Transcriptomic Analysis at Organ and Time Scale Reveals Gene Regulatory Networks Controlling the Sulfate Starvation Response of Solanum Lycopersicum.

Javier Canales (✉ javier.canales@uach.cl)

Universidad Austral de Chile - Campus Isla Teja   https://orcid.org/0000-0002-6263-6974

Felipe Uribe

Universidad Austral de Chile - Campus Isla Teja

Carlos Henríquez-Valencia

Universidad Austral de Chile - Campus Isla Teja

Carlos Lovazzano

Universidad Austral de Chile - Campus Isla Teja

Joaquín Medina

Centro de Biotecnologia y Genomica de Plantas

Elena A. Vidal

Universidad Mayor

Abstract

Background: Sulfur (S) is a major component of biological molecules and thus an essential element for plants. Although the biochemistry and metabolism of sulfate is well understood, little is known about the regulatory mechanisms underlying plant responses to sulfate deprivation, especially in crop plants such as tomato (*Solanum lycopersicum*). In this work we analyzed the impact of sulfate starvation on the transcriptome of tomato plants to identify regulatory networks and key transcriptional regulators at a temporal and organ scale.

Results: Sulfate starvation reduces the growth of roots and leaves which is accompanied by major changes in the organ transcriptome, with the response being temporally earlier in roots than leaves. Comparative analysis showed that a major part of the Arabidopsis and tomato transcriptomic response to sulfate starvation is conserved between these plants and allowed for the identification of processes specifically regulated in tomato, including the control of internal phosphate levels. Integrative gene network analysis uncovered key transcription factors controlling the temporal expression of genes involved in sulfate assimilation, as well as cell cycle, cell division and photosynthesis during sulfate starvation in tomato roots and leaves. Interestingly, one of these transcription factors presents a high identity with SLIM1, a central component of the sulfate starvation response in Arabidopsis.

Conclusions: Together, our results provide the first comprehensive catalog of sulfate-responsive genes in tomato, as well as novel regulatory targets for future functional analyses in tomato and other crops.

Background

S is an essential nutrient for plants, as main component of important biomolecules such as amino acids (methionine, cysteine), glutathione, proteins and sulfolipids [1]. S is also a main component of ferredoxins, iron-sulfur proteins that participate in important metabolic reactions in plants, such as photosynthesis or nitrate reduction and assimilation [2]. As a macronutrient, the average concentration of S in plant tissues for adequate growth is relatively high (around 0.1 to 0.5% of plant dry weight) [3]. However, in recent years, S deficiency has become a rising problem for crop production worldwide, leading to S deficiency symptoms that result in decreased crop yields and quality parameters [4–6]. S deficiency in soils is attributed to decreases in atmospheric S inputs caused by strict industrial emission regulations and changes in agronomic practices, including the use of fertilizers with reduced S compositions, reduction in the use of S-containing fungicides and intensive cropping systems that remove S from the soils [3]. Prolonged S deficiency leads to a decrease in photosynthetic rate, reduced chlorophyll levels and diminished amino acid contents. Moreover, S-starvation alters the acquisition of other nutrients such as nitrate, phosphate, molybdenum, selenium, and iron [7–10]. In this framework, understanding the regulation of the S-starvation response and how it impacts the acquisition and metabolism of S, as well as other processes in plants has become a major interest for research and crop improvement.

Plant acquire S preferentially in the inorganic form of sulfate, which is taken up by root epidermal cells via the action of high affinity sulfate transporters (SULTRs), mainly SULTR1;1 and SULTR1;2 in Arabidopsis [11]. Sulfate can then be transported to the aerial tissues by specific transporters such as SULTR2;1 and SULTR3;5 or stored in vacuoles through the action of SULTR4;1 and SULTR4;2 [12]. The first step in sulfate assimilation is catalyzed by ATP sulfurylase (ATPS), generating adenosine 5'-phosphosulfate (APS) [13]. At this point, APS can be reduced by APS reductase (APR) and sulfite reductase to sulfide, which is incorporated into O-acetylserine (OAS) to form cysteine, or can be phosphorylated by APS kinase to 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which can be utilized as sulfate donor to synthesize a variety of sulfated metabolites .

Sulfate deficiency or starvation triggers important changes in sulfate acquisition and metabolism in plants, such as the catabolism of S-storage compounds and repression of processes involved in the biosynthesis of secondary S metabolites [14]. Moreover, to cope with sulfate limitation, plants can activate high-affinity sulfate uptake transporters and accumulate OAS. A recent meta-analysis showed that cell wall organization, regulation of proteolysis and carbon/nitrogen metabolism are also regulatory targets of the sulfate-starvation response [15]. This complex rearrangement of plant metabolism is partly explained by changes that occur at the transcriptional and post-transcriptional levels. At the transcriptional level, sulfate starvation can induce the expression of SULTRs through S-responsive cis-acting elements present in their promoter regions [16]. To date, most information on possible transcription factors (TFs) mediating the S-starvation response comes from studies performed in the model plant *Arabidopsis thaliana.* The EIL family TF ETHYLENE-INSENSITIVE-LIKE3 (EIL3)/SULFUR LIMITATION 1 (SLIM1), is a central regulator of S-limitation responses, controlling sulfate acquisition and degradation of glucosinolates (GSLs), the major S-containing secondary metabolites present in cruciferous plants [17]. GSL contents are controlled by transcriptional repression of GSL biosynthetic genes by a complex between the SULFUR DEFICIENCY 1 (SD1) and MYB28 TFs [18]. Different MYB TFs have also been reported as important for controlling the homeostasis of GSLs [19–21]. Furthermore, the LONG HYPOCOTYL 5 (HY5) TF has been shown to be an important transcriptional regulator of APR genes APR1 and APR2 [22]. At the post-transcriptional level, the S-starvation-induced expression of microRNA395 (miR395) leads to suppression of ATPS1, ATPS3 and ATPS4 genes and also of sulfate transporter SULTR2;1 in phloem companion cells [23]. Moreover, sulfate homeostasis is controlled at the epigenetic level by DNA methylation of S-deficiency responsive genes by MORE SULPHUR ACCUMULATION 1 (MSA1) [24]. Despite all this accumulated knowledge, the regulatory factors that control sulfate starvation responses in plants -especially in crops- are still largely unknown.

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops in the world [25]. In 2018, the total world area harvested for tomatoes was 4.7 million ha, with an annual production quantity of 182 million tonnes (http://fao.org/faostat/en/#data/QC). Tomato produces fleshy fruits important for the human diet as a source of vitamins and carotenes such as beta-carotene and lycopene [26]. Given its importance as a food source, tomato has been extensively studied, and many genetics and genomics web resources exist today [25]. To date, only a few studies have addressed the impact of sulfate availability on tomato physiology and growth. These studies have shown that sulfate starvation

decreases sulfate accumulation and plant fresh and dry weight, especially in aerial tissues. Sulfate starvation decreases chlorophyll a and b contents, leading to chlorosis on the youngest leaves. Sulfate starvation also leads to a decrease in yield, reducing the weight of immature fruits and the total number of fruits [27–29]. Given the economic importance of tomato worldwide, understanding how tomato responds to sulfate starvation at the molecular level and identifying key regulatory components is of paramount importance for sustainable tomato production in the present and future agricultural scenarios.

In this work, we identified genes and biological processes that participate in the sulfate starvation response of tomato, at a temporal and organ scale using transcriptomics and network analysis. Integration of expression and regulatory TF-target interaction data allowed us to pinpoint key candidate TFs that might coordinate the temporal growth response of roots and leaves to external sulfate availability.

Results

Sulfate starvation has a major impact on leaf and root growth in early stages of tomato development

As previously reported for other tomato cultivars [27–30], we found that the growth of *Solanum lycopersicum* cv. Moneymaker seedlings was severely impacted by sulfate starvation. As shown in Fig. 1A, lack of external sulfate has a clear negative effect on the growth of the aerial tissue, as compared with the full nutrient condition. This effect is noticeable from the third week after sowing. Multifactorial analysis of leaf fresh weight and total leaf area showed that there is a strong interaction between sulfate availability and time factors (p-value <0.001, two-way ANOVA, Fig. 1B and Figure S1), indicating that the growth response of leaves to sulfate availability depends on the plant developmental stage. Accordingly, no significant differences between control and sulfate-starved plants were observed at 2 weeks after sowing (p-value=0.81), while a strong dependence of external sulfate on leaf fresh weight and area was observed 3 and 4 weeks after sowing (p-value <0.001) (Fig. 1B and Figure S1). The same interaction between sulfate and time was observed for root weight, with differences between control and sulfate-starved plants being significant only after 3 and 4 weeks of sowing (p-value <0.001, Fig. 1C). These results indicate that whole-plant growth is severely affected by the lack of external sulfate from 3 weeks after sowing.

Consistent with leaf weight and leaf area measurements over time, there is a steady accumulation of sulfate in leaves, that is abolished in sulfate-starved plants in weeks 3 and 4 (Fig. 1D). In contrast, the increase in root weight over time of plants grown in control condition is not explained by a concurrent increase in root sulfate levels (Fig. 1E). Moreover, no major differences in sulfate accumulation are observed in the roots of full nutrient or sulfate-starved plants (Fig. 1E). Together, these results indicate that additional factors besides sulfate content might be controlling root growth response to sulfate starvation.

Sulfate starvation promotes major changes in leaf and root transcriptomes in a time-dependent manner

In order to gain insights into the molecular mechanisms underlying leaf and root growth repression in response to sulfate starvation, we performed a transcriptomic analysis at a temporal and organ scale, using RNA-seq. To perform this analysis, total RNA from roots and leaves was extracted at 2, 3 and 4 weeks after sowing, considering three independent biological replicates. The RNA-Seq data was pseudo-aligned to the ITAG3.20 transcriptome annotation using kallisto [31] as detailed in Materials and Methods. On average, each sample had 30 million reads, of which 27 million reads (90%) were pseudo-aligned to the tomato transcriptome (Figure S2).

Principal component analysis (PCA) of the RNA-seq data showed that samples are well separated along the first component, which explains more than 50% of the variability in leaf and root samples (Fig. 2). Interestingly, full nutrient and sulfate-starved samples from leaves are grouped together at 2 weeks after sowing, indicating similar transcript expression profiles at this time point (Fig. 2). In contrast, control and sulfate-starved samples from root tissue at 2 weeks are well separated, suggesting that sulfate starvation alters the plant transcriptome in an organ- and time-dependent fashion, with an earlier response in root tissue.

Accordingly, no genes were found differentially expressed between control and sulfate-starved leaves at two weeks after sowing, while 438 genes are differentially expressed in roots in this time point ($\log_2$ Fold Change>1 and q-value<0.05) (Figure S3A and Table S1). In addition, 55% of these 438 genes are subsequently regulated in leaves (241 genes, Figure S3B), which point out an orchestrated whole organism response to sulfate deprivation over developmental time.

A considerable number of genes were differentially expressed by sulfate starvation in roots and leaves: 7024 genes at 3 weeks after sowing and 6163 at 4 weeks after sowing, with most genes being differentially expressed in leaves (Figure S3A and Table S1). This major reprogramming of the plant transcriptome correlates with the observed differences in leaf and root growth of control and sulfate-deprived plants between weeks 3 and 4, suggesting changes in gene expression are partly responsible for the observed phenotypes (Fig. 1A and B).

In order to identify genes that are differentially expressed in response to sulfate starvation, time or a sulfate starvation-time interaction, we performed a multifactorial analysis of RNA-seq data in each tissue using sleuth [32]. In the case of roots, 6052 genes were significantly affected by time, 3755 genes by sulfate starvation and 714 by the interaction of both factors (q-value <0.05, and $\log_2$FC >1) (Fig. 3A). In the case of leaves, 6084 genes were affected by sulfate starvation, 7062 genes by time and 4571 genes by the interaction of both factors (q-value <0.05, and $\log_2$FC >1) (Fig. 3B). In contrast to root tissue, these results indicate that the response to sulfate starvation in leaves has a strong dependence on the plant age. In fact, about 90% of sulfate-responsive genes in leaves are significantly modulated by time (Fig. 3B).

To validate the results of the RNA-seq analysis with an alternative RNA quantification methodology, we selected 10 sulfate-regulated genes in root and leaves involved in different molecular and biological

functions including sulfate metabolism, transport and transcriptional regulation, as well as a group of genes with unknown functions (Table S2). The expression patterns obtained by qPCR analysis showed a significant positive correlation with RNA-seq data (P <0.0001; 0.89 Pearson correlation value; Figure S4), indicating that most of genes analyzed showed a similar expression patterns than the ones identified by RNA-seq analysis.

Comparative analysis of the tomato and Arabidopsis sulfate starvation-responsive transcriptome

In a previous study, we identified 2046 genes regulated by sulfate availability in *Arabidopsis thaliana* using an integrative meta-analysis approach of transcriptomic data [15]. This meta-analysis included samples of roots and leaves at different stages of development (from seedling to juvenile plants). In the case of tomato plants, our multifactorial analysis identified 7589 sulfate-responsive genes in roots and leaves. In order to compare the sulfate starvation responsive transcriptome of both Arabidopsis and tomato, we first performed an orthology analysis of sulfate responsive genes. The identification of orthologous groups allows cross-referencing of genes from multiple species [33]. We used the PLAZA 4.0 database [34] to identify the ortholog group to which each sulfate-regulated gene belongs. Thus, we identified 4262 orthologous gene families out the 7589 sulfate-responsive genes in tomato and 1338 orthologous gene families out the 2046 sulfate-responsive genes in Arabidopsis (Table S3). We found that 70% of the Arabidopsis orthologous gene families are associated with sulfated-responsive genes were also present in tomato plants (Fig. 4A). This result indicates that the RNA-seq analysis in tomato plants captured most of the sulfate-responsive gene families previously detected in Arabidopsis.

Over-representation analysis of biological functions shared by both species revealed several GO terms that are expected to be conserved in the sulfate response as "cellular oxidant detoxification", "sulfate transmembrane transport" or "sulfur utilization" (Table S4). Interestingly, we also found a significant enrichment (adjusted p-value <0.05) in GO terms which have not been previously studied in the context of the response to this nutrient such as those related to cell wall or cytokinin transport (Table S4). On the other hand, specific GO terms of the Brassicaceae family such as GSL biosynthesis are among the most over-represented biological processes in the case of sulfate-responsive genes in Arabidopsis (Fig. 6B and Table S4). The intersection between the lists of orthologues genes of both species also detected sulfate-responsive processes that are found exclusively in tomato plants. The most over-represented biological process in this group of genes was "cellular response to phosphate starvation" suggesting that sulfate deficiency also affects internal phosphate levels in tomato plants. Specifically, we found 52 genes in this biological process including *SPX* genes [35], *phosphatases* and those encoding enzymes involved in phosphate mobilization by membrane lipid remodeling such as sulfoquinovosyl-diacylglycerol [36, 37] (Figure S5). In order to verify the response of this set of genes to sulfate starvation in tomato plants, we computed the average fold of change between sulfate starved and control samples in both tissues and we then performed a hierarchical clustering analysis. Thus, we found that 60% of the genes involved in the phosphate starvation response (30 out 52 genes) showed lower expression levels in sulfate-starved plants at 3 and 4 weeks after sowing in both tissues (Cluster 3, Figure S5), suggesting that sulfate starvation might alter internal phosphate levels. To address this hypothesis, we determined the internal

phosphate levels at 3 weeks after sowing in both tissues. The results shown in Fig. 4D, indicated that sulfate-starved plants exhibited significantly higher phosphate levels that control plants in both tissues, with a greater phosphate accumulation in leaves.

Temporal dynamics of gene expression of tomato roots and leaves are altered by sulfate starvation

In order to get further insights about the temporal patterns of expression of the genes and associated biological processes affected by sulfate starvation, we computed Pearson correlation indexes for each pair of sulfate-regulated genes in roots and leaves. We performed a hierarchical clustering analysis using Dynamic TreeCut [38], and identified two co-expression clusters for genes exclusively regulated by sulfate and six co-expression clusters for genes regulated by sulfate and time in roots and leaves (Fig. 5A, Fig. 5B, Figure S6, Figure S7). We found that the majority of sulfate-responsive genes (60% in roots and 72% in leaves) are contained in Clusters 1 and 2 of genes regulated by both factors (Fig. 5A and Fig. 5B). We thus decided to analyze these clusters in more detail.

As shown in Fig. 5C and Fig. 5D, the expression profiles of Clusters 1 and 2 in roots and leaves show similar trends. Genes in Cluster 1 are repressed over time, with an earlier decrease in mRNA levels under sulfate starvation conditions, between weeks 2 and 3. On the other hand, genes in Cluster 2 slightly increase between weeks 3 and 4 in control conditions, while an important increase in gene expression is shown between weeks 2 and 3 under sulfate starvation conditions (Fig. 5C and Fig. 5D). Although the expression patterns of the genes contained in Clusters 1 and 2 are similar between roots and leaves, the identity of these sulfate-responsive genes differs between these organs (Table S5 and Table S6). As such, we found different enriched biological processes associated with Clusters 1 and 2 in roots and leaves. In roots, Cluster 1 is enriched in genes associated to growth, such as "epidermal cell division" and "plant-type secondary cell wall biogenesis", as well as processes related to redox activity, such as "hydrogen peroxide catabolic process" and "cellular oxidant detoxification" (Fig. 5C and Table S7). In leaves, we found an enrichment of genes involved in photosynthesis, microtubule-based movement and several GO terms related with cell division such as "mitotic cell cycle checkpoint" or "mitotic spindle organization" (Table S8). GO terms related to photosynthesis are especially abundant in this cluster, so we analyzed in more detail the distribution of these genes across different categories of primary metabolism using Mapman4 annotation framework [39]. In the case of photosynthesis, most genes are involved in light reactions, followed by the Calvin-Benson cycle and sucrose/starch metabolism (Figure S8). Cluster 1 genes are also involved in other aspects of primary metabolism such as nitrogen assimilation and amino acid biosynthesis as well as fatty acid biosynthesis (Figure S8). In the case of Cluster 2, and consistent with the marked induction in gene expression triggered by sulfate starvation shown in this cluster, we found an overrepresentation of biological processes related to the cellular response to S starvation such as *5'-adenylylsulfate reductase* genes (Solyc02g032860, Solyc02g080640 and Solyc03g031620), *RESPONSE TO LOW SULFUR* (*LSU*) genes (Solyc03g096760, Solyc03g096770, Solyc03g096780) or *sulfate transporters* (Solyc05g054740, Solyc04g054730, Solyc04g072760, Solyc12g056930) (Fig. 5C and Table S7). Cluster 2 in leaves is enriched in GO terms related with the response to stress and protein phosphorylation (Fig. 5D and Table S8). Interestingly, we did not find an overrepresentation of S-

starvation-related processes in Cluster 2 in leaves, as observed in roots. In fact, classical genes involved in the sulfate starvation response such as *SULTR*, *ATPS, APR* or *LSU* belong to Cluster 5 (Figure S7), suggesting an organ-specific expression of these genes in response to external S during development.

In summary, sulfate starvation promotes significant changes in the temporal expression patterns of a large number of genes in roots and leaves, of which many are associated with functions related to cellular growth, photosynthesis and sulfate uptake and metabolism. Moreover, the comparative analysis of major gene co-expression clusters between roots and leaves indicates an organ-specific response of the tomato plants to sulfate starvation.

Identification of transcription factors involved in the sulfate starvation response in tomato

Our results point at major transcriptomic changes occurring in tomato roots and leaves in response to sulfate starvation. As described in Arabidopsis, some of these changes may be attributed to transcriptional regulation by TFs. Although some TFs controlling sulfate responses have been identified in Arabidopsis [40], to date no information about sulfate-responsive TFs and their role in responses to sulfate starvation in tomato. In order to identify key TFs controlling the response to sulfate starvation of tomato, we constructed gene regulatory networks for roots and leaves [41] as explained in Methods.

In the case of roots, we obtained a gene regulatory network consisting of 24 TFs and 172 targets (Figure S9 and Table S9). TFs of the root regulatory network belong to 17 different families (Table S10), with MYB and bZIP TFs being the most abundant families (4 and 3 members, respectively). In the same way, we constructed a TF-target network using the identified sulfate-regulated genes in leaves which was composed by 45 TFs and 566 targets (Figure S10 and Table S11). Specifically, ERF, TCP and WRKY were the most abundant TF families in the leaves network with 7 and 5 members, respectively (Table S10). Notably, the most abundant TF families in the regulatory network of leaves were not shared with the root network (Table S11), suggesting organ-specific regulation of the transcriptional response to sulfate starvation.

We next focused our analysis on TFs whose predicted target genes have overrepresented biological functions (adjusted p-value <0.05), in order to correlate TF regulation to changes in functional processes. As shown in Fig. 6A and Fig. 6B, we found 3 TFs in roots and 10 TFs in leaves that potentially control genes involved in the sulfate starvation response, phytohormone biosynthesis and signaling, senescence, among others biological processes. Most connected factors in terms of outdegree were a MYB TF (Solyc11g071300) in roots and TCP 21 TF (Solyc03g006800) in leaves, showing 40 and 71 targets respectively (Fig. 6A and Fig. 6B). Notably, MYB target genes are involved in S utilization, while TCP21 ones are involved in photosynthesis (Fig. 6A and Fig. 6B, Table S12).

As mentioned above, our results discovered important changes in gene expression in response to sulfate starvation, suggesting the involvement of different sets of TFs acting in both roots and leaves. However, we found one TF shared by both root and leaf gene regulatory networks which is early induced in roots but later in leaves (Fig. 6C). This TF is member of the EIL family and corresponds to "Ethylene Insensitive

3-Like3" (EIL3), Solyc01g006650. The closest Arabidopsis gene in terms of amino acid identity is SLIM1 (Table S13), which is a key regulatory factor of the sulfate starvation response [17]. In addition, GO enrichment analysis showed that the identified tomato EIL3 TF might control the expression of a set of genes related to sulfate starvation, including classical sulfate-responsive genes such as *LSU*s as well as genes encoding enzymes involved in the reductive steps of sulfate assimilation (Table S14). Furthermore, we found that 60% of the tomato TF target genes are shared with targets predicted for Arabidopsis *SLIM1,* according to PlantRegMap, including sulfate-response genes such as *LSU*s, *APR*s and *SDI*s (Table S14). Overall, we identified several candidate TFs to mediate the transcriptome reprogramming that occurs during sulfate starvation response in roots and leaves, highlighting the possible key regulatory role of the EIL3 TF in tomato.

Discussion

Temporal and organ-specific transcriptomic response to sulfate starvation in tomato plants

Our current understanding of the transcriptomic response of plants to sulfate availability is mostly based on knowledge gathered in the model plant *Arabidopsis thaliana* [40]. Most of the analyses carried out in Arabidopsis do not contain whole transcriptome information since they were performed using microarray technology [15, 40]. On the other hand, the tissue-specific response to sulfate availability over time has been poorly explored so far and most studies have been performed at the seedling stage in Arabidopsis. To overcome these limitations, we performed a comprehensive transcriptome analysis of the sulfate starvation response of tomato plants using RNA-seq, with a temporal and organ resolution.

We found that sulfate starvation has a strong impact on the growth of roots and leaves, starting from 3 weeks after sowing. The inhibition of growth was accompanied with drastic changes in gene expression in roots and leaves. Analysis of enriched GO terms associated with sulfate-responsive genes allowed us to discover several biological processes related to plant growth that were altered by sulfate starvation in both organs. To date, several studies have demonstrated that plants activate sulfate uptake by inducing the expression of high-affinity sulfate transporters in response to sulfate starvation [14]. Moreover, it is well established that plants activate the sulfate assimilatory pathway and the degradation of glutathione and other S compounds such as GSLs during sulfate starvation [14]. As expected, genes involved in sulfate transport and metabolism are differentially expressed according to external sulfate availability in tomato plants, being induced over time in response to sulfate starvation. Specifically, we identified 12 sulfate transporter genes, 3 *ATPS* and 3 *APR* genes significantly regulated by sulfate starvation in at least one sample (q-value <0.05 and log2FC>1) (Table S15).

Our expression analysis also identified several genes involved in cell wall biosynthesis and modification whose expression was altered by sulfate deprivation. It is well known that cell wall is a crucial component for plant biomass accumulation and cell expansion, which are complementary processes that sustain plant growth [42]. However, how nutrient availability regulates cell wall biosynthesis and organization remains poorly understood [43]. In this work we found that the expression of 6 *XET* genes increases over

time in control plants, while it decreases over time in sulfate-starved plants (Figure S6A and Table S6). *XET* genes code for enzymes that have the capacity to loosen cell walls, affecting cell expansion and growth [44]. Thus, repression of *XET* genes by sulfate starvation can partly explain the observed phenotypes. Accordingly, XET knock out mutants have reduced cell sizes [45–48] and overexpression or exogenous application of XET proteins decrease cell expansion [49], suggesting that the regulation of *XET* genes by sulfate starvation might partly explain the observed phenotypes.

Our expression analyses also reveal a significant level of organ-specific response related to the growth inhibition phenotype. Sulfate starvation triggers a strong decrease in the transcript levels of genes related to photosynthesis in leaves, including those related to light and carbon reactions. Taking into account the well-known relationship between plant growth and photosynthesis [50], lower expression of photosynthetic genes likely translates into decreased growth as we observed in the case of sulfate starvation. Interestingly, a similar inhibitory effect of the sulfate starvation on photosynthesis and growth has been demonstrated in other photosynthetic organisms such as microalgae *Dunaliella salina* [51] and *Chlamydomonas reinhardtii* [52], suggesting that the regulation of photosynthetic genes by sulfate starvation might be conserved in photosynthetic organisms.

Phosphate levels are regulated by sulfate starvation in tomato

In order to identify novel and specific biological processes in the response to sulfate starvation of tomato plants, we intersected the orthologous groups of genes regulated by sulfate availability in this work with those reported in a previous meta-analysis in Arabidopsis [15]. Remarkably 70% of the Arabidopsis sulfate-responsive orthologous genes were also regulated by this nutrient in tomato plants (Fig. 4A) indicating a conserved response to sulfate between Arabidopsis and tomato plants. On the other hand, we also found a group of orthologous genes exclusively regulated by sulfate in tomato plants, which showed over-represented biological process including "cellular response to phosphate starvation" "photorespiration", "photosynthetic electron transport in photosystem", "ornithine metabolic process", "sucrose metabolic process", among others (Fig. 4C and Table S4), suggesting additional mechanisms might be involved in sulfate starvation responses in tomato.

Remarkably, the most over-represented biological process of orthologous genes exclusively regulated by sulfate in tomato plants was "cellular response to phosphate starvation", suggesting that sulfate deficiency also affects the internal phosphate levels in tomato plants. In agreement with this result, phosphate content analysis revealed an increase of this nutrient in sulfate-starved plants in both organs. A similar alteration of phosphate levels by sulfate starvation has been reported in Arabidopsis [9, 10], with plants showing a 3-fold increase of free phosphate in response to S-deficiency. This increase in phosphate levels is not triggered by deficiency of other nutrients such as potassium or iron [9], indicating a specific interaction of sulfate and phosphate homeostasis.

The increase of internal phosphate levels can be achieved by an activation of phosphate transport during sulfate starvation, as has been previously demonstrated in Arabidopsis [10]. Although we found a higher phosphate content in both organs in sulfate-starved plants, phosphate accumulation was higher in

leaves, suggesting an increased translocation of phosphate from roots to leaves. In Arabidopsis, phosphate transport from root to shoot is mediated by the PHO1 transporter [53], whose expression is controlled by phosphate starvation at the transcriptional and post-translational levels [8]. PHO1 together with PHT1;9, a member of the PHT1 family of phosphate transporters, are involved in the increased phosphate translocation from roots to shoots during sulfate starvation in Arabidopsis [10]. We found that several members of the PHO1 and PHT1 gene families (Solyc02g088220, Solyc09g066410 and Solyc09g090080; Figure S10) are induced by sulfate starvation in tomato, suggesting that sulfate starvation promotes phosphate transport from roots to leaves in tomato. These results are consistent with previous works in potato and tomato showing that transcript levels of the phosphate transporters StPT2 and LePT1 increases under sulfate starvation [54, 55].

In Arabidopsis, the transcript levels of the main *phosphate uptake transporters (PHT1;1, PHT1;2, and PHT1;4*) and *PHO1* genes are not affected by sulfate starvation [10], which suggest significant differences in the mechanism of control of phosphate allocation between Arabidopsis and tomato during sulfate starvation. The biological role of increasing phosphate levels in response to sulfate starvation is not fully understood. A rapid replacement of sulfolipids by phospholipids has been reported to occur during the early stage of sulfate starvation [56]. This increased demand of phosphate might require an enhanced acquisition of this nutrient in later stages of sulfate starvation response, involving a higher phosphate demand that later might requires a greater uptake of this nutrient. Recent work in Arabidopsis has shown that the re-addition of sulfate to the medium restores phosphate levels in shoots and xylem sap [10], indicating a direct relation between sulfate and phosphate levels. However, further research is required to unravel the complex crosstalk between sulfate and phosphate starvation.

Identification of key TFs involved in response to sulfate-starvation in tomato plants

Gene regulatory network analysis integrating gene expression data and regulatory TF-target data allowed us to identify candidate TFs involved in the response to sulfate starvation during early development of tomato plants. Among the TFs regulated by sulfate starvation in both tissues, we identified a member of the EIL family, whose closest Arabidopsis homolog is SLIM1, a key regulatory factor in the sulfate starvation response [17]. Transcriptome analysis of *slim1* mutants has shown reduced expression of sulfate transporters and increased expression of genes involved in GSL synthesis [17]. Moreover, SLIM1 is required for plant growth under sulfate starvation conditions [17]. Notably, SLIM1 homologs from rice have been reported to complement the *slim1* mutants from Arabidopsis, indicating a functional conservation of this group of EIL TFs in controlling the sulfate starvation response [17]. Our network analysis highlighted that the identified tomato *EIL3* might control an important group of genes involved in sulfate transport and metabolism, like Arabidopsis SLIM1 (Table S14). Specifically, we found that 60% of the predicted targets of the tomato *EIL3* are also described as SLIM1 ones.

Interestingly, the mRNA levels of Arabidopsis *SLIM1* seems to be not controlled by sulfate starvation [14, 57]. The observation that neither nuclear localization nor abundance of SLIM1 protein are changed by S conditions [14, 57], may indicate that its function is mainly controlled by post-translational mechanisms

Arabidopsis. In contrast, we found that the transcript levels of tomato *EIL3* are dramatically increased by sulfate starvation in roots and leaves of tomato plants. However, we cannot rule out that sulfate starvation might also control EIL3 function at the post-translational level. Further work is therefore required to assess the role of EIL3 in the sulfate response of tomato plants.

Besides EIL3, our analysis identified a Myb TF (Solyc11g071300) as the most connected TF of the root regulatory network. According to the functional analysis of its target genes, the identified Myb TF might be involved in the control of cell division, which is an important biological process for plant growth. In agreement, Arabidopsis orthologues MYB3R-1 and MYB3R-4 act as transcriptional activators regulating cytokinesis [58]. In fact, 53.3% of the genes from the G2/M-specific class were downregulated in double mutant Arabidopsis plants. Besides, it was uncovered that both MYB3R4 TFs bind to MSA motifs in vitro, which are common cis-elements of G2/M phase-specific genes [59]. All these data suggest that the MYB might play an important role controlling cell division under sulfate starvation conditions.

On the other hand, the most connected TF of the leaf regulatory network was TCP21 (Solyc03g006800). According to the GO enrichment analysis of its target genes, this TF is related to the control of photosynthesis, which is an essential metabolic process for vegetative growth. Interestingly, several TCP TFs in Arabidopsis have been shown to regulate leaf growth and development [60]. The closest homolog gene of the tomato TCP in Arabidopsis is TCP19, which is involved in the control of leaf senescence in a redundant fashion with TCP20 [61]. Our transcriptomic analysis shows that the expression of the identified tomato TCP is reduced during sulfate starvation in leaves along with genes related to photosynthesis, leading to a strong reduction in plant growth. Therefore, TCPs might play important functions in the control of leaf growth during sulfate starvation in tomato plants.

Conclusions

Our results shed lights into the extensive transcriptome reprogramming that is elicited during sulfate starvation in tomato roots and leaves and highlight the role of TFs such as MYB, TCP and EIL3 in coordinating this complex response. We hope this work can serve as a basis for further studies addressing the role of specific genes and processes to further our understanding of the regulatory mechanisms underlying sulfate starvation responses in tomato and other crops.

Methods

Plant material and growth conditions

Seeds of the *Solanum lycopersicum* 'Moneymaker' cultivar (obtained commercially from Semilias, Chile) were grown for 4 weeks in 0.5X Murashige and Skoog (MS) salts [62] (pH 5.7) using rockwool as support material. This liquid culture medium was replaced twice a week to maintain constant nutrient concentrations. For plants grown in sulfate starvation conditions, sulfate salts contained in the MS medium were replaced with equivalent chloride salts [63]. Tomato plants were cultivated in a controlled

growth cabinet (Bioref-19, Pitec, Chile) with LED lights (200 µmol m^{-2} s^{-1}) at 22°C under 16-h-light/8-h-dark cycles. Three independent experiments were performed using 3 to 5 different plants per replicate.

Plant growth quantification

 For determination of growth-related parameters, leaves and roots were scanned using an Epson Perfection V600 photo scanner. Total leaf area and primary root length were determined from scanned images using the ImageJ software (v1.52) (https://imagej.nih.gov/ij/). Total root and leaf fresh weights were determined using an analytical balance.

Library construction and RNA-Seq data analysis

Leaves and roots samples were frozen in liquid nitrogen at 2, 3 and 4 weeks after sowing and RNA was isolated using the mirVana miRNA Isolation Kit (Invitrogen), following the manufacturer´s instructions for total RNA isolation. The extraction was subjected to on-column DNase I treatment (TURBO DNase, Invitrogen). One microgram of DNase I-treated RNA was used to generate poly-A-enriched sequencing libraries using the TruSeq Stranded mRNA Library Prep kit (Illumina). Libraries were sequenced as 75-nt single-end reads on a NextSeq500 system (Illumina).

The sequenced reads were quality-checked using FastQC version 0.11.5. Taking into account the high quality of the sequenced reads, we skipped the trimming process in order to avoid any potential biases as has been previously described [64]. To quantify the mRNA levels of annotated genes, sequenced reads were pseudo-aligned to the publicly available *Solanum lycopersicum* transcriptome using kallisto (v0.44) [31]. Transcript indices for kallisto were generated from tomato annotation version ITAG3.2 (https://solgenomics.net/organism/Solanum_lycopersicum/genome) which included 35,768 cDNAs. The estimated read counts and calculated transcripts per million (TPM) were used for differential expression analysis [32]. Differential expression analysis of the sulfate starvation response in each time and organ was performed using the likelihood ratio test (LRT) implemented in sleuth (v.0.30.0) [32] with three independent replicates. We defined differential gene expression by setting an arbitrary q-value threshold of 0.05 and absolute log$_2$ Fold Change (FC)>1.

We performed a multivariate linear model to test whether the expression of a given gene could be explained by the sulfate availability, time or the interaction of both factors using the R package sleuth (v.0.30.0) [32]. In order to identify significantly regulated genes for each factor, we also applied a q-value threshold of 0.05 and absolute log$_2$ FC>1 in at least one level of each factor. The identification of gene co-expression clusters associated to each factor was performed using the R package dynamicTreeCut (v.1.63-1) [38], which is a hierarchical clustering method for automatic identification of gene co-expression clusters. Principal component analysis (PCA) was performed on the expression data using pcaExplorer (v.2.14.0) [65].

Gene Ontology enrichment analysis

Gene Ontology (GO) enrichment analyses were performed using BiNGO (v.3.0.3) [66] with *Solanum lycopersicum* GO annotations obtained from PLAZA 4.0 [34]. Benjamini and Hochberg false discovery rate (FDR) correction (<0.05) was applied on the over-represented GO terms obtained in the respective gene set after performing a hypergeometric test with BiNGO. We filtered out the GO terms with less than three annotated genes and used only GO terms associated to biological processes. To reduce the redundancy between GO terms, we selected the overrepresented GO terms with the higher level in the hierarchical GO tree obtained with BiNGO.

Comparative transcriptomics analyses

In order to determine the sulfate-starvation responsive genes that are shared between Arabidopsis and tomato, we used the ortholog database PLAZA 4.0 [34] to assign a common identification code for the genes of both species (orthologous gene family ID). We intersected both lists using VennPainter software [67] to identify conserved and species-specific sulfate-responsive gene families. The group of Arabidopsis sulfate-responsive genes was obtained from a recent transcriptomic meta-analysis by our group [15]. In the case of tomato, the set of sulfate-responsive genes was obtained from the RNA-seq analysis performed in this work, considering genes significantly regulated by sulfate factor or by the interaction of sulfate and time in roots or leaves (q-value threshold of 0.05 and absolute $\log_2$ FC>1).

Gene regulatory network analysis

We constructed a gene regulatory network for each organ considering the following criteria: first, we only included in this analysis genes that showed a significant regulation by sulfate or by the interaction of sulfate and time factors (q-value <0.05 and absolute $\log_2$ FC>1). Second, we only considered genes that have evidence of TF-target interaction according to the PlantRegMap [41] in order to predict direct regulations. Third, pairs of genes with a TF-target interaction must have a Pearson correlation greater than 0.9 to obtain similar expression profiles between TFs and their target genes. In this manner, we focused on coherent temporal responses of TFs and their targets to sulfate starvation. Pearson correlation was determined from the RNA-seq data obtained in each tissue using the R package rsgcc (v.1.0.6) [68].

Gene regulatory networks were visualized and analyzed using Cytoscape 3.7 [69]. Candidate TFs were selected considering their connectivity and overrepresented GO terms of their target genes.

Quantitative real-time PCR (qPCR)

For qPCR analyses, cDNA was generated from 500ng of total RNA using 5X All-In-One RT MasterMix (Applied Biological Materials, Canada) following the manufacturer's instructions. qPCR reactions were performed with 25 ng of cDNA using the PowerUp SYBR Green Master Mix (Applied Biosystems™) in a CFX96 Real-Time PCR Detection System (Bio-Rad). Raw fluorescent data was analyzed using the Real-time PCR Miner 4.0 software [70] to obtain cycle threshold values and gene amplification efficiencies. The expression of each target gene of interest was normalized by the Actin-7 gene (Solyc11g005330), which

showed stable mRNA levels across all samples analyzed by RNA-seq. Sequences of the qPCR primers used in this study are provided in Table S16.

Sulfate and phosphate content analysis

Sulfate content was quantified using a turbidimetric method described by Tabatabai and Bremner [71, 72]. Phosphate content was determined using the Malaquite Green Phosphate Assay Kit (Sigma-Aldrich, Catalog Number MAK307) according to the manufacturer's recommendations.

Abbreviations

Sulfur (S)

3'-phosphoadenosine 5'-phosphosulfate (PAPS)

High affinity sulfate transporters (SULTRs)

Transcription factors (TFs)

ETHYLENE-INSENSITIVE-LIKE3 (EIL3)/SULFUR LIMITATION 1 (SLIM1)

Glucosinolates (GSLs)

SULFUR DEFICIENCY 1 (SD1)

LONG HYPOCOTYL 5 (HY5)

MORE SULPHUR ACCUMULATION 1 (MSA1)

Murashige and Skoog salts (MS)

Likelihood ratio test (LRT)

Transcripts per million (TPM)

Fold Change (FC)

Gene Ontology (GO)

False discovery rate (FDR)

Quantitative real-time PCR (qPCR)

Analysis of Variance (ANOVA)

Principal Component Analysis (PCA)

XET (xyloglucan endotransglucosylase/hydrolase)

RESPONSE TO LOW SULFUR (LSU)

Ethylene Insensitive 3-Like3 (EIL3)

Declarations

References

1. Takahashi H, Kopriva S, Giordano M, Saito K, Hell R. Sulfur Assimilation in Photosynthetic Organisms: Molecular Functions and Regulations of Transporters and Assimilatory Enzymes. Annu Rev Plant Biol. 2011;62:157–84.

2. Barker AV, Pilbeam DJ, Pilbeam DJ. Handbook of Plant Nutrition. CRC Press; 2015. doi:10.1201/b18458.

3. Dick WA, Kost D, Chen L. Availability of Sulfur to Crops from Soil and Other Sources. In: Sulfur: A Missing Link between Soils, Crops, and Nutrition. John Wiley & Sons, Ltd; 2015. p. 59–82.

doi:10.2134/agronmonogr50.c5.

4. McGrath SP, Sidoli CMD, Baker AJM, Reeves RD. The Potential for the Use of Metal-Accumulating Plants for the in Situ Decontamination of Metal-Polluted Soils. In: Eijsackers HJP, Hamers T, editors. Integrated Soil and Sediment Research: A Basis for Proper Protection. Dordrecht: Springer Netherlands; 1993. p. 673–6.

5. McGrath SP, Zhao FJ. Sulphur uptake, yield responses and the interactions between nitrogen and sulphur in winter oilseed rape (Brassica napus). J Agric Sci. 1996;126:53–62.

6. Bonnot T, Bancel E, Alvarez D, Davanture M, Boudet J, Pailloux M, et al. Grain subproteome responses to nitrogen and sulfur supply in diploid wheat Triticum monococcum ssp. monococcum. Plant J. 2017;91:894–910.

7. White PJ, Bowen HC, Parmaguru P, Fritz M, Spracklen WP, Spiby RE, et al. Interactions between selenium and sulphur nutrition in Arabidopsis thaliana. J Exp Bot. 2004;55:1927–37.

8. Briat JF, Rouached H, Tissot N, Gaymard F, Dubos C. Integration of P, S, Fe, and Zn nutrition signals in Arabidopsis thaliana: Potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). Front Plant Sci. 2015;6 APR:290.

9. Forieri I, Sticht C, Reichelt M, Gretz N, Hawkesford MJ, Malagoli M, et al. System analysis of metabolism and the transcriptome in Arabidopsis thaliana roots reveals differential co-regulation upon iron, sulfur and potassium deficiency. Plant Cell Environ. 2017;40:95–107.

10. Allahham A, Kanno S, Zhang L, Maruyama-Nakashita A. Sulfur Deficiency Increases Phosphate Accumulation, Uptake, and Transport in Arabidopsis thaliana. Int J Mol Sci. 2020;21:2971.

11. Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K. Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. Plant J. 2002;29:465–73.

12. Takahashi H. Sulfate transport systems in plants: functional diversity and molecular mechanisms underlying regulatory coordination. J Exp Bot. 2019;70:4075–87.

13. Kopriva S, Mugford SG, Baraniecka P, Lee B-R, Matthewman CA, Koprivova A. Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis. Front Plant Sci. 2012;3. doi:10.3389/fpls.2012.00163.

14. Maruyama-Nakashita A. Metabolic changes sustain the plant life in low-sulfur environments. Curr Opin Plant Biol. 2017;39:144–51.

15. Henríquez-Valencia C, Arenas-M A, Medina J, Canales J. Integrative Transcriptomic Analysis Uncovers Novel Gene Modules That Underlie the Sulfate Response in Arabidopsis thaliana. Front Plant Sci. 2018.

16. Takahashi H. Chapter 4 - Regulation of Sulfate Transport and Assimilation in Plants. In: Jeon KW, editor. International Review of Cell and Molecular Biology. Academic Press; 2010. p. 129–59. doi:10.1016/S1937-6448(10)81004-4.

17. Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H. Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell. 2006;18:3235–51.

18. Aarabi F, Kusajima M, Tohge T, Konishi T, Gigolashvili T, Takamune M, et al. Sulfur deficiency–induced repressor proteins optimize glucosinolate biosynthesis in plants. Sci Adv. 2016;2:e1601087.

19. Celenza JL, Quiel JA, Smolen GA, Merrikh H, Silvestro AR, Normanly J, et al. The Arabidopsis ATR1 Myb Transcription Factor Controls Indolic Glucosinolate Homeostasis. Plant Physiol. 2005;137:253–62.

20. Gigolashvili T, Yatusevich R, Berger B, Müller C, Flügge U-I. The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in Arabidopsis thaliana. Plant J. 2007;51:247–61.

21. Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, et al. Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. Proc Natl Acad Sci U S A. 2007;104:6478–83.

22. Lee B-R, Koprivova A, Kopriva S. The key enzyme of sulfate assimilation, adenosine 5'-phosphosulfate reductase, is regulated by HY5 in Arabidopsis. Plant J Cell Mol Biol. 2011;67:1042–54.

23. Liang G, Yang F, Yu D. MicroRNA395 mediates regulation of sulfate accumulation and allocation in Arabidopsis thaliana. Plant J. 2010;62:1046–57.

24. Huang X-Y, Chao D-Y, Koprivova A, Danku J, Wirtz M, Müller S, et al. Nuclear Localised MORE SULPHUR ACCUMULATION1 Epigenetically Regulates Sulphur Homeostasis in Arabidopsis thaliana. PLoS Genet. 2016;12. doi:10.1371/journal.pgen.1006298.

25. Kimura S, Sinha N. Tomato (Solanum lycopersicum): A Model Fruit-Bearing Crop. Cold Spring Harb Protoc. 2008;2008:pdb.emo105.

26. Canene-Adams K, Campbell JK, Zaripheh S, Jeffery EH, Erdman JW. The Tomato As a Functional Food. J Nutr. 2005;135:1226–30.

27. Lopez J, Tremblay N, Voogt W, Dubé S, Gosselin A. Effects of varying sulphate concentrations on growth, physiology and yield of the greenhouse tomato. Sci Hortic. 1996;67:207–17.

28. Zuchi S, Cesco S, Varanini Z, Pinton R, Astolfi S. Sulphur deprivation limits Fe-deficiency responses in tomato plants. Planta. 2009;230:85–94.

29. Zuchi S, Watanabe M, Hubberten H-M, Bromke M, Osorio S, Fernie AR, et al. The Interplay between Sulfur and Iron Nutrition in Tomato. Plant Physiol. 2015;169:2624.

30. Alhendawi RA, Kirkby EA, Pilbeam DJ. Evidence That Sulfur Deficiency Enhances Molybdenum Transport in Xylem Sap of Tomato Plants. J Plant Nutr. 2005;28:1347–53.

31. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 2016;34:525–7.

32. Pimentel H, Bray NL, Puente S, Melsted P, Pachter L. Differential analysis of RNA-seq incorporating quantification uncertainty. Nat Methods. 2017;14:687–90.

33. Li L, Stoeckert CJ, Roos DS. OrthoMCL: Identification of Ortholog Groups for Eukaryotic Genomes. Genome Res. 2003;13:2178–89.

34. Aleksza D, Horváth GV, Sándor G, Szabados L, Barragan V, Leidi EO, et al. PLAZA 4.0: An integrative resource for functional, evolutionary and comparative plant genomics. Nucleic Acids Res. 2016.

35. Duan K, Yi K, Dang L, Huang H, Wu W, Wu P. Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant J. 2008;54:965–75.

36. Okazaki Y, Otsuki H, Narisawa T, Kobayashi M, Sawai S, Kamide Y, et al. A new class of plant lipid is essential for protection against phosphorus depletion. Nat Commun. 2013;4.

37. Sun Y, Jain A, Xue Y, Wang X, Zhao G, Liu L, et al. OsSQD1 at the crossroads of phosphate and sulfur metabolism affects plant morphology and lipid composition in response to phosphate deprivation. Plant Cell Environ. 2020;n/a n/a. doi:10.1111/pce.13764.

38. Langfelder P, Zhang B, Horvath S. Defining clusters from a hierarchical cluster tree: the Dynamic Tree Cut package for R. Bioinformatics. 2008;24:719–20.

39. Schwacke R, Ponce-Soto GY, Krause K, Bolger AM, Arsova B, Hallab A, et al. MapMan4: A Refined Protein Classification and Annotation Framework Applicable to Multi-Omics Data Analysis. Mol Plant. 2019;12:879–92.

40. Watanabe M, Hoefgen R. Sulphur systems biology-making sense of omics data. J Exp Bot. 2019;70:4155–70.

41. Tian F, Yang D-C, Meng Y-Q, Jin J, Gao G. PlantRegMap: charting functional regulatory maps in plants. Nucleic Acids Res. 2020;48:D1104–13.

42. Ivakov A, Flis A, Apelt F, Fünfgeld M, Scherer U, Stitt M, et al. Cellulose synthesis and cell expansion are regulated by different mechanisms in growing arabidopsis hypocotyls. Plant Cell. 2017;29:1305–15.

43. Ogden M, Hoefgen R, Roessner U, Persson S, Khan G. Feeding the Walls: How Does Nutrient Availability Regulate Cell Wall Composition? Int J Mol Sci. 2018;19:2691.

44. Miedes E, Suslov D, Vandenbussche F, Kenobi K, Ivakov A, Van Der Straeten D, et al. Xyloglucan endotransglucosylase/hydrolase (XTH) overexpression affects growth and cell wall mechanics in etiolated Arabidopsis hypocotyls. J Exp Bot. 2013;64:2481–97.

45. Osato Y, Yokoyama R, Nishitani K. A principal role for AtXTH18 in Arabidopsis thaliana root growth: a functional analysis using RNAi plants. J Plant Res. 2006;119:153–62.

46. Liu Y-B, Lu S-M, Zhang J-F, Liu S, Lu Y-T. A xyloglucan endotransglucosylase/hydrolase involves in growth of primary root and alters the deposition of cellulose in Arabidopsis. Planta. 2007;226:1547–60.

47. Sasidharan R, Chinnappa CC, Staal M, Elzenga JTM, Yokoyama R, Nishitani K, et al. Light Quality-Mediated Petiole Elongation in Arabidopsis during Shade Avoidance Involves Cell Wall Modification by Xyloglucan Endotransglucosylase/Hydrolases. Plant Physiol. 2010;154:978.

48. Ohba T, Takahashi S, Asada K. Alteration of fruit characteristics in transgenic tomatoes with modified expression of a xyloglucan endotransglucosylase/hydrolase gene. Plant Biotechnol. 2011;28:25–32.

49. Maris A, Suslov D, Fry SC, Verbelen J-P, Vissenberg K. Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of Arabidopsis and their effect on root growth and cell wall extension. J Exp Bot. 2009;60:3959–72.

50. Kirschbaum MUF. Does enhanced photosynthesis enhance growth? Lessons learned from CO2 enrichment studies. Plant Physiol. 2011;155:117–24.

51. Giordano M, Pezzoni V, Hell R. Strategies for the allocation of resources under sulfur limitation in the green alga Dunaliella salina. Plant Physiol. 2000;124:857–64.

52. Davies JP, Yildiz FH, Grossman A. Sac1, a putative regulator that is critical for survival of Chlamydomonas reinhardtii during sulfur deprivation. EMBO J. 1996;15:2150–9.

53. Hamburger D, Rezzonico E, Petétot JMDC, Somerville C, Poirier Y. Identification and characterization of the Arabidopsis PHO1 gene involved in phosphate loading to the xylem. Plant Cell. 2002;14:889–902.

54. Leggewie G, Willmitzer L, Riesmeier JW. Two cDNAs from potato are able to complement a phosphate uptake-deficient yeast mutant: identification of phosphate transporters from higher plants. Plant Cell. 1997;9:381–92.

55. Daram P, Brunner S, Persson BL, Amrhein N, Bucher M. Functional analysis and cell-specific expression of a phosphate transporter from tomato. Planta. 1998;206:225–33.

56. Rouached H. Multilevel coordination of phosphate and sulfate homeostasis in plants. Plant Signal Behav. 2011;6:952–5.

57. Wawrzyńska A, Sirko A. To control and to be controlled: understanding the Arabidopsis SLIM1 function in sulfur deficiency through comprehensive investigation of the EIL protein family. Front Plant Sci. 2014;5. doi:10.3389/fpls.2014.00575.

58. Haga N, Kobayashi K, Suzuki T, Maeo K, Kubo M, Ohtani M, et al. Mutations in MYB3R1 and MYB3R4 cause pleiotropic developmental defects and preferential down-regulation of multiple G2/M-specific genes in Arabidopsis. Plant Physiol. 2011;157:706–17.

59. Ito M, Iwase M, Kodama H, Lavisse P, Komamine A, Nishihama R, et al. A novel cis-acting element in promoters of plant B-type cyclin genes activates M phase-specific transcription. Plant Cell. 1998;10:331–41.

60. Danisman S. TCP transcription factors at the interface between environmental challenges and the plant's growth responses. Front Plant Sci. 2016;7:1930.

61. Danisman S, van Dijk ADJ, Bimbo A, van der Wal F, Hennig L, de Folter S, et al. Analysis of functional redundancies within the Arabidopsis TCP transcription factor family. J Exp Bot. 2013;64:5673–85.

62. Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant. 1962;15:473–97.

63. Yoshimoto N, Inoue E, Watanabe-Takahashi A, Saito K, Takahashi H. Posttranscriptional Regulation of High-Affinity Sulfate Transporters in Arabidopsis by Sulfur Nutrition. Plant Physiol. 2007;145:378–88.

64. Williams CR, Baccarella A, Parrish JZ, Kim CC. Trimming of sequence reads alters RNA-Seq gene expression estimates. BMC Bioinformatics. 2016;17. doi:10.1186/s12859-016-0956-2.

65. Marini F, Binder H. pcaExplorer: an R/Bioconductor package for interacting with RNA-seq principal components. BMC Bioinformatics. 2019;20:331.

66. Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinforma Oxf Engl. 2005;21:3448–9.

67. Lin G, Chai J, Yuan S, Mai C, Cai L, Murphy RW, et al. VennPainter: A Tool for the Comparison and Identification of Candidate Genes Based on Venn Diagrams. PloS One. 2016;11:e0154315.

68. Ma C, Wang X. Application of the Gini correlation coefficient to infer regulatory relationships in transcriptome analysis. Plant Physiol. 2012;160:192–203.

69. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 2003;13:2498–504.

70. Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol J Comput Mol Cell Biol. 2005;12:1047–64.

71. Tabatabai MA, Bremner JM. A Simple Turbidimetric Method of Determining Total Sulfur in Plant Materials1. Agron J. 1970;62:805–6.

72. Kanno S, Cuyas L, Javot H, Bligny R, Gout E, Dartevelle T, et al. Performance and Limitations of Phosphate Quantification: Guidelines for Plant Biologists. Plant Cell Physiol. 2016;57:690–706.

Figures
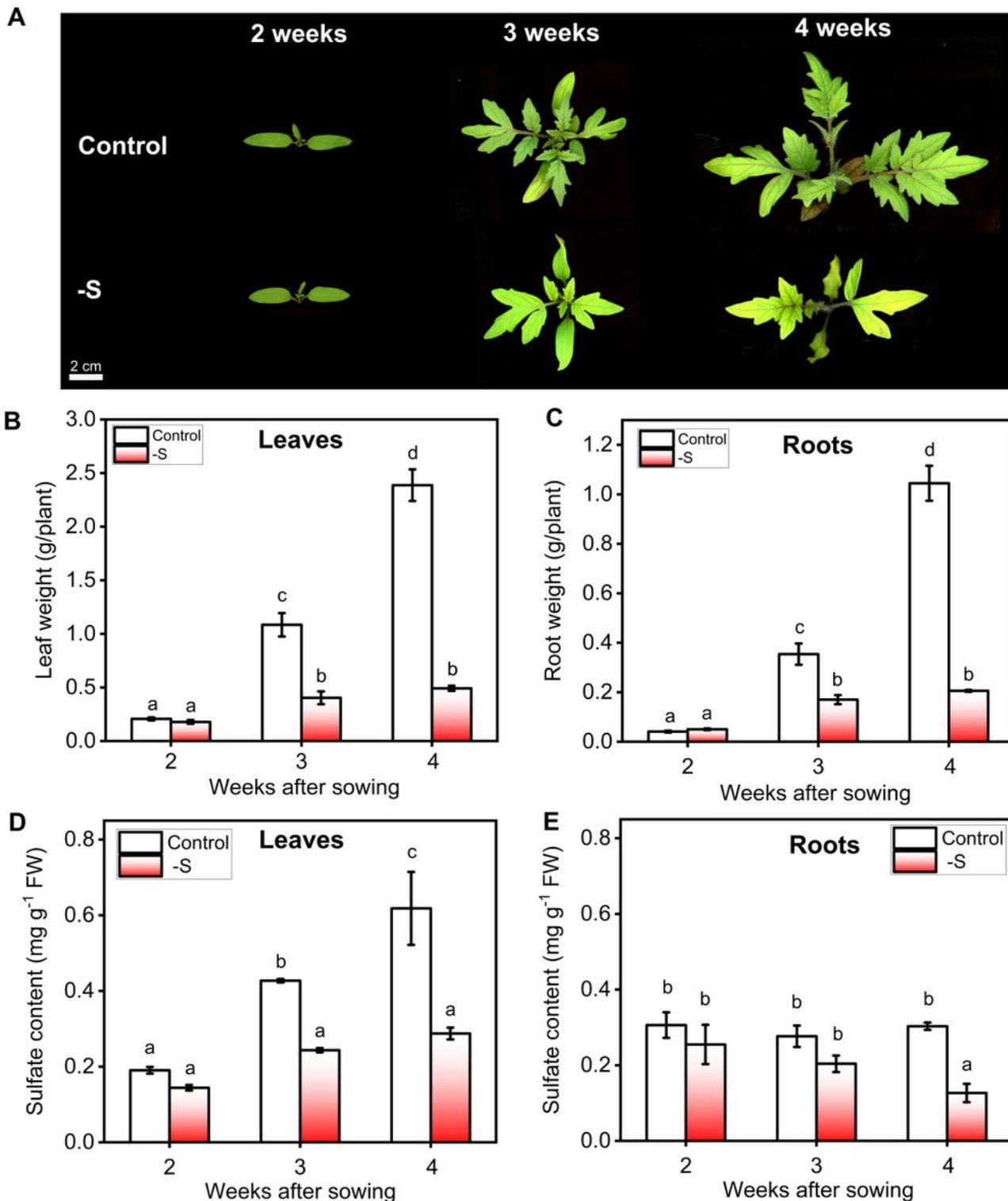
Figure 1

External sulfate availability is required for optimal plant growth. A) Representative images of tomato plants (Solanum lycopersicum cv. Moneymaker) grown under full nutrient or S-limiting conditions for 4 weeks. Biomass accumulation is reduced by sulfate deficiency from 3 weeks after sowing. Fresh weight for leaves (B) and roots (C) were measured from 2 to 4 weeks after sowing. Sulfate content of leaves (D) and roots (E) were determined from 2 to 4 weeks after sowing using the turbidimetric method [23]. Values

plotted correspond to the means of three independent experiments ± the standard error of the mean. Means with different letters indicate significant differences (P<0.05 two-way ANOVA and Tukey's test). 3–5 different plants were measured for each experimental replicate.
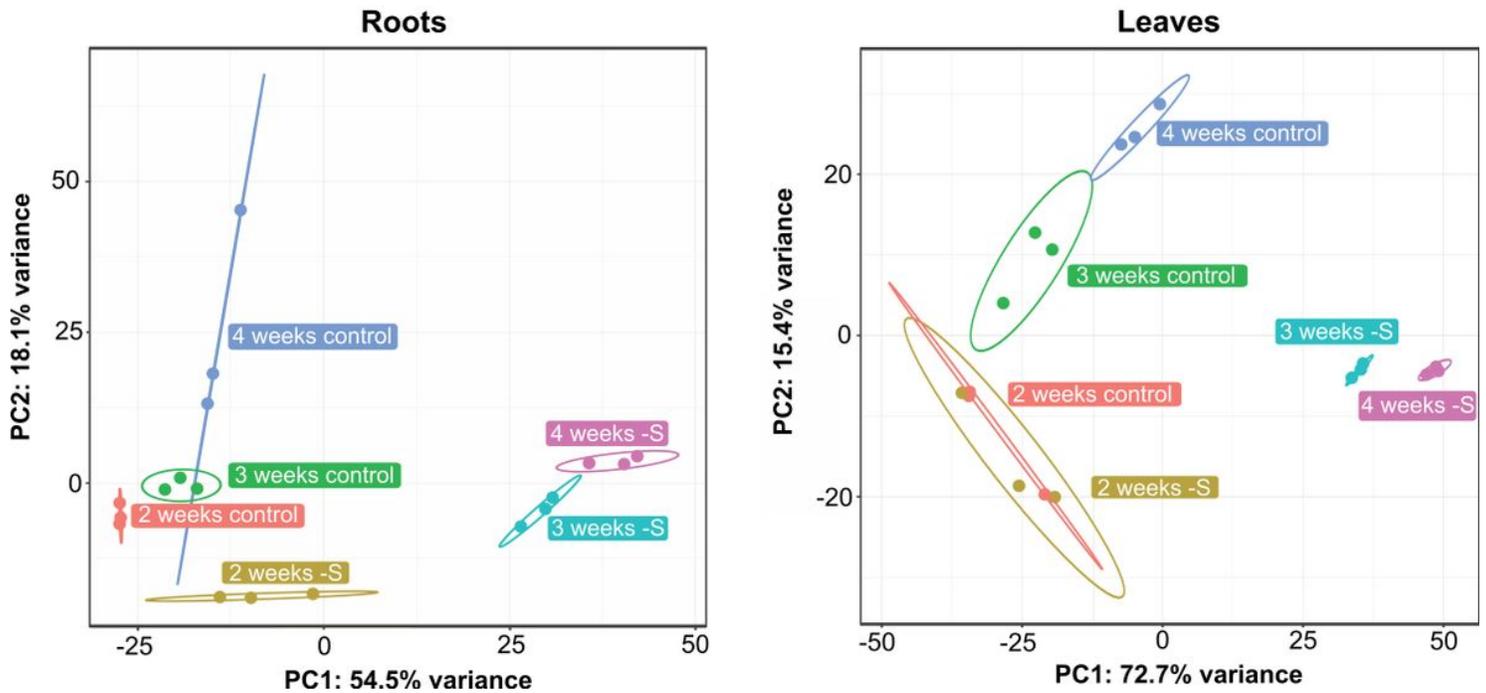
Figure 2

Exploratory data analysis of tomato RNA-seq shows that the response to sulfate starvation begins in root tissue. Principal Component Analysis (PCA) of RNA-seq data of roots (left) and leaves (right) samples. PCA analysis was performed with pcaExplorer R package [65] using log2-transformed normalized expression data. Ellipses represents the 95% confidence interval of 3 independent experiments. Replicates of the same experiment are indicated with the same color.
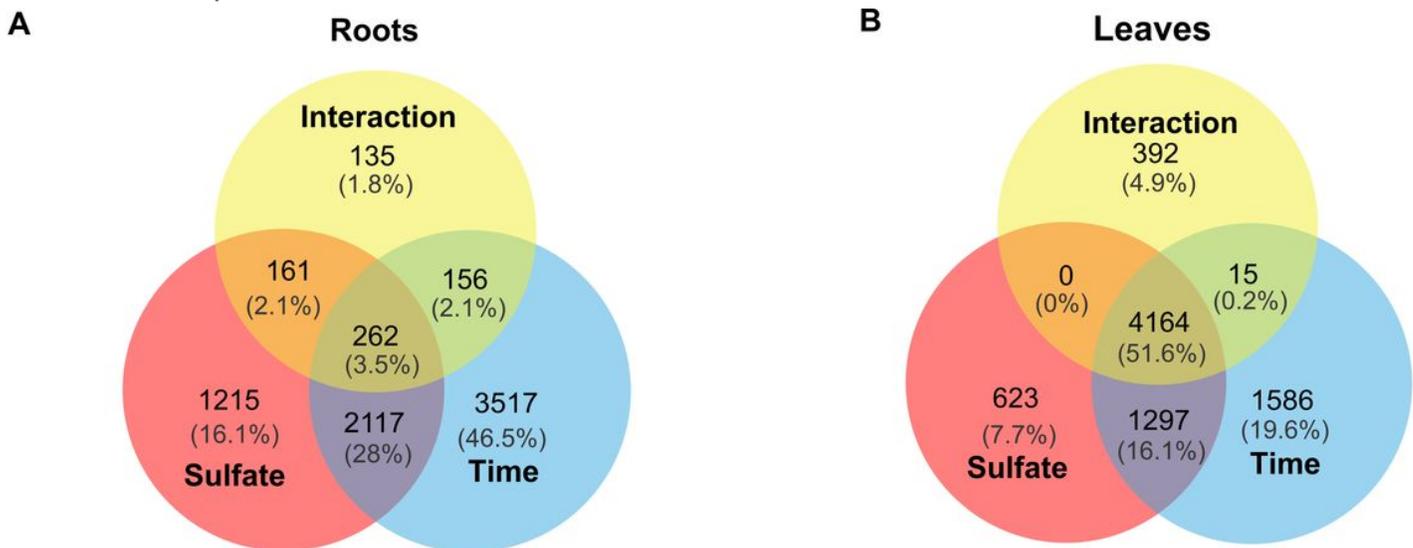
Figure 3

The impact of sulfate starvation on the transcriptome depends on the age of the plants in leaves but not in roots. Venn diagram showing genes significantly regulated by sulfate, time or by interaction of both factors in roots (A) and leaves (B). Multifactorial analysis was performed using sleuth R package [32] with a q-value < 0.05.
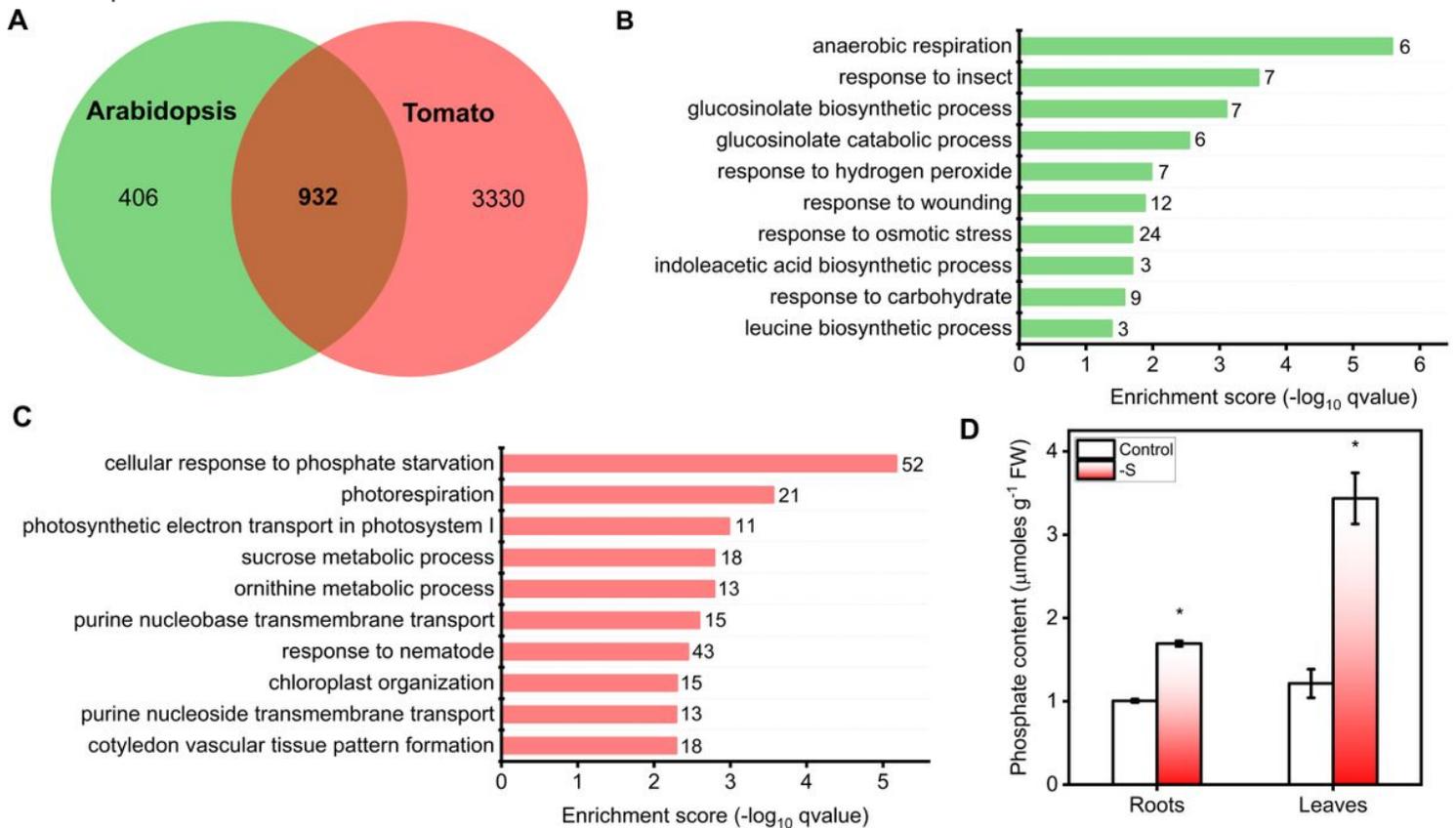
Figure 4

Comparative transcriptome analysis of Arabidopsis and tomato plants in response to sulfate starvation reveal conserved and specific features. A) Venn diagram showing sulfate-responsive genes shared between Arabidopsis and tomato plants. The plant comparative genomics resource PLAZA 4.0 was used to identify the orthologous genes. The Arabidopsis sulfate-responsive genes were obtained from a meta-analysis [15]. B) GO term enrichment analysis of genes exclusively regulated by sulfate in Arabidopsis. The 10 most over-represented biological functions are shown along with the number of genes belongs to each GO term. C) GO term enrichment analysis of genes exclusively regulated by sulfate in tomato plants. The 10 most over-represented biological functions are shown along with the number of genes belongs to each GO term. D) Sulfate starvation increase phosphate content in tomato roots and leaves of plants grown under full nutrient or S-limiting conditions for 3 weeks. Phosphate content was determined by the malachite green assay [72]. A Student's t-test was performed to test the significance ($P < 0.05$) of the differences between sulfate-starved and control samples (*). Values plotted correspond to the means of three independent experiments ± standard deviation. 3–5 different plants were measured for each experimental replicate.
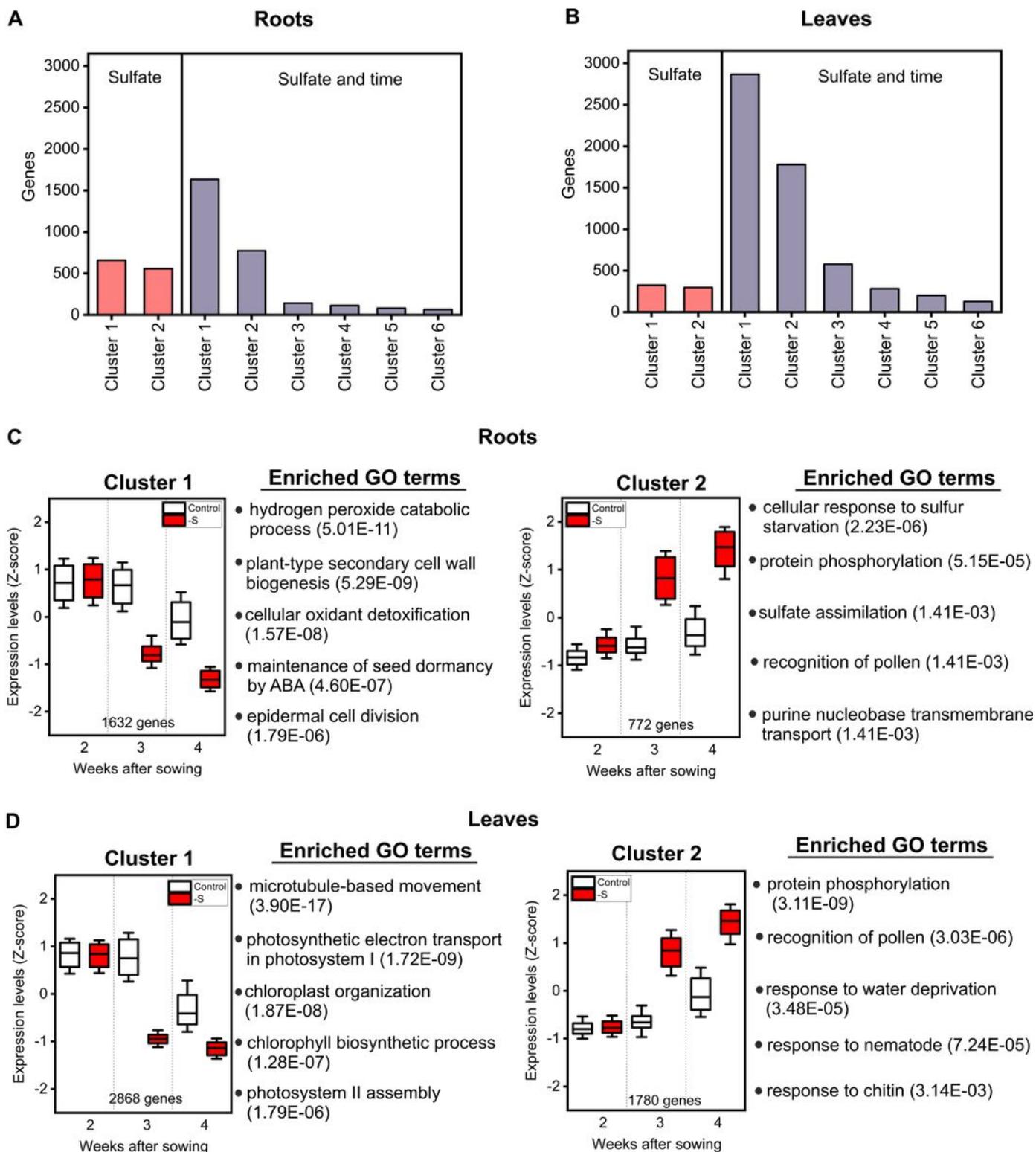
Figure 5

Gene co-expression clusters of roots and leaves during tomato development under sulfate starvation. Co-expression clusters of genes exclusively regulated by sulfate or regulated by sulfate and time in roots (A) or leaves (B). Expression patters of major clusters in roots (C) and leaves (D). On each box, the central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. Whisker indicates standard deviation of the expression data of all genes belongs

to the cluster. The top 5 enriched GO terms are indicated to the right of each box plot with the q-values in brackets.

A

Root regulatory network

Transcription factor	-S response	Targets	
MYB (Solyc11g071300)	↓	**40**	sulfur utilization
E2F (Solyc03g113760)	↓	25	DNA replication initiation
EIL3 (Solyc01g006650)	↑	19	cellular response to S starvation

Number of enriched GO terms of targets

B

Leaf regulatory network

Transcription factor	-S response	Targets	
TCP21 (Solyc03g006800)	↓	**71**	chlorophyll biosynthetic process
bHLH36 (Solyc05g006650)	↓	54	regulation of auxin biosynthetic process
GAGA-binding (Solyc04g081170)	↑	46	gibberellic acid mediated signaling pathway
Zinc finger (Solyc02g085580)	↑	43	gibberellic acid mediated signaling pathway
WRKY71 (Solyc02g071130)	↑	26	cellular response to antibiotic
MYB60 (Solyc10g081490)	↓	21	cutin biosynthetic process
WRKY6 (Solyc02g080890)	↑	18	response to boron-containing substance
NAC (Solyc07g045030)	↑	18	leaf senescence
EIL3 (Solyc01g006650)	↑	17	cellular response to S starvation
NAC (Solyc01g009860)	↑	11	positive regulation of leaf senescence

Number of enriched GO terms of targets

C

EIL3 (Solyc01g006650)

Figure 6

A gene regulatory network analysis identifies new TFs involved in the sulfate starvation response in tomato plants. A). TFs from the root regulatory network that have over-represented biological functions in their target genes. The root regulatory network was constructed considering TF-target interaction

according to the information available in PlantRegMap and Pearson correlation threshold >0.9 in root samples. B) TFs of the leaf regulatory network with over-represented biological functions in their target genes. TFs and their potential targets were identified using the information available in PlantRegMap and Pearson correlation threshold >0.9 in leaf samples. C) RT-qPCR analysis of EIL3 TF (Solyc01g006650) in tomato plants. A Student's t-test was performed to test the significance (P < 0.05) of the differences between sulfate-starved and control samples (*). Values plotted correspond to the means of three independent experiments ± standard deviation. 3–5 different plants were measured for each experimental replicate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS9.xls
- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx
- TableS11.xls
- TableS4.xlsx
- FigureS2.jpg
- TableS5.xlsx
- TableS6.xlsx
- TableS7.xlsx
- FigureS1.jpg
- TableS8.xlsx
- FigureS4.jpg
- FigureS5.jpg
- TableS10.xlsx
- FigureS3.jpg
- FigureS7.jpg
- FigureS6.jpg