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Plant responses to sulphur deficiency and the genetic manipulation of sulphate transporters to improve S-utilization efficiency

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Abstract

Decreased inputs of S have increased the incidence of S-deficiency in crops, resulting in decreased yields and quality. Remediation by fertilizer application is not always successful because this often results in an uneven supply of S. The ability to respond to S-deficiency stress varies between crops and this is a target for the genetic improvement of S-utilization efficiency. Improved capture of resources, the accumulation of greater reserves of S and improved mechanisms for the remobilization of these reserves are required. It is an inability to over-accumulate S and subsequently, effectively remobilize S-reserves, which restricts optimum S-use efficiency. Genetic manipulation of the transporters and their expression will contribute to overcoming these limitations. Control of gene expression limits excess uptake and activity of the assimilatory pathway: the endogenous expression of sulphate transporters is regulated by S-supply, with negative regulation from reduced S-containing compounds and positive regulation by *O*-acetylserine, the C/N skeleton precursor of cysteine. Constitutive expression of the transporter will remove this control and may enable the accumulation of sulphate reserves. Sulphate in the vacuole and other pools of reduced sulphur, such as glutathione or protein may be remobilized under S-limiting conditions. Low efficiencies of these remobilization processes, particularly the remobilization of vacuolar sulphate, suggest that the transporters involved in the remobilization are a target for modification. Transporters are involved in facilitating the multiple trans-membrane transport steps between uptake of sulphate from the soil solution, and delivery to the site of reduction in the chloroplast or plastid. A gene family has been identified and phylogenetic relationships based on primary sequence information indicate multiple sub-groups. Groups which are expressed in roots, in shoots and in both tissue types are postulated, however, the functional roles for these groups and the identification of transporters involved in recycling remain to be confirmed.

Key words: Sulphate transporter, sulphur, S-limitation, S-inputs, S-mobilization, cereal nutrition.

Introduction

Why is S-deficiency a problem?

In recent years S-deficiency has become an increasing problem for agriculture resulting in decreased crop quality parameters and yields (McGrath *et al.*, 1996). Appropriate applications of fertilizer can remedy deficiencies in many instances, however, there remain considerable uncertainties regarding timing and type of S-application, which in turn influence the persistence of the S in the soil and the availability to the plant. A common situation is one in which there is a substantial seasonal variation in S available to the plant and, ideally, crops will be engineered to maximize uptake when S is abundant and therefore be better able to tolerate periods of low S-availability. Studies on the mechanisms for controlling sulphate uptake and assimilation suggest approaches for the genetic manipulation of expression of the transporters to engineer crops with improved S-utilization efficiency and S-deficiency stress tolerance

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Abbreviations: OAS, *O*-acetylserine; SATase, serine acetyl transferase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SHST1 etc, *Stylosanthes hamata* sulphate transporter; HVST1, *Hordeum vulgare* sulphate transporter, TTST1 etc., *Triticum tauschii* sulphate transporter.

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(Clarkson and Hawkesford, 1993; Hawkesford and Smith, 1997; Smith *et al.*, 1997).

In recent decades considerable progress has been achieved in reducing emissions of S to the atmosphere, which has resulted in a consequent decrease in atmospheric depositions of S onto agricultural land (McGrath *et al.*, 1996). Whilst these depositions were once sufficient to support crop requirements for S, they now fall well below the recommended requirements for cereal and oilseed crops. Predictive modelling has shown that the occurrence of agricultural land at risk from S-deficiency will increase. Deficiencies are predicted for cereals and are more likely for oilseed rape crops, which have a higher requirement for S (McGrath *et al.*, 1996). Research into plant adaptation to S-related stresses has shifted from an emphasis on excessive inputs and acidification to the other extreme of how deficiencies are impacting on crop production. This paper will review the effects on plant growth and crop quality associated with S-deficiency and the increased occurrence of this 'hostile environment'. Plant responses to inadequate S-supply will be examined and specific targets for genetic manipulation to engineer S-deficiency stress tolerance are identified. The importance of the sulphate transporters, which have pivotal roles influencing whole plant S-metabolism, will be reviewed.

Plant responses to S-limitation stress

Effects of S-deficiency in plants, symptoms and effects on yield consequences

The agronomic consequences of insufficient S are well documented with decreased yields and a substantial impact on S-content under extreme deficiency (for a review, see Zhao *et al.*, 1999). In many cases of mild S-deficiency stress there may be little impact on yield but important consequences for quality, with substantially modified N : S ratios (Zhao *et al.*, 1996). A shift to higher N : S ratios has been observed in the years 1981 to 1993 in British wheat grain (Zhao *et al.*, 1995), closely mirroring the decrease in atmospheric outputs and subsequent deposition of S (McGrath *et al.*, 1996). Limiting S availability has been shown to favour the synthesis and accumulation of S-poor or low-S storage proteins such as ω-gliadin and high molecular weight subunits of glutenin at the expense of S-rich proteins in wheat (Moss *et al.*, 1981; Wrigley *et al.*, 1984; Fullington *et al.*, 1987). These changes in protein composition are associated with alterations of dough rheology and bread quality.

Physiological responses

Sulphate deficiency in young wheat plants has an early effect on CO_2 assimilation rates and on Rubisco enzyme activity and protein abundance (Gilbert *et al.*, 1997). This is a result of decreased synthesis of new protein under S-limiting conditions and, additionally, some degradation was observed in response to S-limitation in the older leaves. The lack of synthesis of Rubisco and the chlorosis of the young leaves due to decreased chlorophyll content (Burke *et al.*, 1986), reflect a general inhibition of *de novo* synthesis of the photosynthetic apparatus.

Another metabolic effect of S-stress is a depression of the root hydraulic conductivity (Karmoker *et al.*, 1991), an early response which may have a role in signalling nutrient starvation from root to shoot. It is proposed that stomatal closure restricts CO_2 uptake, limiting carbon assimilation and thus restricting the metabolic need for S.

An obvious indication of S-deficiency is the reduction in the internal S pools (see below), but additionally there are rises in soluble nitrogen pools including nitrate and amides as a consequence of the N : S imbalance (Karmoker *et al.*, 1991; Zhao *et al.*, 1996; Prosser *et al.*, 1997; Warrilow and Hawkesford, 1998). These metabolite fluctuations have been proposed as possible diagnostic indicators of S-deficiency (Zhao *et al.*, 1996).

Storage and remobilization

Several distinct pools of S occur in plant tissues, with the most occurring as sulphate or in the protein fraction. The relative abundance of these two fractions depends upon the specific tissue and the previous nutritional history of the plant (Blake-Kalff *et al.*, 1998). Other smaller pools include free amino acids, cysteine and methionine, the tri-peptide glutathione, sulpho-lipids and other secondary compounds such as the glucosinolates found in the Brassicaceae. If present, the most significant and readily mobilized form is sulphate. Whilst cytoplasmic concentrations of sulphate are kept relatively constant, sulphate taken up by the plant, which is surplus to immediate requirements for growth, is stored in the vacuole. Reports on the effectiveness of mobilization of this vacuolar sulphate pool vary, and may reflect species differences or the ability of the remobilization processes to keep pace with growth rates. The mobilization of this vacuolar pool has been reported to be a slow process in roots (Bell *et al.*, 1994, 1995*a*) in mature leaves (Bell *et al.*, 1995*b*), and particularly so in oilseed rape (Blake-Kalff *et al.*, 1998).

The patterns of S-accumulation and redistribution in *Brassica napus* have been described recently in detail (Blake-Kalff *et al.*, 1998). When supplied with adequate S, the concentrations of glutathione and glucosinolates accounted for 2% and 6% of the S-content in the youngest leaves, respectively. In the older leaves these compounds accounted for an even smaller proportion of the total S, and it was concluded that they are not major reserves of S during deficiency. The concentration of insoluble (protein S) was similar for all leaves (around 50%). In the mature leaves 70–90% of the total S could be accumulated as sulphate. If S-supply was withdrawn, these pools all

decreased, although the decrease in concentration could be accounted for mainly by growth. There was little evidence of the large reserves of sulphate being redistributed to the younger growing parts of the plant. This inefficiency in managing S-reserves is suggested to be part of the reason for the high S requirement for oilseed rape crops.

Pulse-labelling experiments have investigated fluxes of sulphate in barley (Adiputra and Anderson, 1992, 1995). These studies demonstrated redistribution of sulphate, but showed no evidence for enhanced redistribution stimulated under S-limiting conditions. In soybean, the greatest redistribution of S occurs when N-limitation induces proteolysis (Sunarpi and Anderson, 1997). Studies on the remobilization of S in the flag leaf and delivery to the developing grain of wheat indicate that when there are adequate reserves of sulphate, this can be remobilized to the grain (MJ Hawkesford, unpublished results). Plants grown on an adequate S-supply (1.0 mM sulphate in the nutrient solution, applied on alternate days) until anthesis when the supply of S was terminated, were able to maintain the S-content of the grain at near control levels (S-supply maintained after anthesis), at the expense of S-pools in the flag leaf. This was in contrast to plants grown with a sub-optimal S-supply, with for example, 0.1 mM sulphate in the nutrient solution, where little S-accumulated in the flag leaf and grain contained substantially less S than the control plants.

Sulphate transporters and the S-assimilatory pathway

Sulphate transporters and metabolite control of expression

Prior to identifying targets for genetic manipulation it is necessary to understand the biology of sulphate uptake and assimilation in higher plants, and to have cloned the genes encoding the relevant components. In recent years genes or cDNAs encoding sulphate transporters and enzymes of the assimilatory pathway have been cloned. Identification of these genes has enabled the resolution of long-standing controversies concerning the components of this pathway and a generally accepted pathway is shown in Fig. 1. In addition, the availability of the cloned genes has facilitated the investigation of the underlying mechanisms controlling flux of S through the assimilatory pathway.

As shown in Fig. 1, control of flux of S is proposed to be by both allosteric regulation of enzyme activity and regulation of levels of gene expression of components of the pathway. The most highly regulated components are the root-expressed transporters (Smith *et al.*, 1995*b*, 1997; Takahashi *et al.*, 1997). The sulphate transporters, expressed in the plasma membranes of root cells are proton/sulphate co-transporters (Hawkesford *et al.*, 1993) comprising a single polypeptide of around 70–74 kDa. The precise location of their expression with respect to the root zone relative to the root tip, or where in the cortex or stele they are expressed remains to be elucidated. In one study there is an indication that one of the *Arabidopsis* transporters (AST68) is expressed within the stele region (Takahashi *et al.*, 1997). These polypeptides encoded by the cloned cDNAs or genes have a proposed 12 trans-membrane helix structure. Although the amino acid sequences of all of the cloned sulphate transporters are related and have homology with other sulphate transporters identified from yeast, fungal and mammalian sources, they form a unique group of transporters with no identified sequence motifs linking this group to any other solute transporter groups.

Sulphate after entering the cell is first activated by ATP sulphurylase to APS, which is then reduced by APS reductase and sulphite reductase to form sulphide, which is then incorporated into cysteine catalysed by the cysteine synthase complex. The cysteine synthase complex comprises two component enzymes, the OAS-thiol lyase which catalyses the conjugation of sulphide with *O*-acetylserine (OAS) to form cysteine, and serine acetyl transferase (SATase) which catalyses the acetylation of serine by acetyl-CoA to form OAS. Provision of serine is dependent upon adequate C and N metabolism, and this point of convergence of the assimilatory pathways represents an opportunity for the co-ordination of S-assimilation with C and N metabolism. The OAS-thiol lyase is present in excess compared to SATase and it has been suggested that only when in this complexed state is the SATase fully active. The presence of high levels of OAS can act to disrupt this complex and limit further OAS synthesis (Hell, 1998). An additional cysteine allosteric feedback loop prevents excess cysteine being formed in the cell when both serine and sulphide are abundant.

A model to control expression of the genes for the sulphate transporters and components of the assimilatory pathway such as APS reductase (Takahashi *et al.*, 1997) and possibly other components of the pathway is outlined in Fig. 1. In the model, metabolic intermediates of the pathway are the first components of signal transduction pathways, which regulate expression of genes encoding the key components of the pathway. It is proposed that the levels of expression of these genes regulate the flux of S through the assimilatory pathway (Kredich, 1993; Hawkesford and Smith, 1997). These feedback loops prevent excess uptake and reduction of S which would result in an accumulation of potentially toxic sulphide if OAS is limiting. A surplus of reduced sulphur-containing compounds act to repress expression of the transporter and the APS reductase. Although there is some regulation of expression of many components of the assimilatory pathway, most notably the APS reductase, by far the greatest regulation seems to be at the level of the trans-

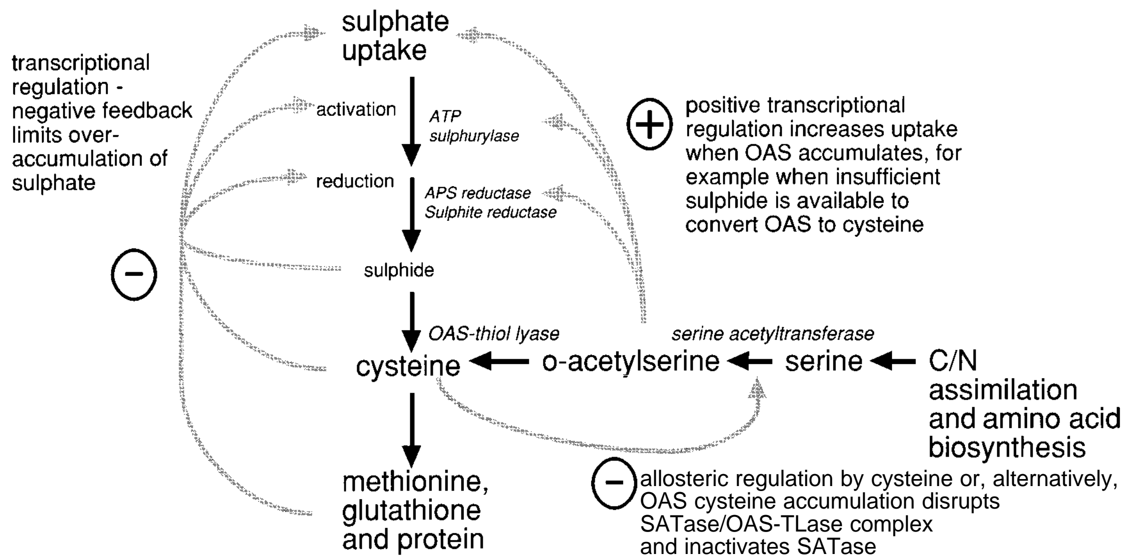

Fig. 1. Metabolite control of sulphate uptake and assimilation. A series of feedback loops are proposed in which cellular concentrations of pathway intermediates may act as part of a signal transduction pathway to repress or activate expression of the genes encoding the proteins controlling some of the individual steps in the pathway. In addition, there is also allosteric feedback regulation of SATase by OAS and cysteine. Solid lines represent metabolite fluxes, grey lines are feedback control loops.

porters, with mRNA pool sizes and transporter activity being regulated by S-availability (Smith *et al.*, 1997). Following re-supply of sulphate to S-starved hydroponically grown barley plants, repression occurs very rapidly. The mRNA pools for the transporter decreased within 1 h, and activity and protein abundance decreased within 2–4 h. The identity of the negative regulatory compounds which are involved in this repression mechanism awaits confirmation and may be cysteine or glutathione or even sulphide itself. It is this feedback loop primarily, which limits excess accumulation of sulphate, but which has a secondary effect in limiting reduction.

In a situation where S is limiting, a second feedback control loop may act to facilitate de-repression. In this case when serine is in excess and S is limiting, there will be no sulphide present for the biosynthesis of cysteine or for the allosteric inhibition of OAS synthesis, and therefore OAS accumulates. Experimental evidence (Smith *et al.*, 1997) suggests that OAS is a positive transcription regulator of transporter expression in plant, a situation analogous to that found for prokaryotes (Kredich, 1993). Furthermore the presence of OAS partially overrode the negative feedback provided by the reduced S-compound. When repression occurs, this may be due to both the negative feedback activity of the reduced S-compound, reinforced by the absence of the activator, OAS. The regulation is an adaptive strategy to maximize resource capture, maximizing flux to cysteine under S-limiting condition, but with built-in controls to prevent the system running away with itself. Thus sulphate uptake is intrinsically linked to availability, demand for reduced S and also the supply of C/N skeletons. A consequence of this fine regulation is that sulphate is unlikely to be accumulated to any degree.

A multitude of transporters—gene families and sites of expression

The first plant sulphate transporters, from *Stylosanthes hamata* (Smith *et al.*, 1995*b*) and from barley (Smith *et al.*, 1997), were cloned by phenotypic complementation of a yeast mutant (Smith *et al.*, 1995*a*). The nature of the selection process, and the subsequent functional analysis of the transporters when expressed in yeast, confirmed that these were transporters for sulphate. Both high (10 μM) and low (100 μM) affinity transporters were cloned from *Stylosanthes hamata* (SHST1/2 and SHST3, respectively). Subsequently, other transporters have been identified, either fortuitously or by screening libraries or EST databases, most notably in *Arabidopsis*. Two wheat transporters (TTST1 and TTST2) have been isolated by heterologous screening (IM Prosser and MJ Hawkesford, unpublished results) bringing the total number of plant transporters belonging to this family to around 20, including seven different members in *Arabidopsis*. All of these sequences were isolated or identified by exploiting sequence homology and are therefore related at the sequence level, however, not all have been confirmed to be sulphate transporters by functional analysis. The amino acid sequence similarity clearly defines this group of transporters, and the complete absence of homology to any other known transporter family makes this transporter group a completely unique type. Following sequence comparison and display of the degree of similar-

ity as a phylogenetic tree, clear sub-groups become apparent (Fig. 2). The sub-groups do not follow strict phylogenetic divisions and it is proposed that they represent functional sub-groups. For example, the seven *Arabidopsis* types (underlined) are relatively unrelated and are found dispersed around the tree. Possible functional sub-groupings for some sequences are indicated on the figure, although these grouping are very speculative, as current information on sites of expression is rather limited. A high affinity group, specifically expressed in the root, is defined by SHST1, SHST2, HVST1, and TTST1 (Smith *et al.*, 1995*b*, 1997; IM Prosser and MJ Hawkesford, unpublished results). TTST2 is an anomaly in this group in that whilst it shows high homology to TTST1, it is not highly expressed in the roots (Prosser and Hawkesford, unpublished results). The closest *Arabidopsis* homologue in this sub-group is AST101. A second group is expressed in both root and shoot. Expression studies show that AST68 is expressed near to root and shoot vascular tissues (Takahashi *et al.*, 1997) and is highly regulated by S-availability in the root. AST56 is also expressed in the root and the shoot (Takahashi *et al.*, 1996). SHST3 is shoot expressed (Smith *et al.*, 1995*b*) and a low affinity type. NOD70 is a soybean root nodule expressed gene (Kouchi and Hata, 1993; Sandal and Marcker, 1994). Members of the third group, with putative leaf specific expression, and whose expression has been confirmed, are the *Sporobolus* transporter cDNA, which came from a shoot expressed library (Ng *et al.*, 1996) and ATD631 which is homologous to the EST clone, 76E7T7, reported to be leaf specific (Takahashi *et al.*, 1996). The remaining three sequences do not fall within any of these tentative groupings.

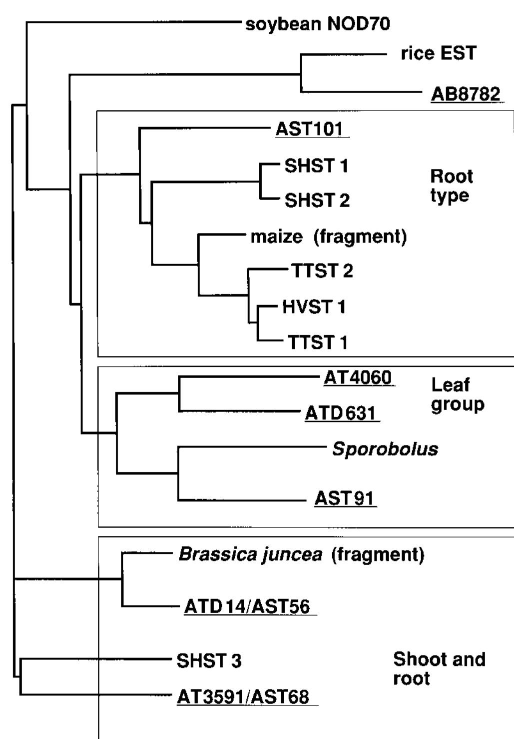

Fig. 2. Similarity analysis of the amino acid sequences of plant sulphate transporters. All of the related sequences from higher plants, currently available in the databases, were aligned using PILEUP in the Wisconsin GCG package (Version 8). The resulting MSF file was analysed to produce a tree using ClustalX version 1.5b and displayed using TreeView version 1.4 for the Macintosh. Accession numbers of the sequences analysed were SHST1, X82255; SHST2, X82256; SHST3, X82454; HVST1, X96431; TTST1, AJ238244; TTST2, AJ238245; *Sporobolus*, X96761; A8782, AB008782; AST101, D1034907 or O22277; AT4060, AB004060; ATD631, D89631 (also known as ATST1), or AB012048 (AST12); AST91, O49307; ATD14, D85415 or AST56, S74246; AT3591 (also known as AST68), AB003591; soybean NOD70, Q02920; maize fragment, O48889; rice EST fragment, D25000; *Brassica juncea* fragment, AJ223495. All *Arabidopsis* sequences are shown underlined. Groups of sequences are enclosed in boxes to indicate possible functional groupings.

Why are there so many transporters?

In between entering the symplast of the plant somewhere in the root and reaching the primary site for reduction, generally considered to be the chloroplast in the leaf tissues, multiple cell-to-cell transfers and transfers across intracellular membranes for the sulphate ion may be envisioned. Balanced against this scenario is the substantial energetic cost of multiple active membrane transport steps (Clarkson, 1993) and at least some cell-to-cell transfer may involve plasmodesmata or transport via channels. Some possible sites for trans-membrane transport of sulphate are listed in Table 1. It is possible that the same transporter (encoded by a single gene) is expressed in different locations and could catalyse several of these steps. It is also very likely that more than one transporter is involved, and that a multi-gene family exists

Table 1. *A list of possible sites of trans-membrane sulphate transport in higher plants*

Examples 1–9 all involve transport across the plasma membrane, whilst examples 10–12 involve transport across intracellular membranes.

Example	Possible site of trans-membrane sulphate transport
1.	Uptake into cells of the cortex or stele of the root.
2.	Efflux from these cells (1) prior to xylem loading.
3.	Xylem loading in root.
4.	Xylem efflux (predominantly aerial parts of the plant).
5.	Uptake into bundle sheaf, mesophyll and epidermal cells etc.
6.	Efflux from these same cells (5).
7.	Phloem loading in leaf tissues for export from the leaf.
8.	Phloem unloading to sink tissues such as young leaves, seeds etc.
9.	Sink tissue uptake, for example, seed-specific uptake and transfer between cells within generative tissues.
10.	Transport across tonoplast (uptake into vacuole).
11.	Transport across tonoplast (efflux from the vacuole).
12.	Chloroplast uptake.

in all higher plants, although this has only been convincingly demonstrated in *Arabidopsis* (seven types), and to a much lesser extent in cereals and *Stylosanthes* (two types found in each). Another unexplored possibility is that there are other sulphate transporters, with unrelated primary amino acid sequences, operating at some of these sites, which have not as yet been cloned or recognized. Such a scenario is quite likely as the bioenergetics of transfers, particularly across endo-membrane systems is quite different to that found at a root plasma membrane. It has been suggested that the phosphate exchange transporter in the plastid catalyses sulphate transport across the chloroplast membrane (Hampp and Ziegler, 1977; Mourioux and Douce, 1979). Alternatively, as ABC-type transporters exist in plants (reviewed in Rea *et al.*, 1998), there is the possibility that systems analogous to the principal prokaryotic sulphate transport systems are also functioning in plants.

Potential targets for genetic engineering

In order to achieve the objective of enhancing the efficiency for sulphate uptake and storage/remobilization, specific targets need to be identified, and transgenic plants made and tested. The clearly defined functions of the sulphate transporters makes these potential sites for manipulation.

Targets for engineered improvement of S-utilization efficiency may be split into two levels as summarized in Fig. 3. The first level is aimed at improving resource capture. Maximized uptake will lead to increased S-reserves, and the second level is aimed at efficient utilization of the increased uptake. The first targets for enhancing uptake are the transporter systems. From a functional viewpoint the plant sulphate transporter system has evolved to be an extremely efficient uptake system, with a high affinity for sulphate in the low micromolar range (Smith *et al.*, 1995*b*, 1997). This corresponds to typical soil solution sulphate concentrations and engineering for higher affinities may not be particularly useful even if technically feasible. Expression of the transporters is controlled by the nutritional status of the plant and the transporters are most highly expressed under S-limiting conditions. This control represents a mechanism evolved to maximize capture under nutrient-limiting conditions, but also is a mechanism to limit uptake when sulphate is abundant. Quite why this should be the case is not clear, but may be a mechanism to avoid wasteful expenditure of energy in transporting ions not immediately required for growth. Overriding this control might be achieved by expressing the transporter under the control of an appropriate constitutive promoter. A limitation to this approach may be achieving the appropriate targeted expression in specific cells of the root. As multiple steps in the uptake and assimilatory pathway seem to be co-ordinately controlled, removing the control only for the transporter would allow sulphate accumulation but leave intact the regulatory mechanisms preventing over-accumulation of sulphide.

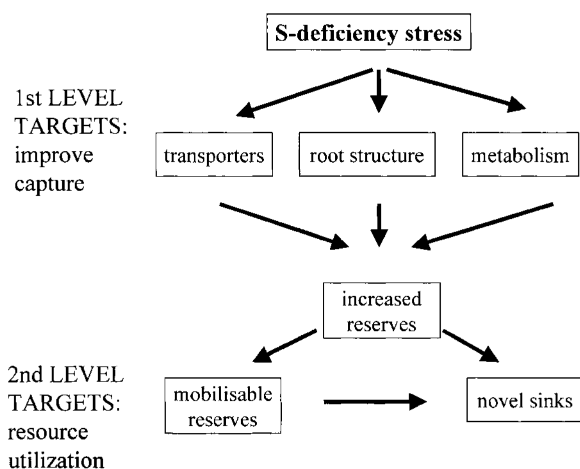

Fig. 3. Summary of strategies for the manipulation of the efficiency of sulphate uptake and utilization in crop plants. Possible targets to enhance the ability of a plant to respond to S-deficiency stress are split into two levels. The inter-dependencies of these targets are indicated by arrows.

An alternative target is root structure and proliferation. It is well known that lateral roots proliferate with localized application of some nutrients, principally nitrate, phosphate and potassium (Drew, 1975), although the response to sulphate has not been investigated. The recent cloning of a MADS-box type gene involved in the control of this root proliferation has opened up the possibilities of adopting this type of strategy (Zhang and Forde, 1998). Shifts in root-to-shoot ratios have been observed under nutrient stress, but again there is no consistent picture for the response to S-stress. As a general response to nutrient deficiency, a shift to produce a greater root proliferation is a sensible adaptation to maximize resource capture, however, carried too far may lead to detrimental influence on shoot production. Modifying root architecture by selection of varieties carrying this trait is a sensible approach in some cases. Specific modification of genes controlling proliferation is much more complex.

Modifying metabolism to utilize available-sulphate or even to stimulate further sulphate uptake by introducing increased demand for sulphate is another clear option. In the 'second level targets' it is proposed that novel sinks (high S-containing proteins) are introduced to act as strong sinks. This is a clear strategy for improved nutritional quality of crops. The limitation of the processes of sulphate remobilization have been discussed, particularly the apparent non-mobile nature of vacuolar reserves. If this were the case, then along with enhanced uptake mechanisms, these processes would also need manipulation. The limitation here is that at present, almost nothing

is known of the transporters catalysing sulphate fluxes into and out of the vacuole.

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