Phyton (Austria) Special issue: "Grill"	**Vol. 45**	Fasc. 3	()–()	x.x.2005

Understanding the Regulation of Sulfur Nutrition – from Sulfate Transporter Genes to the Field

By

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K e y w o r d s : Sulfur nutrition, sulfate transport, transcriptomics.

S u m m a r y

HAWKESFORD M. J., BUCHNER P., HOWARTH J. R. & LU C. 2005. Understanding the regulation of sulfur nutrition – from sulfate transporter genes to the field. - Phyton (Horn, Austria) 45 (3): (x) – (x).

Sulfur is taken up by the plant and transported in the cell and around the plant mainly as sulfate. These processes are dependent on the sulfate transporters, and therefore the transporters have a central role in the management of plant sulfur nutrition for optimisation of growth. A large number of plant sulfate transporters have been cloned and comparative sequence analysis indicates that although they are all related, they cluster into a number of discrete sub-types. Within any plant species there appears to be approximately 14 members of this gene family; functional and expression data suggest that there is little redundancy and that each transporter has a specialised role. Furthermore the expression of many of the transporters is regulated by the sulfur-nutritional status of the plant; the regulation serves to optimize acquisition and utilization of sulfate. The mechanisms facilitating this regulation have been subject to intense investigation. One generally accepted model based on metabolite feedback regulation of gene expression is presented and critically evaluated. Genomic approaches focussed on identification of sensing and signal transduction pathways are described; transcriptome analysis of both field and controlled environment grown wheat has enabled the identification of many nutrient-regulated genes, including potential candidates for regulatory components.

I n t r o d u c t i o n

Sulfur deficiency in crops is becoming increasingly widespread and has substantial impacts on yield and quality. Deficiency may be remedied by appropriate application of S-fertilisers, although form and timing of application need to be

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assessed for each situation and are susceptible to prevailing weather conditions. There is increasing interest in minimising inputs and avoiding damage to the environment from the over use of inorganic fertilisers. Nutrient (including sulfur) use efficiency is a desirable trait for crop improvement, and for sulfur, the sulfate transporters are genetic targets as they play a central role in both efficient acquisition and in management of internal reserves (HAWKESFORD 2000).

Apart from the initial influx of sulfate from the soil across plasma membranes of cells in the root epidermis and cortex, there are further requirements for trans-membrane transport of sulfate in the processes of cell to cell transport to the vascular tissue as well as for long distance transport from the root to the shoot, for intracellular transport of sulfate into the vacuole as the main storage pool and into plastids where sulfate reduction takes place. Central to these processes are the membrane-located sulfate transporters, encoded by a multi-gene family (HAWKESFORD 2003). Importantly the initial uptake will determine efficiency of acquisition; however regulation of expression of all of these transporters is essential for the overall management of the sulfur economy of the plant.

In recent years, great progress has been made on the knowledge of S uptake and assimilation, including the identification of all of the respective genes (HAWKESFORD 2005). The next challenge is the delineation of the control pathways and an understanding of plant responses to limiting or changing nutritional conditions. Transcriptional control plays a major part in the control of these processes; however the sensing and transduction mechanisms are still largely unknown.

In addition to molecular approaches conventionally undertaken with laboratory grown material, it is beneficial to sample field-grown plant materials which are habituated to defined nutrient inputs. Such an experiment is the Broadbalk field at Rothamsted in South East England, which is the oldest continuously running agricultural experiment in the world. On Broadbalk, winter wheat is grown under well-defined nutrient supply conditions including a range of nutrient deficiencies, of which sulfur is one example. Using materials sampled from this experiment, impacts on crop yield and quality may be related directly to variation in expression of specific genes. Using non-biased genomic approaches, unexpectedly large numbers of genes show differential expression to limiting sulfur availability. In addition to the up-regulation of genes directly involved in S-metabolism, recent transcriptomic and metabolomic profiling studies in *Arabidopsis* also revealed far reaching effects on the expression of flavonoid, auxin and jasmonate biosynthetic pathway genes under S-deficiency (HIRAI & al. 2003, 2004, MARUYAMA-NAKASHITA & al. 2003, NIKIFOROVA & al. 2003).

The Plant Sulfate Transporter Gene Family

Since the first reported identification of a plant sulfate transporter in *Stylosanthes hamata* (SMITH & al. 1995a), isolated with the aid of a sulfate transporter-deficient yeast mutant (SMITH & al. 1995b), many genes encoding sulfate transporters have been isolated and characterized (BOLCHI & al. 1999, BUCHNER &

al. 2004a, b, HOWARTH & al. 2003, SMITH & al. 1997, VIDMAR & al. 1999, 2000, SHIBAGAKI & al. 2002, TAKAHASHI & al. 1996, 1997, 1999a, 1999b, 2000, YOSHIMOTO & al. 2002, 2003). With the subsequent analysis of whole plant genomes (The *Arabidopsis* Genome Initiative, 2000, FENG & al. 2002, GOFF & al. 2002, SASAKI & al. 2002, YU & al. 2002) it is now clear that sulfate transport in plants is carried out by a complex system of transporters encoded by a large gene family.

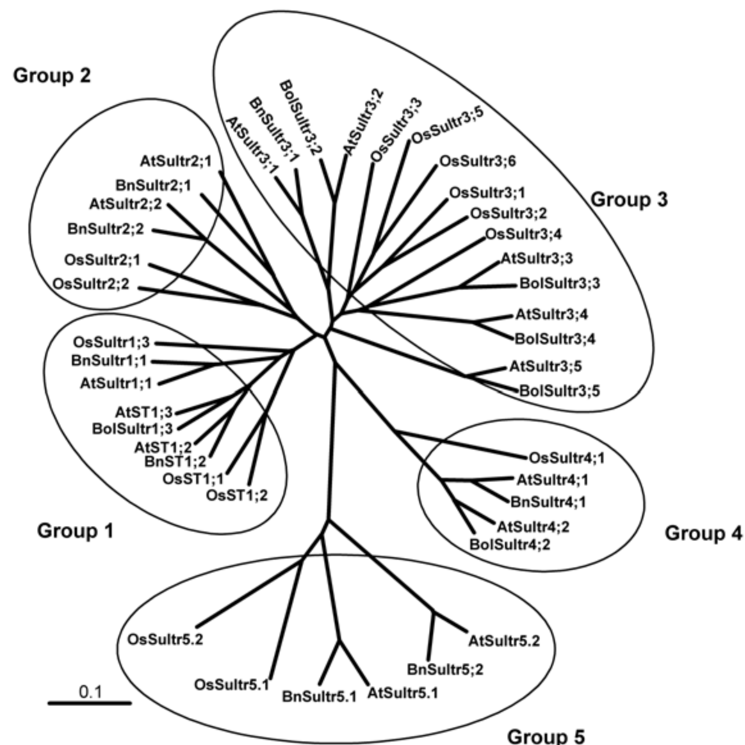

Fig. 1. Phylogeny of selected sulphate transporter cDNAs of *Arabidopsis thaliana*, *Brassica* sp and *Oryza sativa* (rice): Neighbour-joining tree (MEGA V. 2.1, KUMAR & al. 2001) from the multiple alignment (ClustalX V.1.81, THOMPSON & al. 1997) of the coding cDNAs of the *Arabidopsis* (AB018695, AB042322, AB049624, AB003591, D85416, D89631, AB004060, AB023423, AB054645, AB061739, AB008782, AB052775, AC018848, AC006053), *Brassica oleracea* (AJ416460, AJ311388, AJ633707, AJ633705, AJ581745; AJ601439, AJ704373, AJ704374, AJ633706, AJ416461, AJ555124 (BUCHNER & al. 2004b), AJ223495 (HEISS & al. 1999)) and *O. sativa* sulphate transporter family (from genomic sequences - FENG & al. 2002, GOFF & al. 2002, SASAKI & al. 2002, YU & al. 2002, accession and protein ID AF493790, AAN59764.1, BAC98594, AAN59769, AAN59770, NP_921514, AAN06871, AK104831, AK067270, NM_192602, NM_191791, AF493791, BAC05530, BAB03554).

Alignment and phylogeny of the 14 putative sulfate transporter genes present in the *Arabidopsis*, *Brassica* and rice genomes (Fig. 1) subdivides the plant sulfate transporter family into four closely related groups and a fifth more diverse, but clearly related group. The Group 1, 2 and 3 sulfate transporters are plasma

membrane located (SMITH & al. 1995a, TAKAHASHI & al. 1996, 2000, YOSHIMOTO & al. 2002, 2003, KATAOKA & al. 2004a). Transport kinetics as well as spatial and expression analysis indicate different functions of the individual groups and of the individual isoforms within the Groups. The Group 1 and 2 transporters are able to complement a yeast sulfate-transporter deficient mutant, but differ in their transport kinetics; Group 1 transporters are high affinity transporters with K_ms in the range of 1.5 to 10 µM whereas Group 2 transporters have a lower affinity for sulfate with K_ms between 99.2 µM and 1.2 mM (HOWARTH & al. 2003, SMITH & al. 1995a, 1997, TAKAHASHI & al. 2000, SHIBAGAKI & al. 2002, YOSHIMOTO & al. 2002, 2003). For the Group 3 transporters, functional data is only available for SULTR3;5: SULTR3;5 was only able to complement the yeast mutant in combination with SULTR2;1 (Group 2) as a double transformant, interpreted as a co-operative interaction (KATAOKA & al. 2004a).

Transporter Isoforms are Differentially Regulated

A systematic analysis of expression of 12 of the *Brassica* isoforms has been reported (BUCHNER & al. 2004b and Fig. 2). It is clear that the different isoforms show tissue specificity and differential responses to sulfur availability. Expression of the Group 1 types is predominantly in the roots with some expression in the leaves of BSultr1;2 after several days S-deprivation. Studies in *Arabidopsis* have located AtSultr1;3 to the phloem and shown that it is up-regulated by S-starvation (YOSHIMOTO & al. 2003). The induction of the high affinity Group 1 sulfate transporters has been correlated with increased capacity for uptake in roots in response to limiting S-availability (for example SMITH & al. 1997). This response maximises the ability of the plant to capture sulfate from the pedosphere and may be associated with root proliferation.

Group 2 sulfate transporters have been localised to vascular tissues and it is suggested that they are involved in xylem loading and unloading and hence transport around the plant (TAKAHASHI & al. 1997, 2000). BSultr2;1 is only expressed in the root under S-limitation and is expressed under all conditions in both stems and leaves (Fig. 2). BSultr2;2 is only expressed in the root and has an increased expression under S-limitation. An interpretation of this pattern of expression is that translocation processes from the root must be maximised under these conditions.

The Group 3 transporters have not been confirmed to transport sulfate. In *Brassica*, the different isoforms show tissue specificity but expression is not responsive to S-nutrition (Fig. 2).

The Group 4 transporters have been reported to be tonoplast located and responsible for sulfate efflux from the vacuole (KATAOKA & al. 2004b). In *Brassica* (Fig. 2), both isoforms are expressed in all tissues under S-limiting conditions, ensuring efficient efflux of stored sulfate from the vacuole for subsequent assimilation. Expression of BSultr4;1 occurs in the roots even under adequate S-supply indicating a need for constant turnover of this pool in the roots, possibly as a consequence of root cell aging or development.

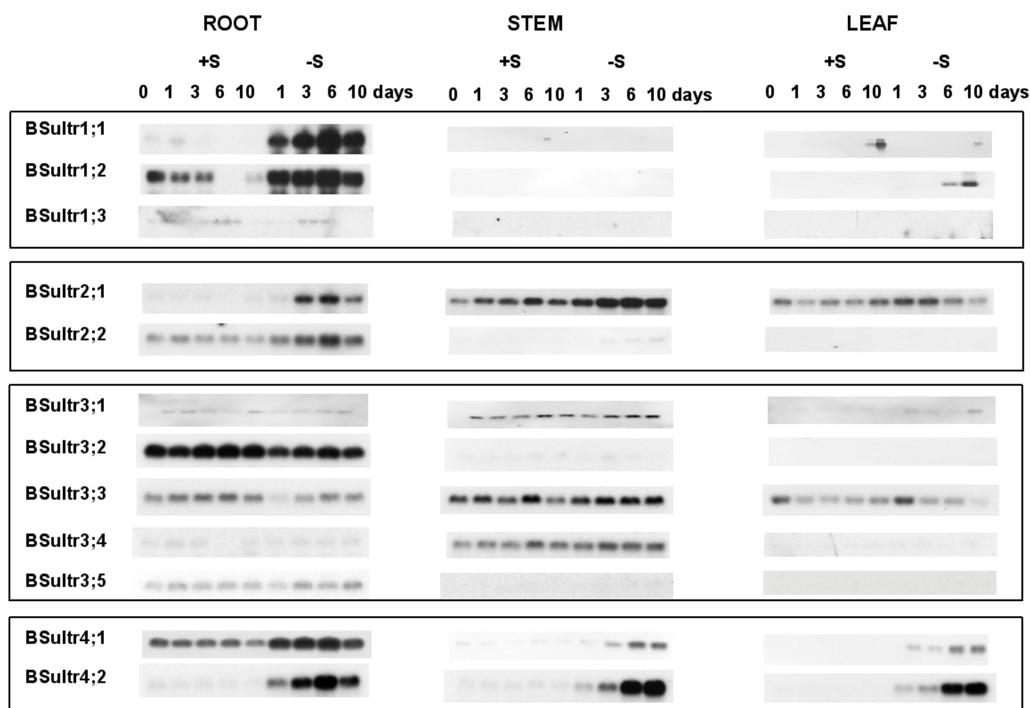

Fig. 2. Expression of isoforms. Northern blots showing expression of 12 sulfate transporter isoforms in *Brassica oleraceae* in response to limiting sulfur availability in root, stem and leaf tissues. Plants were grown on 25% Hoaglands prior to S-starvation for up to 10 d. Equal loading of RNA was determined by ethidium bromide staining of the ribosomal RNA (data not shown). Data is taken from BUCHNER & al. 2004b.

Most regulatory models have focussed on the simple situation of increased expression in response to S-limitation (see below), however it is apparent that transcription of individual genes is modulated quite specifically and that additional layers of regulation operate. A criticism of this kind of approach (Fig. 2) is that no account of cell specific responses is measured and that this will give a misleading impression of relative expression in different tissues under different conditions. To resolve this promoter:reporter constructs are being employed in studies on *Arabidopsis* (see for example TAKAHASHI & al. 2000).

The Model for Regulation

A currently favoured model of metabolite feedback regulation which acts at the transcriptional level is shown in Fig. 3. When S supply is in excess, or demand for S is low, a metabolite whose identity is not confirmed but which could be a reduced sulfur compound such as sulfide, cysteine or glutathione accumulates and acts to repress expression of genes encoding some of the transporters and APS

reductase. Under conditions of limited S supply or high sink demand, this pool diminishes and the repression is relieved. This model has been suggested many times and had been broadly accepted in the literature. An additional aspect, first suggested by BRUNOLD and co-workers (NEUENSCHWANDER & al. 1991), based on the prokaryotic model, is that the cysteine precursor, *O*-acetylserine (*O*AS) acts as an inducer of gene expression. Feeding experiments have demonstrated that an excess of *O*AS induces sulfate transporter and APS reductase gene expression. This occurs in the presence of adequate supply of sulfate and is concomitant with increasing concentrations of internal cysteine and glutathione, normally expected to repress expression (SMITH & al. 1997). The accepted viewpoint is that *O*AS acts as an overriding inducer of gene expression (HAWKESFORD & WRAY 2000). This molecule is expected to accumulate if sulfur is limiting, although recent analyses have indicated that accumulation occurs only after an extended period of S-limitation and follows rather than precedes changes in gene expression (BUCHNER & al. 2004b, HOPKINS & al. 2005).

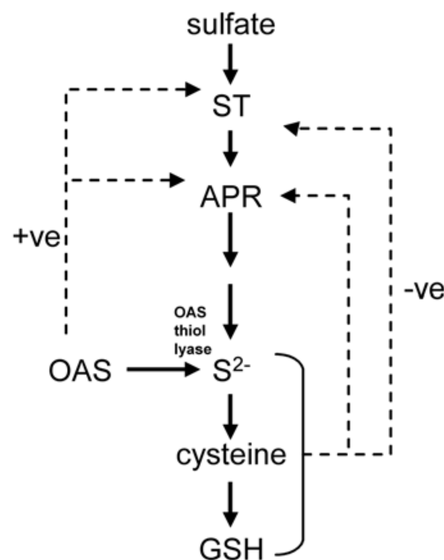

Fig. 3. A simple representation of metabolite feedback loops thought to be involved in the control of gene expression of the sulfate transporter (ST) and APS reductase (APR). OAS: O-acetylserine; GSH: glutathione. Solid lines represent metabolite fuxes, broken lines the putative regulatory influences.

Regulatory Components and the Transcriptome

Transacting factors such as transcription factors bind to regulatory regions of gene sequences and modulate the expression of specific genes. More than 1500 transcription factors have been identified in *Arabidopsis*, and similar numbers are to be expected in crop species. In addition to controlling expression of individual or groups of genes, the expression of many transcription factors themselves are also

regulated during development and in response to environmental factors including nutritional factors. It is likely that the activity of individual transcription factors could modulate whole biochemical pathways or aspects of physiology, so called 'global-regulators'. Due to their importance in controlling nutritional pathways, and because of the clear role of transcription processes in regulating sulfate transporters, these trans-acting factors are of particular interest in relation to sulfur nutrition.

One approach to identify regulatory components is to take a holistic, non-biased approach to gene discovery. An example is transcriptome analysis provide a large scale indication of gene expression occurring in sampled tissues. The mRNA populations in samples are analysed in terms of at least presence/absence and often with additional quantitative information. Sets of transcripts (profiles) are associated with and diagnostic of particular tissues/treatments, and comparisons of multiple samples allow interactions between profiles and sets of genes to be identified. With such an approach associations between expression patterns of genes of known and previously unknown function can be identified. We have employed both cDNA AFLP and microarray approaches to examine responses to nutritional stresses in both the laboratory and in the field in *Arabidopsis* and wheat. In addition to identifying potential regulatory components, this should provide many new targets for crop breeding improvement programmes.

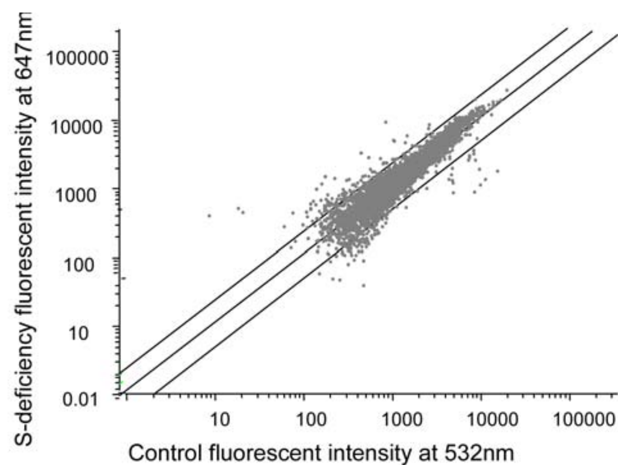

Fig. 4. Transcriptome analysis of the effect of sulfur deficiency on the transcriptome of field-grown wheat endosperm. Gene expression was evaluated using a cDNA microarray with approximately 10,000 elements (see http://www.cerealsdb.uk.net/wheat.htm). The microarray was probed with fluorescently labelled cDNA derived from S-sufficient and S-deficient endosperm RNA harvested (14 days post-anthesis) from the Broadbalk field at Rothamsted in southern England in 2003. For the sulfur deficient plot, no sulfur had been applied since 1999. The scatter plot shows relative expression of S-deficiency versus control. The solid lines show 1:1 expression and 2-fold higher expression in either the –S or control. Values are means of 3 biological replicates and 2 technical replicates (dye-swap). Data from C. LU, M.J. HAWKESFORD and K. J. EDWARDS, unpublished.

Data from a microarray analysis of the transcriptome of developing wheat endosperm is shown in Figure 4. Plant material was sampled from the Broadbalk field from a sulfur deficient plot and compared with a sulfur sufficient control plot. Large numbers of genes differ in their expression level by a factor of 2-fold (up- or down-regulated) in the sulfur-deficient compared to the control. Such a result is typical for this kind of analysis. Identification of the individual genes is possible and reveals genes involved in sulfur metabolism, protein synthesis and transcription factors. Taken as a whole the two large gene subsets (up- and down-regulated) may be analysed for common promoter elements. Such an approach has been described for *Arabidopsis* and has resulted in the identification of the 'SURE' (sulfur responsive element) element (MARUYAMA-NAKASHITA & al. 2005).

Future Prospects

A case for the importance of the roles and the regulation of sulfate transporters has been presented. Models for regulation have evolved, from simple feedback repression by molecules such as glutathione, to more sophisticated dual metabolite control involving both feedback repression and induction. The details of these pathways remain elusive.

Transcriptional control is of major importance; however there are also roles for post transcriptional control in fine tuning the network of sulfur distribution and utilisation in the context of specific metabolic pathways and the whole plant. Repression or de-repression of expression are controlled by the balance of sulfate supply and sink demand. One or more signals for sink demand, whose identity is unclear, act at the cellular level. In a situation where cysteine, or some associated metabolite pool size is perturbed, either through insufficient sulfur supply or increased demand, the cell responds accordingly. A problem with these models is that generally cellular responses to fluctuating S availability are quite rapid and occur before the onset of major measurable changes in metabolite pools, particularly *O*AS (see BUCHNER & al. 2004b, HOPKINS & al. 2005). These same metabolites are likely candidates in the signal transduction pathways regulating gene expression. The sink tissues will be actively growing tissue and includes developing seeds. Source tissues will be the green tissues in which active assimilation is taking place or the roots where acquisition occurs. Apparent sink to source communication is likely to be the result of a sequential perturbation of these essential metabolites, firstly in cells of sink tissues and ultimately in the roots cells involved in acquisition. The actual identity of the signal metabolites still remains elusive. Progress is being made on the identification of cis elements linking metabolite pools to gene expression and identification of the linking trans-acting factors will be the next major breakthrough, arising from either transcriptomic or specifically targeted approaches.

Acknowledgments

This project was supported by DEFRA (AR0911) and by a grant from the Biotechnology and Biological Sciences Research Council (BBSRC) to MJH, P.B. BARRACLOUGH (Rothamsted Research) and K.J. EDWARDS (University of Bristol). Rothamsted Research receives grant-aided support from the BBSRC. MJH also thanks Professor Dieter GRILL for encouragement over many years and for practical assistance in the analysis of glutathione.

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