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Review

Sulfate assimilation and glutathione synthesis in C_4 plants

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Abstract

Sulfate assimilation and glutathione synthesis were traditionally believed to be differentially compartmentalised in C_4 plants with the synthesis of cysteine and glutathione restricted to bundle sheath and mesophyll cells, respectively. Recent studies, however, showed that although ATP sulfurylase and adenosine 5′ phosphosulfate reductase, the key enzymes of sulfate assimilation, are localised exclusively in bundle sheath in maize and other C_4 monocot species, this is not true for the dicot C_4 species of *Flaveria*. On the other hand, enzymes of glutathione biosynthesis were demonstrated to be active in both types of maize cells. Therefore, in this review the recent findings on compartmentation of sulfate assimilation and glutathione metabolism in C_4 plants will be summarised and the consequences for our understanding of sulfate metabolism and C_4 photosynthesis will be discussed.

Introduction

Sulfate assimilation is an essential pathway of plant primary metabolism delivering reduced sulfur for synthesis of amino acids, co-enzymes, and a variety of secondary compounds. Among the sulfur-containing metabolites, glutathione (GSH) has a special place as it plays an important role in plant defence against biotic and abiotic stress and in maintaining a cellular redox status. As reviewed in this issue and several current reviews (Kopriva and Koprivova 2003, 2004; Leustek et al. this issue), sulfate taken up in the cells by sulfate transporters is first activated by ATP sulfurylase (ATPS) to adenosine 5-phosphosulfate (APS). In plastids, APS is reduced to sulfite by APS reductase (APR) and further reduced to sulfide by sulfite reductase (SiR). Sulfide is then incorporated into the amino acid skeleton of *O*-acetylserine (OAS) to form cysteine by OAS thiolyase (OASTL). OAS itself is produced from serine by acetylation catalysed by serine acetyltransferase (SAT). The synthesis of cysteine, a final product of sulfate assimilation, is a reaction in which the pathway merges with the assimilation of nitrogen and carbon. It is therefore not surprising that sulfate assimilation is highly regulated by light, carbon, and nitrogen compounds (Neuenschwander et al. 1991; Brunold 1993; Kopriva et al. 1999; Koprivova et al. 2000; Kopriva et al. 2002; Kopriva and Koprivova 2003; Kopriva and Rennenberg 2004). Indeed, APR activity is greatly reduced in the dark or in the absence of CO_2 but can be restored upon feeding sugars or OAS (Neuenschwander et al. 1991; Kopriva et al. 1999; Kopriva et al. 2002; Hesse et al. 2003). Similarly, it has been long known that sulfur and nitrogen assimilatory pathways are well-coordinated; a deficiency for one element represses the other pathway (Reuveny et al. 1980; Koprivova et al. 2000; Migge et al. 2000; Prosser et al. 2001). Several reports revealed that APR is more susceptible to regulation than other enzymes of the pathway and has the highest control over the flux through sulfate assimilation

(Koprivova et al. 2000; Westerman et al. 2001; Vauclare et al. 2002). The reduction of sulfate occurs predominantly in the leaf, and reduced sulfur compounds are distributed to sink tissues via phloem (Herschbach and Rennenberg 2001). However, other tissues, including developing seeds, are capable of sulfate reduction (Tabe and Droux 2002). This is not surprising since, given the absolute requirement of plant tissues for reduced sulfur, most cells can be expected to cover their needs for reduced sulfur by reduction of sulfate instead of relying on long distance transport of organic sulfur compounds, such as glutathione or *S*-methylmethionine (Herschbach and Rennenberg 2001). Accordingly, available microarray data in the Genevestigator database reveals the presence of APR transcript in all *Arabidopsis* organs, including flowers and siliques (Zimmermann et al. 2004). However, there is a group of plants that lacks the ability to reduce sulfate in a great portion of their cells, the monocot C_4 plants. This review thus concentrates on the special features of sulfate assimilation and glutathione synthesis in maize and other C_4 plants.

C_4 photosynthesis

C_4 photosynthesis is characterised by spatial separation of a primary CO_2 fixation step into C_4 acids from their decarboxylation and refixation of the released CO_2 by Rubisco. Until very recently this separation of the photosynthetic reactions was linked with the occurrence of two distinct cell types – the bundle sheath cells (BSC) and mesophyll cells (MC) arranged around vascular tissue in a radial pattern known as Kranz anatomy (Laetsch 1974). CO_2 is initially fixed by phosphoenolpyruvate carboxylase (PEPCase) in the MC to form a C_4 compound oxaloacetate which is subsequently converted to malate and/or aspartate. These C_4 acids diffuse to BSC, where they become decarboxylated by one of three enzymes – NADP-dependent malic enzyme (ME), NAD-dependent malic enzyme, and phosphoenolpyruvate carboxykinase (PEP-CK), thus defining the three C_4 photosynthesis subtypes. The CO_2 released from these decarboxylation reactions is refixed by Rubisco in the Calvin cycle, similarly as in C_3 plants (Edwards and Huber 1981; Edwards and Walker 1983; Hatch 1987). The advantage of this

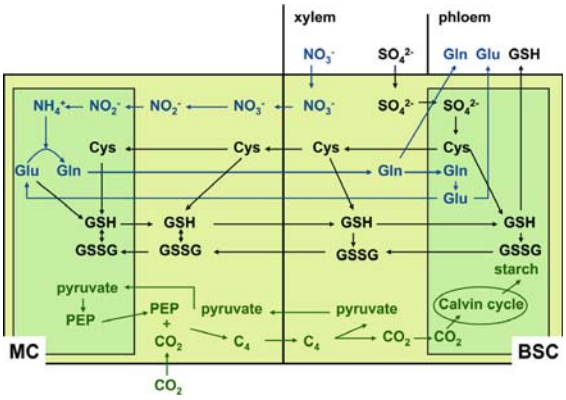

Figure 1. Schematic representation of distribution of major steps in assimilation of carbon (green), nitrogen (blue), and sulfur (black) between mesophyll (MC) and bundle sheath (BSC) of maize.

scheme (Figure 1) is that CO_2 concentration is increased at the site of its photosynthetic fixation and thus eliminates the oxygenation reaction of Rubisco and, therefore, photorespiration. The spatial separation of PEPCase and Rubisco is achieved by a morphological differentiation of MC and BSC and cell-specific localisation of the enzymes. BSC have thick cell walls and numerous large starch-containing chloroplasts whereas MC have smaller randomly distributed chloroplasts that do not accumulate starch. Besides the distinct leaf anatomy, C_4 plants exhibit a low photosynthetic CO_2 compensation point, a lack of O_2 sensitivity in photosynthesis, high maximal rates of CO_2 fixation, high water use efficiency, low values of carbon isotope discrimination, and improved nitrogen use efficiency. The pathway is therefore advantageous especially in hot and dry conditions.

A characteristic feature of C_4 plants is a cell-specific localisation of many enzymes of primary metabolism in BSC or MC. Clearly, the enzymes involved in the primary CO_2 fixation and malate and/or aspartate synthesis, such as cytosolic carbonic anhydrase, PEPCase, pyruvate phosphate dikinase, and NADP-malate dehydrogenase, are localised predominantly in the MC, whereas NAD(P)-ME, Rubisco, Rubisco activase, and some enzymes of the Calvin cycle are found exclusively in BSC (reviewed in Sheen 1999; Edwards et al. 2001). In most C_4 species analysed, including maize and *Sorghum*, BSC chloroplasts lack Photosystem II and therefore exhibit very little oxygen evolution (Hatch and Osmond 1976). Consequently, noncyclic electron flow and the

capacity for NADPH formation are restricted in BSC chloroplasts. In addition, glycine decarboxylase (GDC), a key enzyme of photorespiration, is localised exclusively in BSC of C_4 and C_3–C_4 intermediate plants (Hylton et al. 1988). The C_3–C_4 intermediate plants were originally identified by having a CO_2 compensation point intermediate between C_3 and C_4 species and can be considered as evolutionary intermediates in the path from C_3 to C_4 photosynthesis (Monson and Moore 1989; Kopriva et al. 1996). The intermediate species possess a Kranz-like anatomy and their apparent photorespiration rate is reduced, due to the confinement of GDC to the BSC and efficient refixation of photorespired CO_2 in these cells (Hylton et al. 1988; Rawsthorne 1992). Some C_3–C_4 plants are able to fix CO_2 into malate and aspartate to some extent (Bassüner et al. 1984; Monson et al. 1986) as in C_4 species, but the compartmentalisation of the photosynthetic enzymes is not complete (Bauwe 1984). The presence of C_3–C_4 intermediates and C_4 plants in several orders of dicots and monocots reveals that C_4 photosynthesis evolved several times independently. This surely contributed to the great diversity of C_4 plants reaching from the three classical Kranz-type subtypes namely NAD-ME, NADP-ME, and PEP-CK, to recently discovered single cell C_4 photosynthesis without Kranz anatomy in two species of Chenopodiaceae (Voznesenskaya et al. 2001), aquatic C_4 photosynthesis of *Hydrilla* and *Egeria* (Bowes et al. 2002), and even a C_4 cycle in marine alga *Udotea flabellum* (Bowes et al. 2002) and perhaps in a diatom *Thalassiosira weissflogii* (Reinfelder et al. 2000).

The higher nitrogen use efficiency observed in C_4 plants was initially attributed to a BSC restricted, and thus lower, synthesis of Rubisco (Brown 1978). However, differences exist also between the C_4 subtypes; the photosynthetic N-use efficiency in NADP-ME plants, measured as assimilation rate per unit leaf N, was consistently greater than in NAD-ME ones due to higher Rubisco catalytic turnover rates (Ghannoum et al. 2005). In addition, majority of leaf N was allocated to BSC in NAD-ME plants but to MC in the NADP-ME ones. Interestingly, enzymes participating in the assimilation of nitrogen are also localised in cell-specific manner in various C_4 plants (Figure 1); nitrate reductase (NR) and nitrite reductase are specifically localised in MC, whereas glutamine synthetase is equally distributed between MC and BSC, and glutamate synthetase and glutamate dehydrogenase are predominantly but not exclusively BSC localised (Rathnam and Edwards 1976; Moore and Black 1979). These results were corroborated by immunolocalisation studies demonstrating an exclusive localisation of NR in cytosol of MC (Vaughn and Campbell 1988) and the presence of both cytosolic and plastidic isoforms of glutamine synthetase in MC and BSC (Becker et al. 1993). Ferredoxin-dependent glutamate synthase, however, was found by immunolocalisation to be BSC-specific (Becker et al. 1993). If the compartmentalisation of nitrogen metabolism contributes to improved nitrogen use efficiency in C_4 plants and what the mechanism could be is, however, still not known.

Sulfate assimilation in C_4 plants

Given the number of cellular processes spatially distributed in C_4 plants, it was not a great surprise that enzymes of sulfate assimilation were also found to be differentially localised (Gerwick and Black 1979; Gerwick et al. 1980; Passera and Ghisi 1982; Schmutz and Brunold 1984). Several groups reported that 75–100% of total leaf ATP sulfurylase activity in maize was confined to BSC (Gerwick and Black 1979; Passera and Ghisi 1982; Schmutz and Brunold 1984; Burnell 1984). These findings were extended to 17 other C_4 species of all three C_4 subtypes, where 95–100% of total leaf ATPS activity was localised in chloroplasts of BSC (Gerwick et al. 1980). Also APS reductase was found almost exclusively in BSC of maize (Schmutz and Brunold 1984; Burgener et al. 1998), while the activities of SiR and OASTL were found in MC and BSC at comparable levels (Passera and Ghisi 1982; Burnell 1984; Schmutz and Brunold 1985). The mRNA levels for APR, ATPS, and SiR, are accumulated in BSC only, whereas the mRNA for OASTL was detected in both MC and BSC (Kopriva et al. 2001). Therefore, the cell-specific localisation of enzymes of sulfate assimilation in maize seems to be regulated transcriptionally, at least under standard growth conditions. When maize plants were subjected to chilling stress APR activity and mRNA level was greatly increased in BSC, however, only mRNA but not enzyme activity was also detectable in MC,

showing that post-transcriptional mechanisms also participate in the compartmentalisation of sulfate assimilation in maize (Kopriva et al. 2001). The localisation of ATPS and APR in BSC of C_4 plants requires an efficient transport of reduced sulfur compounds from BSC to MC. As MC possess OASTL and are thus capable of cysteine synthesis, a variety of compounds, such as sulfide, cysteine, or glutathione, could represent the transport form of reduced sulfur. However, since feeding isolated bundle sheath strands with [^{35}S]sulfate resulted in secretion of radioactive cysteine into the nutrient solution (Burgener et al. 1998), cysteine (or its oxidised form cystine) is the most probable transport metabolite (Figure 1). Interestingly, the [^{35}S]sulfate feeding experiments also revealed that glutathione synthesis was predominantly in MC (Burgener et al. 1998).

Glutathione in maize

The tripeptide glutathione (GSH) is the most abundant low molecular weight thiol in plants. GSH plays an important role in the defence against various biotic and abiotic stress conditions and in redox buffering of the cell (May et al. 1998; Noctor et al. 1998). Moreover, GSH is the main storage and transport form of reduced sulfur and is involved in the regulation of sulfate assimilation (Foyer and Rennenberg 2000). GSH is synthesised from its constituent amino acids via the consecutive action of γ-glutamylcysteine synthetase (γ-ECS), synthesizing γ-glutamylcysteine (γ-EC) from glutamate and cysteine, and glutathione synthetase (GSHS), adding glycine to γ-EC. Although GSH is present in both the cytosol and plastids, and GSH synthesis was long believed to take place in both compartments (Hell and Bergmann 1990; Noctor et al. 1998), recently it was shown that the two steps of GSH biosynthesis may be spatially separated. A detailed analysis of subcellular localisation of γ-ECS and GSHS in *Arabidopsis* and *Brassica juncea* revealed that at least in Brassicaceae γ-ECS is exclusively localised in the plastids whereas GSHS is present in both plastids and cytosol (Wachter et al. 2005). These novel results indicate that γ-EC may not only be a precursor of GSH but also play important roles in transport of reduced sulfur from plastids to the cytosol and possibly in signalling of redox status of the chloroplast, at least in Brassicaceae. On the other hand, γ-ECS and GSHS were detected by immunohistochemistry in both chloroplasts and cytosol of maize (Gómez et al. 2004). It thus demonstrates that there are species-specific differences in the localisation of GSH biosynthetic enzymes which possibly result in different regulatory mechanisms for sulfur assimilation, as seen e.g. with the conflicting data on cysteine inhibition of SAT in plastids and cytosol in various plants (Noji et al. 1998; Droux 2003).

GSH is most important for the regeneration of the primary reactive oxygen scavenger, ascorbate, catalysed by GSH dependent dehydroascorbate reductase (Noctor and Foyer 1998). The oxidised glutathione (GSSG, glutathione disulfide) formed in this reaction is subsequently reduced by glutathione reductase (GR), using NADPH as reductant. Glutathione is particularly important in low temperature sensitive C_4 plants, such as maize, where it protects against chilling stress by detoxification of H_2O_2. At low temperatures, GSH content and reduction state were higher in chilling tolerant genotypes of tomato, Sorghum, wheat, and maize (Badiani et al. 1993; Walker and McKersie 1993; Koczy et al. 1996, 2000a). Indeed, Brunner et al. (1995) demonstrated that in maize, chilling induced foliar activities of APR, γ-ECS, and GSHS, and consequently the content of thiols. In addition to total GSH content, the activities of APR and GR were increased in the chilling tolerant maize genotype compared to a sensitive one (Koczy et al. 1997). A direct link between chilling tolerance and GSH synthesis was revealed by manipulation of GSH content in maize in two approaches. In the first set of experiments, GSH was depleted by treatment of the chilling-tolerant maize with buthionine sulfoximine (BSO), a potent inhibitor of γ-ECS (Koczy et al. 2000b). Under normal temperature, treatment with 1 mM BSO decreased GSH content to very low levels but fresh weight and relative injury, based on the extent of shoot necrosis after a 7 day recovery, were unaffected. However, application of 1 mM BSO to plants grown at 5 °C resulted in reduction of both fresh weight and dry weight and in visible leaf injury (Koczy et al. 2000b). Addition of GSH or γ-EC together with BSO protected the plants from the chilling injury by increasing GSH content and GR activity. In a second approach, increasing GSH content in the chilling sensitive maize by

treatment with herbicide safeners significantly reduced the chilling induced injury (Koczy et al. 2001). Again, a simultaneous addition of BSO counteracted the protection resulting from treatment with safeners. Altogether these experiments clearly showed that, at least in maize, sensitivity to chilling is a trait connected with GSH content and/or reduction state.

Although GSH is essential for protection not only against a chilling injury, but also against many other stresses, it is not equally distributed between MC and BSC in maize. GSHS activity is greater in MC than in BSC leading to GSH synthesis predominantly in the MC (Burgener et al. 1998) and higher GSH levels in this cell type (Doulis et al. 1997; Burgener et al. 1998; Kopriva et al. 2001). Doulis et al. (1997) did not find GSSG in MC, whereas Kopriva et al. (2001) found GSSG in both the cell types at comparable levels. The reason for the discrepancy is probably due to the different methodology used. Whereas in the former report thiols were measured in whole leaf and mesophyll sap and their distribution between MC and BSC was calculated according to the distribution of Rubisco as a marker, Kopriva et al. (2001) determined the thiols directly in isolated bundle sheath strands and mesophyll extracts. Probably due to the low capacity for NADPH formation in BSC, GR was found exclusively in MC of maize (Doulis et al. 1997; Pastori et al. 2000). The MC specific localisation of GR is dependent on a post-transcriptional regulation, since the corresponding mRNA was found in both cell types (Pastori et al. 2000). Very recently, the enzymes of GSH synthesis and corresponding mRNAs were localised in both MC and BSC of maize by immunohistochemistry and *in situ* RNA hybridisation, respectively (Gómez et al. 2004). Chilling caused in an induction of γ-ECS mRNA in BSC but not in MC; correspondingly, foliar GSH content increased in cold-treated plants (Gómez et al. 2004). It seems, therefore, that at least in maize both cell types possess the capacity to synthesize GSH, but the enzymes in BSC are more affected by stress. Although these recent results seem to be in contrast with the previous data (Doulis et al. 1997; Burgener et al. 1998) there may be marked differences in the enzymatic properties and activities in the two cell types leading to the different distribution of GSH. The distinct stress response might be explained by the fact that cysteine, the limiting factor in GSH synthesis, is synthesised only in BSC and can be used for GSH synthesis without the necessity for transport (Burgener et al. 1998; Kopriva et al. 2001). On the other hand, the oxidised form of GSH can probably be reduced only in MC (Figure 1; Pastori et al. 2000). Consequently, GSSG formed during the stress in BSC has to be transported to MC for reduction and thus the GSH pool in MC increases while the BSC pool becomes depleted. This results in increased demand for GSH synthesis in BSC but not in MC.

Sulfate assimilation in *Flaveria*

As the significance of the cell-specific distribution of sulfate assimilation is not clear, the question whether the exclusive localisation of ATPS and APR in BSC is a pre-requisite or a consequence of C_4 photosynthesis, is of great importance. Koprivova et al. (2001) therefore addressed the distribution of these enzymes in *Flaveria* species with different types of photosynthesis. The dicot genus *Flaveria* (*Flaveriinae Asteraceae*) is an excellent model to study the evolution of C_4 photosynthesis because, besides C_3 and C_4 species, a relatively large number of C_3–C_4 intermediates occur in this genus (Ku et al. 1991) and a continuous gradation both in the physiology and biochemistry of photosynthesis exists among *Flaveria* species (Monson and Moore 1989). Indeed, based on phylogenetic analysis of the H-protein subunit of GDC, Kopriva et al. (1996) showed that C_3–C_4 intermediate species of *Flaveria* are true evolutionary intermediates in the path from C_3 to C_4 photosynthesis.

Interestingly, APR activity, cysteine, and GSH levels are significantly higher in leaves of C_4-like and C_4 species than in those of C_3 and C_3–C_4 species (Koprivova et al. 2001). When CO_2 compensation point was used as a measure of the degree of C_4 photosynthetic characteristics, there was a clear correlation with foliar APR activity, cysteine and GSH content and the development of C_4 photosynthesis (Figure 2). The absolute concentrations of GSH vary significantly among different plant species and in one species due to environmental conditions. Nevertheless, the clear tendency in *Flaveria*, which were grown at identical conditions, towards higher APR activity and

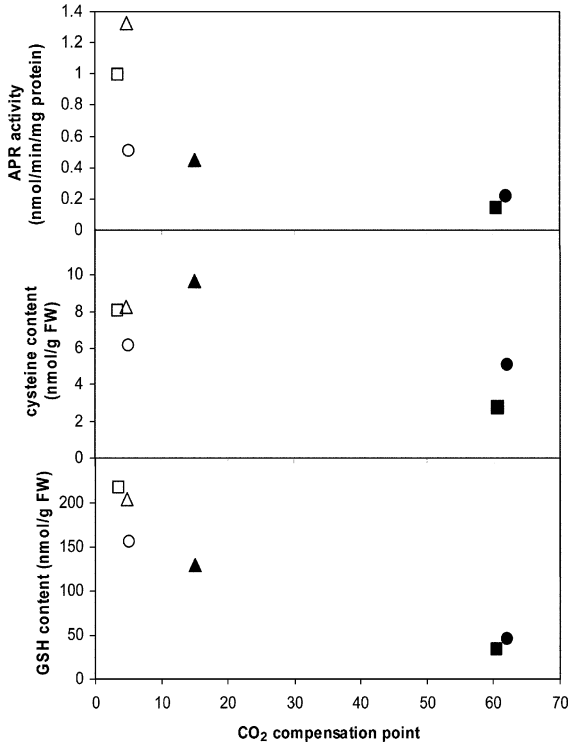

Figure 2. Correlation of APR activity and cysteine and GSH contents in leaves with the CO_2 compensation points of *Flaveria trinervia* (□; C_4), *F. australasica* (△; C_4), *F. palmeri* (○; C_4-like), *F. anomala* (▲; C_3–C_4), *F. pringlei* (■; C_3), and *F. cronquistii* (●; C_3). The CO_2 compensation points are taken from Ku et al. (1991) and the APR activity, cysteine and GSH contents data are derived from Koprivova et al. (2001).

GSH content with increasing C_4 photosynthesis syndrome could result from the adaptation of these plants to different habitats. C_4 photosynthesis is especially advantageous in dry and warm conditions which, on the other hand, are connected with increased oxidative stress. The higher GSH contents in C_4 *Flaveria* might thus be a mechanism to cope with this increased stress and the elevated APR activity might be necessary to supply sufficient cysteine for the increased GSH synthesis.

The major goal of the study with *Flaveria* was to compare the intercellular distribution of sulfate assimilation in C_3, C_3–C_4, and C_4 species. Surprisingly, however, northern analysis of cell-specific RNA and *in situ* hybridisation revealed that ATPS and APR mRNAs were present at comparable levels in both MC and BSC of the C_4 species *Flaveria trinervia*. Also immunogold electron microscopy confirmed the presence of APR protein in chloroplasts of both cell types (Koprivova et al. 2001). Consequently, the localisation of assimilatory sulfate reduction in BSC is not ubiquitous among C_4 plants. How does the study of Koprivova et al. (2001) relate to the previous ones, most notably to Gerwick et al. (1980)? Gerwick et al. (1980) analysed distribution of ATPS in 17 C_4 species of all three subtypes, however, all those species were monocots. *Flaveria trinervia* and *F. australasica* analysed by Koprivova et al. (2001) were thus the first and only C_4 dicots where the subcellular localisation of sulfate assimilation was addressed. Therefore, it was concluded that the exclusive BSC localisation of sulfate assimilation occurs only in C_4 monocots and is thus neither a pre-requisite nor a consequence of C_4 photosynthesis (Koprivova et al. 2001). Moreover, the sole fact that maize is a monocot does not explain the cell-specific compartmentation since in wheat, a C_3 monocot, ATPS and APR occur in all cell types (Schmutz and Brunold 1984). The results of Koprivova et al. (2001) also throw a shadow on the other proteins described as differentially distributed between BCS and MC in C_4 plants after analysis of maize and a few C_4 grasses. Indeed, Ketchner and Sayre (1992) demonstrated that in contrast to maize, in C_4 *Flaveria* species Photosystem II is present in BSC chloroplasts to the same extent as in MC. Therefore, especially the nitrogen assimilation pathway should be revisited in C_4 dicot species to examine whether it is exclusively MC localised and whether this indeed has consequences for improved N use efficiency as a part of the general C_4 photosynthesis trait.

Physiological significance of BSC localisation of sulfate assimilation

Whether linked to C_4 photosynthetic mechanism or not, in maize sulfate is reduced in the BSC only. This, however, is in conflict with the previous discussions about the low rate of NADPH production in BSC chloroplast being the driving force of MC localisation of nitrate assimilation and GSH reduction (Doulis et al. 1997). Following the same argument one would actually expect sulfate reduction to be localised in MC. What is then the significance of this compartmentation? Burgener et al. (1998) speculated that

a low concentration of oxygen in BSC, due to the lack of water-splitting complex of Photosystem II in this cell type, could prevent oxidation of intermediates of sulfate assimilation, sulfite and sulfide. But if such oxidation would be a drawback of sulfate assimilation, the pathway would not be functional in chloroplasts of C_3 plants and elaborate structures would have to be built, perhaps with the help of bacterial symbiosis, similar to N_2 fixation in legumes. Another possible explanation would be the co-localisation with photorespiration, namely with GDC, the major source of serine in plants. After activation by acetylation serine is the acceptor of sulfide and a direct precursor of cysteine. However, serine synthesised in BSC must be transported into MC for protein synthesis and a shortage of this amino acid in MC seems unlikely. Moreover, a proposed link between photorespiration and sulfate assimilation through co-localisation is not supported by the data for *Flaveria*, where in C_4 species GDC is BSC-specific but sulfate assimilation is not (Koprivova et al. 2001). MC specific ferredoxin FdI (Matsumura et al. 1999) was an efficient donor for sulfite reductase (Yonekura-Sakakibara et al. 2000), so cell-specific differences in electron flux can also be excluded.

To find out the significance of the BSC specific distribution of sulfate assimilation, one should ask what advantage maize may have gained by this change. With high probability a simple answer would be "none". On one hand, maize plants probably invest less into synthesis of the proteins of sulfate assimilation pathway, but on the other hand they must possess an efficient transport system for cysteine and GSH. Maize does not require significantly more or less sulfate than other plant species and is not known for especially good or poor sulfur use efficiency. It is not particularly resistant or sensitive to heavy metals, physiological condition with a high demand for reduced sulfur (Nussbaum et al. 1988). On the other hand, maize is chilling sensitive, another trait connected with GSH and sulfate assimilation, so the BSC localisation might even be a limitation of the capacity to reduce sulfate and provide enough GSH to cope with the chilling stress. It seems, therefore, that the significance of compartmentation of sulfate assimilation in maize will further remain an open question.

The second major question to ask is about consequences. Maize was a favourite subject of investigations of assimilatory sulfate reduction in the pre-Arabidopsis age. The results on regulation of sulfate assimilation obtained with maize fitted well to the general hypothesis of demand driven control (Lappartient and Touraine 1996). Coordinate increase in mRNA levels for sulfate transporters, ATPS, and APR was observed in maize roots and leaves upon sulfate starvation (Bolchi et al. 1999; Hopkins et al. 2004) and the ATPS mRNA level was repressed in presence of reduced sulfur compounds (Bolchi et al. 1999). Accordingly, ATPS and APR activities were increased upon treatments of maize with cadmium or chilling, which result in higher demand for reduced sulfur (Nussbaum et al. 1988; Rüegsegger and Brunold 1992; Brunner et al. 1995). ATPS activity was depressed by nitrogen deficiency (Saccomani et al. 1984) and APR induced by NO_2 fumigation (Kast et al. 1995). In all these reports, the regulation of sulfate assimilation in maize is not distinguishable from other plants. Bolchi et al. (1999) however, described an interesting result which differentiates maize from other plant species analysed to date. In order to identify the thiol compound responsible of feedback repression of sulfate assimilation, plants can be treated with cysteine, glutathione, and cysteine together with BSO to prevent its conversion to GSH. Such analysis performed with *Brassica napus*, *Arabidopsis*, and poplar clearly identified GSH as the molecular regulator (Lappartient and Touraine 1996; Vauclare et al. 2002; Kopriva S., unpublished) whereas in maize cysteine is acting directly without conversion to GSH (Bolchi et al. 1999). Nothing is known about the molecular mechanisms of this feedback inhibition, but it is reasonable to expect that the responsible trans-factor in maize binds cysteine in contrast to its orthologues in other species being GSH specific. This variation might well be a consequence of the BSC localisation of sulfate assimilation. As GSH can be synthesised both in MC and BSC (Gómez et al. 2004) and only Cys but not GSH is transported from BSC protoplasts (Burgener et al. 1998) it is probable that the GSH pools in MC and BSC are not rapidly interchangeable. On the other hand, Cys pools in the two cell types are in permanent connection to enable efficient protein and GSH synthesis in MC and rapid modulation of Cys biosynthesis in BSC upon even subtle changes in demand for reduced sulfur in the whole leaf.

Therefore, Cys is much better suited as a signal of sulfur status at the site of sulfate assimilation than GSH. No other specific variations in regulation of sulfate uptake and assimilation have been described to date.

Concluding remarks

The recent investigations of the plant sulfate assimilation pathway and application of molecular methods have resolved many of the old open questions. Unfortunately, the function of the cell-specific distribution of sulfate assimilation in maize does not belong to them and remains to be elucidated. The variation in localisation of assimilatory sulfate reduction in maize is clearly not linked to C_4 photosynthesis *per se*. On the other hand, the previous belief that GSH is synthesised predominantly in MC is not valid; GSH biosynthetic enzymes were clearly localised in both cell types. The finding of different compartmentation of ATPS and APR in maize and *Flaveria* revealed how dangerous generalisation is and that some of the conclusions about C_4 plants derived from work on monocots might be worth revisiting in a C_4 dicot system.

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References

Badiani M, Paolacci AR, D'Annibale A and Sermanni GG (1993) Antioxidants and photosynthesis in the leaves of *Triticum durum* L. seedlings acclimated to low, non-chilling temperature. J Plant Physiol 142: 18–24

Bassüner B, Keerberg O, Bauwe H, Pyarnik T and Keerberg H (1984) Photosynthetic CO_2 metabolism in C_3–C_4 intermediate and C_4 species of *Flaveria* (*Asteraceae*). Biochem Physiol Pflanzen 179: 631–634

Bauwe H (1984) Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose-1, 5-bisphosphate carboxylase/oxygenase in leaves of C_3, C_4, and C_3–C_4 intermediate species of *Flaveria* (Asteraceae). Biochem Phys Pflanzen 179: 253–268

Becker TW, Perrot-Rechenmann C, Suzuki A and Hirel B (1993) Subcellular and immunocytochemical localization of the enzymes involved in ammonia assimilation in mesophyll and bundle-sheath cells of maize leaves. Planta 191: 129–136

Bolchi A, Petrucco S, Tenca PL, Foroni C and Ottonello S (1999) Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine. Plant Mol Biol 39: 527–537

Bowes G, Rao SK, Estavillo GM and Reiskind JB (2002) C_4 mechanisms in aquatic angiosperms: comparisons with terrestrial C_4 systems. Funct Plant Biol 29: 379–392

Brown RH (1978) A difference in N use efficiency in C_3 and C_4 plants and its implications in adaptation and evolution. Crop Sci 18: 93–98

Brunner M, Kocsy G, Rüegsegger A, Schmutz D and Brunold C (1995) Effect of chilling on assimilatory sulfate reduction and glutathione synthesis in maize. J Plant Physiol 146: 743–747

Brunold C (1993) Regulatory interactions between sulfate and nitrate assimilation. In: De Kok LJ (ed) Sulfur Nutrition and Sulfur Assimilation in Higher Plants, pp 61–75. SPB Academic Publishing, The Hague

Burgener M, Suter M, Jones S and Brunold C (1998) Cyst(e)ine is the transport metabolite of assimilated sulfur from bundle-sheath to mesophyll cells in maize leaves. Plant Physiol 116: 1315–1322

Burnell JN (1984) Sulfate assimilation in C_4 plants. Plant Physiol 75: 873–875

Doulis AG, Debian N, Kingston-Smith AH and Foyer CH (1997) Differential localization of antioxidants in maize leaves. Plant Physiol 114: 1031–1037

Droux M (2003) Plant serine acetyltransferase: new insights for regulation of sulphur metabolism in plant cells. Plant Physiol Biochem 41: 619–627

Edwards GE, Franceschi VR, Ku MS, Voznesenskaya EV, Pyankov VI and Andreo CS (2001) Compartmentation of photosynthesis in cells and tissues of C_4 plants. J Exp Bot 52: 577–590

Edwards GE and Huber SC (1981) The C_4 pathway. In: Hatch MD and Boardman NK (eds) Biochemistry of Plants, pp 238–281. Academic Press, New York/London

Edwards GE and Walker DA (1983) C_3, C_4: Mechanisms and Cellular and Environmental Regulation of Photosynthesis, Blackwell Scientific, Oxford

Foyer CH and Rennenberg H (2000) Regulation of glutathione synthesis and its role in abiotic and biotic stress defence. In: Brunold C (ed) Sulfur Nutrition and Sulfur Assimilation in Higher Plants: Molecular, Biochemical and Physiological Aspects, pp 127–153. Paul Haupt, Bern, Switzerland

Gerwick BC and Black CC (1979) Sulfur assimilation in C_4 plants. Plant Physiol 64: 590–593

Gerwick BC, Ku SB and Black CC (1980) Initiation of sulfate activation: a variation in C_4 photosynthesis plants. Science 209: 513–515

Ghannoum O, Evans JR, Chow WS, Andrews TJ, Conroy JP and Caemmerer Svon (2005) Faster Rubisco is the key to superior nitrogen-use efficiency in NADP-malic enzyme relative to NAD-malic enzyme C_4 grasses. Plant Physiol 137: 638–650

Gómez LD, Vanacker H, Buchner P, Noctor G and Foyer CH (2004) Intercellular distribution of glutathione synthesis in

maize leaves and its response to short-term chilling. Plant Physiol 134: 1662–1671
Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy, and ultrastructure. Biochim Biophys Acta 895: 81–106
Hatch MD and Osmond CB (1976) Compartmentation and transport in C_4 photosynthesis. In: Stocking CR and Heber U (eds) Encyclopedia of Plant Physiology, New Series, pp 144–184. Springer-Verlag, Berlin
Hell R and Bergmann L (1990) γ-glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localisation. Planta 180: 303–312
Herschbach C and Rennenberg H (2001) Significance of phloem-translocated organic sulfur compounds for the regulation of sulfur nutrition. Prog Bot 62: 177–192
Hesse H, Trachsel N, Suter M, Kopriva S, Ballmoos Pvon, Rennenberg H and Brunold C (2003) Effect of glucose on assimilatory sulfate reduction in roots of *Arabidopsis thaliana*. J Exp Bot 54: 1701–1709
Hopkins L, Parmar S, Bouranis DL, Howarth JR and Hawkesford MJ (2004) Coordinated expression of sulfate uptake and components of the sulfate assimilatory pathway in maize. Plant Biol 6: 408–414
Hylton CM, Rawsthorne S, Smith AM, Jones DA and Woolhouse HW (1988) Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C_3–C_4 intermediate species. Planta 175: 452–459
Kast D, Stalder M, Rüegsegger A, Galli U and Brunold C (1995) Effects of NO_2 and nitrate on sulfate assimilation in maize. J Plant Physiol 147: 9–14
Ketchner SL and Sayre RT (1992) Characterization of the expression of the Photosystem II-oxygen evolving complex in C_4 species of *Flaveria*. Plant Physiol 98: 1154–1162
Kocsy G, Brunner M, Rüegsegger A, Stamp P and Brunold C (1996) Glutathione synthesis in maize genotypes with different sensitivities to chilling. Planta 198: 365–370
Kocsy G, Galiba G and Brunold C (2001) Role of glutathione in adaptation and signalling during chilling and cold acclimation in plants. Physiol Plant 113: 158–164
Kocsy G, Owttrim G, Brander K and Brunold C (1997) Effect of chilling on the diurnal rhythm of enzymes involved in protection against oxidative stress in a chilling-tolerant and a chilling-sensitive maize genotype. Physiol Plant 99: 249–254
Kocsy G, Szalai G, Vagujfalvi A, Stehli L, Orosz G and Galiba G (2000) Genetic study of glutathione accumulation during cold hardening in wheat. Planta 210: 295–301
Kocsy G, Ballmoos Pvon, Suter M, Rüegsegger A, Galli U, Szalai G, Galiba G and Brunold C (2000) Inhibition of glutathione synthesis reduces chilling tolerance in maize. Planta 211: 528–536
Kopriva S, Chu C-C and Bauwe H (1996) Molecular phylogeny of *Flaveria* as deduced from the analysis of H-protein nucleotide sequences. Plant Cell Environ 19: 1028–1036
Kopriva S, Jones S, Koprivova A, Suter M, Ballmoos Pvon, Brander K, Flückiger J and Brunold C (2001) Influence of chilling stress on the intercellular distribution of assimilatory sulfate reduction and thiols in *Zea mays*. Plant Biol 3: 24–31
Kopriva S and Koprivova A (2003) Sulphate assimilation: a pathway which likes to surprise. In: Abrol YP and Ahmad A (eds) Sulphur in Higher Plants, pp 87–112. Kluwer Academic Publishers, Dordrecht/Boston/London
Kopriva S and Koprivova A (2004) Plant adenosine 5′phosphosulfate reductase – the past, the present, and the future. J Exp Bot 55: 1775–1783
Kopriva S, Muheim R, Koprivova A, Trachsler N, Catalano C, Suter M and Brunold C (1999) Light regulation of assimilatory sulfate reduction in *Arabidopsis thaliana*. Plant J 20: 37–44
Kopriva S, Suter M, Ballmoos Pvon, Hesse H, Krähenbühl U, Rennenberg H and Brunold C (2002) Interaction of sulfate assimilation with carbon and nitrogen metabolism in *Lemna minor*. Plant Physiol 130: 1406–1413
Kopriva S and Rennenberg H (2004) Control of sulfate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Bot 55: 1831–1842
Koprivova A, Melzer M, Ballmoos Pvon, Mandel T, Brunold C and Kopriva S (2001) Assimilatory sulfate reduction in C_3, C_3–C_4, and C_4 species of *Flaveria*. Plant Physiol 127: 543–550
Koprivova A, Suter M, Opden Camp R, Brunold C and Kopriva S (2000) Regulation of sulfate assimilation by nitrogen in Arabidopsis. Plant Physiol 122: 737–746
Ku MSB, Wu JR, Dai ZY, Scott RA, Chu C and Edwards GE (1991) Photosynthetic and photorespiratory characteristics of *Flaveria* species. Plant Physiol 96: 518–528
Laetsch WM (1974) The C_4 syndrome: a structural analysis. Annu Rev Plant Physiol 25: 27–52
Lappartient AG and Touraine B (1996) Demand-driven control of root ATP sulphurylase activity and SO_4^{2-} uptake in intact canola. The role of phloem-translocated glutathione. Plant Physiol 111: 147–157
Matsumura T, Kimata-Ariga Y, Sakakibara H, Sugiyama T, Murata H, Takao T, Shimonishi Y and Hase T (1999) Complementary DNA cloning and characterization of ferredoxin localized in bundle-sheath cells of maize leaves. Plant Physiol 119: 481–488
May MJ, Vernoux T, Leaver C, Montagu Mvan and Inzé D (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. J Exp Bot 49: 649–667
Migge A, Bork C, Hell R and Becker TW (2000) Negative regulation of nitrate reductase gene expression by glutamine or asparagines accumulating in leaves of sulfur-deprived tobacco. Planta 211: 587–595
Monson RK and Moore BD (1989) On the significance of C_3–C_4 intermediate photosynthesis to the evolution of C_4 photosynthesis. Plant Cell Environ 12: 689–699
Monson RK, Moore BD, Ku MSB and Edwards GE (1986) Co-function of C_3- and C_4-photosynthetic pathways in C_3, C_4 and C_3–C_4 intermediate *Flaveria* species. Planta 168: 493–502
Moore R and Black CC (1979) Nitrogen assimilation pathways in leaf mesophyll and bundle sheath cells of C_4 photosynthesis plants formulated from comparative studies with *Digitaria sanguinalis* (L.) Scop. Plant Physiol 64: 309–313
Neuenschwander U, Suter M and Brunold C (1991) Regulation of sulfate assimilation by light and *O*-acetyl-L-serine in *Lemna minor* L. Plant Physiol 97: 253–258
Noctor G, Arisi A-CM, Jouanin L, Kunert KJ, Rennenberg H and Foyer CH (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49: 623–647
Noctor G and Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249–279
Noji M, Inoue K, Kimura N, Gouda A and Saito K (1998) Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J Biol Chem 273: 32739–32745

Nussbaum S, Schmutz K and Brunold C (1988) Regulation of assimilatory sulfate reduction by cadmium in *Zea mays* L. Plant Physiol 88: 1407–1410

Passera C and Ghisi R (1982) ATP sulphurylase and *O*-acetylserine sulphydrylase in isolated mesophyll protoplasts and bundle sheath strands of S-deprived maize leaves. J Exp Bot 33: 432–438

Pastori GM, Mullineaux PM and Foyer CH (2000) Post-transcriptional regulation prevents accumulation of glutathione reductase protein and activity in the bundle sheath cells of maize. Plant Physiol 122: 667–675

Prosser IM, Purves JV, Saker LR and Clarkson DT (2001) Rapid disruption of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate. J Exp Bot 52: 113–121

Rathnam CKM and Edwards GE (1976) Distribution of nitrate-assimilating enzymes between mesophyll protoplasts and bundle sheath cells in leaves of three groups of C-4 plants. Plant Physiol 57: 881–885

Rawsthorne S (1992) C_3–C_4 intermediate photosynthesis – linking physiology to gene expression. Plant J 2: 267–274

Reinfelder JR, Kraepiel AM and Morel FM (2000) Unicellular C_4 photosynthesis in a marine diatom. Nature 407: 996–999

Reuveny Z, Dougall DK and Trinity PM (1980) Regulatory coupling of nitrate and sulfate assimilation pathways in cultured tobacco cells. Proc Natl Acad Sci USA 77: 6670–6672

Rüegsegger A and Brunold C (1992) Effect of cadmium on γ-glutamylcysteine synthesis in maize seedlings. Plant Physiol 99: 428–433

Saccomani M, Cacco G and Ferrari G (1984) Efficiency of the first steps of sulfate utilization by maize hybrids in relation to their productivity. Physiol Plant 53: 101–104

Schmutz D and Brunold C (1984) Intercellular localization of assimilatory sulfate reduction in leaves of *Zea mays* and *Triticum aestivum*. Plant Physiol 74: 866–870

Schmutz D and Brunold C (1985) Localization of nitrite and sulfite reductase in bundle sheath and mesophyll cells of maize leaves. Physiol Plant 64: 523–528

Sheen J (1999) C_4 gene expression. Annu Rev Plant Physiol Plant Mol Biol 50: 187–217

Tabe LM and Droux M (2002) Limits to sulfur accumulation in transgenic lupin seeds expressing a foreign sulfur-rich protein. Plant Physiol 128: 1137–1148

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, Ballmoos Pvon, Krähenbühl U, Opden Camp R and Brunold C (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible to negative control by thiols than ATP sulphurylase. Plant J 31: 729–740

Vaughn KC and Campbell WH (1988) Immunogold localization of nitrate reductase in maize leaves. Plant Physiol 88: 1354–1357

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H and Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414: 543–546

Wachter A, Wolf S, Steininger H, Bogs J and Rausch T (2005) Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the *Brassicaceae*. Plant J 41: 15–30

Walker MA and McKersie BD (1993) Role of the ascorbate-glutathione antioxidant system in chilling resistance of tomato. J Plant Physiol 141: 234–239

Westerman S, Stulen I, Suter M, Brunold C and De Kok JL (2001) Atmospheric H_2S as sulphur source for *Brassica oleracea*: consequences for the activity of the enzymes of the assimilatory sulphate reduction pathway. Plant Physiol Biochem 39: 425–432

Yonekura-Sakakibara K, Onda Y, Ashikari T, Tanaka Y, Kusumi T and Hase T (2000) Analysis of reductant supply systems for ferredoxin-dependent sulfite reductase in photosynthetic and nonphotosynthetic organs of maize. Plant Physiol 122: 887–894

Zimmermann P, Hirsch-Hoffmann M, Hennig L and Gruissem W (2004) GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox. Plant Physiol 136: 2621–2632