Natural Variation in the ATPS1 Isoform of ATP Sulfurylase Contributes to the Control of Sulfate Levels in Arabidopsis\textsuperscript{1[W]}

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Sulfur is an essential macronutrient for all living organisms. Plants take up inorganic sulfate from the soil, reduce it, and assimilate it into bioorganic compounds, but part of this sulfate is stored in the vacuoles. In our first attempt to identify genes involved in the control of sulfate content in the leaves, we reported that a quantitative trait locus (QTL) for sulfate content in Arabidopsis (Arabidopsis thaliana) was underlain by the APR2 isoform of the key enzyme of sulfate assimilation, adenosine 5’-phosphosulfate reductase. To increase the knowledge of the control of this trait, we cloned a second QTL from the same analysis. Surprisingly, the gene underlying this QTL encodes the ATPS1 isoform of the enzyme ATP sulfurylase, which precedes adenosine 5’-phosphosulfate reductase in the sulfate assimilation pathway. Plants with the Bay allele of ATPS1 accumulate lower steady-state levels of ATPS1 transcript than those with the Sha allele, which leads to lower enzyme activity and, ultimately, the accumulation of sulfate. Our results show that the transcript variation is controlled in cis. Examination of ATPS1 sequences of Bay-0 and Shahadara identified two deletions in the first intron and immediately downstream the gene in Bay-0 shared with multiple other Arabidopsis accessions. The average ATPS1 transcript levels are lower in these accessions than in those without the deletions, while sulfate levels are significantly higher. Thus, sulfate content in Arabidopsis is controlled by two genes encoding subsequent enzymes in the sulfate assimilation pathway but using different mechanisms, variation in amino acid sequence and variation in expression levels.

Sulfur is an essential macronutrient for all organisms, as it is part of the amino acids Cys and Met, a great range of coenzymes and prosthetic groups, and other essential cellular components. In these metabolites, sulfur is usually in its reduced form as organic sulfide; however, the most accessible source of sulfur in nature is inorganic sulfate. Plants, algae, yeast, fungi, and many prokaryotes can take up the sulfate, reduce and assimilate it, and so provide animals and humans with the sulfur necessary for life. In sulfate assimilation, sulfate has to be first activated by ATP sulfurylase to adenosine 5’-phosphosulfate (APS), which is reduced in two steps, by adenosine 5’-phosphosulfate reductase (APR) to sulfite and subsequently by sulfite reductase to sulfide. Sulfide is incorporated into the amino acid skeleton of O-acetylseryne to form Cys, the first product of sulfate assimilation and the donor of reduced sulfur for other metabolites (for review, see Takahashi et al., 2011). Alternatively, APS can be activated by a second ATP to form 3’-phosphoadenosine 5’-phosphosulfate, the activated sulfite donor for a range of sulfations in secondary metabolism. The major class of sulfated compounds, at least in plants of the Brassicaceae, are the glucosinolates, compounds important for plant defense against numerous pathogens and herbivores but also responsible for the taste and smell of the cruciferous vegetables and their health benefits (for review, see Sonderby et al., 2010; Takahashi et al., 2011).

However, not all sulfate entering the plant cell is metabolized; part of it is stored in the vacuole. This sulfate can be remobilized through the action of group 4 sulfate transporters localized in the tonoplast and enter the assimilation pathway (Kataoka et al., 2004). The portion of sulfur stored as vacuolar sulfate is highly variable among environmental conditions and plant species but also between variants of the same species (Durenkamp et al., 2007; Koralewska et al., 2007; Loudet et al., 2007). Since the consequence of

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the high sulfate content is high demand for sulfate in the soil, leading to the need for sulfate fertilization, the control of plant sulfate homeostasis is of high interest. Given the variability of sulfate concentration in different plant species, an approach to tackle this issue has been quantitative genetics (Loudet et al., 2007). Quantitative trait locus (QTL) analysis of recombinant inbred lines (RILs) derived from the cross between two Arabidopsis (Arabidopsis thaliana) wild accessions, Bay-0 and Shahdara (Loudet et al., 2002), revealed two major and several minor QTLS responsible for the variation of sulfate content in the population (Loudet et al., 2007). One QTL was cloned, showing that the underlying gene encodes the APR2 isoform of APR. Detailed biochemical analysis of the two APR2 alleles revealed that a single nucleotide polymorphism leads to an amino acid change close to an active center of the enzyme, making the Shahdara-derived APR2 protein lose 99% of its enzyme activity compared with the Bay-0 counterpart (Loudet et al., 2007). The diminished sulfate-reducing capacity thus results in an accumulation of the unused substrate of the pathway, sulfate.

Here, we show that the gene underlying the second major QTL encodes the ATPS1 isoform of the preceding enzyme of the pathway. We show that, in contrast to APR2, the basis for variation at this locus is the expression level of the ATPS1 gene. The difference in expression affects the total ATP sulfurylase (ATPS) activity in plants and results in a limited capacity to reduce sulfate and variation in sulfate level. Interestingly, the variation in expression seems to be linked to two deletions in the ATPS1 gene in Bay-0, deletions common among several Arabidopsis accessions, where it can also be related to lower ATPS1 expression level and higher accumulation of sulfate.

RESULTS

ATPS1 Underlies the SO10.2 QTL

QTL analysis within the Bay-0 × Shahdara RIL set revealed two major QTLS: one on chromosome 1, which was apparent at both nitrate-sufficient and nitrate-limiting nutrition (SO3.1 = SO10.1), and one on the top arm of chromosome 3, present at normal nitrate only (SO10.2; Loudet et al., 2007). Under the normal nitrate supply, the two QTLS are independent and fully additive in their effect on sulfate levels (Table I). SO3.1/SO10.1 has been previously identified as APR2, the proof obtained by exploiting the remaining heterozygosity in heterogenous inbred families (HIFs; Loudet et al., 2007). The SO10.2 QTL was localized between markers ATHCHIB2 and MSAT3.19. In the HIF collection, line HIF004 was identified as being heterozygous at the QTL locus; thus, the Bay and Shahdara alleles of the QTL, derived from Bay-0 and Shahdara accessions, respectively, could be analyzed in an otherwise homoyzogous genetic background. Using the same approach as previously, within HIF004, the segregating interval (ATHCHIB2–MSAT3.19) was confirmed to cause variation in sulfate content (Fig. 1A).

Conspicuously, among the genes in this interval, the ATPS1 isoform of ATPS (At3g22890) has been identified as a good candidate. This gene encodes one of four isoforms of the enzyme in the sulfate assimilation pathway preceding APR, which was shown to be responsible for the control of variation in sulfate levels (Loudet et al., 2007). More importantly, the atps1 mutant in the Columbia (Col-0) background accumulates sulfate compared with the wild type (Liang et al., 2010; Kawashima et al., 2011).

Indeed, the sulfate levels in HIF004 containing the Bay allele of ATPS1 (004B) were higher compared with those with the Sha allele (004S), although not as pronounced as the difference between atps1 and wild-type Col-0 (Fig. 1A). Correspondingly, sulfate levels were higher in the Bay-0 parental accession compared with Shahdara. Disruption of the ATPS1 gene in Col-0 resulted in the reduction of ATPS enzyme activity by approximately 50% (Fig. 1B). The enzyme activity in Bay-0 was lower than in both Col-0 and Shahdara, and the same was true for 004B and the corresponding 004S (Fig. 1B). This strongly suggests that ATPS1 underlies the variation in sulfate content between the two parental accessions. To assess the variation in this gene, we sequenced ATPS1 from the two accessions, including 1,800 bp upstream from the translation start and 600 bp downstream of the stop codon (Supplemental Fig. S1). Only one silent single-nucleotide variation (SNP) between Bay-0 and Shahdara was found in the coding region of ATPS1, and five SNPs were present in the promoter region. In addition, compared with Col-0, three short insertions/deletions and 10 SNPs are common in the 1,800 bp of Bay and Sha ATPS1 upstream sequence (Supplemental Fig. S1). However, two regions of major variation were found in Bay compared with both Sha and Col-0. In Bay, a 13-bp deletion and a highly polymorphic region occur in the middle part of intron 1 while a 71-bp deletion and a few additional substitutions are located 249 bp downstream of the stop codon (Supplemental Fig. S1). Thus, the difference in ATPS activity between Bay-0 and Shahdara cannot be caused by different kinetic properties of the ATPS1 protein but should be based on differences in expression. Indeed, the steady-state levels of ATPS1 mRNA were lower in 004B and

Table 1. Interaction of APR2 (SO10.1) and ATPS1 (SO10.2) in the control of sulfate accumulation

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<th>Sha</th>
<th>Bay</th>
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<tr>
<td>SO10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sha</td>
<td>145 ± 3</td>
<td>+18%</td>
</tr>
<tr>
<td></td>
<td>−31%</td>
<td>−29%</td>
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<tr>
<td>Bay</td>
<td>100 ± 3</td>
<td>+21%</td>
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Sulfate levels (mmol mg⁻¹ DM) in leaves of RILs with different combinations of alleles at the two QTLS are shown in bold as means ± se. The percentages show the relative effect of the Bay alleles with respect to the Sha allele.
Bay-0 than in 004S, Shahdara, or Col-0 (Fig. 1C). In an alternative approach using pyrosequencing, the difference in ATPS1 transcript accumulation levels between the two accessions was confirmed and revealed to be controlled in cis: in F1 plants from reciprocal crosses between Bay-0 and Shahdara (as well as in the original RIL004 heterozygous individuals), the ratio of Sha versus Bay ATPS1 transcripts was 1.4 (1.3 in the heterozygous RIL004), which is significantly different from 1 at $P < 10^{-4}$ ($P < 10^{-3}$ in RIL004). This unequivocally confirms a differential cis-acting regulation at ATPS1 and indicates that ATPS1 is indeed a strong candidate gene.

To confirm that SO10.2 is ATPS1, we performed a quantitative genetic complementation (Mackay, 2004; Loudet et al., 2007) comparing the effects of Bay and Sha alleles of ATPS1 in hybrids from crosses of both HIF004 lines with the atps1 mutant and its corresponding Col-0 wild type. Using the HIF line rather than the parental accessions ensures a near isogenic background of the tested genotypes and limits the genetic differences in the F1 plants to the SO10.2 region in one set of parents and on a single gene (ATPS1) in the second tester genotype. Indeed, in contrast to the Sha allele, the Bay ATPS1 allele was not able to fully complement the high-sulfate phenotype of the atps1 mutant (Fig. 2A). The same was true for ATPS enzyme activity and ATPS1 transcript levels; there was no difference in the Bay or Sha allele of ATPS1 when crossed with Col-0, but both were lower in 004B × atps1 crosses than in 004S × atps1 F1 plants (Fig. 2, B and C). These results confirm that ATPS1 is the best candidate for the SO10.2 QTL and is at least partly responsible for the variation in sulfate content in the Bay-0 × Shahdara population.

To better understand the biochemical mechanisms linking ATPS1 and sulfate content, we analyzed more aspects of sulfate assimilation in the HIF004 lines, the parental accessions, and the atps1 mutant. Disruption of ATPS1 in the Col-0 background did not affect APR activity (Fig. 3A). The activity, however, was very low in Sha and the HIF004 lines, in agreement with their possessing a Sha allele at APR2 (Loudet et al., 2007). The contents of glutathione, the major low-M, thiol, were not affected by the disruption of ATPS1 in Col-0 but were lower in Shahdara and both HIF004 lines compared with Bay-0 and the Col-0 genotypes (Fig. 3B), most probably linked to the lower APR activity. An important measure of disturbance in sulfur metabolism is the flux through sulfate assimilation, measured as the incorporation of $^{35}$S from $[^{35}$S]sulfate into thiols and proteins for the primary pathway or into glucosinolates for the secondary sulfur metabolism (Vauclare et al., 2002; Scheerer et al., 2010; Mugford et al., 2011). The flux through the primary assimilation was lower in the atps1 mutant compared with Col-0, higher in Bay-0 than in Shahdara, and, importantly, lower in 004B than in 004S (Fig. 3C). Interestingly, disruption of ATPS1 in the Col-0 background diminished the synthesis rate of glucosinolates by approximately 55%, but no difference was observed between the 004B and 004S lines (Fig. 3D). These results thus support the conclusion that the SO10.2 QTL is caused by variation in ATPS1 transcript levels. In the isogenic background of the HIF004 lines, the lower transcript levels of Bay ATPS1 lead to lower ATPS activity compared with the Sha allele, which results in lower flux through the sulfate assimilation pathway and, consequently, in sulfate accumulation.
Deletion in Bay-0 of the ATPS1 Gene Is Linked to High Sulfate Levels

Having established that the difference in ATPS1 transcript levels between Bay-0 and Shahdara contributes to the control of variation in sulfate levels, we were interested in the extent to which this variation is common among Arabidopsis accessions. We interrogated the Arabidopsis 1001 Genomes database (http://signal.salk.edu/atg1001/3.0/gebrowser.php) for variation in ATPS1 sequence among worldwide Arabidopsis accessions. Given that the major difference between Bay and Sha ATPS1 is the presence of the two deletions in intron 1 and downstream of the stop codon in Bay-0 (Supplemental Fig. S1), we focused on accessions sharing similar deletions (Fig. 4). Indeed, from the approximately 500 accessions available so far, 50 had a deletion downstream of the ATPS1 gene similar to Bay-0. Among these, 28 also shared a deletion in the first intron. While the deletions were not necessarily predicted to be of the same size according to resequencing data, their positions were well aligned with the deletions in Bay-0 (Fig. 4A). To confirm these deletions, corresponding DNA fragments were amplified from six accessions representing each haplotype and sequenced (Supplemental Fig. S2). All predicted deletions were confirmed in the selected accessions, although the exact borders of the deletions were often slightly different from what was indicated in the Arabidopsis 1001 Genomes browser. We have chosen several accessions from each of the three haplotypes, with two deletions (including Bay-0), one deletion, and no deletion (including Shahdara and Col-0), and determined sulfate levels in the leaves (Fig. 4B). The accessions from the haplotype with two deletions appeared to have higher sulfate levels than the other haplotypes. Indeed, the mean sulfate level in 10 accessions with two deletions was significantly (by approximately 30%) higher than a mean value of sulfate levels from seven accessions with one deletion and from 12 accessions without any deletion (Fig. 4D). The ATPS1 transcript levels, when presented relative to the constitutively expressed APR2 to account for differences in the expression of the sulfate assimilation pathway, were also lower in the accessions with two deletions than in the other groups (Fig. 4, C and E).

On the other hand, the five SNPs in the ATPS1 promoter appear not to be linked to the difference in sulfate levels. The G in the Bay sequence at position 239 (Supplemental Fig. S1) is much more common among the accessions than the A found in Col and Sha, as are the three linked SNPs at positions 643, 675, and 680 (Supplemental Fig. S1). From the 29 accessions analyzed, 11 share with Bay-0 the A at position 1,264 (Supplemental Fig. S1), while 17 accessions, including Col-0 and Shahdara, possess G at this position. However, there was no significant difference in sulfate levels between these two groups. It seems, therefore, that the deletion in intron 1 of the ATPS1 gene, possibly together with the downstream deletion, causes a lower accumulation of ATPS1 transcript and underlies the SO10.2 QTL in the Bay-0 × Shahdara RIL population but also generally contributes to the variation in sulfate contents among Arabidopsis accessions.

DISCUSSION

The levels of sulfate stored in plant vacuoles are dependent on genetic as well as environmental factors.
and are tightly controlled. While in leaves of many species, sulfate constitutes about 50% of total sulfur, in others, such as *Brassica oleracea*, it can reach up to 90% (Durenkamp et al., 2007; Koralewska et al., 2007). Sulfate homeostasis is dependent on the interplay between supply and utilization. Sulfate is depleted in plants exposed to sulfate deficiency (Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003) or in plants deficient in sulfate uptake (Hirai et al., 2003; Lee et al., 2012). Interestingly, sulfate levels are low in a mutant of a *FIERY1* gene encoding a bifunctional enzyme possessing 3(29),5'-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities (Lee et al., 2012). However, the low sulfate levels in these mutants were only accompanying other phenotypes used for the genetic screens. The only direct investigation of the genetic control of sulfate homeostasis so far resulted in the identification of two strong and several weak QTLs (Loudet et al., 2007). The first QTL was cloned, and the APR2 gene encoding an isoform of APR was found to underlie the SO3.1/SO10.1 QTL (Loudet et al., 2007). The identification of *ATPS1* as the gene underlying the second major QTL thus adds another piece to the puzzle of molecular mechanisms controlling sulfate homeostasis among natural populations. It is also another example of variation in a metabolic trait being caused by variation in a metabolic gene, such as the accumulation of glucosinolates (Kliebenstein et al., 2001) or fumarate (Brotman et al., 2011).

Among the genes present in the QTL interval, *ATPS1* has been the obvious candidate, as it encodes an enzyme involved in sulfate assimilation. Indeed, disruption of the gene in the Col-0 genetic background resulted in a reduction of ATPS activity and substantially increased sulfate levels (Fig. 1). ATPS activity was also lower in the parental accession Bay-0 than in Shahdara. However, the genetic diversity between the two accessions is high, and the difference between the Bay and Sha alleles of *ATPS1* has to be analyzed in an otherwise homogenous genetic background. This was achieved by exploiting the residual heterozygosity in the HiFs (Tuinstra et al., 1997); indeed, the Bay allele of *ATPS1* in the HiF004 family has shown lower enzyme activity and higher sulfate accumulation than the Sha allele. The final evidence that *ATPS1* underlies the SO10.2 QTL was achieved through genetic complementation, as the Bay allele of *ATPS1*, unlike

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**Figure 3.** Effects of variation in *ATPS1* on sulfate assimilation. Col-0, atps1, Bay-0, Shahdara, 004B, and 004S plants were grown for 2 weeks on Murashige and Skoog-agarose vertical plates in a controlled-environment room. A and B, APR activity (A) and glutathione (GSH) content (B) were measured in shoots. C and D, The plants were incubated for 4 h with 0.2 mM [35S] sulfate, and the incorporation of 35S into glutathione, Cys, and proteins (C) and glucosinolates (GLS; D) was quantified. Results are presented as means ± so from three pools of four plants. Different letters mark significantly different values at *P* < 0.05. FW, Fresh weight.
the Sha allele, failed to complement the high-sulfate phenotype of the atps1 mutant (Fig. 2).

The analysis of atps1 mutants also revealed that the ATPS1 isoform has the highest contribution to total ATPS activity in leaves, as the activity in atps1 leaves was less than half of the wild-type values. The presence of another three isoforms of ATPS ensures that, apart from the high sulfate levels, the atps1 mutants do not show any developmental or growth phenotypes. The reduced ATPS activity in atps1 results in a

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**Figure 4.** Species-wide natural variation in ATPS1. A, Representative schemes of the three types of ATPS1 haplotypes as obtained from the Arabidopsis 1001 Genomes browser. B and C, Sulfate levels (B) and expression of ATPS1 (C) in leaves of individual accessions belonging to three haplotypes according to the number of deletions at ATPS1. D and E, Sulfate levels (D) and expression of ATPS1 (E) in leaves of the three haplotypes of Arabidopsis accessions. Results are presented as means ± SD from three plants. Different letters mark significantly different values at \( P < 0.05 \). Accessions without any deletions (Sha allele) are presented in black, those with one deletion are in dark gray, and those with two deletions (Bay allele) are shown in light gray. FW, Fresh weight.
reduction of the flux through sulfate assimilation, which consequently leads to the accumulation of sulfate. This is the same mechanism by which sulfate levels are increased in apr2 mutants (Loudet et al., 2007); lower APR activity also results in lower flux through the pathway (Mugford et al., 2011). APR was previously shown to possess almost complete control over the flux through primary sulfate assimilation (Vauclare et al., 2002); the analysis of atps1 mutants, however, shows that at least under some conditions, the flux can be limited also by ATPS. The experimental conditions in which the control coefficient of APR was determined were characterized by a 90% decrease in APR activity (Vauclare et al., 2002), which probably masked the contributions of other components of sulfate assimilation to the control of the flux. At comparable APR activity, reduction in ATPS can also result in a reduction in flux and so participate in the control of the pathway, as in the atps1 mutant (Fig. 2). Since APR is more strongly regulated than ATPS, at least in Arabidopsis (Takahashi et al., 2011), in most conditions it has greater control over the flux. ATPS becomes important when its levels are genetically manipulated, as in the atps1 mutant. However, this simple relationship between APR or ATPS and flux is true only within the same genetic background (e.g. apr2 or atps1 and the Col-0 wild type or the Bay and Sha alleles of HIF004; Figs. 1 and 2). For example, Shahdara and Col-0 differ significantly in APR activity, but not in the flux, whereas having the same APR activity, Bay-0 shows lower ATPS activity than Col-0 but higher flux (Fig. 2). This indicates that the control of flux through sulfate assimilation is much more complex than anticipated from control analyses focused on APR and ATPS and that other components, possibly not directly participating in sulfate reduction, also play an important role.

Interestingly, the levels of Cys and glutathione, the products of sulfate assimilation, were not affected in the atps1 plants, despite the reduced flux (Fig. 4). The effects of ATPS1 disruption on sulfuryl metabolism are thus very similar to the effects of the mutation in APR2, as in both atps1 and apr2 mutants glutathione levels in leaves do not differ from wild-type levels but the unused substrate, sulfate, accumulates (Loudet et al., 2007). A major difference between the two mutants, however, is the effect on glucosinolate synthesis rate. While the incorporation of 35S to these secondary compounds is not affected by the loss of APR2 (Mugford et al., 2011), the disruption of ATPS1 results in a significant reduction of the flux to glucosinolates (Fig. 4). This agrees with ATPS1 being a part of the glucosinolate synthesis network (Yatusevich et al., 2010). In contrast to the clear effect of reduced ATPS activity on glucosinolates in the atps1 mutant in the Col-0 background, their synthesis rate is identical in both ATPS1 alleles of HIF004. Given the large qualitative and quantitative variation in glucosinolate contents in Arabidopsis (Chan et al., 2011), the control of glucosinolate synthesis may differ in these different genetic backgrounds, so that ATPS and the primary assimilation are more important in Col-0 than in Bay-0 or Shahdara.

The identification of ATPS1 as the gene underlying the second major QTL is remarkable, as it reveals two consecutive steps in the same metabolic pathway to control variation in sulfate accumulation in the same RIL population. Each parent accession possesses one allele causing sulfate accumulation through a negative effect on the total activity of one of the two enzymes, in Shahdara APR2 and in Bay-0 ATPS1. The two alleles are independent and fully additive in their effect on sulfate levels (Table I). This was surprising, because previous analyses of flux through sulfate metabolism pointed to APR having a much stronger control than ATPS (Vauclare et al., 2002; Scheerer et al., 2010), so that a larger effect of the Bay allele of ATPS1 was expected in genotypes with the Sha allele of APR2 and so with lower APR activity. This would be analogous to the effect of nitrogen nutrition on sulfate levels in this RIL population: the effect of the Sha allele of APR2 was much stronger at low nitrate supply, when the total APR was down-regulated (Loudet et al., 2007). But this was not the case, showing that the two enzymes affect sulfate assimilation independently and that ATPS makes an important contribution to the flux control of the pathway.

The sulfate accumulation phenotype of genotypes with low APR or ATPS activity indicates an uncoupling of sulfate accumulation and the control of sulfate uptake. While low sulfate levels in leaves seem to be the trigger of the sulfate starvation response, including the induction of sulfate uptake (Lee et al., 2012; Matthewman et al., 2012), high sulfate levels are not a part of negative feedback regulation. Most probably, the sulfate accumulates in the vacuoles and is not available for cellular signaling. The adjustment of sulfate uptake to the plant’s need for sulfur thus seems to be mediated by compounds containing reduced sulfur, such as Cys or glutathione (Datko and Mudd, 1984; Vauclare et al., 2002). Thus, sulfate does not seem to possess the same signaling function as nitrate, despite many similarities between sulfate and nitrate assimilation and their regulation (Kopriva and Rennenberg, 2004).

While allelic variation in APR2 and ATPS1 contributes to the variation of sulfate levels, the mechanisms by which the two genes achieve this are different. The APR2 gene from Sha contains several SNPs in the coding region, with one of them leading to an amino acid change close to an active center of the corresponding enzyme. This alters the kinetic properties of the enzyme and leads to an almost complete loss of its activity (Loudet et al., 2007). Since APR2 is the major isoform of APR, the total activity in Shahdara is about 30% of the APR activity in Bay-0 or Col-0. On the other hand, the reduction in ATPS activity in Bay-0 is caused by a lower transcript accumulation of the major ATPS1 isoform. Interestingly, in an isogenic background, there seems to be a direct correlation between ATPS1
expression and ATPS activity. The complete loss of ATPS1 transcripts in the atps1 mutant (Col-0 background) reduced ATPS activity by 56%, whereas in the HIF004 lines, a 35% difference in the transcript levels of ATPS1 resulted in a 21% loss of activity. The lower mRNA accumulation in turn seems to be caused by two deletions in the ATPS1 gene, as the presence of these two deletions is correlated with high sulfate levels and lower ATPS1 expression among Arabidopsis accessions. This is similar to observations of a link between variation in the expression of ATPS1 and lower ATPS activity. Lower ATPS activity leads to a reduction in flux through primary sulfate assimilation and, consequently, to sulfate accumulation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Bay-0 × Shahdara RIL population of Arabidopsis (Arabidopsis thaliana) has been described previously (Loudet et al., 2002, 2007). For this work, the complete set of 411 genotyped RILs and the original 38 microsatellite markers map have been used, and QTL analyses and results were described earlier (Loudet et al., 2007). HIFs were employed as near isogenic lines as described previously (Loudet et al., 2005, 2007). RIL004 was still heterozygous for markers ATHCHIB2 and MSAT3.19; by screening the F7 seeds with these two markers, we have fixed the region for each parental allele, thereby generating a series of lines that differ only at the region of interest (HIF004-Bay, HIF004-Sha). Bay-0 × Shahdara material was obtained from the Versailles Stock Center (http://publiclines.versailles.inra.fr/). The transfer DNA insertion mutant in At3g22890 (atps1; GABI850C05) as well as wild-type Arabidopsis accessions were obtained from the Nottingham Arabidopsis Stock Centre. F1 plants for the quantitative complementation assay were generated by reciprocally crossing 004B to Col-0, 004B to Col-0, and 004S to Col-0. F1 plants were directly used for analysis and genotyped to verify that they were real F1 plants.

For the initial QTL analysis, the plants were grown for 35 d under a short-day photoperiod on a nonfertilized peat soil watered every other day as described (Loudet et al., 2007). For further analysis, plants were grown for 5 weeks in a controlled-environment room under a short-day 10 h-light/14 h-dark cycle at constant temperature of 22°C, 60% relative humidity, and light intensity of 160 µE s⁻¹ m⁻². For the sulfate uptake and flux analysis, the plants 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. For each experiment, at least two independent sets of plants were grown and analyzed.

Measurements of Sulfur-Containing Metabolites

For the QTL analysis, sulfate was measured in freeze-dried leaf material by HPLC as described earlier (Loudet et al., 2007). For further analysis, sulfate was determined as described (Lee et al., 2012). Thiols were determined in 20 to 30 mg of leaf material by a standard HPLC method as described (Koprivova et al., 2008).

Determination of Flux through Sulfate Assimilation

The flux through sulfate assimilation was measured as the incorporation of 35S from [35S]sulfate to thiols and proteins and to glucosinolates essentially as described (Mugford et al., 2011). Two-week-old plants were transferred onto 48-well plates containing 1 mL of Murashige and Skoog nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with 5.6 µCi of [35S]sulfate (Hartmann Analytic) and incubated in light for 4 h. After the 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. The quantification of 35S in different sulfur-containing compounds was performed exactly as described (Mugford et al., 2011).

Expression Analysis

To determine mRNA levels, total RNA was isolated by standard phenol/chloroform and LiCl precipitation. First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the Quant iTect Reverse Transcription Kit (Qiagen), which includes a DNase step to remove possible DNA contamination. Quantitative real-time reverse transcription (RT)-PCR was performed using gene-specific primers (Supplemental Table S1) and the fluorescent intercalating dye SYBR Green (Applied Biosystems) as described (Lee et al., 2011). All quantifications were normalized to the TIP41 gene. RT-PCR was performed in duplicate for each of three independent samples.

Pyrosequencing was used to assess the relative contribution of each allele to the population of mRNA in F1 individuals from reciprocal crosses between Bay-0 and Shahdara, as described previously (Pineau et al., 2012; Silveira et al., 2013). In summary, a pyrosequencing reaction using the primers F1 (PCR), 5′-AACCTCCGAGGTTCTATG-3′, R1-biotin (PCR), 5′-CCTTCTTTTGATGGTTATAAAGTCCACTCGGAGGTTTCATG-3′ (biotinylated), and S2 (pyrosequencing), 5′-CAGGTGCTGCTCTTT-3′, was set up on a coding SNP polymorphic between parents (5′-TTT[C/T]ATTCGGATGTG-3′). Pyrosequencing was performed on F1 cDNA as well as on 1:1 pools of parent cDNA to establish the allelic contribution to ATPS1 expression. F1 genomic DNA is used as a pyrosequencing control to normalize against possible pyrosequencing biases. Similarly, HIF004 heterozygous individuals were used to confirm the regulation. Anything significantly driving allele-specific expression in hybrids is, by definition, acting in cis, since F1 nuclei contain a mix of all trans-acting factors (Wittkopp et al., 2004).

Enzyme Assays

APR activity was determined as the production of [35S]urate, assayed as acid-volatile radioactivity formed in the presence of [35S]APS and dithioerythritol as reductant (Lee et al., 2011). ATPS was measured as the APS and pyrophosphate-dependent formation of ATP (Kawashima et al., 2011). The protein concentrations were determined according to Bradford with bovine serum albumin as a standard (Bradford, 1976).

Supplemental Data

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

Supplemental Figure S1. Sequence comparison of the ATPS1 gene from Bay-0, Sha, and Col-0.

Supplemental Figure S2. Sequence comparison of the two deletions in the ATPS1 gene from nine Arabidopsis accessions.

Supplemental Table S1. Primer sequences for expression analysis by qPCR.

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LITERATURE CITED


