restricted to consider only the SNPs within APR2 (The Arabidopsis Information Resource 10 build; <http://www.arabidopsis.org/servlets/TairObject?type=locus&name=At1g62180>) or to data within 100 kb of DNA centered on the APR2 gene (chromosome 1 centered at 22,976,566 bp in The Arabidopsis Information Resource 10). Seven SNPs were found in the APR2 gene and 245 were identified within 100 kb of DNA.

Map-Based Cloning

The F2 mapping population was derived from an outcross of *Arabidopsis* accessions Hod and Col-0. Rough mapping was performed using SNP tiling array-based XAM as described previously (Becker et al., 2011; Chao et al., 2012). In brief, 328 F2 individuals were phenotyped by ICP-MS and ranked by leaf sulfur concentrations. The 57 individuals with the highest leaf sulfur concentration and the 61 individuals with the lowest leaf sulfur concentration were pooled separately. Genomic DNA from each pool was extracted using a DNeasy plant

mini kit (Qiagen) and labeled using the BioPrime DNA labeling system (Invitrogen Life Technologies). The labeled DNA from each pool was separately hybridized to the Affymetrix SNP tiling array Atsnptile 1. The signal intensities for all probes were extracted and spatially corrected using R scripts written by Borevitz et al. (2003). The original CEL files containing raw signal intensity data used in this study have been submitted to the Gene Expression Omnibus under accession number GSE61922. Polymorphic SNPs between the two parents were used for linkage mapping. Based on signal intensity differences among four probes for each SNP, antisense and sense probes for two alleles, the allele frequency differences between the two pools were calculated for each SNP. The whole process was performed using R scripts available at <http://ars.usda.gov/mwa/bsanp>.

The XAM generated a rough mapping position for the causal gene, so the candidate region was further narrowed using PCR-based markers. Multiple PCR-based CAPS and simple sequence length polymorphism markers were developed (Supplemental Table S2). All 328 F2 individuals used for XAM were genotyped at these markers, and recombinants between the markers were selected for further analysis. The F2 recombinants with high leaf sulfur were used directly to narrow the mapping interval, while the recombinants with low leaf sulfur content were used for determination of the causal region in the F3 generation. Fine-mapping was performed in a similar manner using an enlarged F2 population of 1,084 individuals.

Sequencing of Candidate Genes and Screening of APR2 Variants

The genomic region of Hod APR2 was sequenced using overlapping fragments amplified by PCR as described previously (Chao et al., 2012). First, overlapping DNA fragments (0.7–1.2 kb) covering the APR2 genomic region, including the promoter, gene body, and 3' terminus, were amplified using KOD Hot Start DNA polymerase (Toyobo) using Hod genomic DNA as the template. Specific primers for the reactions were designed using Overlapping Primersets (http://pcrsuite.cse.ucsc.edu/Overlapping_Primers.html) and are listed in Supplemental Table S3. Each fragment was sequenced in two directions using the same primers for amplification. The sequences were assembled using SeqMan Lasergene software (DNASTAR; <http://www.dnastar.com>), and the assembled contigs was aligned using the Col-0 APR2 sequence as a reference to identify polymorphisms between Hod and Col-0.

The DNA sequences of APR2 variants were obtained from the database containing the genome sequences of 855 *Arabidopsis* accessions (<http://signal.salk.edu/atg1001/3.0/gebrowser.php>). First, the predicted protein sequence of APR2 in each of the 855 accessions was extracted. Sequences were copied into a Microsoft Word document. Each sequence was separated by a paragraph marker, and each amino acid in the same sequence was separated with a tab using the Replace function. These data were copied into Microsoft Excel. In the Excel file, each row represented one APR2 amino acid sequence of an accession, each cell represented one amino acid of the protein, and each column represented a position of the amino acid. Positions at which at least one amino acid varied were identified using the Filter function. Candidate hypofunctional APR2 variants were further screened for based on the presence of variant amino acids with conserved domain.

Plant Transformation

In order to complement the high leaf sulfur phenotype of Hod, a binary vector for the expression of the Col-0 APR2 fragment was constructed. First, a 4.4-kb DNA fragment containing the APR2 promoter, gene body, and 3' terminus was amplified from Col-0 genomic DNA using KOD Hot Start DNA polymerase (Toyobo) using specific primers listed in Supplemental Table S3. This fragment was cloned into the pCR-XL-TOPO vector (Invitrogen Life Technologies) for DNA sequencing, followed by recombination into the pCambia1301 binary vector using *SalI* and *BamHI* restriction enzymes. This vector was transformed into *Agrobacterium tumefaciens* strain GV3101. Transgenic transformation of *Arabidopsis* was achieved using this transgenic *A. tumefaciens* strain following the floral dip method (Clough and Bent, 1998). Positive transgenic lines were identified using one-half-strength Murashige and Skoog medium solidified with 0.7% agar containing 50 $\mu\text{g mL}^{-1}$ hygromycin and 1% Suc.

APS Reductase Activity

APS reductase activity was determined as the production of [³⁵S]sulfite and assayed as acid volatile radioactivity formed in the presence of [³⁵S]APS and GSH as the reductant (Loudet et al., 2007). The protein concentrations were

determined using a Bio-Rad protein kit with bovine serum albumin as a standard. Recombinant APR2 proteins were prepared and analyzed as described by Loudet et al. (2007). In short, the APR2 coding regions were amplified from RNA isolated from the corresponding accessions, cloned into pET14b plasmids, and their identity was verified by sequencing. The proteins were purified at 4°C from triplicate 100-mL cultures of *Escherichia coli* BL21(DE3) grown overnight at 30°C using Ni²⁺ affinity chromatography. The APR reaction mixture contained 5 µg of recombinant APR2 from Sha, Hod, and Lov-5 or 4 ng of enzyme from Col-0 and Bay-0, 50 mM Tris-HCl, pH 9, 500 mM MgSO₄, and varying concentrations of [³⁵S]APS (specific activity, 50 Bq nmol⁻¹; 1–150 µM with 10 mM GSH) and GSH (1–100 mM with 75 µM APS) in a volume of 250 µL and was incubated for 30 min at 37°C. For the calculation of kinetic parameters, the data were linearized according to Lineweaver and Burk.

Leaf Sulfate Quantification and Analysis of Sulfate Uptake and Assimilation

Leaf sulfate contents were determined by ion-exchange HPLC as described by Koprivova et al. (2013). Approximately 50 mg of plant tissue was homogenized and extracted in 1 mL of sterile water for 1 h at 4°C. The extracts were heated for 15 min at 95°C and centrifuged for 15 min at 13,000 rpm. Twenty microliters of the extracts was analyzed on an IC-PAK Anion HR 4.6 × 75-mm column (Waters) using a lithium borate/gluconate eluent at 1 mL min⁻¹ in isocratic mode and a Waters 432 conductivity detector. Sulfate uptake and sulfur flux through sulfate assimilation were measured as incorporation of ³⁵S from [³⁵S] sulfate to thiols and proteins essentially as described by Mugford et al. (2011). The seedlings were transferred onto 24-well plates containing 1 mL of Mura-shige and Skoog nutrient solution adjusted to a sulfate concentration of 0.2 mM and supplemented with 5.6 µCi of [³⁵S]sulfate (Hartmann Analytic) to a specific activity of 1,860 Bq nmol⁻¹ sulfate and incubated in light for 4 h. After incubation, the seedlings were washed three times with 2 mL of nonradioactive nutrient solution, carefully blotted with paper tissue, weighed, transferred into 1.5-mL tubes, and frozen in liquid nitrogen. Total ³⁵S accumulation in seedlings was used to calculate the sulfate uptake rate. The quantification of total ³⁵S in plant material and in different thiols and proteins was performed exactly as described by Mugford et al. (2011).

Reciprocal Grafting Experiment

The grafting of Arabidopsis plants was performed as described previously (Chao et al., 2012). On day 7 after grafting, grafted unions were examined with the stereoscope. Healthy grafted plants without any adventitious root were transferred to potting mix for a further 4 weeks of growth in a controlled environment as described above. Graft unions were again examined after leaf samples were harvested for ICP-MS analysis, and grafted plants with adventitious roots or without a clear graft union were removed from the final analysis.

Expression Analysis of APR2

Total RNA was extracted using the TRIzol Plus RNA Purification kit (Invitrogen Life Technologies) according to the manufacturer's protocol. First-strand cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen Life Technologies). First-strand cDNA was used as a template for subsequent qRT-PCR. Primers for APR2 quantification were designed using Primer Express Software version 3.0 (Applied Biosystems) such that the reverse primer spanned the third exon-exon junction. qRT-PCR was performed using a real-time PCR system (ABI StepOnePlus; Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). The qRT-PCR results were analyzed as described previously (Livak and Schmittgen, 2001).

Statistical Analyses

To calculate the broad sense heritability, the environmental variance (V_E) was first estimated based on replicates of each reference and tested accession, as $V_E = (1/n) \sum_{i=1}^n V_i$, where V_i is the phenotypic variance within the individuals of the i accession and n is the number of accessions tested. The genotypic variance was calculated as $V_G = V_P - V_E$, where V_P is the phenotypic variance of the whole GWAS population. The broad sense heritability was then calculated as $H^2 = V_G/V_P$. We used normalized data across trays for the above estimates. ANOVA was performed in Microsoft Excel by using the Anova: Single Factor

function of the Data Analysis Tools. Student's t test was also performed in Microsoft Excel by using the TTEST function assuming equal variances.

Supplemental Data

- The following materials are available in the online version of this article.
- Supplemental Figure S1.** Genome-wide association analysis of leaf sulfur concentration across 349 Arabidopsis accessions.
 - Supplemental Figure S2.** Genome-wide association analysis of leaf selenium concentration across 349 Arabidopsis accessions.
 - Supplemental Figure S3.** Sulfur uptake in natural accessions and *apr2* alleles.
 - Supplemental Table S1.** Associations of APR2-linked genotypes with leaf sulfur and selenium.
 - Supplemental Table S2.** Polymorphisms on APR2 region among Col-0, Hod, and Ct-1.
 - Supplemental Table S3.** Information on primers used in this study.

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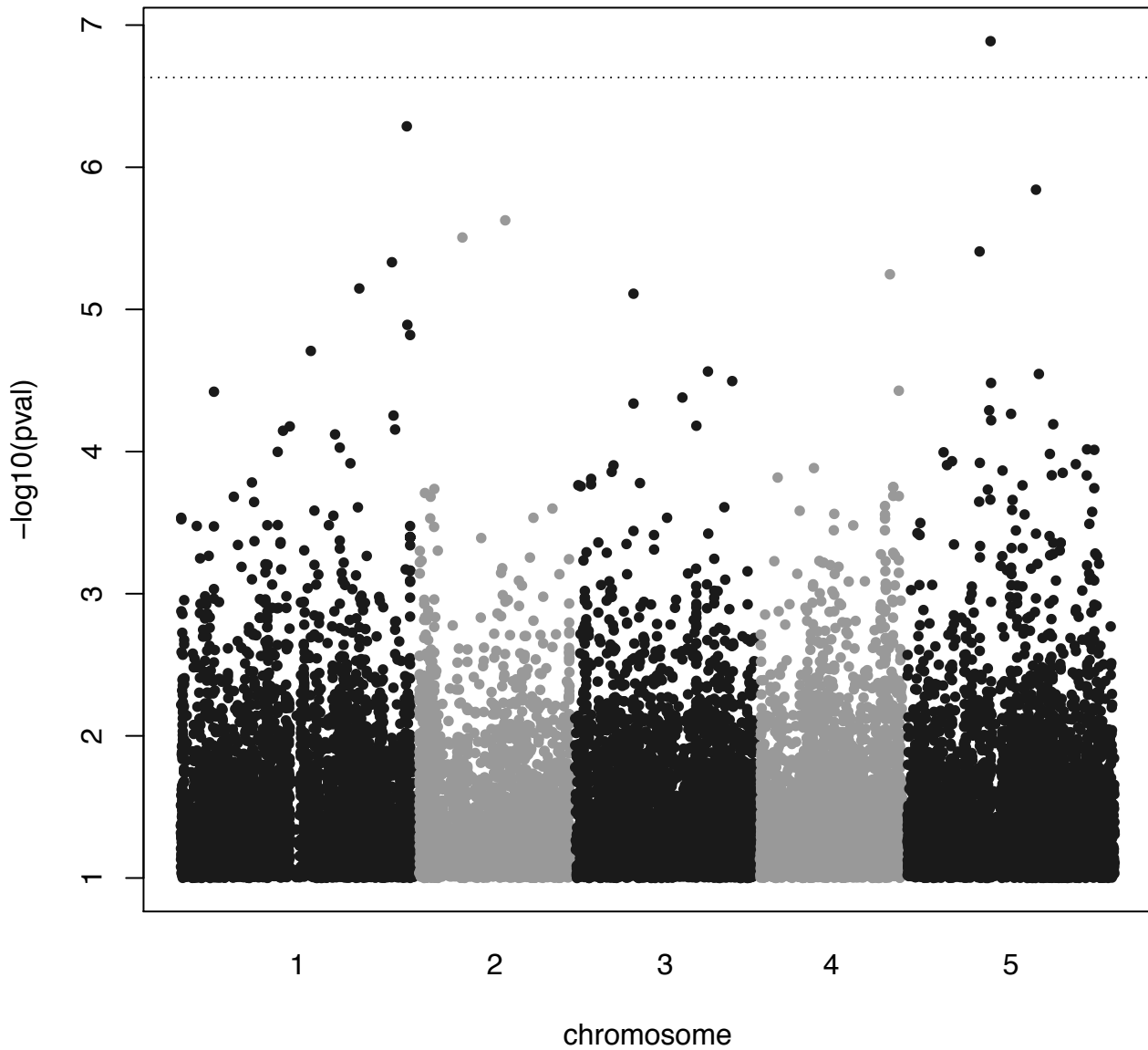
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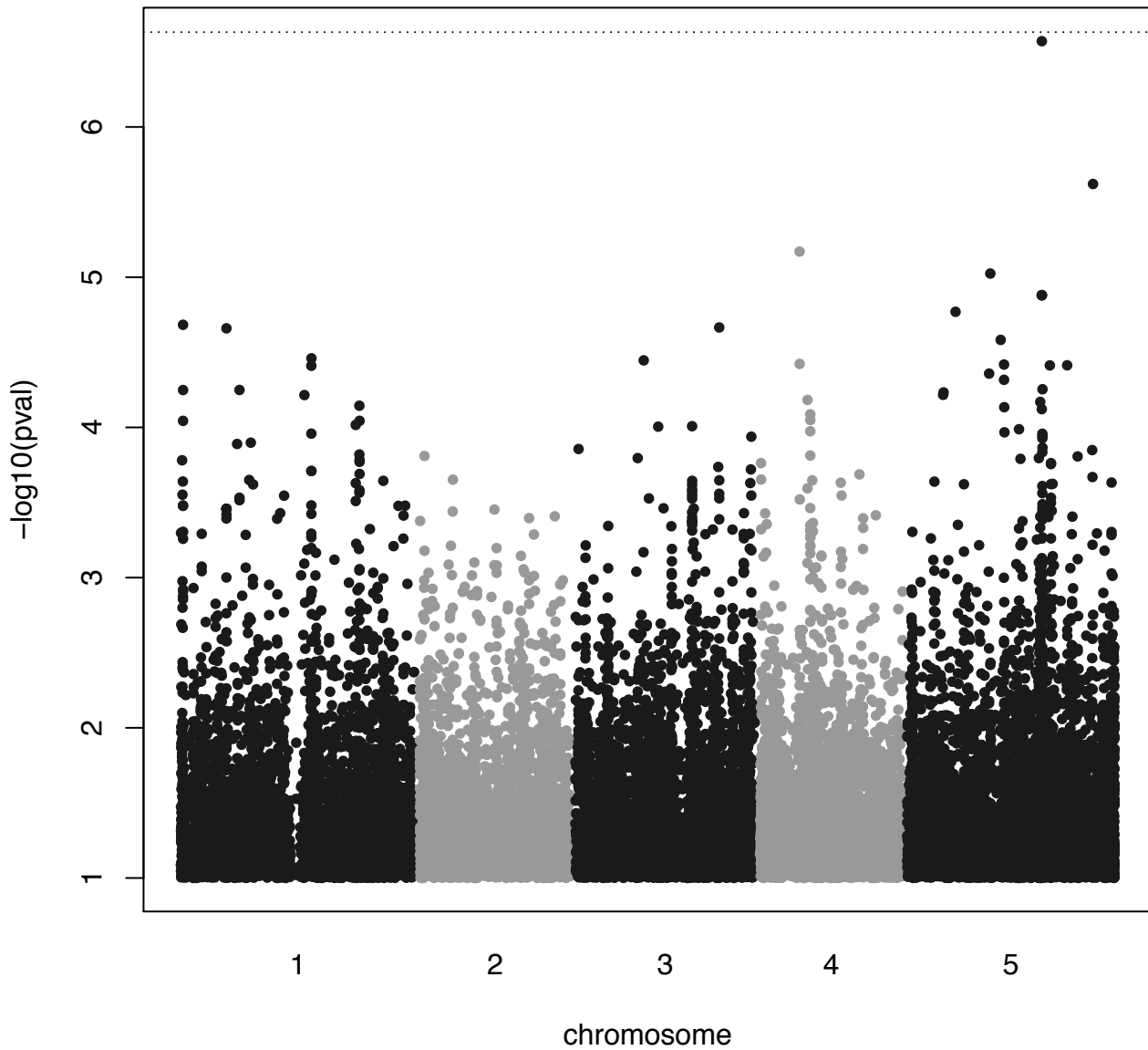
LITERATURE CITED

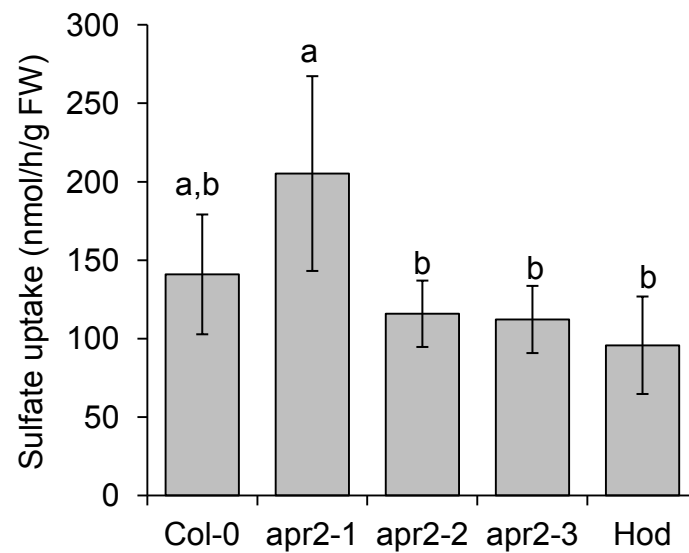
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Supplemental Figure 3. Sulfate uptake in natural accessions and *apr2* alleles. Data represents the mean values \pm standard errors ($n = 3$). Letters above each bar indicate statistically significant groups using a one-way ANOVA with groupings by Tukey's HSD using a 95% confidence interval. Sulfate uptake quantified as the accumulation of ^{35}S in whole seedlings incubated with [^{35}S]sulfate. FW – fresh weight