

# Natural variation for sulfate content in *Arabidopsis thaliana* is highly controlled by APR2

Olivier Loudet<sup>1</sup>, Vera Saliba-Colombani<sup>2</sup>, Christine Camilleri<sup>1</sup>, Fanny Calenge<sup>2</sup>, Virginie Gaudon<sup>2</sup>, Anna Koprivova<sup>3</sup>, Kathryn A North<sup>3</sup>, Stanislav Kopriva<sup>3</sup> & Françoise Daniel-Vedele<sup>2</sup>

**Most agronomic traits of importance, whether physiological (such as nutrient use efficiency) or developmental (such as flowering time), are controlled simultaneously by multiple genes and their interactions with the environment. Here, we show that variation in sulfate content between wild *Arabidopsis thaliana* accessions Bay-0 and Shahdara is controlled by a major quantitative trait locus that results in a strong interaction with nitrogen availability in the soil. Combining genetic and biochemical results and using a candidate gene approach, we have cloned the underlying gene, showing how a single-amino acid substitution in a key enzyme of the assimilatory sulfate reduction pathway, adenosine 5'-phosphosulfate reductase, is responsible for a decrease in enzyme activity, leading to sulfate accumulation in the plant. This work illustrates the potential of natural variation as a source of new alleles of known genes, which can aid in the study of gene function and metabolic pathway regulation. Our new insights on sulfate assimilation may have an impact on sulfur fertilizer use and stress defense improvement.**

**np** *Brassica* species are among the crops with the highest demand for sulfur and need for sulfur fertilizer<sup>1</sup>, which is associated with high sulfate content in green tissues<sup>2</sup>. To improve the efficiency of sulfur use in *Brassica*, we must understand the genetic and environmental controls of sulfate accumulation. Therefore, we turned to *Arabidopsis thaliana*, which has proven to be an excellent model for *Brassica* crops and also accumulates sulfate. As a quantitative trait, sulfate content variation can be analyzed through quantitative trait locus (QTL) analysis in recombinant inbred line (RIL) sets derived from crosses between wild accessions, which show tremendous variation for most physiological traits studied<sup>3</sup> and are the material of choice to analyze genotype-environment interaction.

In our analysis of natural variation for shoot sulfate content in *A. thaliana*, we find substantial variation among lines originating from the cross between Bay-0 (collected near Bayreuth, Germany) and Shahdara (from the high Shokhdara Valley in Tajikistan), using an RIL population previously established by our group<sup>4</sup> and already

extensively used to characterize growth and nitrogen metabolism (for example, ref. 5). Transgression in the phenotypic values of the RILs compared with the parents was obvious in both a high-nitrogen and a low-nitrogen environment. However, the distribution of phenotypic values in the latter showed signs of bimodality around parental phenotypes (Fig. 1a), suggesting the role of a major-effect locus. The QTL analysis (Fig. 1b and Supplementary Table 1 online) confirmed that among many detected loci, several of which were found in both environments and showed phenotypic effects in both directions, one had a predominant role, especially in the low-nitrogen environment: SO3.1, which explained as much as 48% of the total phenotypic variance. The same locus explained only 21% of the variation in a high-nitrogen environment (SO10.1) (Supplementary Table 1), demonstrating a marked QTL × nitrogen environment interaction pattern confirmed by analysis of variance (ANOVA) ( $P < 0.01$ ; data not shown). As this cannot be totally accounted for by changes in other QTL effects, the SO3.1 locus clearly has an increased impact on sulfate content when nitrogen availability is limited in the soil, becoming a major-effect QTL under strongly growth-limiting nitrogen conditions.

Taking advantage of the residual heterozygosity segregating in some of the F<sub>7</sub> RILs, we developed heterogeneous inbred families<sup>6</sup> (HIFs) to quickly obtain appropriate nearly isogenic lines (NIL) and confirm the importance of the candidate marker interval (NGA128–F5I14), which includes the QTL support interval (Fig. 1c,d). Sulfate content variation was linked to the allelic segregation of this 4-Mb region, showing that the Shahdara allele at this locus is responsible for sulfate accumulation and acts in a recessive manner (Fig. 1d). In a search for candidate genes in the interval, we extrapolated the position of SO3.1 on the physical map from the relative genetic positions of the LOD score peak and the initial flanking markers (NGA128–F5I14) and realized that it was predicted just 20 kb away from the gene encoding adenosine 5'-phosphosulfate reductase (*APR2*, also known as *At1g62180*), one of the three isoforms of a key enzyme of assimilatory sulfate reduction pathway<sup>7,8</sup>. To investigate the possible role of this gene in sulfate content variation between Bay-0 and Shahdara, we measured its expression in the rosette of HIF plants and did not find any significant difference (data not shown). We then

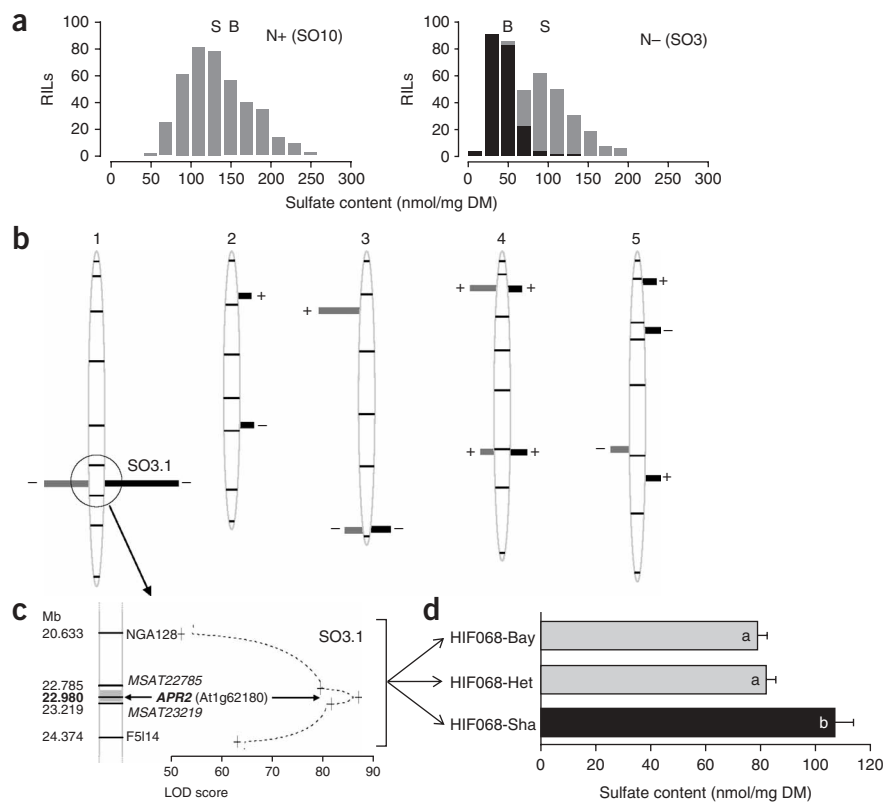
<sup>1</sup>Station de Génétique et Amélioration des Plantes (SGAP), Institut National de la Recherche Agronomique (INRA), Rte. de St. Cyr, Versailles 78026, France.

<sup>2</sup>Laboratoire de Nutrition Azotée des Plantes (NAP), Institut National de la Recherche Agronomique (INRA), Rte. de St. Cyr, Versailles 78026, France. <sup>3</sup>John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK. Correspondence should be addressed to O.L. (loudet@versailles.inra.fr).

Received 9 April; accepted 25 April; published online 24 June 2007; doi:10.1038/ng2050

**Figure 1** Mapping and confirmation of the SO3.1 shoot sulfate content QTL. (a) Distribution of the phenotypic values among the 411 Bay-0 × Shahdara RILs. Left, frequency of phenotypes in control nitrogen environment (N+); right, frequency of phenotypes in nitrogen-limiting environment (N-). Parental values are indicated above bars ('B', Bay-0; 'S', Shahdara). To help in visualizing N- phenotype bimodality and the major QTL effect, we have *a posteriori*

represented the distribution of Bay-0 (black) and Shahdara (gray) genotypes at the SO3.1 locus among RILs. (b) QTL map obtained from QTL Cartographer 'composite interval mapping' results for sulfate content in each environment. QTLs are depicted by bars beside each of the five chromosomes (N+: gray bars, left; N-, black bars, right) at the most probable positions. Bar length is proportional to the QTL's contribution to total phenotypic variation ( $R^2$ ). The sign of the allelic effect (2a) is indicated for each QTL ('2a' represents the mean effect of the replacement of both Shahdara alleles by Bay-0 alleles at the QTL; see **Supplementary Table 1**). The framework genetic map (where horizontal marks indicate marker positions) is from ref. 4. Major-effect QTL SO3.1 is circled. (c) Mapping SO3.1 and the candidate gene. SO3.1 is shown between markers NGA128 and F5114. Left: a physical representation of chromosome 1. Right: a LOD score curve (dashed line). Three additional markers in the interval were used to draw a more precise map around the *APR2* candidate gene (MSAT22785 and MSAT23219 on each side and one dCAPS marker placed directly on the *APR2* gene). Marker positions are indicated by thin horizontal lines on the chromosome (with physical positions in Mb) and '+' on the LOD score curve. Gray area indicates the two-LOD drop support interval drawn from the analysis of the LOD score curve around the peak (located directly at the *APR2* locus). (d) Confirmation of SO3.1 in nearly isogenic lines. RIL068 still segregates for the NGA128–F5114 interval, including the SO3.1 support interval. Sulfate content in a low-nitrogen environment is shown as mean ± s.e.m. of several plants (24 plants pooled in six replicates), where the segregating region was fixed for each of the two homozygous alleles or was still heterozygous (HIF068-Bay, -Sha or -Het). Letters on bars indicate significantly different means ( $P < 0.01$ ; least significant difference test). DM, dry matter.

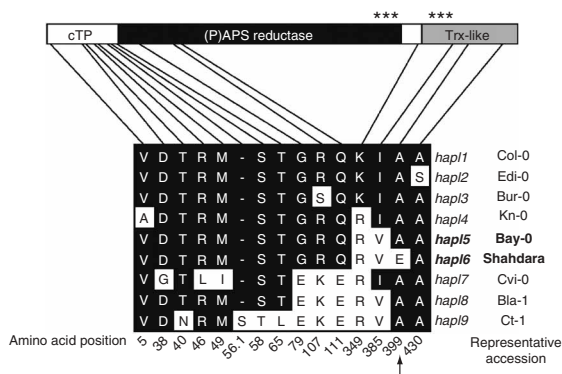


sequenced the Bay-0 and Shahdara alleles of *APR2* and found only one single-SNP difference in the coding sequence, which resulted in an amino acid change in a conserved domain of the protein (**Fig. 2**). This substitution of alanine with glutamate might interfere with binding of the reductant to the thioredoxin-like active site<sup>7</sup>. To confirm that this gene was a good positional candidate, we included three additional markers in the QTL mapping process: one on each side of *APR2* (just 200 kb away) and one using a dCAPS marker on the *APR2* SNP. The maximum of the reanalyzed LOD score curve (**Fig. 1c**) coincided perfectly with the *APR2* gene and dropped very rapidly as we moved away from it (even by a few dozen kilobases), suggesting that *APR2* was a very good candidate for SO3.1. To assess the importance of the sequence change detected in this gene, we sequenced 32 more accessions from a core collection defined to represent the greatest diversity within the species<sup>9</sup>. From this, we identified more amino acid variation in the *APR2* protein (some potentially affecting major domains of the protein and some shared across haplotypes), defining a total of nine distinct haplotypes, including the Col-0 reference protein sequence. Haplotype #6 was present only in Shahdara. To increase our understanding of the sequence variation, we focused on the unique polymorphism between the Bay-0 and the Shahdara amino acid sequences and extended the typing for this nucleotide to the complete core collection of 48 accessions<sup>9</sup>. In addition, we sequenced a series of recently collected populations from Central Asia (described at <http://www.inra.fr/vast/collections.htm>) to increase the chance of

capturing more accessions related to the Tajik Shahdara. Among a total of 62 accessions, the Shahdara polymorphism remains a singleton (data not shown).

With such a strong positional and functional candidate gene identified, we used a transgenic complementation strategy to directly test the hypothesis that *APR2* is the SO3.1 QTL. Knowing that the Bay-0 allele at SO3.1 is dominant over the Shahdara allele, we transformed the original HIF068 containing the Shahdara allele in the QTL region (HIF068-Sha) with the Bay-0 allele of *APR2* (with the resulting lines denoted HIF068-Sha [*APR2*-Bay]). This completely restored Bay-0-level sulfate content in the plants (**Fig. 3**), as none of the six independent transformants tested had a significantly different sulfate content from HIF068-Bay in a low-nitrogen environment. We conclude that SO3.1 can be fully explained by *APR2* allelic variation. In the absence of expression differences between the Shahdara and Bay-0 alleles, the unique amino acid change in the Shahdara *APR2* protein is very likely to represent the causative quantitative trait nucleotide (QTN).

To confirm these findings independently, we analyzed a mutant with a transfer DNA (T-DNA) insertion in *APR2* (*apr2-1*) available in a different genetic background (Col-0) and found that the homozygous mutant accumulated 2.5 times more sulfate than the wild-type (data not shown). This indicates that the absence of the *APR2* protein results in greater sulfate accumulation in this *apr2-1* mutant (relative to its wild-type background) than is caused by variation of the *APR2* sequence in Shahdara (relative to Bay-0). To further confirm that



**Figure 2** Amino acid changes along the APR2 protein sequence detected within a core collection of 32 *Arabidopsis thaliana* accessions designed to represent a maximum of the total SNP diversity<sup>9</sup>. Upper panel shows different domains of the protein (cTP = transit peptide; APS reductase = adenosine phosphosulfate reductase; Trx-like = thioredoxin-like domain); active centers within these domains are indicated by asterisks<sup>7</sup>. Below is a table summarizing all polymorphic amino acids (columns) detected among accessions. Each row represents a haplotype (hapl) illustrated by one representing accession. Numbers at bottom are the polymorphic amino acid positions along the protein. Identical amino acids are shaded in black; substitutions and one insertion (56.1) are shown in white; Col-0 sequence serves as reference. The arrow indicates the unique polymorphism between Bay-0 and Shahdara (amino acid 399).

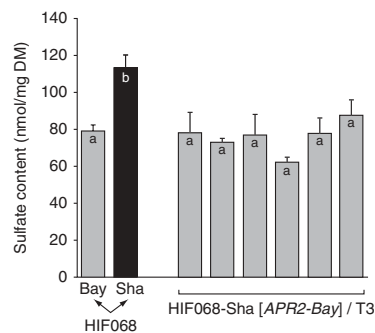
APR2 is SO3.1, we performed a quantitative complementation test<sup>10</sup> by comparing the different alleles in F<sub>1</sub> plants obtained after crossing each HIF068 genotype to either the T-DNA mutant line or its wild-type background. Here we compare the relative complementation of the APR2 mutant phenotype, by either a Bay-0 or a Shahdara allele, with respect to the wild-type, on a one-for-one allelic basis and in a nearly isogenic (heterozygous) genetic background. The Shahdara allele at SO3.1 was unable to restore low-sulfate contents like the Bay-0 allele does (Fig. 4). This failure to complement was statistically confirmed by the very high significance of the QTL allele  $\times$  APR2 genotype interaction term (ANOVA;  $P < 0.00001$ ). This result further proves that SO3.1 and the APR2 gene either interact or are identical, and, when considered with the mapping and transgenic complementation data, it leads us to conclude that APR2 is SO3.1. Again, the F<sub>1</sub> plant bearing a Shahdara allele with a T-DNA genotype accumulated much less sulfate than the homozygous T-DNA line (data not shown), probably indicating that the Shahdara allele of APR2 is not completely inactive. This leaves the following question unanswered: at what level does the change in Shahdara explain the observed variation? The discovery of both the gene and the QTN underlying the sulfate content QTL placed us in a favorable situation to answer this question and identify the functional mechanism(s) resulting in such a quantitative phenotype<sup>11</sup>.

Measuring total APR enzyme activity *in vitro* in protein extracts from leaves of NILs and the parental accessions showed that the enzyme is three to four times less active in genotypes containing the Shahdara allele at the QTL than in genotypes containing the Bay-0 allele. Transforming HIF068-Sha with the Bay allele of APR2 consistently increased APR activities above HIF068-Sha values in all transformants tested (Fig. 5), confirming the functional link between the level of APR activity (probably with respect to some threshold) and the accumulation of sulfate in the plant. Consistent with the sulfate content results, the *apr2-1* mutant showed a more marked reduction in APR activity than the HIFs (Fig. 5). Thus, the QTN causes the reduction in total APR activity in Shahdara despite affecting only one of three isoforms of the enzyme.

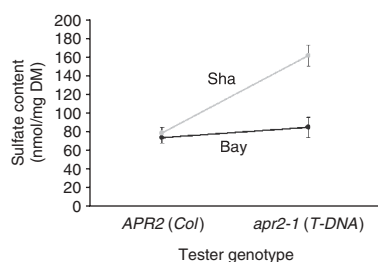
To understand how the QTN alters the property of the enzyme, we heterologously expressed APR2 from Bay-0 and Shahdara in *Escherichia coli* and analyzed the purified recombinant proteins. The Shahdara APR2 retained some enzymatic activity; its  $V_{max}$  was approximately 0.2% of the  $V_{max}$  of recombinant proteins derived from Bay-0 or Col-0. As predicted from the nature of the amino acid change caused by the QTN, the lower velocity was caused by less efficient interaction of the recombinant Shahdara protein with the electron donor for APS reduction. This was demonstrated by a higher  $K_M$  for glutathione for the Shahdara APR2 (32 mM) than for the Bay-0 APR2 ( $K_M = 13$  mM) but an unchanged binding of APS between the two (Supplementary Fig. 1 online).

The biochemical analysis clearly shows that the SO3.1 sulfate content QTL is fully explained by a modulation in APR2 activity due to a single-amino acid change in Shahdara. The allelic effect tends to be stronger with nitrogen limitation in the soil, probably owing to the tight interconnection of sulfate assimilation with nitrogen metabolism<sup>12</sup>, providing an interesting example of physiological QTL-environment interaction. Indeed, under conditions of low nitrogen availability, APR activity and mRNA accumulation of all three APR isoforms are reduced compared with accumulation under high-nitrogen conditions<sup>12</sup>. Notably, it is only under nitrogen-limiting conditions that the parental accession Shahdara accumulates more sulfate than Bay-0 (Fig. 1a) and many other accessions (data not shown). Furthermore, only the Shahdara APR2 haplotype substantially reduced the total APR activity (data not shown). It seems that under high-nitrogen conditions, the APS reduction capacity in Shahdara is adequate to prevent sulfate accumulation, but when the expression of APR is reduced owing to the effect of low-nitrogen conditions, the activity is no longer sufficient, and sulfate accumulates. In addition, the effect of SO3.1 cannot be balanced by the other segregating sulfate QTLs.

Furthermore, accumulation of the major low-molecular weight thiol, glutathione, which is important for plant stress defense<sup>13</sup>, was lower in Shahdara than in Bay-0 leaves (Supplementary Fig. 2 online). Glutathione synthesis is dependent on availability of cysteine, so this finding could be seen as an indirect effect of reduction of APR activity in Shahdara<sup>14</sup>. Notably, however, we did not observe any difference in glutathione content in the NILs (Supplementary Fig. 2),



**Figure 3** Transgenic complementation test for SO3.1 QTL. Shoot sulfate content measured in the low-nitrogen environment for the nearly isogenic lines (HIF068) and for six independent transformants. Transformants represent the original nearly isogenic line fixed for the Shahdara allele at the QTL region (HIF068-Sha) transformed with the Bay-0 allele of APR2. Data are shown as mean  $\pm$  s.e.m. Different letters on bars indicate significantly different means ( $P < 0.05$ ; least significant difference test). DM, dry matter.



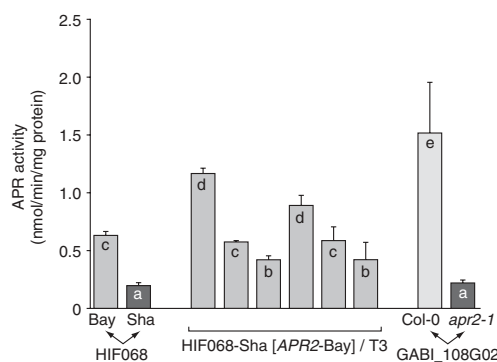
**Figure 4** Quantitative complementation test for SO3.1 QTL. Shoot sulfate content measured in a low-nitrogen environment for each of the four allelic combinations obtained in  $F_1$  plants, generated from the crossing of HIF068-Bay or HIF068-Sha with an *apr2-1* mutant or with its wild-type background (Col-0). Each data point represents the mean  $\pm$  s.e.m. of six plants pooled in three replicates for each reciprocal cross (12 plants total). The SO3.1 allele  $\times$  APR2 genotype interaction term is very highly significant ( $P < 0.00001$ ). DM, dry matter.

showing that the control of thiol content is at least partly uncoupled from the control of sulfate reduction and implying an even more important role for *O*-acetylserine, the acceptor of sulfide in cysteine synthesis, in the control of the pathway than previously anticipated<sup>15</sup>. This finding should be taken into account when developing strategies to increase glutathione content in plants in order to improve stress tolerance and detoxification capacity, and it shows that the flux control of sulfate assimilation<sup>8</sup> must be revisited.

In summary, we have (i) described a QTL for sulfate content, (ii) identified the QTN in the *APR2* gene using a candidate gene approach and (iii) demonstrated its effect on the enzymatic properties of the corresponding gene product. Thus, major variation in a quantitative physiological trait is clearly related to molecular and biochemical changes in a single protein. These results shed new light on the relationships between sulfate reduction and accumulation, as we have found that naturally occurring variation at one of the main sulfate assimilation enzymes directly affects sulfate homeostasis in the plant. The analysis of the Shahdara *APR2* allele illustrates the power of exploring natural variation at already known genes as a source of new alleles complementary to loss-of-function mutants. Moreover, the Shahdara QTL allele would not have been detected by association studies, as it is a very rare allele (if not a singleton). Of note, the previously cloned 'EDI' QTL in *A. thaliana* (corresponding to the *CRY2* gene) also represents a very rare allele with large effect<sup>16</sup>. With the recently described genome-wide excess of rare polymorphisms<sup>17</sup> in *A. thaliana* that are likely to result in singletons or at least in genetic heterogeneity, quantitative genetics must be thought of as a combination of both approaches: QTL mapping in specific crosses and association studies among accessions<sup>18</sup>. This will lead to a better understanding of natural variation at the phenotypic and molecular levels. Finally, the identification of a modulation in *APR2* activity as responsible for controlling sulfate accumulation is a notable step toward increasing the efficiency of sulfur use in crop plants, as efficiency is negatively affected by high sulfate content, especially in *Brassica* crops. Where needed, this kind of variation could be induced or detected in crop species using a TILLING<sup>19</sup> or EcoTILLING<sup>20</sup> strategy, respectively.

## METHODS

**Plant material.** The Bay-0  $\times$  Shahdara RIL population has been fully described and is available to the community from the VNAT website (<http://dbsgap.versailles.inra.fr/vnat/>). It was generated from a cross between single seed



**Figure 5** Total adenosine 5'-phosphosulfate reductase (APR) activities. Enzymatic activities (mean  $\pm$  s.e.m.) were measured *in vitro* on the same genotypes and material as in **Figure 3**, plus a T-DNA insertion line in *APR2* (*apr2-1* mutant) and a wild-type plant of the same background (Col-0). Bars labeled with different letters differ significantly from each other ( $P < 0.05$ ; least significant difference test).

descent (SSD) lines for accessions Bay-0 (collected near Bayreuth, Germany; stock center reference N954) and Shahdara (from the high Shokhdara Valley in Tajikistan; stock center reference N929). Six generations of selfing through SSD were performed until  $F_7$ . For this work, we have used the complete set of 411 genotyped RILs and the original 38-microsatellite marker map<sup>4</sup>.

Many of the  $F_7$  RILs are still individually segregating for one specific region. We took advantage of this to generate nearly isogenic lines in the form of heterogeneous inbred families (HIFs) as previously described<sup>21</sup>. RIL068 is still heterozygous for markers NGA128 and F5114; by screening the  $F_7$  seeds with these two markers, we have fixed the region for each parental allele, thereby generating two lines that differ only at the region of interest (HIF068-Bay and HIF068-Sha). HIF068-Het plants (used in **Fig. 1d**) were obtained by direct progeny testing (genotyping and phenotyping) of RIL068.

The T-DNA insertion mutant in At1g62180 (*apr2-1*) was ordered from the GABI\_Kat program<sup>22</sup> as GABI\_108G02.

Other accessions used for sequencing the At1g62180 coding sequence were as described previously<sup>9</sup> and can be ordered from the VNAT website (<http://dbsgap.versailles.inra.fr/vnat/>).

$F_1$  plants for the quantitative complementation assay were generated by reciprocally crossing HIF068-Bay to *apr2-1*, HIF068-Sha to *apr2-1*, HIF068-Bay to Col-0 and HIF068-Sha to Col-0.  $F_1$  seeds were used directly for phenotyping, and each individual plant was genotyped to ensure it was a real  $F_1$  with the expected allelic combination at SO3.1 and *APR2*.

**Plant growth and sulfate content measurements.** Plants were grown under the same conditions as described earlier<sup>5</sup>. In summary, they were grown under a short-day photoperiod for 35 d on a nonfertilized peat soil watered every other day. Two environments were compared in each experiment depending on the amount of nitrate in the watering solution: 'N+' conditions (10 mM nitrate) did not limit plant growth, whereas 'N-' conditions (3 mM nitrate) corresponded to a growth-limiting environment. After harvesting the vegetative shoot tissue, we freeze-dried the material and ground it for ethanol-water extraction of the anions, the concentrations of which were estimated by HPLC, as described earlier<sup>5</sup>. Sulfate amounts were measured on the same extracts as were used for other anion estimates in previously reported Bay-0  $\times$  Shahdara QTL mapping studies<sup>5,23</sup>. Sulfate contents were expressed in terms of nanomoles of sulfate per milligram of shoot dry matter.

**QTL mapping and statistical analysis.** For each of the 411 RILs included in the analysis, the phenotypic value was the mean of the phenotype obtained from three complete and independent replications of the cultivation cycle and sulfate content measurements. Again, the trait was analyzed as previously described<sup>5</sup> using QTL Cartographer (<http://statgen.ncsu.edu/qtlcart/>). Composite Interval Mapping (CIM) was performed on the trait measured in each environment, including a maximum of six cofactors. The LOD significance threshold

(2.3 LOD) was estimated from permutation test analyses, as suggested in ref. 24. The contribution of each identified QTL ( $R^2$  in **Supplementary Table 1**) was calculated by ANOVA, using the closest markers as random factors. When significant, QTL  $\times$  QTL epistatic interactions were also included in the linear model via the corresponding marker  $\times$  marker interaction component. Allelic effects (referred to as '2a' in **Supplementary Table 1**) were obtained from QTL Cartographer. The SO3.1 confidence interval was estimated from the conservative two-LOD drop support interval. QTL  $\times$  environment interaction was tested in an ANOVA including phenotypic data from both environments and the genotype at the marker linked to SO3.1 and SO10.1 (that is, F5114).

Reanalysis of QTL mapping (**Fig. 1c**) was performed using the same protocol after the following polymorphic markers were genotyped and added to the genetic map interval: MSAT22785, MSAT23219 and dCAPS-APR2 (digested with *TaqI*). See **Supplementary Table 2** online for primer sequences.

**Sequencing APR2.** The APR2 gene was amplified in three overlapping fragments from 32 accessions of the core collection from ref. 9. Genomic DNA was extracted from leaves as described previously<sup>4</sup>. Pairs of primers used for PCR and sequencing were APR2Seq1, APR2Seq2 and APR2Seq3 (**Supplementary Table 2**).

**Transgenic complementation.** Primers APR2Seq1-F and APR2Seq3-R (**Supplementary Table 2**) were used to amplify a complete APR2 gene from genomic DNA extracted from Bay-0 leaves. PCR products (3,135 bp) were cloned into the pGEMT-easy vector, and one clone was fully sequenced and compared with the Bay-0 APR2 sequence. After digestion with *NotI*, the insert was then purified and subsequently cloned in a pGREEN vector<sup>25</sup>. Binary recombinant vectors were then introduced into *Agrobacterium tumefaciens* C58C1 (pMP90), and HIF068-Sha plants were transformed as already described<sup>26</sup>. Homozygous transformants containing one copy of the resistance gene were selected on hygromycin B (20 mg/ml).

**APR activity, recombinant protein assays and thiol measurements.** Total APR activity was measured as the production of [<sup>35</sup>S]sulfite, assayed as acid volatile radioactivity formed in the presence of [<sup>35</sup>S] adenosine 5'-phosphosulfate (APS) and dithioerythritol (DTE) or glutathione as reductants<sup>27</sup>. The protein concentrations were determined according to the Bradford method with BSA as a standard.

The coding regions of APR2 from *A. thaliana* accessions Col-0, Bay-0, and Shahdara were amplified from total RNA isolated from leaves of these ecotypes by RT-PCR with primers APR2EN and APR123EC (**Supplementary Table 2**), which included restriction sites for *NdeI* and *BamHI*, respectively, to enable cloning into the expression vector pET-14b (Novagen). After amplification and digestion, the fragment was ligated into pET-14b plasmid and transformed into *Escherichia coli* BL21(DE3) cells. The APR2 coding insert in recombinant pET plasmid was completely sequenced to ensure that no mutations had been introduced in the course of PCR amplification. The recombinant mature APR2 proteins, carrying an N-terminal extension coding for a six-histidine (His) tag, were purified from 50-ml overnight cultures grown at 30 °C on the pET His Tag System (Novagen) according to the manufacturer's instructions. To determine the biochemical parameters of APR2 proteins, the APR activity was measured at different concentration of APS (2.5–37.5  $\mu$ M) and GSH (2–20 mM) with 4 ng (Bay-0) or 1  $\mu$ g (Shahdara) purified recombinant APR2. To calculate apparent  $K_M$  and  $V_{max}$ , we linearized the data according to Lineweaver and Burk.

Glutathione was extracted from leaves by grinding 0.1 g of frozen material in 1 ml of 0.1 M HCl. After centrifugation at 20,000g for 10 min, the supernatant was used to measure the content of glutathione by HPLC using the monobromobimane derivatization method as described in ref. 28.

Note: Supplementary information is available on the Nature Genetics website.

#### ACKNOWLEDGMENTS

We thank J. Talbotec for taking care of the plants. We thank J. Chory, D. Weigel and T.P. Michael for discussions and comments on the manuscript. This work was supported by a European grant to F.D.-V. ('Natural' project number QLRT-2000-01097, 2002–2005). Research in S.K.'s laboratory at the John Innes Centre is supported by the Biotechnology and Biological Sciences Research Council (BBSRC).

#### AUTHOR CONTRIBUTIONS

O.L., S.K. and F.D.-V. conceived the experiments; O.L., V.S.-C., C.C., F.C., V.G., A.K., K.A.N. and S.K. performed the experiments; O.L., V.S.-C., C.C., S.K. and F.D.-V. analyzed the data and O.L., S.K. and F.D.-V. wrote the manuscript.

#### COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Published online at <http://www.nature.com/naturegenetics>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

- Hawkesford, M.J. & de Kok, L.J. Managing sulphur metabolism in plants. *Plant Cell Environ.* **29**, 382–395 (2006).
- McGrath, S.P. & Zhao, F.J. Sulphur uptake, yield response and the interactions between N and S in winter oilseed rape (*Brassica napus*). *J. Agric. Sci.* **126**, 53–62 (1996).
- Koornneef, M., Alonso-Blanco, C. & Vreugdenhil, D. Naturally occurring genetic variation in *Arabidopsis thaliana*. *Annu. Rev. Plant Biol.* **55**, 141–172 (2004).
- Loudet, O., Chaillou, S., Camilleri, C., Bouchez, D. & Daniel-Vedele, F. Bay-0  $\times$  Shahdara recombinant inbred line population: a powerful tool for the genetic dissection of complex traits in *Arabidopsis*. *Theor. Appl. Genet.* **104**, 1173–1184 (2002).
- Loudet, O., Chaillou, S., Merigout, P., Talbotec, J. & Daniel-Vedele, F. Quantitative Trait Loci analysis of nitrogen use efficiency in *Arabidopsis*. *Plant Physiol.* **131**, 345–358 (2003).
- Tuinstra, M.R., Ejeta, G. & Goldsborough, P.B. Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci. *Theor. Appl. Genet.* **95**, 1005–1011 (1997).
- Kopriva, S. & Koprivova, A. Plant adenosine 5'-phosphosulphate reductase: the past, the present, and the future. *J. Exp. Bot.* **55**, 1775–1783 (2004).
- Vauclaire, P. *et al.* Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5'-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. *Plant J.* **31**, 729–740 (2002).
- McKhann, H.I. *et al.* Nested core collections maximizing genetic diversity in *Arabidopsis thaliana*. *Plant J.* **38**, 193–202 (2004).
- Mackay, T.F. Complementing complexity. *Nat. Genet.* **36**, 1145–1147 (2004).
- Salvi, S. & Tuberosa, R. To clone or not to clone plant QTLs: present and future challenges. *Trends Plant Sci.* **10**, 297–304 (2005).
- Koprivova, A., Suter, M., Op den Camp, R., Brunold, C. & Kopriva, S. Regulation of sulfate assimilation by nitrogen in *Arabidopsis thaliana*. *Plant Physiol.* **122**, 737–746 (2000).
- Noctor, G. *et al.* Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *J. Exp. Bot.* **49**, 623–647 (1998).
- Strohm, M. *et al.* Regulation of glutathione synthesis in leaves of transgenic poplar (*Populus tremula*  $\times$  *P. alba*) overexpressing glutathione synthetase. *Plant J.* **7**, 141–145 (1995).
- Hesse, H., Nikiforova, V., Gakiere, B. & Hoefgen, H. Molecular analysis and control of cysteine biosynthesis: integration of nitrogen and sulphur metabolism. *J. Exp. Bot.* **55**, 1283–1292 (2004).
- El-Din El-Assal, S., Alonso-Blanco, C., Peeters, A.J., Raz, V. & Koornneef, M. A QTL for flowering time in *Arabidopsis* reveals a novel allele of *CRY2*. *Nat. Genet.* **29**, 435–440 (2001).
- Nordborg, M. *et al.* The pattern of polymorphism in *Arabidopsis thaliana*. *PLoS Biol.* **3**, e196 (2005).
- Weigel, D. & Nordborg, M. Natural variation in *Arabidopsis*: how do we find the causal genes? *Plant Physiol.* **138**, 567–568 (2005).
- Slade, A.J., Fuerstenberg, S.I., Loeffler, D., Steine, M.N. & Facciotti, D. A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nat. Biotechnol.* **23**, 75–81 (2005).
- Mejhed, N. *et al.* EcoTILLING for the identification of allelic variation in the powdery mildew resistance genes *mlo* and *Mla* of barley. *Plant Breed.* **125**, 461–467 (2006).
- Loudet, O., Gaudon, V., Trubuil, A. & Daniel-Vedele, F. Quantitative trait loci controlling root growth and architecture in *Arabidopsis thaliana* confirmed by heterogeneous inbred family. *Theor. Appl. Genet.* **110**, 742–753 (2005).
- Rosso, M.G. *et al.* An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol. Biol.* **53**, 247–259 (2003).
- Loudet, O., Chaillou, S., Krapp, A. & Daniel-Vedele, F. Quantitative trait loci analysis of water and anion contents in interaction with nitrogen availability in *Arabidopsis thaliana*. *Genetics* **163**, 711–722 (2003).
- Churchill, G.A. & Doerge, R.W. Empirical threshold values for quantitative trait mapping. *Genetics* **138**, 963–971 (1994).
- Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. & Mullineaux, P.M. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* **42**, 819–832 (2000).
- Clough, S.J. & Bent, A.F. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
- Brunold, C. & Suter, M. Adenosine 5'-phosphosulfate sulfotransferase. In *Methods in Plant Biochemistry* Vol. 3 339–343 (P. Lea, ed.) (Academic, London, 1990).
- Creissen, G. *et al.* Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *Plant Cell* **11**, 1277–1291 (1999).