Managing sulphur metabolism in plants

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ABSTRACT

Resolution and analysis of genes encoding components of the pathways of primary sulphur assimilation have provided the potential to elucidate how sulphur is managed by plants. Individual roles for members of gene families and regulatory mechanisms operating at gene, cellular and whole plant levels have been recognized. Sulphur is taken up and transported around the plant principally as sulphate, catalysed for the most part by a single gene family of highly regulated transporters. Additional regulation occurs in the pathway of reduction of sulphate to sulphide and its incorporation into cysteine, which occurs principally within the plastid. Cellular and whole-plant regulation of uptake, and the assimilatory pathway attempt to balance supply with demand for growth and include mechanisms for remobilization and redistribution of sulphur. Furthermore, optimization of sulphur assimilation requires coordination with carbon and nitrogen pathways, and multiple processes have been proposed to contribute to this balance. Present studies on cis and trans elements are focusing on transcriptional regulation, but this regulation still needs to be linked to apparent metabolite sensing. Whilst the components of the assimilatory pathways have been resolved after many years of controversy, uncertainties remain concerning roles of individual genes in gene families, their sub-cellular localization and their significance in balancing sulphur flux to sulphur demand of the plant for growth under variable environmental conditions.

Key-words: cysteine synthesis; metabolic regulation; nutrition; sulphate transporters.

INTRODUCTION

The focus of this review is to summarize the new developments and key open questions in the current state of understanding of regulation of sulphur metabolism in plants. An integration of ideas from the level of the control of gene expression to the regulation of whole plant demand and partitioning of sulphur is presented. How a plant effectively manages one nutrient, in this case sulphur, may be a paradigm for how plants manage nutritional resources in general.

There has been substantial progress on the elucidation of the pathways involved in sulphur uptake and assimilation with several comprehensive reviews appearing in recent years (Leustek 1996; Bick & Leustek 1998; Leustek & Saito 1999; Hawkesford & Wray 2000; Leustek et al. 2000; Saito 2000; Hell & Hillebrand 2001; Droux 2004; Saito 2004; Hawkesford 2005). Furthermore, as a result of recent transcriptome studies, an additional apparent complexity in plant responses to sulphur nutrition has been documented (Hirai et al. 2003; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003). There are complex responses for the adaptation to available sulphur supply to fulfill the demands for growth, and the interactions between shoot and root in the regulation of sulphur assimilation versus uptake and distribution are still poorly understood. Responses to sulphur deprivation (starvation) have been extensively studied; however, the limitation with this approach is that it represents a gross change of circumstance from luxurious abundance to severe limitation. Conversely, in some circumstances, sulphur may be present in abundance, and regulatory mechanisms will favour a limitation of uptake and assimilation. In most natural and agronomic scenarios, a steady-state acclimatization to an intermediary abundance is likely, and demands will depend on a variety of additional factors, both environmental and developmental. Responses to insufficient sulphur availability to match demand may be graded into initial responses specific for sulphur nutrition, followed by more complex responses which may be less specific and invoked by other nutrient deficiencies depending on circumstance (Fig. 1). The complexity of the adaptation is confirmed by the large numbers of nutrient-responsive genes observed in the transcriptome studies and in the numbers of interacting pathways that can be discerned when combined with additional data sets (Nikiforova et al. 2003, 2005; Hirai et al. 2004). The large numbers of regulated processes, although heavily interconnected, form a logical and sequential response and will involve both overlapping and discrete regulatory signals and pathways. Complex responses, such as a shift in the shoot:root ratio (Clarkson, Saker & Purves 1989), or changes in root architecture are likely to involve changes in expression of many genes. Proliferation of roots occurs in response to localized nutrient availability or there may be general root proliferation in response to limiting nutrient availability. Arabidopsis plants grown on low sulphur have an increased lateral proliferation (Kutz et al. 2002), possibly linked to a sulphur limitation-induced nitritease...
which in turn may contribute to the control of indole-3-acetic acid (IAA) levels.

Although root plastids contain all of the enzymes of the sulphate assimilatory pathway, it is evident that the sulphate assimilated in the shoot chloroplasts is the primary source of reduced sulphur in the plant. Multiple points of control within the sulphate assimilatory pathway have been described with much emphasis on the cellular control of flux through the pathway (Vauclare et al. 2002; Wirtz, Droux & Hell 2004). Recent research has begun to determine the molecular components of the signal transduction pathway responsible for the transcriptional regulation controlling the expression of sulphate transporters (Maruyama-Nakashita et al. 2005). This review attempts to put these levels of control in the context of whole plant sulphur nutrition.

THE NEED TO ADAPT TO AND MANAGE SULPHUR SUPPLY

In many industrialized regions, anthropogenic sulphur emissions have been restricted in recent years as a result of environmental legislation. A direct consequence has been a decrease in aerial dry and wet deposition from levels that were previously adequate to supply the sulphur needs of most crops to suboptimal inputs. Added to this is the use of high-analysis triple superphosphate fertilizers with no sulphur content, and the net result has been an increasing incidence of sulphur deficiency in crops worldwide. Sulphur deficiency decreases yields and has consequent effects on nitrogen uptake efficiency as well as specific effects on quality in terms of sulphur amino acid (and hence, protein) content. Decreased content of certain sulphur-rich proteins in wheat has detrimental impacts on flour and baking quality (Zhao, Hawkesford & McGrath 1999). In crops, remedial action is best achieved by early diagnosis, and novel protocols for early detection to supplant difficult visual diagnosis have evolved from research on metabolite responses to sulphur limitation (Blake-Kalff et al. 2000).

All plants regulate sulphur uptake and are able to adapt to a variable or fluctuating sulphur supply, for example:

1. **Acquisition when sulphur is in short supply**. This is probably a common scenario in many environments, and increasingly a situation occurring in agro-ecosystems if no sulphur fertilizer is provided.

2. **Redistribution around the plant**. Re-mobilization of reserves and redistribution around the plant are employed to maximize the usefulness of a limited resource. In cereals during grain filling, sulphur along with nitrogen is re-mobilized from vegetative tissues to the grain as vegetative tissues senescence. Redistribution of protein sulphur is driven by the need to redistribute nitrogen; however, in some circumstances, redistribution can occur as a direct result of sulphur limitation. Redistribution is important if the overall supply is limiting or if the supply is intermittent.

3. **Avoiding excess uptake**. This is a well-studied scenario in the laboratory which at cellular level involves the repression of uptake and assimilation. The rationale is to avoid excess uptake, which is energetically wasteful and/or to avoid potential osmotic imbalances. This is not always successful as with a high external sulphate supply, homeostasis may be overwhelmed. In some plants, for example in *Brassica*, there is a high demand for sulphur, and the sulphate content of vegetative tissues tends to be high.

**BALANCING SULPHUR NEEDS FOR THE WHOLE PLANT**

The sulphur demand varies strongly between species and may be dependent upon the developmental stage of the plant (vegetative growth, seed production). In the perspective of whole plant sulphur metabolism, the requirement is the provision of adequate sulphur to optimize vegetative plant growth, and hence, reproductive potential, and ultimately to provide sulphur for seed tissues to maximize fecundity. In grain crops, the delivery of adequate sulphur to seed tissues is needed for maximal production and for quality aspects in terms of maximizing sulphur amino acid content.

At a whole plant perspective, the uptake of sulphate, its distribution and assimilation will be coordinated and balanced with the actual sulphur demand for growth. During vegetative growth, sulphur uptake is optimized for growth, and when supply is in excess, uptake mechanisms are down-regulated. Sulphate taken up in excess is loaded into vacuoles from where it can be subsequently re-mobilized. The apparent efficiency of this re-mobilization varies between
plant species and depends upon leaf maturity. In *Brassica*, the highest content of sulphate was found in the more mature leaves, and upon cessation of sulphur supply, a short lag period was apparent before this was re-mobilized (Blake-Kalff *et al.* 1998). In some cases, the rate of re-mobilization may cause young developing leaves to be sulphur deficient (Clarkson, Smith & Vandenberg 1983); however, a controlled rate of redistribution may be necessary to optimize availability in the whole plant context. In wheat and soybean, the patterns of redistribution have been extensively documented (summarized in Anderson & Fitzgerald 2003), although the roles of the specific transporters and the cues that control partitioning remain to be determined.

The mechanisms for the regulation of distribution of sulphate taken up by the root to the various sink tissues and then the subsequent redistribution are unclear. Although initial distribution occurs via the xylem, it is not simply related to transpiration, rather sulphate is directed to leaves approaching full expansion (Adiputra & Anderson 1992; Sunarpi & Anderson 1996a). Furthermore, this short-term redistribution is suggested not to be dependent upon sulphur status, but developmentally programmed. A crucial aspect would be xylem to phloem transfer, which might be achieved via Sultr1;3 or members of the group 2 sulphate transporters, which have been reported to be phloem localized (Yoshimoto *et al.* 2003). This mechanism to facilitate favouring younger leaves over older leaves for newly acquired sulphate would require a developmentally controlled process, possibly in the transfer cells (Gunning 1977), selectively removing sulphate from the transpiration stream in the xylem into these leaves.

Pulse-labelling studies with barley and soybean (Adiputra & Anderson 1992; Sunarpi & Anderson 1996a, b) indicate substantial redistribution of sulphur from mature leaves to younger leaves or generative tissues, even when plants are sulphur sufficient. Sulphur that is incorporated into protein during early leaf development is less mobile, and is predominantly re-mobilized only during senescence. Redistribution may be promoted by nitrogen stress (Sunarpi & Anderson 1997a, b) rather than sulphur stress (Adiputra & Anderson 1995; Sunarpi & Anderson 1996b), although ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) breakdown specifically in response to sulphur stress also occurs (Gilbert *et al.* 1997).

In addition to sulphate, sulphur is moved around the plant in the reduced form as glutathione (Rennenberg, Schmitz & Bergmann 1979) or as S-methylmethionine (Bourgis *et al.* 1999). Recently, a gene for a glutathione transporter has been identified, which is part of a larger family of oligopeptide transporters (Zhang *et al.* 2004) and is responsible for the cellular uptake of glutathione. Nutritional conditions will determine the relative importance of these reduced pools as a means of transporting sulphur within the plant. If sulphur supply has been low and vacuolar sulphate pools are not significant, then the relative contribution of reduced sulphur compounds, translocated in the phloem, becomes much greater. Generally, sulphate is a much larger pool than glutathione and S-methylme-thionine, and although they may be important in sulphur delivery to sink tissues such as seed, these tissues have been shown to be fully competent for sulphate assimilation (Tabe & Droux 2001).

### Sulphate Transporter Gene Family

Transport of sulphate into the cell is considered to be a major regulated step (Vauclare *et al.* 2002). In fact, there are several distinct transport steps that are independently, but probably coordinately regulated and which serve to maintain constant cytoplasmic sulphate levels and prevent excess accumulation. To facilitate the complex movements of sulphate around the plant, the sulphate transporters themselves are encoded by a gene family consisting of 14 members in *Arabidopsis*, probably with little redundancy (Hawkesford 2003). There is clear evidence that transcription of the genes encoding the transporters involved in initial uptake at the soil–root interface, cell to cell transfer and vascular transportation as well as the vacuolar efflux transporter is controlled by plant sulphur status (Buchner *et al.* 2004a). The coordinated expression of this gene family facilitates optimum management of plant sulphur under varying conditions of supply and demand.

The sulphate transporter family comprises 14 genes in *Arabidopsis* and probably a similar number in other species (Hawkesford 2003). Based on sequence comparisons, these genes may be aligned into at least five clusters (referred to as groups 1–5, see Fig. 2). It has been proposed that the different groups represent functional sub-types; however, this may be an oversimplification. Broadly, group 1 represents high-affinity transporters, predominantly but not exclusively expressed in the roots (Smith *et al.* 1995; 1997). Many of this group are transcriptionally regulated in response to sulphur availability. A unique specific localization of one isoform in this group, AtSultr1;3, to the sieve elements–companion cell in the phloem is indicative of a specialized role in the redistribution of sulphur from source to sink tissues (Yoshimoto *et al.* 2003).

Group 2, when expressed in yeast, has a lower affinity for sulphate. Expression studies indicate a vascular tissue location, and therefore, of potential significance in considering tissue distribution of sulphate. Details of the expression patterns of the two genes found in this group differ substantially between the two plant species investigated in detail. In *Brassica*, only isoform AtSultr2;1 is expressed substantially in root, stem and leaves, while AtSultr2;2 is also expressed in roots. In *Arabidopsis*, both isoforms are expressed in roots and leaves. In *Brassica*, (Buchner *et al.* 2004a; Buchner, Takahashi & Hawkesford 2004b), the expression of AtSultr2;1 only occurs under sulphur starvation in the roots; however, in the leaves expression occurs also under sulphur-replete conditions, but is increased upon sulphur starvation. AtSultr2;2 expression in the root is increased by sulphur starvation. In *Arabidopsis*, (Takahashi *et al.* 2000), AtSultr2;1 is noticeably induced by sulphur starvation in the roots, as found in *Brassica*. There is little influence of sulphur nutrition on AtSultr2;2 expression.

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Many studies have taken the impact of sulphur-nutritional status on sulphate-influx capacity as a model for dissecting the regulation of sulphur nutrition. Classic physiological studies indicated an increased capacity for uptake into intact plants following a period of sulphur limitation (Lee 1982; Clarkson et al. 1983). Evidence that this was a function of a plasma membrane activity was shown in studies with plasma membrane vesicles isolated from control and sulphur-starved plants (Hawkesford, Davidian & Grignon 1993). It was proposed that a repression mechanism operated in which some downstream reduced sulphur compound acted to repress uptake, probably acting on the transcription of the genes for the uptake transporters (Rennenberg, Kemper & Thoene 1989). When sulphur supply becomes limiting, the levels of these compounds fall and the repression is relieved. Indirect evidence using inhibitors supported a rapid turnover of the sulphate transporter proteins and the importance of transcriptional regulation (Rennenberg et al. 1989; Clarkson et al. 1992).

An interaction with cytokinin-mediated regulation of gene expression is indicated as cytokinins down-regulate the inducible high-affinity sulphate transporters, Sultr1;1 and Sultr1;2 in Arabidopsis (Maruyama-Nakashita et al. 2004). This response is moderated in the mutant, cytokinin response1 (cre1), which is deficient in a two-component cytokinin receptor. This parallels the response reported for a phosphate transporter (Franco-Zorrilla et al. 2002) and may act antagonistically to sugar induction of expression (Lejay et al. 2003; Franco-Zorrilla et al. 2005). Under conditions that are unfavourable for cell proliferation, transporters may be subject to a general down-regulation.

**REGULATION OF SULPHATE UPTAKE AND TRANSPORT AT GENE AND CELLULAR LEVELS**

**Nutritional status and the regulation of sulphate influx**

By contrast to the plasma membrane location of the sulphate transporters of groups 1–3, groups 4 and 5 putative sulphate transporters have been localized to the tonoplast membrane (Kataoka et al. 2004b) (and Buchner & Hawkesford, unpublished results). The group 4 transporters have been implicated in efflux of sulphate from the vacuole and are up-regulated by sulphur stress, thus favouring the unloading of sulphate from the vacuole. The role of the group 5 transporters has yet to be established.

A greater resolution of expression patterns of individual isoforms will be informative, particularly in the light of the complex patterns of sulphate redistribution, which are both developmentally programmed and also influenced by nutrient availability.

**Sulphate transporter regulation**

After the cloning of the first plant sulphate transporters (Smith et al. 1995, 1997), a clear evidence was obtained confirming that sulphur-nutritional status had a major
impact on the transcription of the high-affinity sulphate transporters in the root. Following the removal of the sulphur supply, an increased abundance of mRNAs for high-affinity transporters was observed in parallel with decreasing tissue contents of sulphate, cysteine and glutathione (Smith et al. 1997). Upon the re-supply of sulphate to the medium, a re-repression occurred within hours with almost parallel decreases in mRNA abundance and transporter activity measured as influx, together with rises in internal sulphur pools. Using an antibody to an expressed C-terminal region, the levels of expression of the transporter protein in the plasma membrane have been determined and shown to parallel the observed levels of actual sulphate uptake capacity (Hawkesford & Wray 2000). Such data led to the generally accepted model that the sulphate transporter is transcriptionally regulated by the sulphurnutritional status of the plant; however, some inconsistencies remain. In barley (Smith et al. 1997), the uptake capacity reached a maximum after 4 d of sulphur deprivation and even decreased after this; however, sulphate transporter mRNA abundance continued to increase. In a similar experiment with potato, the sulphate transporter mRNA abundance increased over an 8 d period; however, the measured increased uptake capacity showed only a transient rise (Hopkins et al. 2005).

Clearly, there is more to gene expression than measuring mRNA pools as mRNA pools, proteins and activities are all quite separate entities. This also underlines the care required when interpreting micro-array data. In addition, post-translational modifications, protein turnover and kinetic properties of the transporters will all influence transporter activity.

Role of O-acetylserine (OAS)

Exogenous supply of OAS leads to an increased thiol content indicating that the supply of OAS may limit cysteine synthesis (Neuenschwander, Suter & Brunold 1991; Smith et al. 1997). In addition, a specific influence on the expression of a gene for a sulphate transporter and for enzymes of the assimilatory pathway in a mechanism analogous with that found in bacteria is suggested. In barley, exogenous supply of the cysteine precursor, OAS, enhanced transcription, seemingly overriding the repression signal, as both cysteine and glutathione were enhanced in this treatment (Smith et al. 1997). In bacteria, OAS and sulphide both bind to the cysteine B (CysB) protein, a trans-acting factor, which modulates RNA polymerase activity; OAS enhances binding while sulphide causes a conformational change releasing binding from the promoter site. By analogy with this work on enteric bacteria (Kredich 1992), and in support of an earlier suggestion that OAS could link nitrogen and sulphur metabolism (Neuenschwander et al. 1991), a dual model of repression and activation was proposed (Hawkesford & Wray 2000) and is now a widely accepted model of regulation in relation to balancing sulphur metabolism to that of carbon and nitrogen. The attraction of this mechanism is that when nitrogen and carbon supply exceeds sulphur availability within the cell, OAS accumulates, and this signals to switch on processes enhancing sulphate uptake and reduction. OAS by itself is not able to fully effect control, and an additional inhibitory role of, for example, sulphide or some other reduced sulphur compounds, which can accumulate when sulphur reduction is in excess, is required. It should be noted that no CysB homologue has been identified in plants.

To further support this model, many studies have analysed OAS levels during sulphur limitation with the expectation of an elevation in OAS paralleling or anticipating an increased expression of the sulphate transporter or other sulphate-regulated genes, for example, the β-subunit of β-conglycinin (Kim et al. 1999). Interestingly, a thiol reductase has been implicated in regulating OAS levels (Ohkama-Ohtsu et al. 2004): a mutant in this gene has increased OAS levels and induced sulphur response genes, including a sulphate transporter (AtSultr2:2) and an adenosine phosphosulphate (APS) reductase (APR1). The accumulation of OAS was seen upon sulphur deprivation (Kim et al. 1999); however, substantially increased OAS concentrations in tissues occurred only after an extended period of sulphur deprivation and appeared to be a consequence of the deprivation, rather than part of an early response signalling pathway (Buchner et al. 2004a; Hopkins et al. 2005). A contrary explanation for the effect of exogenously applied OAS is that this overcomes endogenous controls, providing an excess of an otherwise limiting substrate that will drive flux of reduced sulphur through the reductive pathway and will lead to cysteine and glutathione accumulation as has been reported (Smith et al. 1997). Transcriptome profiling studies have suggested that OAS is a global regulator of large numbers of genes, specifically in many cases the same genes that are regulated by sulphur-nutritional status (Hirai et al. 2003). This is consistent with OAS mimicking sulphur limitation as it creates an increased demand for reduced sulphur. All of these data remain consistent with the idea that OAS accumulation reflects an imbalance of nitrogen and sulphur nutrition, rather than an early metabolic signal which the plant can use to fine-tune these pathways.

Components involved in transcriptional control

Given the apparent importance of transcriptional regulation, a priority is the identification of components of the signal transduction pathway. Potential sulphur-responsive elements (SUREs) in promoter regions have been described, although no consensus has been shown. Soybean embryo factors (SEFs) 3 and 4 have been identified to bind to a 235 bp region of the β-conglycinin promoter, which is known to be sulphur responsive (Awazuharra et al. 2002). Such SEF binding sites are also present in the promoter region of serine acetyltransferase of Citrullus vulgaris, which has also been reported to be sulphur responsive (Saito et al. 1997). The NIT3 gene (nitrilase) is highly sulphur regulated, and a region of 317 bp has been identified to contain essential sulphur-nutrition regulatory elements.
(Kutz et al. 2002). A 5 bp sequence represents the core element in the SURE identified in the AtSultr1;1 gene (Maruyama-Nakashita et al. 2005). This core sequence appears in the promoter regions of many sulphur-responsive genes, but not all. It also occurs in promoter regions of genes, which are not sulphur responsive, leading to the conclusion that additional, as yet unidentified, sequences are additionally required. Both the SURE and SEF elements are found upstream of the highly sulphur-responsive gene, AtSg48850 (Howarth & Hawkesford, unpublished results). The next major step is the identification of trans-acting factors.

Post-translational control

In spite of the rapid responses to sulphur-nutritional status in terms of transcriptional regulation and the observed rapid protein turnover, there appear to be additional levels of regulation acting on the sulphate transporters. The carboxy-terminal region contains a sulphate transporter and anti-sigma antagonist (STAS) domain (Aravind & Koonin 2000; Shibagaki & Grossman 2004; Rouached et al. 2005). Mutations or deletions in this region affect function and plasma membrane targeting. The region has a phosphorylation site, and furthermore, the region may be involved in protein:protein interactions, both of which could contribute to regulation. The in vivo significance of these regulatory mechanisms has yet to be shown. A specific protein:protein interaction of sulphate transporters in relation to multimerization has already been referred to earlier (Kataoka et al. 2004a). In addition, it has been suggested that an interaction with ATP sulphurylase may play a role in regulation (Logan et al. 1996; Hatzfeld et al. 1998).

REGULATION OF SULPHATE UPTAKE AND TRANSPORT AT WHOLE PLANT LEVEL

At whole plant level, the uptake of sulphate by the root and its distribution in the plant are driven presumably by the sulphur demand for growth at any specific developmental stage (De Kok et al. 2002a, b; Anderson & Fitzgerald 2003). The extrapolation of the knowledge on regulation at gene/cellular level, and the signal transduction pathways involved to that at whole plant level are rather complicated. For instance, it is still unclear whether bulk changes in gene expression of the different sulphate transporters in the root mimic an altered regulation of uptake and distribution of sulphate, or to what extent there is an interference of local cellular changes in the sulphur status, perhaps acting only in certain cells. At some point, at or prior to the endodermis, in the epidermis or cortex, transport into the symplast occurs, thus at least, the endodermis is the primary selective cell layer. Sulphate is then transferred into the xylem vessels and transported to the shoot by the transpiration stream. Sulphate transporters are presumably present in the plasma membrane of most cells, and their bulk expression responds to variation in sulphate supply (Hawkesford 2003; Buchner et al. 2004a, b); however, changes in expression in selected cells may have a greater influence on net flux. For example, upon sulphate deprivation, there is generally a mass induction of expression of various members of the sulphate transporters in the root, whereas the increase in the overall capacity of sulphate uptake by the root is often limited (ranging from 1.5- to 15-fold in different species) (Hawkesford 2003; Hawkesford et al. 2003; Buchner et al. 2004b).

Variation of sulphate supply generally induces multiple responses facilitating an increased sulphate uptake efficiency on a whole plant basis. In addition to the general fast induction of expression of the sulphate transporters and sulphate uptake capacity by the roots, more prolonged sulphate deprivation generally results in changes of shoot/root biomass partitioning in favour of root production (Stuiver, De Kok & Westerman 1997; Yang, Stulen & De Kok 2003, 2005; Buchner et al. 2004a) and root morphology by increasing the total absorptive surface of the root system (Kutz et al. 2002; López-Bucio, Cruz-Ramirez & Herrera-Estrella 2003). The patterns of changes in morphology upon nutrient deprivation will be mediated by nutrient-specific signal transduction pathways that sense the external and/or internal nutrient concentrations to modify root development (López-Bucio et al. 2003); however, there are species and varietal differences. Studies on the interaction between atmospheric and pedospheric sulphur nutrition of Brassica showed poor shoot to root signalling in relation of the sulphate uptake efficiency. Plants were able to utilize foliar absorbed and metabolized SO2 or H2S as the sole sulphur source for growth upon sulphate deprivation of the root. Peculiarly, the strongly induced expression of the sulphate transporters in the sulphate uptake capacity of roots (Buchner et al. 2004a) and the decrease in shoot:root biomass ratio of Brassica upon sulphate deprivation were hardly affected by SO2 or H2S exposure (Yang et al. 2003, 2005; Buchner et al. 2004a). There was apparently no strict and direct shoot to root signalling for the regulation of sulphate uptake by the root and transport to the shoot in relation to the need for growth. It is unclear to what extent the external or internal sulphate concentration in the root itself is the sensing factor in the modulation of the sulphate efficiency in general. Recent data suggest that when Brassica was grown at a maintained 5 µM sulphate concentration (the sulphate concentration in Hoagland nutrient solution is 2000 µM), plant growth was quite normal although the sulphate content of the shoots was somewhat lower compared to plants grown at 100 or 500 µM (Posthumus, Koralewska, Stuiver & De Kok, unpublished results). In spite of this, the overall sulphate concentration in the root exceeded 5000 µM, and the sulphate uptake capacity of the roots was strongly enhanced. This means that if sulphate itself is the sensed factor, then there would need to be a huge local variation in inter- and/or intracellular distribution of sulphate in root cells.

It is often stated that after an uptake by the root, sulphate is transported to the shoot where it is reduced in the chloroplast prior to its assimilation into other organic sulphur compounds. However, Pate (1965) demonstrated that roots
were able to reduce sulphur, some of which was transported as methionine and to a lesser extent as cysteine and glutathione to the shoot. All enzymes of sulphate assimilation are present in the roots (Heiss et al. 1999; Lappartient et al. 1999; Lee & Leustek 1999; Yonekura-Sakakibara et al. 2000), their activity and expression (e.g. that of ATP sulphurylase and APS reductase responds to the sulphate supply) (Lappartient et al. 1999) and sulphate reduction most likely occur in the plastids. The production of phytochelatins in response to heavy metal stress increased sulphate assimilation and resulted in an induction of not only the expression of sulphate transporters, but also of the enzymes involved in sulphate assimilation (Heiss et al. 1999; Lee & Leustek 1999). However, by contrast to the reduction of nitrate (Rufty et al. 1986), there is still little information on the abundance and compartmentation of sulphate reduction, in particular groups of cell collectively forming the root sympatry. As with nitrate reduction (Scheuwater et al. 2002), the proportion of sulphate reduction in the root, at least for seedlings of most herbaceous and crop plants, compared to whole plant sulphur assimilation will be restricted, because the shoot:root ratio generally exceeds a factor of 2–5. However, the abundant sulphate reduction and assimilation found in the different root cell types may be important in local tissue and whole plant modulation of sulphate uptake and transport by local signalling by, for example, the in situ sulphate and/or OAS concentration.

CONTROL OF ASSIMILATORY PATHWAY FLUX AT GENE AND CELLULAR LEVELS

After many years of controversy, a plant-specific pathway of sulphate reduction and assimilation into cysteine and methionine has been defined (Fig. 3). Additional complexity arises as multi-gene families exist for most enzymes, and in many cases, specific gene products of these families are localized in individual sub-cellular compartments (Hawkesford 2005). Sulphite reductase is the exception, represented by just a single gene, and localized exclusively in the chloroplast, hence ensuring that the primary site for sulphate reduction and assimilation is in the chloroplast, where adequate ATP and reductant are available. Functions of other isoforms are not fully resolved, but are likely to be involved in providing intermediates for other pathways (Rotte & Leustek 2000), in scavenging ‘leaked’ metabolites, for recycling of metabolite or with specific biosynthetic roles as is the case for cysteine synthase in the mitochondrion (Warrilow & Hawkesford 1998, 2000, 2002).

A major uncertainty in the pathway was resolved with the cloning and description of APS reductase (Gutierrez-Marcos et al. 1996; Setya, Murillo & Leustek 1996). Previously, it had been unclear as to whether there was a plant-specific APS sulphotransferase with bound sulphite intermediates or whether the pathway was analogous to the bacterial pathway and included phosphoadenosine phosphosulphate (PAPS) as an intermediate (Kopriva & Koprivova 2004). It is now clear that APS reductase is the APS sulphotransferase, and reduces APS to produce free sulphite directly. PAPS is part of a biosynthetic pathway branch that supplies sulphur for sulphotransferase catalysed reactions (Klein & Papenbrock 2004). APS reductase has been shown to have a major contribution to the control of pathway flux (Vauclare et al. 2002), that over-expression increases flux (Tsakraklides et al. 2002) and the expression of the respective gene is controlled by sulphate status (Hopkins et al. 2004).

Cysteine synthase complex as a molecular sensor of sulphur status, controlling flux to cysteine

Cysteine synthesis is catalysed by cysteine synthase, a dissociable complex of OAS (thiol)lyase (OASTL) and serine acetyltransferase (SAT). The two component enzymes form a reversible complex comprising a homotetramer of SAT and two dimers of OASTL. The respective activities of the two components are dependent upon their association state, which in turn is controlled by the free concentration of OAS. In effect, flux through this part of the pathway is effectively controlled by OAS concentration, which varies depending upon the sulphur supply. Hence, the dissociable complex becomes a sensor (Fig. 4). At high levels of OAS (which occur under sulphur-limiting conditions), the complex dissociates and SAT are inactivated, thus prevent-
Methionine

Methionine is an important sink for sulphur, required both as a constituent of proteins and as a methyl group donor or precursor for metabolites such as ethylene, polyamines and dimethylsulphoniopropionate. Methionine biosynthesis represents a convergence point of the sulphur-assimilatory pathway and the aspartate-derived amino acid family bio-synthetic pathways. The control of flux of sulphur to methionine, and hence, the control of methionine pools appears to differ between plant species (Hesse & Hoefgen 2003). In potato and Arabidopsis, S-adenosylmethionine (SAM) feedback activates the threonine synthase enzyme in a direct allosteric fashion (Curien et al. 1998), enhancing flux of the common substrate, O-phosphohomoserine (OPHS), to the threonine branch and restricting flow for methionine biosynthesis. In Arabidopsis but not in potato, there is further negative regulation, in a post-transcriptional mechanism, of expression of cystathionine γ-synthase by an end product of the pathway, probably either methionine or SAM (Inaba et al. 1994; Chiba et al. 1999; Kim & Leustek 2000; Hacham, Avraham & Amir 2002; Hesse & Hoefgen 2003). Both methionine and threonine synthases may be limited by homoserine availability (Lee et al. 2005), the precursor for OPHS. This would take a level of control to the lysine branch point in the aspartate pathways.

CONTROL OF ASSIMILATORY PATHWAY FLUX AT WHOLE PLANT LEVEL

As with sulphate uptake and distribution, the assimilation of sulphate in plants is driven and balanced with a demand for growth and development. The overall sulphur flux in the plant at any stage of development can be estimated by multiplying biomass production with time by the plant sulphur content (De Kok et al. 2002a, b). The actual flux of the sulphur assimilatory pathway may be estimated if the organic sulphur content is used in the calculation. There is a large variation in sulphur demand and assimilatory pathway flux between plant species, which in addition, is strongly affected by environmental (growth) conditions. At optimal growth conditions, the sulphur assimilatory pathway flux of different crop plants ranges from 0.03 to 0.2 μmol g−1 plant fresh weight (FW) h−1 (De Kok et al. 2002b; Durenkamp & De Kok 2004). It is evident that
plants have, to some extent, a physiological plasticity to modulate the sulphur flux in order to adapt to an altered sulphur demand and limited or excess sulphur supply by changing the level and expression of sulphate transporters and the enzymes of the sulphur assimilatory pathway.

Despite the tremendous progress in the control of assimilatory pathway flux at gene/cellular level, an insight on the regulation of the sulphur assimilatory pathway in relation to the regulation of the uptake and distribution of sulphur at whole plant level (e.g. shoot:root and source:sink signalling) is largely unclear. Moreover, conclusions of changes in fluxes and regulatory control are often based on observed variation in levels of free specific sulphur metabolites, ignoring the predominant proportion found in proteins, which as cysteine and methionine represent, up to 70% of the total sulphur content of plants. Many questions raised more than a decade ago (Stulen & De Kok 1993) are still valid for the interpretation of sulphur assimilatory pathway regulation at whole plant level: (1) how are the overall reduction and assimilation of sulphate controlled so that under non-limiting conditions of sulphur supply and growth conditions, sulphate reduction reaches a maximum and no more reduced sulphur is accumulated; (2) how does the cell (both root and shoot) control the fluxes of sulphate into the chloroplast (plastid) and vacuole; and (3) how does the sulphur demand for growth regulate the uptake and transport of sulphate, and what signal transduction pathway is involved?

The level of metabolites, which play an important role in the regulation of the sulphate assimilatory pathway, is quite dynamic and has been shown to be dependent on the environmental conditions; however, their sub-cellular distribution is largely unclear. For example, if plants are exposed to atmospheric sulphur gases such as H2S, these gases are assimilated and utilized as a sulphur source. However, exposure also generally results in an increased size and change in composition of the thiol pool of the shoot, dependent on the H2S concentration, leaf age, the exposure temperature, the level of sulphur nutrition and which varies strongly between species. In some species, exposure resulted in a slight increase in the thiol content of the roots. Upon H2S exposure, the glutathione level in the shoot may increase to a new steady-state level up to threefold greater than that of unexposed plants, and the levels of cysteine may increase more than 30-fold, without negative effects on plant growth (De Kok et al. 2002a; Durenkamp & De Kok 2004; Riemenschneider et al. 2005b). Apparently, the thiol accumulation reflects a slight overload of the reduced sulphur supply, but its steady-state level is beyond strict regulatory control. The levels of the precursor of cysteine, OAS and also that of N-acetylserylne in the shoots were strongly decreased upon prolonged H2S exposure (Buchner et al. 2004a; Riemenschneider et al. 2005b).

**Stress-induced demands for sulphur**

A number of stresses appear to increase the plant demand for reduced sulphur, notable amongst these are oxidative stress and heavy metal exposure. An increased expression of sulphate transporters and enzymes of the assimilatory pathway is seen in response to stress (Schäfer, Haag-Kerwer & Rausch 1998; Heiss et al. 1999; Vanacker, Carver & Foyer 2000; Harada et al. 2002; Howarth et al. 2003a). Transgenic lines with increased fluxes to cysteine, and hence, protective and/or metal-chelating compounds such as glutathione and phytochelatins may contribute to an improved tolerance of plants to these stresses (Błaszczyk, Brodzik & Sirko 1999; Domínguez-Solis et al. 2001, 2004; Noji et al. 2001; Howarth et al. 2003b). However, these compounds, even at high stress levels, generally account for only a minor proportion of the total plant sulphur content. It is assumed that modulation of glutathione levels (e.g. upon stress conditions) would require a higher sulphate uptake and assimilatory sulphur flux; however, glutathione accounts only for a minor proportion (about 2%) of the total organic sulphur content and assimilatory sulphur flux (De Kok & Stulen 1993; Stulen & De Kok 1993). Alternatively, an enhanced glutathione level upon stress exposure might be the consequence of an imbalance between the rate of sulphur assimilation and protein synthesis at a stress-induced hampered growth (Stuiver, De Kok & Kuiper 1992).

Several recent studies have underlined the importance of sulphur nutrition in plant defence. Many plant anti-pathogen compounds are sulphur containing, including thionins (Thomma, Cammue & Thevissen 2002), alliins (Jones et al. 2004) and glucosinolates (Griffiths, Birch & Hillman 1998). Profiles of glucosinolates can be modified by pathogen infection (Mikkelsen et al. 2003). Recent observations have shown that elemental sulphur is deposited in xylem parenchyma upon infection with *Verticillium dahliae* in both *Theobroma cacao* and in tomato, and appears to limit the spread of infection (Williams et al. 2002; Howarth et al. 2003a; Cooper & Williams 2004). The biosynthetic pathway for elemental sulphur production is not known. A general mechanism of importance for pathogen resistance may be the release of sulphide from desulphydrase reactions in response to infection (Bloem et al. 2004; Riemenschneider et al. 2005b). Maintaining these defence reactions places a high demand on the plant sulphur pathway and requires an optimum sulphur nutritional availability.

**SULPHUR METABOLISM AS PART OF A METABOLIC NETWORK**

There are several studies employing transcriptome analysis to study global responses to sulphur deprivation. In each case, one or more discrete time points, in some cases together with a sulphur re-supply and/or OAS treatment, have been studied (Hirai et al. 2003; Nikiforova et al. 2003). In one case, a mutant lacking the Sultr1;2 sulphate transporter, which as a consequence had reduced uptake capacity and sulphur pools, was analysed (Maruyama-Nakashita et al. 2003). In this instance, a steady-state reduced sulphur availability was achieved, rather than the typical experimental non-steady state or highly stressed situation. Tran-
Scriptome profiles indicated the up-regulation of antioxidant mechanisms in response to the reduced glutathione availability. Generally in transcriptome studies, large numbers of genes have been shown to vary in their expression in terms of mRNA pool sizes. The regulated genes may be placed into functional categories (Wawrzynska et al. 2005), or into pathways and defined areas of metabolism (Nikiforova et al. 2003, 2004). In most instances, hypotheses may be made to implicate the respective processes in the adaptive response. Large numbers of regulated genes might be expected given the cascade of responses expected as a consequence of sulphur limitation (Fig. 1).

Metabolite profiling provides a similar picture of interacting pathways responding to an external stimulus such as sulphur deprivation (Nikiforova et al. 2005). A coordinating network of adaptation is revealed, which is suggested to result in a re-balancing of plant metabolism as a whole, so called ‘systems re-balancing’. Analysis of multiple time points aids in distinguishing early and late responses in which resources are channelled to optimize metabolism. There is an initial accumulation of certain nitrogen-rich compounds, but ultimately many catabolic pathways are switched on.

An ideal approach is to perform both transcriptome and metabolome analyses, and to combine these data sets (Hirai et al. 2004, 2005). Analysing responses to both nitrogen and sulphur limitation reveals the extent of both parallel responses to these nutrient deficiencies (e.g. photosynthetic pathways, nitrate reductase and glucosinolate metabolism) and those specific for the individual nutrient limitation (Hirai et al. 2004). In such studies, often the imposed deficiencies are quite severe and will result in complex responses (Fig. 1).

A goal of the ‘omic’ approaches is the elucidation of these signalling components; however, these may not be differentially expressed themselves but must be inferred by association of interacting pathways or genes. For example, a gene encoding the v-myb myeloblastosis viral oncogene homologue (MYB) transcription factor, production of anthocyanin pigment 1 (PAP1), was identified because of co-expression with anthocyanin biosynthetic genes, which were all co-regulated by sulphur availability (Hirai et al. 2005; Tohge et al. 2005). The identification of non-regulated components is far more demanding.

SUMMARY

Sulphur is an important nutrient for plant growth and health. Optimizing its assimilation into the many compounds involved in specific aspects of metabolism, as well as coordinating its incorporation into amino acids and proteins by balancing availability and sink demands, requires a complex network of interacting aspects of plant metabolism. Furthermore, sulphur pools are managed, mobilized and distributed through development to optimize fecundity. Understanding the underlying mechanisms at gene, cellular and whole plant levels may enable us to produce crops with improved quality and resistance to stress. As a paradigm for nutritional management, ideas concerning sulphur fertilizers and their efficient use may be helpful for optimizing fertilizer use efficiency in general.

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REFERENCES


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Nikiforova V., Freitag J., Kempa S., Adamik M., Hesse H. &...


Rennenberg H., Kemper O. & Thoene B. (1989) Recovery of


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