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Genome-wide analysis of response to low sulfur (LSU) genes in grass species and expression profiling of model grass species *Brachypodium distachyon* under S deficiency

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Abstract: Sulfur (S) affects the plant life cycle and crop yield and has nutritional importance for human and animal diet. Its deficiency is one of the major problems in agriculture. However, the plant-specific *LSU* (response to Low SUlfur) gene family has not been extensively analyzed in major plant species such as grasses. In this study, we have performed in silico genome-wide analysis of *LSU* genes in 6 grass species, including *Brachypodium distachyon*, *Sorghum bicolor*, *Oryza sativa*, *Zea mays*, *Triticum aestivum*, and *Panicum virgatum*. All identified *LSU* genes contained one exon encoding proteins of acidic character with cytoplasmic localization. In silico analysis of *cis*-elements revealed that sulfur-responsive elements (SURE boxes, SUlfur Response Element, GAGAC motif) were present in all *LSU* genes. In phylogenetic analysis, dicot and monocot *LSU* genes were separated. Expression profiles of *B. distachyon BdLSU1* and *BdLSU2* genes were analyzed by qRT-PCR method. Two *Brachypodium LSU* genes demonstrated different expression patterns when subjected to 48 h of S-depletion treatment. In roots, the *BdLSU2* gene was upregulated, while *BdLSU1* was downregulated. In leaves, expression levels were decreased for both genes. Analysis of the *BdLSU* expression under drought, cold, salt, and heat stresses was carried out based on the *Brachypodium* stress atlas. Results showed that *BdLSU* genes are not specific to S limitation; indeed, they may be involved in different stress conditions by cooperating with their interacting partner proteins. The results of this study could significantly contribute to the understanding of *LSU* genes in plants, particularly in grass species. These results may also support plant molecular studies by aiding the understanding of the sulfur assimilation pathway.

Key words: Sulfur deficiency, *LSU*, gene expression, genome-wide analysis, in silico

1. Introduction

Sulfur is an essential macronutrient for plant growth and development. It is found in amino acids such as cysteine and methionine, thiols of proteins, glutathione, secondary products, peptides, cofactors, membrane sulfolipids, cell walls, and hormones. ATP sulfurylase, adenosine 5'-phosphosulfate, phosphorylation to 3'-phosphoadenosine 5'-phosphosulfate, and sulfite reductase are major enzymes in the sulfate assimilation pathway (Saito, 2004; Takahashi et al., 2011). Sulfur compounds play roles in responses to abiotic and biotic stress, including drought and salinity stresses, detoxification of reactive oxygen species, and production of glucosinolates in defense against herbivores and other pathogens (Davidian and Kopriva, 2010; Gallardo et al., 2014). Sulfur is mainly taken up by plants as inorganic sulfate (SO_4^{2-}) and then converted to sulfite (SO_3^{2-}) and sulfide (S^{2-}). Later, assimilation into cysteine is accepted to be the major point of the natural sulfur cycle (Buchner et al., 2004). Gene expression data of S nutrition are available for *Arabidopsis thaliana* (Hirai et al., 2003), poplar (Honsel et al., 2012), and tobacco (Lewandowska et al., 2005; Wawrzynska et al., 2005). In *A. thaliana*, 4 members of the plant-specific *LSU* (response to Low SUlfur) gene family (*LSU1–4*) have been found. *LSU1* is considered a significant connector in the gene–metabolite hormone-related network for S response, but there is no experimental evidence related to this gene (Nikiforova et al., 2005). The *LSU1* (At3g49580) and *LSU3* (At3g49570) genes are localized on chromosome 3, separated by a distance of about 2250 bp, while *LSU2* (At3g49580) and *LSU4* (At5g24660) are on chromosome 5, separated by about 2060 bp (Sirko et al., 2015). S deficiency and some environmental conditions such as salt stress and $AgNO_3$ treatment have been reported to induce the *LSU1* and *LSU2* genes (Zimmermann et al., 2004, 2008). *LSU2* was also induced by oxidative stress (Davletova et al., 2005), by carbon starvation, and in response to sugar (Usadel et al., 2008). The *Arabidopsis LSU2* gene has been temporarily determined as one of the genes cross-talking between

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several signaling pathways such as iron, sulfur, nitrate, and hormones under various stress conditions including sulfur and iron restriction and nitrate and stress hormone treatments (Omranian et al., 2012). The *LSU1* gene was reported to be constitutively expressed during pollen germination and tube growth (Wang et al., 2008). The level of *LSU1* transcript (but not other LSUs) is connected with levels of O-acetylserine, which plays roles in the regulation of sulfate uptake and assimilation (Hubberten et al., 2012). In silico analysis of *LSU* promoter regions revealed that UPE-box specific elements were detected for induction of these genes under S deficiency, with the exception of the *LSU4* promoter (Wawrzynska et al., 2010). Sulfur-responsive elements (SURE boxes) have been identified in the promoter regions of *LSU1* and *LSU2* (Maruyama-Nakashita et al., 2005). In tobacco, LSU-like proteins could be grouped into 6 clusters (UP9A–F), but the exact number of these genes is not known (Lewandowska et al., 2010). Among these genes, only *UP9C* was investigated. The expression level of *UP9C* was increased in all parts of the plant including stems, young leaves, roots, and mature leaves in S-deficient medium. Additionally, in silico analysis of the *UP9C* promoter region showed that it contains light, salt stress, and phytohormone elements. SURE boxes were detected at 350 bp upstream of the start codon (Wawrzynska et al., 2010). LSU-like proteins play important roles in the ethylene signaling pathway, and therefore ethylene perception contributes to response to S deficiency in tobacco (Moniuszko et al., 2013). In this study, we have performed in silico genome-wide analysis of *LSU* genes in 6 select species of grasses. For this purpose, physicochemical properties, conserved motif sequences, and predicted subcellular localization of proteins were determined; exon/intron organization of genes was analyzed; and a phylogenetic tree using protein sequences was constructed. We have analyzed the expression profile of *Brachypodium distachyon LSU* genes using qRT-PCR. Their digital expressions were calculated under drought, salt, cold, and heat stresses. Results of this study could contribute towards the understanding of *LSU* gene functions in grass species and provide a scientific basis for further studies related to *LSU* genes in various other plant species.

2. Materials and methods

2.1. Retrieval of *LSU* gene sequences

Six grass species, *B. distachyon*, *Sorghum bicolor*, *Oryza sativa*, *Zea mays*, *Triticum aestivum*, and *Panicum virgatum*, were selected for genome-wide analysis. Four *Arabidopsis* LSU protein sequences (AtLSU1: AEE78560, AtLSU2: AED93346, AtLSU3: NP_190526, and AtLSU4: AED93345) were used as queries against proteome datasets of 6 grass species in the Phytozome database, v10.3 (http://phytozome.jgi.doe.gov/pz/portal.html) (Goodstein et al., 2012). Later, redundant sequences were removed from the retrieved genes/proteins manually.

2.2. Sequence analysis of *LSU* genes and proteins

Physicochemical properties of LSU proteins, including sequence length, molecular weight, and isoelectric point (*pI*), were analyzed using the ProtParam tool (http://web.expasy.org/protparam/) (Gasteiger et al., 2005). Subcellular localizations were predicted by the CELLO server (http://cello.life.nctu.edu.tw/) (Yu et al., 2006). Exon numbers of *LSU* genes were analyzed using the Gene Structure Display Server (GSDS) server (http://gsds.cbi.pku.edu.cn/) (Guo et al., 2007). The lengths of open reading frames (ORFs) were analyzed using BioEdit version 7.2.5 (Hall, 1999). Predicted interacting partners of rice LSU protein were identified using the STRING 9.1 server (http://string91.embl.de/) (Franceschini et al., 2013) and an interactome network was generated by Cytoscape (Smoot et al., 2011).

2.3. Conserved motif and phylogenetic analysis of LSU proteins

The conserved motif analysis was performed using the MEME tool (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) (Timothy et al., 2009) with the following parameters: maximum number of motifs to find, 5; minimum width of motif, 6; and maximum width of motif, 50. LSU protein sequences were aligned with ClustalW (Thompson et al., 1994) and edited with the BioEdit sequence alignment editor (Hall, 1999). The phylogenetic tree was constructed with MEGA 6.06 (Tamura et al., 2013), following the maximum-likelihood (ML) method, for 1000 bootstrap values. The phylogenetic tree was constructed with 20 LSU protein sequences from 7 higher plant species, including *A. thaliana* (4), *B. distachyon* (2), *S. bicolor* (3), *Z. mays* (2), *T. aestivum* (3), *O. sativa* (2), and *P. virgatum* (4). For the phylogenetic tree, the substitution model and data treatment were selected as the Jones–Taylor–Thornton (JTT) model and complete deletion, respectively. Initial trees for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model. All positions containing gaps and missing data were eliminated. There were a total of 42 positions in the final dataset.

2.4. Promoters (cis-regulatory elements) of *LSU* genes

The 5' UTR regions (–500 to +0) of 16 *LSU* genes were retrieved from the Phytozome database and a manual search was conducted to identify the binding sites of transcription factors (TFs). The *cis*-regulatory elements (CREs) were analyzed using the plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al., 1999).

2.5. Plant growth and S-depletion treatment

B. distachyon Bd21 seeds were purchased from the RIKEN Institute (Japan). The seeds were surface-sterilized with a 10% bleach solution containing 0.1% Triton X-100 and washed 4 times with distilled water. Sterilized seeds were incubated for 3 days in sterile water at 4 °C under dark conditions and germinated between sterile filter papers at room temperature. Five-day-old seedlings were transferred to a hydroponics medium containing macronutrient solution in mM: 6 KNO_3, 1 $NH_4H_2PO_4$, 2 $MgSO_4$, 4 $Ca(NO_3)_2$; and micronutrient solution in µM: 50 H_3BO_3, 9 $MnCl_2$, 0.3 $CuSO_4$, 25 Fe-EDTA, 0.8 $ZnSO_4$, 0.02 g MoO_3 (85%) (pH 5.5) (Hoagland and Arnon, 1950). The control and treatment plants were grown for 4 weeks in the growth chamber (WiseCube, Lab Constant Temperature & Humidity Chamber, Korea) under the following conditions: 23 °C, 16:8 (light:dark) photoperiod, 60% humidity, and 10,000 lx light. The nutrient solution was renewed every 2 days. Sulfur deficiency was applied as described by Honsel et al. (2012). Accordingly, *B. distachyon* Bd21 seedlings were transferred to the S-free medium: $MgSO_4$ in the Hoagland solution was replaced with an appropriate concentration of $MgCl_2$. Final pH was adjusted to 5.5. The leaves and roots were harvested after 48 h of sulfur starvation. The samples were kept in liquid nitrogen and stored at –80 °C until RNA isolation.

2.6. RNA isolation, primer design, and real-time quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA was extracted from control and 48-h S-starved *B. distachyon* seedlings using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and the purity of RNA were determined by measuring the OD_{260} and OD_{280} (NanoDrop, Thermo Scientific, USA). Total RNA was reacted with RNase-free recombinant DNase I (Roche, Basel, Switzerland) in order to get rid of DNA contamination. The reaction tubes were incubated at 37 °C for 15 min. The reaction was stopped by heating to 75 °C for 10 min. The first strand of cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo, Waltham, MA, USA). Accordingly, 0.2 µg of DNase I-treated RNA, 100 µM oligo$(dT)_{18}$, and nuclease-free water were mixed in a 12-µL reaction tube and incubated at 65 °C for 5 min. The following reagents were then added into the reaction mixture, respectively: 4 µL of 5X reaction buffer, 20 units of RiboLock RNase inhibitor, 10 mM dNTP mix, and 200 units of RevertAid RT enzyme. Reverse transcription reaction was performed at 42 °C for 60 min. The reaction was terminated by heating at 70 °C for 5 min. The final product was kept at –20 °C.

Forward and reverse primers for *BdLSU* genes were designed with Primer3web, version 3.0.0 (http://primer3plus.com/web_3.0.0/primer3web_input.htm). The primer sequences were 5'-CACGGAGAGCAAGATGAGC-3' (forward) and 5'-CTGACGTGCTGCTGGTACT-3' (reverse) for *BdLSU1* (Bradi3g30730.2) and 5'-TCTTGGGTGGAAGAAGCAGA-3' (forward) and 5'-CACCTCCTTCTCCAGCTCC-3' (reverse) for *BdLSU2* (Bradi3g02810.1).

For quantification analysis, the targeted regions of genes were amplified using the SYBR Premix Ex Taq kit (Takara Bio Inc., Japan). Real-time PCR was performed in a Rotor-Gene PCR machine (Corbett Research-QIAGEN). Accordingly, 10 µL of SYBR Premix Ex Taq (2X) (Tli RNaseH Plus) was mixed with 1 µL of gene-specific forward and reverse primers (10 µM each) and 2 µL of cDNA template. Sterile distilled water was added to the mixture up to 20 µL. qPCR conditions consisted of 95 °C for 2 min; 45 cycles of 95 °C for 5 s, 60–62 °C for 15 s, and 72 °C for 10 s; and a melting analysis of 52 to 95 °C with an increasing temperature of 0.5 °C min^{-1}. The relative quantity of the *BdLSU* transcripts was calculated using the efficiency correction model and included the calculation of crossing point (Cp) deviation of a subject versus a control (Pfaffl, 2001). The method was applied with minor changes based on a previous article (Tombuloglu et al., 2012). Three biological and 2 technical replicates were used for each treatment. The data were normalized with *18S* rRNA gene specific primers: 5'-GGCTACCACATCCAAGGAA-3' forward and 5'-CTATTGGAGCTGGAATTACCG-3' reverse.

2.7. Digital expression analysis of *BdLSU* genes

In order to understand the response of *BdLSU* genes against various types of stress conditions, we used an online database that shows gene expression patterns upon drought (desiccation), cold (4 °C), salt (500 mM), and heat (42 °C) stress conditions (http://stress.mocklerlab.org/NetPortal/expression_query.php) (Priest et al., 2014). The database enables digital expression values based on the Affymetrix Brachypodium Genome Array (BradiAR1b520742). Digital expression value (RMA: robust multiarray average) was normalized by the log2 function of the module.

3. Results and discussion

3.1. Identification and characterization of *LSU* genes

In this study, we have identified 16 *LSU* genes in 6 different grass species, including 2 genes from *Brachypodium*, *Oryza*, and *Zea*; 3 genes from *S. bicolor* and *T. aestivum*; and 4 genes from *P. virgatum* (Table). In tobacco, no specific motifs or domains in LSU/UP9 proteins were detected (Moniuszko et al., 2013). Short and strongly evolutionarily conserved regions were found in various plant LSU proteins, but their functions are unknown (Sirko et al., 2015). Based on the LSU protein alignment, some identical and similar regions

Table. Homologs of *LSU* genes in 6 grass species and their gene/protein features.

Gene name	Phytozome sequence ID	Chr	Exon number	ORF length (bp)	Length (aa)	MW (kDa)	*pI*	Predicted subcellular location
BdLSU1	Bradi3g30730	3	1	303	100	10.9	4.79	Cytoplasmic
BdLSU2	Bradi3g02810	3	1	360	119	12.4	5.49	Cytoplasmic
SbLSU1	Sobic.001G198000	1	1	336	111	12.2	4.98	Cytoplasmic
SbLSU2	Sobic.004G028200	4	1	330	109	11.5	4.98	Cytoplasmic
SbLSU3	Sobic.003G237500	3	1	264	87	9.4	4.80	Cytoplasmic
OsLSU1	LOC_Os10g36610	10	1	270	89	9.9	4.77	Cytoplasmic
OsLSU2	LOC_Os02g03710	2	1	309	102	11.0	5.15	Cytoplasmic
ZmLSU1	GRMZM2G339562_T01	2	1	357	118	12.7	4.85	Cytoplasmic
ZmLSU2	GRMZM2G371167_T01	2	1	321	106	11.3	6.16	Cytoplasmic
TaLSU1	Traes_1AL_04C9CFE29	1A	1	312	103	11.1	5.06	Cytoplasmic
TaLSU2	Traes_1BL_A3BAAF32	1B	1	309	102	11.1	5.22	Cytoplasmic
TaLSU3	Traes_5BL_5082542FB	5B	1	213	71	7.8	4.66	Cytoplasmic
PvLSU1	Pavir.Ab00142	-	1	306	101	10.8	5.19	Cytoplasmic
PvLSU2	Pavir.J13198	-	1	351	116	12.6	5.06	Cytoplasmic
PvLSU3	Pavir.J19029	-	1	351	116	12.6	5.06	Cytoplasmic
PvLSU4	Pavir.J11280	-	1	279	93	10.5	4.84	Cytoplasmic

Chr: Chromosome.

were identified (Figure 1). These regions may be related to functions of LSU proteins, but wet-lab studies are required for confirmation.

The lengths of ORFs ranged from 213 bp (*TaLSU3*) to 360 bp (*BdLSU2*). Notably, all *LSU* genes contained one exon. It has been reported that most *LSU* genes have no intron, and the ORFs are relatively small and consist of about 280 bp (Sirko et al., 2015). This was in agreement with our findings. Analysis of LSU protein sequences showed that *LSU* genes encode a polypeptide of 71–119 amino acids with 7.8–12.4 kDa molecular weight. The LSU proteins demonstrated an acidic character with *pI* values of 4.66–6.16. The analysis of predicted subcellular localizations revealed that all LSUs were predicted to be localized in the cytoplasm. In tobacco, the UP9C protein has been reported to show cytosol-nuclear localization (Lewandowska et al., 2010), but *Arabidopsis* LSU proteins did not contain any localization motifs (Sirko et al., 2015). Our data are similar to *Arabidopsis* LSU proteins and there were no other nuclear localization motifs detected in grass LSUs.

3.2. Conserved motif analysis

Five common conserved motifs were detected in grass LSU proteins, including NAELECRVERLRRELGAALRRVAVAEEAEERLCV QLGDLEVEAMTQAIEY (motif 1), QQHVHDLSDRLAFMD (motif 2), MSPPISIGIGIPSWHKGWNKK (motif 3), MMTRTKVTVDAGREAEEMRRR (motif 4), and GVLRSSGIHGF (motif 5) (Figure 2). Only motif 1 was detected in all LSUs, while the other motifs indicated various distribution patterns. Motifs 2, 3, 4, and 5 were detected in 14, 8, 4, and 5 LSU proteins, respectively. Hence, it was suggested that various motif patterns could prove the diverse functions of LSU proteins in plants.

3.3. Predicted cis-regulatory elements of *LSU* genes

To understand *LSU* gene regulation, we performed an in silico investigation of *cis*-elements in their putative promoter regions (upstream region of 500 bp) using the PLACE database. Particularly, sulfur-responsive elements (SURE boxes, SUlfur Response Element, GAGAC motif) were identified in all grass species, but not in all *LSU* genes (11 of 16 *LSU* genes, with 22 sites). The SURECOREATSULTR11 element was identified in *BdLSU1* (2 sites), *BdLSU2* (4 sites), *SbLSU1* (2 sites), *OsLSU1* (one site), *ZmLSUS1* (2 sites), *TaLSU1* (2 sites), *TaLSU2* (2 sites), *TaLSU3* (2 sites), *PvLSU1* (one site), *PvLSU2* (2 sites), and *PvLSU4* (2 sites). Transcription factors and *cis*-acting regulatory sequences play essential roles in transcriptional control of genes (Mignone et al., 2002). The first *cis*-element SURE (sulfur response element) was detected in the promoter region of the *Arabidopsis* sulfur transporter gene (*SULTR1;1*). A 5-nt core of SURE (GAGAC or GTCTC) was able to induce a sulfur deprivation-dependent transcriptional response (Maruyama-Nakashita et al., 2005). Micro- and macroarray experiments revealed that many up- and downregulated genes were identified under sulfur-deficient conditions

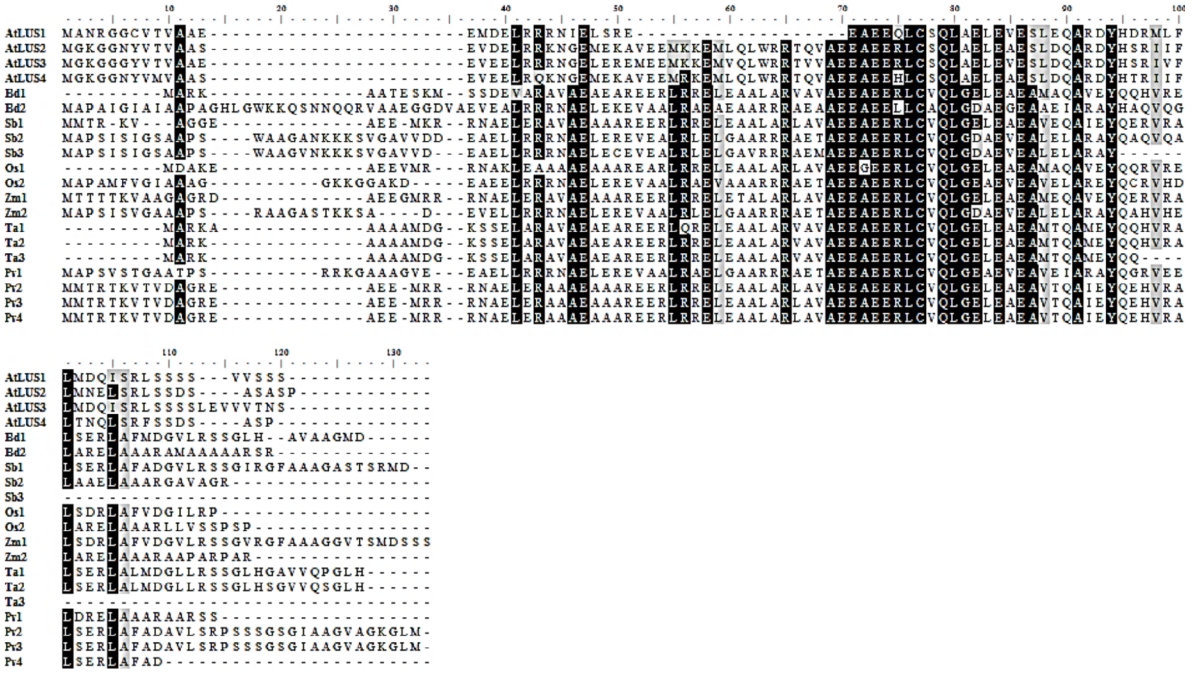

Figure 1. Alignment of grass LSU proteins using BioEdit program. Sequences were aligned by ClustalW. Identical and similar residues were shaded as black and gray, respectively. At: *A. thaliana*; Bd: *B. distachyon*; Sb: *S. bicolor*; Os: *O. sativa*; Ta: *T. aestivum*; Pv: *P. virgatum*.

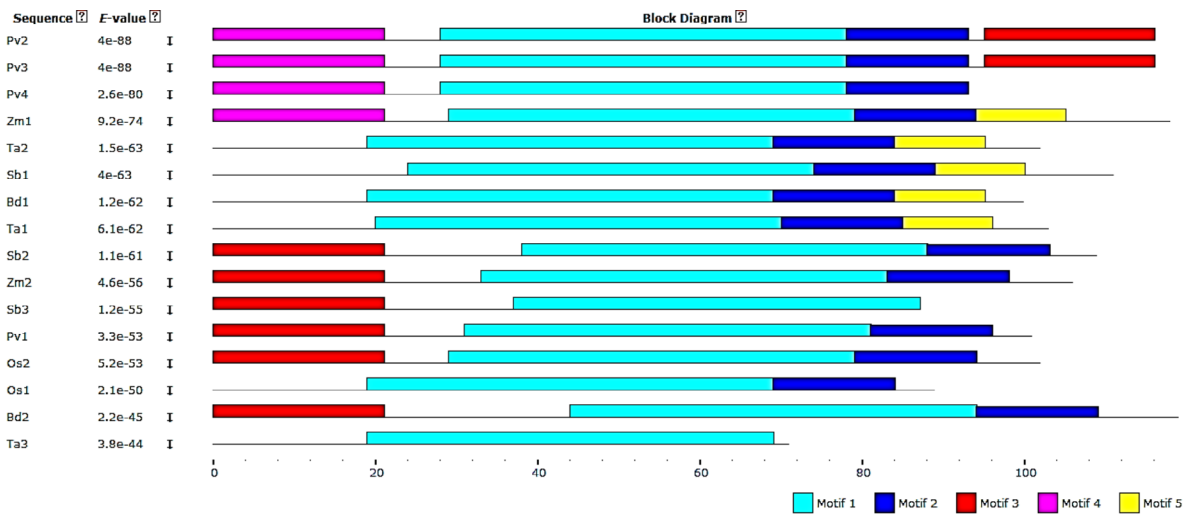

Figure 2. The most conserved protein motifs in LSUs (motif 1, motif 2, motif 3, motif 4, and motif 5, respectively). Each motif is represented in boxes with different colors: motif 1, cyan; motif 2, blue; motif 3, red; motif 4, purple; and motif 5, yellow. At: *A. thaliana*; Bd: *B. distachyon*; Sb: *S. bicolor*; Os: *O. sativa*; Ta: *T. aestivum*; Pv: *P. virgatum*.

in *Arabidopsis* (Hirai et al., 2003; Nikiforova et al., 2003). In this study, various *cis*-elements may indicate that *LSU* genes play roles in different metabolic pathways and/or conditions in grasses.

3.4. Predicted interacting partners of LSU proteins

To understand LSU protein networks, putative interaction partner analysis was performed using an identified sequence of the *OsLSU1* gene (LOC_Os10g36610) (Figure 3). Ten potential interaction partners of the OsLSU1 protein with a high confidence score (≥0.671) were predicted by the STRING 10 server. AP2 domain-containing protein, U3 small nucleolar RNA-associated protein 11, zinc finger, Joka2, C2H2 zinc-finger protein, ethylene-responsive protein, and basic helix-loop-helix

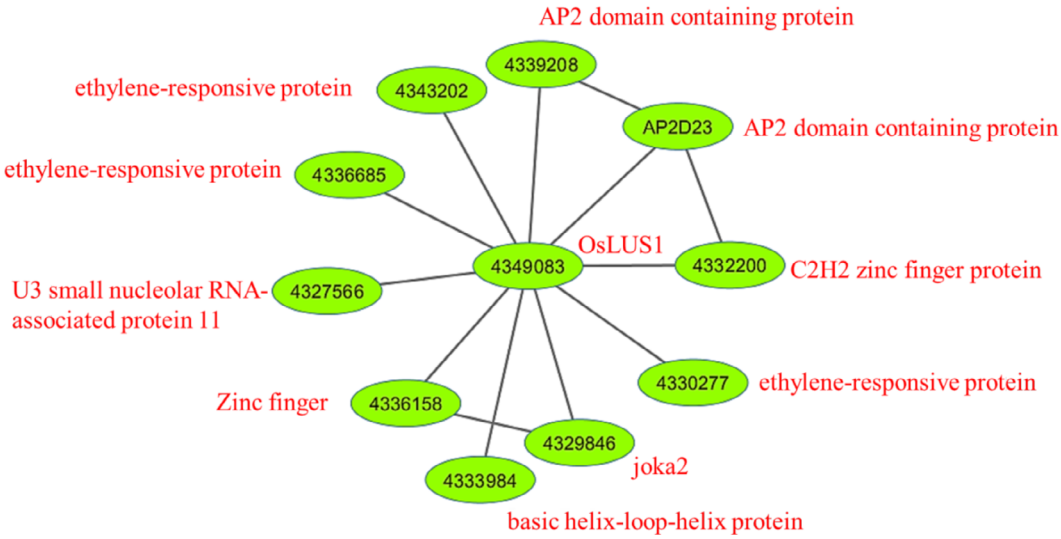

Figure 3. Ten predicted interaction partners of rice OsLSU1 protein. Interactome was generated using Cytoscape with STRING 10 server data.

protein have been predicted among the interaction partners of OsLSU1 protein. AP2/ERF (APETALA2/ethylene-responsive factor) proteins that include at least one DNA-binding domain (named as AP2) have been separated into 4 different families, namely DREB, ERF, AP2, and RAV (Mizoi et al., 2012). These regulatory proteins affect the various metabolic pathways, including primary and secondary metabolism, growth and developmental programs, and responses to environmental stimuli (Licausi et al., 2013). Zinc fingers are small domains that play important structural roles in the stability of the protein and show structural diversity (Krishna et al., 2003). Several types of zinc finger proteins were observed based on the number and order of Cys and His residues that bind to the zinc ion. The C2H2-type zinc finger proteins are one of the largest families of transcriptional regulators in plants with a conserved QALGGH sequence within their zinc finger domain. They play important roles in defense and response to different environmental stress conditions in plants (Ciftci-Yilmaz and Mittler, 2008). The ethylene-responsive element binding protein (EREBP) belongs to a homeobox gene in *Arabidopsis* that encodes a transcription factor (Riechmann and Meyerowitz, 1998). EREBP proteins are involved in the response to ethylene in plants (Ohme-Takagi and Shinshi, 1995). Basic helix-loop-helix (*bHLH*) transcription factors are present in plants, animals, and fungi (Pires and Dolan, 2010). The *bHLH* family of transcription factors is known to be involved in photosynthesis, light signaling, pigment biosynthesis, and seed pod development (Hudson and Hudson, 2015). According to interaction partner analysis, LSU proteins are related to various metabolic pathways, suggesting that they could play important roles in different metabolic pathways in plants. In *Arabidopsis*, the LSU1 partners were found as *ERF/AP2*, *bHLH*, and *myb*-like HTH TF family members (Sirko et al., 2015), and these results were similar to rice LSU1 interacting partners.

3.5. Phylogenetic analysis of LSU proteins

The phylogenetic tree was divided into 2 main groups, namely A and B, according to the clustering of sequences (Figure 4). Group A consisted of 2 subgroups, namely A1 and A2, while group B had 2 subgroups, namely B1 and B2. Dicot LSU proteins from *Arabidopsis* were clustered together in subgroup A2 with 87% bootstrap value. In contrast, monocot LSUs were separated from each other and distributed in groups A and B. However, only wheat LSU proteins were grouped together in subgroup B1 in monocots; the other monocots were separated from each other. This separation may indicate gene variations during *LSU* gene evolution in plant lineages. Notably, dicot LSUs (subgroup A2) were separated from monocot LSUs (subgroup A1) with the highest bootstrap value (100%) in group A. These findings prove the *LSU* gene divergences after the monocot-dicot split in plant lineages.

3.6. Expression profile of *B. distachyon LSU* genes under sulfur deficiency

In order to understand the possible roles of identified *LSU* genes under S-deficient conditions, expression levels of *BdLSU* genes were detected using the qRT-PCR technique (Figure 5). Root and leaf samples treated with S depletion for 48 h were compared with control tissues. The expression levels were normalized with *18S* transcripts. The results showed that in roots, S depletion decreased *LSU1* transcript levels. Similarly, dramatic reduction (approximately 5 times) was observed in leaf samples.

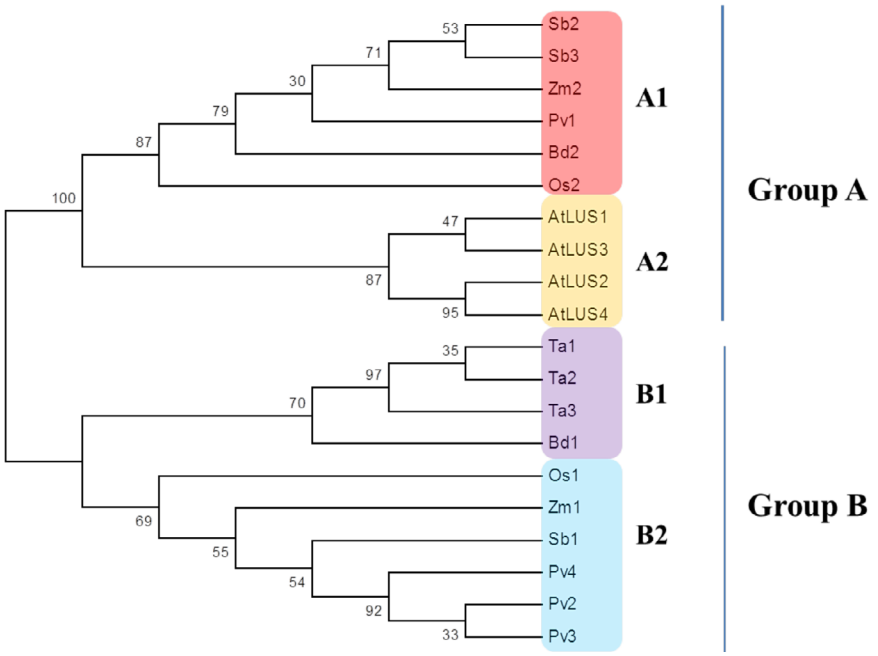

Figure 4. Phylogenetic tree of LSU proteins from 6 grass species. Phylogenetic tree was constructed by MEGA 6.06 software with maximum-likelihood (ML) method for 1000 bootstrap replicates. At: *A. thaliana*; Bd: *B. distachyon*; Os: *O. sativa*; Pv: *P. vurgatum*; Sb: *S. bicolor*; Ta: *T. aestivum*; Zm: *Z. mays*.

For the *BdLSU2* gene, the abundance of transcript was found to be increased approximately 5 times more than that of control roots. However, transcript accumulation was decreased in the leaf tissue (approximately 5 times). The expression profiles of *BdLSU* genes revealed that *BdLSU2* seemed to be upregulated, and the *BdLSU1* gene was negatively regulated by 48 h of S-depletion treatment in root. In leaves, expressions were decreased for both genes, showing tissue-specific reduction of *LSU* genes upon S deficiency. A limited number of studies have shown that only some of the *LSU* genes are induced by S limitation. Our results are in agreement with those studies. For instance, there are 4 *LSU* genes and 3 of them were induced by S deficiency in *Arabidopsis* (Sirko et al., 2015). In tobacco, the *Arabidopsis LSU* homolog *UP9C*-like gene was also strongly upregulated by S deficiency (Maruyama-Nakashita et al., 2005; Nikiforova et al., 2005). However, some S deficiency-related genes (*PR1a*) exhibited reduced expression. It should be noted that UP9-like proteins might interact with many proteins at the same time, showing their indirect effects on plants (Lewandowska et al., 2010). Changes in gene expression profiles could be interpreted with their direct or indirect roles in S deficiency. It is important to note that previous

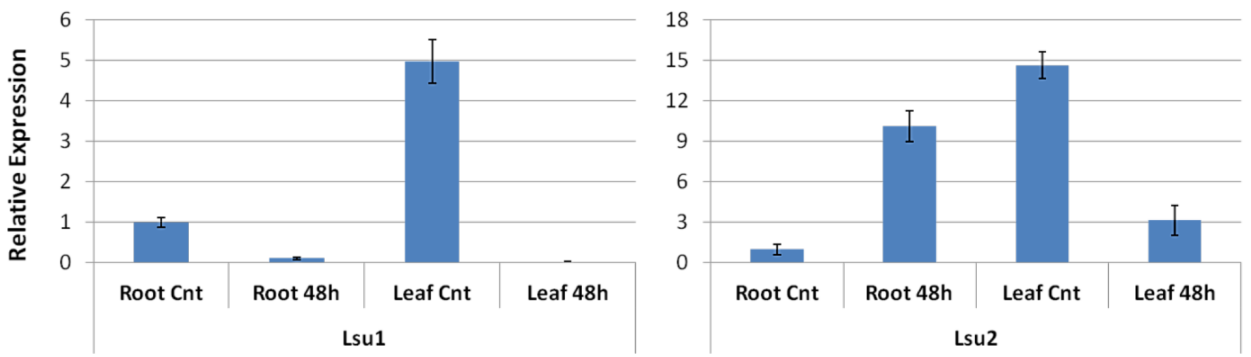

Figure 5. Relative expression of Bd*LSU1* and Bd*LSU2* genes under S-deficient conditions. Error bars represent standard deviations obtained after 3 technical and 2 biological replicates.

studies have revealed that *LSU* and related genes are not only specific to S depletion. Condition-specific or tissue-specific expression was observed under various stresses. In our study, alteration in expression level could be explained by their direct regulatory function in S limitation, or their possible indirect roles in another regulatory metabolism.

In order to assess the indirect roles of BdLSU members in plants, we conducted an in silico analysis including several stress conditions. For this purpose, *BdLSU* gene expressions were detected under drought, salt, cold, and heat stresses. Remarkably, it was found that other than S-deficiency stress, the *BdLSU* genes were regulated by different stress conditions. For instance, in a time-dependent manner (1 h to 24 h), the *BdLSU1* gene exhibited increased expression upon drought, cold, and heat stresses. Salt stress seemed to cause *BdLSU* transcript accumulation, but the relative expression change between control and stressed plants was limited (Figure 6a). Similarly, *BdLSU2* transcripts were found to be overexpressed under cold stress. However, expression was decreased under salt and heat stress conditions (Figure 6b). This result proves that *BdLSU* genes are responsive not only to S deficiency, but can also be regulated against different stress types.

The expression profile of *BdLSU* genes was correlated with protein interactome results in this study. Protein interaction analysis pointed out that the OsLSU1 protein could be related to stress-responsive proteins such as AP2 domain-containing proteins and ethylene-responsive and bHLH proteins. AP2 domain-containing proteins are mostly related to the *AP2/ERF* transcription factor

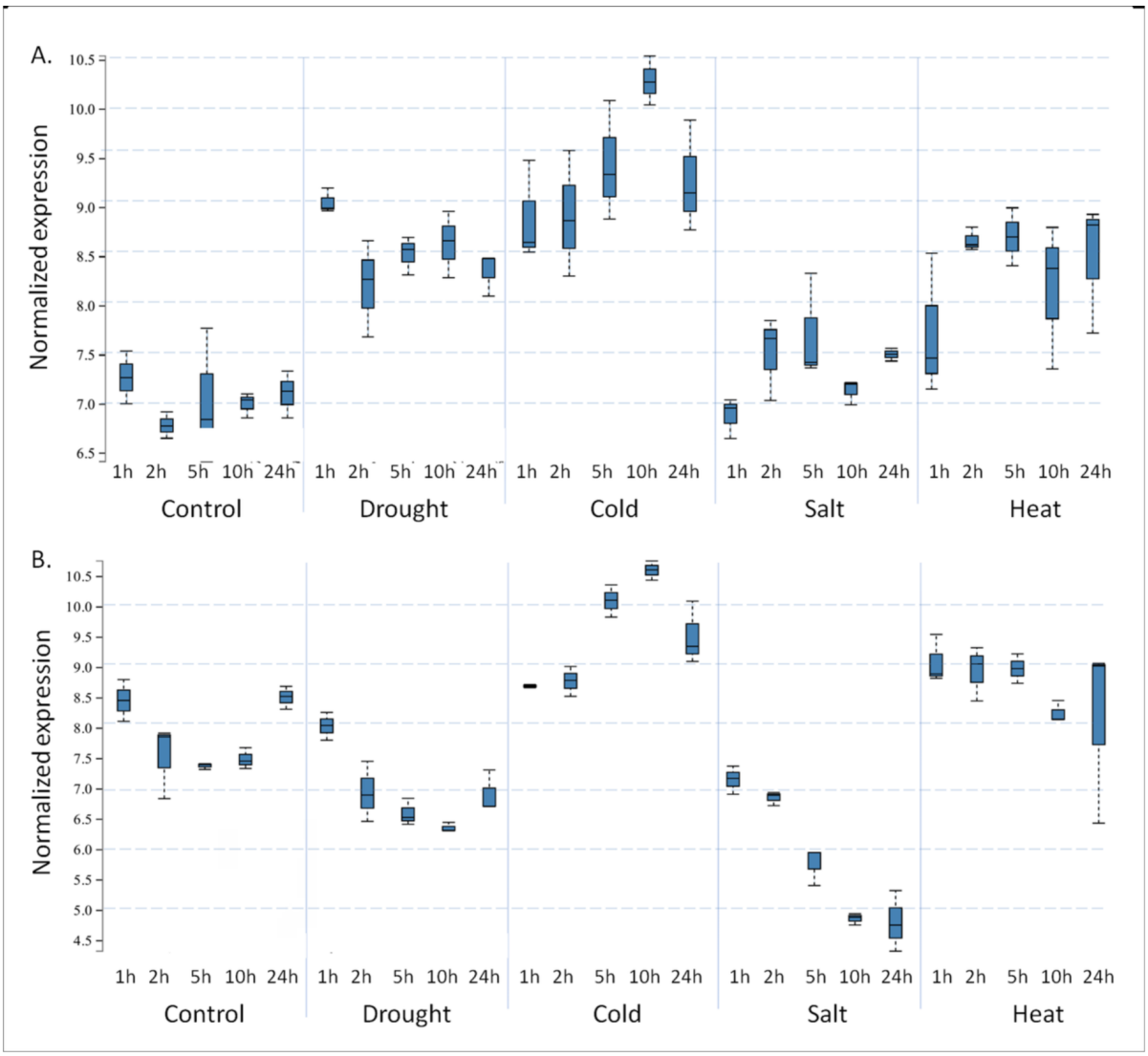

Figure 6. *BdLSU1* and *BdLSU2* gene expressions upon various types of stress conditions. Digital expression value (RMA) was normalized by log2 function.

family. DREBs belonging to this family play a role in many different regulatory bioprocesses, especially in cold stress response. In addition, heat and drought stresses induce DREB2A protein, causing transcriptomic changes in heat- and drought-inducible genes that activate heat and drought stress response (Mizoi et al., 2012). In this study, it was found that cold, heat, and drought stresses increased *BdLSU1* gene expression, which is in agreement with DREB2A (AP2/ERF family member)-mediated gene regulation. Similarly, the bHLH family proteins are related to stresses, especially salt. In the current study, *BdLSU1* was slightly upregulated; in contrast, *BdLSU2* was remarkably downregulated under salt stress. In a previous study, salt stress-responsive *bHLH* genes were evaluated in common bean, and up- and downregulated *bHLH* genes were detected after salt treatment (Kavas et al., 2016). Additionally, bHLH122 was found to be important for drought resistance (Liu et al., 2014). Under drought stress, the *BdLSU1* gene was upregulated and *BdLSU2* was downregulated (Figures 6a and 6b). According to interactome analysis, we conclude that LSU proteins may negatively and/or positively cooperate with AP2/ERF and bHLH proteins for genetic regulation against changing conditions. It is noteworthy that these gene families have many members. In *Brachypodium*, none of them have been identified on a genome-wide scale. Hence, further studies can be conducted to characterize these families that have the potential to work with BdLSU members under changing conditions including, but not limited to, S-depletion, cold, heat, and drought stresses.

In conclusion, in the current study, plant-specific *LSU* genes were analyzed in 6 grass species using bioinformatics tools. We also assessed *BdLSU* gene expressions under 48 h of S-depletion treatment. Expression profiles were detected under drought, cold, salt, and heat stresses. The findings of this study could be used for genomic studies to understand *LSU* gene functions under various types of stress conditions including S depletion. However, further wet-lab studies are required to get a deeper insight into the *LSU* gene family in plants and to understand their direct or indirect roles under various stress conditions, including S limitation.

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