Effects of **fou8/fry1** Mutation on Sulfur Metabolism: Is Decreased Internal Sulfate the Trigger of Sulfate Starvation Response?

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**Abstract**

The **fou8** loss of function allele of adenosine bisphosphate phosphatase **FIERY1** results in numerous phenotypes including the increased enzymatic oxygenation of fatty acids and increased jasmonate synthesis. Here we show that the mutation causes also profound alterations of sulfur metabolism. The **fou8** mutants possess lower levels of sulfated secondary compounds, glucosinolates, and accumulate the desulfo-precursors similar to previously described mutants in adenosine 5′-phosphosulfate kinase. Transcript levels of genes involved in sulfate assimilation differ in **fou8** compared to wild type Col-0 plants and are similar to plants subjected to sulfate deficiency. Indeed, independent microarray analyses of various alleles of mutants in **FIERY1** showed similar patterns of gene expression as in sulfate deficient plants. This was not caused by alterations in signalling, as the **fou8** mutants contained significantly lower levels of sulfate and glutathione and, consequently, of total elemental sulfur. Analysis of mutants with altered levels of sulfate and glutathione confirmed the correlation of sulfate deficiency-like gene expression pattern with low internal sulfate but not low glutathione. The changes in sulfur metabolism in **fou8** correlated with massive increases in 3′-phosphoadenosine 5′-phosphate levels. The analysis of **fou8** thus revealed that sulfate starvation response is triggered by a decrease in internal sulfate as opposed to external sulfate availability and that the presence of desulfo-glucosinolates does not induce the glucosinolate synthesis network. However, as well as resolving these important questions on the regulation of sulfate assimilation in plants, **fou8** has also opened an array of new questions on the links between jasmonate synthesis and sulfur metabolism.


**Editor:** Juergen Kroymann, French National Centre for Scientific Research, Université Paris-Sud, France

**Received** January 13, 2012; **Accepted** May 21, 2012; **Published** June 18, 2012

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**Funding:** SK’s research is supported by British Biotechnology and Biological Sciences Research Council (BBSRC). EEF is supported by the Swiss National Science Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

Arabidopsis gene **At5g63980**, **FIERY1**, encodes a bifunctional enzyme possessing 3′ (2′,3′)-5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities [1]. Among its *in vitro* substrates are several important cellular metabolites: inositol 1,4,5-triphosphate (IP3), which is important for the phospholipid signalling [2], 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the donor of active sulfate for sulfotransferase reactions, and 3′-phosphoadenosine 5′-phosphate (PAP), which is a byproduct of these sulfotransferases [3,4]. Therefore, it is not surprising that the gene has been identified in various genetic screens for a great range of phenotypes and that it possesses a large number of alternative names.

The Arabidopsis gene was first described as **SAL1** in a screen for plant genes increasing Li⁺ tolerance of yeast [1]. It is similar to yeast **Met22** essential for sulfate assimilation in yeast [5], which catalyses the dephosphorylation of PAPS and PAP. This gene is a target for salt toxicity in yeast and is named alternatively as **HAL2** [5]. The genetic screen that gave the Arabidopsis gene the commonly used name **FIERY1** or **FRY1** was designed to identify genes affecting abscisic acid and stress signalling [2]. Afterwards, the gene has been identified in screens for genes affecting cold signalling as **HOS2** (high expression of osmotically responsive genes) [6], for RNA silencing suppressors [7], for elevated expression of ascorbate peroxidase 2 as **ALX8** [8], for genes required for venation patterning as **RON1** [9], for mutants with deregulated fatty acid oxygenation rate as **FOU8** [10], and for mutations affecting expression of phosphate transporter [11].

Nearly all the different phenotypes of **fry1** mutants have been ascribed to disruptions of inositol signalling [5], [6], [9], inhibition of exoribonucleases of the **XRN** family by accumulated PAP [7], [11], [12], or both [8]. However, since the gene product is a metabolic enzyme, we were interested whether disruption of
FIERY1 in the fou8 mutant would also lead to a metabolic phenotype, namely whether it would disrupt the synthesis of sulfur containing molecules like glucosinolates. The glucosinolates are a large group of sulfur-rich amino acid-derived metabolites, found mainly in the Brassicaceae [13], [14] and involved in defence against herbivores and insects, as well as fungi and bacteria [14], [15], [16]. The final step of the core glucosinolate synthesis is sulfation of the desulfo-glucosinolate precursors (Figure 1) [17]. In Arabidopsis, the sulfotransferases (SOT) of group VII, AtSOT16, 17 and 18, are responsible for this reaction [18], [19].

A close link between FIERY1 and glucosinolates is supported by several recent reports. Apart from the substrate of FIERY1, PAP, being produced during glucosinolate synthesis, the gene has been found to be co-regulated with other genes of the pathway [20]. Also, crossing of the fou8 mutant with apk1 apk2, which is characterised by low PAPS production and glucosinolate content, attenuated or prevented a range of phenotypes of the single fou8 mutant [10]. Here we show that indeed, disruption of FIERY1 in the fou8 allele [10] leads to decrease in glucosinolate content with a concomitant accumulation of desulfo-glucosinolate precursors. We report a new phenotype of fou8/fry1, low sulfur content, and show that this is connected with changes in PAP/PAPS rather than phosphoinositols or RNA processing. Importantly, the analysis of fou8 mutant revealed important clues to sensing and signalling in sulfur metabolism.

Results
Disruption of FIERY1 inhibits glucosinolate synthesis
The phenotypes caused by mutations of the FIERY1 gene have mostly been attributed to disruption of signalling [2], [6], [8], [9]. However, as the substrate for the enzyme, PAP, is produced during synthesis of glucosinolates (Figure 1), and the gene is co-expressed with genes involved in glucosinolate synthesis [20], we tested whether disruption of FIERY1 affects glucosinolate levels. Indeed, in the fou8 mutant the total glucosinolate content is significantly lower than in wild type Col-0 plants (Figure 2a). As the mutation affects the last step in glucosinolate synthesis, sulfation of the desulfo-precursors, these precursors accumulate in the fou8 mutants (Figure 2b). Similar, but more profound changes in glucosinolate contents were observed previously in the apk1 apk2 mutant with strongly inhibited synthesis of PAPS (Figure 2) [20]. The triple mutant fou8 apk1 apk2 has the same glucosinolate and desulfo-glucosinolate levels as apk1 apk2, showing that the mutations are indeed affecting the same metabolic step. Interestingly, not all individual glucosinolates were affected to the same level in fou8. The decrease in total glucosinolates was mostly caused by lowering the levels of aliphatic glucosinolates, while the indolic glucosinolates were affected to lesser degree or unaffected (Table 1).

The reduced synthesis of PAPS in the apk1 apk2 mutant resulted in coordinated increases in transcript levels of genes involved in glucosinolate synthesis (Table S1) [20]. Mugford et al. [20]

![Figure 1. Scheme of involvement of FOU8 in sulfur metabolism.](https://doi.org/10.1371/journal.pone.0039425.g001)

![Figure 2. fou8 is affected in glucosinolate synthesis.](https://doi.org/10.1371/journal.pone.0039425.g002)
concluded that this regulation is caused either by accumulation of desulfo-glucosinolates or decrease in glucosinolate levels. Since fou8 also accumulated desulfo-glucosinolates, we tested whether they are responsible for the up-regulation, by comparing mRNA levels of several genes of the glucosinolate synthesis network in fou8 (Figure 2c). In contrast to esk1 esk2 and fou8 esk1 esk2, where transcript levels for all genes tested were up-regulated, in fou8 the tested genes were regulated differently. From the three genes involved in synthesis of glucosinolate backbone tested, one (CYP79F2) was up-regulated whereas two genes (MAM-L, SUR1) were not affected. On the other hand, mRNA levels of genes responsible for the metabolic step directly affected by fou8 mutation, sulfotransferases SOT16, SOT17 and SOT18, were up-regulated co-ordinately. To obtain a better insight into the regulation of the glucosinolate synthesis network, we used available microarray data obtained with various alleles of regulated co-ordinately. To obtain a better insight into the mutation, sulfotransferases SOT16 responsible for the metabolic step directly affected by were not affected. On the other hand, mRNA levels of genes (involved in synthesis of glucosinolate backbone tested, one tested genes were regulated differently. From the three genes transcript levels for all genes tested were up-regulated, in fou8 (Figure 2c). In contrast to esk1 esk2 and fou8 esk1 esk2, where transcript levels for all genes tested were up-regulated, in fou8 the tested genes were regulated differently. From the three genes involved in synthesis of glucosinolate backbone tested, one (CYP79F2) was up-regulated whereas two genes (MAM-L, SUR1) were not affected. On the other hand, mRNA levels of genes responsible for the metabolic step directly affected by fou8 mutation, sulfotransferases SOT16, SOT17 and SOT18, were up-regulated co-ordinately. To obtain a better insight into the regulation of the glucosinolate synthesis network, we used available microarray data obtained with various alleles of fiery1 [8], [9] and compared the gene expression with microarray data of apk1 apk2 mutant [20] (Table S1). From the 42 genes, 36 of the glucosinolate synthesis network as compiled in [21] and 6 MYB factors controlling the network [22], 33 were up-regulated at least 1.5-fold in apk1 apk2. In contrast, in fiy1 [C24 background] and alx8 allele (Col-0) [8], nine and ten genes, respectively were up-regulated while in ron1-1 allele (Col-0) [9] only four genes were affected. Thus, it seems that in mutants with disrupted fiery1 gene the increase in desulfo-glucosinolates per se does not trigger the co-ordinated activation of the biosynthetic network as in apk1 apk2 mutant.

Interaction of FIERY1 with primary sulfate assimilation

We next asked whether the strong interconnection of primary and secondary sulfur metabolism [20], [22], [23], [24] can also be observed in the fou8 mutant. Indeed, the transcript levels for most genes involved in sulfate reduction were found to differ significantly between Col-0 and fou8 (Table 2). Whereas the mRNA levels of APR isoforms were higher in fou8 leaves, the transcripts for ATPS1 and ATPS4 were reduced. Interestingly, these genes are regulated in the same way in sulfate deficient plants [25], [26]. Therefore we also tested the expression levels of two genes, At5g48850 (sulfate deficiency-induced 2; Lys Sulphur 2, LS2) and

Table 1. Levels of individual glucosinolates in Col-0 and fou8.

<table>
<thead>
<tr>
<th>Glucosinolate</th>
<th>Col-0</th>
<th>fou8</th>
<th>Ratio fou8/Col-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M5OB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.65±0.27</td>
<td>0.86±0.02</td>
<td>0.52</td>
</tr>
<tr>
<td>4MTB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18±0.01</td>
<td>0.05±0.01</td>
<td>0.27</td>
</tr>
<tr>
<td>8M5SO&lt;sub&gt;0&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25±0.03</td>
<td>0.1±0.01</td>
<td>0.42</td>
</tr>
<tr>
<td>4OH3M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.018±0.007</td>
<td>0.015±0.002</td>
<td>0.83</td>
</tr>
<tr>
<td>8M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.42±0.07</td>
<td>0.39±0.01</td>
<td>0.94</td>
</tr>
<tr>
<td>4M3M3&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.17±0.01</td>
<td>0.09±0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>1M3M3&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.007±0.002</td>
<td>0.03±0.01</td>
<td>3.83</td>
</tr>
<tr>
<td>total</td>
<td>2.69±0.37</td>
<td>1.53±0.03</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Col-0 and fou8 plants were grown for 5 weeks in controlled environment room. Leaves were harvested and the levels of glucosinolates (μmol/g FW) were determined by HPLC. Results are presented as means ± SD from three individual plants characteristic of four independent experimental repeats. Ratios of individual glucosinolates different from the ratio of total glucosinolates by more than 25% are printed bold.

doi:10.1371/journal.pone.0039425.t001

Figure 3. Glucosinolate synthesis in fou8 mutant with and without cytosolic APS kinase. Col-0, fou8, apk3, and fou8 apk3 plants were grown for 5 weeks in controlled environment room. The total content of A glucosinolates and B desulfo-glucosinolates was measured in leaves. C Total RNA was isolated from leaves and the transcript levels of six genes involved in glucosinolate synthesis was determined by quantitative RT-PCR. The qRT-PCR reactions were performed in triplicate for each biological sample. The values in Col-0 were set to 1 for all genes. Results are presented as means ± SE from six pools of three individual plants grown in two independent experiments. Different letters mark values significantly different at P<0.05; asterisks mark values significantly different from Col-0 at P<0.05.

doi:10.1371/journal.pone.0039425.g003
At5g26220 (Low Sulfur 5, LS5), that belong among genes with the highest degree of up-regulation upon sulfate starvation [25], as markers for sulfate starvation expression response. The mRNAs for these genes were increased in foul similar to sulfate deficient plants (Table 2). These results are in line with multiple signalling defects described for other fry1 alleles [0, 9], and particularly with recently reported symptoms of phosphate deficiency in the fry1 mutant [11]. Therefore, we revisited the previously described microarray data obtained with various alleles of fry1/fou8 [8, 9] and used iterative group analysis [27] to compare genes altered in expression in fry1 alleles with sets of genes showing differential expression by different treatments from the AgGen Express data set. Interestingly, in all three fry1 related microarray experiments, the miss-regulated genes showed significant overlap with the set of genes regulated by sulfate starvation (Table S2). Thus, disruption of FIER1 seems to trigger the same expression response as sulfate starvation.

To test whether the sulfate starvation-like syndrome in foul and other fry1 alleles is restricted to gene expression or manifested also otherwise, we determined the enzyme activity of APR and the levels of sulfur containing metabolites. In agreement with the increased transcript levels, and consistent with response to sulfate starvation, APR activity was higher in leaves of foul than in Col-0 (Figure 4a). While cysteine levels were unaffected, contents of the major thiols, glutathione, were lower in foul leaves, again similar to plants under sulfate starvation (Figures 4b and 4c). Sulfate content was significantly lower in foul and reached only ca. 50% of the levels in Col-0 (Figure 4d). Correspondingly, the total sulfur content in foul leaves was significantly lower than in Col-0 (Table 3). Thus, the changes in expression pattern in foul are not due to alteration in signalling but a genuine response to reduced levels of sulfate and sulfur containing metabolites.

These results strongly indicate that it is not the external sulfate concentration that triggers sulfate deficiency response but instead the levels of internal sulfur-containing compounds. As Figure 4 shows, foul possesses lower levels of sulfate and glutathione and both compounds may represent the sensed molecule. Indeed, reduction of glutathione content, e.g., by the inhibition of its synthesis by buthionine sulfoximine (BSO), induces APS reductase activity, similar to sulfate starvation [28]. We therefore asked whether reduced glutathione content in two independent Arabidopsis mutants in the first enzyme of glutathione synthesis, γ-glutamylcysteine synthetase, cad2 and rax1 [29], [30], also triggered sulfate starvation response. The iterative group analysis employed on microarray data from cad2 and rax1 mutants [30], however, did not show any overlap with sulfate deficiency response (Table S3).

To verify the transcriptomics data biochemically, we compared levels of sulfur-containing metabolites and gene expression of key markers of sulfate deficiency in 2-weeks old seedlings of foul, cad2, rax1, and sultr1;2 mutants. As expected, sulfate content was lower than in Col-0 in shoots and roots of foul and sultr1;2, but not in cad2 or rax1 (Table 4). GSH levels were significantly lower in shoots and roots of cad2 and rax1 and also of sultr1;2. In leaves of foul GSH levels were lower than in Col-0, while in roots GSH accumulation was much greater than in the foul mutant (Table 4). However, this reduction was not accompanied by accumulation of desulfo-glucosinolates in sultr1;2 and these precursors were also at very low levels in cad2 and rax1 (Table 4). Transcript levels for LS2, LS3, and APR1 were elevated and ATPS4 was reduced in both leaves and roots of foul compared to Col-0 (Figure 5), in agreement with the regulation of these genes by sulfate deficiency. On the other hand, in cad2 and rax1 the transcript levels of these genes were not affected or regulated in an opposite way than in sulfate deficient plants. The strong reduction in glucosinolate levels in sultr1;2 did not cause similar co-ordinated up-regulation of the genes of glucosinolate synthesis as in apk1 apk2 or foul mutants (Figure 5e). Thus, diminishing glutathione content to ca. 15% WT levels does not trigger a sulfate deficiency response. In contrast, increased mRNA levels of LS2, LS3, and APR1 correlate with reduced levels of internal sulfate in foul and sultr1;2. Therefore, it seems that sulfate deficiency response in Arabidopsis is triggered by a reduction of internal sulfate levels.

Dissection of the Low Sulfur Phenotype

As the enzyme encoded by FIER1 has been shown to act on two types of substrates, adenosine bisphosphates as well as inositol bisphosphates, and as its mutation leads to defects in many signalling pathways, the low sulfur phenotype of foul mutant can be caused by different mechanisms. To find out which of the FIER1 functions is responsible for the low accumulation of sulfur compounds, we analysed various mutants and transgenic lines related to foul. To confirm that the observed metabolic changes are due to disruption of FIER1, we used foul complemented by the wild type allele (foul/RFT1) [10]. In addition, the reduced levels of glucosinolates and sulfate and accumulation of desulfo-glucosinolates were confirmed in two T-DNA insertion lines in the FIER1 gene (Figure S2). As sulfate assimilation is highly responsive to jasmonate [31], [32], we tested another mutant with a similar fatty acid oxygenation phenotype connected with jasmonate accumulation, fou2 [33], as well as the aos mutant deficient in jasmonate [34]. To test whether changes in accumulation of inositol related compounds might be responsible
for the phenotype we analysed the cvp2 mutant [35] that shares with fou8 the defect in leaf venation pattern [9]. Since mutations in FIERY1 affect processing of small RNAs and since sulfate assimilation is regulated by microRNA miR395 [36], we analysed a mutant in ribonucleases xrn2 xrn3 xrn4 [11].

First we checked the fatty acid oxygenation rates in these genotypes. Both fou8 and fou2, which was isolated in the same screen as fou8, showed increased activity in this assay, whereas aos had an oxygenation rate lower than wild type. The other genotypes tested did not differ from Col-0 (Figure 6).

The expression of the wild type FIERY1 copy in fou8 restored sulfate accumulation to the levels of Col-0 (Figure 7a). While there were no changes in sulfate levels in cvp2 and fou2, sulfate accumulated to higher levels in aos and conversely, its content was lower in xrn2 xrn3 xrn4 than in Col-0. Glutathione content, which was lower in fou8, was not completely restored to wild type levels by expression of FIERY1, however, the difference was much smaller than in the case of sulfate (Figure 7b). Interestingly, aos plants possessed higher GSH levels than other genotypes. Significant differences in glucosinolate levels were observed in mutants affected in jasmonate synthesis. Whereas fou2 possessed higher levels of these metabolites than Col-0, aos contained significantly less glucosinolates than Col-0 and about the same as fou8 (Figure 7c). However, these changes were not accompanied by accumulation of desulfo-glucosinolates, which were detected only in genotypes with disruption of FIERY1 or APK1 and APK2 isoforms of APS kinase (Figure 7d).

The expression profiles of key genes involved in sulfur, glucosinolate, and jasmonate metabolism were compared in these genotypes (Figure 8). Clearly, the sulfate deficiency expression pattern was retained in genotypes possessing the fou8 mutation, as in these plants the sulfate levels did not differ from fou8. Also connected to the fou8 mutation was the increased transcript level of SOT18, whereas other genes involved in glucosinolate synthesis were induced only in fou8 apk1 apk2, where the induction was driven by the apk1 apk2 parent. The differences in glucosinolate levels between Col-0 and jasmonate affected genotypes fou8, fou2, and aos were not reflected in the transcript levels of the genes.
involved in glucosinolate synthesis. For example, mRNAs for MAM-L and SOT18 were lower than in Col-0 in both fou2 and aos, whereas the glucosinolate levels were increased in the former and lower in the latter (comp. Figure 7c). To find out whether the changes in sulfur metabolism are linked to PAP and/or PAPS accumulation we compared the levels of PAP and PAPS in these genotypes. Indeed, fou8 mutation-containing genotypes accumulated PAP whereas this metabolite was not detectable in Col-0 or other lines analysed (Figure 9a). Similar levels of PAP were detected in fou8 apk3 mutant, which is consistent with it being identical in appearance and sulfur metabolism to fou8. Reduced synthesis of PAPS in fou8 apk1 apk2 mutants also prevented accumulation of PAP. The fou8 mutant had higher levels not only of PAP but also of its precursor PAPS (Figure 9b). Disruption of cytosolic APS kinase in fou8 apk3 plants did not affect the increased PAPS levels of fou8, while in fou8 apk1 apk2 plants PAPS content was lower than in Col-0 (Figure 9b). Small changes in PAPS levels compared to Col-0 were observed in other genotypes, but they were much less pronounced than the alterations in PAP levels (Figure 9).

We also tested whether the alternative substrates of FIERY1, inositol polyphosphates, may be associated with the low sulfur metabolism.
The sulfate content in *fou8* can be maintained lower than in Col-0 by reduced uptake or increased utilisation. Therefore we analysed flux through sulfate assimilation in genotypes differing in sulfate content (Figure 10). Sulfate uptake of three weeks old plants was not different between *fou8* and Col-0, however, it was somewhat increased in sulfate accumulating *aos* (Figure 10a). Also the sulfate translocation to shoots was higher in *aos* but identical in *fou8* and Col-0 and somewhat lower in *fou2* (Figure 10b). On the other hand, the flux through sulfate assimilation, determined as the percentage of $^{35}$S from the $[35S]$sulfate taken up incorporated into reduced compounds, thiols and proteins, was higher in *fou8* but identical in Col-0, *fou2*, and *aos* (Figure 10c). Thus, whereas in *aos* sulfate accumulates due to increased uptake and translocation to the leaves, the low sulfate phenotype in *fou8* seems not to be caused by differences in sulfate uptake but rather its reduction and utilisation.

**Discussion**

**Links between FIERY1 and Glucosinolates**

The *FIERY1* gene has been shown to affect a great range of cellular processes [2], [6], [7], [8], [9], [10], [11], [12], [37] and global analyses of transcriptome and metabolome of its mutants have been reported [8], [9]. It is however surprising that the direct metabolic effects of its disruption have been analysed only very recently, showing accumulation of PAP in the mutant [37–38]. We revealed the association of *FIERY1* with glucosinolate metabolism (Figure 1 and 2) [20] and showed that, as expected, the glucosinolate levels are lower in this mutant than in wild type. The rationale for this expectation was the disruption of PAP removal in the mutant. Accumulation of PAP can be expected to directly inhibit sulfotransferases, as it will shift the reaction equilibrium towards the substrates, however, a direct biochemical evidence for this inhibition is not available. Alternatively, PAP may disrupt the transport of PAPS from plastids to the cytosol and reduce its concentration in the cytosol. Lower availability of PAPS in *apk1 apk2* mutants indeed resulted in low glucosinolate levels and accumulation of desulfo-glucosinolates [20]. Although a PAPS transporter has not yet been identified in plants, its presence in chloroplast envelope has been postulated from analyses of APS kinase mutants [20], [39]. To distinguish between the two options the cross between *fou8 apk3* mutants indeed resulted in low glucosinolate levels and accumulation of desulfo-glucosinolates [20]. As the relevant inositol bis- and tri-phosphates are present in very low levels, as a surrogate we compared inositol (hexa)phosphate (IP6) levels in seeds. However, no differences were seen between *fou8* and Col-0 (data not shown), which is consistent with no differences in IP3 labelling between Col-0 and *fry1-6* [37].

**Table 4. Contents of sulfur-containing metabolites in genotypes affected in sulfur metabolism.**

<table>
<thead>
<tr>
<th></th>
<th>Shoots (µmol/g FW)</th>
<th>Roots (µmol/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfate</td>
<td>Cysteine</td>
</tr>
<tr>
<td></td>
<td>(µmol/g FW)</td>
<td>(µmol/g FW)</td>
</tr>
<tr>
<td></td>
<td>Col-0</td>
<td><em>fou8</em></td>
</tr>
<tr>
<td>Sulfate</td>
<td>7.8±0.9 a</td>
<td>3.8±0.5 b</td>
</tr>
<tr>
<td>Cysteine</td>
<td>32±2 a</td>
<td>31±11 a</td>
</tr>
<tr>
<td>GSH</td>
<td>604±1 a</td>
<td>575±7 b</td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>3.37±0.11 a</td>
<td>1.89±0.08 b</td>
</tr>
<tr>
<td>desulfo-glucosinolates</td>
<td>0.0014±0.002 a</td>
<td>3.3±0.4 b</td>
</tr>
</tbody>
</table>

Col-0, *fou8*, *cad2*, *rax1*, and *sultr1;2* plants were grown for 2.5 weeks on MS-agarose plates. Sulfate, cysteine, glutathione in leaves and roots, and total glucosinolates and desulfo-glucosinolates levels in leaves were determined by HPLC. Results from one of two independent experiments are presented as means ± SD from three biological replicates, different letters mark values significantly different at P<0.05. n.d., not determined.

doi:10.1371/journal.pone.0039425.t004

**Figure 6. Variation in fatty acid oxygenation rate in *fou8* related genotypes.** The various Arabidopsis lines were grown for 5 weeks in controlled environment room. Leaf juice was incubated for 2 min with $[^14]$C-18:3. Products were separated by thin layer chromatography and the radioactivity quantified. The rate of oxygenation is expressed as % of radioactivity in 18:3-α-ketol. Results are presented as means ± SE from six individual plants grown in two independent experiments, different letters mark values significantly different at P<0.05.

doi:10.1371/journal.pone.0039425.g006
glucosinolate content and accumulation of desulfo-glucosinolates. A compartment specific quantification of PAP and PAPS in the various combinations of $\texttt{fou8}$ and $\texttt{apk}$ mutants would unequivocally clarify the mechanisms, such measurements are, however, not technically feasible yet. This is supported by detecting increased transcript levels of all three desulfo-glucosinolate sulfotranferases (Figure 2 and 3). However, it has to be noted, that the levels of desulfo-glucosinolates were higher than would correspond to unused substrates, indicating an increased flux through the synthesis of glucosinolate precursors. This increase in flux was, however, much lower than that observed due to the disruption of PAPS synthesis in $\texttt{apk1 apk2}$ mutants. In $\texttt{apk1 apk2}$ almost all genes of glucosinolate synthesis network were co-ordinately up-regulated [20] but this was not the case in $\texttt{fou8}$. In various alleles of this mutant consistently only some genes of the network were induced, which is in accordance with the increased accumulation of desulfo-glucosinolates (Table S1). It is thus likely that the signal for the co-ordinated regulation of the network in $\texttt{apk1 apk2}$ is not the desulfo-glucosinolates, as in such case the network would be induced in $\texttt{fou8}$ to similar extent, unless the trigger is dependent on very high concentration of the precursors. It seems rather that a decrease in levels of one or more glucosinolates causes the up-regulation of the glucosinolate synthesis network. As aliphatic glucosinolates were reduced to much higher degree than indolic ones in $\texttt{fou8}$, it is possible that when one or more indolic glucosinolates decrease under certain level, a feedback mechanism induces the co-ordinated expression of the glucosinolate network. Indolic glucosinolates were indeed shown to exhibit negative feedback control of the pathway [40]. The mechanism of such positive feedback loop is unknown as direct evidence for regulation of gene

Figure 7. Accumulation of sulfur-containing compounds in $\texttt{fou8}$ related genotypes. The various Arabidopsis lines were grown for 5 weeks in controlled environment room. Leaves were harvested and the levels of sulfur-containing compounds were determined by HPLC. A sulfate, B glutathione, C glucosinolates, and D desulfo-glucosinolates. Results are presented as means ± SE from six individual plants grown in two independent experiments with three replicates each. Different letters mark values significantly different at $P<0.05$; ND = not detectable.

doi:10.1371/journal.pone.0039425.g007

Figure 8. Expression analysis of Arabidopsis lines. The various Arabidopsis lines were grown for 5 weeks in controlled environment room. Total RNA was isolated from leaves and the transcript levels of genes involved in sulfur metabolism, glucosinolate synthesis, and jasmonate synthesis were determined by quantitative RT-PCR. The qRT-PCR reactions were performed in triplicate for each of the six independent biological samples from plants grown in two independent experiments. Results are presented as a heat map of relative mRNA levels compared to Col-0. For comparison, sulfate levels are presented in the same way on the far right.

doi:10.1371/journal.pone.0039425.g008
expression by glucosinolates in plants is not available. Since the health promoting effects of glucosinolates is connected to stimulation of gene expression in human cells by glucosinolate degradation products [41], it can be expected that similar mechanisms exist in plants for example for the function of glucosinolates in immunity [15–16] [40]. In addition, the co-incidence of glucosinolate metabolic QTLs with eQTLs of the biosynthesis genes may also indicate an effect of glucosinolates on gene expression [42]. It is possible to speculate that such regulatory glucosinolate-related compound binds to transcription factor(s) and prevents their DNA binding. When the glucosinolate level is low, the inhibition is relieved and genes of the network are increasingly transcribed.

Links of Jasmonate and Sulfur Metabolism

Jasmonate has a prominent role in coordinated regulation of sulfur metabolism [31], [32]. This is not surprising given the many functions of sulfur containing compounds in plant stress defense [43]. It has long been known that jasmonate signalling is important for regulation of glucosinolate synthesis, which is increased upon treatment with jasmonate [44], [45]. The induction is stronger for the indolic group of glucosinolates, which are considered to be more responsive to the environment [45]. Correspondingly, the indolic glucosinolates were much less affected in fou8 than the aliphatic ones (Table 1), presumably since the inhibition of sulfotransferases was counteracted by the up-regulation of indolic glucosinolate synthesis due to increased jasmonate. Indeed, the glucosinolate content was increased in fou2, also with a larger contribution of the indolic group. However, the loss of jasmonate synthesis in aos mutant led to decreases of both groups of glucosinolates, so that there is at least some need for jasmonate for basal synthesis of aliphatic glucosinolates.

Figure 9. Accumulation of PAP and PAPS in fou8 related genotypes. The various Arabidopsis lines were grown for 5 weeks in controlled environment room. Leaves were harvested and the levels of A PAP and B PAPS were determined by HPLC. Results are presented as means ± SD from four individual plants, grown in two independent experiments. Asterisks represents levels under detection limit, different letters mark values significantly different at P<0.05. doi:10.1371/journal.pone.0039425.g009

Figure 10. Sulfate uptake and flux through sulfate assimilation in fou8 and related mutants. WT Col-0 and mutants fou8, fou2, and aos were grown for 3 weeks on MS-phytagel vertical plates in controlled environment room. The seedlings were incubated for four hours with their roots submerged in nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with 6.7 µCi [35S]sulfate. Shoot and root material was harvested separately, and the flux was determined as incorporation of [35S] from [35S] sulfate to thiols and proteins. A sulfate uptake, B Percentage of [35S] transported to leaves from the [35S] sulfate taken up, C relative flux through the sulfate assimilation in the leaves calculated as % of incorporation in thiols and proteins from total [35S] sulfate taken up. Results are presented as means ± SE from six independent pools of 8 seedlings grown in two independent experiments. Values marked with an asterisk show significant (P<0.05) difference from Col-0. doi:10.1371/journal.pone.0039425.g010
starved plants [47]. Interestingly, fou8 plants possess significantly lower levels of potassium than Col-0 (Table 3). Thus, some of the changes in oxylipins and glucosinolates may actually be connected to reduced levels of potassium. The interactions between the mineral nutrients sulfate, phosphate, and potassium on one hand and metabolites such as jasmonate and glucosinolates on another hand are too complex to distinguish their causal relationships. It is possible that the low potassium is a consequence of low accumulation of anions sulfate and phosphate to keep the ionic balance but in the same way, the reduced accumulation of the anions may be secondary to the primary defect in potassium uptake. These findings thus open many exciting questions on the interconnection of plant mineral nutrition with primary and secondary metabolism and the fou8 mutants may be an important tool in their dissection.

Low Sulfur Phenotype of fou8

The effect of disruption of FIERY1 on sulfur metabolism, however, did not stop at the glucosinolates. The transcripts of genes involved in sulfate assimilation, APR, ATPS4 and ATPS4 were regulated in fou8 in the same way as in plants under sulfate starvation (Table 2). As this expression pattern could be due to the close links between primary and secondary sulfur metabolism [22] it was important to confirm the findings in a non-biased way. The iterative group analysis of available data on three independent microarray experiments showed that indeed, there is significant similarity in changes of global transcription of of fou8/fy1 mutant and sulfate starved plants. Because the fou8 mutant possesses higher levels of jasmonate [10], this very intriguing result reinforces the links between regulation of sulfate assimilation and jasmonate signalling: finding of jasmonate synthesis genes among those up-regulated by sulfate starvation [25], [26] and coordinated regulation of sulfur assimilation genes by jasmonate [31], [32]. However, as the sulfur-containing compounds sulfate and glutathione accumulated to lower levels in fou8 than in Col-0 (Figure 4), the mutant indeed suffers from a mild sulfur deficiency. The lower content of sulfur is not connected to sulfate uptake, which is identical in fou8 and Col-0 (Figure 10). This was surprising, since sulfate uptake is normally induced in sulfur deficient plants [4]. It seems therefore, that only a subset of sulfate starvation responses is triggered in fou8.

The analysis of fou8 thus gave an interesting hint about the nature of sulfur sensing. The induction of the sulfate starvation response has always been associated with the decrease of external sulfate availability [25], [26]. Here we show, however, that at least some components of the response react to a decrease in internal levels of sulfate containing compounds. Results with fou8 alone cannot distinguish between decrease of sulfate or glutathione as responsible for the signal, as both compounds were reduced in the mutant. To distinguish between these candidates existing microarray data of plants with reduced glutathione levels were interrogated. Firstly, we utilised the data from Arabidopsis plants where depletion of glutathione content without affecting sulfate levels was achieved by treatment with BSO [48]. Genes up-regulated by BSO treatment were similar to genes both up-regulated and down-regulated by sulfate starvation, thus not showing the clear relation like fy1 mutants and making glutathione an unlikely candidate for the signal. This conclusion was corroborated by analysis of microarray datasets from cad2 and rax1 mutants in γ-glutamylcysteine synthetase [30]. These mutants possess only about 25% of wild type glutathione levels and are thus affected in its content to much greater degree than fou8. Since no overlap in expression between sulfate-starved plants and cad2 or rax1 mutants was observed (Table S3) reduction in internal sulfate levels seems to be the trigger for sulfate starvation response of gene expression in fou8. The correlation of sulfate deficiency-like expression of APR1, ATPS4, and the two marker genes for sulfate deficiency with sulfate levels but not GSH levels in a variety of mutants (Figure 5), strongly corroborates this conclusion. The finding that the induction of low sulfur responsive genes occurs also at normal sulfate supply is an important step in the search for sulfate sensing mechanism in plants. Actually, data allowing the same conclusion has been presented before in transcriptome analysis of sulfate deficient plants alongside the sel1-10 mutant of Sultr1;2 [49], however, the authors did not discuss such signalling.

Interestingly, the analysis of cad2, rax1, and sultr1;2 mutants revealed further links between primary and secondary sulfur metabolism. Surprisingly, disruption of glutathione synthesis in cad2 and rax1 did not affect glucosinolate levels, in contrast to an allelic mutant pad2 [50]. This could be caused by different growth conditions but also by different residual levels of glutathione in the mutants. Despite no effect on total glucosinolate levels, some desulfo-glucosinolates were detected in the mutants and transcript levels for some genes of the glucosinolate synthesis were increased, due to the well-known effect of glutathione deficiency on expression of defense genes [30]. In the sultr1;2 mutant, the low capacity to uptake sulfate was manifested not only by reduced sulfate and glutathione levels, but also by strongly reduced glucosinolate content. In this case, however, only a very small increase in desulfo-glucosinolates and mRNA levels of the biosynthesis genes was observed (Table 4, Figure 5). Presumably, the sulfate starvation response initiated in this mutant prevents the up-regulation of glucosinolate synthesis.

So what triggers the decrease in sulfur accumulation in fou8? Four metabolites, phosphoinositols, PAP, PAPS, or jasmonate, are the main suspects as the signalling molecules. The phosphoinositols are unlikely, as csp2 that accumulates these compounds and shares several phenotypes with fou8 [35] is not affected in sulfur metabolism. In addition, Estavillo et al. [37] did not observe any changes in inositol phosphates in fy1 or other related genotypes. Jasmonate is also unlikely, as no alterations in sulfate or glutathione content was detected in fou2 mutant that was isolated in the same genetic screen as fou8 and no correlation between the fatty acid oxygenation phenotype and glucosinolate levels was observed (Figure 6). PAP and PAPS both accumulate in fou8 and other fy1 alleles [37], [38]. Their levels are reduced by crossing with apkl apk2, but not apk3. However, the sulfate levels do not seem to be correlated with PAP or PAPS content as they remain low in fou8 apk1 apk2 despite a large reduction of PAP and restoration of wild type PAPS levels (Figure 9). The large transcriptome reprogramming of apk1 apk2 plants results in redirecting sulfur flow from secondary to primary metabolism as evidenced by increased flux through sulfate reduction and accumulation of reduced sulfur compounds [20], [22]. Similarly, in fou8 mutant increased APR activity (Figure 4) and increased flux through the pathway (Figure 8) have been detected. It is thus possible that any restoration of sulfate levels in the fou8 apk1 apk2 mutant due to reduced levels of PAP or PAPS is counteracted by the increased reduction rate and the effective sulfate content remains low. Such PAP induced sulfate deficiency resembles the local phosphate starvation in roots of fy1 [11].

In conclusion, this study revealed several new phenotypes caused by disruption of FIERY1, the decrease in glucosinolate content and in accumulation of sulfur and potassium. These newly observed links between sulfur metabolism and jasmonate and between potassium and FIERY1 open up new avenues of research to better understand the integration of plant nutrition
and metabolism. The analysis of fou8 indicated that decreases in indolic glucosinolate(s) may be responsible for feedback up-regulation of glucosinolate synthesis in apk1 apk2 and fou8 mutants. Most importantly, the data presented here show that sulfur starvation responsive gene expression is not linked to external sulfate concentration but correlates with a decrease in internal sulfate levels. This is thus the first step in elucidation the mechanism of how plants sense adequate levels of sulfur supply.

Materials and Methods

Plant material and growth conditions

In this study, Arabidopsis thaliana (ecotype Col-0) were used as wild type. The fou8 mutant and fou8 apk1 apk2 plants were described in [10]. The csp2 and xrn3 xnr3 xrn4 seeds were provided by F. Carland, Yale University and E. Marin, CNRS Aix-Marseille, respectively. T-DNA lines disrupting Fiery1 gene SALK_020882 and SALK_151367 were obtained from T. Gigolashvili, University of Cologne. Plants were grown for 5 weeks in 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⁻¹m⁻². For elemental analyses plants were soil-grown (one seed per 7 cm diameter pot) with 12 h light (100 μE s⁻¹m⁻²), 70% humidity; daytime temperature 22°C and nighttime temperature 18°C. For the sulfate uptake and flux analysis the plants were grown for three weeks on vertical plates with Murashige Skoog media without sucrose supplemented with 0.5% phytagel. The plates were placed in a controlled environment room at 20°C under 16 h light/8 h dark cycle. For each experiment at least two independent sets of plants were grown and analysed, each including fou8 as a control.

Glucosinolate and Desulfo-glucosinolate Analysis

Glucosinolates were extracted from 50 mg frozen leaf material. The extraction and quantification of intact glucosinolates followed the protocol described in [20]. Native desulfo-glucosinolates were determined from the same extracts. However, desulfo-glucosinolates do not bind to DEAE-Sephadex. The flow through from loading the extract onto DEAE-Sephadex columns and the following washing step with water were collected and combined and analysed by HPLC-UV as described for mature glucosinolates [20].

Expression Analysis

To determine mRNA levels total RNA was isolated by standard phenol/chloroform and LiCl precipitation. First-strand cDNA was synthesized from 1 μg of total RNA using Quantitect Reverse Transcription Kit (Qagen, Crawley, UK), which includes a DNAse step to remove possible DNA contamination. Quantitative real-time RT-PCR (qPCR) was performed using gene-specific primers (Table S4) and the fluorescent intercalating dye SYBR Green (Applied Biosystems, Warrington, UK) as described in [51]. All quantifications were normalized to the Tap41 gene. The RT-PCR reactions were performed in duplicate for each of the three independent samples.

Enzyme Assays

APS reductase activity was determined as the production of [³⁵S]sulfite, assayed as acid volatile radioactivity formed in the presence of [³⁵S]APS and dithioerythritol as reductant [52]. The protein concentrations were determined with a Bio-Rad protein kit (Bio-Rad, Hemel Hempstead, UK) with bovine serum albumin as a standard.

A lipoxygenase activity assay employed to compare the fatty acid oxygenation ratios was based on the oxygenation of radiolabeled linoleic acid (18:2) as described previously [53]. The rate of oxygenation has been calculated as % of radioactivity in 18:3-α-ketol from total radioactivity of linoleic acid used.

Measurements of Sulfur-Containing Compounds

Sulfate contents were determined by ion-exchange HPLC method as described in [54]. Cysteine and GSH were analysed by HPLC as described by [52] from 20–30 mg of plant material. PAPS and PAP were extracted from leaves of Arabidopsis plants according to [55]. The adenosine compounds were derivatized with chloroacetaldehyde and separated by HPLC as described in [56]. For elemental analysis plants were harvested on day 30. The rosettes were ground in liquid nitrogen, lyophilized and analysed with a Philips PW2400 X-ray fluorescence spectrophotometer.

Iterative Group Analysis

Original microarray data for fry1 alleles were obtained from [8] and [9] and those for cad2 and rax1 from [30]. The expression data were normalised according to the AtGenExpress recommendations using a global mean normalisation excluding the top and bottom 2% of the data. Fold-changes in expression levels between the fry1 mutants and corresponding wild types were calculated from means of the three biological replicates. The resulting data were compared to publically available transcriptome data using iterative group analysis [27] to identify microarray experiments which resulted in similar sets of regulated genes.

Determination of Flux Through Sulfate Assimilation

The flux through sulfate assimilation was measured as incorporation of [³⁵S] from [³⁵S]-sulfate to thiols and proteins essentially as described in [57] and [58]. Three week old plants were transferred into 48-well plates containing 1 mL of MS nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with 5.6 μCi [³⁵S]sulfate (Hartmann Analytic, Braunschweig, Germany) and incubated in light for 4 hours. After the incubation the seedlings were washed 3 times with 2 mL of non-radioactive 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 S-containing compounds was performed exactly as in [22].

Statistical analysis

The results were analysed for variance by the Genstat software using significance level of P = 0.05. When the two independent experiments were treated as variable in two-way ANOVA, the variances within the experiments were not different and the genotype x experiment interactions were not significant. Where only 2 genotypes were compared Student’s T-test was used. For the figures the two experiments were analysed together (n = 6) and the data are presented as means ± standard error, for tables one experiment with 3 biological replicates is shown.

Supporting Information

Figure S1 Phenotype of fou8 apk3 mutant. (PDF)

Figure S2 Glucosinolate and sulfate accumulation in different alleles of fry1. (PDF)
Table S1 Relative expression of genes involved in glucosinolate synthesis in microarrays of apk1 apk2 mutant [20] and three alleles of foulf/fry1 [8–9].

Table S2 AtGen express treatments which produce similar changes to genes significantly affected in expression in fry1/foul.

Table S3 AtGen express treatments which produce similar changes to genes significantly affected in expression in cad2 and rax1.

Table S4 Primers used for qRT-PCR.

References


