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Manipulation of thiol contents in plants

Review Article

R. Höfgen[1], **O. Kreft**[1], **L. Willmitzer**[1], and **H. Hesse**[2]

[1] Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Federal Republic of Germany
[2] Angewandte Genetik, Institut für Biologie, Freie Universität Berlin, Federal Republic of Germany

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Summary. As sulfur constitutes one of the macronutrients necessary for the plant life cycle, sulfur uptake and assimilation in higher plants is one of the crucial factors determining plant growth and vigour, crop yield and even resistance to pests and stresses. Inorganic sulfate is mostly taken up as sulfate from the soil through the root system or to a lesser extent as volatile sulfur compounds from the air. In a cascade of enzymatic steps inorganic sulfur is converted to the nutritionally important sulfur-containing amino acids cysteine and methionine (Hell, 1997; Hell and Rennenberg, 1998; Saito, 1999). Sulfate uptake and allocation between plant organs or within the cell is mediated by specific transporters localised in plant membranes. Several functionally different sulfate transporters have to be postulated and have been already cloned from a number of plant species (Clarkson et al., 1993; Hawkesford and Smith, 1997; Takahashi et al., 1997; Yamaguchi, 1997). Following import into the plant and transport to the final site of reduction, the plastid, the chemically relatively inert sulfate molecule is activated through binding to ATP forming adenosine-5′-phosphosulfate (APS). This enzymatic step is controlled through the enzyme ATP-sulfurylase (ATP-S). APS can be further phosphorylated to form 3′-phosphoadenosine-5′-phosphosulfate (PAPS) which serves as sulfate donor for the formation of sulfate esters such as the biosynthesis of sulfolipids (Schmidt and Jäger, 1992). However, most of the APS is reduced to sulfide through the enzymes APS-reductase (APR) and sulfite reductase (SIR). The carbon backbone of cysteine is provided through serine, thus directly coupling photosynthetic processes and nitrogen metabolism to sulfur assimilation. L-serine is activated by serine acetyltransferase (SAT) through the transfer to an acetyl-group from acetyl coenzyme A to form *O*-acetyl-L-serine (OAS) which is then sulhydrylated using sulfide through the enzyme *O*-acetyl-L-serine thiol lyase (OAS-TL) forming cysteine. Cysteine is the central precursor of all organic

molecules containing reduced sulfur ranging from the amino acid methionine to peptides as glutathione or phytochelatines, proteines, vitamines, cofactors as SAM and hormones. Cysteine and derived metabolites display essential roles within plant metabolism such as protein stabilisation through disulfide bridges, stress tolerance to active oxygen species and metals, cofactors for enzymatic reactions as e.g. SAM as major methylgroup donor and plant development and signalling through the volatile hormone ethylene. Cysteine and other metabolites carrying free sulfhydryl groups are commonly termed thioles (confer Fig. 1). The physiological control of the sulfate reduction pathway in higher plants is still not completely understood in all details. The objective of this paper is to summarise the available data on the molecular analysis and control of cysteine biosynthesis in plants, and to discuss potentials for manipulating the pathway using transgenic approaches.

Keywords: Amino acids – Thioles – Cysteine – Sulfur – Sulfate – Glutathione

Regulation of sulfur assimilation and cysteine biosynthesis in plants

Sulfate uptake, reduction and cysteine biosynthesis is highly regulated in plants both on gene expression and enzyme activity level and further complicated by the subcellular localisation of enzymes as well as developmental and spatial activity patterns and differences between plant species (Saito, 1999; Leustek and Saito, 1999). Induction of gene epression patterns under sulfur deprived conditions has been systematically and thoroughly investigated in *Arabidopsis thaliana*. Under sulfur deficient conditions expression of sulfur transporters and APR is predominantly induced in roots and expression of SAT and OAS-TL, though the latter to a lower extent, is induced in leaf tissues (Takahashi et al., 1997; Saito, 1999; Leustek and Saito, 1999). The signalling pathways are still under debate mainly discussing OAS as intracellular signal and glutathione (GSH) and/or S-methylmethionin (SMM) as long distance transport molecules of reduced sulfur and as potential signal (Clarkson et al., 1993; Lappartient and Tourraine, 1997; Hell and Rennenberg, 1998; Leustek and Saito, 1999; Bourgis et al., 1999). Investigations of the effect of sulfur starvation on enzyme activity revealed that the activities of sulfate transporters, SAT, APR, and OAS-TL are increased. Metabolic control of sulfate uptake and further steps are shown to be mediated by cysteine, glutathione or low levels of nitrogen: sulfate activation through ATP-S is inhibited by cysteine and methionine, sulfate reduction is reduced by cysteine, and stimulated by OAS and light, respectively sucrose, and SAT activity is inhibited by reduced organic sulfur compunds as cysteine, GSH, and methionine (Saito, 1999; Kopriva, 1999). The final goal of metabolic engineering will be to validate and verify the respective regulatory steps through forward and reverse genetic approaches and to exploit these results for manipulation of metabolite fluxes.

Molecular manipulation of sulfate uptake and activation

Sulfate transport

Under regular nutrient conditions sulfate is not limiting and surplus sulfate is efficiently stored in the plant vacuoles. Studies using cultured tobacco cells revealed inhibition of sulfate uptake through cysteine, methionine, homocysteine and isoleucine (Hart and Filner, 1969) and inhibition of sulfate uptake in barley roots by methionine and seleno-methionine and in tuber discs by methionine and cysteine (Maggioni and Renosto, 1977). In plants a multitude of functionally different sulfate transporters have to be postulated because sulfate is transported throughout the plant and has to cross several types of membranes along it's route from soil to vacuoles for storage, or plastids for reduction or re-routing during mobilisation (Clarkson et al., 1993). An increasing number of sulfate transporters has been cloned recently either through complementation of yeast mutants (Hawkesford and Smith, 1997) or homology searches applying EST data bases (Takahashi et al., 1997). Though different regimes of regulation have to be expected for the individual transporters or between different plant species general features of sulfate transporter regulation for herbacious plants are the stimulation of expression upon sulfur depletion or external supply of OAS, whereas nitrogen depletion or high levels of GSH or cysteine repress gene expression. High levels of OAS, however, are able to overcome repression of high sulfur contents leading to increased transport activity and increased levels of GSH and cysteine (Saito, 1999; Leustek and Saito, 1999; Hawkesford and Smith, 1997). There are no data yet available for genetically engineered plants overexpressing certain sulfate transporter genes but a number of genes have been cloned with detailed molecular and physiological analysis pending. However, genetic manipulation of sulfate transporters can be postulated to result in increased assimilation rates under certain conditions such as limiting soil sulfate or during certain developmental stages such as seed filling where soil derived or vacuolar sulfate has to be quickly mobilised, reduced and transported to the sink organs. There seems to be a limiting window for seed loading as expression of sulfur rich proteins did not always lead to increased overall levels of sulfur amino acids but only to a reallocation from endogenous to the transgenic protein (Fujiwara et al., 1997; Tabe and Higgins, 1998). Molecular and physiological analysis of genetically engineered plants modulating expression of sulfate transporters will provide clues for the subcellular localisation and physiological function of the various types of transporters.

Sulfate activation: ATP-sulfurylase

Activation of the relatively inert sulfate occurs through binding to ATP catalysed by ATP-sulfurylase (ATP-S). ATP-S expression and activity is weakly induced upon sulfur depletion but repressed through glutathione (GSH) or methionine and cysteine (Reuveny and Filner 1977; Ruffet et al.,

1995). In potato ATP-S is expressed in the source organs, leaves and stems but also in roots, stolons or flower buds. However, in tubers no expression of ATP-S could be detected indicating supply of reduced sulfur to this storage organ (Klonus et al., 1994). Subcellular localisation studies revealed a major activity for ATP-S in the chloroplasts and a minor activity in the cytosol and mitochondria which corresponds to the genes cloned from plants (Lunn et al., 1990; Renosto et al., 1993; Schmidt and Jäger, 1992). ATP-S has been assumed as the rate limiting step enabling and initiating sulfur metabolism and thus as an ideal target for molecular manipulation. Overexpression of ATP-S did lead to a 2,5 fold increase of enzymatic activity in tobacco and an 8 fold increase in indian mustard (*Brassica juncacea*) (Hatzfeld et al., 1998; Pilon-Smits et al., 1999). No physiological effects were observed for transgenic tobacco, however, in *Brassica juncacea* ATP-S overproduction led to an increased uptake of selenate and sulfate, higher levels of GSH and thioles, and to an increased selenate tolerance. Antisense inhibition of ATP-S in potato resulted in a 6 fold decrease of enzyme activity accompanied with a mild growth retardation and a reduction of tuber yield; the shoot to root ratio, the sulfate content in leaves, and the sensitivity towards cadmium were increased (unpublished data). Unexpectedly, molecular manipulation of ATP-S activity did not result in severe effects on plant growth and physiology indicating a minor role only of ATP-S in controlling sulfur metabolism in plants. This is probably mirrored by the fact that sulfur depleted growth conditions did not increase expression and activity of ATP-S drastically (Saito, 1999).

Sulfate reduction: APS reductase and sulfite reductase

Sulfate reduction is a costly process as after activation through ATP eight reduction equivalents are necessary to reduce sulfate to sulfide, two more than for the reduction of nitrate to ammonium (Hell und Rennenberg, 1998; Leustek and Saito, 1999). The relevance of APS-Reductase (APR), formerly termed APS sulfotransferase, for the actual biosynthetic steps of sulfur reduction in plants, has been a topic of debate for quite some time as either the free reduction pathway as present in enteric bacteria or the bound reduction pathway has been favoured (Schwenn, 1997; Brunold and Rennenberg, 1997; Hell, 1997). Different APS-reductases have been cloned recently strongly favouring the "bound pathway" via a thiosulfonate intermediate in plants whereas the pathway involving a PAPS intermediate seems to play only a minor role (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Bick and Leustek, 1998). There are no genetically engineered plants available for analysis, yet. However, it can be assumed that manipulation of APR should result in significant effects on sulfur metabolism and pathway fluxes in plants, especially, as APR belongs together with sulfate transporters to the most strongly induced genes upon sulfur starvation indicating a central role in sulfur assimilation (Saito, 1999). Recently, it has been shown that expression and activity of APR is not only regulated through the "sulfur status", but that expression and activity of APR are induced by light, probably mediated through sucrose, thus coupling carbon and sulfur metabolism (Kopriva et al., 1999).

Concerning the following enzyme in the sulfate reduction sequence, sulfite reductase (SIR), there are not many data available. SIR seems not to be controlled strongly neither on the expression nor on the activity level through sulfate depleted growth conditions though it provides the sulfide necessary for cysteine formation (Saito, 1999; Bick and Leustek, 1998).

Cysteine formation

Biosynthesis of cysteine: O-acetylserine(thiol)lyase

O-acetylserine(thiol)lyase (OAS-TL), synonymously termed cysteine synthase, catalyses the terminal step of cysteine biosynthesis. OAS-TL is slightly induced upon sulfur starvation and a 5 fold increase upon simultaneaous depletion of nitrogen and sulfur has been described (Takahashi and Saito, 1996). Distinct isoforms are localised in plastids, the cytosol and in mitochondria (Hesse et al., 1999; Saito, 1999). OAS-TL forms a complex with SAT ("cysteine synthase complex") involved in regulation of cysteine synthesis. The availability of the substrate OAS seems to be limiting for the biosynthetic rate of cysteine biosynthesis which has been shown through feeding experiments with OAS. Overexpression of OAS-TL did not effect cysteine or GSH contents in the respective plants (Saito et al., 1994) though a slight increase in resistance to sulfite and hydrogen sulfide induced stresses have been observed (Youssefian et al., 1993). Therefore, it seems justified to assume that OAS-TL does not have a central role in regulating cysteine synthesis.

Precursor supply: Serine acetyl transferase

Serine acetyl transferase (SAT) activates serine through O-acetylation prior to formation of cysteine catalysed by O-acetylserine(thiol)lyase (OAS-TL). SAT is localised in plastids, the cytosol and in mitochondria and several isoforms have been cloned from various plants (Saito, 1999). In plants, OAS-TL has been shown to be present in excess over SAT ranging from 100 to 400 fold. Sulfur depletion leads to a weak induction of gene expression, whereas cysteine reduces SAT activity of some isoforms through feedback inhibition, while others are not affected. The physiological significance of this differential regulation of various isoforms ist still under debate and speculated to be involved in regulation of gene expression of sulfur status responsive genes (Noji et al., 1998). Constitutive overexpression of a bacterial gene, *E. coli* cys E, and targeting of the protein to the plastids resulted in an increased enzyme activity of SAT in transgenic tobacco and potato plants (Blasczcyk et al., 1999; Harms et al., 2000). This increase is accompanied by elevated levels of free cysteine and glutathione (GSH). SAT, in contrast to it's binding partner OAS-TL, thus proved to be a suitable target for controlled manipulation of metabolite fluxes through the pathway changing significantly the levels of sulfur containing metabolites in plants. Especially, the increase in GSH content will allow further studies with respect to increased tolerance levels versus

stresses such as heavy metal exposure or oxidative stresses. Blasczcyk et al. (1999) could already show an increased resistance towards oxidative stress in transgenic tobacco plants overexpressing the bacterial SAT. With respect to physiological studies it will be interesting to compare the results of the overexpression of the various endogenous genes or the ectopic expression of bacterial genes to these initial results. In summary it seems to be justified to assume that SAT executes a central role in cysteine synthesis.

Conclusions

Genetic modifications with the aim to provide tailor made transgenics will prove to be a valid tool for crop improvement providing valuable agronomic traits such as a balanced amino acid composition of crops to the breeders, to the farmers and, finally, safe and high quality products to the consumers. Manipulation of the biosynthetic capacity of plants will support recent approaches of expressing nutritionally valuable sink proteins (Tabe and Higgins, 1998). Furthermore, the increasing database of reports on genetically engineered plants within the cysteine biosynthetic pathways will help to understand the regulation of the pathway, it's control through internal and external factors as well as the role of the subcellular compartimentation and the regulation of differential activities in different plant tissues such as source and sink organs. Of high interest, furthermore, is the coregulation of different "metabolic kingdoms" as nitrogen, carbon and sulfur metabolism where first results provide valuable information giving guidelines for future research. Sulfur assimilation and cysteine biosynthesis are not isolated within plant metabolism but heavily wired into the network of diverse pathways (Fig. 1). Manipulations of cysteine biosynthesis can be expected to yield various pleiotropic effects which will primarily help to understand coordination of metabolic processes and control of fluxes which will sustain applied approaches as plant breeding aiming at nutritional improvement, increased stress tolerance or phytoremediation. Remarkably, the overexpression of SAT and not OAS-TL lead to an increase in free cysteine and most important of glutathione which provided increased resistance towards heavy metals and oxidative stress (Blasczcyk et al., 1999). This could either be interpreted as detoxification of the free cysteine into a physiologically less toxic metabolite or, that the available amount of cysteine is normally limiting for GSH biosynthesis which is, furthermore, competing with methionine biosynthesis and protein incorporation of cysteine and methionine. Increased levels of GSH were likewise reported for overexpression of ATP-S in *Brassica juncacea* (Pilon-Smits et al., 1999). Overexpression of the GSH synthesising enzymes, gamma-glutamylcysteine synthetase and glutathione synthase, resulted in accumulation of glutathione in transgenic plants (Noctor et al., 1998).

The available data on manipulation of thiol contents in plants already provide a scheme for meaningful manipulation of thiol contents in plants as it seems that uptake and activation of sulfate which is catalysed by sulfate transporters and ATP sulfurylase is sufficiently flexible in plants to react on

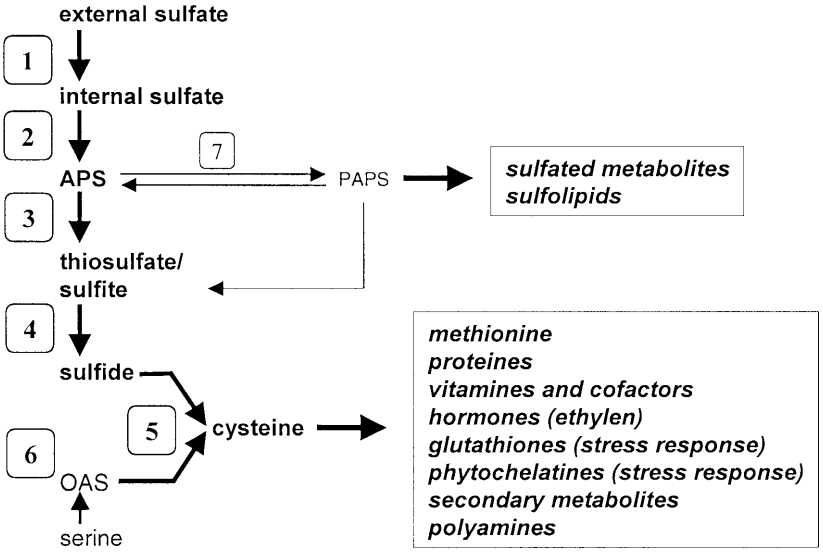

Fig. 1. Schematic representation of the cystein biosynthetic pathway in plants: Sulfate is taken up from the soil through sulfate transporters (*1*) and transported to the plastids for further assimilation, or to the vacuole for storage. Within the plastids sulfate is reduced to sulfide following the bound pathway (*2*, *3*, *4*). Sulfide is incorporated into OAS derived from serine (*6*) forming cysteine (*5*). The reaction steps 5 and 6 do also occur in the cytosol and mitochondria. APS kinase catalyzes the formation of PAPS (*7*) which is the precursor of sulfated metabolites. Cysteine constitutes the first organic molecule carrying reduced sulfur and is the precursor of a wide variety of metabolites. *1* sulfate transporter; *2* ATP sulfurylase; *3* APS reductase; *4* Sulfite reductase; *5* O-acetylserine (thiol) lyase; *6* serineacetyl transferase; *7* APS kinase. *APS* 5′-adenosine phosphosulfate, *PAPS* 3′-phosphoadenosine-5′-phosphosulfate; *OAS* O-acetylserine

changing demands, probably with the exception of sulfate mobilisation from the vacuole, which is still unclear. Likewise, sulfite reductase and cysteine synthase seems to be of lower impact of the metabolite flux if manipulated. Successful strategies for increasing thioles and derived products in plants are the overexpression of serineacetyl transferase to increase cysteine and glutathiones and gamma-glutamyl-cysteine synthase and glutathione synthase for further increase of glutathione in respect to stress physiology.

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Authors' address: Dr. Rainer Höfgen, Max-Planck-Institut für Molekulare Pflanzenphysiologie, D-14424 Potsdam, Federal Republic of Germany,
Fax +49 (0)331 / 567-8201, E-mail: hoefgen@mpimp-golm.mpg.de

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