 doi:10.20944/preprints202204.0208.v1

Article

Evolutionary and Gene Expression Analyses Reveal New Insights into the Role of *LSU* Gene-Family in Plant Responses to Sulfate-Deficiency

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Abstract: *LSU* (*RESPONSE TO LOW SULFUR*) proteins belong to a plant-specific gene family initially characterized by their strong induction in response to sulfate (S) deficiency. However, little is known about the *LSU* gene repertoire and evolution of this family in land plants. In this work, a total of 270 LSU family members were identified using 134 land plant species with whole genome sequence available. Phylogenetic analysis revealed that *LSU* genes belong to a *Spermatophyta*-specific gene family, and their homologs are distributed in three major groups, two for dicotyledons and one group for monocotyledons. Moreover, we analyzed the expression of *LSU* genes in one representative species of each phylogenetic group (wheat, tomato and Arabidopsis) and found a conserved response to S-deficiency, suggesting that these genes might play a key role in S stress responses. Accordingly, Arabidopsis *lsu2* knockout mutant plants showed increased levels of internal sulfate content and lower levels of expression of different key genes involved in S deficiency and metabolism like *SDI2* and *APR3*. In summary, our results indicate that *LSU* genes are evolutionarily conserved in angiosperms and that specific members of this family might play an important role regulation of S transport and assimilation.

Keywords: sulfate deficiency; *Arabidopsis thaliana*; *Solanum lycopersicum; Triticum aestivum;* LSU; response to low sulfur; abiotic stress; sulfur nutrition

1. Introduction

Sulfur (S) is an essential macronutrient for plants, and a constituent of relevant biomolecules such as the aminoacids methionine and cysteine, the antioxidant glutathione, glucosinolates, coenzymes and prosthetic groups [1]. As such, the availability of sulfate, the main source of S in soils, is an important determinant of plant growth, yield and quality.

In recent years, sulfate deficiency has become widespread in many regions of the world, mainly due to anthropogenic factors such as the use of fertilizers with a diminished content of sulfate, intensive agriculture, a diminished use of S-containing fungicides and the advent of environmental policies that limit the emissions of SO_2 [2]. Due to the inability of animals to synthesize S-containing amino acids, plant-derived S-containing compounds are key for nutrition and survival of humans and livestock [3]. Thus, understanding how plants cope with sulfate deficiency is of relevance for improving sulfate content and productivity of crops.

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Plant responses to sulfate deficiency have been studied both at the physiological and molecular levels. At the physiological level, sulfate deficiency leads to reduced S content and reduced metabolic activity, alters photosynthetic activity and produces oxidative stress due to an accumulation of reactive oxygen species. This leads to plants with stunted growth and leaf chlorosis [4]. At the molecular level, several sulfate deficiency-responsive genes have been identified using omics approaches, indicating a massive reprogramming of gene expression in response to the availability of this nutrient [5–14]. A meta-analysis of published transcriptomics data of the sulfate response in Arabidopsis identified genes whose expression is consistently controlled by sulfate deficiency [15], including sulfate transporters and enzymes related to sulfate assimilation, as well as genes participating in cell wall organization, regulation of proteolysis and C/N metabolism. Among the consistently induced genes were *LSU1* and *LSU2*, members of the plant-specific "RESPONSE TO LOW SULFUR (LSU)" family, composed by four members in Arabidopsis (LSU1-4). *LSU1* and *LSU2* were first identified as strongly induced at the transcript level by sulfate deficiency in Arabidopsis [6,16] as well as at the protein level[17]. Analysis of LSU-GFP transgenic lines in Arabidopsis shows that LSU1 is preferentially located in chloroplasts of guard cells and LSU2 in the nucleus of epidermal root cells [17]. Another study showed that LSU2 protein is also localized in chloroplasts [18] and cytosolic localization has also been reported for LSU1 and LSU2 in Arabidopsis [17]. Similar to LSU2, tobacco LSU homolog UP9C, is localized in the nucleus of root cells [16]. UP9C has been described as necessary for tobacco response to sulfate deficiency and possesses a 20-nt DNA motif in its promoter termed UPE-box that confers sulfate-deficiency responsiveness. This element is also present in the promoters of Arabidopsis *LSU1*, *LSU2* and *LSU3*, as well as in promoters from other sulfate-responsive genes such as *APS reductase 1* (*APR1*), *APR3* and sulfate transporter *SULTR2;1* [19]. Mutations within this motif affect the binding of the NtEIL2 transcription factor, as well as the binding of its Arabidopsis homolog SLIM1 [20], a key transcription factor mediating the sulfate-deficiency response [21], further supporting a role for LSU proteins in the response to this nutrient.

LSU are small proteins of approximately 90-100 amino acids. Molecular modeling of LSU structures in Arabidopsis show that these small proteins have a coiled-coil structure [22]. Consistently, circular dichroism analysis of a recombinant LSU protein (UP9C) indicate that this protein is almost fully alpha-helical [16], which suggests that LSUs are flexible and might be able to bind other proteins. Accordingly, LSUs appear as hubs in protein-protein interaction networks in Arabidopsis [22–25]. A recent study also showed that LSU proteins are able to generate multimers, forming hetero- and homodimers [22].

Besides sulfate deficiency, LSU transcript levels change in response to other abiotic stresses like H_2O_2 treatment [26], C-starvation [27], treatment with lincomycin, an inhibitor of chloroplast biogenesis [28], iron deficiency, copper excess, salt stress or high pH [17]. LSUs have also been linked to the plant response to biotic stress. Interactome analysis in Arabidopsis has shown that LSUs are targeted by effectors from different pathogens including the bacterium *Pseudomonas syringae*, the oomycete *Hyalopernosopora arabidopsidis* and the fungus *Golovinomyces orontii* [24,29]. Furthermore, mutant *lsu2* plants present an enhanced susceptibility to infection by these pathogens while *LSU2* overexpression generates a resistant phenotype [24,29]. Consistent with a role of LSUs in plant defense, LSU1 has been shown to bind Fe superoxide dismutase 2 (FSD2), increasing its enzymatic activity and leading to enhanced chloroplastic H_2O_2 production, an important element of pattern-triggered immunity. Binding of pathogen effectors to LSU1 could interfere this process, leading to plant susceptibility [17]. On the other hand, infection by *Pseudomonas syringae* leads to a disruption of the physical interaction between LSU2 and the AtRAP protein in the chloroplast, positively regulating host defense [18]. Interestingly, LSU downregulation results in a higher susceptibility to *Pseudomonas syringae* infection upon sulfate deficiency, indicating that LSU proteins mediate defense responses during this nutritional stress [17]. Consistently, LSU1-4 are able to interact in vivo with the sulfate assimilation enzyme ATP sulfurylase (APS1) in Arabidopsis [22], suggesting a potential direct regulation of sulfate metabolism by LSUs. Furthermore, evidence points at a possible role of

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LSU-like proteins as common regulatory elements of the sulfate and ethylene signaling pathways. UPC9 is able to interact with ACC oxidase and silencing of *UP9C* leads to a reduction in ethylene biosynthesis during sulfate deficiency [30]. Additionally LSU2 is directly regulated by EIN3 [31], a key transcription factor of ethylene signaling.

In terms of genomic organization, LSUs in Arabidopsis are located in pairs of directed gene repeats (*LSU1* and *LSU3* in chromosome 3 and *LSU2* and *LSU4* in chromosome 5) [32], indicating a possible gene duplication event. In tobacco, six LSU homologs have been described (*UP9A-F*). LSU homologs can be found by BLAST search across all higher land plants [17,19], and protein alignment of LSUs from different plants show a short, conserved region, of yet unidentified function [32]. However, no systematic identification and characterization of *LSU* genes in plants has been performed to date. Therefore, the origin and the evolution of *LSU* genes in plants remains unknown. On the other hand, studies on the evolutionary history of genes involved in sulfate transport and metabolism [33,34], have shown that all genes of the sulfate assimilation pathway of higher plants can be found in *Chlamydomonas reinhardtii*, *Physcomitrella patens* and *Selaginella moellendorffii* genomes [33,34]. However, significant differences were found in the genomic organization of these genes in basal land plants, showing a reduced complexity in some aspects of sulfur metabolism, such as cysteine synthesis or synthesis of sulfated metabolites [34].

In this work, we performed a genome-wide identification and evolutionary analysis of *LSU* genes in plants. Our results suggest that different from genes from the sulfate assimilation pathway, the LSU gene family arose in *Spermatophyta*. Phylogenetic analysis reveals 3 major LSU groups in angiosperms, two for dicotyledons and one group for monocotyledons. Moreover, we analyzed the expression of *LSUs* in one representative species of each phylogenetic group and found a conserved response to sulfate-deficiency, suggesting that these genes might play a key role in S-stress responses. Accordingly, we found that Arabidopsis *lsu2* mutant plants show increased levels of internal sulfate content while the expression of several genes involved in S-deficiency response and metabolism are also affected. Overall, these data indicate that *LSU* genes are evolutionarily conserved in angiosperms and members of this family might play a significant role in the regulation of sulfate transport and assimilation.

2. Results

2.1. Genome-wide identification of LSU genes in plants reveals that LSUs are a Spermatophyta-specific gene family

We performed a genome-wide search of *LSU* genes in the PLAZA 5.0 database [35] as a first step towards a phylogenetic analysis of this gene family in plants. This database contains structural and functional annotation of 134 high-quality plant genomes including angiosperms, gymnosperms, non-seed plants and microalgae [35] (Figure 1A). We used the *LSU* genes of *Arabidopsis thaliana* as a reference to identify 270 genes of this family in the PLAZA 5.0 database (Table S1). Interestingly, all *LSU* genes belong to angiosperm and gymnosperm species suggesting that *LSU*s are a *Spermatophyta*-specific gene family (Figure 1A). Accordingly, these homolog groups are annotated as an exclusively *Spermatophyte* family in PLAZA 5.0.

LSU1/*LSU3* and *LSU2*/*LSU4* genes in Arabidopsis are localized close to each other in chromosome 3 and 5 respectively, suggesting that each of these pairs of *LSU* genes arose from a duplication event [32]. To investigate the potential expansion of the LSU family in plants, we analyzed the number of genes of this family across angiosperm genomes. The number of *LSU* genes is variable ranging from 1 to 9 members (Table S1). In general, the species with a higher number of *LSU* genes were polyploids, so we performed a correlation analysis to verify the statistical significance of this observation. We found a significant positive correlation between the *LSU* gene number and the total number of coding genes (Figure S1), suggesting that the variation of gene copy number in the *LSU* family depends on genome size. Considering this correlation, we normalized the *LSU* copy number by the

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total number of coding genes to compare between major taxonomic groups of angiosperms. As shown in Figure 1B, the distribution of normalized number of *LSU* genes is very similar in monocotyledons and eudicotyledons species, with an average number of 2 *LSU* genes per 34.075 coding genes. Indeed, no significant differences in the number of *LSU* sequences was obtained between these taxonomic groups (*p-value* =0.2; Figure S2). Therefore, these results suggest no evolutionary trend toward expansion or reduction of this family in angiosperm plants.

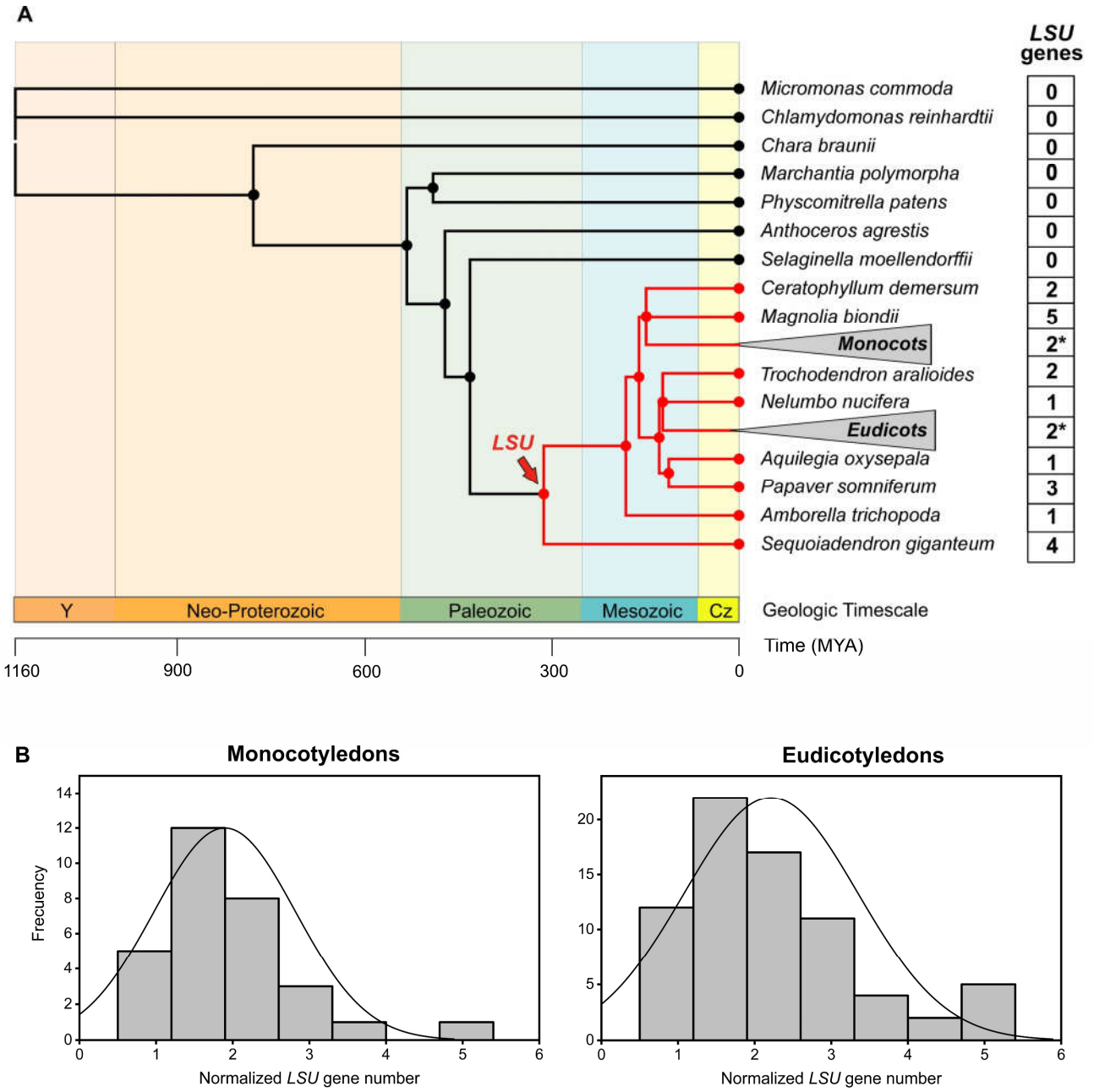

Figure 1. *LSUs* belong to a *Spermatophyta*-specific gene family**.** A) The phylogeny of the 134 plants of PLAZA 5.0 database used in this study. To improve visualization, 73 eudicotyledon and 31 monocotyledon species were collapsed in the phylogenetic tree (triangle). The order of tree branches and divergence time were obtained from the TimeTree database (http://timetree.org/) [36]. (B) The normalized number of *LSU* genes identified in angiosperms plants. The number of *LSU* genes of each specie was divided per 34,075 coding genes, which is average total number of genes in analyzed species.

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2.2. Phylogenetics analysis of the LSU gene family

To reveal the evolutionary relationships of the 270 *LSU* genes identified in the PLAZA 5.0 database [35] among the seed-plant lineages, we constructed a phylogenetic tree using the maximum likelihood method implemented in IQ-TREE 2 [37] with protein sequences. The tree showed that these LSU members can be divided into three major phylogenetic groups: Group A, which included most of monocotyledon species; Group B, which included most of malvid species and Group C, which included most of rosid species (Figure 2). This organization of *LSU* genes fits very well with the reported phylogeny of angiosperm plants [38]. One important exception was the case of basal angiosperms, which are clustered together with species of Group C, however the bootstrap support for this case is very low (aLRT<70%).

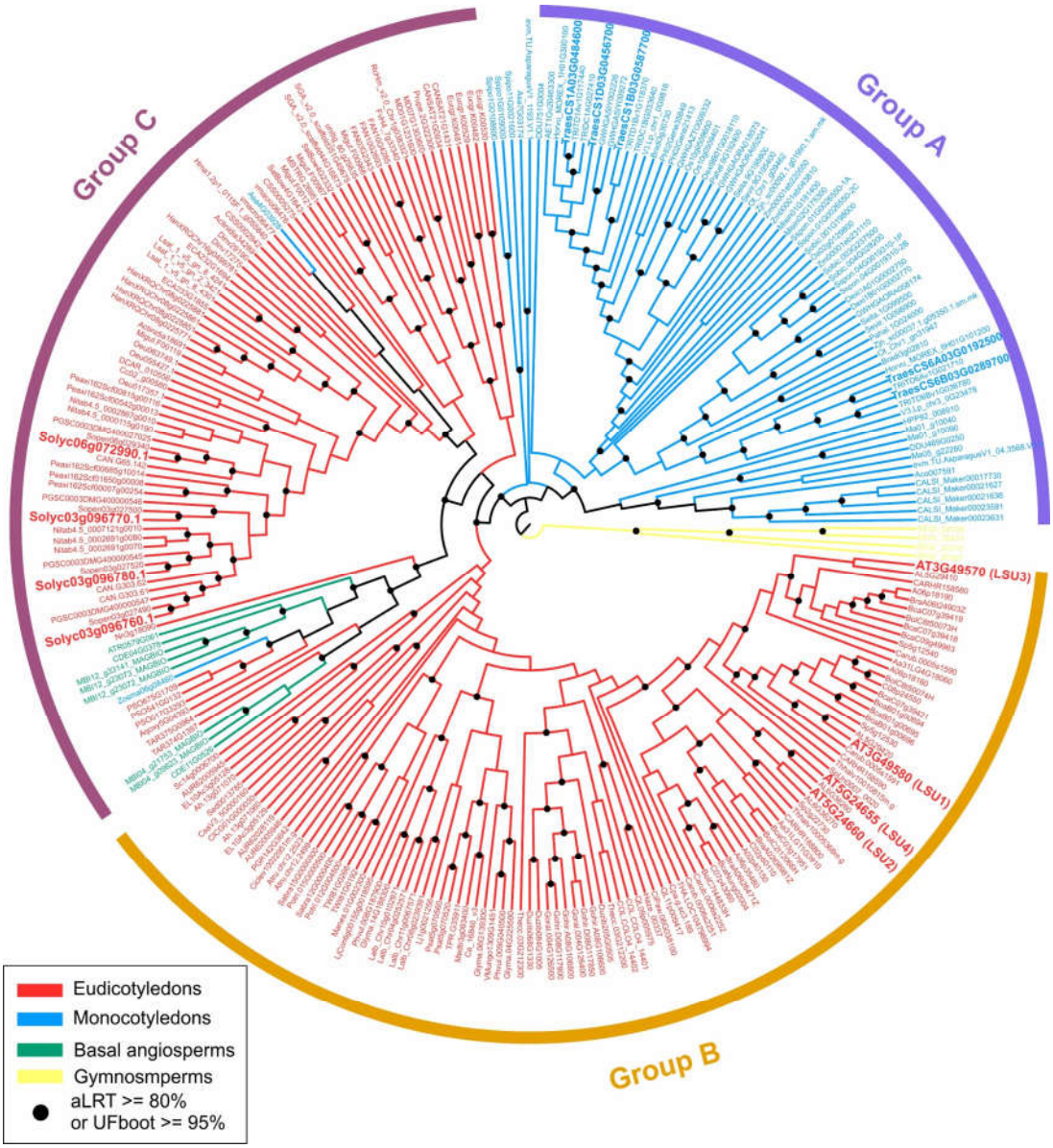

Figure 2. Phylogenetic analysis of *LSU* genes in angiosperm plant linages. A phylogenetic tree was constructed using the maximum likelihood method with IQ-TREE 2 [37] supported by 10,000 bootstrap samples. Yellow lines represent gymnosperms, green lines represent basal angiosperms, blue lines represent monocotyledons and red lines represent eudicotyledons. The gene identifiers correspond to PLAZA 5.0 database [35] and the complete list of *LSU* genes is shown Table S1.

The phylogenetic analysis also showed that all *LSU* members of the same species in Group B and Group C are clustered very close, suggesting a low intraspecific sequence

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divergence in the species of these groups. In contrast, most of *LSU* genes of the same species in the Group A are separated in two different clusters (Figure 2). To elaborate on this observation, we computed the evolutionary distance between each pair of sequences of the same species using MEGA 11 with the JTT matrix-based model [39]. As shown in Figure 3A, the intraspecific distance of *LSU* genes of the Group A is significantly higher (*p-value* <0.0001) than those of Group B and Group C. Then, we selected one species per each group to visualize these differences more clearly. We selected bread wheat (*Triticum aestivum*) for Group A whose *LSU* genes cluster into two homoeolog groups: 3 on chromosome 1 (*TraesCS1A03G0484600*, *TraesCS1B03G0587700*, *TraesCS1D03G0456700*) and two on chromosome 6 (*TraesCS6A03G0192500*, *TraesCS6B03G0289700*). As shown in Figure 3B, the percent of amino acid identity is approximately 37-39% between the two homoeolog groups indicating a high degree of divergence between members of this family in wheat. In contrast, for Arabidopsis in Group B, the lower percentage of identity between LSUs is 47-50%, with most of the LSUs sharing 60% or more of identity. The same trend is seen for tomato in Group C, where most LSUs share more than 70% of identity (Figure 3B). These results suggest a significant differences in the intraspecific evolution of *LSU* genes between monocotyledons and eudicotyledons.

Multiple sequence alignment revealed that LSU proteins of the selected species exhibited significant differences at the N-terminal and C-terminal regions (Figure 3C). In contrast, the central region of LSU proteins showed two highly conserved domains, one from the amino acid positions 31 to 49 and the other one from 59 to 95 (Figure 3C). To extend this analysis to the whole 270 LSU proteins and reveal the structural variation of *LSU* genes in angiosperms, we predicted putative motifs using the MEME tool from the MEME Suite [40]. As shown in Figure 4A, we found a total of 5 distinct motifs with different degree of distribution between phylogenetic groups. Motif 1 and motif 2 were widely present in most members (>90%) of the 3 phylogenetic groups and these conserved motifs were found in the central region of LSU proteins (Figure 4B). It is important to note that motif 1 match with the only reported motif of the LSU family (A-x-x-x-E-E-x-L-C-x-x-L-x-[E/D]-x-[E/D]) [22]. In contrast, motif 3 was present in the C-terminal regions of LSU proteins and showed a limited distribution in Group A compared to Group B or C (62% versus 76-89%). The motifs found in the N-terminal regions (motif 4 and motif 5) also showed different degree of distribution between phylogenetic groups (Figure 4A). These results suggest that the amino acid residues outside of the motif 1 and motif 2 have significantly changed during plant evolution.

2.3. Expression analyses of LSU family in wheat, tomato and Arabidopsis reveals a conserved response to S-deficiency.

To elucidate if the response of *LSU* genes to S deficiency is conserved in angiosperms, one species of each phylogenetic group was selected for mRNA expression analysis: wheat from Group A, Arabidopsis from Group B and tomato from Group C. We grew plants in liquid medium with all nutrients available (control condition) or lacking sulfate (S-deficiency condition) as previously described [14] until emergence of the second true leaves. In this growth stage, transcript levels in roots and leaves samples were analyzed by qRT-PCR. First, we evaluated the mRNA levels of classical sulfate-responsive genes as a positive control of the nutritional treatment in each species (Figure S3). In Arabidopsis and tomato plants, a significant increase in mRNA levels of of *SULTR1.2* and *APR2* genes, encoding a sulfate transporter and a sulfate assimilation enzyme respectively, was observed in both organs in response to S deficiency. In the case of wheat, we analyzed the orthologs of Arabidopsis *MORE SULPHUR ACCUMULATION1* (*MSA1*) and APR2, which also showed higher expression levels under S deficiency (*p-value* <0.05; Figure S3) in both root and leaves. These results indicate that S deficiency response is triggered at the selected developmental stage in all species.

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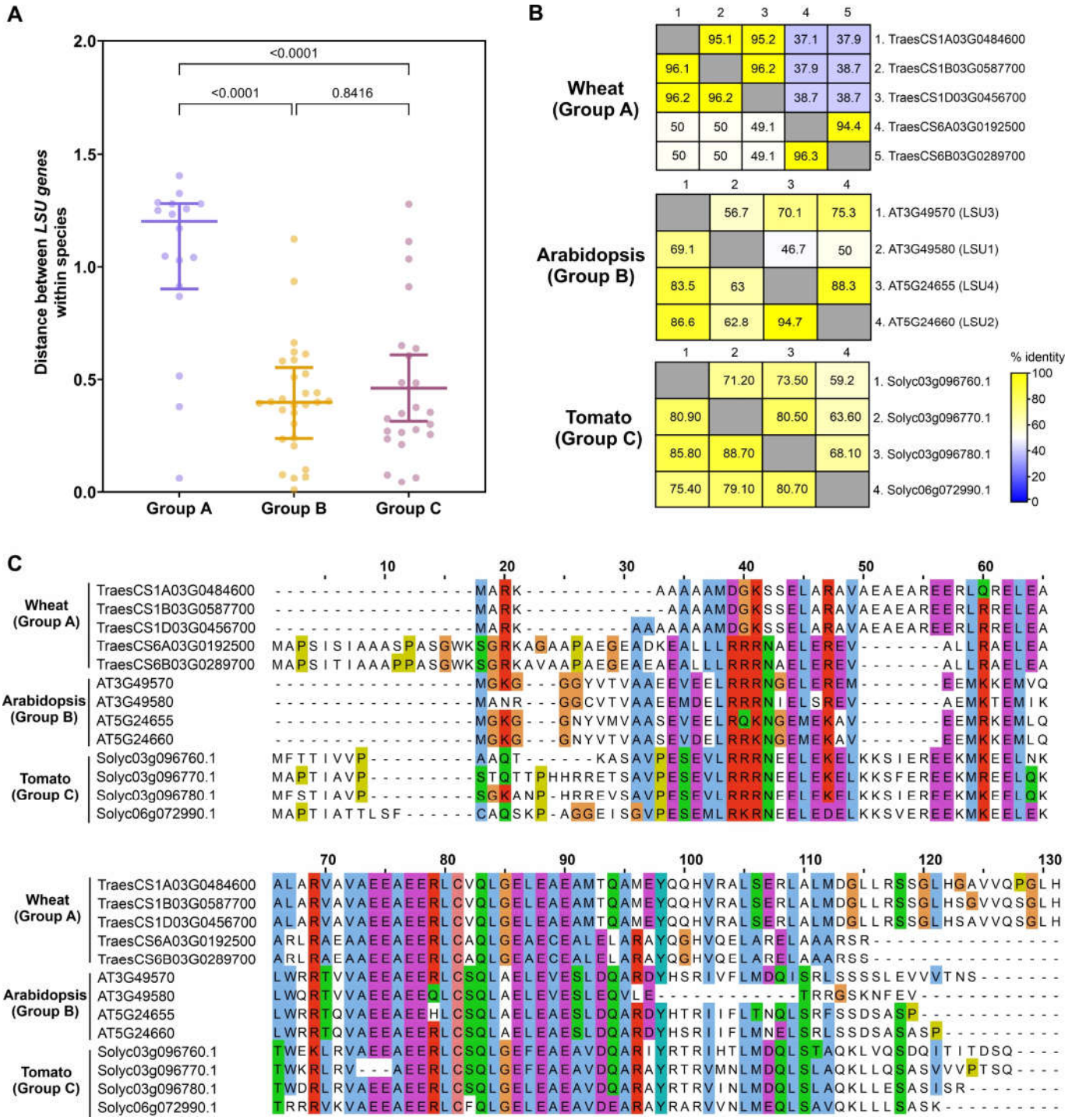

Figure 3. Comparative sequence analysis between representative species of the 3 phylogenetic groups of LSU family. A) The evolutionary distance between each pair of *LSU* sequences of the same species was obtained using MEGA 11 [39]. B) The percentage of similarity and identity between every pair of *LSU* sequences within a representative specie of each phylogenetic group. The upper matrix represents the identity and the lower is the similarity. C) Multiple sequence alignment of LSU proteins from wheat, tomato and Arabidopsis. Amino acid sequences were aligned using MAFFT [41] and visualized with Jalview 2 using Clustal X color scheme [42].

In the case of Arabidopsis, all *LSU* genes showed significantly increased mRNA levels under S deficiency (p-value <0.05) in root and leaves, with *LSU4* showing a lower expression than the other *LSU* genes in all conditions (Figure 5). A similar result was observed in tomato plants, in which all *LSU* genes showed increased mRNA level under S

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deficiency in both root and leaves, with one of them (*Solyc03g096770.1*) showing a lower expression in all samples (Figure 5).

In the case of wheat, we analyzed the expression of the two homoeolog groups, *TaLSU1* corresponding to homoeologs of chromosome 1 and *TaLSU2* in the case of homoeologs of chromosome 6. As shown in Figure 5, we found a significant induction of *TaLSU1* by S deficiency in both organs. On the other hand, the mRNA levels of *TaLSU2* were very low in all analyzed samples; this could be due to a developmental effect, since public data from the Wheat eFP Browser [43] shows that the expression of this gene is very low in the seedling stage (Figure S4).

Taken together these results indicate that the response of *LSU* genes to S deficiency is evolutionary conserved in the representative examples of plant species of the 3 phylogenetic groups, suggesting that these genes present conserved functions in S responses in plants.

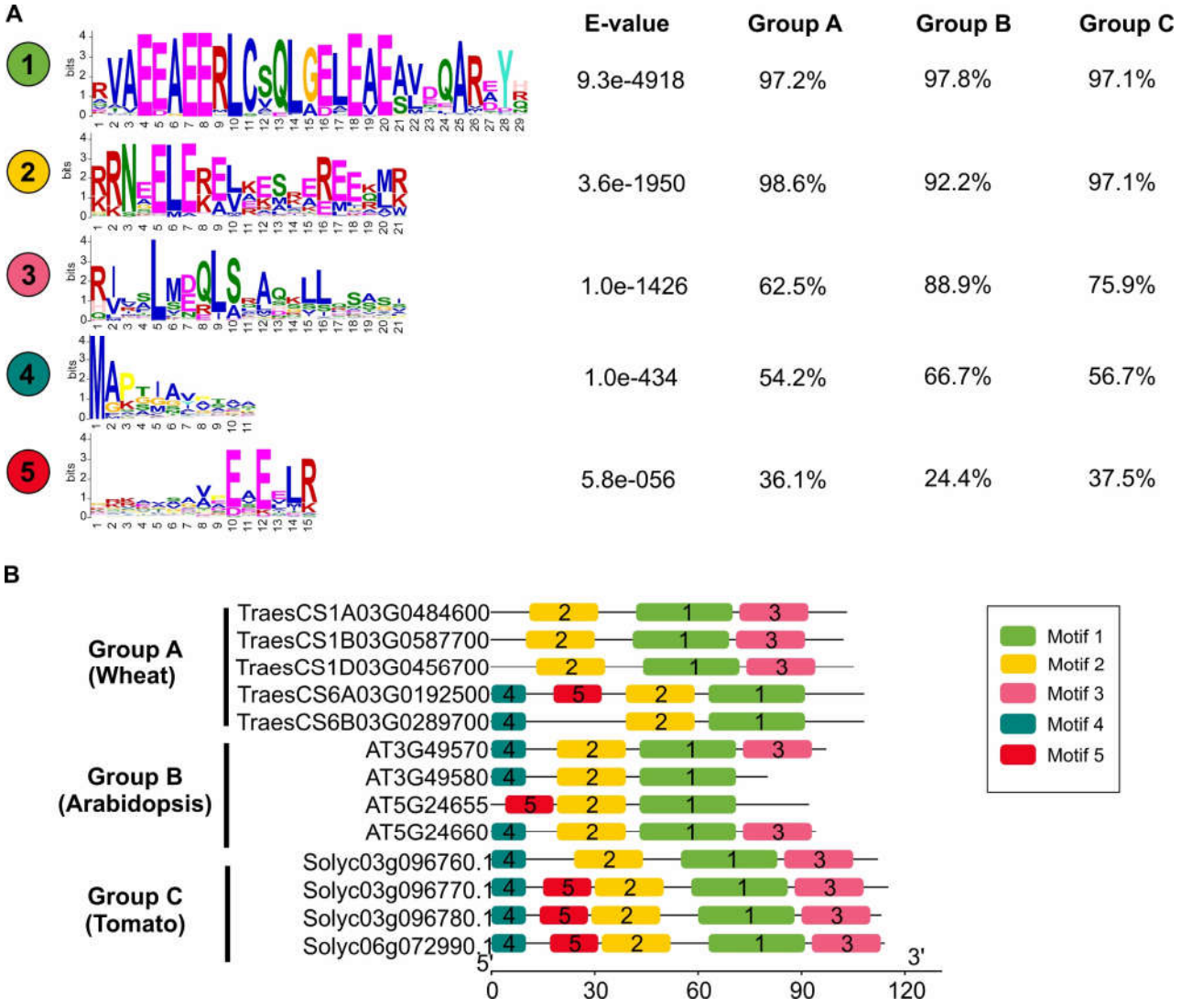

Figure 4. Conserved motif compositions of the LSU gene family**.** A) The conserved motifs were detected using the MEME tool [40] and the distribution (%) across the 3 phylogenetic groups is indicated right to the motif. B) Five putative motifs are indicated by colored boxes in representative species of each phylogenetic group.

2.4. Functional analysis of LSU2 in response to sulfate deficiency in Arabidopsis.

To get new insights into the role of *LSUs* in the S-deficiency response, we analyzed the loss-of-function of an *LSU* gene at the phenotypic and molecular level in Arabidopsis. We previously found that *LSU1* and *LSU2* were in the top 10 of the most consistent S-

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deficiency responsive genes in Arabidopsis [15] and showed a strong response to this nutrient deficiency in roots and leaves (Figure 5). The best characterized LSU mutant to date is *lsu2* (SALK_31648C) [32], which presents an altered response to biotic and abiotic stress, as compared to wild-type plants [24,44]. Considering the consistency of the S-deficiency response and the availability of a previously characterized mutant, we selected *LSU2* as a representative example for a further functional analysis in the context of the S-response.

The homozygosity of the *lsu2* mutant line was confirmed by PCR (Figure S5A) and the expression of *LSU2* was significantly reduced in all analyzed samples of *lsu2* mutants compared to WT plants (p-value <0.05, t-test). The reduction of *LSU2* mRNA levels in mutants was from 12-fold up to 35-fold in the case of leaves, indicating that this T-DNA insertional line is a *knockout* mutant of *LSU2* gene (Figure S5B) as previously shown for this mutant.

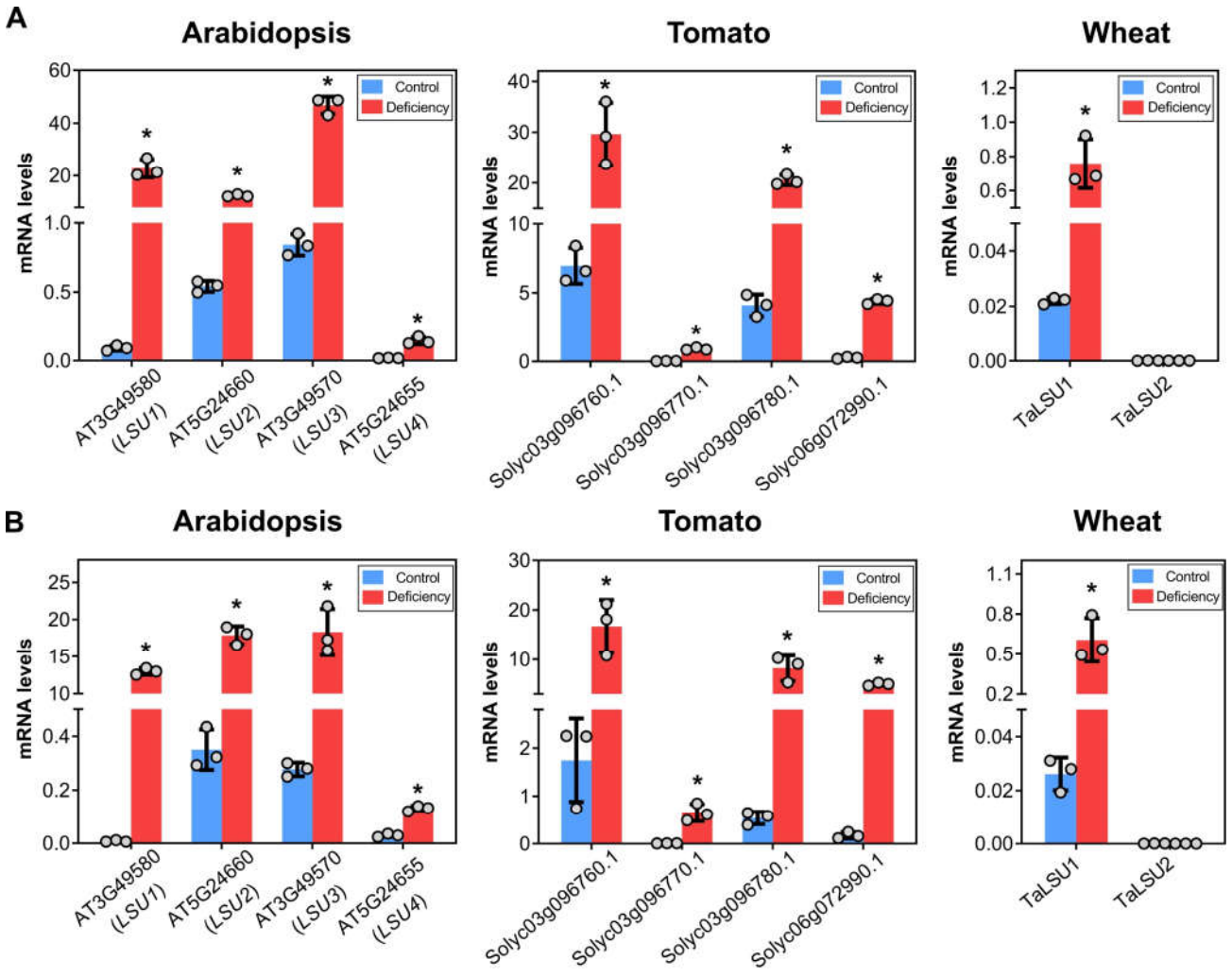

Figure 5. Expression profiles of *LSU* genes of wheat, Arabidopsis and tomato under S-deficiency. The mRNA levels of the *LSU* gene family were measured by RT-qPCR in leaves and roots in leaves (**A**) and roots (**B**) of each species grown for 3 weeks in liquid medium under control and S-deficiency conditions. The expression levels were normalized with the *ADAPTOR PROTEIN-4-MU-ADAPTIN* gene (AT4G24550) in the case of Arabidopsis [45], TIP4I-like family protein (TIP4I, SGN-U584254) for tomato [46] and the Ubiquitin-conjugating enzyme gene (TraesCS4A02G414200) for wheat [47]. Each treatment was performed using 3 independent experiments. The bars represent the arithmetic mean and the asterisks indicate the mean of the measurements that are significantly different according to a t-student test with $p < 0.05$.

At the phenotypic level, we observed a significant decrease (p-value <0.05, t-test) in the shoot area of *lsu2* mutants compared to wild-type plants grown in full nutrient

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condition for three weeks (Figure 6A and Figure 6B). However, no significant differences were observed between genotypes when plants were grown under S-deficiency condition. A similar result was obtained in the case of primary root length (Figure 6B). Furthermore, the sulfate content was also analyzed in the same samples, and we found that *lsu2* mutants had a significantly higher sulfate content (p-value <0.05, t-test) in S-starved leaves. Additionally, the roots of *lsu2* mutants showed significantly higher levels of internal sulfate in both nutrient conditions. These results suggest that *LSU2* might be involved in the regulation of internal sulfate levels.

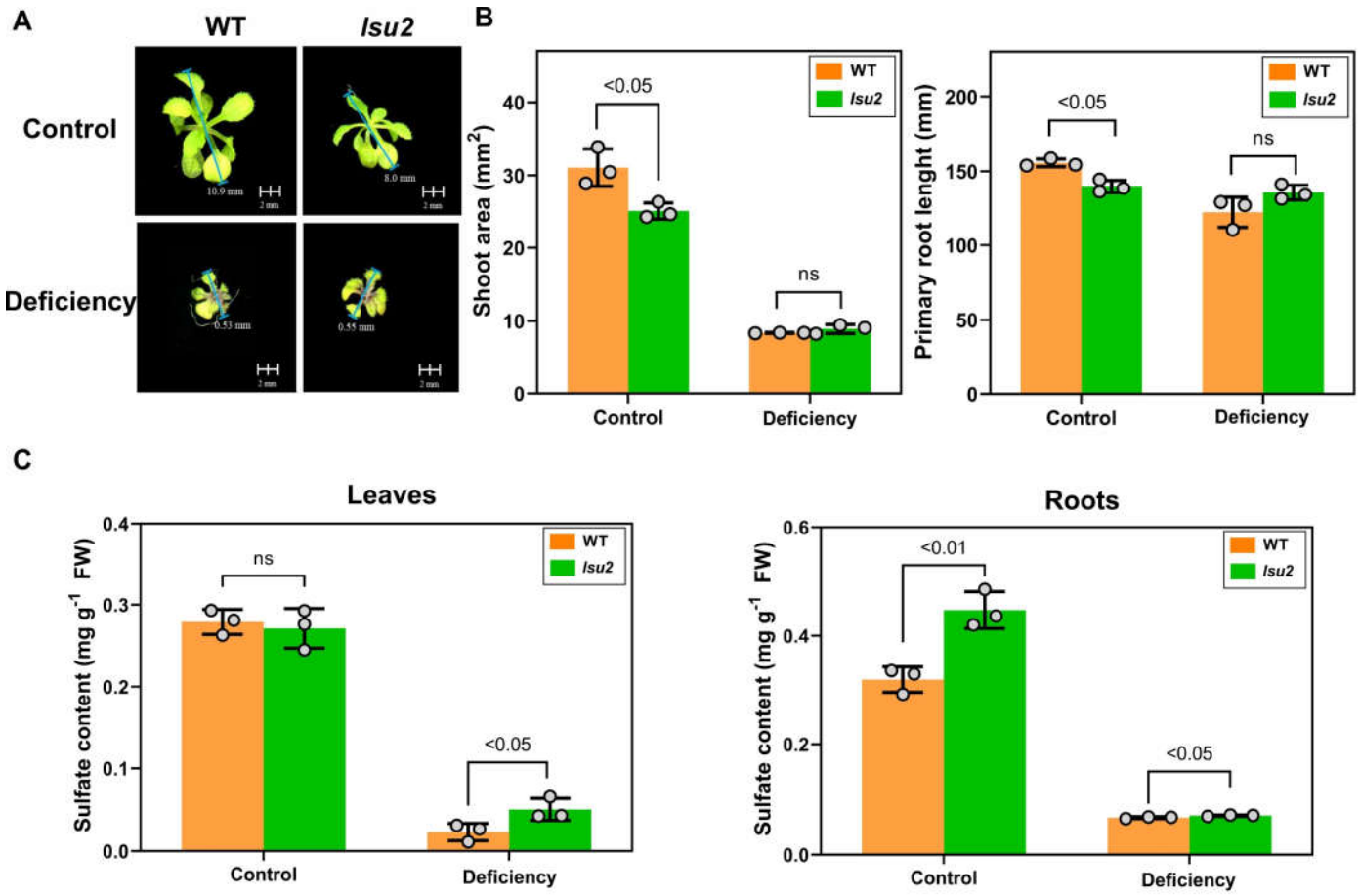

Figure 6. Phenotypic and molecular analyses of *lsu2* mutant plants under S deficiency**. A)** Representative images of wild-type and *lsu2* knock-out mutant shoots grown on agar plates under control or sulfate deficiency condition for 3 weeks. **B)** Total shoot area and primary root length. **C)** Sulfate content of leaves and roots were determined using the turbidimetric method [48]. Values plotted correspond to the means of three independent experiments ± the standard deviation. A Student's t-test was performed to test the significance (p<0.05) of the differences between genotypes.

To investigate the molecular function of *LSU2* in the Arabidopsis S-deficiency response, we decided to analyze the expression of several genes related to sulfate transport (*SULTR1.2*, *SULTR4.1*), sulfate metabolism (*APS1*, *APS2*, *APS3*, *APR2*, *APR3*, *SiR*, *APK1*) and S-deficiency response (*LSU1*, *LSU2*, *LSU3*, *LSU4*, *SDI1*, *SDI2*, *BGLU28*, *MSA1*) by qRT-PCR in wild-type and *lsu2* mutant plants.

As shown in Figure 7A, two classical marker genes of the S-deficiency response, *SDI2* and *MSA1*, were strongly affected by the knock-out mutation of the *LSU2* gene. Specifically, the mRNA levels of *SDI2* were significantly lower (p-value <0.05) in control and S-starved conditions in roots and shoots of *lsu2* mutants (Figure 7B). In the case of *MSA1*, the significantly lower expression (p-value <0.05) only occurs in sulfate-starved roots (Figure 7A). Interestingly, the transcript levels of *LSU4* are significantly higher (p-value <0.05) in *lsu2* mutants under S-deficiency condition (Figure 7A), suggesting a genetic

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compensation in response to the loss of *LSU2* gene expression. In addition, the transcript levels of one of the isoforms of the adenosine 5'-phosphosulfate reductase (APR3), which is the key enzyme of sulfate assimilation [49], were significantly reduced in *lsu2* mutants (Figure 7B). However, other genes related to sulfate metabolism (*APS1*, *APS2*, *APS3*, *SiR*, *APK1*) and sulfate transport (*SULTR1.2*, *SULTR4.1*) were not affected by *LSU2* mutation.

In conclusion, the most affected genes by the knock-out mutation of *LSU2* gene are those associated with S-deficiency response while most genes related to sulfate transport and assimilation are not affected.

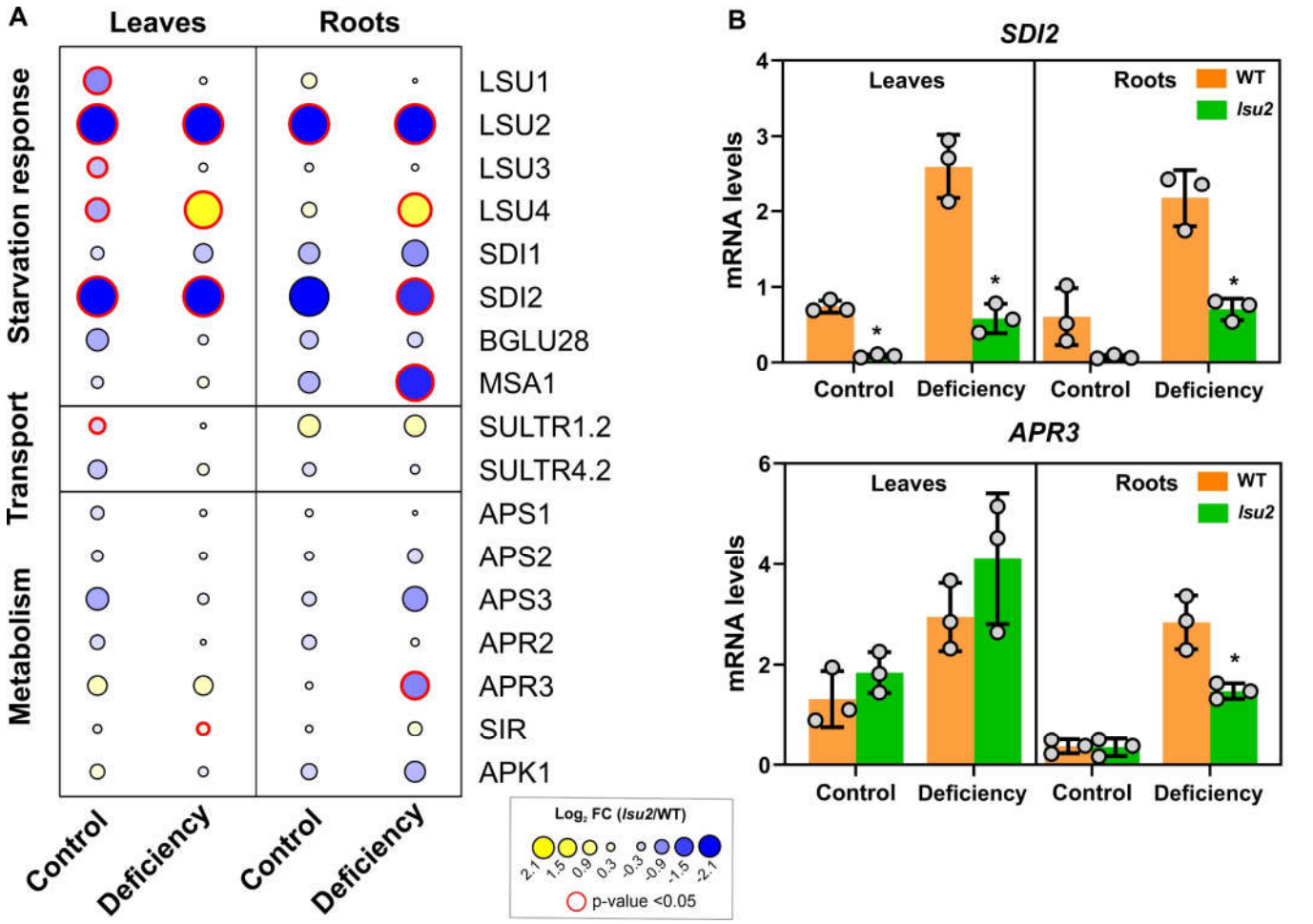

Figure 7. Expression profiles of genes involved in sulfate assimilation and response in *lsu2* mutants. A) Bubble plot showing the average log-fold change (FC) between the expression values of *lsu2* mutants and wild-type plants determined by RT-qPCR in leaves and roots. Blue color indicates down-regulation and yellow up-regulation, the magnitude of FC is proportional to the bubble size. Red circles indicate significant differences between genotypes ($p<0.05$, Student's t-test). B) Expression profiles of *SDI2* and *APR3* in response to S-deficiency in *lsu2* mutants and wild-type plants determined by qRT-PCR in leaves and roots. Values plotted correspond to the means of three independent experiments ± standard deviation. The expression levels were normalized with the *ADAPTOR PROTEIN-4-MU-ADAPTIN gene* (AT4G24550).

3. Discussion

3.1. Molecular evolution of LSU family

In this work, we performed an evolutionary analysis of the *LSU* family in plants using the 134 high-quality genomes available in PLAZA 5.0 database. We found *LSU* homolog sequences in gymnosperm and angiosperm species, indicating that *LSU* genes are a *Spermatophyta*-specific gene family. However, typical S-responsive genes such as sulfate transporters or genes encoding enzymes of S assimilation such as *ATP sulfurylase* (*ATPS*) or *APS reductase* (*APR*) are present in all Viridiplantae from microalgae to angiosperms

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[34], indicating that the evolutionary appearance of *LSU* family is recent compared to S assimilation genes. Interestingly, several experimentally-verified interactors of *LSU* genes in Arabidopsis, such as *APS1*, *GAPC1*, *RAF2*, *FSD2* and *RAP1* [17,18,22], are also present in all analyzed Viridiplantae genomes (HOM05D001870, HOM05D000557, HOM05D002852, HOM05D002348 and HOM05D006787 homologous gene family respectively in PLAZA 5.0), suggesting that LSU-target regulatory interactions do not require the same evolutionary history.

A recent study analyzed the evolution of land plants using 208 complete genomes [50] and identified 1432 *Spermatophyta*-specific genes. GO ontology enrichment analysis revealed that *Spermatophyta*-specific genes are over-represented in GO terms associated to developmental processes and phytohormone responses. In this regard, several lines of evidence have related *LSU* genes and phytohormes [30,31]. For instance, it has been proposed that *LSU* genes may establish a communication between the phytohormone ethylene and S assimilation in response to cadmium stress [31]. Moreover, the overexpression of *LSU1* increases primary root length [31] which is in accordance with the diminished root length of *lsu2* mutants that we observed in this work. Therefore, it is possible that *LSU* genes are related to phytohormone responses and root development, similar to other *Spermatophyta*-specific genes.

The analysis of *LSU* sequences across angiosperms also reveals that the evolutionary distance between *LSU* genes of the same species in monocotyledons is significantly higher than eudicotyledons, suggesting a potential functional divergence of *LSU* genes within monocotyledon species such as wheat. In plants, functional divergence is also reflected in contrasting expression profiles of divergent genes due to the well-known correlation between gene expression and function in plants [51–53]. In fact, the expression patterns of the two *LSU* genes with lower percentage of sequence identity in wheat (*TraesCS1A03G0484600* and *TraesCS6A03G0192500*) shows important differences during development according to WheatOmics database [54]. *TraesCS1A03G0484600* is preferentially expressed in roots and spikes whereas *TraesCS6A03G0192500* shows higher transcript levels in leaves and stems at flowering stage (Figure S6). Consistent with these expression patterns, we found that the expression of *TraesCS1A03G0484600* was significantly higher than *TraesCS6A03G0192500* in all analyzed samples of early seedling stage.

It has been previously reported that LSU proteins have a small-conserved motif of 16 amino acids (A-x-x-x-E-E-x-L-C-x-x-L-x-[E/D]-x-[E/D]) [32]. However, this motif was deduced from a reduced set of sequences (42 protein sequences from 15 species) and therefore, other relevant domains in the evolution of these proteins could be identified by expanding this analysis to other plant species. Indeed, our conserved motif analysis identified 4 new putative motifs based on 270 LSU protein sequences from 112 species (Figure 4). Interestingly, one of these new motifs (motif 2) also showed a high degree of conservation suggesting that LSU proteins might have two essential domains. Our results also suggest that the known motif is of much greater extent than previously reported [32] (from 16 to 29 amino acids). The significance of these strongly evolutionarily conserved regions is unknown, but a recent study suggest that particular motifs in LSU proteins are not required for the interaction the interaction of LSU with other molecular targets [22].

3.2. Expression profiling unveils a conserved function in the control of S responses

Previous studies have shown that *LSU1-3* of Arabidopsis strongly respond to S-deficiency as well as the *LSU* genes of tobacco plants [32]. In this work we extended these expression analyses to wheat and tomato plants using the same experimental design and similar developmental stages. We found that all *LSU* genes were induced in response to S-deficiency in roots and leaves of Arabidopsis and tomato plants (Figure 3), suggesting a functional redundancy in the response to this nutritional stress. Accordingly, a recent study of the interactome of LSU proteins in Arabidopsis showed similar binding properties of LSU1-4 [22]. On the other hand, the fold change and expression levels of *LSU4*, *TaLSU2* and *Solyc03g096770.1* were remarkably lower than the other *LSU* genes in our

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experimental conditions. These exceptions may be due to a different temporal response to S-deficiency or by developmental effect. This last hypothesis is consistent with the developmental expression pattern of the *LSU4* gene in Arabidopsis, which is preferentially expressed in flowers according to ePlant database [55] and it has been reported that mutation in *LSU4 gene* affects flower development in Arabidopsis [56]. A similar case occurs with the wheat as discussed below. These evidences suggest that some members of the *LSU* family may have a tissue-specific response to S-deficiency.

3.3. LSU2 involvement in S-stress responses

In this work, it was conducted a cross-species genome wide expression analyses that showed a significant induction by S-deficiency of at least one *LSU* gene member in all analyzed plant species, suggesting that they might play a conserved role in response to S stress conditions in angiosperms. It is important to note that the selected species belongs to the 3 major *LSU* clades detected in the phylogenetic analysis (Figure 2). Moreover, the induction of *LSU* genes in tobacco plants has been also reported [16]. This conserved response to sulfate availability strongly suggests a functional role of LSUs in this nutritional response. Interestingly, TAP-MS approaches in Arabidopsis have identified ATP sulfurylase 1 (APS1) as a protein directly interacting with LSU1, and this interaction has also been validated by *in planta* BiFC assays [22]. In this work we used a reverse genetic strategy in Arabidopsis to get new insights into this functional role of *LSU*s. Knock-out mutation of *LSU2* significantly reduce the growth of aerial tissues and roots under control conditions, which is consistent with the phenotype recently reported for the amiR-LSUc line, which is a transgenic line expressing artificial microRNAs targeting the four *LSU* genes of Arabidopsis [57]. The downregulation of *LSU* genes reduced the total fresh weight in 30% compared to wild-type plants under control conditions [57]. Moreover, we found that the internal sulfate content of *lsu2* mutants was significantly higher than wild-type plants in roots and shoots under S-deficiency conditions, suggesting that *LSU2* is involved in the regulation of internal sulfate levels. Accordingly, we also found that *lsu2* mutants showed reduced *APR3* transcript levels in S-starved roots. However, other genes involved in sulfate transport and metabolism were not affected by *LSU2* mutation. The relatively low effect of this mutation on the expression of sulfate assimilation genes and internal sulfate levels may be due to a compensatory effect with other members of the *LSU* family. Consistently, our analyses showed a significant induction of LSU4 in *lsu2* mutants under S-deficiency in roots and shoots. Genetic compensation in response to knockout mutations is a widespread phenomenon in Arabidopsis and other model organisms [58].

In summary, we found that *LSU* genes belong to *Spermatophyta*-specific gene family and their response to S-deficiency is conserved in angiosperms. The functional analysis of *LSU2* in Arabidopsis indicates that these genes may be involved in the regulation of internal sulfate levels and plant growth. Further research should be undertaken in other angiosperm species such as wheat or tomato to verify this potential role of *LSU* genes in regulating plant growth and sulfate levels.

4. Materials and Methods

4.1. Identification of LSU genes and phylogenetic analysis

The sequence and gene codes of Arabidopsis LSU proteins were retrieved from the Arabidopsis Information Resource (https://www.arabidopsis.org/). *LSU* genes of other plant species were identified by BLASTP searches across the set of protein sequences obtained from the 134 species publicly available in the PLAZA 5.0 database (http://bioinformatics.psb.ugent.be/plaza/) [35]. In this manner, we found a total 270 LSU sequences corresponding to the PLAZA homologous gene family HOM05D003381, HOM05M005606 and HOM05M009560 (Table S1).

Multiple sequence alignments were performed using the MAFFT software [41] and the alignments were visualized with Jalview 2 [42]. To identify and retain parsimony-informative sites, the alignments were trimmed using ClipKIT version 1.3.0 with default

 doi:10.20944/preprints202204.0208.v1

parameters [59]. IQ-TREE 2 [37] was used to perform phylogenetic analyses for maximum likelihood (ML) trees using the trimmed multiple alignments. The implemented ModelFinder function in IQ-TREE 2 software determined Q.plant+R5 amino acid replacement matrix [60] to be the best substitution model for tree inference. A total of 10,000 bootstrap replicates were performed using aLRT [61] and UFBoot tests [62] implemented in IQ-TREE 2 [37] for support estimation of reconstructed branches. The evolutionary distance between each pair of LSU sequences was estimated using MEGA 11 with the JTT matrix-based model [39].

4.2. Conserved motif analysis

Conserved protein motifs of the deduced LSU protein sequences were detected using the MEME tool from the MEME Suite v.5.4.1 [40]. The number of potential motifs was set up to 10 with having an optimum motif width ranging between 11 and 50 residues. The distribution of conserved motifs was visualized using TBtools v.0.665 [63].

4.3. Plant material and growth conditions

For the gene expression analysis of LSU gene family in response to S-deficiency, we used seeds of the *Arabidopsis thaliana* ecotype Columbia (Col-0), *Solanum lycopersicum (cv. Moneymaker)* and *Triticum aestivum* L. in liquid medium with all nutrients available or lacking sulfate as we previously described [14] until emergence of the second set of true leaves, which occurs 3 weeks after sowing for these plants under our experimental conditions.

For the functional analysis of *LSU2* gene in Arabidopsis, we have used the T-DNA insertion line SALK_031648C (*lsu2*) [18,28,44] obtained from the Arabidopsis Biological Resource Center (ABRC) (https://abrc.osu.edu/). According to the SALK database (http://signal.salk.edu/cgi-bin/tdnaexpress), the T-DNA of SALK_031648C was inserted in the coding region of *LSU2* gene, close to 5' end. Homozygous lines of the *lsu2* mutant were screened by PCR using a left border primer of the T-DNA insertion (LBb1.3) and two gene-specific primers whose sequences are shown in Table S2. The confirmed homozygous seeds of *lsu2* and Col-0 seeds were surface sterilized with 50% (v/v) bleach and 0.1% (v/v) Triton X-100 for 10 minutes and then thoroughly washed four times with sterile water. After stratification at 4° C for 24 h in darkness, Arabidopsis seeds were grown on agar plates with half-strength MS medium containing 0.5% (w/v) sucrose for 3 weeks. In the case of S-deficiency conditions, the sulfate salts contained in the MS medium were replaced with equivalent chloride salts as we previously described [14].

4.4. Plant growth quantification

Arabidopsis shoots and roots were scanned using an Epson Perfection V600 photo scanner as we previously described [14]. Total shoot area and primary root length were determined from scanned images using the ImageJ software (v1.52) [64].

4.5. Sulfate content analysis

The internal sulfate content was determined using 100 mg of powder ground tissue in liquid nitrogen and incubated with 1mL of 0.1 M HCl for 2 hours at 25ºC. The samples were centrifuged at 12,000 g for 15 minutes at 20 ºC and the obtained supernatants were recovered and used for sulfate determination using the turbidimetric method described previously [48].

4.6. RNA extraction and qRT-PCR

Total RNA extraction was performed from 200 mg of frozen leaves or roots of Arabidopsis, wheat and tomato plants using the Spectrum Plant Total RNA kit (Sigma) and DNAase treatment with TURBO DNase (Invitrogen), according to the manufacturer's protocols. cDNA was synthesized using 1000 ng of total RNA with All-In-One RT MasterMix 5X (abm). qPCR reactions were performed with 25 ng of cDNA using the PowerUp SYBR

Green Master Mix (Thermo Fisher Scientific) and CFX96 Touch Real-Time PCR Detection System (BioRad) according to the manufacturer's instructions. The expression levels were normalized with the *ADAPTOR PROTEIN-4-MU-ADAPTIN* gene (AT4G24550) in the case of Arabidopsis [45], TIP4I-like family protein (TIP4I, SGN-U584254) for tomato [46] and the *Ubiquitin-conjugating enzyme* gene (*TraesCS4A02G414200*) for wheat [47]. Raw fluorescence data derived from each qPCR reaction was analyzed using the Real-time PCR Miner 4.0 software to obtain Ct values and gene amplification efficiencies [65]. Sequences of the qRT-PCR primers used in this study are provided in Table S2. In the case of wheat, conserved primers for each homoeolog group were designed (*TaLSU1* for *TraesCS1A03G0484600*, *TraesCS1B03G0587700*, *TraesCS1D03G0456700*; *TaLSU2* for *TraesCS6A03G0192500*, *TraesCS6B03G0289700*) as the sequences of *LSU* genes of all the respective homoeologs were almost the same in wheat sub-genomes. Moreover, we verified that *LSU* genes of the same homoeolog group have a significant high correlation in public RNA-seq datasets (p-value<0.05, Figure S7), indicating redundant expression profiles within the homoeolog group as previously reported for ~70% of wheat homoeolog triads [43].

Supplementary Materials: Figure S1: scatter plot showing the positive correlation between total coding genes and *LSU* gene number; Figure S2: Comparative analysis of LSU gene number across phylogenetic groups; Figure S3: Expression profiles of S-marker genes in wheat, Arabidopsis and tomato; Figure S4: Expression profiles of *TaLSU1* and *TaLSU2* in different tissues and growth stages of wheat obtained from Wheat eFP Browser; Figure S5: Validation of the *lsu2* mutant line; Figure S6: Comparative expression profiles of TraesCS1A03G0484600 and TraesCS6A03G0192500 in different developmental stages obtained from WheatOmics database; Figures S7: Heatmap of Pearson correlation coefficient matrix of the wheat *LSU* gene expression levels obtained from Wheat eFP Browser; Table S1: list of *LSU* genes and corresponding species; Table S2: Primers used in quantitative real-time PCR analysis.

Author Contributions: Conceptualization, F.U., J.M, E.A.V and J.C.; methodology, F.U., C.H-V., A.A.-M.; writing—original draft preparation, J.C.; writing—review and editing, A.A.-M., E.A.V., J.M., and J.C.; funding acquisition, E.A.V., J.M. and J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the National Agency for Research and Development (ANID) Chile with Program FONDECYT Regular 1190812 and FONDECYT Regular 1211130 and Spanish Science Ministry (PID2020-114165RR-C21 to J.M). J.C. and E.A.V. was supported by ANID—Millennium Science Initiative Program—ICN17_022. We also want to acknowledge the "Severo Ochoa Program for Centres of Excellence in R&D" (CEX2020-000999-S) supported by MCIN/AEI /10.13039/501100011033 for supporting the scientific services used in this work.

Data Availability Statement: Not applicable.

Acknowledgments: We thank the Arabidopsis Biological Resource Center for T-DNA mutant lines used in this study.

Conflicts of Interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Zenda, T.; Liu, S.; Dong, A.; Duan, H. Revisiting Sulphur—The Once Neglected Nutrient: It's Roles in Plant Growth, Metabolism, Stress Tolerance and Crop Production. *Agriculture* **2021**, *11*, 626, doi:10.3390/agriculture11070626.
2. Haneklaus, S.; Bloem, E.; Schnug, E. History of Sulfur Deficiency in Crops. In *Sulfur: A Missing Link between Soils, Crops, and Nutrition*; John Wiley & Sons, Ltd, 2008; pp. 45-58–6 ISBN 978-0-89118-186-6.
3. Ufaz, S.; Galili, G. Improving the Content of Essential Amino Acids in Crop Plants: Goals and Opportunities. *Plant Physiol.* **2008**, *147*, 954–961, doi:10.1104/pp.108.118091.
4. Li, Q.; Gao, Y.; Yang, A. Sulfur Homeostasis in Plants. *Int. J. Mol. Sci.* **2020**, *21*, 8926, doi:10.3390/ijms21238926.
5. Forieri, I.; Sticht, C.; Reichelt, M.; Gretz, N.; Hawkesford, M.J.; Malagoli, M.; Wirtz, M.; Hell, R. 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, doi:10.1111/pce.12842.

6. Maruyama-Nakashita, A.; Nakamura, Y.; Watanabe-Takahashi, A.; Inoue, E.; Yamaya, T.; Takahashi, H. Identification of a Novel Cis-Acting Element Conferring Sulfur Deficiency Response in Arabidopsis Roots. *Plant J.* **2005**, *42*, 305–314, doi:10.1111/j.1365-313X.2005.02363.x.
7. Maruyama-Nakashita, A.; Inoue, E.; Watanabe-Takahashi, A.; Yamaya, T.; Takahashi, H. Transcriptome Profiling of Sulfur-Responsive Genes in Arabidopsis Reveals Global Effects of Sulfur Nutrition on Multiple Metabolic Pathways. *Plant Physiol.* **2003**, *132*, 597–605, doi:10.1104/pp.102.019802.
8. Bielecka, M.; Watanabe, M.; Morcuende, R.; Scheible, W.-R.; Hawkesford, M.J.; Hesse, H.; Hoefgen, R. Transcriptome and Metabolome Analysis of Plant Sulfate Starvation and Resupply Provides Novel Information on Transcriptional Regulation of Metabolism Associated with Sulfur, Nitrogen and Phosphorus Nutritional Responses in Arabidopsis. *Front. Plant Sci.* **2015**, *5*, 805, doi:10.3389/fpls.2014.00805.
9. Nikiforova, V.; Freitag, J.; Kempa, S.; Adamik, M.; Hesse, H.; Hoefgen, R. Transcriptome Analysis of Sulfur Depletion in Arabidopsis Thaliana: Interlacing of Biosynthetic Pathways Provides Response Specificity. *Plant J. Cell Mol. Biol.* **2003**, *33*, 633–650, doi:10.1046/j.1365-313x.2003.01657.x.
10. Hirai, M.Y.; Fujiwara, T.; Awazuhara, M.; Kimura, T.; Noji, M.; Saito, K. Global Expression Profiling of Sulfur-Starved Arabidopsis by DNA Macroarray Reveals the Role of O-Acetyl-l-Serine as a General Regulator of Gene Expression in Response to Sulfur Nutrition. *Plant J. Cell Mol. Biol.* **2003**, *33*, 651–663, doi:10.1046/j.1365-313x.2003.01658.x.
11. Howarth, J.R.; Parmar, S.; Jones, J.; Shepherd, C.E.; Corol, D.-I.; Galster, A.M.; Hawkins, N.D.; Miller, S.J.; Baker, J.M.; Verrier, P.J.; et al. Co-Ordinated Expression of Amino Acid Metabolism in Response to N and S Deficiency during Wheat Grain Filling. *J. Exp. Bot.* **2008**, *59*, 3675–3689, doi:10.1093/jxb/ern218.
12. Gupta, S.; Yadav, B.S.; Raj, U.; Freilich, S.; Varadwaj, P.K. Transcriptomic Analysis of Soil Grown T. Aestivum Cv. Root to Reveal the Changes in Expression of Genes in Response to Multiple Nutrients Deficiency. *Front. Plant Sci.* **2017**, *8*.
13. Dai, Z.; Plessis, A.; Vincent, J.; Duchateau, N.; Besson, A.; Dardevet, M.; Prodhomme, D.; Gibon, Y.; Hilbert, G.; Pailloux, M.; et al. Transcriptional and Metabolic Alternations Rebalance Wheat Grain Storage Protein Accumulation under Variable Nitrogen and Sulfur Supply. *Plant J. Cell Mol. Biol.* **2015**, *83*, 326–343, doi:10.1111/tpj.12881.
14. Canales, J.; Uribe, F.; Henríquez-Valencia, C.; Lovazzano, C.; Medina, J.; Vidal, E.A. Transcriptomic Analysis at Organ and Time Scale Reveals Gene Regulatory Networks Controlling the Sulfate Starvation Response of Solanum Lycopersicum. *BMC Plant Biol.* **2020**, *20*, 385, doi:10.1186/s12870-020-02590-2.
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**, *9*, 470.
16. Lewandowska, M.; Wawrzyńska, A.; Moniuszko, G.; Łukomska, J.; Zientara, K.; Piecho, M.; Hodurek, P.; Zhukov, I.; Liszewska, F.; Nikiforova, V.; et al. A Contribution to Identification of Novel Regulators of Plant Response to Sulfur Deficiency: Characteristics of a Tobacco Gene UP9C, Its Protein Product and the Effects of UP9C Silencing. *Mol. Plant* **2010**, *3*, 347–360, doi:10.1093/mp/ssq007.
17. Garcia-Molina, A.; Altmann, M.; Alkofer, A.; Epple, P.M.; Dangl, J.L.; Falter-Braun, P. LSU Network Hubs Integrate Abiotic and Biotic Stress Responses via Interaction with the Superoxide Dismutase FSD2. *J. Exp. Bot.* **2017**, *68*, 1185–1197, doi:10.1093/jxb/erw498.
18. Wang, H.; Seo, J.-K.; Gao, S.; Cui, X.; Jin, H. Silencing of AtRAP, a Target Gene of a Bacteria-Induced Small RNA, Triggers Antibacterial Defense Responses through Activation of LSU2 and down-Regulation of GLK1. *New Phytol.* **2017**, *215*, 1144–1155, doi:10.1111/nph.14654.
19. Wawrzynska, A.; Moniuszko, G.; Sirko, A. Links Between Ethylene and Sulfur Nutrition—A Regulatory Interplay or Just Metabolite Association? *Front. Plant Sci.* **2015**, *6*, 1053, doi:10.3389/fpls.2015.01053.
20. Wawrzyńska, A.; Lewandowska, M.; Sirko, A. Nicotiana Tabacum EIL2 Directly Regulates Expression of at Least One Tobacco Gene Induced by Sulphur Starvation. *J. Exp. Bot.* **2010**, *61*, 889–900, doi:10.1093/jxb/erp356.
21. 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*, 3235–3251, doi:10.1105/tpc.106.046458.
22. Niemiro, A.; Cysewski, D.; Brzywczy, J.; Wawrzyńska, A.; Sieńko, M.; Poznański, J.; Sirko, A. Similar but Not Identical—Binding Properties of LSU (Response to Low Sulfur) Proteins From Arabidopsis Thaliana. *Front. Plant Sci.* **2020**, *11*.
23. Arabidopsis Interactome Mapping Consortium Evidence for Network Evolution in an Arabidopsis Interactome Map. *Science* **2011**, *333*, 601–607, doi:10.1126/science.1203877.
24. Mukhtar, M.S.; Carvunis, A.-R.; Dreze, M.; Epple, P.; Steinbrenner, J.; Moore, J.; Tasan, M.; Galli, M.; Hao, T.; Nishimura, M.T.; et al. Independently Evolved Virulence Effectors Converge onto Hubs in a Plant Immune System Network. *Science* **2011**, *333*, 596–601, doi:10.1126/science.1203659.
25. Vandereyken, K.; Van Leene, J.; De Coninck, B.; Cammue, B.P.A. Hub Protein Controversy: Taking a Closer Look at Plant Stress Response Hubs. *Front. Plant Sci.* **2018**, *9*, 694, doi:10.3389/fpls.2018.00694.
26. Davletova, S.; Rizhsky, L.; Liang, H.; Shengqiang, Z.; Oliver, D.J.; Coutu, J.; Shulaev, V.; Schlauch, K.; Mittler, R. Cytosolic Ascorbate Peroxidase 1 Is a Central Component of the Reactive Oxygen Gene Network of Arabidopsis. *Plant Cell* **2005**, *17*, 268–281, doi:10.1105/tpc.104.026971.
27. Usadel, B.; Bläsing, O.E.; Gibon, Y.; Retzlaff, K.; Höhne, M.; Günther, M.; Stitt, M. Global Transcript Levels Respond to Small Changes of the Carbon Status during Progressive Exhaustion of Carbohydrates in Arabidopsis Rosettes. *Plant Physiol.* **2008**, *146*, 1834–1861, doi:10.1104/pp.107.115592.

28. Ruckle, M.E.; Burgoon, L.D.; Lawrence, L.A.; Sinkler, C.A.; Larkin, R.M. Plastids Are Major Regulators of Light Signaling in Arabidopsis. *Plant Physiol.* **2012**, *159*, 366–390, doi:10.1104/pp.112.193599.
29. Weßling, R.; Epple, P.; Altmann, S.; He, Y.; Yang, L.; Henz, S.R.; McDonald, N.; Wiley, K.; Bader, K.C.; Gläßer, C.; et al. Convergent Targeting of a Common Host Protein-Network by Pathogen Effectors from Three Kingdoms of Life. *Cell Host Microbe* **2014**, *16*, 364–375, doi:10.1016/j.chom.2014.08.004.
30. Moniuszko, G.; Skoneczny, M.; Zientara-Rytter, K.; Wawrzyńska, A.; Głów, D.; Cristescu, S.M.; Harren, F.J.M.; Sirko, A. Tobacco LSU-like Protein Couples Sulphur-Deficiency Response with Ethylene Signalling Pathway. *J. Exp. Bot.* **2013**, *64*, 5173–5182, doi:10.1093/jxb/ert309.
31. Kong, X.; Li, C.; Zhang, F.; Yu, Q.; Gao, S.; Zhang, M.; Tian, H.; Zhang, J.; Yuan, X.; Ding, Z. Ethylene Promotes Cadmium-Induced Root Growth Inhibition through EIN3 Controlled XTH33 and LSU1 Expression in Arabidopsis. *Plant Cell Environ.* **2018**, *41*, 2449–2462, doi:10.1111/pce.13361.
32. Sirko, A.; Wawrzyńska, A.; Rodríguez, M.C.; Sęktas, P. The Family of LSU-like Proteins. *Front. Plant Sci.* **2014**, *5*, 774, doi:10.3389/fpls.2014.00774.
33. Takahashi, H.; Buchner, P.; Yoshimoto, N.; Hawkesford, M.J.; Shiu, S.-H. Evolutionary Relationships and Functional Diversity of Plant Sulfate Transporters. *Front. Plant Sci.* **2012**, *2*, 119, doi:10.3389/fpls.2011.00119.
34. Kopriva, S.; Wiedemann, G.; Reski, R. Sulfate Assimilation in Basal Land Plants - What Does Genomic Sequencing Tell Us? *Plant Biol. Stuttg. Ger.* **2007**, *9*, 556–564, doi:10.1055/s-2007-965430.
35. Van Bel, M.; Silvestri, F.; Weitz, E.M.; Kreft, L.; Botzki, A.; Coppens, F.; Vandepoele, K. PLAZA 5.0: Extending the Scope and Power of Comparative and Functional Genomics in Plants. *Nucleic Acids Res.* **2021**, gkab1024, doi:10.1093/nar/gkab1024.
36. Kumar, S.; Stecher, G.; Suleski, M.; Hedges, S.B. TimeTree: A Resource for Timelines, Timetrees, and Divergence Times. *Mol. Biol. Evol.* **2017**, *34*, 1812–1819, doi:10.1093/molbev/msx116.
37. Minh, B.Q.; Schmidt, H.A.; Chernomor, O.; Schrempf, D.; Woodhams, M.D.; von Haeseler, A.; Lanfear, R. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol. Biol. Evol.* **2020**, *37*, 1530–1534, doi:10.1093/molbev/msaa015.
38. Li, H.-T.; Luo, Y.; Gan, L.; Ma, P.-F.; Gao, L.-M.; Yang, J.-B.; Cai, J.; Gitzendanner, M.A.; Fritsch, P.W.; Zhang, T.; et al. Plastid Phylogenomic Insights into Relationships of All Flowering Plant Families. *BMC Biol.* **2021**, *19*, 232, doi:10.1186/s12915-021-01166-2.
39. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027, doi:10.1093/molbev/msab120.
40. Bailey, T.L.; Johnson, J.; Grant, C.E.; Noble, W.S. The MEME Suite. *Nucleic Acids Res.* **2015**, *43*, W39–W49, doi:10.1093/nar/gkv416.
41. Katoh, K.; Rozewicki, J.; Yamada, K.D. MAFFT Online Service: Multiple Sequence Alignment, Interactive Sequence Choice and Visualization. *Brief. Bioinform.* **2019**, *20*, 1160–1166, doi:10.1093/bib/bbx108.
42. Waterhouse, A.M.; Procter, J.B.; Martin, D.M.A.; Clamp, M.; Barton, G.J. Jalview Version 2--a Multiple Sequence Alignment Editor and Analysis Workbench. *Bioinforma. Oxf. Engl.* **2009**, *25*, 1189–1191, doi:10.1093/bioinformatics/btp033.
43. Ramírez-González, R.H.; Borrill, P.; Lang, D.; Harrington, S.A.; Brinton, J.; Venturini, L.; Davey, M.; Jacobs, J.; van Ex, F.; Pasha, A.; et al. The Transcriptional Landscape of Polyploid Wheat. *Science* **2018**, *361*, eaar6089, doi:10.1126/science.aar6089.
44. Luhua, S.; Hegie, A.; Suzuki, N.; Shulaev, E.; Luo, X.; Cenariu, D.; Ma, V.; Kao, S.; Lim, J.; Gunay, M.B.; et al. Linking Genes of Unknown Function with Abiotic Stress Responses by High-Throughput Phenotype Screening. *Physiol. Plant.* **2013**, *148*, 322–333, doi:10.1111/ppl.12013.
45. Czechowski, T.; Stitt, M.; Altmann, T.; Udvardi, M.K.; Scheible, W.-R. Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. *Plant Physiol.* **2005**, *139*, 5–17, doi:10.1104/pp.105.063743.
46. Paolacci, A.R.; Celletti, S.; Catarcione, G.; Hawkesford, M.J.; Astolfi, S.; Ciaffi, M. Iron Deprivation Results in a Rapid but Not Sustained Increase of the Expression of Genes Involved in Iron Metabolism and Sulfate Uptake in Tomato (Solanum Lycopersicum L.) Seedlings. *J. Integr. Plant Biol.* **2014**, *56*, 88–100, doi:10.1111/jipb.12110.
47. Borrill, P.; Ramirez-Gonzalez, R.; Uauy, C. ExpVIP: A Customizable RNA-Seq Data Analysis and Visualization Platform. *Plant Physiol.* **2016**, *170*, 2172–2186, doi:10.1104/pp.15.01667.
48. Tabatabai, M.A.; Bremner, J.M. A Simple Turbidimetric Method of Determining Total Sulfur in Plant Materials1. *Agron. J.* **1970**, *62*, 805–806, doi:10.2134/agronj1970.00021962006200060038x.
49. Davidian, J.-C.; Kopriva, S. Regulation of Sulfate Uptake and Assimilation—the Same or Not the Same? *Mol. Plant* **2010**, *3*, 314–325, doi:10.1093/mp/ssq001.
50. Bowles, A.M.C.; Bechtold, U.; Paps, J. The Origin of Land Plants Is Rooted in Two Bursts of Genomic Novelty. *Curr. Biol. CB* **2020**, *30*, 530-536.e2, doi:10.1016/j.cub.2019.11.090.
51. Niehrs, C.; Pollet, N. Synexpression Groups in Eukaryotes. *Nature* **1999**, *402*, 483–487, doi:10.1038/990025.
52. Hu, W.; Hua, X.; Zhang, Q.; Wang, J.; Shen, Q.; Zhang, X.; Wang, K.; Yu, Q.; Lin, Y.-R.; Ming, R.; et al. New Insights into the Evolution and Functional Divergence of the SWEET Family in Saccharum Based on Comparative Genomics. *BMC Plant Biol.* **2018**, *18*, 270, doi:10.1186/s12870-018-1495-y.
53. Zhong, H.; Zhang, H.; Guo, R.; Wang, Q.; Huang, X.; Liao, J.; Li, Y.; Huang, Y.; Wang, Z. Characterization and Functional Divergence of a Novel DUF668 Gene Family in Rice Based on Comprehensive Expression Patterns. *Genes* **2019**, *10*, 980, doi:10.3390/genes10120980.

54. Ma, S.; Wang, M.; Wu, J.; Guo, W.; Chen, Y.; Li, G.; Wang, Y.; Shi, W.; Xia, G.; Fu, D.; et al. WheatOmics: A Platform Combining Multiple Omics Data to Accelerate Functional Genomics Studies in Wheat. *Mol. Plant* **2021**, *14*, 1965–1968, doi:10.1016/j.molp.2021.10.006.
55. Waese, J.; Fan, J.; Pasha, A.; Yu, H.; Fucile, G.; Shi, R.; Cumming, M.; Kelley, L.A.; Sternberg, M.J.; Krishnakumar, V.; et al. EPlant: Visualizing and Exploring Multiple Levels of Data for Hypothesis Generation in Plant Biology. *Plant Cell* **2017**, *29*, 1806–1821, doi:10.1105/tpc.17.00073.
56. Myakushina, Yu.A.; Milyaeva, E.L.; Romanov, G.A.; Nikiforova, V.Yu. Mutation in LSU4 Gene Affects Flower Development in Arabidopsis Thaliana. *Dokl. Biochem. Biophys.* **2009**, *428*, 257–260, doi:10.1134/S1607672909050093.
57. Andrés-Barrao, C.; Alzubaidy, H.; Jalal, R.; Mariappan, K.G.; de Zélicourt, A.; Bokhari, A.; Artyukh, O.; Alwutayd, K.; Rawat, A.; Shekhawat, K.; et al. Coordinated Bacterial and Plant Sulfur Metabolism in Enterobacter Sp. SA187-Induced Plant Salt Stress Tolerance. *Proc. Natl. Acad. Sci. U. S. A.* **2021**, *118*, e2107417118, doi:10.1073/pnas.2107417118.
58. El-Brolosy, M.A.; Stainier, D.Y.R. Genetic Compensation: A Phenomenon in Search of Mechanisms. *PLOS Genet.* **2017**, *13*, e1006780, doi:10.1371/journal.pgen.1006780.
59. Steenwyk, J.L.; Buida, T.J.; Li, Y.; Shen, X.-X.; Rokas, A. ClipKIT: A Multiple Sequence Alignment Trimming Software for Accurate Phylogenomic Inference. *PLoS Biol.* **2020**, *18*, e3001007, doi:10.1371/journal.pbio.3001007.
60. Minh, B.Q.; Dang, C.C.; Vinh, L.S.; Lanfear, R. QMaker: Fast and Accurate Method to Estimate Empirical Models of Protein Evolution. *Syst. Biol.* **2021**, *70*, 1046–1060, doi:10.1093/sysbio/syab010.
61. Guindon, S.; Dufayard, J.-F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Syst. Biol.* **2010**, *59*, 307–321, doi:10.1093/sysbio/syq010.
62. Minh, B.Q.; Nguyen, M.A.T.; von Haeseler, A. Ultrafast Approximation for Phylogenetic Bootstrap. *Mol. Biol. Evol.* **2013**, *30*, 1188–1195, doi:10.1093/molbev/mst024.
63. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202, doi:10.1016/j.molp.2020.06.009.
64. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9*, 671–675, doi:10.1038/nmeth.2089.
65. Zhao, S.; Fernald, R.D. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. *J. Comput. Biol. J. Comput. Mol. Cell Biol.* **2005**, *12*, 1047–1064, doi:10.1089/cmb.2005.12.1047.