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Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism

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Abstract

Sulphate assimilation is an essential pathway being a source of reduced sulphur for various cellular processes and for the synthesis of glutathione, a major factor in plant stress defence. Many reports have shown that sulphate assimilation is well co-ordinated with the assimilation of nitrate and carbon. It has long been known that, during nitrate deficiency, sulphate assimilation is reduced and that the capacity to reduce nitrate is diminished in plants starved for sulphate. Only recently, however, was it shown that adenosine 5′ phosphosulphate reductase (APR), the key enzyme of sulphate assimilation, is regulated by carbohydrates. In plants treated with sucrose or glucose APR was induced, whereas the activity was strongly reduced in plants grown in CO_2-free air. The availability of cysteine is a crucial factor in glutathione synthesis, but an adequate supply of glutamate and glycine are also important. The molecular mechanisms for the co-ordination of S, N, and C assimilation are not known. *O*-acetylserine, a precursor of cysteine, was proposed to be the signal regulating sulphate assimilation, but most probably is not the outgoing signal to N and C metabolism. cDNA arrays revealed the induction of genes involved in auxin synthesis upon S-starvation, pointing to a possible role of phytohormones. Clearly, despite significant progress in understanding the regulation of sulphate assimilation and glutathione synthesis, their co-ordination with N and C metabolism achieved, and several potential signal molecules identified, present knowledge is still far from being sufficient.

Key words: Glutathione synthesis, nitrate deficiency, nitrate reduction, sulphate assimilation, regulation.

Introduction

Cysteine, the initial product of sulphate assimilation in plants, and its immediate metabolite glutathione (GSH) are both cellular constituents that originate from the three most important pathways of plants' primary metabolism, i.e. photosynthesis, nitrogen assimilation, and sulphate assimilation (Leustek *et al.*, 2000; Brunold *et al.*, 2003). The sulphur in cysteine originates from sulphate that is reduced in the chloroplasts or plastids of heterotrophic tissues in the pathway of sulphate assimilation. In this pathway, sulphate that is transported into plant cells by sulphate transporters, is activated by adenylation to adenosine 5′ phosphosulphate (APS) in a reaction catalysed by ATP sulphurylase (ATPS; EC 2.7.7.4). APS is reduced to sulphite by APS reductase (APR; EC 1.8.4.9); the electrons are derived from GSH. Sulphite is further reduced by a ferredoxin-dependent sulphite reductase (SiR; EC 1.8.7.1) to sulphide which is incorporated by *O*-acetylserine (thiol)lyase (OAS-TL; EC 2.5.1.47) into the amino acid skeleton of *O*-acetylserine (OAS) to form cysteine. OAS is synthesized by acetylation of serine with acetyl-CoenzymeA catalysed by serine acetyltransferase (SAT; EC 2.3.1.30) (Leustek *et al.*, 2000; Suter *et al.*, 2000; Kopriva and Koprivova, 2003). The carbon skeleton and the nitrogen of cysteine thus originate from serine, produced by photorespiration in photoautotrophic tissues or by a plastid localized 'phosphorylated pathway' from 3-phosphoglycerate in non-photosynthetic tissues (Ho and Saito, 2001). The nitrogen in serine is a product of nitrogen reduction and assimilation. Since nitrogen assimilation in plants is achieved by the GS-GOGAT pathway that requires 2-oxo-glutarate, catabolic reactions of the Citric Acid Cycle in the mitochondria are also involved. In plants that assimilate nitrogen in the roots, for example, forest trees such as Norway spruce (*Picea abies* L. [Karst.]) and European beech (*Fagus sylvatica* L.),

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nitrogen in photorespiratory serine will further depend on amino acid delivery from the roots to the shoot by xylem transport (Geßler *et al.*, 1998).

The biosynthesis of GSH (γ-glutamylcysteinylglycine) depends on the linking and the availability of its constituent amino acids cysteine, glutamic acid, and glycine. Linking is achieved in two ATP-dependent steps catalysed by separate enzymes, i.e. a γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) that synthesizes γ-glutamylcysteine (γ-EC) from cysteine and glutamate and a glutathione synthetase (GSHS; EC 6.3.2.3) that binds glycine at the C-terminal site of γ-EC (May *et al.*, 1998*a*; Noctor *et al.*, 1998). The synthesis of GSH will therefore be regulated not only by the dependency of cysteine availability on sulphur, nitrogen, and carbon metabolism, but also on the availability of glutamate and glycine. Glutamic acid, the primary product of the GS–GOGAT pathway of nitrogen assimilation, is produced in photoautotrophic cells in significant amounts and is a major component of the amino acids transported from the roots to the leaves by xylem transport in plants that assimilate nitrogen in the roots, (Geßler *et al.*, 1998). Thus, a shortage of Glu for GSH synthesis appears unlikely in photoautotrophic cells. Glycine, the C-terminal amino acid of GSH, is (like the serine used for cysteine synthesis) an intermediate of photorespiration in photoautrophic cells. Its availability is, therefore, also dependent on nitrogen assimilation and the Citric Acid Cycle in the mitochondria. Thus, GSH synthesis has an even higher degree of complexity than cysteine synthesis in its interaction between sulphur, nitrogen, and carbon metabolism.

Obviously, a complex regulatory crosstalk between sulphur, nitrogen, and carbon metabolism is required for the control of both sulphate assimilation into cysteine and GSH synthesis from its constituent amino acids. This crosstalk has to integrate chloroplastic, peroxisomal, mitochondrial, and cytosolic metabolism since, in addition to sulphate and nitrogen reduction and assimilation, photorespiration and the Krebs Cycle are involved. The present review aims to summarize current knowledge about this crosstalk in the control of sulphate assimilation and glutathione biosynthesis.

Regulation of sulphate assimilation

Regulation of S-assimilation by sulphur compounds

Sulphate assimilation pathways provides plants with cysteine, which is further used for protein synthesis and as a source of reduced sulphur for the biosynthesis of methionine, GSH, coenzymes, and many secondary compounds. As these compounds are essential for plant growth and survival and several products and intermediates of sulphate assimilation are toxic, the pathway undergoes strict regulation by the sulphur status of the plant (Brunold, 1990; Leustek *et al.*, 2000). Although the molecular mechanisms are not known, sulphate assimilation seems to be controlled by demand (Lappartient and Touraine, 1996; Lappartient *et al.*, 1999; Herschbach *et al.*, 2000; Westerman *et al.*, 2001; Vauclare *et al.*, 2002). This means that the pathway is repressed under normal levels of external sulphate and de-repressed by sulphate limitation or by an enhanced sulphur demand for growth and development. On the other hand, even the repressed pathway may be further reduced when reduced sulphur is made available to the plant. It has long been known that sulphate starvation results in a strong increase of sulphate uptake and activity of ATPS and APR in plant cells (Reuveny and Filner, 1977; Reuveny *et al.*, 1980; Smith, 1980; Haller *et al.*, 1986) and on the whole plant level (Smith *et al.*, 1997; Lappartient *et al.*, 1999). The increase in steady-state levels of mRNAs for high affinity sulphate transporters, ATPS, and APR upon sulphur starvation, detected by northern analysis (Takahashi *et al.*, 1997; Yamaguchi *et al.*, 1999) or cDNA arrays (Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003), reveals that the de-repression is regulated at the level of transcription. This de-repression correlates with the time of exposure to S deficiency; when plants are supplied with sulphate again, APR and ATPS activity quickly return to the normal levels (Reuveny and Filner, 1977; Brunold *et al.*, 1987; Lappartient and Touraine, 1996). The consequences of sulphate starvation are most pronounced on the enzymes involved in the uptake and reduction of sulphate, but the synthesis of the amino acid acceptor *O*-acetylserine also seems to be influenced. The mRNA levels for the chloroplastic isoform of SAT and for the cytosolic isoform of OAS-TL were induced by sulphur starvation (Barroso *et al.*, 1995; Takahashi *et al.*, 1997). On the other hand, OAS-TL activity was reported either to be reduced by sulphur deficiency in spinach (Warrilow and Hawkesford, 1998) or not to be affected (Smith, 1980; Takahashi and Saito, 1996).

Too much sulphur, on the other hand, results in even greater repression of sulphate assimilation. In *Lemna minor* a ten times increased sulphate concentration in the nutrient solution reduced extractable APR activity, whereas ATPS was not affected (Brunold *et al.*, 1987). A strong decrease in sulphate uptake and assimilation is observed in plants fed reduced forms of sulphur, such as SO_2, H_2S, cysteine, or GSH (Brunold and Schmidt, 1976, 1978; Wyss and Brunold, 1979, 1980; Rennenberg *et al.*, 1988; Lappartient *et al.*, 1999; Westerman *et al.*, 2001). Fumigation with SO_2 resulted in a decrease of APR but not ATPS activity and, since sulphate uptake was not affected, in increased sulphate content in the leaves (Wyss and Brunold, 1980; Brunold *et al.*, 1983). H_2S exposure results in the accumulation of thiols, reduction in sulphate uptake, and reduction in activity of APR (Brunold and Schmidt, 1976; Wyss and Brunold, 1979; Herschbach *et al.*, 2000; Westerman *et al.*, 2001). Other enzymes of the sulphate assimilation pathway are regulated by H_2S differently in various plants. ATPS, SiR, and OAS-TL were affected by fumigation in pea (von

Arb and Brunold, 1986) but not in *Brassica oleracea* (Westerman *et al.*, 2001). Treatment of plants with cysteine leads to responses very similar to that of fumigation with H_2S (Brunold and Schmidt, 1978; von Arb and Brunold, 1986). This shows that it is not the sulphide itself that is regulating sulphate uptake and assimilation, but that it has to be incorporated into organic sulphur compounds.

In investigations on the molecular mechanisms of the feedback regulation of sulphate assimilation by thiols, attention was first paid to ATPS. In *A. thaliana*, ATPS activity and mRNA levels of the APS1 isoform were decreased by GSH treatment (Lappartient *et al.*, 1999). However, a major contribution of ATPS for the regulation of sulphate assimilation was questioned because several reports showed that APR was more susceptible to regulatory signals than ATPS (Koprivova *et al.*, 2000; Westerman *et al.*, 2001). In *A. thaliana* root cultures APR activity and transcript levels were decreased by feeding cysteine and GSH, whereas ATPS was not affected (Vauclare *et al.*, 2002). Control flux analysis revealed that the flux through sulphate assimilation from internal sulphate is from 90% controlled by APR, but that sulphate transport exerts a significant part of the control when the uptake is taken into account (Vauclare *et al.*, 2002). However, since the recent development in the investigations of sulphate transporters is given elsewhere in this issue, emphasis is given here to the regulation of sulphate reduction.

Because external GSH supply increases also the accumulation of cysteine, both Cys and GSH might be responsible for the control of SO_4^{2-} uptake and assimilation. Phloem sap analysis indicated that GSH rather than cysteine was the signal acting in *Brassica napus* (Lappartient and Touraine, 1996) and poplar (Herschbach *et al.*, 2000). In *Brassica* and *Arabidopsis*, blocking GSH synthesis by L-buthionine [S, R] sulphoximine, an inhibitor of γ-glutamylcysteine synthetase, relieved the repression of ATPS and APR mRNA accumulation, respectively (Lappartient *et al.*, 1999; Vauclare *et al.*, 2002). By contrast, in maize, cysteine was able to regulate the level of ATPS mRNA without the need for conversion to GSH (Bolchi *et al.*, 1999). Cysteine regulates both pathway branches of its synthesis because it also exerts feedback regulation on SAT (Fig. 1B). In *A. thaliana* only one SAT isoform, which was localized to cytosol, was susceptible to this regulation (Noji *et al.*, 1998); however, in pea and spinach the chloroplast located SAT activity was inhibited by cysteine (Brunold and Suter, 1982; Noji *et al.*, 2001; Droux, 2003). In addition, H_2S has an effect on SAT, since it stabilizes the complex with OAS-TL and, thus, increases the activity (Droux *et al.*, 1998). In addition to GSH, another potential signal in the regulation of sulphate assimilation is the precursor of cysteine, OAS. The addition of OAS increases sulphate uptake and APR activity (Neuenschwander *et al.*, 1991; Smith *et al.*, 1997). OAS accumulates during sulphur starvation and may thus serve as a signal of the sulphur status (Kim *et al.*, 1999; Ohkama

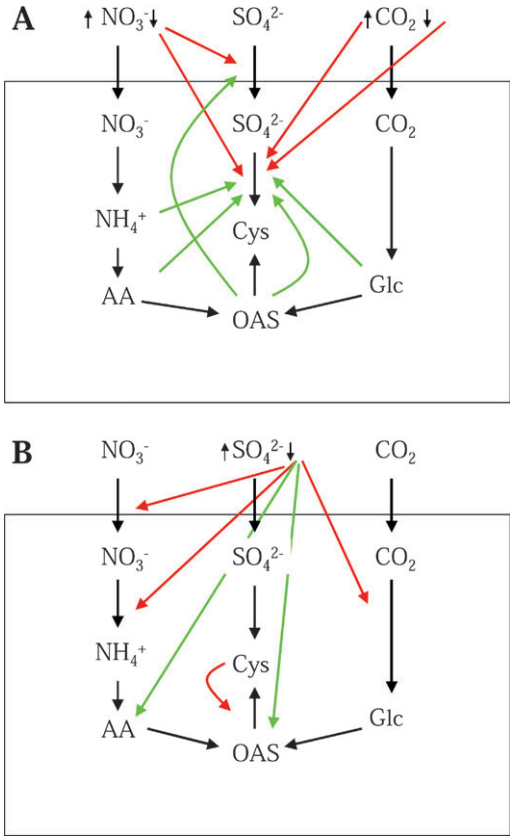

Fig. 1. Schematic representation of interaction between S, N, and C assimilation. (A) Regulation of sulphate uptake and reduction by N and C assimilation. (B) Regulation of C and N assimilation by S availability and S compounds. Red arrows represent repression of the metabolic steps, green ones mark induction of the processes or increase in metabolite content (arrow towards the metabolite). AA, free amino acids; Glc, glucose.

et al., 2002). OAS acts most probably as a transcriptional regulator, since its addition strongly increases mRNA levels of all three APR isoforms and also those of SiR, chloroplastic OAS-TL, and cytosolic SAT (Koprivova *et al.*, 2000). More than 100 genes were induced and 548 genes were repressed in leaves of *A. thaliana* treated with 1 mM OAS for 48 h (Hirai *et al.*, 2003). However, the correlation between the response to OAS and the response to sulphur starvation was not sufficient to confirm that OAS is indeed the molecular signal.

Until now, genes involved in signal transduction of sulphur starvation were identified only in the green alga *Chlamydomonas reinhardtii* (Davies *et al.*, 1994). Mutations in sac genes (for sulphur acclimation) result in a lack of response to sulphur limitation (Davies *et al.*, 1994). The *Sac*1 gene encodes a protein homologous to ion channels which seems to function as a sulphate sensor (Davies *et al.*, 1996). The *sac*1 mutants are lacking both general and specific responses to sulphur starvation, including the effects on photosynthesis, whereas in *sac*2 and *sac*3 only sulphate uptake and assimilation are disturbed. The *Sac*3 encodes an Snf1-like kinase; its disruption leads to de-repression of the

expression of arylsulphatases (Davies *et al.*, 1999; Ravina *et al.*, 2002). *Sac*2 is involved in post-transcriptional regulation of APR, but has not been identified, yet (Ravina *et al.*, 2002). In higher plants, molecular analysis of the seed-specific promoter of the β subunit of β-conglycinin led to the identification of a 235 bp sulphur-regulated element which also confers a sulphur deficiency response in non-seed tissues (Awazuhara *et al.*, 2002).

Although the exact mechanisms are still not known, a simple regulatory circuit was proposed to explain the demand-driven control of sulphate assimilation. At normal sulphur levels the pathway is repressed by a counter-balanced action of the positive and negative signals OAS and GSH. At low sulphur levels, OAS accumulates and activates the pathway, whereas after an increase in reduced sulphur, OAS synthesis is inhibited and the repression effect of GSH becomes stronger (Leustek *et al.*, 2000). However, in poplars overexpressing γ-ECS which possess 2–4 times elevated GSH levels sulphate assimilation is not affected, but in wild-type poplars a 2-fold increase of GSH due to external GSH feeding strongly represses APR (Hartmann *et al.*, 2004). This repression is attenuated by the simultaneous addition of OAS, but changes in OAS alone cannot explain the lack of regulation in the transgenic poplars. Clearly, another, as yet unidentified, signal or an additional level of regulation must be postulated to explain the molecular mechanism of regulation of sulphate assimilation by sulphur availability.

Regulation of S-assimilation by nitrogen

The major part of reduced sulphur is found in proteins with the relatively stable S to N molar ratio of *c*. 1:25 (Rennenberg, 1984). It is thus not surprising that regulatory interactions between assimilatory sulphate and nitrate assimilation in plants were long established (Reuveny and Filner, 1976; Reuveny *et al.*, 1980; Smith, 1980; Brunold, 1993; Yamaguchi *et al.*, 1999; Koprivova *et al.*, 2000). The two assimilatory pathways are well co-ordinated, so that a deficiency for one element represses the other pathway. Accordingly, the activities of ATPS, APR, and OAS-TL decreased under nitrogen deficiency in *Lemna minor* and cultured tobacco cells and were restored when nitrate or ammonia were resupplied (Reuveny *et al.*, 1980; Smith, 1980; Brunold and Suter, 1984) (Fig. 1A). When more amino acids are available for protein synthesis, for example, due to the addition of ammonium to the nutrient solution, the flux through sulphate assimilation, measured as the incorporation of ^{35}S into proteins after feeding [^{35}S]sulphate, increased (Brunold and Suter, 1984). The increased flux resulted from an increase in APR activity while ATPS and OAS-TL were not affected (Brunold and Suter, 1984). Similarly, APR activity increased upon the addition of the amino acids Arg, Asn, or Gln (Suter *et al.*, 1986). Adding ammonia or Gln, but not nitrate, to nitrogen-starved *Arabidopsis* plants resulted in an increased flux through sulphate assimilation, mostly due to the increased incorporation of ^{35}S into the proteins (Koprivova *et al.*, 2000). Nitrogen nutrition seems to regulate sulphate assimilation at the transcriptional level, since, in *A. thaliana*, a decrease of APR activity in nitrogen-deficient plants correlated with decreased mRNA and enzyme levels (Koprivova *et al.*, 2000) and nitrogen deficiency affected mRNA accumulation of several other genes of sulphate assimilation (Yamaguchi *et al.*, 1999). In addition, the mRNA of the mitochondrial isoform of OAS-TL was increased in spinach plants deprived of nitrogen (Takahashi and Saito, 1996). Again, little is known about the molecular signals co-ordinating N and S assimilation. Since [^{35}S]sulphate feeding experiments with N-starved *Arabidopsis* plants demonstrated that the closer the N-sources were metabolically related to OAS, the higher was the incorporation of ^{35}S, OAS was proposed to play an important role in the co-ordination of sulphate and nitrate assimilation (Koprivova *et al.*, 2000).

The effects of nitrogen nutrition on sulphate assimilation were corroborated by transcriptome analyses. In the cyanobacterium *Anabena* PCC7120, nitrogen deprivation resulted in reduced mRNA levels of sulphate transporters (Ehira *et al.*, 2003). On the other hand, nitrate added to plants grown on ammonium as the sole nitrogen source induced the accumulation of mRNA for high affinity sulphate transporters and APR (Wang *et al.*, 2003). This was rather unexpected since APR is actually activated by ammonium (Brunold and Suter, 1984; Suter *et al.*, 2000). These results might be interpreted considering a report of Ohkama *et al.* (2002) who showed an increase in APR mRNA levels upon the application of cytokinins. Cytokinins are known to respond to nitrogen nutrition and their biosynthesis seems to be dependent on nitrate (Walch-Liu *et al.*, 2000). Thus, the nitrate effect on APR and/or sulphate transporters might be mediated by an increased level of cytokinins induced by nitrate. Cytokinins would thus be a potential alternative to OAS in delivering the N-status of the plants for the regulation of sulphate assimilation (Fig. 2A). The co-ordination of S and N assimilation on the molecular level was further substantiated by the fact that the sulphur response element of the β-conglycinin promoter is also regulated by nitrogen (Awazuhara *et al.*, 2002).

Interaction of S-assimilation with carbon metabolism

Although an amino acid acceptor of sulphide is necessary for sulphate assimilation, only little attention was paid to the regulation of S assimilation by carbon metabolites. It has long been known that cysteine production from sulphate is stimulated by light (Schmidt and Trebst, 1969) and the activities and mRNA levels of enzymes of sulphate assimilation are higher in green leaves than in etiolated tissues (von Arb and Brunold, 1986; Hell *et al.*, 1997). Furthermore, activities of ATPS and APR were shown to be light induced (Passera *et al.*, 1989; Neuenschwander *et al.*, 1991) and a diurnal rhythm of APR with maximal activity during the

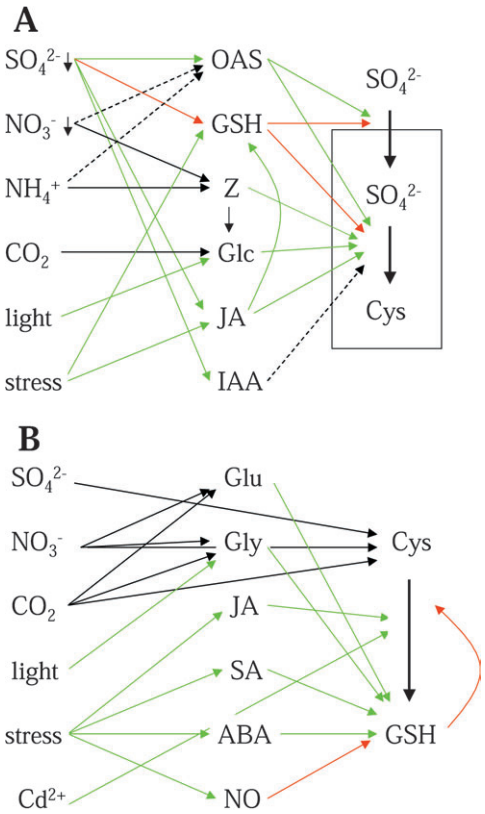

Fig. 2. Schematic representation of nutritional and environmental conditions and putative signals involved in the regulation of sulphate assimilation and GSH synthesis. (A) Conditions and signals involved in the regulation of sulphate uptake and reduction. (B) Conditions and signals involved in regulation of GSH synthesis. Red arrows represent reduction of the metabolite content (arrow towards the metabolite) or repression of the metabolic step; green arrows mark increase in the metabolite accumulation and induction of the reaction. Black arrows represent concentration dependent changes in the corresponding metabolites; the dashed lines mark proposed regulation which is not satisfactorily confirmed. Glc stays for glucose, Z for zeatine (cytokinins).

light period was observed in maize and *Arabidopsis* (Koczy *et al.*, 1997; Kopriva *et al.*, 1999). Indeed, a co-ordinate circadian cycling of mRNA levels of two sulphate transporters, APR, and SAT, peaking at the beginning of the light period, was detected in *Arabidopsis* in a search for genes undergoing a circadian control of expression (Harmer *et al.*, 2000). All these observations could be ascribed to a direct influence of light or to an indirect regulation by carbohydrates synthesized in photosynthesis. Kopriva *et al.* (1999) showed that reillumination of *Arabidopsis* plants which had been kept in darkness for 36 h induced APR mRNA accumulation and activity. This induction was not mediated by phytochrome but could be mimicked by treatment of the roots with sucrose (Kopriva *et al.*, 1999). Glucose was also able to induce APR activity in dark-adapted plants whereas sorbitol, mannitol, and 2-deoxyglucose were not, revealing that (i) sugars act on APR directly and not via osmotic stress and (ii) hexokinase-mediated signalling is not responsible for this regulation (Hesse *et al.*, 2003). Sugars thus affect APR in the same way as OAS, which also induced APR activity in *Lemna* plants in the dark (Neuenschwander *et al.*, 1991). Hesse *et al.* (2003) compared the effects of single and combined applications of glucose and OAS and tested whether glucose also affects sulphate assimilation at nitrogen-deficient conditions. Feeding plants with glucose and OAS simultaneously resulted in a more than additive induction of APR activity and APR was also induced by glucose in N-deficient plants, revealing that sugars regulate sulphate assimilation independently of OAS and nitrate assimilation (Hesse *et al.*, 2003) (Fig. 2A).

The hypothesis that sulphate assimilation is regulated by carbohydrates rather than by light was corroborated by a finding that, in *Lemna* plants cultivated in an atmosphere without CO_2, APR activity and mRNA level rapidly decreased (Kopriva *et al.*, 2002) (Fig. 1A). This reduction in APR activity, but not in mRNA accumulation, was attenuated by supplementing the nutrient solution with sucrose. The addition of OAS, on the other hand, also delayed the decline in the APR mRNA level. $^{35}SO_4^{2-}$ feeding showed that exposure to an atmosphere without CO_2 severely inhibited sulphate uptake and the flux through sulphate assimilation, which could then be restored by the resupply of ambient air or the addition of sucrose, but not by OAS (Kopriva *et al.*, 2002). Increased CO_2 concentrations were also reported to affect sulphate assimilation; exposure of holm oak (*Quercus ilex*) to 700 ppm CO_2 resulted in a decrease in thiol concentration and APR activity (Schulte *et al.*, 2002). Interestingly, when oaks originating from a natural spring site with elevated pCO_2 were subjected to increased pCO_2, APR and thiols were not affected, showing a possibility for a genetic acclimation of sulphate assimilation to high CO_2. The decreased content of thiols in the high CO_2-treated oaks is most probably derived from low APR activity and thus a reduced flux through sulphate assimilation. Whether this regulation is a general feature found in other plant species as well and what is the mechanism by which APR is down-regulated, still remains to be elucidated.

Carbohydrates not only provide the acceptor of sulphide for cysteine biosynthesis, they are the source of reductants for sulphate reduction in non-photosynthetic tissues. GSH for reduction of APS to sulphite is regenerated from oxidized glutathione (GSSG) by NADPH-dependent GSH reductase. Ferredoxin providing electrons for the reduction of sulphite to sulphide can be reduced by ferredoxin NADP reductase (FNR). In maize a root-specific FNR with a ferredoxin III isoform supported a higher rate of sulphite reduction than photoreduced ferredoxin (Yonekura-Sakakibara *et al.*, 2000). The major source of the NADPH in non-photosynthetic tissue is the oxidative pentose phosphate cycle (Neuhaus and Emes, 2000). The availability of electrons from glucose catabolism thus represents another step in the regulation of sulphate assimilation in non-photosynthetic tissues. When glucose oxidation is disturbed, for example, by anoxia caused by flooding, sulphate assimilation is reduced as demonstrated by the highly

diminished activity and mRNA level of APR in flooded poplar roots (Herschbach, 2003).

Another major group of carbohydrate metabolites which can interact with sulphate assimilation are phytohormones. Ohkama *et al.* (2002) used transgenic *Arabidopsis* plants, expressing GFP under the control of a chimeric promoter containing the sulphur-responsive element of β-conglycinin (Awazuhara *et al.*, 2002), to test the influence of phytohormones on the sulphur-deficiency response. Whereas abscisic acid (ABA), indole-3-acetic acid (IAA), aminocyclopropane carboxylic acid (ACC, precursor of ethylene), gibberelic acid (GA_3), and jasmonic acid (JA) were not able to induce the expression of GFP derived from the sulphur-responsive element and, thus, mimic the sulphur-starvation response, *trans*-zeatin caused an increase in GFP synthesis both in sulphur-sufficient and sulphur-deficient conditions. In addition, zeatin treatment resulted in an increased accumulation of mRNA for APR and a low-affinity sulphate transporter (Ohkama *et al.*, 2002). Since in the cytokinin-treated plants the sucrose, but not the OAS content was elevated, the authors speculated on sucrose being the mediator of cytokinin regulation of sulphate assimilation (Fig. 2A). JA did not affect the expression of the sulphur-responsive promotor element, but is nevertheless involved in the regulation of sulphate assimilation. Treatment of *Arabidopsis* with methyljasmonate resulted in a fast, but transient increase in mRNA levels of ATPS, APR, APS kinase, γ-ECS, and GSHS (Harada *et al.*, 2000). The mRNA for sulphate transporters was not affected, confirming that JA does not participate in the regulation by sulphur nutrition. The induction of sulphate assimilation by JA is not surprising, since JA is known to participate in the transduction of stress responses (Reymond and Farmer, 1998) and sulphur compounds often play an important role in plant stress defence (Foyer and Rennenberg, 2000). The role of phytohormones in the regulation of sulphate assimilation, however, still remains to be elucidated.

Regulation of GSH synthesis

The potential mechanisms that control GSH biosynthesis include (i) regulation of the availability of its constituent amino acids cysteine, glutamate, and glycine; (ii) transcriptional regulation of the enzymes of glutathione biosynthesis γ-ECS and GSHS; (iii) regulation of the activity of these enzymes, and (iv) hormonal control. A large body of evidence indicates that regulation of GSH biosynthesis includes all these potential mechanisms which may reflect the requirement for different mechanisms of control for different functions of GSH.

Regulation of GSH synthesis by sulphur compounds

The GSH synthesis of plant cells is dependent on and regulated by the sulphur supply of the plants. During sulphur deficiency GSH content rapidly decreased in tobacco cell cultures but was rebuilt upon the resupply of sulphate or cysteine (Smith, 1980). GSH is reduced in plants subjected to sulphur deficiency (Nikiforova *et al.*, 2003), as well as in *Arabidopsis* plants with reduced sulphur supply, due to a mutation in a high affinity sulphate transporter Sultr1;2 (Maruyama-Nakashita *et al.*, 2003). On the other hand, GSH in leaves is slightly enhanced by SO_2 exposure and is significantly enhanced by fumigation with H_2S (De Kok and Tausz, 2001). Apparently, these atmospheric sulphur compounds that circumvent the APR-mediated control of sulphate assimilation are used as substrates for the increased synthesis of cysteine that is incorporated into GSH. It can therefore be concluded that the cysteine concentration limits glutathione biosynthesis. This assumption is supported by studies in which cysteine was supplied to leaf discs of poplar plants. Irrespective of the level of activity of γ-ECS and GSHS, incubation with cysteine enhanced foliar GSH contents (Strohm *et al.*, 1995; Noctor *et al.*, 1996). This observation has been explained by cellular cysteine concentrations being low compared with K_M-values of γ-ECS for cysteine or close to these values (Bergmann and Rennenberg, 1993).

Overexpression of γ-ECS, but not of GSHS, causes a 2–4-fold constitutive increase in foliar glutathione levels (Strohm *et al.*, 1995; Noctor *et al.*, 1996; Creissen *et al.*, 1999). Since the foliar cysteine pool does not become depleted as a consequence of overexpression γ-ECS, up-regulation of GSH synthesis at the level of the γ-ECS protein is connected with enhanced cysteine synthesis. However, the activities and mRNA levels of enzymes of sulphate reduction and assimilation are not significantly affected by γ-ECS overexpression (Hartmann *et al.*, 2004); thus, the mechanism by which the overexpression stimulates cysteine synthesis remains to be elucidated.

Little is known about the regulation of expression of the genes of γ-ECS and GSHS, *gsh1* and *gsh2*, in plants. Apparently, S-compounds and S deficiency do not control the corresponding mRNA accumulation. Although mRNA for *gsh1* and *gsh2* were regulated in a co-ordinated manner by heavy metals and jasmonate (Fig. 2B), GSH or GSSG were not directly involved in the control of expression of these genes (Xiang and Oliver, 1998). A microarray analysis of the sulphate-starvation response did not suggest the transcriptional regulation of γ-ECS and GSHS (Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003). Correspondingly, evidence for post-transcriptional activation of γ-ECS during the stress response was reported (May *et al.*, 1998*b*); however, the expression data contradict the results of Xiang and Oliver (1998). Nevertheless, as also observed in animal cells (Huang *et al.*, 1993), the activity of plant γ-ECS seems to be regulated at the post-translational level. *In vitro* the enzyme is inhibited non-allosterically by the GSH concentrations frequently found in plant cells (Bergmann and Rennenberg, 1993; Hell and Bergmann, 1990; May and Leaver, 1994; Schneider and Bergmann,

1995). Inhibition of γ-ECS by GSH was found to be competitive to glutamate and may, therefore, be overcome at high glutamate concentrations (Schneider and Bergmann, 1995). Mammalian γ-ECS also seems to be regulated by protein phosphorylation (Sun *et al.*, 1996), but data on the phosphorylation of the plant enzyme have not been reported. Several pieces of evidence suggest that feedback regulation of γ-ECS by GSH also operates *in vivo* and exerts control over GSH synthesis under steady-state conditions. γ-EC levels in plant cells are generally low and can limit GSH synthesis (Schneider and Bergmann, 1995; Strohm *et al.*, 1995). Enhanced metabolization of GSH, for example, for phytochelatin synthesis at heavy metal exposure, transiently lowers the GSH level and, at the same time, enhances the rate of glutathione synthesis (Bergmann and Rennenberg, 1993; Schneider and Bergmann, 1995). This observation can be explained by a release of the control of GSH synthesis at the level of feedback inhibition of γ-ECS by GSH. Still other regulatory factors, such as improved cysteine availability, enhanced γ-ECS activity, and enhanced abundance of *gsh1*and *gsh2* transcript levels contribute to enhanced GSH synthesis upon heavy metal exposure (Rüegsegger and Brunold, 1992; Schneider and Bergmann, 1995; Xiang and Oliver, 1998). Obviously, feedback inhibition of γ-ECS by GSH can by overruled by other regulatory mechanisms, since a whole range of environmental factors can enhance cellular GSH levels (Noctor *et al.*, 1998) and overexpression of γ-ECS (Noctor *et al.*, 1996), SAT (Blasczyk *et al.*, 1999; Harms *et al.*, 2000), or APR (S Kopriva *et al.*, unpublished data) result in elevated GSH contents.

Regulation of GSH synthesis by nitrogen

Since GSH contains three moles of nitrogen per mole of sulphur, it may be assumed that GSH synthesis is also dependent on the availability of nitrogen precursors and thus on nitrogen nutrition of the plants. However, the sink strength of GSH synthesis for nitrogen may be low compared with other major nitrogen sinks, such as the synthesis of proteins or nucleotides. Changes in inorganic and organic nitrogen nutrition did not affect the GSH level in *Arabidopsis* (Koprivova *et al.*, 2000) and nitrogen deficiency affected the GSH level of poplar only when plants were simultaneously deprived in sulphur (Kopriva *et al.*, 2004). It is therefore unlikely that nitrogen metabolism *per se* can limit the availability of the nitrogen precursors for GSH synthesis. Because the generation of these precursors also involves other metabolic pathways, nitrogen precursors may still become limiting for GSH synthesis, when these other pathways are impaired, in particular, when photorespiration is diminished.

Interaction of GSH synthesis with photosynthesis

In spruce foliar GSH levels undergo diurnal changes with increasing levels in the morning, maximum levels at midday, decreasing levels in the afternoon, and low levels during the night (Schupp and Rennenberg, 1989, 1990). These diurnal changes were not mediated by a circadian rhythm, but were entirely dependent on illumination. Increasing GSH levels cause a concomitant decrease in γ-EC, decreasing GSH levels a recovery of the γ-EC pool of the leaves (Schupp and Rennenberg, 1990). In maize, on the other hand, GSH concentration did not vary significantly during the day and night, but γ-EC was also elevated in the dark (Masi *et al.*, 2002). Since the accumulation of γ-EC in the dark is prevented by supplying glycine to the leaves (Noctor *et al.*, 1997*a*, *b*), it may be concluded that, in the absence of light, incorporation of γ-EC into GSH is limiting GSH synthesis. This limitation may be a consequence of a decreasing availability of photorespiratory glycine for GSH synthesis with decreasing light intensity. Serine may not become rate-limiting for cysteine synthesis under these conditions, because it is not only produced by photorespiration, but also by an alternative plastidic pathway (Ho and Saito, 2001). This assumption is consistent with a lack of diurnal changes in cellular cysteine concentrations (Schupp and Rennenberg, 1990; Masi *et al.*, 2002). The limitation of GSH synthesis by glycine availability in the dark was found to be of particular significance under conditions of enhanced cysteine synthesis, either as a consequence of exposure to H_2S (Buwalda *et al.*, 1990), or as a consequence of overexpression of γ-ECS (Noctor *et al.*, 1997*a*, *b*). Since enhanced cysteine synthesis for increased GSH production is crucial under various stress conditions (Rennenberg and Brunold, 1994), photorespiratory glycine seems to be an important factor for GSH-mediated stress compensation in plants. The accumulation of γ-EC in the night seems to be a general feature of plant sulphur metabolism, whereas the significance of the diurnal changes in GSH content has yet to be demonstrated in other plant species.

Regulation of GSH synthesis by phytohormons

As already mentioned the accumulation of mRNA for γ-ECS and GSHS is regulated by JA in a dose-dependent manner (Xiang and Oliver, 1998). The increase in transcript level is not reflected by the GSH content, which is not affected by JA treatment, pointing out again a post-transcriptional/post-translational regulation of GSH synthesis. However, the lack of an effect of JA on steady-state GSH concentration does not exclude the possibility that the rate of GSH synthesis and turnover are affected, especially when mRNAs for sulphate assimilation enzymes are also increased by JA (Harada *et al.*, 2000). The interaction of GSH synthesis with stress defence is further corroborated by the finding that GSH contents increased in plants treated with abscisic acid (Jiang and Zhang, 2001) and salicylic acid (Fodor *et al.*, 1997) (Fig. 2B). ABA plays an important role in adaptive responses to environmental stresses (Chandler and Robertson, 1994) and leads to increased production of reactive oxygene species (Guan *et al.*, 2000). It

is, therefore, not clear whether GSH synthesis is regulated by ABA itself or by the oxidative stress resulting from ABA treatment. Salicylic acid (SA) plays a central role in plant defence against pathogens. SA accumulates upon pathogen attack, induces expression of pathogenesis-related genes, and is a necessary component of systemic acquired resistance (Kunkel and Brooks, 2002). Treatment of tobacco leaves with SA as well as infection with tobacco mosaic virus resulted in an increase in GSH content in inoculated, but not in systemic leaves (Fodor *et al.*, 1997). A treatment with the biologically active SA analogue 2,6-dichloroisonicotinic acid also increased the GSH level leading to a reduction of NPR1, a regulator of systemic acquired resistance, and expression of the *PR1* gene for a pathogenesis-related protein (Mou *et al.*, 2003). If SA regulates the expression of *gsh1* and *gsh2* or utilizes another mechanism to increase GSH synthesis remains to be clarified. Another phytohormone which interacts with GSH metabolism is nitric oxide (NO) (Fig. 2B). It seems to be another key component in signalling during plant defence against pathogens (Durner and Klessig, 1999). NO interacts with GSH forming *S*-nitrosoglutathione, which may represent a transport form of NO. Accordingly, treatment with the NO-generating compound sodium nitroprusside resulted in decreased GSH levels without disturbing the GSH/GSSG ratio (de Pinto *et al.*, 2002). Depletion of GSH by conjugating NO most probably cannot fully account for the reduction of GSH content; thus, it is also probable that this substance is able to modulate the rate of GSH synthesis or degradation, albeit by an unknown mechanism. Apparently, phytohormones play important roles in the regulation of GSH synthesis that have to be investigated in detail in future studies.

Consequences of S starvation for N and C assimilation

The interaction of S, N, and C assimilation is clearly demonstrated by the effects that sulphur starvation has on overall plant metabolism (Fig. 1B). Sulphur deficiency results in reduced protein synthesis and, therefore, in the accumulation of amino acids and the inhibition of photosynthesis leading to retarded growth (Klapheck *et al.*, 1982; Gilbert *et al.*, 1997). The major target seems to be nitrate reductase. The activity of this enzyme is significantly decreased in S-starved plants, but nitrate transport is also reduced (Reuveny *et al.*, 1980; Migge *et al.*, 2000; Prosser *et al.*, 2001). Plants have developed several strategies to cope with a reduced availability of sulphur, such as derepression of sulphate transport and assimilation, induction of enzymes for the utilization of alternative sulphur sources (de Hostos *et al.*, 1988), or the synthesis of protein isoforms with a lower Met and Cys content (Takahashi *et al.*, 2001). In the green alga *Dunaliella salina* phosphoenolpyruvate carboxylase activity decreased 11-fold upon sulphur starvation, suggesting significant changes in the allocation of carbon (Giordano *et al.*, 2000). Sulphur deficiency induces nitrilase3, an enzyme involved in auxin biosynthesis, pointing to the possibility that the responses of sulphur starvation are also mediated by phytohormones (Kutz *et al.*, 2002).

The extent to which sulphur deficiency affects plant metabolism was revealed by expression profiling (Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003). Approximately 1500 genes were affected by sulphur starvation, however, due to different experimental setup, not all the genes were identified. Therefore, the total number of S-responsive genes might be even higher. As expected, the genes induced by S deficiency included the genes coding for sulphate transporters and APR; other genes of sulphate assimilation were not significantly and/or consistently affected. However, the genes involved in jasmonate and auxin biosynthesis were induced consistently, confirming the results obtained by previous investigations (Harada *et al.*, 2000; Kutz *et al.*, 2002). A strong induction of NADPH oxidoreductase, which is involved in oxidative stress defence, was also observed in all three experiments. Several genes were strongly regulated in one experiment but not in others. Examples of such genes are thioglucosidase, which was strongly induced in the roots after 24 h of S deficiency (Maruyama-Nakashita *et al.*, 2003) and was suggested to be involved in providing sulphur from alternative S sources (degradation of glucosinolates; Wittstock and Halkier, 2002), or phenylalanine ammonia-lyase, strongly reduced after 6 d and 10 d of S starvation (Nikiforova *et al.*, 2003). The interesting link between S deficiency and auxin biosynthesis was further corroborated by the finding of a 6–28-fold higher tryptophan content in sulphate-starved plants (Nikiforova *et al.*, 2003). Altogether, transcriptome analyses revealed the complexity of the interactions between S, N, and C metabolism and opened new perspectives for dissecting the molecular mechanisms of regulation of sulphate assimilation. In this context, the finding of 49 transcription factor genes that specifically responded to sulphur deficiency is of great importance (Nikiforova *et al.*, 2003).

Conclusions

The complexity of C, N, and S interactions can be seen in a relatively simple graphic representation. Figure 1A summarizes the regulation of sulphate assimilation by N and C assimilation. Obviously, the products of the assimilation pathways promote sulphate reduction whereas nutrient deficiencies generally result in reduced sulphate uptake and assimilation. Figure 1B shows how sulphur compounds and sulphate assimilation influence N and C metabolism. Here, the major part of regulation is exerted on the level of sulphate availability. Sulphur deficiency leads to inhibition of both C and N assimilation, but also to the accumulation of amino acids, due to reduced availability of

Cys and Met for protein synthesis. The nutritional/environmental conditions and compounds proposed to act as molecular signals in the regulation of sulphate assimilation and GSH synthesis are presented in Fig. 2. Again, the regulation of sulphate assimilation is very complex and interactions of multiple signals may be expected. Only little is known about the regulation of GSH synthesis since most reports deal merely with metabolite contents. Therefore, the schemes of regulation are far from being complete and the finding of new signal molecules and signalling pathways can be expected. Another major gap in current knowledge of the regulation of sulphate assimilation and GSH synthesis is the identity of the *trans* elements. Therefore the finding of 49 transcription factor genes responding to sulphur deficiency (Nikiforova *et al.*, 2003) is of great importance. However, the progress achieved recently in the identification of molecular signals and the sulphur-responsive element of the β-conglycinin promoter (Awazuhara *et al.*, 2002) are an excellent basis for searching transcription factors and elements of signalling pathways. Given the importance of sulphate assimilation in plant primary metabolism and the importance of sulphur for crop productivity, rapid progress may be expected.

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