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The Sulfate Transporter Family in Wheat: Tissue-Specific Gene Expression in Relation to Nutrition

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ABSTRACT Sulfate uptake and distribution in plants are managed by the differential expression of a family of transporters, developmentally, spatially, and in response to sulfur nutrition. Elucidation of the signaling pathways involved requires a knowledge of the component parts and their interactions. Here, the expression patterns of the full complement of sulfate transporters in wheat, as influenced by development and sulfur nutrition, are described. The 10 wheat sulfate transporters characterized here are compared to the gene families for both rice and *Brachypodium*, for whom full genome information is available. Expression is reported in young seedlings with a focus on roles in uptake from nutrient solution and differential expression in relation to sulfate deprivation. In addition, patterns of expression in all organs at the grain filling stage are reported and indicate differential responses to nutritional signals of the individual transporters in specific tissues and an overall coordination of uptake, storage, and remobilization to deliver sulfur to the developing grain.

Key words: Sulfate transporter; wheat; transcriptional regulation; sulfur nutrition; grain filling.

INTRODUCTION

The SulP gene family in plants comprises a small family of, for example, 14 genes in *Arabidopsis* and 15 genes in rice, which are responsible for trans-membrane transport of sulfate and other oxy-anions. The gene family may be sub-divided on the basis of primary sequence into five or more distinct clades, designated Groups 1–5 (Hawkesford, 2003; Buchner et al., 2004c). Furthermore, functional and expression-based patterns for the Groups are indicative of differences in roles within the plant. Plasma membrane-located high-affinity sulfate transporters, which show sulfate-deprivation-induced expression in the roots, belong to Group 1, whilst low-affinity transporters expressed in vascular tissues represent Group 2 (Smith et al., 1995, 1997; Takahashi et al., 2000). Group 3 is enigmatic, probably representing multiple 'types', with one example being involved in modulation of the activity of the *Arabidopsis* Group 2 sulfate transporter, SULTR2;1 (Kataoka et al., 2004a). Group 4 represents transporters involved in vacuolar efflux (Kataoka et al., 2004b), whilst one Group 5 transporter appears to be involved specifically in molybdenum accumulation (Baxter et al., 2008; Tomatsu et al., 2007).

A distinctive feature of several members of the family is a modulation of expression dependent upon sulfur nutrition: high demand or limited availability results in increased expression of some sulfate transporters, manifested by increased mRNA abundance. A widely held belief is that one or more metabolite pools involved in cysteine and/or glutathione biosynthesis are the primary signals in a transduction pathway, which results in the increased mRNA pools (Hawkesford and Wray, 2000) or post-transcriptional regulation of these pools (Yoshimoto et al., 2007).

In wheat, an adequate supply of sulfur is required for optimum yield and quality. Sulfur is a constituent of amino acids, redox compounds, and many secondary metabolites contributing to both abiotic and biotic stress responses. The composition of seed storage proteins is influenced by sulfur availability and affects dough functionality (Zhao et al., 1999). An adequate supply without fertilization is by no means guaranteed in agronomic systems, particularly with diminished deposition due to decreased industrial emissions. An additional and important role of sulfate transporters is in the acquisition of other nutritionally important micronutrients

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such as selenium (Hawkesford and Zhao, 2007) and molybdenum (Baxter et al., 2008; Fitzpatrick et al., 2008; Tomatsu et al., 2005, 2007), which are both taken up as oxyanions by the same sulfate transporters and whose rate of uptake is likely to be influenced by both transporter expression levels and competition with bio-available sulfate.

Understanding the controls that influence optimal uptake and partitioning will be essential for selecting crops and designing crop management systems with high yields and quality; fundamental to this is an understanding of plant responses to nutritional conditions. De-convoluting nutritional responses includes the identification of the important triggering environmental cues and an approach to this is via correlation of changing nutritional status with specific plant responses, which include potential signal molecules and pathway component activity, including gene expression, in a complex system; here, we describe the correlation of sulfate transporter expression with specific tissue nutritional information.

Manipulation of regulatory systems to enhance nutrient acquisition and/or utilization efficiency via plant transgenesis or breeding will enable the design of crops for the future.

RESULTS

The Wheat Sulfate Transporter Gene Family

A combination of analysis of public databases and reverse transcription (RT) as well as genomic PCR approaches revealed 10 different Group 1–4 sulfate transporter genes (Buchner et al., 2004a; Table 1 for accession and gene index numbers). For Group 5, a partial cDNA sequence homologous to the rice Sultr5;1 (AK100928) was identified in the database (CF554492). The Group 5 transporter is likely to be involved in molybdate transport (Baxter et al., 2008; Tomatsu et al., 2007) and is only distantly related to Group 1–4 sulfate transporters on the basis of sequence; the Group 5 was not included into the phylogenetic analysis. Phylogenetic comparison of the wheat Group 1–4 sulfate transporters to the rice and *Brachypodium* sulfate transporter gene families indicated differences in three groups (Figure 1). Although Group 1, the high-affinity sulfate transporter Group, contains three genes in wheat similar to rice, the Sultr1;1 type is duplicated into Sultr1;1a and 1;1b (Buchner et al., 2004a) and the Sultr1;2 type is absent. The corresponding gene in wheat was not identified via RT–PCR or genomic PCR using degenerated primers homologous to the Sultr1;2-type genes (based on sequences derived from other monocotyledonous and dicotyledonous plant species). The Sultr1;3 type is present in wheat as well as in rice and *Brachypodium distachyon*. Group 2 contains two genes in rice but only one in wheat or *Brachypodium*. Approaches using RT–PCR or genomic PCR, using degenerated primers homologous to the Sultr2;2-type genes based on sequences derived from other monocotyledonous and dicotyledonous plant species, were not able to identify a second gene from wheat; genomic database analysis of the published *Brachypodium* genome also failed to identify a homolog. The rice genome contains six Group 3 sulfate transporter genes, whilst the *Brachypodium* genome contains only five, as found for *Arabidopsis*. In wheat, five Group 3 sulfate transporter genes were identified (Figure 1).

General Expression Pattern of the Wheat Sulfate Transporter Family in Seedlings and the Influence of Sulfur Deprivation

The influences of sulfate deprivation on plant growth, sulfate content, and sulfate transporter gene expression were studied in 2–3-week-old wheat plants in a hydroponic culture system. When the plants were grown under sufficient sulfur supply, more than two-fold increases in shoot and root fresh weights were observed during the 7 d of culture. Root but not shoot fresh weight increased in the S-deprived plants. A significant reduction for the shoot growth of more than 50% was found

Table 1. Sulfate Transporter Gene Names, Accession, and Gene Index Numbers of Identified cDNA/Genomic Sequences and Respective Primer Sequences Used for sq-RT–PCR Expression Analysis.

		Oligonucleotide primer sequences used for sq-RT–PCR – 5′ to 3′	
	Accession/gene index numbers	Forward	Reverse
TaeSultr1;1a	AJ512821*	ACGTATCCATCTGCACATAGG	GACCGATGGCTATATCCCTGG
TaeSultr1;1b	AJ512820*	n.d.	n.d.
TaeSultr1;3	BT009249#	GGATTGACCATCGCAAGTCTCT	CCAGGAAAGATACGCCAATCAC
TaeSultr2;1	TC366953/ TC291347#	CCGGATCTCTATCCTCGTGCTA	GATGAAAGTCGCGTTGATGAAGC
TaeSultr3;1	FN432835*	CGCCATGTTCAAGAACTACCAC	CGTCGTGTACTGGTCCATCCT
TaeSultr3;2	FN599528*	n.d.	n.d.
TaeSultr3;3	TC272130/ TC259376#	GTCGAGTCGGCCATCTACTTC	CCCTAACCACACTCTGCATCC
TaeSultr3;4	TC318325/ TC314180#	GATGGACAAGATGGATTTCCTCG	GATTGTGCGCGTCATTCGCTC
TaeSultr3;5	AM747385*	CCAACATCCTCGTCTTGCAGC	CTTCGTGTTTGCTCGCAGCTC
TaeSultr4;1	BT009340#	GCTGTCACTGGCCTGGTAGATT	CGCTATAGCAATCTGGATGTCG
TaeActin 2	TC234027#	CCTTCAATGTTCCAGCCATGTA	ATAGTTGAGCCACCACTGAGCA

*Sequences derived by cDNA/genomic DNA isolation and sequencing; # sequences derived from database analysis. TC gene index numbers were derived from the TIGR gene indices (Quackenbush et al., 2000).

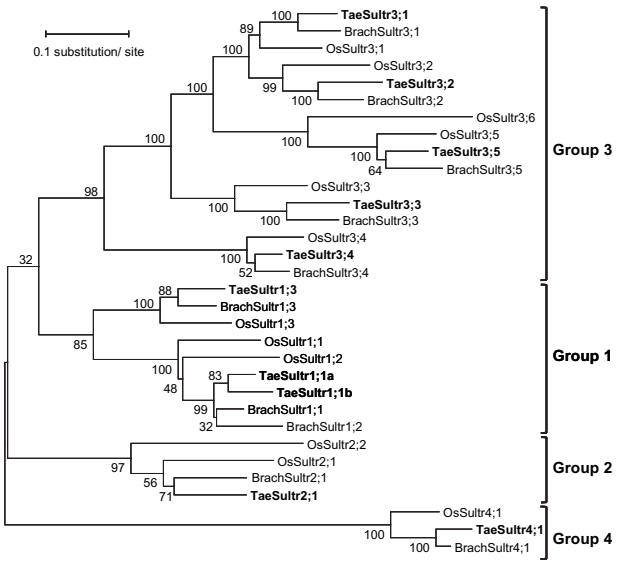

Figure 1. Phylogenetic Analysis: Neighbour-Joining Tree (Mega 3, Kumar et al., 2004) from the Multiple Alignment (ClustalX V.1.81, Thompson et al., 1997) of the Coding cDNAs of the *Triticum aestivum*, *Oryza sativa*, and *Brachypodium distachyon* Sulfate Transporter Gene Family.

The *Brachypodium distachyon* coding sulfate transporter sequences were extracted from genomic sequence data that were produced by the US Department of Energy Joint Genome Institute (www.jgi.doe.gov/). The bootstrap values, expressed as a percentage, were obtained from 1000 replicate trees.

at day 7 compared with the +S culture (Figure 2A and 2B). Under continuous sulfur supply, the sulfate content also increased in both roots and shoots. In contrast, a drastic decrease in the tissue sulfate concentrations were seen in the S-deprived culture in both roots and shoots (Figure 2).

Expression patterns of the individual members of the wheat sulfate transporter family were analyzed in roots and shoots of the hydroponically grown plants (non-sulfur-depleted) at day 0. In roots, the highest relative transcript amounts were found for TaeSultr1;1 and TaeSultr4;1 (Figure 3A), followed by TaeSultr2;1. The expression of TaeSultr1;3 and TaeSultr3;4 were similar to each other and 7–10-fold lower compared to TaeSultr1;1. The relative transcript amount of TaeSultr3;5 was 18-fold lower compared to TaeSultr1;1. Very weak expression of TaeSultr3;1 and TaeSultr3;3 was detected in the root. In shoots, TaeSultr1;3, TaeSultr2;1, and TaeSultr4;1 were the highest expressed sulfate transporter genes, with lower expression of TaeSultr1;1 and TaeST3;4 (Figure 3A). Expression of TaeSultr3;1, TaeSultr3;3, and TaeSultr3;5 in the shoot was very low, as found for the root. TaeSultr4;1 expression was similar in roots and shoots. Higher root expression was found for TaeSultr1;1, whilst TaeSultr2;1 and TaeSultr3;5 showed higher transcript amounts in the shoot compared to the root; TaeSultr3;4 showed higher amounts of transcripts in the shoot compared to the root. Although the transcript levels of TaeSultr3;1, TaeSultr3;3, and TaeSultr3;5 were very low, a differential patterns was observed between roots and shoots

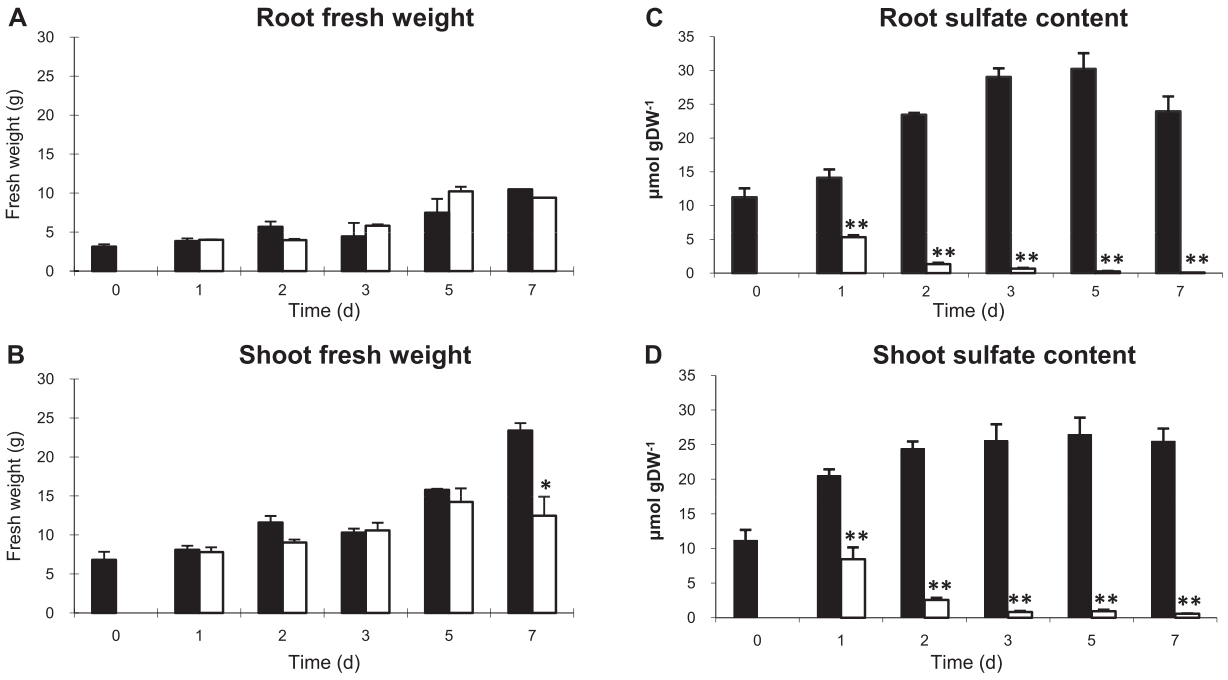

Figure 2. Influence of Sulfate Deprivation on Growth and Sulfate Content in Hydroponically Cultured Plants.

(A, B) Root and shoot fresh weight in relation to + (filled bars) and – (open bars) sulfate supply.

(C, D) Sulfate content in roots and shoots in relation to + (filled bars) and – (open bars) sulfate supply. Each bar represents the mean ± SE of at least three replicates. The data were submitted to *t*-test variance analysis with two-tailed distribution and two-sample equal variance and the stars represent *p*-values: * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. Time (d) = days.

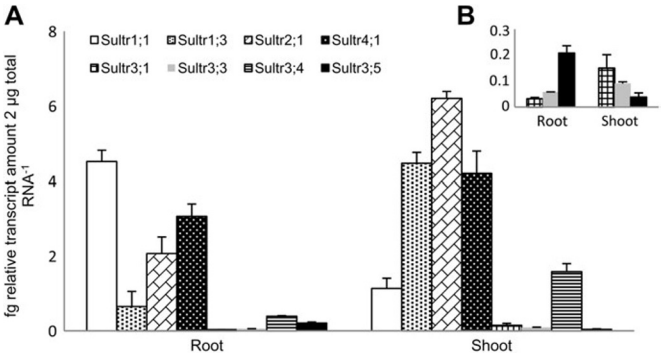

Figure 3. Sulfate Transporter Gene Expression in Roots and Shoots of Wheat.

(A) Transcript levels of wheat sulfate transporters in roots and shoots of hydroponically cultivated plants. Sq–PCRs were performed with 30 cycles for all sulfate transporters apart from TaeST3;3, which required 40 cycles for amplification. Transcript levels were calibrated as described in the Methods section.

(B) Scale enlargement of transcript levels of TaeSultr3;1, 3;3, and 3;5. Each bar represents the mean ± SE of at least three replicates.

(Figure 3B). The transcript level of TaeSultr3;1 was three times higher in the shoot; the opposite was found for the level of TaeSult3.5 transcript, with 16-fold higher transcript in the root; finally, the expression of TaeSultr3;3 was similar in roots and shoots. For the Group 3 sulfate transporter, TaeSultr3;2, no transcript could be amplified from either from root or shoot RNA.

Sulfur starvation induced significant changes in expression of TaeSultr1;1, TaeSultr2;1, and TaeSultr4;1 (Figure 4) in roots: within 2 d of sulfate deprivation, the expression level of both TaeSultr1;1 and TaeSultr4;1 increased up to eight-fold compared to day 0 and remained high throughout the 7 d of the starvation experiment. A significant increase in TaeSultr2;1 expression was detected only after 5 d of sulfate deprivation. TaeSultr1;3, for which the *Arabidopsis* homologous gene is upregulated by diminished sulfate supply (Yoshimoto et al., 2003), did not show a significant change in expression in roots or shoots. As shown in Figure 3, there was expression of TaeSultr1;1 in shoots; in sulfate-starved plants, an increased expression was seen from day 2 of deprivation that was up to 30 times higher at day 7 compared with day 0. An increase in expression from day 2 was found for TaeSultr4;1, with an approximate six to seven-fold increase under sulfate deprivation compared with the +S treatment. The increase in expression of TaeSultr2;1 in shoots was not statistically significant (Figure 4). As in Brassica (Buchner et al., 2004b), no influence of sulfate supply on the relative expression of all Group 3 sulfate transporters was found in roots or shoots (Figure 4).

Influence of Sulfate Starvation on Sulfate Levels and Gene Expression of Sulfate Transporters during Grain Development

Wheat plants were grown under sufficient sulfur supply until anthesis and, thereafter, the influence of the removal of a sulfate supply on tissue sulfate content and specific sulfate transporter gene expression in defined tissues was analyzed for 4 weeks, corresponding to a critical period of grain filling. A significant reduction in tissue sulfate content was found in the glume/lemna tissues 1 week after anthesis (Figure 5G). Although the supply of sulfate ceased at anthesis, a significant reduction in sulfate was not detected in leaf and sheath tissues until 2 weeks after anthesis (Figure 5A–5D). In stems, rachis, and grain, a significant reduction was not found until 3 weeks after anthesis (Figure 5E, 5F, and 5H). Compared with the plants with sufficient sulfate supply, the sulfate content in all leaves and sheaths was reduced down to less than 10 and 16%, respectively, by 4 weeks after anthesis. A similar reduction was found in the rachis tissue. In the stems, as well as in the glume/lemna tissues and additionally in the grain, the reduction in sulfate content was less than in the other tissues. In the stems, 4 weeks after anthesis, the sulfate content was 33% compared to sufficient sulfate supply, which was three-fold more than in S-deprived leaves. In the sink tissues comprising the glumes/lemnas and the grain, the sulfate content was reduced to 49 and 55%, respectively, compared to the controls, which probably represents remobilized sulfate from tissues such as the leaves and sheaths.

Sulfate deprivation had a small influence on the total sulfate content in grains. In mature grain, the total S content was 87% in the sulfur-starved compared to S-sufficient material. In the S-deprived plants, all sulfur delivered to the grain must be released via remobilization from other plant tissues. Furthermore, no sulfur starvation-related plant phenotype was observed during the post-anthesis starvation period, indicating that sufficient sulfate accumulation had occurred prior to anthesis to allow normal further development until senescence and grain ripening.

To verify how sulfate transporter gene expression may be coupled to the remobilization process, the relative expression levels in the different tissues were analyzed. As in the hydroponic seedling root/shoot experiment, the expression of all analyzed sulfate transporter genes could be detected in all tissues apart from the grain, in which no expression of the TaeSultr2;1 could be found.

The relative transcript amounts of the individual sulfate transporters were compared in leaves, sheath, stem, and rachis at anthesis and in glume/lemnas and the grain at 1 week post anthesis (Figure 6). With few exceptions, a similar pattern of expression to that found for hydroponically grown plants was observed (compare Figure 6 with Figure 3). With the exception of the grain, where TaeSultr2;1 appeared to be not expressed, TaeSultr2;1 was the most abundant transcript in all tissues. The transcript levels of TaeSultr1;3 and 4;1 were similar in all tissues, including the grain. The level of TaeSultr3;4 was eight-fold reduced compared with the highest TaeSultr2;1 level, but was very consistent in all tissues apart from the glume/lemnas, where much lower expression was seen. Transcripts of TaeSultr1;1 could be detected in all tissues but at variable abundance. In the glume/lemna fraction, the TaeSultr1;1 transcript abundance was similar to that of TaeSultr1;3 and

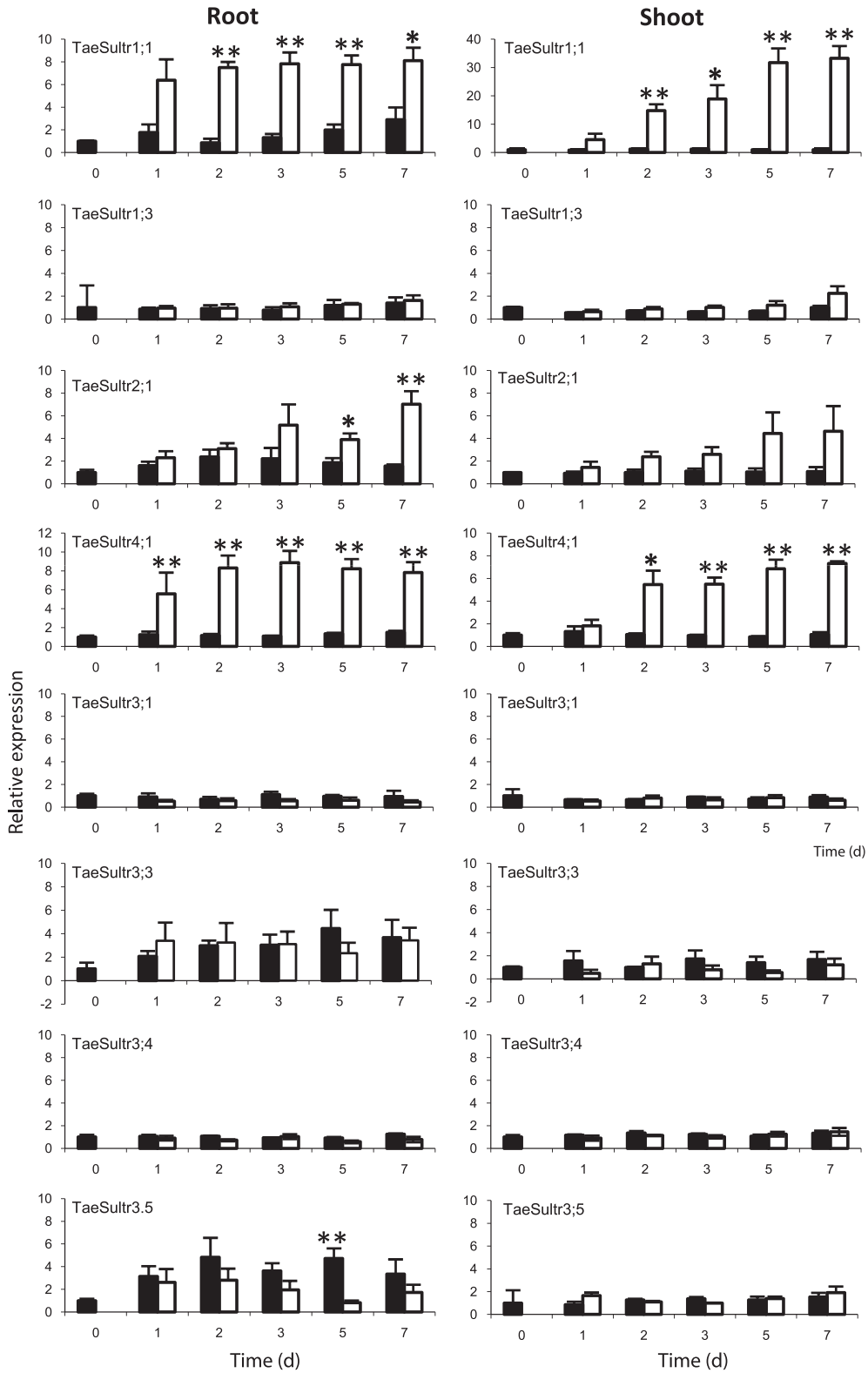

Figure 4. Influence of Sulfur Deprivation (+ (Filled Bars) and – (Open Bars) Sulfate Supply) on Sulfate Transporter Gene Expression in Hydroponically Grown Wheat Plants.

Semi-quantitative expression analysis in relation to day 0 expression (value set to 1). Each bar represents the mean ± SE of at least three replicates. The data were submitted to *t*-test variance analysis for the comparison between + and – sulfate supply with two-tailed distribution and two-sample equal variance; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. Time (d) = days.

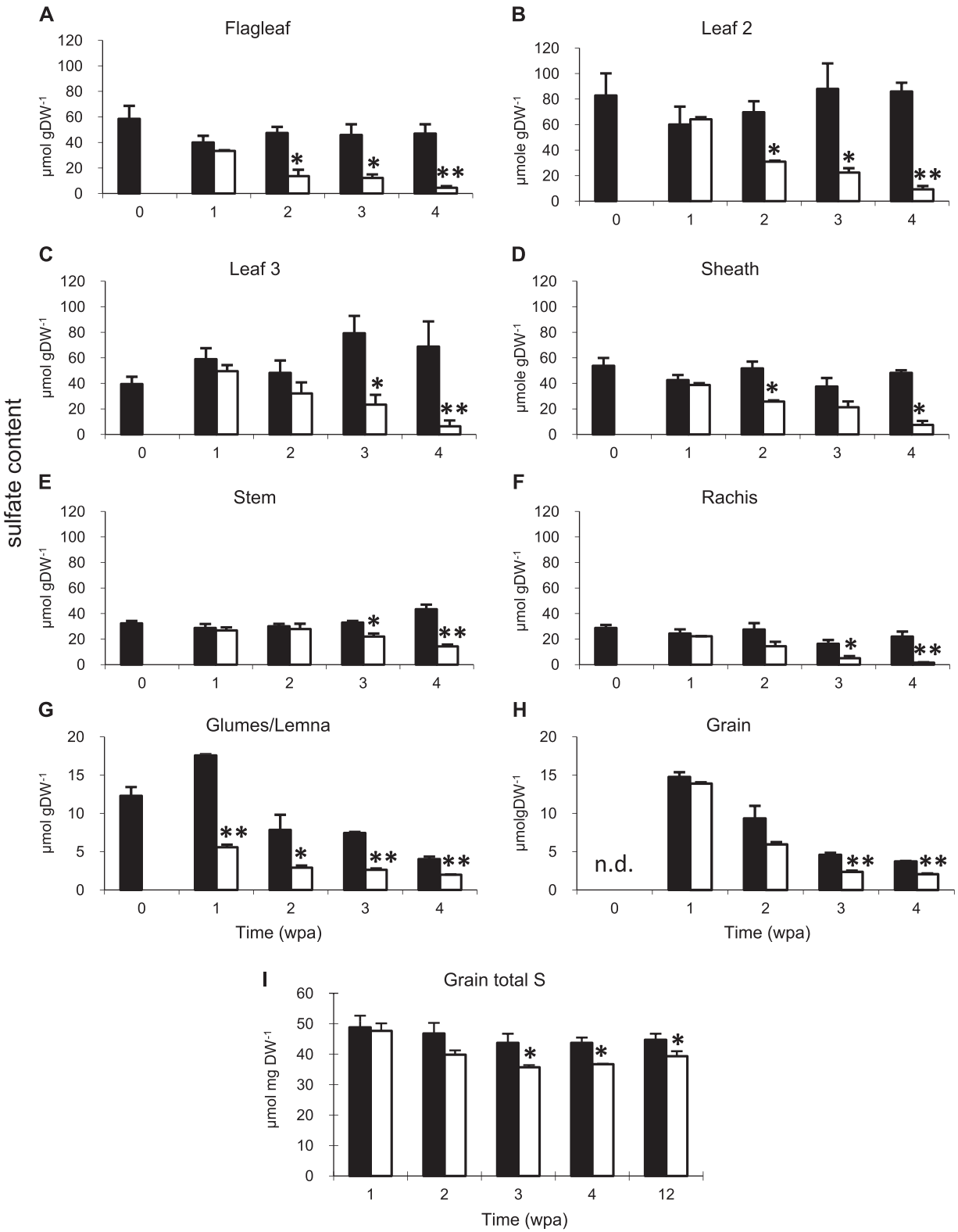

Figure 5. Influence of Sulfur Deprivation (+ (Filled Bars) and – (Open Bars) Sulfate Supply) on the Sulfate Content and Total S (Grain Only) in Sand-Perlite-Grown Wheat Plants during Grain Development.

Each bar represents the mean ± SE of at least three biological replicates. The data were submitted to *t*-test variance analysis of the comparison between + and – sulfate supply with two-tailed distribution and two-sample equal variance and the stars represent *p*-values: * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. wpa, weeks post anthesis.

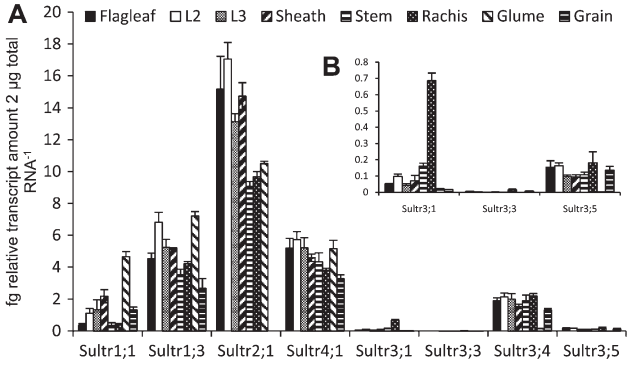

Figure 6. Sulfate Transporter Gene Expression in Specific Wheat Tissues.

(A) Transcript levels of wheat sulfate transporters in different tissue for sand/perlite-grown pot plants at anthesis (leaves, sheath, stem, and rachis) and 1 week post anthesis (glume/lemna and grain). Sq–PCRs were performed with 33 cycles for all sulfate transporters apart from TaeST3;3, which required 40 cycles for amplification. Transcript levels were calibrated as described in the Methods section.

(B) Scale enlargement of transcript levels of TaeSultr3;1, 3;3, and 3;5. Each bar represents the mean ± SE of at least three replicates.

TaeSultr4;1, but much reduced in the flag leaf, the stem, and the rachis. Slightly higher transcript abundance was detected in the other leaves and in the grain. As for the hydroponically grown plants, the transcript levels of TaeSultr3;1, TaeSultr3;3, and TaeSultr3;5 were low compared to the other sulfate transporter genes (Figure 6A and 6B). The transcript levels of those three sulfate transporters were consistent in most tissues, with the exception of TaeSultr3;1 in rachis, with a nearly six-fold increase in transcript level and for TaeSultr3;5 with a reduction in transcript abundance in the glumes/lemna fractions compared to the other tissues (Figure 6B). As found for hydroponically grown wheat plants, no transcript of TaeSultr3;2 was amplified from the RNA derived from the different tissues.

For all tissues analyzed, no influence of sulfate supply on the relative expression of Group 3 sulfate transporter genes was identified, which was consistent with the hydroponic culture experiment. In leaves, the strongest influence of sulfate starvation was found on the expression of TaeSultr1;1, which was generally correlated with the tissue sulfate content. In flag leaves of sulfur-starved plants, a significant increase in the expression of TaeSultr1;1 was found 2 weeks after anthesis, with a further increase 3 weeks after anthesis (Figure 7). In the second leaf (the leaf below the flag leaf), an increase in TaeSultr1;1 expression occurred from 2 weeks after anthesis, with a continued increase thereafter. In the third leaf, a similar pattern was seen, although the induction was only statistically significant 4 weeks after anthesis (Figure 7). For all the time points showing increased TaeSultr1;1 expression, the tissue sulfate content was below 30% compared to the sulfate-supplied control plants (Figure 5). As in the hydroponic experiment, the expression level of the second Group 1 sulfate transporter, TaeSultr1;3, was not influenced by sulfate deprivation. Changes in the expression of TaeSultr2;1 in relation to sulfate deprivation was only visible in the second leaf, with a statistically significant increase occurring from 2 weeks after anthesis. In flag leaves and in the third leaf, the expression of TaeSultr2;1 was not influenced by sulfate starvation (Figure 7), indicating that in addition to the nutritional effects on gene expression, a tissue-specific component of gene regulation was also involved. A similar pattern was found for the relative expression level of TaeSultr4;1, which increased in flag leaves and the second leaves at 3 and 4 weeks post anthesis, but not in the third leaf (Figure 7).

In sheaths, the expression levels of most sulfate transporter genes, apart from TaeSultr4;1, were not significantly affected by sulfate deprivation (Figure 8); a slightly increased expression of TaeSultr4;1 was detected 4 weeks after anthesis. In stems, the highest reduction in sulfate content was found 4 weeks after anthesis (Figure 5); in correlation, an increased expression was seen for TaeSultr1;1 and TaeSultr4;1 (Figure 8). In rachis tissue, the sulfate level also decreased by 3 weeks after anthesis, but an influence on gene expression was only found for the expression of TaeSultr1;1; the slight increase in TaeSultr4;1 expression was not significant. All other sulfate transporter genes of Groups 1 and 2 did not show any response in relation to expression in relation the sulfur supply in the sheath, stems, and rachis.

In the glume/lemna fraction, there was a small reduction in the sulfate content under sufficient sulfate supply between 2 and 4 weeks post anthesis (Figure 5G), which may indicate the high demand of the grain. A small increase was also found for the expression levels of TaeSultr1;1 and TaeSultr4;1, between 1 and 4 weeks after anthesis (Figure 9). In sulfur-starved plants, the increased expression levels of TaeSultr1;1 and TaeSultr4;1 were statistically significantly further increased compared to sufficient sulfate supply. In addition, the expression level of TaeSultr2;1 was significantly higher in the glume/lemna fraction in sulfur-starved plants (Figure 9), which may be an indication of the importance of the grain hull tissue as a sulfate reserve supply to the grain, as was found for nitrogen (Simpson et al., 1983). The detection of transcript levels of most of the sulfate transporter genes in grains indicates the importance of sulfate transport in the grain itself; however, no significant influence of the sulfur supply on the relative expression levels was found. Some sulfate transporter expression in the grain seemed to be developmentally regulated during development: there was a drastic increase in TaeSultr1;1 relative expression levels between the first and second weeks after anthesis, and a slight reduction thereafter in plants with sufficient sulfur supply. This reduction was not seen in sulfur-starved plants because of the high standard errors resulting in no *t*-test significance. For TaeSultr3;1, a substantial decrease in expression was observed between the first and second weeks after anthesis.

Spatial Expression Patterns of TaeSultr1;1 in Different Tissues in Relation to Sulfate Supply

Due to the surprising expression pattern of the high-affinity Group 1 TaeSultr1;1, which is in contrast to published

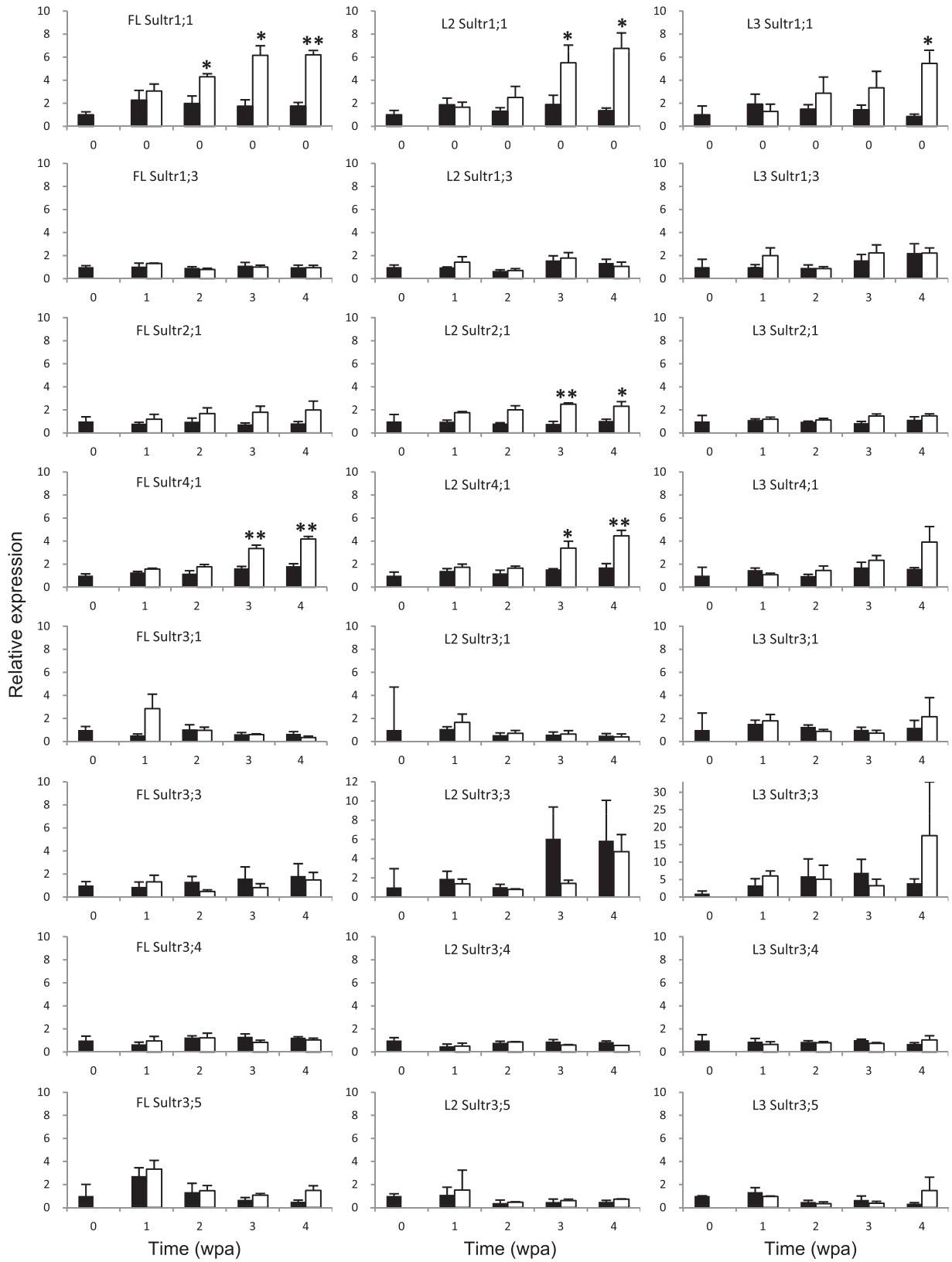

Figure 7. Influence of Sulfur Deprivation (+ (Filled Bars) and – (Open Bars) Sulfate Supply) on the Expression Patterns of Wheat Sulfate Transporters in Flag Leaves (FL), Second Leaf (L2), and Third Leaf (L3) during Seed Development.

expression data on homologous Sultr1;1 genes from dicotyledonous plant species (Takahashi et al., 1996, 1997, 2000; Buchner et al., 2004b; Hopkins et al., 2005; Howarth et al., 2003), spatial expression analysis using *in situ* hybridization was performed. Sections from different tissues and whole mount root tips (up to 1 cm from the root tip) were hybridized with digoxigenin-labeled TaeSultr1;1 antisense and, as a negative control, sense RNA.

Sections of roots approximate 3–6 cm from the root tip of sulfate-sufficient roots (Figure 10B) and sulfate-deprived roots (Figure 10C) showed expression in all cell layers when labeled with the antisense RNA probe. In sulfate-starved roots, the signal was stronger compared to the sulfate-sufficient roots. To verify the expression in roots, particularly in root hairs, whole mount *in situ* hybridizations on approximately 0.5–1-cm root tips were performed. A hybridization signal was found throughout the section including the root hairs but not the root tip itself (Figure 10E). A similar pattern was also found in stem sections of plants 4 weeks after anthesis: in sulfate-sufficient stems, a weak signal was seen in the chlorenchyma as well as in the ground parenchyma and part of the vascular bundle; sulfate deprivation led to an enhanced transcript signal in nearly all cell types (Figure 10G and 10H). In young, not fully developed leaves, the weak expression signal seemed to be restricted to parenchymal cells in sulfate-sufficient plants compared to a strong signal increase in parenchymal and vascular tissue in sulfate-deprived plants (Figure 10J and 10K). In cross-sections of rachis of sulfate-deprived plants, a transcript signal was visible throughout in ground parenchymal tissues, as well as in vascular tissues (Figure 10M). In cross-sections of grains of sulfate-sufficient plants, the TaeSultr1;1 transcript signal was also seen in different cell types: a labeling was visible in the endosperm, the aleurone including the modified aleurone, the nucellar projection, and in the vascular tissue (Figure 10O).

DISCUSSION

There are many possibilities for achieving regulation of gene expression; modulation of transcriptional activity resulting in changes in specific mRNA pools is one fundamental mechanism and the focus of this study. Previously, it was shown that upon sulfur re-supply, a mRNA abundance for a Group 1 sulfate transporter decreased rapidly (Smith et al., 1997), indicative of rapid mRNA turnover. Additionally, rapid protein turnover occurs as implied by studies using protein synthesis inhibitors (Clarkson et al., 1992). This implies that regulated transcription, rapid mRNA, and protein turnover are regulatory mechanisms controlling expression, at least of the high-affinity Group 1 sulfate transporters in root tissues in response to sulfur-nutritional status. It is possible, but there is no evidence, that these are all coordinated.

Any signal transduction pathway would need to reflect sulfate availability and/or metabolic demand for sulfur, at least resulting in a direct influence on transcription; the pathway would need to down-regulate quickly in the event of re-supply as well as up-regulate upon deficiency. Additional mechanisms also operate; for example, when sulfate transporters were expressed with constitutive promoters, in the absence of sulfur nutrition-induced modification of mRNA abundance, an accumulation of sulfate transporter protein upon sulfur deprivation still occurred, indicating post-transcriptional regulation (Yoshimoto et al., 2007). However, irrespective of whether either modified abundances of mRNA or protein are observed, interpretation would be influenced by morphological changes in root structure or cell proliferation induced by nutritional demands, such as if there were a proliferation of root hairs.

The 10 sulfate transporters identified in wheat (Buchner et al., 2004a and this paper) are unlikely to be the complete wheat gene family, but reflect the major expressed isoforms in the tissues examined, and include representatives in the major clades or groups identified (Hawkesford, 2003). On the other hand, the appearance of a second Group 4 sulfate transporter gene in the genomes of all dicotyledonous plant species studied to date, but which seems to be absent in monocotylenous plants, and the absence of a second Group 2 subtype gene in *Brachypodium* and wheat compared to rice and other plant species, suggest that there are species-specific aspects to the regulation of sulfate partitioning within plants. A study of the specificity of tissue expression and the patterns of expression in relation to developmental and environmental cues will help unravel the complex signaling mechanisms required to achieve this. In addition, knowledge of the expression patterns will provide a view on whole-plant sulfur management.

The expression pattern of TaeSultr1;1 in wheat reveals a species-specific aspect of sulfate transporter expression. In most dicotyledonous plants, there are two Group 1 sulfate transporters, and the expression of the Sultr1;1 homologs is, with a few exceptions, restricted to the root tissue and only induced in other tissues by extended sulfur deprivation. The second, usually referred to as Sultr1;2, is the constitutive sulfate transporter present in root tissues (Takahashi et al., 1996, 1997, 2000; Buchner et al., 2004b; Hopkins et al., 2005; Howarth et al., 2003). In *Arabidopsis*, expression of Sultr1;1 was identified in auxiliary buds and the hydathode of cotyledons (Takahashi et al., 2000). In maize, low Sultr1;1 expression was detected in S-sufficient-grown plants (Hopkins et al., 2004). Furthermore, in barley and tomato roots, expression is not restricted to the epidermis and root hairs (Rae and

Semi-quantitative expression analysis in relation to expression at anthesis (value at anthesis, time 0, set to 1). Each bar represents the mean $\pm$ SE of at least three replicates. The data were submitted to *t*-test variance of the comparison between + and – sulfate supply with two-tailed distribution and two-sample equal variance analysis and the stars represent *p*-values: * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. wpa, weeks post anthesis.

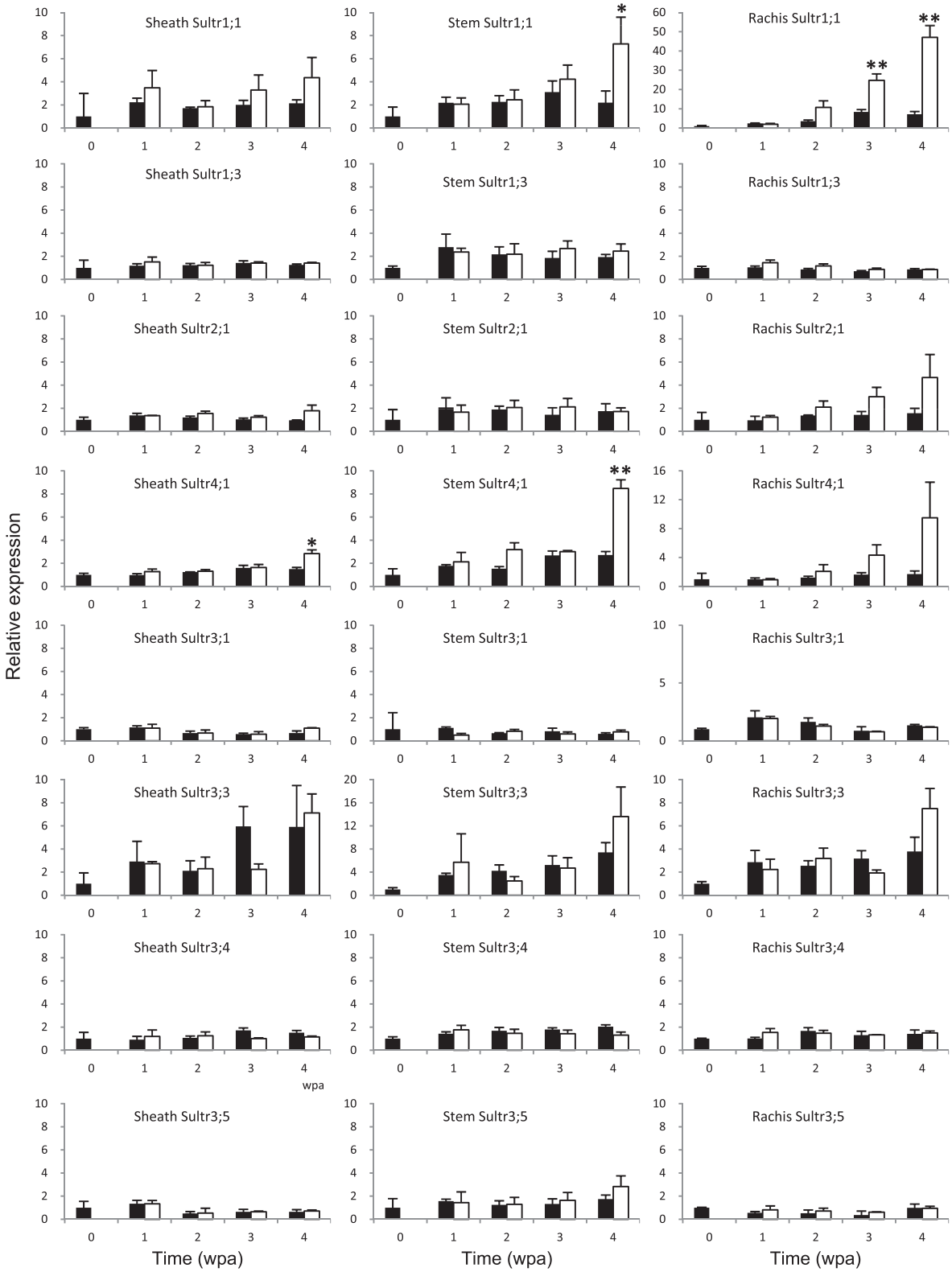

Figure 8. Influence of Sulfur Deprivation (+ (Filled Bars) and – (Open Bars) Sulfate Supply) on the Expression Patterns of Wheat Sulfate Transporters in Sheath, Stem, and Rachis Tissues during Grain Development.

Semi-quantitative expression analysis in relation to expression at anthesis (value set to 1). Each bar represents the mean ± SE of at least three replicates. The data were submitted to *t*-test variance analysis of the comparison between + and – sulfate supply with two-tailed distribution and two-sample equal variance and the stars represent *p*-values: * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. wpa, weeks post anthesis.

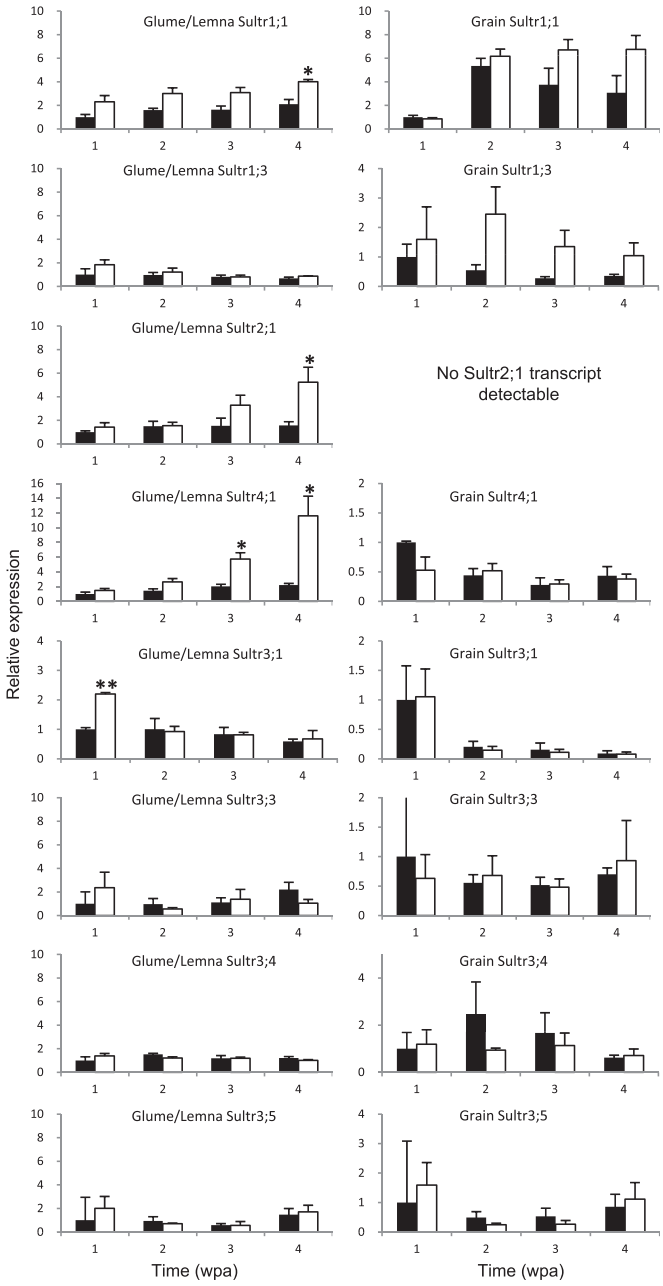

Figure 9. Influence of Sulfur Deprivation (+ (Filled Bars) and – (Open Bars) Sulfate Supply) on the Expression Patterns of Wheat Sulfate Transporters in Glumes/Lemna and Grain Tissues during Grain Development.

Semi-quantitative expression analysis in relation to expression at 1 week post anthesis (value set to 1). Each bar represents the mean ± SE of at least three replicates. The data were submitted to *t*-test variance analysis of the comparison between + and – sulfate supply with two-tailed distribution and two-sample equal variance and the stars represent *p*-values: * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. wpa, weeks post anthesis.

Smith, 2002; Howarth et al., 2003), but also occurs in the vascular tissue. The wheat Sultr1;1 fulfils the function of both Sultr1;1 and Sultr1;2, which occur in dicotyledonous plants, and is reflected in being the most abundant transcript of all sulfate transporters in roots under normal nutritional conditions and showing a sulfur deprivation-induced increase in expression. The spatial expression patterns of Sultr1;1 in wheat suggest a general function of high-affinity uptake not only for the initial uptake of sulfate by the root, as suggested in *Arabidopsis* (Takahashi et al., 2000), but also for a more widespread uptake into different cell types in multiple tissues, which includes parenchymal and vascular tissues.

Patterns of expression may be a reflection of tissue specificity, variation in development or nutritional status dependent upon any of these factors in combination with environmental availability. The data present in Figures 2, 4, and 6–9 indicated that different isoforms are expressed differentially and, furthermore, in a tissue-specific manner. Signal transduction pathways need to accommodate this variation and provide for the observed responses to sulfur availability and demand. The sulfur nutrition-induced response of expression may depend on a tissue or cellular threshold value of available sulfate. For the expression of TaeSultr1;1 and TaeSultr4;1 in leaves and sheathes, a sulfate-starvation-related increase in transcript abundance occurred below a tissue sulfate content of less than 40 μmol sulfate g dry weight^{-1} (Figure 11). However, this putative threshold value for leaves and sheathes is not directly applicable to all tissues. For stem, rachis, glume/lemnas, and grains, the sulfate content was always below 40 μmol and a sulfur starvation-related increase in expression of Sultr1;1 and 4;1 was found at sulfate contents of less than 20, 10, and 5 μmol, respectively (data not shown). On the other hand, total tissue analysis does not reflect the cellular sulfate concentration or, similarly, the individual cellular expression of the different sulfate transporter. For a determination of the cellular sulfate threshold value triggering individual sulfate transporter gene expression, a target cell-specific analysis would be necessary.

Sulfur nutrition-responsive elements are required and a means to modulate them (Maruyama-Nakashita et al., 2005). In addition, tissue-dependent expression levels will require further regulatory elements. For example, in *Arabidopsis*, the Group 2 Sultr2;1 and 2;2 are differentially expressed in the root xylem parenchyma and phloem as well as in relation to sulfur nutrition in shoots and roots, indicating a regulation of the root–shoot sulfate distribution by two Group 2 transporters. Only one Group 2 gene is found in wheat and *Brachypodium*, and implies a different kind of regulation of long-distance sulfate transport. This may explain the observed absence of sulfur regulation of the wheat Sultr2;1.

The analysis of tissue sulfate contents and mRNA abundances is always limited by spatial and, to a lesser degree, in this instance, temporal resolution. The *in situ* analysis shown indicates that a major regulated sulfate transporter, TaeSultr1;1, has a widespread distribution of expression; for example, in both root and stem, all cells show increased expression levels in response to imposed sulfur limitation. This indicates the approach of analysis of whole tissue fractions in many cases; interestingly, in the stem, it is likely that sulfate may be localized in xylem cells

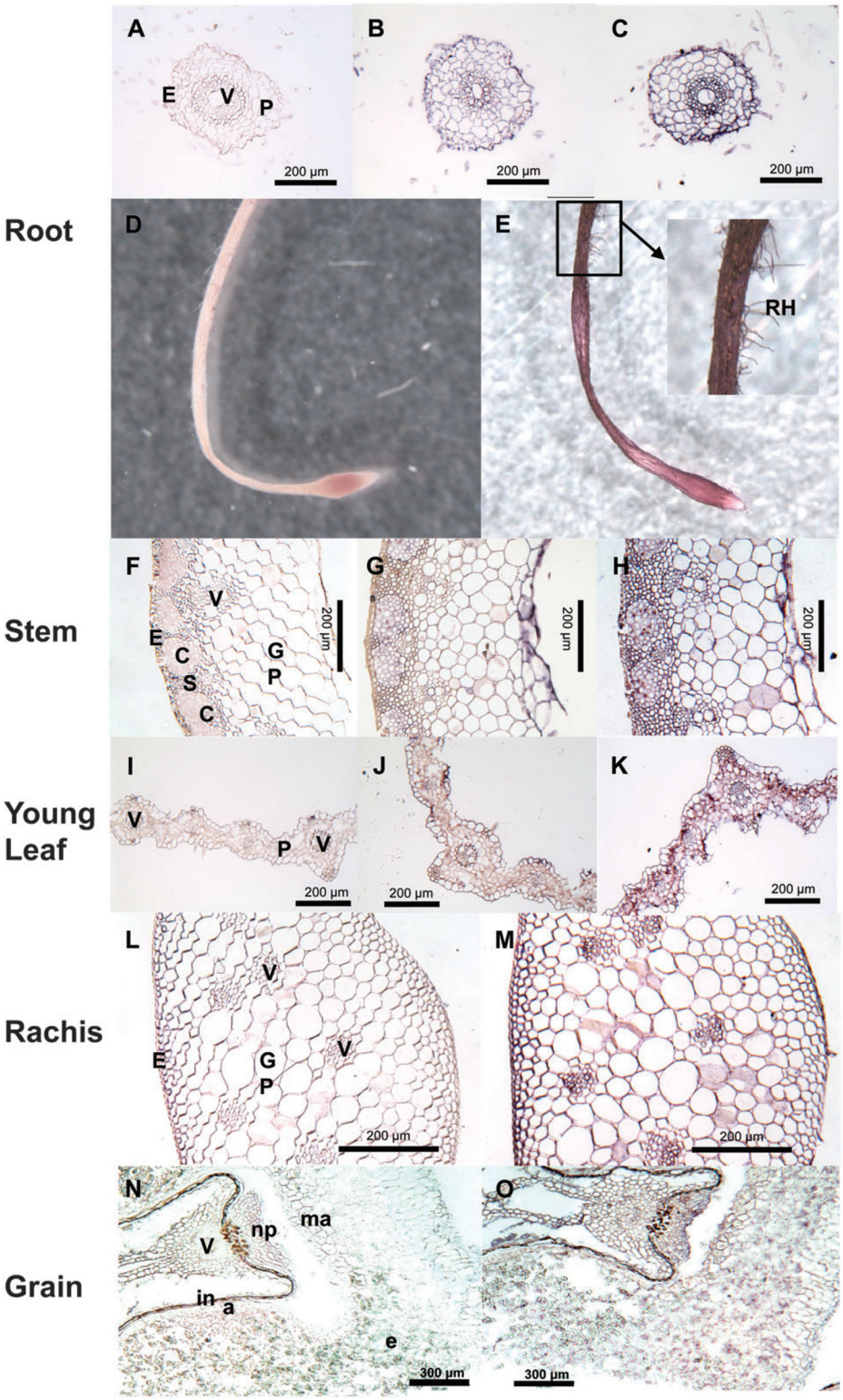

Figure 10. Spatial Expression Analysis of TaeST1;1 in Different Tissues by *In Situ* Hybridization Probed with Digoxigenin-Labeled Sense (A, D, F, I, L, N) and Antisense (B, C, E, G, H, J, K, M, O) TaeST1;1 RNA and Viewed under Bright Field Optics.

(A–C) Cross-sections of aeroponically +S (B) and –S (C) grown roots, approximately 3–6 cm from the root tip.
(D, E) Whole mount *in situ* hybridization of root tips.
(F–H) Stem cross-sections 4 weeks post anthesis from +S (G) and –S (H) grown plants.
(I–K) Cross-sections of young leaves of 3-week-old hydroponic +S (J) and 5 d –S (K) grown plants.

and may not be available to many stem cells, hence the observed widespread induction of expression (Figure 10).

Surplus sulfate is undoubtedly stored in the vacuole. Depletion of cytoplasmic sulfate must trigger, directly or as a consequence of another altered metabolite signal, such as *O*-acetylserine (Smith et al., 1997), changes in gene expression. Modification of expression of both the high-affinity plasma membrane-located transporter (Sultr1;1), which will maximize uptake, and of the vacuolar efflux transporter (Sultr4;1), which will enhance remobilization of vacuole-stored sulfate, occurs in parallel. Increased expression is observed upon a reduction in tissue sulfate content, but prior to complete depletion (compare Figures 2C and 4). This may indicate tissue variation, but this is unlikely, as indicated by the *in situ* analysis, as already described–more likely, the sulfate partitioned to the vacuole. Vacuolar sulfate that is transferred to the cytoplasm and that is utilized over a period of days (dependent upon tissue status and growth rates) does not prevent the induction of the sulfate transporter gene expression. This is indicative of a level or mechanism of control that can distinguish between an adequate external sulfur supply and a situation of depleting reserves.

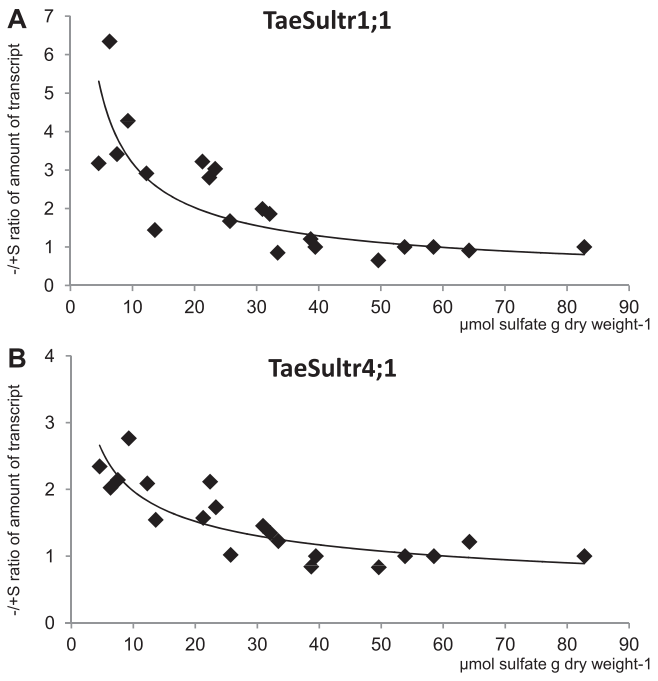

Figure 11. Gene Expression of TaeSultr1;1 and TaeSultr4;1 in Relation to Leaf and Sheath Tissue Sulfate Content.

Scatter analysis of ratio expression in –/+ sulfur-starved tissue versus sulfate content including trendline.

The grain tissues show the most complex patterns of expression, reflecting the complexity of this tissue. Grain sulfate levels are always low, as it is probably rapidly assimilated into amino acids and grain protein, as described for lupin (Tabe and Droux, 2001). However, Sultr1;1 is induced by sulfur deprivation during development, and the *in situ* data indicate that the induced expression is widespread, even in this complex tissue. The putative vacuolar efflux carrier, TaeSultr4;1, was down-regulated during development in the grain tissue, probably due to loss of vacuolate cells in the endosperm. The expression of sulfate transporters in grain tissue during grain filling is indicative of the need for sulfur for cysteine and methionine biosynthesis, both of which are required for storage proteins (Zhao et al., 1999).

In conclusion, patterns of expression of the sulfate transporter gene family are complex when considered in relation to the whole plant during development. Plasticity of expression is targeted at optimizing uptake and allocation when sulfur supply fails to match demand for growth and for optimal expression of seed storage proteins. Identifying the regulatory pathways remains a major challenge but is necessary to aid in the rationale design of crops for optimal resource utilization.

METHODS

Plant Material

Hydroponic culture: seeds of wheat (*Triticum aestivum*) cv Paragon were germinated on sterile tap water-soaked soft paper tissue for 3–4 d before transfer to single plant hydroponic culture. Plants were cultivated including aeration for the first 3 d on half strength before changing to full-strength-modified Letcombe liquid nutrient solution (1.5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $NaNO_3$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 25 μM FeEDTA, 160 nM $Cu(NO_3)_2$, 9.2 μM H_3BO_3, 3.6 μM $MnCl_2$, 16 nM$(NH_4)_6Mo_7O_{24}$, 5 μM KCl, and 770 nM $ZnCl_2$). Nutrient solutions were replaced twice per week. After 2 weeks, $MgSO_4$ was replaced by $MgCl_2$ for sulfate-starvation experiments. Plants were harvested in triplicate at each time point. The roots were rinsed three times in de-ionized water and dried briefly on paper towels. The total shoot and root weights were measured before freezing in liquid nitrogen.

Aeroponic seedling culture: seeds were surface-sterilized with 5% sodium hypochloride solution for 15 min. Seeds were germinated and grown on soft paper tissue soaked with full Letcombe solution with or without sulfate in semi-transparent boxes for up to 7 d, with nutrient solution exchange every 2 d. Approximately 10-cm roots of 5–7-day-old plants were divided into sections of 0–3, 3–6, and 6 cm to shoot and processed for *in situ* hybridization.

(L, M) Cross-sections of –S grown 4 weeks post anthesis rachis (middle region).
(N, O) Partial cross-section of 2 weeks post anthesis grain from +S grown plants.
The main tissue parts are annotated as follows: epidermis (E), parenchymal tissue (P), vascular tissue (V), root hairs (RH), chlorenchyma (C), sclerenchyma (S), ground parenchyma (GP), integuments (in), aleurone (a), nucellar projection (np), modified aleurone (ma), endosperm (e).

Sand/perlite greenhouse culture: germination was as described above. After germination, three plants per 15-cm diameter pot were cultivated in de-ionized water-washed sand/perlite (1:1). In the first week, the plants received three times 500 ml per pot half-strength modified Letcombe nutrient solution three times weekly. Thereafter, the nutrient solution was changed to full-strength. Apart from the first and second tillers, all further tillers were regularly removed. At anthesis, $MgCl_2$ replaced $MgSO_4$ for sulfate starvation experiments. Flag leaf, second and third leaf, sheath, stem, rachis, glumes/lemna, and grain tissue of the main shoot were harvested 1–4 weeks after anthesis and frozen directly in liquid nitrogen and stored at –80°C before further processing. Further grain sections as well as stem and rachis sections were harvested 2 and 4 weeks after anthesis, respectively, and processed for *in situ* hybridization.

Total RNA Isolation

Total RNA from roots, stems, and leaves was isolated by a method based on Verwoerd et al. (1989), which involved an additional phenol–chloroform–isoamyl alcohol extraction of the aqueous phase after the first centrifugation. Possible genomic DNA contamination was removed by a RNase-free DNase treatment. The final air-dried pellet was dissolved in an appropriate volume of diethyl pyrocarbonate-treated water.

cDNA Isolation, Sequencing, and Sequence Analysis

Partial or full-length cDNAs corresponding to the nine sulfate transporter genes were isolated by RT–PCR from total RNA. TaeSultr3;2 could only be isolated and identified by genomic PCR amplification of partial DNA fragments. Oligonucleotide primer including degenerated primers were designed based on database-published wheat sulfate transporter cDNA sequences or based on highly homologous regions identified in sequence alignments of published sulfate transporter genes from different plant species, corresponding to the respective sulfate transporter Groups. First-strand cDNA synthesis was performed according the Invitrogen (Paisley, UK) Superscript II or III Reverse Transcriptase manual with 1-μg aliquots of total root and leaf RNA and antisense primer. Following PCR was performed according the Promega (Southampton, UK) Taq-DNA polymerase or the Sigma (Gilligham, UK) RedTaq DNA polymerase mix standard protocol for 50-μL reaction by using a 1-μl aliquot of each of the first-strand cDNA solutions and specific sense/antisense primer combinations. 5′- and 3′-region of the sulfate transporter transcripts were isolated by 5′- and 3′-Race according the Invitrogen 5′-Race and 3′-Race kit manuals and sequence-specific oligonucleotide primers based on the sequence results from the partial cDNA fragment isolation (data not shown). Finally, the full-length cDNAs containing the full coding region of some of the sulfate transporters were generated via RT–PCR and sequence-specific primers obtained from the 5′- and 3′-Race fragments (data not shown), using proofreading *Pfu*-DNA polymerase. All PCR fragments were verified by sequencing in both directions. ClustalX V.1.81 (Thompson et al., 1997) was used for multiple sequence alignment of the coding cDNA sequences. MEGA V. 3.0 (Kumar et al., 2004) was used for calculation of phylogenetic trees (the neighbor-joining method) on the basis of the coding sequences. Bootstrap values for the trees were calculated as a percentage of 1000 trials, with a seed number for the random number generator of 1000.

SO_4^{2-} Determination

Anions were measured by extracting approximately 20 mg of homogenized, freeze-dried plant material in 1 ml of de-ionized water at 80°C for 2–4 h. Anion concentrations in the filtered (0.45-μm) extracts were determined by ion chromatography using an AS9SC separation column (Dionex, Sunnyvale, CA, USA). The eluent solution consisted of 1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$.

Total S Analysis

0.2–0.5 g of homogenized freeze-dried plant tissue samples were digested in 5 ml nitric acid:perchloric acid (87:13, v/v) (70% concentration, trace analysis grade, Fisher Scientific, Loughborough, UK), for 4 h at room temperature followed by 195°C for 5 h. 5 ml of 20% (v/v) nitric acid was added to the cooled solution and the tubes were reheated for 30 min at 80°C. Ultra pure water (>18 MΩ) was added to approx 15–18 ml, mixed well and re-warmed for a further 30 min at 80°C. After cooling, the solutions were made up to a final volume of 20 ml with ultra pure water. After filtering (Whatman, no. 42 (GE Healthcare, Maidstone, UK)), ICP analysis was carried out using an Accuris ICP-AES (ARL, Vallaire, Ecublens, Switzerland).

Semi-Quantitative RT–PCR

Sulfate transporter gene expression was analyzed by two-step semi-quantitative RT–PCR. First-strand cDNA synthesis was performed from 2-μg aliquots of total RNA and dT-adapter primer using Invitrogen Superscript III, according the standard protocol but using a 2-h synthesis time. Subsequently, sqPCR was performed as a 15-μl reaction using 1 μl of each first-strand cDNA solution, specific primer combinations (Table 1) for the respective sulfate transporters, and Red Taq mix (Sigma-Aldrich). To equalize PCR conditions, the Tm of all primers were in the range of 61–63°C, and the amplicon size of the PCR fragments was between 480 and 520 bp, apart from Actin2, with 538 bp. The PCR amplification was stopped after 25 cycles, which corresponded to the linear amplification phase of the expressed analyzed sulfate transporters, which, for most tissues, was in the range of 20–30 cycles for most sulfate transporter genes, besides TaeSultr3;3 (linear amplification in the range of 35–38 cycles) and TaeSultr1;1and 3.5 in non-root tissues (linear amplification in the range of 30–36 cycles). In wheat grains, the linear amplification was in the range of 30–38 cycles for all sulfate transporter genes apart from TaeSultrt2;1, for which no expression in grains could be detected. As a constitutive control, semi-quantitative RT–

PCR was performed using wheat actin2-specific oligonucleotide sense and antisense primers for all tissues apart from grain tissue. The grain expressions of sulfate transporters were normalized to a DSS1/SEM1 proteasome subunit family protein (GenBank Accession: BQ806121) gene, which showed consistent expression in micro array assays of wheat grain development (Wan et al., 2008).

Equal amounts of PCR products separated by electrophoresis were analyzed by a digital image system using Gene snap software (Syngene, Synoptics Ltd, Cambridge, UK). Fluorescence value of products was analyzed by Gene Tools software (Syngene, Synoptics Ltd, UK). Gene expression value was determined as peak volume. Specific gene expression value was normalized to the constitutive control expression values.

PCR efficiencies and kinetics of the semi-quantitative expression analysis by PCR may vary by length and sequences of the amplicons and primers for each individual sulfate transporter. For quantitative comparison of the expression of the individual sulfate transporter gene in different tissues, the amount of amplified PCR product was calibrated on a standard curve generated by a serial dilution and subsequent PCR of defined amounts of plasmid DNA containing the specific amplicon cDNA fragment of the individual sulfate transporter, under the same cycle regime as for the expression analysis. Based on this calibration, the relative amount of target sulfate transporter transcript in the cDNA derived from total RNA was calculated.

In Situ Hybridization

cDNA probes for *in situ* hybridization were labeled using a digoxigenin (DIG) *in vitro* Transcription kit (Roche, Burgess Hill, UK). Sulfate transporter cDNA fragments were subcloned into the pGEM-T Easy Vector system (Promega, Southampton, UK). After linearization by restriction, *in vitro* antisense as well as sense DIG-labeled RNA was synthesized using T7 or Sp6 RNA polymerase. After purification, the *in vitro* synthesized RNAs were carbonate hydrolyzed to an average size of 200–300 base fragments size. All tissue sections were fixed in 4% paraformaldehyde, embedded in paraplast paraffin wax, and processed and hybridized under the conditions previously described by Buchner et al. (2002). Colorimetric alkaline phosphatase detection of hybridization was carried out as described in the DIG non-radioactive detection kit protocol (Roche). Slides were developed at room temperature in the dark for between 16 h and 3 d. Whole mount *in situ* hybridization of root tips was performed according to Drea et al. (2009) with an additional 5 μg ml^{-1} proteinase K treatment for 30 min.

Sequence Accessions

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: wheat sulfate transporter cDNA sequences AJ512821; AJ512820; BT009249; TC366953/TC291347; FN432835; FN599528; TC272130/TC259376; TC318325/TC314180; AM747385; BT009340; TC234027 (for further details, see Table 1). Wheat actin cDNA sequence TC234027. The rice sulfate transporter accessions AF493792, NM_001055796, AP004691, AK111395, AK067353, NM_001055577, NM_196532, AK104831, AK067270, NM_192602, NM_191791, and AF493793 for cDNA sequences were based on genomic sequences and their analysis (Feng et al., 2002; Goff et al., 2002; Yu et al., 2002). The *Brachypodium distachyon* coding sulfate transporter sequences were extracted from genomic sequence data that were produced by the US Department of Energy Joint Genome Institute (www.jgi.doe.gov/).

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