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Generation and characterization of single and multigene *Arabidopsis thaliana* mutants in *LSU1-4* (*RESPONSE TO LOW SULFUR*) genes

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ABSTRACT

In *Arabidopsis thaliana*, there are four members of the *LSU* (*RESPONSE TO LOW SULFUR*) gene family which are tandemly located on chromosomes 3 (*LSU1* and *LSU3*) and 5 (*LSU2* and *LSU4*). The LSU proteins are small, with coiled-coil structures, and they are able to form homo- and heterodimers. LSUs are involved in plant responses to environmental challenges, such as sulfur deficiency, and plant immune responses. Assessment of the role and function of these proteins was challenging due to the absence of deletion mutants. Our work fulfills this gap through the construction of a set of *LSU* deletion mutants (single, double, triple, and quadruple) by CRISPR/Cas9 technology. The genomic deletion regions in the obtained lines were mapped and the level of expression of each *LSUs* was assayed in each mutant. All lines were viable and capable of seed production. Their growth and development were compared at several different stages with the wild-type. No significant and consistent differences in seedlings' growth and plant development were observed in the optimal conditions. In sulfur deficiency, the roots of 12-day-old wild-type seedlings exhibited increased length compared to optimal conditions; however, this difference in root length was not observed in the majority of lsu-KO mutants.

1. Introduction

The genome of *Arabidopsis thaliana* encodes four LSU (*RESPONSE TO LOW SULFUR*) proteins. The *LSU* genes that are localized in pairs of direct repeats on two chromosomes, *LSU1* and *LSU3* (At3g49580 and At3g49570, respectively) on chromosome 3 separated by about 2250 bp; the *LSU2* and *LSU4* (At5g24660 and At5g24655, respectively) on chromosome 5 separated by about 2060 bp. The length of these open reading frames is about 280 base pairs. Only for *LSU1* three splicing forms were reported, while other *LSU* genes are intronless. The LSUs proteins have coil-coil structures and can form homo and heterodimers, and possibly also multimers (Niemiro et al., 2020). Despite the growing interest in these proteins, their precise role and their significance in plant growth and development remain unknown.

LSU genes were described in the context of sulfur (S) deficiency response in *A. thaliana* by two independent research groups. In the earlier study (Maruyama-Nakashita et al., 2005), the *LSU1* and *LSU2* genes were identified as two of 15 genes significantly upregulated after plants were transferred to S-free medium. In the other work (Nikiforova et al., 2005) the *LSU1* gene appeared as an important element of the gene-metabolite network of plant response to S-deficiency. A recent study showed that mRNA levels of all *LSUs* are increased under S-deficiency in roots and leaves (Uribe et al., 2022). The *LSU*-like genes are up-regulated by S-deficiency not only in *A. thaliana* but also in tobacco (Lewandowska et al., 2010), tomato, and wheat (Uribe et al., 2022). This observation suggests that they might have evolutionarily conserved functions in angiosperms relevant to S availability and/or requirement for S. On the other hand, regulation of *LSUs* expression by other abiotic stresses, such as increased salinity, iron (Fe) deficiency, copper (Cu) excess, or basic pH was also reported (Garcia-Molina et al., 2017). Additionally, *LSU1* was identified as a part of OAS cluster genes which are induced when O-acetylserine (OAS) accumulates (Hubberten et al., 2012). And recently, an extended OAS cluster co-expression network was proposed which among other genes includes also *LSU3* (Apodiakou and Hoefgen, 2023).

The latest findings indicate that the overexpression of *LSU1* and *LSU2* improved Cd tolerance and simultaneously promoted Cd accumulation, presumably by coordinating the flow of S from glucosinolates,

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to detoxify metabolites under Cd stress (Li et al., 2023). Similarly, overexpression of *LSU1* and *LSU2* from broccoli led to better Arabidopsis plants performance under S-deficiency (Yang et al., 2023). BoLSU1 and BoLSU2 not only hindered the production of glucosinolates but also encouraged their breakdown, consequently boosting the levels of glutathione. Nevertheless, most of the up-to-date research on these proteins was based on the characterization of T-DNA insertion mutants or amiRNA LSU lines with reduced levels of all *LSU* transcripts. There are T-DNA insertional mutants for *LSU2* (e.g. SALK_31648, SALK_070105), *LSU3* (e.g. GABI_207B03) and *LSU4* (e.g. SALK_069114) but not for *LSU1*. Most available data relate to the analysis of *lsu2* or *lsu4* mutants. For example, SALK_31648C seemed to be more tolerant to osmotic stress than the wild type (Luhua et al., 2013), SALK_031648 and SALK_070105 exhibited enhanced susceptibility to two evolutionarily distinct pathogens, *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Mukhtar et al., 2011), while defects in flower and inflorescence development were observed in SALK_069114 when grown under short-day conditions (Myakushina et al., 2009). The construction of amiRNA LSU lines provided some insight into the role of LSU proteins in the regulation of the activity of superoxide dismutase FSD2 and the author's conclusion on the role of LSU in integrating the biotic and abiotic responses via LSU-FSD2 interaction (Garcia-Molina et al., 2017).

The lack of individual and multiple *lsu* mutants retards the progress in establishing the function of these small coiled-coil proteins. Therefore, the aim of this study was the creation of a set of single and multiple *lsu* knockouts (lsu-KO lines) which would enable future analysis of the individual or overlapping functions of LSU quadruple pack in *A. thaliana*, including their relation to S metabolism and plant response to S-deficiency stress.

2. Materials and methods

2.1. Plant lines, construction of the single and multiple KO mutants, and methods related to their verification

All plant lines used in this work were derivatives of *Arabidopsis thaliana* Col-0 ecotype (denoted in this work as wild type, WT). The mutants were obtained *via* CRISPR/Cas9 method (Fauser et al., 2014). In the first step, the pairs of sgRNAs were designed by CRISPR-P 2.0 software (Liu et al., 2017), annealed, and cloned into the pChimera plasmid. Next, the complete sgRNA construct was transferred to a binary vector pDe-CAS9 that contains the Cas9 expression cassette. The obtained vectors, after their validation by sequencing, were introduced into Arabidopsis plants *via Agrobacterium tumefaciens*-mediated transformation using the floral dip method (Clough and Bent, 1998). T1 generation plants were screened for the presence of transgene using 15 mg/l phosphinothricin (PPT) and for the presence of deletions in *LSUs* by PCR and sequencing. All mutants, except lsu1lsu3-KO, were obtained by the above approach. The lsu1lsu3-KO mutant was obtained as described by (Stuttmann et al., 2021). The pairs of sgRNAs were cloned into shuttle vectors pDGE332 and pDGE334 using a *BpiI* Golden Gate reaction and subsequently mobilized into a recipient vector pDGE347 in a Golden Gate reaction using *Bsa*I. After the transformation of *Agrobacterium tumefaciens* the vector was introduced to Arabidopsis using the floral dip method (Clough and Bent, 1998). In this approach, the seeds of transformants were selected based on the presence of the FAST (fluorescence-accumulating seed technology) marker using binocular under UV illumination with an RFP filter. T1 generation plants were screened for the presence of the mutations by PCR and sequencing. The plasmids used for the generation of the mutants are listed in Suppl. Table 1. The line lsu1lsu3-KO was not included in most series of analyses discussed in this study because it was selected much later than the other lines. The lsu1lsu3-KO has no apparent morphological differences from the WT or other mutants.

The triple (lsu1lsu2lsu4-KO) and the quadruple (lsu1lsu3lsu2lsu4-KO; q-lsu-KO) mutants were obtained by generation of additional CRISPR/Cas9 mutants in previously obtained lsu2lsu4-KO mutant. The T3 generations of the mutants were screened by PCR for homozygous deletions and the lack of the transformation cassette (a marker gene was *bar* conferring resistance to the herbicide Basta). All mutants were confirmed to be homozygous and all, except lsu4-KO, lacked the marker gene. In all screened by PCR T3-individuals of lsu4-KO the product corresponding to the marker gene was detected and the selection of marker-free lsu4-KO line is continued. The LSU-OX transgenic Arabidopsis lines constitutively overproducing individual LSUs in fusion with the TAP tag have been described earlier (Niemiro et al., 2020).

2.2. Plant growth conditions

For seed production, the plants were grown in soil in greenhouse conditions. For phenotypic and molecular analysis, seedlings were cultivated *in vitro*. Seeds were dry sterilized as described previously (Zientara et al., 2009), plated out onto plates with half-strength Hoagland's medium containing 0.8% agar, and stratified at 8°C for 2 days. Plates were placed vertically in a plant growth chamber with a photoperiod of 8 h of light and 16 h of darkness. The 0.5 x Hoagland medium containing 1 mM sulfate was treated as sulfur-sufficient medium [nS]; in sulfur-deficient medium [dS] $MgS0_4$ was replaced by equimolar $MgCl_2$. Additionally, media with low sulfur ([LS]; 0,01 mM sulfate), low nitrogen ([LN]; 2 mM nitrate) or low phosphorus ([LP]; 0,1 mM phosphate) content were used. Seedlings were either grown for 10 days in [nS] and transferred for 2 days into [nS] and [dS] (transcriptomic studies) or they were germinated and grown in parallel in [nS], [dS], [LS], [LP], or [LN] media for 6, 12 or 18 days (phenotypic studies). For stomatal measurements, seedlings were grown for 12 days in either [nS] or [dS] conditions in a chamber with a photoperiod of 16 h of light and 8 h of darkness. To measure abscisic acid (ABA) content seedlings were grown for 15 days in [nS] and then transferred for an additional 5 days to either [nS] or [dS] conditions. Only shoots were collected for ABA measurements. All media compositions are listed in Suppl. Table 2

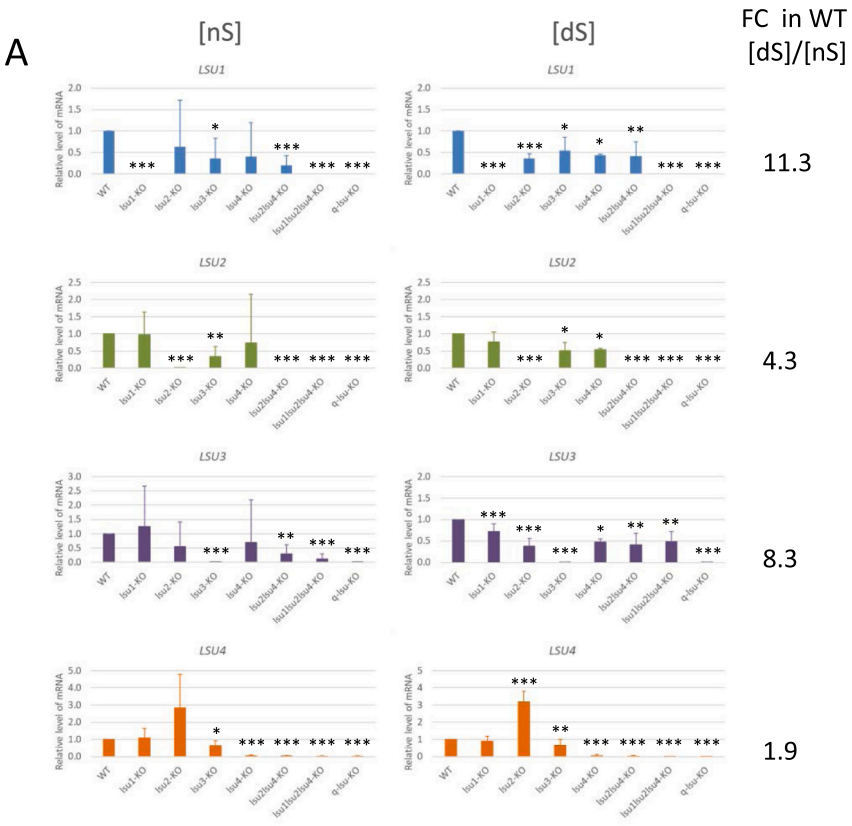
2.3. Yeast two-hybrid assay

The coding regions of *LSU* genes were amplified using specific primers described in Suppl. Table 3 and cloned into pENTR/D-TOPO using TOPO cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, the entry clones encoding full-length LSU proteins (and open reading frames amplified from lsu2-KO and lsu3-KO mutants) were recombined into binary vectors pDEST22 or pDEST32 using Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific, Waltham, MA, USA). All cloning reactions were performed as described in the manufacturer's protocols. All clones were validated by sequencing. *Saccharomyces cerevisiae* Y2HGold strain (Takara Bio, Shiga, Japan) was transformed according to standard procedures. The transformants were selected on plates with synthetic media lacking leucine and tryptophan (−LT), and protein-protein interactions were tested on media without histidine in the presence of 5 mM 3-amino-1,2,4- triazole at (−LTH + 3AT), or without adenine (−LTA). The plates were usually incubated for three days at 28°C.

2.4. Isolation of plant DNA and RNA

Genomic DNA was extracted from plants by grinding one leaf in 400 µl of Extraction Buffer (0,5% SDS, 200 mM Tris pH 7,5, 25 mM EDTA pH 8, 250 mM NaCl). After vortexing and centrifugation for 1 min at 13 000 rpm, the supernatant (300 µl) was transferred to a new tube, and 300 µl of isopropanol was added, followed by centrifugation for 5 min at 13 000 rpm. The supernatant was removed and the DNA pellets were washed twice with 70% ethanol, dried, and re-suspended in 100 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

Total RNA was isolated separately from shoots and roots using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to



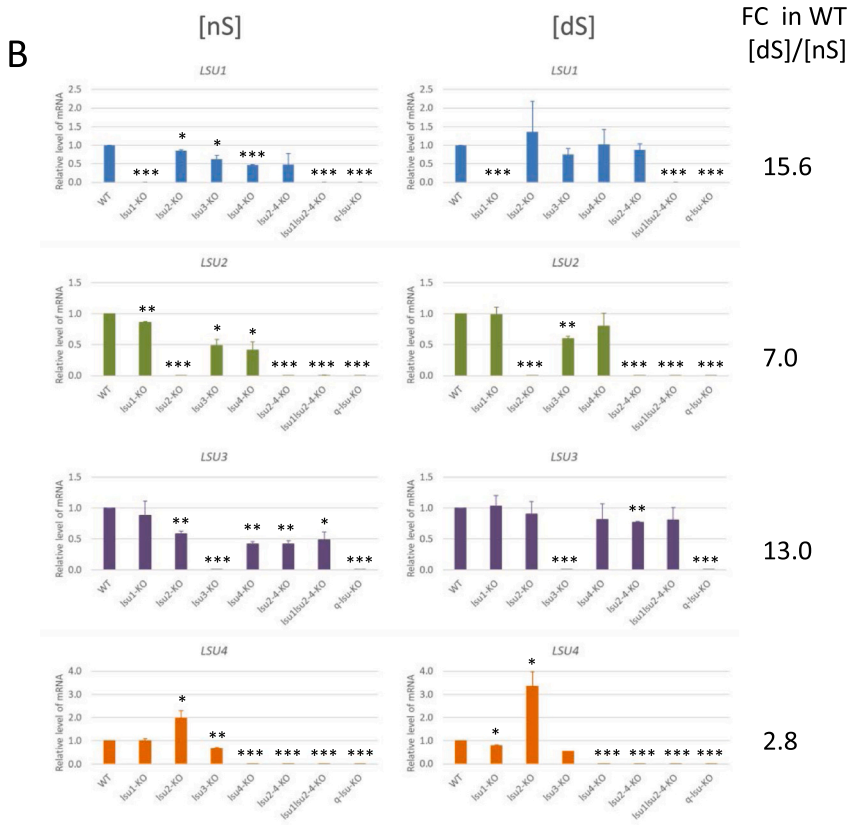


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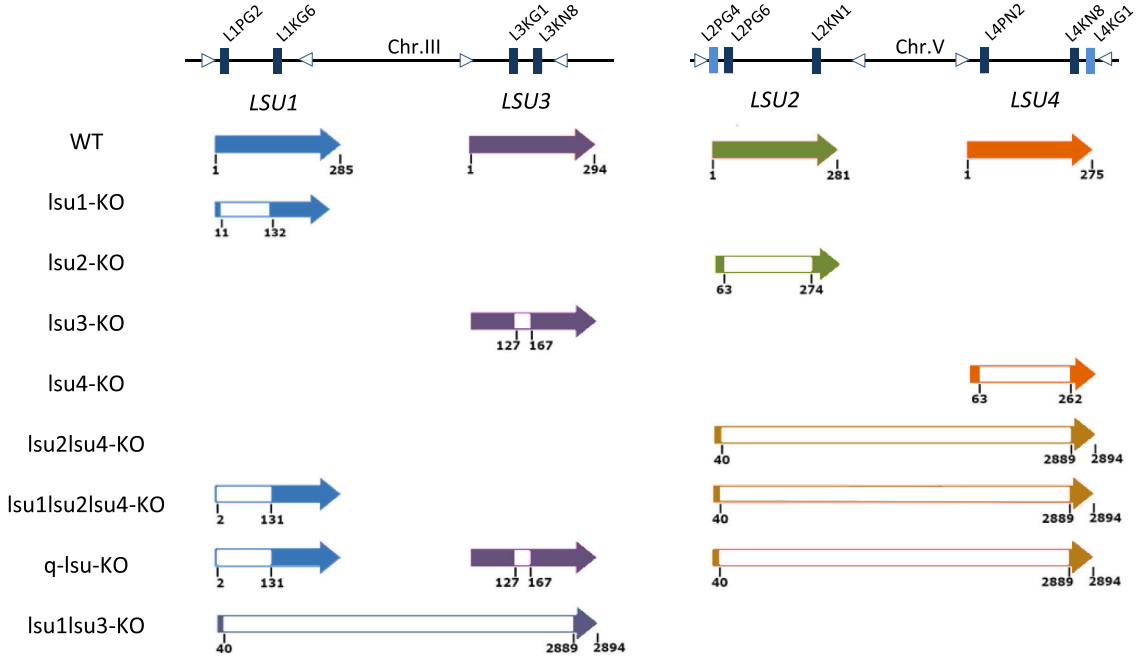
Fig. 1. The expression level of LSU genes in wild type (WT) and *lsu* knock-out lines (lsu-KO) grown in sulfur sufficient [nS] conditions for 10 days and transferred for two additional days to either [nS] or sulfur deficient [dS] conditions. Results of reverse transcription quantitative PCR (RT-qPCR) using the shoots and roots material were normalized to WT. The numbers on the right indicate the fold change of the level of expression of a particular gene in the indicated plant part in WT in [dS] versus [nS]. Error bars represent the standard deviation calculated from six independent experiments conducted for shoots (A) and two independent experiments conducted for roots (B). The observed large scatter of results in shoots in [nS] is due to an extremely low level of *LSUs* expression. The asterisks mark statistical differences between the given line and WT in the same conditions calculated with the Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

the manufacturer's protocol (Chomczynski and Sacchi, 1987). Plant tissue was collected from at least 10 seedlings. The quality and the concentration of RNA were evaluated using a NanoDrop ND-100 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer.

2.5. Genomic PCR and gene expression analysis by RT-qPCR

The presence of expected deletions in obtained plant lines was confirmed by a series of genomic PCR reactions with oligonucleotides specific to each *LSU* gene (Suppl. Table 3). PCR reaction consisted of 1 µl of genomic DNA as a template, 0.4 µl of each forward and reverse 10 µM primers, and 5 µl of Dream Taq Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 10 µl. The initial denaturation for 2 minutes at 95 °C was followed by 30 cycles composed of: denaturation for 30 s at 95 °C, annealing for 30 s at 56 °C, and elongation for 30 s. For the real-time quantitative PCR (RT-qPCR) analysis, 2 µg of RNA

A



B

LSU1 in the lines:		
WT	MANRGGCVTVAAEEMDELRRRNIELSREVAEMKTEMIKLWQRTVVAEEAEEQLCSQLAELEVESLEQARDYHDRMLFLMDQISRLSSSSVVSSS	94aa
lsu1-KO	MANRGGGGGRATLLAAGGAGGRVFRTGT	28aa
lsu1lsu2lsu4-KO	MAEEAEEQLCSQLAELEVESLEQARDYHDRMLFLMDQISRLSSSSVVSSS	50aa
LSU2 in the lines:		
WT	MGKGGNYVTVAASEVDELRRKNGEMEKAVEEMKKEMLQLWRRTQVAEEAEERLCSQLAELEAESLDQARDYHSRIIFLMNELSRLSSDSASASP	94aa
lsu2-KO	MGKGGNYVTVAASEVDELRRNLSVELDRYLLLKKDRFFRLFDVSSYRLISMPICRVVFKTNMEVSIKKKNRCCYRLIFAINDE	83aa
lsu2lsu4-KO	MGKGGNYVTVAASEA	15aa
LSU3 in the lines:		
WT	MGKGGGYVTVAAEEVEELRRRNGELEREMEEMKKEMVQLWRRTVVAEEAEERLCSQLAELEVESLDQARDYHSRIVFLMDQISRLSSSSLEVVVTNS	97aa
lsu3-KO	MGKGGGYVTVAAEEVEELRRRNGELEREMEEMKKEMVQLWRRTGGARGRVFRSGA	55aa
lsu1lsu3lsu2lsu4-KO	MGKGGGYVTVAAEEVEELRRRNGELEREMEEMKKEMVQLWRRTGGARGRVFRSGA	55aa
LSU4 in the lines:		
WT	MGKGGNYVMVAASEVEELRQKNGEMEKAVEEMRKEMLQLWRRTQVAEEAEEHLCSQLAELEAESLDQARDYHTRIIFLTNQLSRFSSDSASP	92aa
lsu4-KO	MGKGGNYVMVAASEVEELRQKLCLSL	26aa
lsu2lsu4-KO	----------	-------

Fig. 2. Characteristic of the genomic changes in lsu-KO mutants. (A) The schematic location of the deletions in the single and multiple lsu-KO lines. The *LSU* genes are represented by arrows. The deletions are marked by blank spots on arrows, and the numbers below indicate the number of base pairs from ATG of the most upstream corresponding *LSU*, while the other *LSUs* are like in the WT. The lines above represent chromosomes III and V (not in scale); the blue rectangles on the lines stand for designed sgRNAs with their names. The corresponding sequences of sgRNA are shown in Suppl. Table 1. The approximate location of the primers used for verification of the presence of deletions in the genomic DNA of the mutants are indicated as white arrowheads and their sequence is indicated in Suppl. Table 3. The lsu1lsu3-KO line was obtained later than all other KO lines. (B) The open reading frames predicted from the sequences of the PCR products corresponding to the genomic fragments covering the regions of deletions in the indicated mutants.

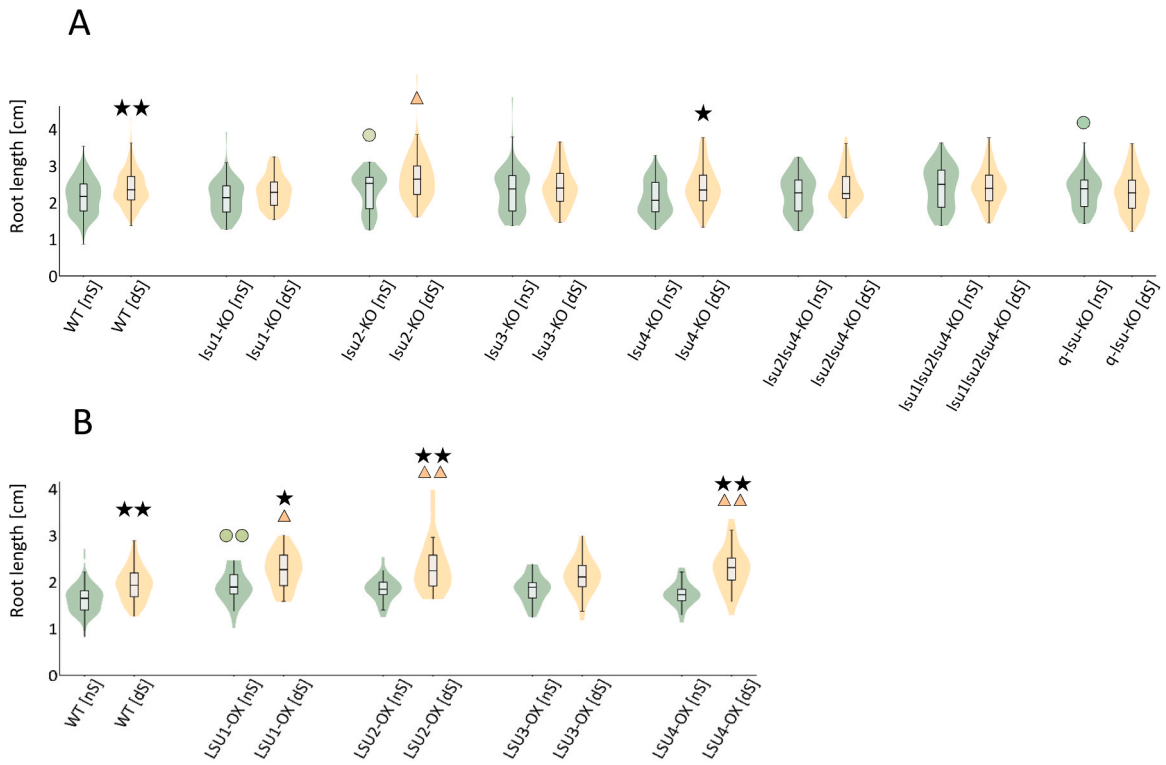


Fig. 3. Comparison of the root length of the lsu-KO (A) and LSU-OX (B) seedlings germinated and grown in [nS] and [dS] conditions for 12 days. The boxes extend from the first quartile to the third quartile with the line at the median. The lower and upper whiskers are at the lowest datum above Q1 – 1,5*(Q3 – Q1) and the highest datum below Q3 + 1,5*(Q3 – Q1), respectively. Statistically significant differences between [dS] and [nS] of the same genotype are shown by asterisks, while differences between genotypes in [nS] and [dS]are indicated by green circles and red triangles, respectively. Significance levels were denoted by either a double symbol ($p<0.001$) or a single symbol ($p<0.05$). The number of individual roots measured: in panel A, for WT n> 490, for lsu-KO n> 60; in panel B, for WT n>100, for LSU-TAP n> 20. The results were calculated from six or two biological repeats (independent germination of the seeds) for panel A or panel B, respectively. Two batches of seeds were used, each batch was collected from all plant lines grown at the same time in the same conditions.

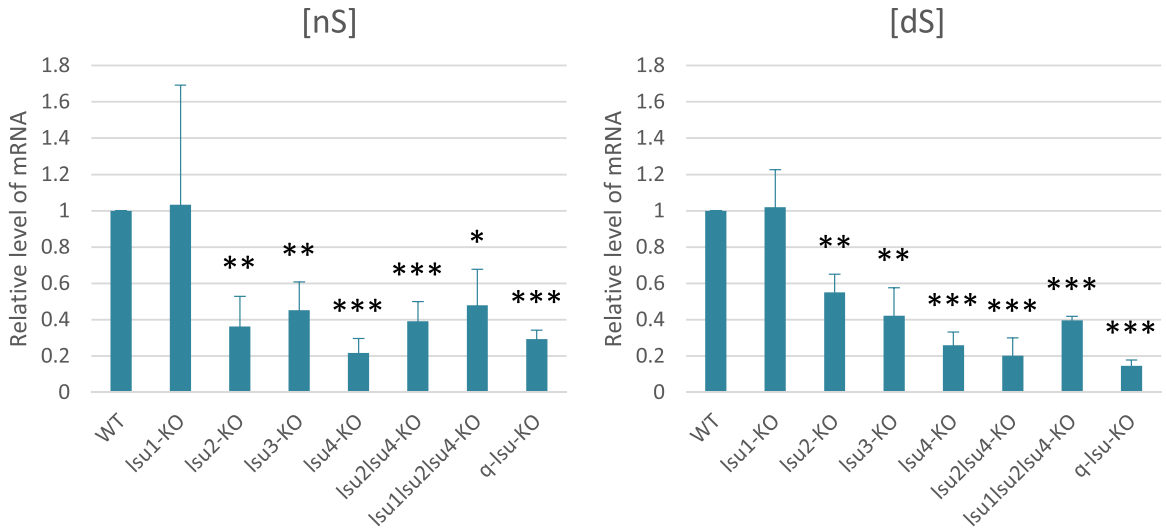


Fig. 4. The expression level of *BGLU28* in wild type (WT) and *lsu* knock-out lines (lsu-KO) grown in sulfur sufficient [nS] conditions for 10 days and transferred for two additional days to either [nS] or sulfur deficient [dS] conditions. The results of reverse transcription quantitative PCR (RT-qPCR) in the shoots were normalized to WT. The level of expression of *BGLU28* in the WT in [dS] increased 113-fold in comparison to the level of expression in [nS]. Error bars represent the standard deviation calculated from three independent experiments. The asterisks mark statistical differences between the given line and WT in the same conditions calculated with the Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

was reverse-transcribed using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the product manual. The obtained cDNA was diluted 100-fold. The RT-qPCR was performed in a Roche Lightcycler 480 (Roche, Basel, Switzerland) with Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The initial denaturation for 6 minutes at 95 °C was followed by 40 cycles composed of: denaturation for 5 s at 95 °C and annealing for 30 s at 60 °C. The content of individual cDNA was

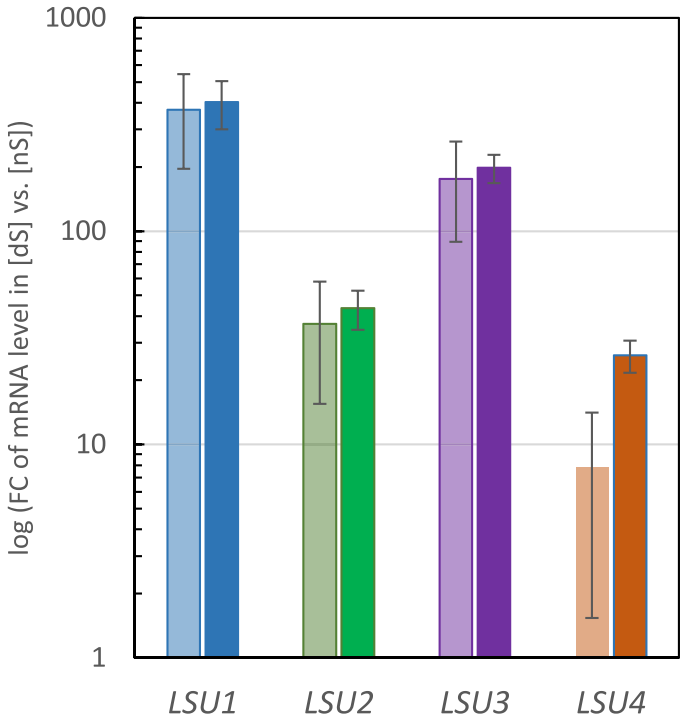


Fig. 5. The relative level of *LSU1-4* expression in WT seedlings grown in [dS]. The expression of each gene was monitored in two stages of seedling growth, 6-day-old (transparent bars) and 12-day-old (full-color bars). Results are shown in relation to the expression level of the same gene in seedlings grown in [nS] for the same time.

normalized relative to the tubulin *TUA3* gene expression level. In two initial experiments, *TIP4* was used as a second reference gene. The expression profiles of *TIP4* and *TUA3* were comparable which was also in agreement with our previous data (Wawrzynska et al., 2022). Primers used for qRT-PCR are listed in Suppl. Table 3.

2.6. ABA concentration measurement

The abscisic acid (ABA) levels in shoots were analyzed following a previously published procedure (Liu et al., 2014), employing the Phytodetek ABA Test Kit (Agdia-Biofords, Grigny, France). The assay consisted of three biological replicates, each conducted with two technical replicates.

2.7. Software used and statistical analysis

Relative gene expression levels were calculated using the delta-delta Ct method (Livak and Schmittgen, 2001) as the transcription level under [dS] treatment compared to the transcription level in control conditions [nS]. Statistical differences were analyzed using Student's t-test.

The root lengths, rosette diameters, stem lengths, seed sizes, and stomatal aperture were measured using ImageJ software (NIH, Madison, WI, USA; https://imagej.nih.gov/ij/). The Violin Plots were created using Matplot library in Python. For the statistical analysis of root length differences, R software was employed, utilizing either a two-way ANOVA or its non-parametric equivalent, ARTANOVA (Wobbrock et al., 2011), followed by a significantly modified procedure detailed at the following link: https://rcompanion.org/handbook/F_16.html. Additional information of the results of statistical analysis is provided in Suppl. Table 4.

Seed sizes, rosette diameters, and length of stem were analyzed by the post-hoc HSD test for unequal N included in Statistica (StatSoft, Cracow, Poland). Statistical distinctions in mass, shoot and root ratio, as well as root lengths between WT and q-lsu-KO grown on [LS], [nS], [LP], [LN] media, were assessed using Student's t-test. The differences in stomata aperture and ABA levels between WT and q-lsu-KO mutant were statistically examined using Student's t-test.

3. Results and discussion

3.1. Generation of the plant lines with a deletion within LSU genes and verification of the obtained deletions

The series of lsu-KO lines with single and multigene *lsu* deletions were generated in this work. Four single (lsu1-KO, lsu2-KO, lsu3-KO, and lsu4-KO), two double (lsu2lsu4-KO and lsu1lsu3-KO), one triple (lsu1lsu2lsu4-KO) and one quadruple (q-lsu-KO) mutant lines were obtained. All lines except lsu4-KO are marker-free. After confirmation of the presence of expected deletions in the genomic DNA of the mutants (Suppl. Fig. 1), the expression level of each *LSU* gene was monitored by RT-qPCR (Fig. 1). As expected, no transcripts corresponding to the genes covered by the deletions were detected. Interestingly, in many cases, the levels of expression of the other (non-targeted by deletion) *LSU* members were lower than in the wild type (WT). The only exception was the elevated expression of *LSU4* in the lsu2-KO mutant. Since the deletion in lsu2-KO does not cover the *LSU2* promoter region, the most plausible explanation of this phenomenon (higher level of *LSU4* in lsu2-KO) is placing the *LSU4* gene under the control of the *LSU2* promoter. However, more studies are required to understand the presumed reciprocal regulatory effects of LSU on each other expression and to identify the *cis*-acting sequences activating *LSU4* transcription in the lsu2-KO mutant.

Subsequently, the PCR fragments obtained with the primers flanking the expected deletions were sequenced and analyzed to reveal the precise location of the deletions (Fig. 2A). Genomic deletions in lsu2-KO and lsu3-KO lines generated the new open reading frame with the predicted sequence partially overlapping with the sequence of the corresponding LSU proteins in the WT (Fig. 2B). To exclude the possibility of maintaining the functionality of these predicted novel proteins (i.e. their interaction with other LSU proteins in the KO lines) the corresponding DNA fragments were cloned and used in yeast two-hybrid experiment to examine interactions (Suppl. Fig. 2). The results indicated that the chimeric proteins from the corresponding deletion regions of lsu2-KO and lsu3-KO mutants are not capable to interact with LSU1 in contrast to the LSU2 and LSU3 of the WT.

3.2. Growth characteristics of the lsu-KO lines and their response to S deficiency

Next, a series of experiments comparing the morphology and selected phenotypes of the generated lsu-KO lines with WT have been conducted. All obtained mutants were viable and were producing seeds. We particularly decided to concentrate our observations on the root morphology. S deficiency was observed to decrease auxin accumulation (Dan et al., 2007). Low S levels downregulate the genes involved in auxin biosynthesis and transport thus regulating root elongation pace (Zhao et al., 2014). Moreover, *LSU* genes were shown to be induced by SLIM1 transcription factor during S-deficiency (Wawrzynska et al., 2010). It was shown that the *slim1* mutant has a reduced ability to extend roots in [dS], though the molecular basis of this observation was never explained (Maruyama-Nakashita et al., 2006). Therefore, to verify the hypothesis that LSUs might function in that process we decided to observe the root growth of lsu-KO lines. The careful analysis of the seedlings grown in S-sufficient [nS] and S-deficient [dS] conditions indicated some differences in root lengths of 12-day-old seedlings. The statistical tests showed that only WT and lsu4-KO had significantly longer roots in [dS] than in [nS], while all other mutants do not have significantly different lengths in [dS] and [nS] at this stage of seedlings growth (Fig. 3A). The observed phenomenon is in agreement with the previously reported results for tobacco seedlings overexpressing *UP9*

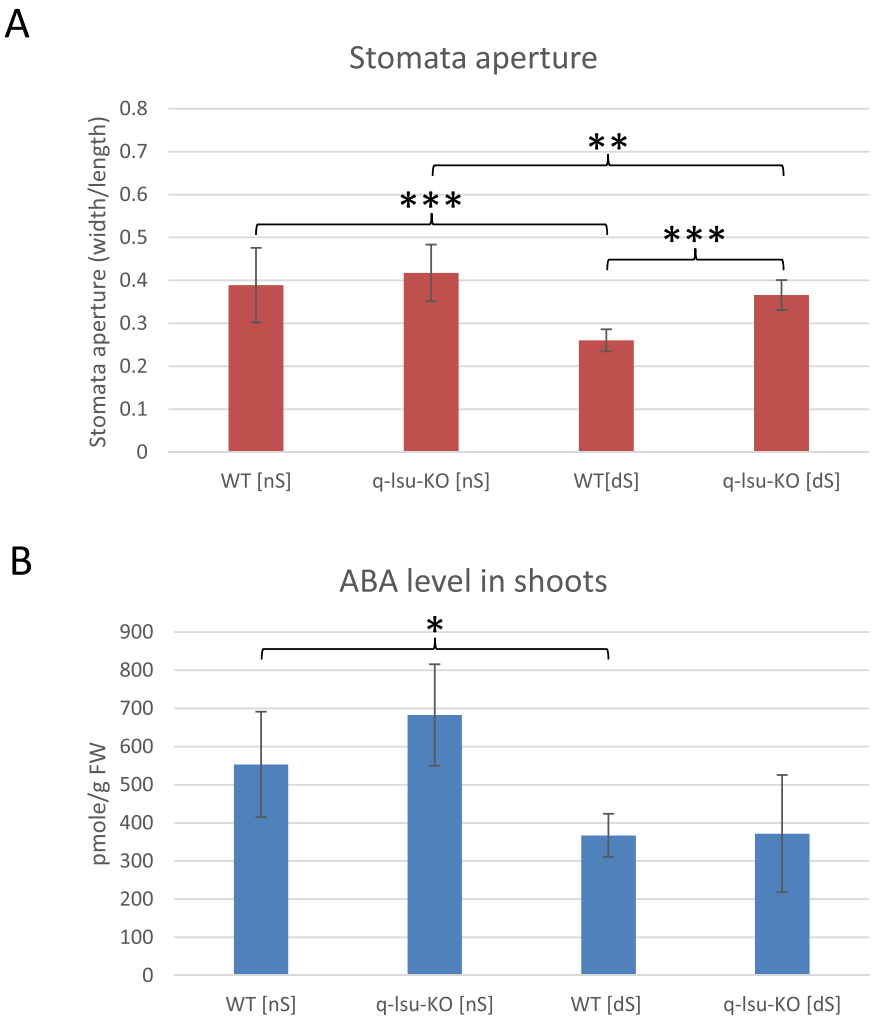


Fig. 6. Stomatal aperture (A) and ABA level (B) in twelve-day-old WT and q-lsu-KO seedlings grown in [nS] or [dS] conditions. Stomatal aperture was quantified as the ratio of ostiol width to length. Error bars correspond to the SD of 3 biologically independent measurements (n ≥35 stomata each), and significant differences were assessed by the Student's t-test. ABA level was assayed in shoots of three independent biological replicas and significant differences were assessed by the Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

(*LSU* homologue) in an antisense orientation (Lewandowska et al., 2010). The UP9-antisense seedlings had shorter roots in [dS] than in [nS], which was in apparent contrast to the control line having longer roots in [dS] than in [nS]. Then, downregulation or deletion of *LSU*-like genes has a similar influence on the ability of seedlings to respond to S-deficit in (at least) two plant species. Additionally, we observed that lsu2-KO mutant had statistically longer roots in both [nS] and [dS] and this was also visible for q-lsu-KO in [nS]. Statistical analyses failed to detect any differences in root length between the WT and other lines grown in the same conditions (either [nS] or [dS]).

We also tested if constitutive overexpression of the individual *LSU*s had any influence on the root length ratio in 12-day-old seedlings. The difference was statistically significant in WT and three overexpressors, LSU1 (LSU1-OX), LSU2 (LSU2-OX) and LSU4 (LSU4-OX). Additionally, these three lines had statistically longer roots in [dS] than the WT, while LSU1-OX had longer roots than WT in [nS] (Fig. 3B).

Interestingly, the differences among genotypes in root length ratio ([dS]/[nS]) were not observed in 6-day-old seedlings. At this earlier stage all lines had longer roots in [dS] than in [nS] (Suppl. Fig. 3). The plausible explanation is that the younger seedlings are relying on the material accumulated in the seeds for growth, while in the 12-day-old seedlings, it is critical to adjust metabolism (and root morphology) to the S-availability in the medium. It could be linked to the transition from heterotrophic to autotrophic growth of seedlings which takes place in about 4-day-old seedlings (Vidal et al., 2014). Additionally, we decided to challenge WT and q-lsu-KO plants with long-term S-deficiency (18 days). For that experiment, we needed to grow plants on a limiting S medium ([LS]; 1% of S present in [nS]) since the total S removal prevents plant growth over day 12. Interestingly, there were no statistical differences in roots length or shoot-to-root ratio, though we detected that the average mass of q-lsu-KO plants is a little bit smaller than the WT in [nS] conditions (Suppl. Fig. 4B). The differences in root length ratio in seedlings grown in S deficient and S sufficient conditions ([dS]/[nS]) were previously observed in Arabidopsis *ggct2* mutant defective in gamma-glutamylcyclotransferase, however, that mutant had longer roots in [dS] than the WT (Joshi et al., 2019). The *GGCT2* gene, similarly to *LSU*s, belongs to the OAS cluster (Hubberten et al., 2012). The authors explain the observed phenotype by the involvement of GGCT in the control of the glutathione level. Recently, it was demonstrated that overexpression of *LSU1* and *LSU2* from broccoli in Arabidopsis results in the degradation of glucosinolates thus providing more glutathione (Yang et al., 2023). Such plants showed improved tolerance to [dS] by growing longer roots than WT. Moreover, the authors also showed that under S-deficiency, BoLSU1 and BoLSU2 induce the expression of myrosinase genes *BGLU28* and *BGLU30* involved in glucosinolates catabolism. In the double bglu28/30 mutant the root growth retardation in [dS] was particularly noticeable (Zhang et al., 2020).

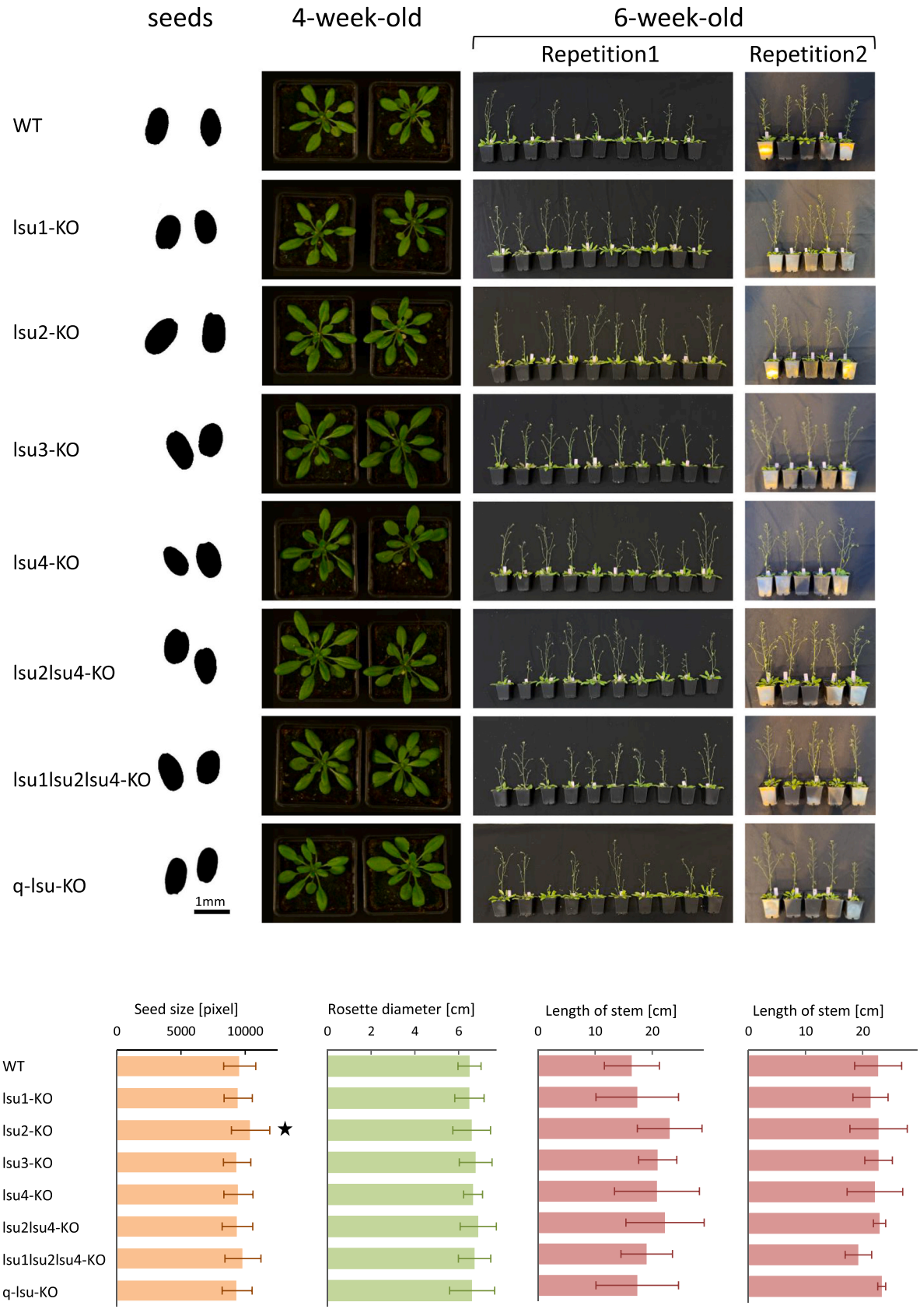


Fig. 7. Soil-grown lsu-KO lines and WT do not have any significant developmental or morphological differences. However, in some sets, the seeds collected from lsu2-KO were larger than the seeds from other lines, and (in one per two repetitions) the 6-week-old plants of lsu2-KO line had longer stems than the other lines.

To assess whether the lack of any of the LSUs might influence glucosinolates metabolism gene expression we assessed the level of *BGLU28* in all mutants grown either in [nS] or [dS] conditions (Fig. 4). We observed a significantly lowered level of the expression of this gene in all lsu-KO lines grown not only in [dS] but also in [nS]. This, together with the literature data, suggests that LSU proteins might influence the remobilization of S from secondary metabolites. Interestingly, the removal of a single *LSU* is sufficient to lower the expression of *BGLU28*. The mechanism of the transcriptional regulation of *BGLU28* by LSU proteins is yet unknown. It might be one of the factors responsible for the observed difference (shorter than WT roots in [dS]). It is worth emphasizing that the effect was strictly connected with the lack of S in the media as we did not observe any differences in root length between the lines when plants were challenged with limiting nitrogen [LN] or phosphorus [LP] (Suppl. Fig. 5).

It has been reported that the level of *LSU1* transcript increases gradually during S starvation (Wawrzynska and Sirko, 2016). To exclude the possibility that *LSU* genes are not yet fully induced in 6-day-old seedlings grown in [dS] we decided to monitor the level of different *LSU*s in WT seedlings grown in either [nS] or [dS] for 6 and 12 days. As expected, all *LSU*s were induced by S-deficit. In 6-day-old seedlings, the mRNA levels were about 380, 37, 175, and 8-fold higher for *LSU1*, *LSU2*, *LSU3,* and *LSU4*, respectively in [dS] than in [nS], and these levels of increase in [dS] *versus* [nS] was similar (except *LSU4* where it riched 26-fold elevation) in 12-day-old seedlings (Fig. 5). As expected and as previously was shown, *LSU1* and *LSU3* were most strongly responding to S-deficit (Bielecka et al., 2014; Uribe et al., 2022).

One of the reported phenotypes of the amiR-LSU mutants is the lack of response of stomata to the conditions of S deficiency (Garcia-Molina et al., 2017). While WT seedlings reacted to the stress by increasing stomata closure, the mutant with a lowered expression of all *LSUs* seems unresponsive. We decided to check whether we could observe similar phenotype in q-lsu-KO plants. The seedlings were grown for 12 days in either [nS] or [dS] and the stomatal aperture was measured. Similarly to the previous report, we observed that q-lsu-KO plants do not respond to [dS] stress with stomatal closure to the extent observed in WT (Fig. 6A). Additionally, we measured the level of abscisic acid (ABA) in the shoots. Both WT and q-lsu-KO mutant reacted to [dS] conditions by lowering the content of ABA (Fig. 6B). However; no significant differences between genotypes were observed in either [nS] or [dS] conditions. It is known that sulfate availability affects the ABA content in plant tissues, as the ABA level is reduced in sulfate-deprived plants (Cao et al., 2014). Though the involvement of ABA in the stomata movements is well described (Bharath et al., 2021), the observed differences in stomata aperture in [dS] between WT and q-lsu-KO are ABA-independent. It is in agreement with the observation that stomata of amiR-LSU lines react normally to the exogenous ABA application. The plausible explanation is that LSU proteins regulate stomata movement by modulating ROS production through interaction with FSD2 in chloroplasts of the guard cells (Garcia-Molina et al., 2017).

Notably, we failed to detect statistically significant developmental or morphological (such as seed size, rosette size, stem length, bolting, and flowering time) differences between the WT and lsu-KO lines (Fig. 7 and Suppl. Fig. 6). Some differences, like seed size and stem length, could be noticed for lsu2-KO which might reflect from the higher *LSU4* expression, resulting probably from placing this gene under additional regulatory elements of *LSU2* promoter, caused during mutant construction. Moreover, all lsu-KO lines had similar to the WT seed germination rate and germination efficiency regardless of the status (fresh or stratified) of the seeds (results not shown).

4. Conclusions

Several marker-free single and multiple lsu-KO lines, including the quadruple mutant, were generated and analyzed. All mutants are morphologically similar to the parental lines in optimal conditions, and differences, such as delays in flowering, are not consistent. Therefore, the most significant finding of this study is that the function of LSUs is not essential for plants and those proteins rather act as modulators of the S-deficiency response. However, in contrast to WT seedlings which have (at the age of 12 days) longer roots in S-deficient than in S-sufficient conditions, the seedlings of most obtained lsu-KO lines have a strong tendency for maintaining similar lengths of roots in both conditions. A similar phenomenon was previously observed in tobacco *LSU*-like (UP9) antisense mutant and might suggest difficulties in appropriate adaptation to S deficient conditions in the absence of LSU. All up-to-date data suggest that LSUs might be involved in adjusting S-flow from glucosinolates during S-deficiency (Li et al., 2023; Yang et al., 2023). The generated single and multigene knockout lines provide a useful tool for future studies on the precise function of LSU proteins in plants.

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CRediT authorship contribution statement

Anna Wawrzyńska: Writing – review & editing, Writing – original draft, Supervision, Methodology. **Agnieszka Sirko:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Marcin Olszak:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Hubert Salamaga:** Software, Methodology. **Anna Niemiro:** Writing – original draft, Methodology, Investigation. **Marzena Sieńko:** Visualization, Methodology, Investigation. **Justyna Piotrowska:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2024.112063.

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