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Boron toxicity induces sulfate transporters at transcriptional level in *Arabidopsis thaliana*

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Abstract: Plants activate glutathione (GSH)-dependent detoxification pathways at biochemical and molecular levels under boron (B) toxicity. Sulfate uptake and transport are necessary for GSH biosynthesis in plants. Therefore, the transcriptional regulation of some sulfate transporters was determined in this study to clarify the importance of these transporters in leaf and root tissues of *Arabidopsis thaliana* under toxic B conditions. The expression level of *SULTR1;3* was dramatically increased in leaf and root tissues under moderate and severe toxic B conditions, suggesting source-to-sink sulfate translocation under B toxicity. Stable expression levels of *SULTR2;1*, *SULTR2;2*, and low *SULTR3;5* expression might restrict the sulfate movement into the xylem in leaves. *SULTR3;1*, *SULTR3;2*, *SULTR3;3*, *SULTR3;4*, *SULTR4;1 and SULTR4;2* were induced in root tissues under toxic B conditions, indicating an induction of root-to-shoot sulfate translocation. These results showed that B toxicity might disrupt the homogeneous distribution of sulfate and sulfur-containing compounds in both tissues of *A. thaliana*. Moreover, we performed in silico analysis of microarray experiments to determine the common differentially expressed genes (DEGs) under B toxicity and sulfur deficiency. Gene ontology, hierarchical clustering, and coexpression network analyses of these DEGs demonstrated the requirement of sulfate transporters under B toxicity. A set of genes involved in sulfur metabolism coexpress with sulfate transporters under B toxicity. To the best of our knowledge, this is the first report focusing on the molecular regulation of sulfate transporters in *Arabidopsis thaliana* under B toxicity.

Keywords: *Arabidopsis thaliana*, boron toxicity, gene expression, sulfate transporter

1. Introduction

Boron (B) is an essential micronutrient for plant development and growth. It can form strong complexes with biological molecules containing cis-hydroxyl groups such as ribose, sorbitol, and apiose (Ralston and Hunt, 2001). The pectic polysaccharide rhamnogalacturonan II (RGII) is the first B-containing compound determined in the plant (Voxeur and Fry, 2014). Wang et al. (2015) have suggested that B participates in the complexes of Glycosylinositol phosphorylceramides (GIPCs)-RGII and this provides bridging the cell membrane with the cell wall. In addition to this structural role in the plant cell wall, B has other roles in plasma membrane integrity, seed improvement, reproductive tissue stimulation, transportation of sugar, phenol, and ascorbate metabolism in plants (Landi et al., 2019). However, accumulation of B at levels slightly higher than the concentration required for normal growth can become toxic to plants (Mengel and Kirkby, 2001). Geothermal and volcanic processes as well as weathering are the major causes of excess B in the soil. Moreover, evaporation from the oceans contributes to B accumulation (Landi et al., 2019). Because of the poor drainage in arid and semiarid countries such as Morocco, Syria, Egypt, Iraq, Italy, and Turkey, B can easily accumulate in the soil (Nable et al., 1997; Pennisi et al., 2006).

B toxicity causes limitation in crop yield, and it affects the product quality in many regions of the world (Brdar-Jokanović, 2020) because B accumulation at a toxic level in the soil leads to impairment of growth and plant metabolism, causing chlorosis and necrosis in leaf tissues (Reid et al., 2004; Landi et al., 2012; Camacho-Cristóbal et al., 2018). Furthermore, excess B causes changes in the cell wall structure and disturbs cell division (Reid et al., 2004). A decrease in photosynthetic pigments is another effect of toxic B in plants (Kayıhan et al., 2016). The uptake of excess B causes photo-oxidative stress, in turn, inhibiting the overall plant growth (Reid et al., 2004; Aquea et al., 2012). Oxidative stress might be caused by these physiological disorders due to over-accumulation of reactive oxygen species (ROS), causing cell death by oxidizing pigments, lipids, nucleic acids, proteins, and inactivating enzymes (Blokhina et al., 2003). Plants have scavenging mechanisms including antioxidant enzymes against ROS accumulation. In our previous study, severe B toxicity promoted the

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nonenzymatic antioxidants including proline, flavonoid, and anthocyanin, and dramatically enhanced superoxide dismutase expression and activity that resulted in hydrogen peroxide (H_2O_2) accumulation in *Arabidopsis thaliana* (Kayıhan et al., 2016). It was also suggested that the ascorbate-glutathione cycle might be regulated at the transcriptional level under B toxicity. For this reason, we have recently studied the molecular regulation of B toxicity responses via glutathione (GSH)-dependent detoxification pathways in *A. thaliana* (Kayıhan et al., 2019). Accordingly, we suggest that glutathione S-transferases (GST), such as GSTU19 and GSTZ1, might have roles in the dramatic increase of the total GST activity under B toxicity, and GST can have a special protective role in B toxicity tolerance in plants. In other words, our findings can support an internal B detoxification mechanism via GSH-GST conjugation in plants. This information can be validated by the suggestion of Landi et al. (2015).

The ubiquitous tripeptide GSH (γ-glutamyl-cysteinyl-glycine) is often termed as nonprotein reduced sulfur and is a strong water-soluble antioxidant. Conjugation of sulfhydryl groups of GSH is responsible for its protective antioxidant mechanism. The synthesis of GSH starts from inorganic sulfate and sulfate is required for further sulfur (S) assimilation and cysteine (Cys) biosynthesis (Hell and Wirtz, 2011). It was shown that the levels of Cys and GSH decline under S deficiency (Panthee et al., 2006; Reinbold et al., 2008). Moreover, sulfate transporters having roles for sulfate uptake and transport are necessary for Cys and GSH biosynthesis in plants (Takahashi et al., 2012). Taken together, these data suggest that proper functioning of sulfate transporters might be required for the B detoxification mechanism via GSH-GST conjugation in plants. Sulfate transporters are divided into five groups in *A. thaliana* based on their phylogenic relationships (Buchner et al., 2004; Zuber et al., 2010). They are localized to different tissues, cells, and subcellular compartments, and they are regulated at the transcriptional and posttranscriptional levels (Takahashi et al., 2012). SULTR1;1, SULTR1;2, and SULTR1;3 are the members of group 1 sulfate transporters. Among them, SULTR1;1 and SULTR1;2 are active transport systems for sulfate influx and are responsible for preventing sulfate leakage from the epidermal and cortical cells in the roots whereas SULTR1;3 has a role in the transfer of sulfate from shoot to root (Yoshimoto et al., 2003). SULTR1;3 is expressed in the phloem companion cells and has a role in loading of sulfate to phloem, facilitating the source to sink translocation of sulfate and related compounds in *A. thaliana* (Yoshimoto et al., 2003). SULTR2;1 and SULTR2;2 are found in group 2 and expressed in parenchyma cells of xylem and are involved in long-distance transport in *A. thaliana* (Takahashi et al., 2000). SULTR2;1 interacts with SULTR3;5 to increase sulfate uptake capacity in yeast (Davidiana and Kopriva, 2010). Found in group 3, SULTR3 isoforms are localized to the outer membrane of the chloroplast and involved in sulfate influx into chloroplasts (Cao et al., 2013). Moreover, they contribute to sulfate transport across the chloroplast membrane (Chen et al., 2019). In group 4, SULTR4 isoforms are localized in the vacuolar membrane of root vasculature and function in the efflux of sulfate out of the vacuole, mediating the root-to-shoot sulfate transport (Kataoka et al., 2004b). Finally, SULTR5.1 (MOT1;2) and SULTR5.2 (MOT1;1) are classified together in group 5 and involved in molybdate transport but not sulfate transport (Tomatsu et al., 2007; Gasber et al., 2011). In this study, the expression levels of sulfate transporters were examined to clarify the excess B responsive regulation of these transporters in leaf and root tissues of *A. thaliana*. Furthermore, we performed in silico analysis of microarray experiments to determine the common differentially expressed genes (DEGs) under B toxicity and S deficiency. Then, gene ontology (GO), hierarchical clustering, and coexpression network analyses of these DEGs were performed to demonstrate the requirement of sulfate transporters under B toxicity and identify a set of genes coexpressed with sulfate transporters under B toxicity.

2. Materials and methods

2.1. Growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) were surface sterilized as explained in our previous report (Kayıhan et al., 2016), and they were sown on ½ x MS medium (supplemented with 1% of sucrose and 0.8% of agar) (Murashige and Skoog, 1962) containing 100 µM of boric acid (control) and toxic levels of B (supplemented as 1 mM and 3 mM of boric acid dissolved in sterile distilled water). Plates (each plate contained 15 seeds) were stratified at 4 °C in dark for 3 days for synchronized germination, then they were horizontally transferred to a controlled growth chamber (21 ± 2 °C, 60% of relative humidity) under long photoperiod (16–8 h light-dark). Seedlings were grown for 14 days and then, leaves and roots were separately harvested for the gene expression analyses. Toxic B concentrations and their durations have been chosen based on our previous articles where they were optimized (Kayıhan et al., 2016; Kayıhan et al., 2019; Kayıhan, 2021).

2.2. Total RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from leaf and root tissues of 14-day-old *Arabidopsis thaliana* according to Chomczynski and Sacchi (1987). After DNase I treatment, agarose gel electrophoresis was used for the integrity of RNAs. The quality and quantity of RNAs were determined by using

a NanoDrop spectrophotometer (Denovix, USA). One microgram of total RNA was used to prepare the first strand complementary DNA (cDNA) by iScript cDNA Synthesis Kit (Bio-Rad). Roche LightCycler 480 was used for qRT-PCR experiments. Every sample contained 1 µL of cDNA, 10 µL of iTaq universal SYBR Green super mix (2x) (Bio-Rad), 1µL of each forward and reverse primers (0.5 µM final concentration), and 7 µL of PCR-grade water. Primers were designed from exon-exon boundaries of the sequences of each sulfate transporter gene of *Arabidopsis thaliana* by Primer 3 software (Rozen and Skaletsky, 2000) and they were shown in Table 1. Conditions of qRT-PCR include the preincubation at 95 °C for 30 s, following 95 °C for 10 s and 59 °C for 1 min for 40 cycles. To determine the specific amplification of each gene product, a melting curve analysis was performed following the amplification by incubating at 95 °C for 5 s, at 65 °C for 1 min, and cooling to 40°C for the 30 s. Expression levels of sulfate transporter genes were normalized by using the actin (*ACT2*) gene (Czechowski et al., 2005). The relative fold changes of each sulfate transport gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3. In silico analysis of microarray experiments

To determine the differentially expressed genes (DEGs) under boron toxicity and sulfur deficiency, first raw gene expression data for one B toxicity (Aquea et al., 2012) and two sulfur deficiency (Maruyama-Nakashita et al., 2006; Iyer-Pascuzzi et al., 2011) microarray experiments were obtained from the Gene Expression Omnibus database (Edgar et al., 2002). Then, DEGs from each experiment were determined by Genespring GX (Agilent) according to the user's manual (Aksoy et al., 2013). A fold-change of ≤1.5 was considered a differential expression, with $p \leq 0.05$ for significant expression.

2.4. Gene annotation enrichment analysis

Enrichment of gene sets was performed by gene ontology (GO) enrichment analysis using PANTHER version 16 (Mi et al., 2021) against the GO Ontology database. The reference list includes all available Arabidopsis genes. Results of only the GO biological process was given after Fisher's exact testing followed by Bonferroni correction for multiple testing ($p < 0.05$).

2.5. Hierarchical clustering and coexpression network analysis

Gene clustering was performed by using Genevestigator (Zimmermann et al., 2004) according to Pearson correlation distance and the optimal leaf ordering (Eisen et al., 1998). Coexpression networks of sulfate transporters were generated by using Atted II (Obayashi et al., 2006).

2.6. Gene set enrichment analysis (GSEA)

GSEA was performed using GeneTrail (Backes et al., 2007; Schuler et al., 2011). Briefly, 22,811 probes on ATH1 microarray data sets obtained from public databases were ranked and sorted according to fold change from the most induced to the most suppressed by each stress treatment. Subsequently, GSEA analyses were performed for each

Table 1. qRT-PCR primer sequences for sulfate transporter genes in *Arabidopsis thaliana*.

Locus	Gene name	Primer sequences (5' – 3')	
		Forward	Reverse
AT4G08620	*SULFATE TRANSPORTER 1;1 (SULTR1;1)*	CAAGAAACCCACCAGTCG	CGCTTTCGGAGGAGCTAG
AT1G78000	*SULFATE TRANSPORTER 1;2 (SULTR1;2)*	GCATTCCTCAGGATATTGGATACGC	CGAAACCACAGCGACAGGTCCT
AT1G22150	*SULFATE TRANSPORTER 1;3 (SULTR1;3)*	CGAGCCGACAAGAAAGGAGT	CTACAGCTTCCGTCAAGGCA
AT5G10180	*SULFATE TRANSPORTER 2;1 (SULTR2;1)*	GGTGTGAAGACAGTGAGGCA	ATCGCCTCGGTTAGAGCAAC
AT1G77990	*SULFATE TRANSPORTER 2;2 (SULTR2;2)*	TCCAATGCTGAGTCACGAGG	ATTGCTTCCGTTAGGGCGAT
AT3G51895	*SULFATE TRANSPORTER 3;1 (SULTR3;1)*	ACTCACGAGTGGAGATGGGA	GCCGCCACCCAAAAGAATTT
AT4G02700	*SULFATE TRANSPORTER 3;2 (SULTR3;2)*	ATGCTCAGCTCGCTAATCTCCC	CCAACATCGCAGCCGTCAA
AT1G23090	*SULFATE TRANSPORTER 3;3 (SULTR3;3)*	ATCCGACGTCGTTTCAGGTC	AGCTCGAGTATAGACCAACGA
AT3G15990	*SULFATE TRANSPORTER 3;4 (SULTR3;4)*	CCTGATGATCCGTTACAGAGGT	TGATTCCCTGAGGAATGGCG
AT5G19600	*SULFATE TRANSPORTER 3;5 (SULTR3;5)*	CTCGACCATAACGGGCTTCA	TTTGCCACTTCCACTCAGCC
AT5G13550	*SULFATE TRANSPORTER 4;1 (SULTR4;1)*	CGAACTTACCGATGGAGCGA	TACGACATTGCCTGGGGAAC
AT3G12520	*SULFATE TRANSPORTER 4;2 (SULTR4;2)*	GGATTCGGACTTACCGGTGG	TACGACATTGCCTGGGGAAC
AT1G80310	*SULFATE TRANSPORTER 5;1 (SULTR5;1)*	GAGACAACTACAACTCCTCTGCTCC	CTAGAGTTAGTGTAAGGACGATGGG
AT2G25680	*SULFATE TRANSPORTER 5;2 (SULTR5;2)*	GGAGTCTCAGTCTCAGAGAGGTCA	AGTACCAAGATCACCCATTGCAC
AT3G18780	*ACTIN2 (ACT2)*	CTTGACCTTGCTGGACGTGA	AATTTCCCGCTCTGCTGTTG

sorted data set using gene sets created from an analysis of B toxicity (Aquea et al., 2012), and S deficiencies (Maruyama-Nakashita et al., 2006; Iyer-Pascuzzi et al., 2011) and osmotic stress (Kilian et al., 2007). As a reference, gene sets consisting of constitutively expressed genes in Arabidopsis were analyzed (Czechowski et al., 2005). The false discovery rate was used as the *p*-value adjustment and the values are presented (Benjamini and Hochberg, 1995).

2.7. Statistical analysis

Gene expression analyses were performed as three biological replicates (with two technical replicates). The data were statistically analyzed by using nonparametric versions of the *t*-test ($p \leq 0.05$). They were presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Changes in the expression levels of sulfate transporters under B toxicity in leaf and root tissues of *Arabidopsis thaliana*

In leaf tissues of *A. thaliana*, the expression level of *SULTR1;3* increased nine-fold under 1B condition and increased four-fold in response to 3B condition with respect to the control plants (Figure 1). However, the transcript levels of *SULTR2;1*, *SULTR2;2*, *SULTR3;1*, and *SULTR4;2* were not significantly affected by both 1B and 3B treatments. *SULTR3;3* expressions significantly increased more than two-fold following 1B and 3B treatments. Furthermore, the expression levels of *SULTR3;4* were dramatically induced under both toxic B treatments while the expression levels of *SULTR3;5* were significantly decreased under both treatments as compared to the control. Finally, *SULTR4;1* expression was significantly increased more than three-fold under 1B treatment; however, it was slightly increased after 3B treatment, but it was not significant (Figure 1).

1B treatment did not cause any significant changes in the expression levels of *SULTR1;1*, *SULTR1;2*, *SULTR2;1*, *SULTR3;1*, *SULTR5;1*, *SULTR5;2* in root tissues of *A. thaliana* (Figure 2). However, the expression levels of *SULTR1;3*, *SULTR2;2*, *SULTR3;2*, *SULTR3;3*, *SULTR3;4*, *SULTR3;5*, *SULTR4;1*, and *SULTR4;2* were significantly increased under 1B condition. 3B treatment did not cause any significant change in the expression levels of *SULTR1;2*, *SULTR2;1* and *SULTR5;2* while the expression levels of *SULTR1;1 SULTR1;3*, *SULTR2;2*, *SULTR3;1*, *SULTR3;2*, *SULTR3;3*, *SULTR3;4*, *SULTR3;5*, *SULTR4;1*, *SULTR4;2*. *SULTR5;1* were significantly upregulated in root tissues (Figure 2).

3.2. B toxicity and sulfur deficiency cause differential expression of the same set of genes

The same set of genes can be differentially expressed under one mineral deficiency and another mineral toxicity since similar metabolic pathways may function in plant stress tolerance against both conditions. Although differentially expressed genes (DEGs) were identified separately from Arabidopsis roots treated with 5 mM H_3BO_3 (Aquea et al., 2012) and Arabidopsis roots treated with sulfur deficiency (Maruyama-Nakashita et al., 2006; Iyer-Pascuzzi et al.,

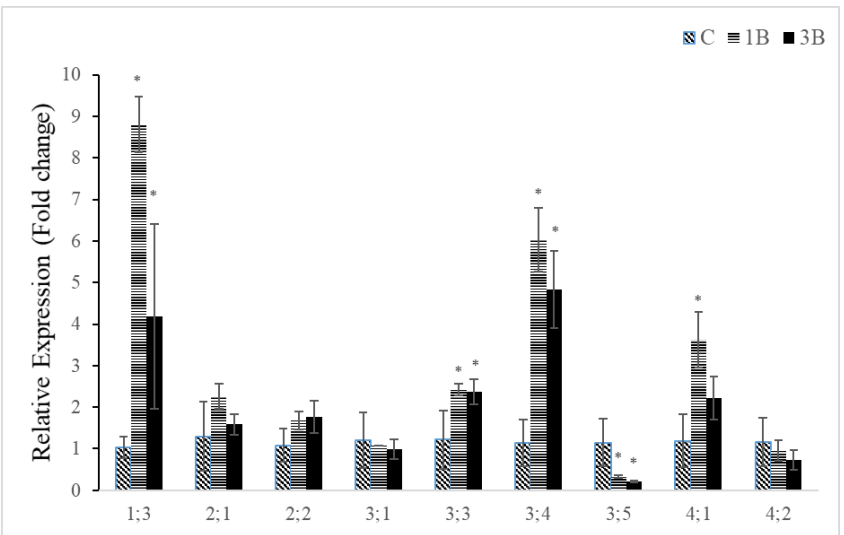

Figure 1. Relative expression levels of sulfate transporters in the leaf tissues of *A. thaliana* in response to toxic B. C: Control, 1B: 1 mM of H_3BO_3, 3B: 3 mM of H_3BO_3. 1;3: *SULTR1;3*, 2;1: *SULTR2;1*, 2;2: *SULTR2;2*, *3;1: SULTR3;1*, 3;3: *SULTR3;3*, 3;4: *SULTR3;4*, 3;5: *SULTR3;5*, 4;1: *SULTR4;1*, 4;2: *SULTR4;2*. Values represent mean ± SEM (n = 3). An asterisk above the bars represents significant differences between the control and B toxicity-treated plants ($p \leq 0.05$).

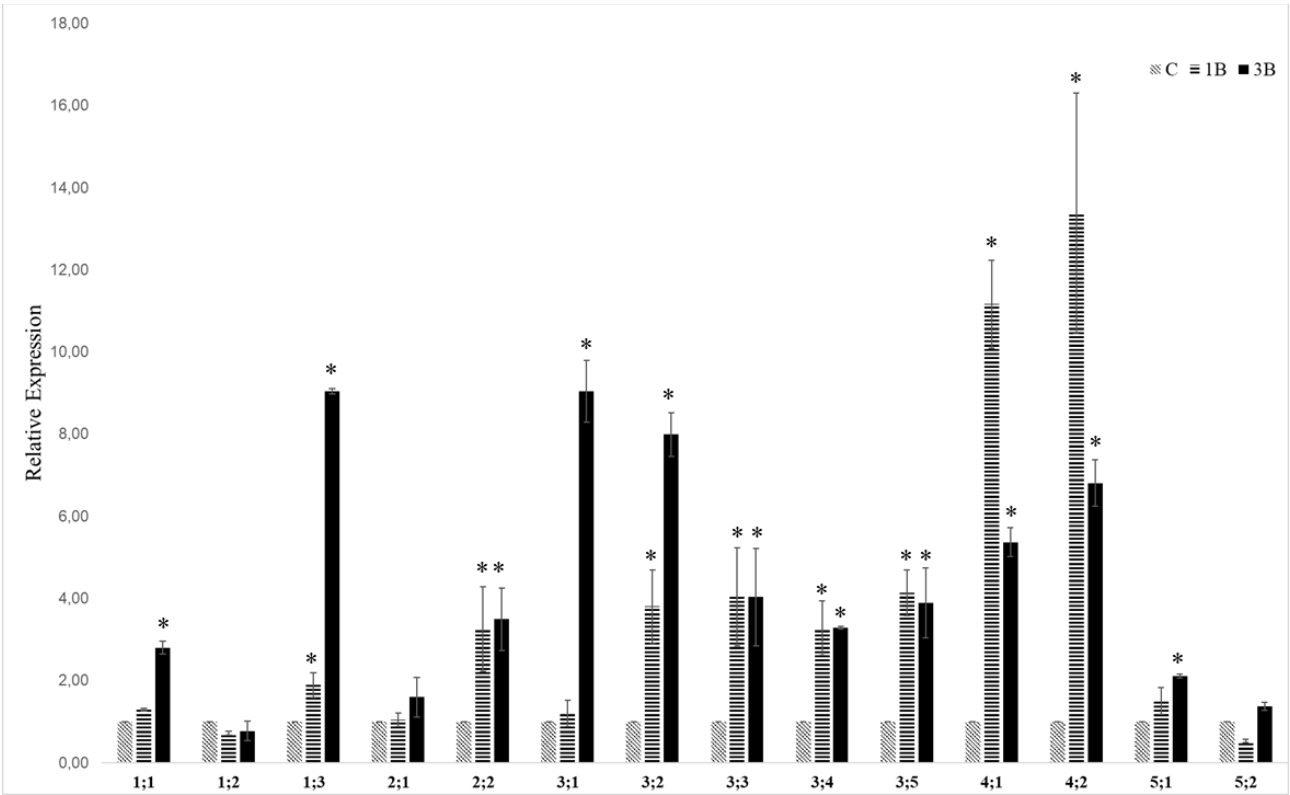

Figure 2. Relative expression levels of sulfate transporters in the root tissues of *A. thaliana* in response to toxic B. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. 1;1: *SULTR1;1*, 1;2: *SULTR1;2*, 1;3: *SULTR1;3*, 2;1: *SULTR2;1*, 2;2: *SULTR2;2*, *3;1: SULTR3;1*, *3;2: SULTR3;2*, 3;3: *SULTR3;3*, 3;4: *SULTR3;4*, 3;5: *SULTR3;5*, 4;1: *SULTR4;1*, 4;2: *SULTR4;2*. 5;1: *SULTR5;1*, 5;2: *SULTR5;2.* Values represent mean ± SEM (n = 3). An asterisk above the bars represents significant differences between control and B-toxicity treated samples ($p \leq 0.05$).

2011), common DEGs of both stress conditions have not been identified before. Therefore, we determined the DEGs under boron toxicity and sulfur deficiency. A comparison of DEGs identified a set of 21 genes differentially expressed in both mineral stress conditions (Figure 3 and Table 2). These genes were enriched in gene ontologies (GO) mainly related to S-glycoside biosynthetic process, sulfur metabolism, glucosinolate biosynthetic processes, and osmotic stress (Table 3). Besides these 21 common genes, 72 and 667 DEGs were determined in individual B toxicity and S deficiency conditions, respectively. 72 DEGs in individual B toxicity were enriched in GOs related to adventitious root development, ammonium homeostasis, hydrogen peroxide and chloride transport, indoleacetic acid biosynthesis, suberin biosynthesis and defense response by callose deposition in cell wall, glucosinolate biosynthesis and sulfate transport (Table S1). On the other hand, 667 DEGs in individual S deficiency were enriched only in one GO (sulfate transmembrane transporter activity), suggesting S deficiency-related genes are not very specific as compared to B-toxicity-related genes. Taken together, these results suggest a possible interaction between B toxicity and sulfur metabolism in the Arabidopsis roots.

3.3. DEGs in B-treated Arabidopsis roots are clustered in two major groups under sulfur deficiency according to their expression patterns

To understand the expression profile of DEGs in B-treated Arabidopsis roots under sulfur deficiency, we first checked the in silico expression levels of DEGs identified by Aquea et al. (2012) from 5-day-old Arabidopsis roots treated with 5 mM H_3BO_3 under two different sulfur deficiency experiments. Then, we performed hierarchical clustering of all 93 DEGs to determine two major clusters including genes down- (Clusters 1) or upregulated (Cluster 2) in the roots of sulfur deficiency-treated Arabidopsis plants (Figure 4). There were 51 and 42 genes in Cluster 1 and Cluster 2, respectively (Table 4). Interestingly, the genes in Cluster 1 were suppressed under B toxicity, especially in longer exposure times, while the ones in Cluster 2 were generally induced, indicating similar effects of B toxicity and S deficiency on the expression patterns of these genes in Arabidopsis roots. The genes involved in sulfur-assimilation, glucosinolate production, auxin biosynthesis,

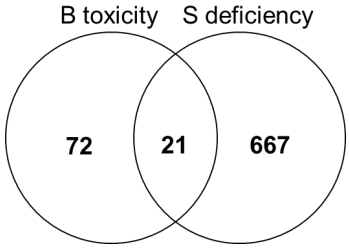

Figure 3. Venn diagram comparison of differentially expressed genes in boron toxicity (Aquea et al., 2012) and the sulfur deficiency (Maruyama-Nakashita et al, 2006; Iyer-Pascuzzi et al., 2011) in Arabidopsis roots.

Table 2. Common DEGs in Arabidopsis roots under boron toxicity and sulfate deficiency.

LOCUS	**Gene**	
	Abbreviation	**Name/Annotation**
Upregulated		
AT1G04220	*KCS2*	*3-KETOACYL-COA SYNTHASE2 / DAISY*
AT1G52690	*LEA7*	*LATE EMBRYOGENESIS ABUNDANT 7*
AT1G72770	*HAB1*	*HYPERSENSITIVE TO ABA1*
AT2G35300	*LEA18 / LEA4-2*	*LATE EMBRYOGENESIS ABUNDANT18 / LATE EMBRYOGENESIS ABUNDANT4-2*
AT3G17520	-	Late embryogenesis abundant protein (LEA) family protein
AT3G50400	-	GDSL-motif esterase/acyltransferase/lipase
AT3G60140	*BGLU30 / DIN2 / SRG2*	*BETA GLUCOSIDASE30 / DARK INDUCIBLE2 / SENESCENCE-RELATED GENE2*
AT3G61890	*HB12*	*HOMEOBOX12*
AT4G28110	*MYB41*	*MYB DOMAIN PROTEIN41*
AT5G06760	*LEA4-5*	*LATE EMBRYOGENESIS ABUNDANT4-5*
AT5G57050	*ABI2*	*ABA INSENSITIVE2*
AT5G59220	*HAI1 / SAG113*	*HIGHLY ABA-INDUCED PP2C GENE1 / SENESCENCE ASSOCIATED GENE113*
Downregulated		
AT1G18590	*SOT17/ST5C*	*SULFOTRANSFERASE17 / SULFOTRANSFERASE5C*
AT1G62280	*SLAH1*	*SLAC1 HOMOLOGUE1*
AT3G58990	*IPMI1*	*ISOPROPYLMALATE ISOMERASE1*
AT4G12030	*BAT5*	*BILE ACID TRANSPORTER5*
AT4G13770	*CYP83A1 / REF2*	*CYTOCHROME P450, FAMILY 83, SUBFAMILY A, POLYPEPTIDE1 / REDUCED EPIDERMAL FLUORESCENCE2*
AT4G39940	*AKN2 / APK2*	*APS-KINASE2*
AT4G39950	*CYP79B2*	*CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE2*
AT5G23010	*IMS3 / GSM1 / MAM1*	*2-ISOPROPYLMALATE SYNTHASE3 / GLUCOSINOLATE METABOLISM1 / METHYLTHIOALKYLMALATE SYNTHASE1*
AT5G23020	*IMS2 / MAM3*	*2-ISOPROPYLMALATE SYNTHASE2 / METHYLTHIOALKYLMALATE SYNTHASE3*

A fold-change of ≤1.5 was considered differential expression, with $p \leq 0.05$ for significant expression.

glutathione metabolism, and ion transport were found specifically in Cluster 1 while the genes involved in processes of fatty acid metabolism, suberin, cutin, and phenylpropanoid biosynthesis, and osmotic stress response were grouped in Cluster 2 (Figure 4 and Table 5). There were no genes involved in sulfur assimilation,

Table 3. Enrichment of top 15 biological process GO terms of common DEGs in Arabidopsis roots under boron toxicity and sulfate deficiency.

GO biological process term	GO number	Expected %	Fold enrichment	p-value
S-glycoside biosynthetic process	GO:0016144	0.03	>100	1.95E-09
glucosinolate biosynthetic process	GO:0019761	0.03	>100	1.95E-09
glycosyl compound biosynthetic process	GO:1901659	0.05	>100	2.84E-08
response to insect	GO:0009625	0.02	>100	6.74E-03
glycosinolate metabolic process	GO:0019760	0.09	82.09	7.06E-09
secondary metabolite biosynthetic process	GO:0044550	0.11	79.87	3.78E-12
sulfur compound biosynthetic process	GO:0044272	0.10	77.26	2.39E-10
glycosyl compound metabolic process	GO:1901657	0.13	54.08	1.18E-07
secondary metabolic process	GO:0019748	0.24	41.30	4.24E-11
response to water deprivation	GO:0009414	0.26	34.77	5.28E-09
response to water	GO:0009415	0.26	33.97	6.47E-09
sulfur compound metabolic process	GO:0006790	0.27	33.11	8.09E-09
carbohydrate derivative biosynthetic process	GO:1901137	0.27	21.89	6.60E-04
response to osmotic stress	GO:0006970	0.41	16.87	3.10E-04
carbohydrate derivative metabolic process	GO:1901135	0.48	14.69	7.82E-04

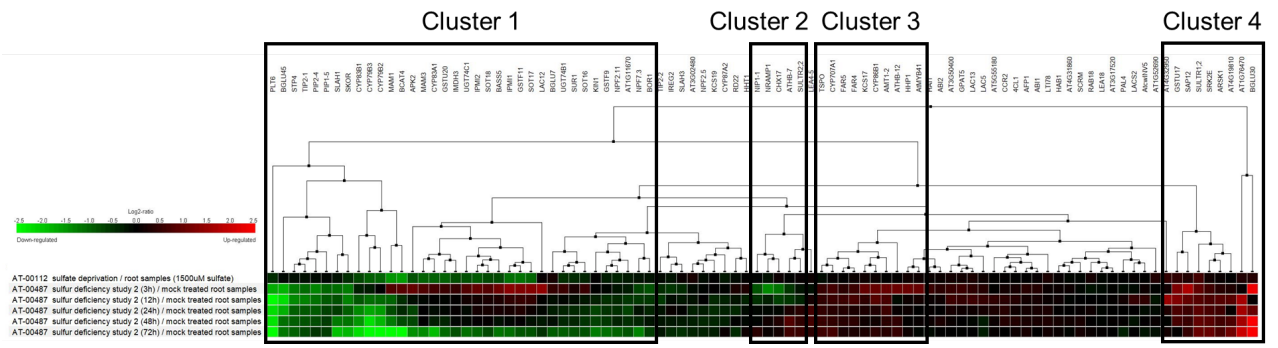

Figure 4. Hierarchical clustering of differentially expressed genes of boron toxicity under sulfur deficiency. Generated with Genevestigator (see the methods) by using 93 DIGs obtained from Aquea et al. (2011) against two sulfur deficiency microarray experiments (Maruyama-Nakashita et al., 2006; Iyer-Pascuzzi et al., 2011) in Arabidopsis roots.

glucosinolate production, or glutathione metabolism in Cluster 2. Three *GST* genes, namely *GSTU20*, *GSTF6/GSTF11*, and *GSTF9* were identified in Cluster 1, whereas *ERD9/GST30B/GSTU17* was identified in Cluster 2, indicating the requirement of GSTs in B toxicity response in Arabidopsis. Although many genes involved in cell wall modification and ABA signaling were upregulated in Cluster 2 under S deficiency, some of them (such as *BGLU7*, *BGLU45*, *KIN1*, *RD22*, *ABR*, and *LEA4-5*) were also downregulated in Cluster 1 under S deficiency. These data suggest that there is a similarity between B toxicity and sulfur deficiency responses in terms of differentially expressed genes in Arabidopsis roots.

3.4. Sulfate transporters coexpress with a large set of genes overrepresented in B toxicity

As some sulfate transporters are suppressed under B toxicity in Arabidopsis roots, the genes coexpress with sulfate transporters were identified to further evaluate the connection between S metabolism and boron toxicity. Accordingly, the coexpression network of sulfate transporters was analyzed. According to the results, *SULTR4;1*, *SULTR4;2*, *SULTR3;1*, *SULTR3;5*, *and SULTR5;2* (another name is *MOT1*) grouped together in Cluster 1 (Figure 5). *SULTR2;1* and *SULTR3;4* were found together in Cluster 2, whereas *SULTR2;2* and *SULTR3;3* were identified in Cluster 3. The rest of the sulfate transporters

Table 4. Expression levels of Clusters 1 and 2 genes in Arabidopsis roots under B toxicity (Aquea et al., 2012).

Locus	Gene		Expression in B toxicity
	Abbreviation	**Name/Annotation**	
Cluster 1			
Transporter proteins			
AT4G36670	*PLT6 / PMT6*	*POLYOL TRANSPORTER6 / POLYOL/MONOSACCHARIDE TRANSPORTER6*	–1.84
AT3G19930	*STP4*	*SUGAR TRANSPORTER4*	–1.45
AT3G16240	*AQP1 / TIP2;1*	*DELTA TONOPLAST INTEGRAL PROTEIN / DELTA-TIP1*	–2.12
AT5G60660	*PIP2;4*	*PLASMA MEMBRANE INTRINSIC PROTEIN2;4*	–1.51
AT4G23400	*PIP1;5*	*PLASMA MEMBRANE INTRINSIC PROTEIN 2;4*	–1.79
AT1G62280	*SLAH1*	*SLAC1 HOMOLOGUE1*	–2.36
AT3G02850	*SKOR*	*STELAR K+ OUTWARD RECTIFIER*	–2.02
AT4G12030	*BAT5*	*BILE ACID TRANSPORTER5*	–2.48
AT5G62680	*GTR2 / NPF2.11*	*GLUCOSINOLATE TRANSPORTER2 / NRT1-PTR FAMILY2.11*	–1.67
AT1G11670	-	MATE efflux family protein	–1.59
AT1G32450	*NPF7.3 / NRT1.5*	*NITRATE TRANSPORTER1.5 / NRT1-PTR FAMILY 7.3*	–2.09
AT2G47160	*BOR1*	*REQUIRES HIGH BORON1*	–1.48
AT4G17340	*TIP2;2*	*TONOPLAST INTRINSIC PROTEIN2;2*	–1.69
AT5G03570	*IREG2 / FPN2*	*IRON-REGULATED PROTEIN2, FERROPORTIN2*	–1.72
AT5G24030	*SLAH3*	*SLAC1 HOMOLOGUE3*	–1.78
AT3G45710	*NPF2.5*	A chloride permeable transporter	–1.85
AT1G80830	*NRAMP1*	*NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN1*	–1.76
AT4G19030	*NIP1-1*	*NOD26-LIKE INTRINSIC PROTEIN1;1*	–2.62
AT1G77990	*SULTR2;2*	*SULPHATE TRANSPORTER2;2*	–1.91
Sulfur metabolism			
AT4G31500	*CYP83B1 / ATR4 / RED1 / RNT1 / SUR2*	*CYTOCHROME P450, FAMILY 83, SUBFAMILY B, POLYPEPTIDE1 / ALTERED TRYPTOPHAN REGULATION4 / RED ELONGATED1 /RUNT1 / SUPERROOT 2*	–2.16
AT2G22330	*CYP79B3*	*CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE3*	–3.58
AT4G39950	*CYP79B2*	*CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE2*	–3.21
AT5G23010	*GSM1 / IMS3 / MAM1*	*2-ISOPROPYLMALATE SYNTHASE3 / GLUCOSINOLATE METABOLISM1 / METHYLTHIOALKYLMALATE SYNTHASE1*	–4.12
AT3G19710	*BCAT4*	*BRANCHED-CHAIN AMINOTRANSFERASE4*	–5.26
AT4G39940	*AKN2 / APK2*	*APS-KINASE2 KINASE2*	–2.29
AT5G23020	*IMS2 / MAM3*	*2-ISOPROPYLMALATE SYNTHASE2 / METHYLTHIOALKYLMALATE SYNTHASE3*	–5.95
AT4G13770	*CYP83A1 / REF2*	*CYTOCHROME P450, FAMILY 83, SUBFAMILY A, POLYPEPTIDE1 / REDUCED EPIDERMAL FLUORESCENCE2*	–3.61
AT1G78370	*GSTU20*	*GLUTATHIONE S-TRANSFERASE TAU20*	–1.56
AT5G14200	*IMD1*	*ISOPROPYLMALATE DEHYDROGENASE1*	–1.89
AT2G31790	*UGT74C1*	*UDP-GLUCOSYL TRANSFERASE 74C1*	–1.54
AT2G43100	*IPMI2*	*ISOPROPYLMALATE ISOMERASE2*	–2.67
AT1G74090	*SOT18*	*DESULFO-GLUCOSINOLATE SULFOTRANSFERASE18*	–1.87

Table 4. (Continued).

AT3G58990	*IPMI1*	*ISOPROPYLMALATE ISOMERASE 1*	–4.41
AT3G03190	*GSTF6 / GSTF11*	*GLUTATHIONE-S-TRANSFERASE6 / GLUTATHIONE S-TRANSFERASE F11*	–1.87
AT1G18590	*ST5C / SOT17*	*SULFOTRANSFERASE 5C / SULFOTRANSFERASE17*	–1.68
AT1G24100	*UGT74B1*	*UDP-GLUCOSYL TRANSFERASE 74B1*	–1.22
AT2G20610	*ALF1 / HLS3 / RTY1 / SUR1*	*BERRANT LATERAL ROOT FORMATION1, HOOKLESS 3, ROOTY1, SUPERROOT 1*	–2.18
AT1G74100	*SOT16 / ST5A / CORI-7*	*SULFOTRANSFERASE16 / SULFOTRANSFERASE 5A / CORONATINE INDUCED-7*	–1.53
AT2G30860	*GSTF9*	*GLUTATHIONE S-TRANSFERASE PHI9*	–1.96
AT1G12740	*CYP87A2*	*CYTOCHROME P450, FAMILY 87, SUBFAMILY A, POLYPEPTIDE2*	–1.78
Cell wall modification			
AT1G61810	*BGLU45*	*BETA-GLUCOSIDASE45*	1.61
AT5G05390	*LAC12*	*LACCASE12*	2.48
AT3G62740	*BGLU7*	*BETA GLUCOSIDASE7*	2.88
AT5G43760	*KCS19*	*3-KETOACYL-COA SYNTHASE 20*	1.89
AT5G41040	*ASFT / HHT1 / RWP1*	*ALIPHATIC SUBERIN FERULOYL-TRANSFERASE, ASFT, HYDROXYCINNAMOYL- COA:&OMEGA;-HYDROXYACID O-HYDROXYCINNAMOYLTRANSFERASE, REDUCED LEVELS OF WALL-BOUND PHENOLICS1*	2.37
ABA Signaling and osmotic stress response			
AT5G15960	*KIN1*	*cold and ABA inducible protein KIN1*	2.31
AT3G02480	*ABR*	*ABA-RESPONSE PROTEIN*	5.99
AT5G25610	*RD22*	*RESPONSIVE TO DESICCATION 22*	1.61
AT4G23700	*CHX17*	*CATION/H+ EXCHANGER17*	2.50
AT2G46680	*HB-7*	*HOMEOBOX7*	3.43
AT5G06760	*LEA4-5*	*LATE EMBRYOGENESIS ABUNDANT4-5*	4.97
Cluster 2			
Transporter proteins			
AT1G64780	*AMT1;2*	*AMMONIUM TRANSPORTER1;2*	–1.60
AT1G78000	*SEL1 / SULTR1;2*	*SELENATE RESISTANT1 / SULFATE TRANSPORTER 1;2*	–2.79
Cell wall modification			
AT3G44550	*FAR5*	*FATTY ACID REDUCTASE5*	2.14
AT3G44540	*FAR4*	*FATTY ACID REDUCTASE4*	1.71
AT1G04220	*KCS2*	*3-KETOACYL-COA SYNTHASE2 / DAISY*	2.15
AT5G23190	*CYP86B1*	*CYTOCHROME P450, FAMILY 86, SUBFAMILY B, POLYPEPTIDE1*	2.37
AT3G50400	-	A lipase protein	3.42
AT3G11430	*GPAT5*	*GLYCEROL-3-PHOSPHATE SN-2-ACYLTRANSFERASE5*	1.96
AT5G07130	*LAC13*	*LACCASE13*	2.61
AT2G40370	*LAC5*	*LACCASE5*	1.60
AT5G55180	-	*O-Glycosyl hydrolases family 17 protein*	2.06
AT1G80820	*CCR2*	*CINNAMOYL COA REDUCTASE2*	1.77
AT1G51680	*4CL1*	*4-COUMARATE:COA LIGASE1*	1.67
AT3G10340	*PAL4*	*PHENYLALANINE AMMONIA-LYASE4*	2.29
AT1G49430	*LACS2 / LRD2*	*LATERAL ROOT DEVELOPMENT2, LONG-CHAIN ACYL-COA SYNTHETASE2*	1.68

Table 4. (Continued).

AT3G13784	*CWINV5*	*CELL WALL INVERTASE5*	1.98
AT4G19810	*CHIC*	*CLASS V CHITINASE*	2.07
AT1G76470	-	NAD(P)-binding Rossmann-fold superfamily protein	2.42
AT3G60140	*BGLU30 / DIN2 / SRG2*	*BETA GLUCOSIDASE30 / DARK INDUCIBLE2 / SENESCENCE-RELATED GENE2*	3.22
ABA Signaling and osmotic stress response			
AT2G47770	*TSPO*	*OUTER MEMBRANE TRYPTOPHAN-RICH SENSORY PROTEIN-RELATED*	2.98
AT4G19230	*CYP707A1*	*CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE1*	1.73
AT3G61890	*HB12*	*HOMEOBOX12*	3.37
AT5G20270	*HHP1*	*HEPTAHELICAL TRANSMEMBRANE PROTEIN1*	2.52
AT4G28110	*MYB41*	*MYB DOMAIN PROTEIN41*	2.30
AT5G59220	*HAI1 / SAG113*	*HIGHLY ABA-INDUCED PP2C GENE , SENESCENCE ASSOCIATED GENE113*	4.08
AT5G57050	*ABI2*	*ABA INSENSITIVE2*	1.83
AT1G69260	*AFP1*	*ABI FIVE BINDING PROTEIN*	3.02
AT4G26080	*ABI1*	*ABA INSENSITIVE1*	1.56
AT5G52310	*COR78 / LTI78 / RD29A*	*COLD REGULATED78, LOW-TEMPERATURE-INDUCED78, RESPONSIVE TO DESICCATION29A*	2.08
AT1G72770	*HAB1*	*HYPERSENSITIVE TO ABA1*	1.51
AT4G31860	-	Protein phosphatase 2C family protein	1.53
AT3G26744	*SCRM / ICE1*	*SCREAM / CBP EXPRESSION1*	1.56
AT5G66400	*DI8 / RAB18*	*DROUGHT-INDUCED8, RESPONSIVE TO ABA18*	1.62
AT2G35300	*LEA18 / LEA4-2*	*LATE EMBRYOGENESIS ABUNDANT18, LATE EMBRYOGENESIS ABUNDANT4-2*	1.53
AT3G17520	*SSLEA*	Late embryogenesis abundant protein (LEA) family protein	1.74
AT1G52690	*LEA7*	*LATE EMBRYOGENESIS ABUNDANT7*	3.79
AT4G32950	-	Protein phosphatase 2C family protein	3.08
AT1G10370	*ERD9 / GST30B / GSTU17*	*EARLY-RESPONSIVE TO DEHYDRATION9 / GLUTATHIONE S-TRANSFERASE30B / GLUTATHIONE S-TRANSFERASE TAU 17*	2.26
AT3G28210	*SAP12*	*STRESS-ASSOCIATED PROTEIN12*	1.98
AT4G33950	*OST1 / SNRK2.6*	*OPEN STOMATA1 / SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE2.6*	1.79
AT2G26290	*ARSK1*	*ROOT-SPECIFIC KINASE1*	1.76

Microarray results were analyzed by Genespring GX (Agilent) with $p \leq 0.05$ for significant expression.

did not cluster with any other sulfate transporters; yet, they had their own coexpression networks. As expected, all sulfate transporters coexpressed with the genes involved in S metabolism (Cluster 1), glucosinolate synthesis (Cluster 2), and arabinan metabolism (Cluster 3) according to the GO enrichment analysis (Table 6). Although the remaining transporters (*SULTR1;1*, *SULTR1;2*, *SULTR1;3*, *SULTR5;2*, and *SULTR3;2*) had their own coexpression networks, any GO enrichments were not identified in their analysis since the coexpression networks had no connection with each other. When the expression of these genes in each cluster was analyzed under sulfur deficiency, the majority of the Cluster 1 genes were upregulated while the majority of the genes in Cluster 2 were downregulated (Figure 6). Among Cluster 3 genes, especially *BETA GALACTOSIDASE1 (BGAL1)*, *BETA-XYLOSIDASE1 (BXL1)*, and *BETA-XYLOSIDASE4 (BXL4)* were highly suppressed under sulfur deficiency.

Table 5. Enrichment of top 10 biological process GO terms in Clusters 1 and 2.

Cluster	GO biological process term	GO number	Expected %	Fold enrichment	p-value
1	glucosinolate biosynthetic process	GO:0019761	0.07	>100	4.37E-30
	chloride transport	GO:0006821	0.01	>100	8.67E-07
	indoleacetic acid biosynthetic process	GO:0009684	0.02	>100	1.19E-08
	leucine biosynthetic process	GO:0009098	0.02	>100	1.66E-10
	hydrogen peroxide transmembrane transport	GO:0080170	0.01	>100	8.64E-05
	defense response by callose deposition in cell wall	GO:0052544	0.03	>100	4.21E-08
	iron ion transmembrane transport	GO:0034755	0.02	>100	2.03E-04
	sulfur compound biosynthetic process	GO:0044272	0.25	67.57	1.84E-26
	sulfur compound transport	GO:0072348	0.04	50.95	8.37E-04
	glutathione metabolic process	GO:0006749	0.14	21.84	3.96E-04
2	long-chain fatty-acyl-CoA metabolic process	GO:0035336	0.01	>100	7.87E-05
	suberin biosynthetic process	GO:0010345	0.03	>100	2.63E-10
	regulation of stomatal opening	GO:1902456	0.02	>100	2.09E-04
	cutin biosynthetic process	GO:0010143	0.02	82.47	3.30E-04
	fatty acid derivative metabolic process	GO:1901568	0.05	60.18	2.14E-05
	phenylpropanoid biosynthetic process	GO:0009699	0.12	59.04	4.44E-11
	negative regulation of abscisic acid-activated signaling pathway	GO:0009788	0.07	58.21	9.00E-07
	secondary metabolite biosynthetic process	GO:0044550	0.19	36.08	1.17E-09
	negative regulation of signal transduction	GO:0009968	0.14	28.27	1.39E-05
	response to osmotic stress	GO:0006970	0.71	18.27	1.33E-13

The differential regulation of Clusters 1-3 under boron toxicity was confirmed by Gene Set Enrichment Analysis (GSEA). As shown in Table 7, the genes in all three clusters were significantly enriched under boron toxicity as well as sulfur deficiency ($p < 0.05$). However, the genes in Clusters 1 and 3 were not significantly enriched under osmotic stress, suggesting that the cluster of genes obtained from sulfate transporter coexpression networks are specifically overrepresented in B toxicity. The genes in Cluster 2 are overrepresented under osmotic stress and this indicates the potential involvement of cell wall modifications in osmotic stress as expected. Taken together, our coexpression network analysis followed by GSEA proved that the sulfur transporters coexpress with a large set of genes involved in S metabolism were overrepresented under B toxicity in Arabidopsis roots.

4. Discussion

4.1. Sulfate transporters are induced under B toxicity in leaf and root tissues of *A. thaliana*

B at toxic level is one of the major limiting factors for crops in the world, especially in semiarid and arid regions. Therefore, it is important to determine the regulation of excess B in order to develop B-tolerant plants. The findings in our previous studies support an internal B detoxification mechanism via GSH-GST conjugation in plants (Kayıhan et al., 2019; Kayıhan, 2021). The synthesis of GSH begins with uptake of inorganic sulfate, and sulfate is also used for sulfur assimilation and the Cys biosynthesis. The uptake of sulfate is performed through sulfate transporters, which also function in translocation and distribution. Since B toxicity downregulates the genes involved in sulfur and glucosinolate metabolisms, and the high-affinity sulfate transporters, namely *SULTR1;2* and *SULTR2;2* (Aquea et al., 2012), it suggests that the sulfate uptake together with primary sulfur metabolism have a pivotal function in tolerance to B toxicity. For this reason, fine-tune regulation of sulfate uptake and transport can be critical for B tolerance in plants. In this study, we primarily focused on the transcriptional regulation of sulfate transporters in leaf and root tissues of *A. thaliana*. The group 1 sulfate transporters consist of the high-affinity transporters *SULTR1;1*, *SULTR1;2*, and *SULTR1;3*. *SULTR1;1* and *SULTR1;2* are expressed in the epidermis and cortex of roots and facilitate the initial uptake of sulfate from the soil (Yoshimoto et al., 2002). In this study, in root tissues

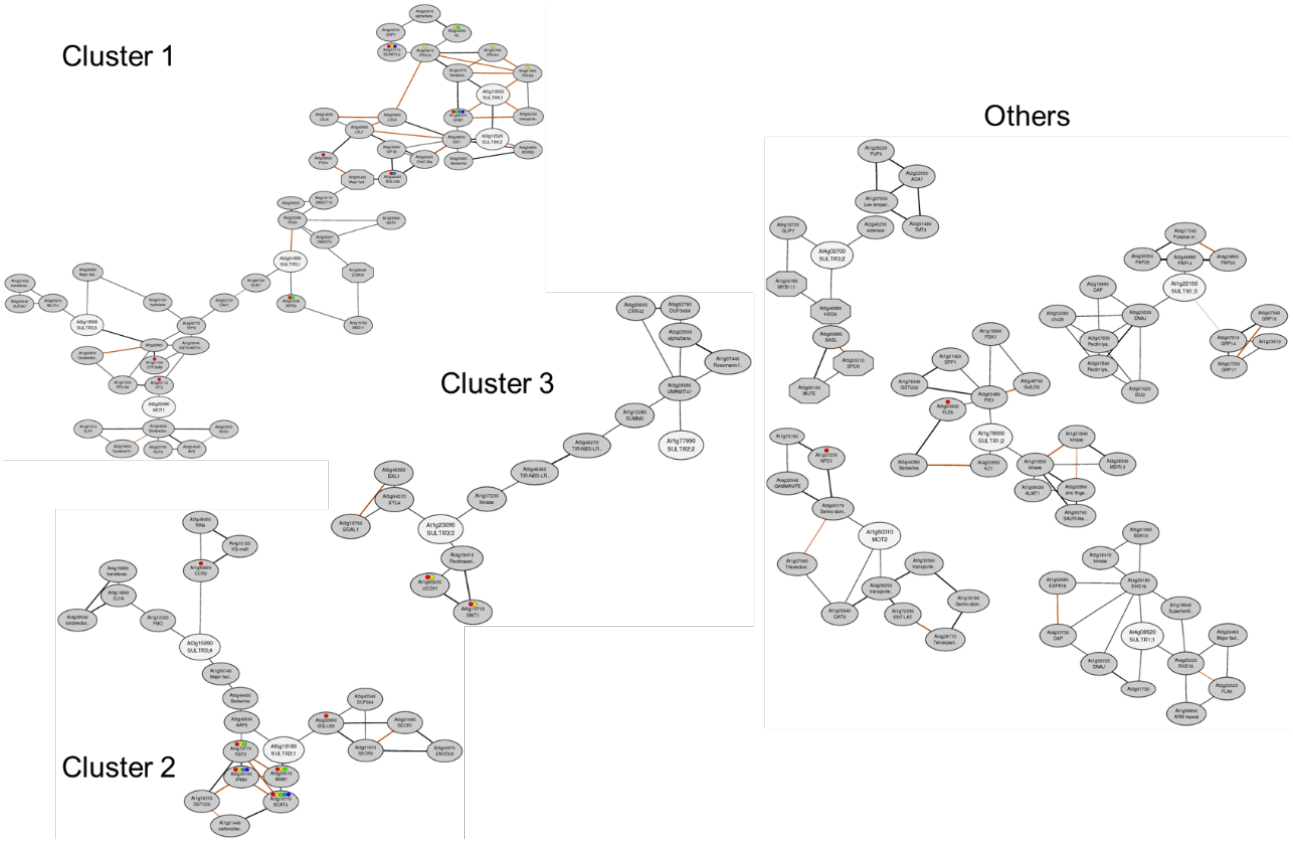

Figure 5. Coexpression network of sulfur transporters in Arabidopsis. The Clusters were generated in Atted II (Obayashi et al., 2006).

Table 6. Enrichment of top 5 biological process GO terms in coexpression clusters.

Cluster	GO biological process term	GO number	Expected %	Fold enrichment	p-value
1	sulfate reduction	GO:0019419	0.01	>100	5.03E-04
	cellular response to sulfur starvation	GO:0010438	0.01	>100	1.20E-03
	cysteine biosynthetic process	GO:0019344	0.04	>100	2.49E-04
	sulfate assimilation	GO:0000103	0.04	>100	2.96E-04
	cysteine metabolic process	GO:0006534	0.05	83.39	7.31E-04
2	glucosinolate biosynthetic process	GO:0019761	0.03	>100	1.01E-04
	glycosyl compound biosynthetic process	GO:1901659	0.05	79.60	6.19E-04
	glucosinolate metabolic process	GO:0019760	0.09	44.77	5.63E-03
	secondary metabolite biosynthetic process	GO:0044550	0.12	42.35	3.30E-04
	sulfur compound biosynthetic process	GO:0044272	0.11	36.87	1.19E-02
3	arabinan catabolic process	GO:0031222	0.00	>100	3.41E-02
	arabinan metabolic process	GO:0031221	0.00	>100	4.17E-02

of *A. thaliana*, *SULTR1;1* expression was significantly upregulated under only 3B condition; however, the expression level of *SULTR1;2* did not significantly change under 1B and 3B conditions. As expected, they were not detected in the leaves. On the other hand, both toxic B treatments caused a sharp increase in the expression levels of *SULTR1;3* in leaf and root tissues. Similarly, its expression was increased both in leaves and roots and was abundantly

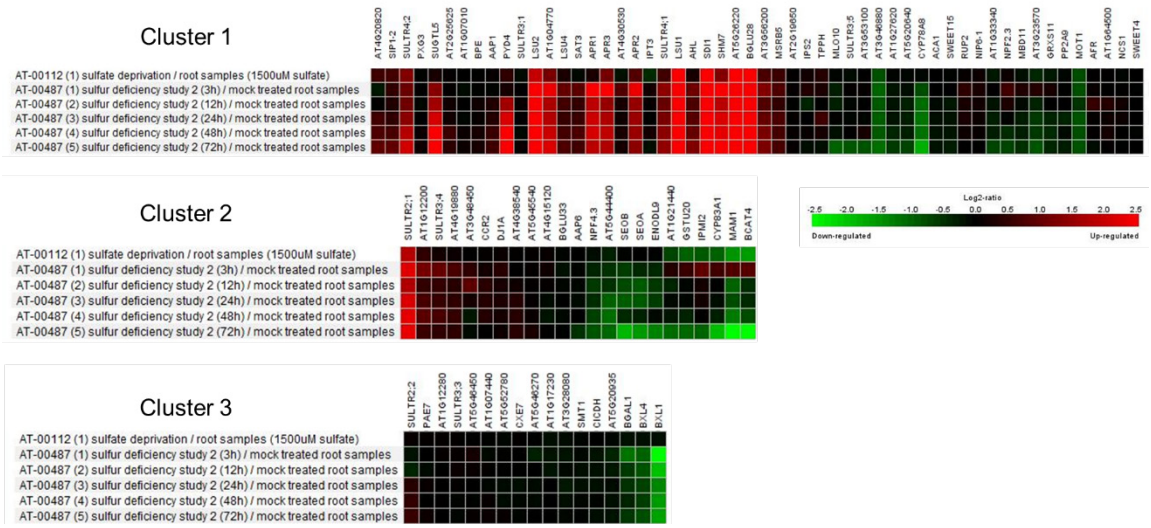

Figure 6. Expression levels of genes involved in the coexpression networks of sulfur transporters under sulfur deficiency. Generated in Genevestigator (see the methods) by using the gene lists in Clusters 1-3 of Figure 5 against two sulfur deficiency microarray experiments in Arabidopsis roots (Maruyama-Nakashita et al., 2006; Iyer-Pascuzzi et al., 2011).

Table 7. Gene Set Enrichment Analysis (GSEA) of Cluster 1-3 Gene Sets under B toxicity and S deficiency

Gene set (Number of genes in the set)	**B toxicity** [a]	**S deficiency** [b]	**S deficiency** [c]	**Osmotic stress** [d]
	p-value			
Cluster 1 (49)	0.025*	0.001*	0.011*	0.179
Cluster 2 (21)	0.008*	0*	0*	0.028*
Cluster 3 (16)	0.048*	0.035*	0.042*	0.072
Reference (20) [a]	0.245	0.158	0.216	0.099

* Indicates a specific gene set significantly enriched ($p < 0.05$) in top-ranked genes.
[a] A reference gene set contains constitutively expressed genes (Czechowski et al., 2005), and was used as a negative control.
[b] Aquea et al., 2012
[c] Maruyama-Nakashita et al., 2006
[d] Iyer-Pascuzzi et al., 2011
[e] Kilian et al., 2007

expressed under sulfur deficiency, particularly in the leaves (Yoshimoto et al., 2003). Moreover, it was upregulated under phosphate deficiency in *A. thaliana* (Rouached et al., 2011). The increased accumulation of *SULTR1;3* mRNA by sulfur limitation were comparable with those observed in *SULTR1;1* and *SULTR1;2* expressions. In this study, the expression levels of *SULTR1;3* were compatible with the expression level of *SULTR1;1* under 3B treatment. SULTR1;3 transporter is more likely responsible for the retrieval of sulfate within the transport phloem in *A. thaliana*. The analysis of the *sultr1;3* mutant suggests that recovery or retrieval of sulfate within the transport phloem significantly promotes the interorgan translocation of sulfate (Yoshimoto et al., 2003). SULTR1;3 in the root phloem helps sulfate uptake directly to the companion cells. Therefore, upregulation of *SULTR1;3* gene expression under B toxicity might be related to overaccumulation of B and ROS. Both of them might trigger the need for additional sulfate for the GSH synthesis. In addition, similar to phosphate deficiency (Rouached et al., 2011), B toxicity might disrupt the homogeneous distribution of sulfate and sulfur-containing compounds in leaf and root tissues of *A. thaliana*.

In this work, the expression levels of *SULTR2;1*, *SULTR2;2* were not significantly affected by both 1B and 3B treatments, and the expression levels of *SULTR3;5*

were significantly decreased under both treatments when compared to the control in leaf tissues. In contrast to *SULTR1;3*, which has a role in loading of sulfate to phloem, low expression levels of *SULTR2;1*, *SULTR2;2*, and *SULTR3;*5 might be related to the restriction of sulfate movement to the xylem because SULTR3;5 has a role in the root-to-shoot transportation of sulfate with SULTR2;1 in *A. thaliana* (Kataoka et al., 2004a). Supportively, Kawashima et al. (2009) suggest that specific miR395 targets *SULTR2;1* and miR395 expression excludes completely SULTR2;1 from phloem and restricts sulfate movement to the xylem. Similar to leaf tissues, *SULTR2;1* expression did not significantly change in the root tissues under both toxic B treatments. Kataoka et al. (2004a) reported that *SULTR3;5* is expressed in the root vasculature of *A. thaliana* showing the same expression pattern as the low-affinity *SULTR2;1*. However, in this study, *SULTR3;5* and *SULTR2;2* mRNAs were expressed in the root tissues under both toxic B conditions. It was found that *SULTR2;2* may play a role in the transport of sulfate via root phloem (Takahashi et al., 2000). Thus, it might be suggested that SULTR3;5 may help SULTR2;2 contributing to root-to-shoot sulfate translocation in *A. thaliana* under B toxicity.

In our study, the expression levels of *SULTR3;3* and *SULTR3;4* were induced in leaf tissues of *A. thaliana* exposed to 1B and 3B conditions. These results suggest that transcriptional regulation of *SULTR3* isoforms for chloroplast sulfate uptake under B toxicity might have a significant influence on Cys, GSH, and even abscisic acid (ABA) biosynthesis because group 3 sulfate transporters are functional for most of the chloroplast sulfate uptake and they affect sulfate assimilation and ABA biosynthesis (Chen et al., 2019). However, both 1B and 3B treatments did not significantly alter the expression level of *SULTR3;1* in *A. thaliana*. This implies the *SULTR3* transporters respond differentially to diverse stresses (Gallardo et al., 2014). On the other hand, SULTR3 and SULTR4 subfamily members such as *SULTR3;1*, *SULTR3;2*, *SULTR3;3*, *SULTR3;4*, *SULTR4;1*, and *SULTR4;2* were induced in the roots under toxic B conditions. They are known to control root-to-shoot sulfate transport in *A. thaliana* (Takahashi et al., 2019). SULTR4;1 and SULTR4;2 facilitate the unloading of sulfate from the vacuoles to increase the flux of sulfate directed toward the xylem in Arabidopsis roots (Takahashi et al., 2019). This suggests that B toxicity might cause induction of root-to-shoot sulfate translocation in *A. thaliana*. Moreover, in this study, *SULTR4;1* expression was significantly increased more than three-fold in the leaves under the 1B treatment. This data implies that the molecular function of this transporter is related to the vacuolar sulfate unloading and this is used for local and long-distance sulfate needs in plants (Takahashi, 2019). Accordingly, *SULTR4;1* might have a role in the regulation response mechanism to enhance the amount of sulfate to be delivered from shoot to root under toxic B conditions. However, the transcript levels of *SULTR4;2* were significantly altered by both 1B and 3B treatments because SULTR4;1 has a primary role in remobilizing sulfate reserves as opposed to SULTR4;2, which was shown to have a slight contribution (Zuber et al., 2010).

4.2. B toxicity and sulfur deficiency affect the expression of common genes involved in sulfur metabolism

The important role of sulfur metabolism in plant stress tolerance was identified by Rausch and Wachter (2005). Afterward, the involvement of S metabolism in tolerance mechanisms of various biotic and abiotic stresses has been investigated in detail (Capaldi et al., 2015; Chan et al., 2019; Samanta et al., 2020). Since the S metabolism is involved in tolerance against some metal toxicities, such as cadmium (Cd), lead (Pb), zinc (Zn), and iron (Fe) (Nocito et al., 2007; Hardulak et al., 2011; Kaur and Hussain, 2020), it was speculated that it could be involved in B toxicity tolerance in plants. Since the same set of genes can be differentially expressed under the deficiency of one mineral and toxicity of another mineral, we first determined that a set of 21 genes were differentially expressed in both boron toxicity and sulfur deficiency in Arabidopsis roots (Figure 3 and Table 2), suggesting similar metabolic pathways may function in plant stress tolerance against both conditions. These genes were enriched in S metabolism, S-glycoside and glucosinolate biosynthetic process, and osmotic stress response (Table 3). Since the biosynthesis of S-glycosides, including glucosinolates, involves the S metabolism (Sønderby et al., 2010), it is no surprise that the common DEGs of B toxicity and sulfur deficiency were enriched in these GOs. As both B toxicity and sulfur deficiency alter ROS production (Ghori et al., 2019), the common set of genes also includes the ones involved in osmotic stress response.

Seventy-two genes expressed uniquely under B toxicity were enriched in GOs related to suberin biosynthesis, hydrogen peroxide transport, and defense response by callose deposition in cell wall, which leads to cell wall thickening (Table S1). These findings are not interesting since B toxicity causes oxidative stress and lipid oxidation of membranes. In parallel, toxic B concentrations change the cell wall composition and integrity (Riaz et al., 2021), and in the long term or under excessive toxicity, suberin and lignin levels increase in the cell wall stiffening the cell wall matrix (Reid et al., 2004). Under B toxicity, S transporters and glucosinolate biosynthesis genes are strongly downregulated (Aquea et al., 2012). Our results prove that enrichment of GOs related to S transporters and glucosinolate biosynthesis under individual B toxicity is an essential phenomenon requiring more detailed investigation.

4.3. B toxicity and sulfur deficiency simultaneously affect the genes involved in sulfur, GSH, and glucosinolate metabolisms

The expression patterns of DEGs in B-treated Arabidopsis roots under sulfur deficiency grouped them in two clusters (Figure 3 and Table 4). The DEGs in Cluster 1 were downregulated when exposed to longer periods of sulfur deficiency and were involved in sulfur, glutathione, and glucosinolate metabolisms, and ion transport (Table 5). The genes involved in glucosinolate metabolisms were shown to be downregulated under sulfur deficiency (Hirai et al., 2004; Falk et al., 2007; Hoefgen and Nikiforova, 2008). These genes were also suppressed under B toxicity. A similar observation was shown by Aquea et al. (2012) that B toxicity downregulates the genes involved in sulfur and glucosinolate metabolisms. Therefore, our findings indicate that the S metabolism, especially glucosinolate biosynthesis, has a pivotal function in tolerance to boron toxicity. A regulatory network among sulfur deficiency, primary metabolism, and glucosinolate metabolism was shown before to be centered around O-acetylserine (Hirai et al., 2004), and it included several transcription factors (Hirai et al., 2005). Glucosinolates are produced from primary sulfur metabolism in Brassica family plants, especially against herbivores (Sønderby et al., 2010). Therefore, sulfur uptake from the rhizosphere is very essential for glucosinolate biosynthesis. However, neither the functions of glucosinolates nor the transcription factors in the network have been investigated in B toxicity tolerance yet.

Although there are no specific studies on the importance of S metabolism under B toxicity, it is known that the S metabolism is essential for heavy metal toxicity tolerance in plants via the production of cysteine (Cys) (Domínguez-Solís et al., 2004), methionine (Shahid et al., 2014), glucosinolates (Sun et al., 2009), and the major antioxidant GSH (Amist and Singh, 2020). Moreover, several studies showed the positive effects of sulfate and sulfur metabolites in the alleviation of heavy metal toxicity symptoms (Dixit et al., 2015; Ahikari et al., 2018; Ding et al., 2019; Huang et al., 2019; Lu et al., 2019), signifying the essentiality of primary and secondary sulfur metabolism in metal stress tolerance in different plant families (Babula et al., 2012). A recent RNA-sequencing study in alfalfa revealed the induction of the genes involved in sulfur and glutathione metabolisms, and oxidative stress (Cui et al., 2020). The synthesis of GSH and phytochelatins (PCs) is increased under proper S supply, which confers the tolerance to Cd (Rabêlo et al., 2018). Moreover, the application of S significantly enhanced the tolerance of oilseed rape exposed to chromium stress by activating several detoxification mechanisms including the ascorbate-glutathione enzyme defense system and GSH production (Zhang et al., 2018). It was shown that the metabolic engineering of *Brassica napus* via overexpression of a tobacco serine acetyltransferase (SAT), the rate-limiting enzyme of Cys biosynthesis, enhanced the Cys (3.5-fold) and GSH (5.3-fold) levels; therefore, enhanced tolerance against hydrogen peroxide- and Cd-based oxidative stress (Rajab et al., 2020). Therefore, our results indicate the importance of balanced S metabolism in efficient protection against B toxicity in plants.

4.4. Genes involved in cell wall modification and ABA-based osmotic stress tolerance are induced under B toxicity and sulfur deficiency

The genes involved in Cluster 2 were involved in phenylpropanoid biosynthesis, cell wall modification, ABA signaling, and osmotic stress tolerance, and they were significantly upregulated under both sulfur deficiency and B toxicity (Figure 4). This result indicates that i) cell wall modifications are one of the important cellular responses against both stresses, ii) general osmotic stress response mechanisms via ABA signaling is activated under both stress conditions. It is known that B is essential for cross-linking of cell wall rhammogalacturonan II (RGII) and pectin; therefore, it is required for cell wall structure and function (O'Neill et al., 2004). This is why more than 80% of B is located in the cell wall of vascular plants (Hu and Brown, 1994). Hence, B homeostasis is necessary to regulate the cell wall structure and plant development. B deficiency causes an alteration in cell wall composition such that the soluble polyamines increase and cell wall pectins are modified while its toxicity inhibits the cell wall expansion (Camacho-Cristóbal et al., 2008a). It was also shown that the genes involved in cell wall biosynthesis and integrity were downregulated under B deficiency (Camacho-Cristóbal et al., 2008b) while upregulated under B toxicity (Day and Aasim, 2020). The same situation was also observed under S deficiency. Cell wall structural proteins accumulated more (Fernandes et al., 2013), and the genes encoding for them were highly induced under S deficiency in *Vitis vinifera* callus (Fernandes et al., 2016). The same results were also observed in *Chlamydomonas reinhardtii* (Takahashi et al., 2001). Additionally, the cell wall-related transcripts were differentially expressed in *serat* quadruple mutants, which have altered Cys levels (Watanabe et al., 2010). Taken together, our results show that B toxicity and S deficiency affect the plant cell wall integrity and function.

Upregulation of the genes involved in ABA signaling and osmotic stress tolerance under B toxicity was predicted as a general stress response of plants (Aquea et al., 2012); however, they were later shown to be important in tolerance against B toxicity (Macho-Rivero et al., 2017). Under B toxicity, the gene responsible for ABA biosynthesis, *AtNCED3*, was highly induced in the roots and ABA

levels were increased in the shoots in Arabidopsis. ABA-deficient *nced3* mutants accumulated more B in the shoots (Macho-Rivero et al., 2017). ABA application decreased the B level in the shoots under B toxicity. Since ABA is required for decreasing the oxidative damage caused by ROS under environmental stress conditions, upregulation of genes involved in ABA signaling and osmotic stress tolerance indicates the enhancement of oxidative stress tolerance mechanisms under B toxicity. The Cluster 2 genes involved in ABA signaling and osmotic stress tolerance were also induced in sulfur deficiency. Sulfate supply affects the synthesis and steady-state levels of ABA, and ABA induces the expression of S-metabolism-related genes in Arabidopsis (Cao et al., 2014). Taken together, our results suggest coregulation of S-metabolism and ABA biosynthesis that operates to ensure sufficient Cys, GSH, and glucosinolate levels to tolerate B toxicity. However, further studies are required to elucidate this mechanism.

4.5. GSTs are involved in B toxicity response in Arabidopsis and affected by S availability

According to our hierarchical clustering, *GSTU20*, *GSTF6/GSTF11*, and *GSTF9* were suppressed under S deficiency and B toxicity in Cluster 1, whereas *ERD9/GST30B/GSTU17* was induced under S deficiency and B toxicity in Cluster 2 (Table 4). Identification of these GSTs under B toxicity indicates their involvement in tolerance mechanisms. In addition to *GSTU19* and *GSTZ1*, these identified GSTs might have some special protective roles in B toxicity tolerance via GSH-GST conjugation (Landi et al., 2015; Kayıhan et al., 2019). Induction of some GSTs, including *GSTU20* and *GSTF6/GSTF11* were shown under arsenate toxicity in maize (Mylona et al., 1998) and Arabidopsis (Abercrombie et al., 2008). Moreover, GSTF9 protein was enriched in Arabidopsis roots after exposure to Cd for 24 h (Roth et al., 2006). Interestingly, an in silico analysis demonstrated the importance of *GSTU20* and *AtGSTF11* as the hub of methionine and tryptophan-derived glucosinolate biosynthesis in Arabidopsis (Buxdorf et al., 2013). Therefore, these GSTs may not only function in B toxicity tolerance via GSH-GST conjugation but also by regulating glucosinolate production (Aarabi et al., 2020). The GSTs in Cluster 1 was also shown to be suppressed under S deficiency in previous studies (Henríquez-Valencia et al., 2018; Watanabe and Hoefgen, 2019), demonstrating their potential functions in S deficiency tolerance in plants. Opposite to the GSTs in Cluster 1, induction of *ERD9/GST30B/GSTU17* under S deficiency can be explained by its roles in ABA-based oxidative stress tolerance, most probably in relation to GHS-based ROS scavenging activities under abiotic stresses (Chen et al., 2012; Hahn et al., 2013). Taken together, our results point out the significance of S metabolism to keep a constant level of total GST activities in the cell to support an internal B detoxification mechanism via GSH-GST conjugation and/or glucosinolate biosynthesis in plants.

4.6. S transporter coexpression networks are divided in three clusters which are overrepresented in B toxicity

B toxicity downregulates the high-affinity sulfate transporters, namely *SULTR1;2* and *SULTR2;2* (Aquea et al., 2012), which are induced by S deficiency in *Arabidopsis thaliana* (Takahashi, 2019). *SULTR* orthologs were also highly suppressed under B toxicity in sensitive barley cultivar (*Hordeum vulgare* cv. Hamidiye) (Öz et al., 2009). On the other hand, our gene expression analysis showed induction in *SULTR1;1* under high B toxicity in the roots while the *SULTR1;2* expressions were not changed (Figure 2), suggesting the significance of sulfur level in the plant required to activate the appropriate metabolic pathway to ensure tolerance. Similar to our observations, many *SULTR* genes were upregulated under Cd in different plant species (Ferri et al., 2017; Yamaguchi et al., 2017; Akbudak et al., 2018; Yamaguchi et al., 2020) since the biosynthesis of GSH and glucosinolates starts with the Cys and S assimilation, and the levels of Cys and GSH decline under S deficiency (Panthee et al., 2006; Reinbold et al., 2008). Therefore, proper functioning of proteins involved in sulfate uptake, assimilation, and conjugation is required for the B detoxification mechanism via GSH-GST conjugation in plants. The coexpression network of sulfate transporters identified three main clusters (Figure 5).

The genes in the Cluster 1 are related to sulfate assimilation and Cys biosynthesis while the ones in Clusters 2 and 3 are involved in glucosinolate biosynthesis and arabinan metabolism, respectively (Table 6). Although an alteration in B homeostasis decreases the cell wall integrity by affecting the RG-II cross-linking (Camacho-Cristóbal et al., 2008b; Day and Aasim, 2020), our GO enrichment and GSEA results suggest the involvement of arabinan metabolism in B toxicity tolerance. The plant cell wall is made up of rhamnogalacturonan type I (RG-I) residues in addition to RG-II, and RG-I is composed of D-galactose-rhamnose (Rha) units, in which the Rha residues can be substituted with different side chains including type-I arabinogalactan, arabinan, and galactan (Atmodjo et al., 2013). Our detailed in silico analyses determined three genes, namely *BGAL1*, *BXL1*, and *BXL4*, as highly suppressed under S deficiency (Figure 6). *BGAL1* encodes for a β-galactosidase that functions in β-(1,4)-galactan remodeling in Arabidopsis cell walls (Moneo-Sánchez et al., 2019). BXL1 and BXL4 are β-D-xylosidase/α-L-arabinofuranosidases thought to remove L-arabinofuranose from RG-I (Arsovski et al., 2009); therefore, they are required for pectic arabinan modification by avoiding RG-I cross-linking (Showalter and Basu, 2016). Barley orthologs of *BGAL1*, *BXL1*, and *BXL4* were shown to be highly downregulated under boron

toxicity (Öz et al., 2009). Therefore, it is no surprise that the genes linked to the arabinan biosynthesis coexpress together with sulfate transporters and are overrepresented under B toxicity. Recent studies also showed that boron cross-link glycosyl inositol phosphorylcer amides of the plasma membrane with arabinogalactan proteins (AGPs) of the cell wall, thereby attaching the membrane to the cell wall (Tenhaken, 2015). The expression of these genes was shown to be downregulated in Arabidopsis roots under B deficiency (Camacho-Cristóbal et al., 2008b). Furthermore, the expression of *AGP* genes was shown to be downregulated under B deficiency in tobacco cells (Sardar et al., 2006). AGPs may play an essential role in the B deficiency signal transduction by binding Ca^{2+} and altering the actin structure (González-Fontes et al., 2014), and accumulate throughout the pollen tube under B toxicity in apple (Fang et al., 2016). On the other hand, in another study, the expression of xyloglucan endotransglycosylase/hydrolases, expansins, and pectate lyases were shown to be induced in oilseed rape leaves under deficient and excessive B conditions (Hua et al., 2017). These results indicate the essential connection between the sulfate uptake and cell wall integrity under B toxicity; therefore, it should be further studied in the future.

5. Conclusion

In conclusion, here we provided strong evidence for the involvement of sulfur uptake and metabolism under B toxicity. Firstly, the B toxicity and sulfur deficiency caused differential expression of the same set of genes involved in glucosinolate biosynthetic processes, sulfur metabolism, and osmotic stress. Additionally, a subset of differentially expressed genes in B-treated Arabidopsis roots was downregulated altogether under sulfur deficiency and

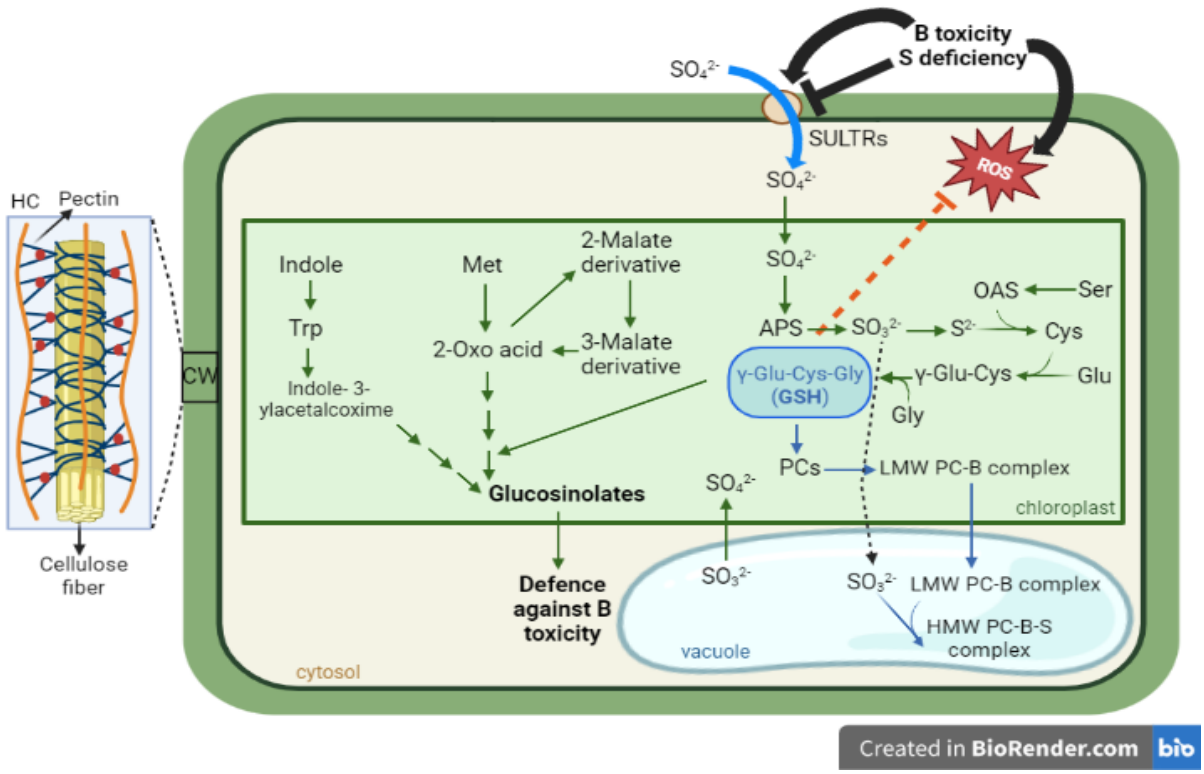

Figure 7. Proposed model of action under B toxicity or S deficiency in plant cells. S deficiency inhibits the SULTR transporters and therefore decreases the S metabolism. Reduced glutathione (GSH), phytochelatin (PC), and glucosinolate production is inhibited. B toxicity activates SULTR transporters, increasing the sulfate (SO_4^{2-}) influx in the cell, conversion to sulfite (SO_3^{2}) and sulfide (S^{2-}) in the chloroplast. S^{2-} is used to produce cysteine (Cys) and GSH through addition of glutamate (Glu) and glycine (Gly) in a two-step biosynthesis pathway. GSH is used to produce PCs that complex with excessive B and sequester it in the vacuole by conjugation with SO_3^{2-}. Meanwhile, tryptophan driven from indole metabolism and methionine are used in the production of glucosinolates, secondary metabolites required for B toxicity tolerance. GSH is also involved in modification steps of glucosinolates. GSH is also used as a ROS scavenger under both B toxicity and S deficiency by forming complexes with glutathione S-transferases (GSTs). Finally, excessive B alters the cell wall (CW) structure by remodeling it via affecting the rhamnogalacturonan type (RG-II) cross-linking. Ser: serine. LMW: low molecular weight. HMW: high molecular weight. HC: hemicellulose. Modified from Gigolashvili and Kopriva (2014), Gao et al. (2014), and Chia (2021). Created with BioRender.com.

was related to sulfur assimilation, glucosinolate and GSH production, and ion transport. We proved that some sulfur transporters were induced under B toxicity in Arabidopsis leaves and roots. Finally, sulfur transporters were coexpressed with a large set of genes involved in sulfur metabolism and glucosinolate biosynthesis as well as cell wall modification, and they were overrepresented in B toxicity. We suggest that B toxicity can cause vacuolar sulfate unloading, chloroplast sulfate uptake, and loading of sulfate to the phloem to raise the amount of sulfate and thus transport the sulfate from shoot to root and might induce root to shoot sulfate translocation because B toxicity might disrupt the homogeneous distribution of sulfate and sulfur-containing compounds in leaf and root tissues of *A. thaliana* (Figure 7). Although some clues have been presented here on the molecular regulation of sulfate transporters under B toxicity in plants, further studies related to the changes in sulfate uptake, transport, and distribution caused by excess B are required at biochemical and molecular levels.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Table S1. Enrichment of top 10 biological process GO terms in DEGs under only B toxicity, or S deficiency.

GO biological process term	GO number	Expected %	Fold enrichment	p-value
Only under B toxicity (72 genes)				
adventitious root development	0048830	0.02	>100	1.87E-04
ammonium homeostasis	0097272	0.02	>100	1.87E-04
hydrogen peroxide transmembrane transport	0080170	0.02	>100	1.87E-04
indoleacetic acid biosynthetic process	0009684	0.03	>100	6.13E-06
chloride transport	0006821	0.02	95.24	3.00E-04
suberin biosynthetic process	0010345	0.06	86.58	8.24E-09
glucosinolate biosynthetic process	0019761	0.11	81.64	9.58E-15
defense response by callose deposition in cell wall	0052544	0.04	71.43	1.62E-05
sulfate transport	0008272	0.03	69.27	5.17E-04
defense response by cell wall thickening	0052482	0.04	67.23	1.90E-05
Only under S deficiency (667 genes)				
sulfate transmembrane transporter activity	0015116	0.33	15.22	4.28E-02