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REVIEW PAPER

Integration of sulfate assimilation with carbon and nitrogen metabolism in transition from C_3 to C_4 photosynthesis

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Abstract

The first product of sulfate assimilation in plants, cysteine, is a proteinogenic amino acid and a source of reduced sulfur for plant metabolism. Cysteine synthesis is the convergence point of the three major pathways of primary metabolism: carbon, nitrate, and sulfate assimilation. Despite the importance of metabolic and genetic coordination of these three pathways for nutrient balance in plants, the molecular mechanisms underlying this coordination, and the sensors and signals, are far from being understood. This is even more apparent in C_4 plants, where coordination of these pathways for cysteine synthesis includes the additional challenge of differential spatial localization. Here we review the coordination of sulfate, nitrate, and carbon assimilation, and show how they are altered in C_4 plants. We then summarize current knowledge of the mechanisms of coordination of these pathways. Finally, we identify urgent questions to be addressed in order to understand the integration of sulfate assimilation with carbon and nitrogen metabolism particularly in C_4 plants. We consider answering these questions to be a prerequisite for successful engineering of C_4 photosynthesis into C_3 crops to increase their efficiency.

Keywords: C_4 photosynthesis, cysteine, evolution, *Flaveria*, primary metabolism, sulfate assimilation.

Introduction

Sulfur is an essential element for all living organisms, and the amino acid cysteine is perhaps the most important sulfur-containing compound in biology. Cysteine represents a remarkably versatile, highly reactive molecule involved in a number of physiological reactions, bestowing upon it a pivotal role in plant primary and secondary metabolism (Takahashi *et al.*, 2011). The chemical reactivity of cysteine is a consequence of the thiol moiety present in its molecular structure, derived from a series of reductive enzymatic reactions in the sulfate assimilation pathway. The large atomic radius of sulfur in addition to the low dissociation energy of the S–H bond confer on cysteine the unique ability to perform both nucleophilic and redox-active functions (Pace and Weerapana, 2013).

Furthermore, cysteine biosynthesis is the merging point of carbon, nitrogen, and sulfur assimilation pathways. In a simplified view, cysteine biosynthesis can be depicted in three subprocesses involving: (i) the photosynthetic assimilation of carbon dioxide (CO_2), that provides the carbon backbone; (ii) the assimilation of nitrogen and its incorporation into the carbon backbone, resulting, among others, in the amino acid serine; and, finally, (iii) the reduction of inorganic sulfate into sulfide and its incorporation into the serine-derived organic compound *O*-acetylserine (Fig. 1; reviewed in Takahashi *et al.* 2011). The resulting cysteine represents the first form of reduced organic sulfur from primary metabolism in plant cells, and is therefore an important

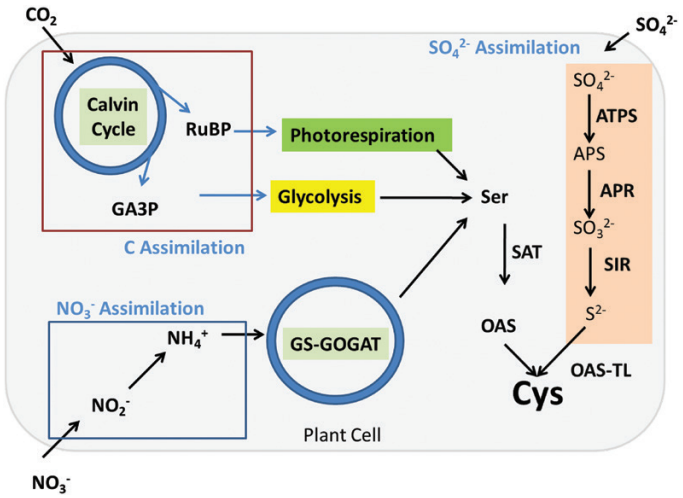

Fig. 1. Conceptualized plant cell showing the convergence of carbon assimilation through the Calvin cycle, nitrate assimilation through reduction and the GS–GOGAT cycle, and sulfate assimilation. These three pathways converge at cysteine synthesis, making cysteine a keystone metabolite connecting primary metabolism.

bioavailable source of sulfur for subsequent metabolic reactions (Pivato *et al.*, 2014).

The convergence of these three essential assimilatory pathways in plant metabolism raises a number of underexplored questions, starting with the coordination of mineral nutrition, namely sulfur and nitrogen, with photosynthetic capacity and performance. While the molecular components of the assimilatory pathways of carbon, nitrogen, and sulfur metabolism were uncovered in the last decades, the regulatory mechanisms integrating these pathways and their interaction are still relatively poorly understood. Scarce information exists regarding the simultaneous coordination and regulation of these pathways in the whole plant system.

In addition, evolutionary divergence of carbon assimilation strategies in higher plants imposes an extra layer of complexity on interactions with the assimilation of nitrate and sulfate, the main inorganic sources of nitrogen and sulfur in the soil. Although all plants ultimately use the enzyme Rubisco to fix CO_2 into organic acid molecules, some species have evolved additional carbon-concentrating mechanisms (CCMs) to minimize the oxygenase activity of Rubisco and consequently increase carbon assimilation efficiency(Leegood, 2002).

C_4 photosynthesis is the most extensively studied example of such adaptation, and can best be described as an interdependent blend of modified biochemistry, anatomy, and structural mechanisms coordinated by complex molecular entities that ultimately lead to a cell-specific spatial compartmentalization of photosynthesis-related biochemical processes (Hibberd and Covshoff, 2010). Interestingly, the cell-specific distribution of the biochemical CO_2 assimilation apparatus between bundle sheath (BS) and mesophyll (M) cells observed in species employing C_4 photosynthesis seems to extend to enzymatic mechanisms involved in nitrogen and sulfur assimilation, which segregate their components between these two cell types(Kopriva and Koprivova, 2005). Hence, a high degree of coordination among these processes must take place to achieve optimal growth and development under fluctuations in any of the components. While the spatial separation of sulfur assimilation machinery in C_4 plants was first observed >40 years ago and has been revisited more recently with a molecular emphasis, its physiological relevance as well as its regulation and coordination with CO_2 assimilation are still unknown (Weckopp and Kopriva, 2014).

Considering the number of key roles reduced sulfur compounds play in biological processes, one could argue that cysteine, while often overlooked from a nutritional perspective, is a keystone nutritional compound linking together sulfur, carbon, and nitrogen metabolism, not just in plants but in all living organisms. In this review, we will qualify this assertion by briefly presenting the biochemistry linking together carbon, nitrogen, and sulfur metabolism in plants. We will then highlight the implications this may have for C_4 plants, in which these three pathways are spatially separated between two distinct cell types. Finally, we will discuss critical knowledge gaps in our understanding of plant sulfur metabolism in the context of C_3 and C_4 metabolism and beyond.

Cysteine: a keystone nutritional compound

Cysteine synthesis connects the assimilation of three major nutrients: carbon, nitrogen, and sulfur (Fig. 1). Carbon required for the carbon backbones of all amino acids and organic compounds is provided by the carbon reduction reactions of photosynthesis. Key among these metabolites with respect to sulfur assimilation is the amino acid serine. Serine synthesis can occur in both photosynthetic and non-photosynthetic plant cells. In plants, there are three different biochemical pathways producing serine: photorespiration, glycolysis, and the so-called 'phosphorylated pathway' (Fig. 1) (Ros *et al.*, 2014; Krueger *et al.*, 2017). For both glycolysis and the phosphorylated pathway, serine biosynthesis begins following the generation of 3-phosphoglycerate (3-PGA) derived either from the Calvin cycle or from the oxidation of sugars in glycolysis. The phosphorylated pathway is particularly important in heterotrophic tissues (Ros *et al.*, 2014); however, in green leaves, the major pathway of serine synthesis is photorespiration, which forms one molecule of serine from two glycine molecules by the concerted action of glycine decarboxylase (GDC) and serine hydroxymethyltransferase in the mitochondria. Correspondingly, disruption of photorespiratory serine synthesis in the Arabidopsis *bou-2* mutant affected leaf sulfur metabolism to a higher degree than disruption of the phosphorylated pathway (Samuilov et al., 2018a, b), which played an important role in controlling sulfur fluxes in non-photosynthetic tissues (Anoman *et al.*, 2019).Once serine is produced, the enzyme serine acetyltransferase catalyzes the acetylation of serine to provide *O*-acetylserine (OAS), which is a direct precursor of cysteine (Fig. 1).

For the nitrogen found in amino acids, including serine, and other metabolites, plants take up nitrate from the soil and reduce it to ammonium, which is incorporated into amino acids. The first enzyme involved in nitrate assimilation is nitrate reductase, which catalyzes the reduction of nitrate to nitrite.

Nitrite is toxic for plant cells, and is immediately transported from the cytosol into plastids, where it is reduced by nitrite reductase into ammonium. The ammonium generated is then used by the glutamate synthetase–glutamine oxoglutarate aminotransferase (GS–GOGAT) cycle to produce glutamate and glutamine (Krapp, 2015).

The last constituent of cysteine is sulfur. Plant sulfate assimilation requires a complex series of biochemical reactions (reviewed by Koprivova and Kopriva, 2014). Sulfate, which is taken up at the plant root by sulfate transporters, is very stable and requires activation by ATP to form adenosine 5'-phosphosulfate (APS). The enzyme involved in this step of the pathway is ATP sulfurylase. There are two divergent pathways utilizing APS, one for the creation of reduced sulfur compounds and one for the synthesis of sulfated compounds; however, the pathway leading to reduced sulfur compounds is the dominant pathway in most circumstances and occurs exclusively in the plastids. To produce sulfite, two electrons are transferred from glutathione (GSH) to APS by APS reductase (APR). Reduction of sulfite is catalyzed by sulfite reductase (SIR), and the resultant sulfide reacts with OAS for cysteine synthesis. The cysteine-producing reaction is catalyzed by *O*-acetylserine (thiol)lyase (OAS-TL) (Fig. 1).

Cysteine as an indicator of evolution

Cysteine residues usually account for one of the less abundant amino acids in proteins, although evolutionary studies show an increased frequency of cysteine incorporation into proteins in different taxa over time. Evidence suggests that cysteine enrichment in proteomes may reflect the evolution of the genetic code itself, and consequently the amino acid composition relative to ancestral proteins, implying that the usage of cysteine may further expand in descendants (Brooks *et al.*, 2002; Jordan *et al.*, 2005; Liu *et al.*, 2010; Yampolsky *et al.*, 2017). One plausible explanation for this evolutionary increase in cysteine abundance in the amino acid composition of proteins might be related to the physicochemical properties of this molecule. Despite the low frequency, cysteine residues are more frequent and prevalent at functionally important sites within protein scaffolds, such as the CxxC motifs characteristic of zinc fingers and oxidoreductase active sites, or in regions that are not functionally characterized yet, representing a vast area to explore regarding the possible numerous functional aspects involving cysteine (Pe'er *et al.*, 2004; Poznański *et al.*, 2018).

Examples can be found already in the sulfate assimilation pathway. APR in plants possesses an iron–sulfur cluster as cofactor, which is bound to the protein by two cysteine pairs (Kopriva *et al.*, 2001). These cysteine pairs are not present in related enzymes from yeast and most cyanobacteria, which instead need a second activation step for sulfate reduction from APS to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Kopriva *et al.*, 2002a). In addition, several sulfate assimilation enzymes are known to be redox regulated in plants but not in other organisms (Hell and Bergmann, 1990; Bick *et al.*, 2001; Jez *et al.*, 2004, 2016; Hothorn *et al.*, 2006). Redox regulation seems to control the partitioning of sulfur between primary sulfate reduction and secondary sulfation pathways (Koprivova and Kopriva, 2016). While APR is activated by oxidation, the enzyme catalyzing entry of sulfate into secondary metabolism, APS kinase, is activated by reduction (Hothorn *et al.*, 2006). However, while APS kinase is a ubiquitous enzyme, its redox regulation has been described only for plants and, correspondingly, only plant APS kinases possess the redox-active cysteine pair (Ravilious *et al.*, 2012). Another enzyme connected to secondary sulfur metabolism present in all kingdoms of life is the phosphoadenosine phosphate phosphatase SAL1. SAL1 is inactivated by oxidation in a retrograde stress signaling process only in plants and not in other organisms, again relying on additional cysteine residues conserved only in the plant proteins (Chan *et al.*, 2016). The glutamate-cysteine ligase (γ-ECS), which is the first and rate-limiting enzyme in GSH biosynthesis, also exhibits several layers or redox regulation that correlate well with the evolutionary history of the enzyme. While one redox-active cysteine pair is present in γ-ECS proteins from all sources, plant enzymes possess another level of redox regulation by a second cysteine pair. Under oxidizing conditions, the disulfide bridges bring the dimeric complex together and dramatically increase the enzymatic activity, while under reducing conditions the disulfide bridges are reduced, causing the dissociation of the dimer into monomers with substantially decreased activity (Hothorn *et al.*, 2006). Thus, cysteine residues can be indicators of increasing regulatory complexity and evolutionary advancement.

Coordination of carbon, nitrogen, and sulfur metabolism

The need for regulatory interconnections between the metabolism of carbon, nitrogen, and sulfur is obvious, but the mechanisms, sensors, and signals are far from understood (Koprivova and Kopriva, 2014). Sulfur nutrition is strongly coordinated with nitrogen due to the need for both in protein synthesis (Fig. 2). Thus, sulfate deficiency reduces nitrate uptake and reduction, and, vice versa, nitrogen deficiency leads to diminished sulfate uptake and reduction rate (Fig. 2). On the other hand, the key assimilatory enzymes APR and nitrate reductase are induced by reduced nitrogen (ammonium and amino acids) and sulfur (cysteine) compounds, respectively (Kopriva and Rennenberg, 2004). In the very few available reports, the coordination of sulfate assimilation with carbon metabolism seems to be similar to nitrogen—the pathway was downregulated by low CO_2 availability and induced by glucose and sucrose (Fig. 2) (Kopriva *et al.*, 2002b; Hesse *et al.*, 2003). Sulfate deficiency results in a general slowdown of metabolism, leading to a decrease in carbon assimilation and photosynthesis, and to a reprogramming of metabolism and developmental programs towards economizing resources for seed production (Nikiforova *et al.*, 2005).

While the interconnection of these pathways is well described on physiological as well as on systems biology levels, the sensors and signals triggering the metabolic adaptations are unknown. Among the signals discussed, the cysteine precursor OAS was previously and controversially considered a mediator of plant sulfur status (Hirai *et al.*, 2003; Hopkins *et al.*, 2005) or

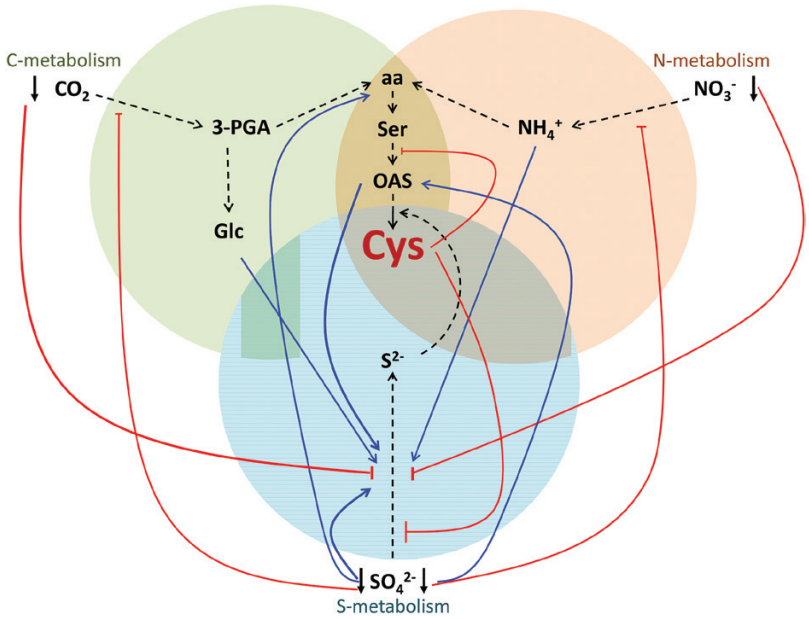

Fig. 2. Carbon and nitrogen metabolism impact sulfur signaling. Red lines signify repression or down-regulation, while blue lines represent activation or up-regulation. Black lines represent biochemical pathways

a signal connecting nitrogen and sulfur metabolism (Koprivova *et al.*, 2000). Using a systems biology approach, Hubberten *et al.* (2012) identified six genes whose expression was highly correlated with accumulation of OAS, the 'OAS cluster', which included genes shown previously to be strongly up-regulated by sulfate deficiency. Thus, OAS displays a signaling function leading to changes in transcript levels of a specific gene set irrespective of the sulfur status of the plant, and seems to play a specific part in the sulfate response. Given the increase in OAS accumulation and OAS cluster transcript levels in conditions not connected to sulfur deficiency (Espinoza *et al.*, 2010; Caldana *et al.*, 2011), the true function of OAS as a signal and the OAS cluster genes might be less linked to sulfur deficiency response but might instead function in more general coordination of the assimilatory pathways.

Another mechanism coordinating sulfur assimilation with other metabolic processes and plant growth has recently been uncovered (Dong *et al.*, 2017). It has long been known that growth of eukaryotic cells is regulated by amino acid availability through a gene called target of rapamycin (TOR). However, the well-established transducing molecules (TOR-interacting proteins: RAG GTPase, TSC1/2, and RHEB) for TOR signaling are absent in plants. Thus, how plants sense amino acids and regulate TOR signaling has been an open question in the field. Interestingly, a recent publication has shown that, unlike in mammalian systems that sense amino acids directly, plants may sense amino acid precursors (Dong *et al.* 2017). More specifically, in the case of cysteine, the precursors OAS and sulfide (S^{2-}) appear to be selectively sensed and can activate TOR signaling via two distinct pathways. Under conditions of low carbon or nitrogen status, decreased levels of OAS can activate TOR signaling in a GCN2- (general control nonderepressible 2) mediated manner, while under low sulfur status decreased sulfide levels can activate TOR signaling via glucose–TOR signaling (Dong *et al.*, 2017). While uncovering TOR signaling mechanisms represents a major breakthrough in understanding the regulatory integration of sulfur metabolism in global plant metabolism, the downstream mechanisms and transcription factors remain to be identified.

Cysteine synthesis: C_4 plants are different

The group of plants with probably the highest divergence in primary metabolism compared with the model plant Arabidopsis is C_4 plants. CO_2 fixation in C_3 photosynthesis is inherently inefficient due to Rubisco's poor ability to discriminate between CO_2 and O_2. The oxygenation results in generation of a two-carbon product, 2-phosphoglycolate. Since this compound is toxic, it is rapidly metabolized and a portion of the carbon is regenerated through photorespiration, which incurs significant energy costs (Hagemann and Bauwe, 2016). To overcome this inefficiency, plants using C_4 photosynthesis fix CO_2 initially into a four-carbon compound (oxaloacetate) via the enzyme phosphoenolpyruvate (PEP) carboxylase, which is not affected by oxygen (Gilbert and Wilhelm, 2019). CO_2 is then released from the C_4 compounds for re-fixation by Rubisco.

However, for C_4 photosynthesis to be more efficient than C_3 photosynthesis, these steps must be separated into two compartments—one where PEP carboxylase can capture CO_2 from a high O_2 environment, and one where Rubisco can fix CO_2 in a low O_2 environment (Fig. 3a). In most C_4 plants, these two processes have been spatially separated into two distinct photosynthetic cell types. C_4 M cells perform the C_4 carbon capture reactions, while BS cells perform carbon assimilation using Rubisco and the Calvin cycle. Importantly, this compartmentalization requires major alterations in typical C_3 leaf structure giving rise to a repeating pattern of cells within C_4 leaves. While C_3 leaves are structurally characterized by having many M cells between the BS cells and the veins (V), C_4 plants

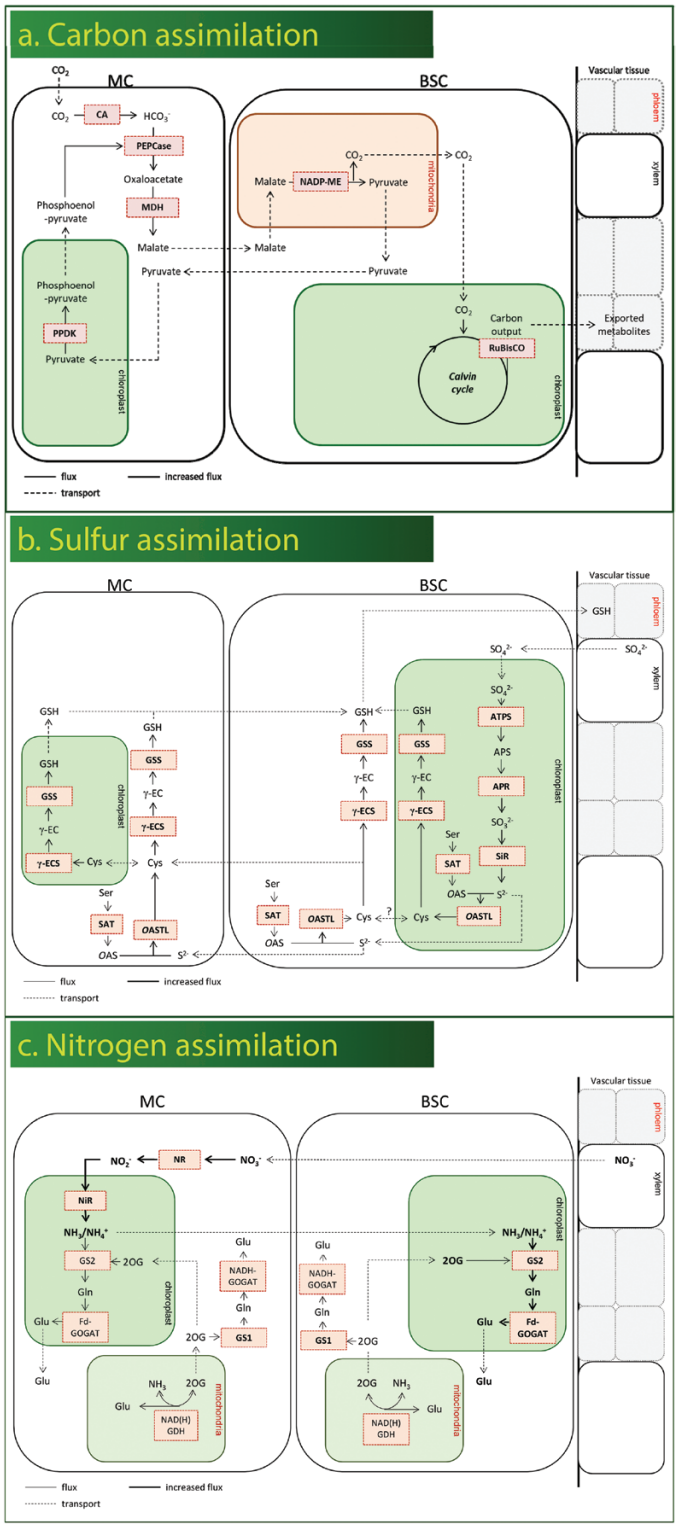

Fig. 3. Model showing separation of enzymes between the M and BS in carbon assimilation (a), nitrogen assimilation (b), and sulfate assimilation (c) in C_4 plants.

tend to form a repeating pattern of V–BS–M–M–BS–V. This cellular arrangement results in fewer M cells, decreased M area, and higher V density in C_4 plants compared with C_3 plants. Finally, in addition to this cellular rearrangement, C_4 plants have more abundant chloroplasts in the BS cells compared with C_3 plants. Collectively, these changes in tissue arrangement and BS chloroplast number are referred to as Kranz anatomy (Lundgren *et al.*, 2014). Thus, C_4 metabolism requires highly coordinated changes in tissue structure to achieve Kranz anatomy as well as the development of cell type-specific photosynthetic biochemistry to achieve spatial separation of carbon fixation and carbon assimilation (Junqueira *et al.*, 2018). A detailed analysis of monocot species revealed that C_4 photosynthesis evolved in lineages with higher BS:M ratios; that is, the anatomical pre-adaptation was the prerequisite for evolution of a full C_4 cycle (Christin *et al.*, 2013).

C_4 photosynthesis conveys improved water use efficiency, making it more efficient than C_3 carbon assimilation, particularly in hot, dry, and/or saline environmental conditions where photorespiration is high (Sage *et al.*, 2018). Despite its complexity, this extraordinarily successful metabolic trait has evolved independently >60 times and is present in many of the most successful arid and semi-arid grasses, eudicot herbs and shrubs in low to mid latitudes, and major crops, such as maize, sorghum, and sugar cane (Sage *et al.*, 1999; Still *et al.*, 2003; Edwards *et al.*, 2010). Since its discovery >50 years ago, there has been substantial interest in C_4 metabolism, much of which transcends plant metabolism (e.g. see Beerling and Osborne, 2006 for C_4 effects on climate; Bobe and Behrensmeyer, 2004; Sage and Zhu, 2011 for information on C_4 influence on meat and sugar production).

More recently, interest has grown in engineering C_4 metabolism into agronomically important C_3 crops, such as rice and wheat, to improve stress tolerance and carbon fixation efficiency (Hibberd *et al.*, 2008; Jones, 2010; von Caemmerer *et al.*, 2012; von Caemmerer and Furbank, 2016; Wang *et al.*, 2016). However, for these approaches to be successful, we must first recognize and understand the metabolic, biochemical, and structural consequences of spatially separating carbon capture from carbon assimilation, which is the hallmark of C_4 metabolism. To this end, extensive scientific literature examining these consequences exists for carbon assimilation and, to a much lesser extent, for nitrogen assimilation.

In addition to spatial separation of carbon assimilation, C_4 plants have also been shown to spatially separate nitrate assimilation (Fig. 3b). The reduction of nitrate to nitrite occurs exclusively in M cells, while the incorporation of reduced nitrogen into glutamate and glutamine (GS–GOGAT) occurs either in the BS or in the M and BS (Rathnam and Edwards, 1976; Moore and Black, 1979; Becker *et al.*, 2000). C_4 species have also been shown to exhibit higher nitrogen use efficiency than C_3 species, reportedly due to the strict localization of Rubisco in BS leading to a decreased Rubisco quantity per leaf area (Brown, 1999; Ghannoum *et al.*, 2010). It is unclear if spatial separations of nitrate reduction or the strong decrease in photorespiratory ammonium recycling contribute to improved nitrogen use efficiency in C_4 plants. Also, cysteine synthesis might be affected: the restriction of photorespiration to BS of C_4 plants could limit the ability of M cells to synthesize serine—an essential precursor of cysteine. Indeed, very little is known regarding how the massive biochemical and structural rearrangements that accompany the evolution of C_4 metabolism impact sulfate assimilation and cysteine synthesis.

Sulfate assimilation in C_4 plants

As early as the 1970s, there was evidence suggesting that sulfate assimilation in C_4 grasses is somewhat different from that in C_3 plants. Gerwick and Black (1979) demonstrated, using enzymatic assays, that 90% of the ATPS activity in crabgrass leaves was found specifically in the BS, suggesting that the bulk of sulfate assimilation was occurring in these cells. An extension of this work showed BS localization of ATPS in a broader assessment of 18 different C_4 plant species (Gerwick *et al.*, 1980). Shortly afterwards, Schmutz and Brunold (1984) confirmed the ATPS observations of Gerwick *et al.* in maize and wheat, and further showed that APR and SIR were also preferentially expressed in the BS, while OAS-TL was expressed in both BS and M cells. Burgener *et al.* (1998) then showed that isolated BS strands export cysteine into the medium, suggesting that in C_4 plants, sulfate assimilation and cysteine synthesis occur exclusively in the BS (Fig. 3c).

In an attempt to find out whether the BS localization of sulfate assimilation is a consequence or prerequisite of C_4 photosynthesis, Koprivova *et al.* (2001) analyzed the pathway in plants of the genus *Flaveria*, which contains species with C_3 and C_4 photosynthesis as well as a number of C_3–C_4 intermediate plants that show different degrees of C_4 characteristics (Ku *et al.*, 1991). Surprisingly, in both C_3 and C_4 species of *Flaveria*, APR transcript and protein localized to both the M and the BS. Because *Flaveria* is a eudicot species, these results could not be easily reconciled with previous observations in monocot systems and were interpreted as a difference between eudicot and monocot C_4 species. Further questions regarding the importance of spatial compartmentalization of sulfate assimilation in C_4 plants later came from the C_3 model organism *Arabidopsis thaliana*. Using a systems biology approach, Aubry *et al.* (2014) obtained a translatome of Arabidopsis BS cells to identify transcripts enriched in this cell type. Surprisingly, they found preferential expression of ATPS and APR, as well as transcripts associated with secondary sulfur metabolism and transport of sulfur-containing compounds in Arabidopsis BS (Aubry *et al.*, 2014). While it has been previously proposed that BS spatial separation of key metabolic enzymes is a pre-condition for evolution of C_2 photosynthesis and has been observed for GDC in several Brassicaceae species (Adwy *et al.*, 2015), the significance of BS-specific expression of sulfur enzymes is unclear. Indeed, a recent review proposes that anatomical modifications are the rate-limiting step in the C_4 trajectory, not metabolic changes (Edwards, 2019). When viewed in context with the results from *Flaveria*, two major questions arising from these studies remain: (i) what is the ancestral state/characteristics of plant sulfur metabolism, especially regarding preferential expression in the BS; and (ii) why do we not observe compartmentalization of sulfur assimilation genes in C_4 *Flaveria*, the only C_4 eudicot to be evaluated to date?

A partial answer to the second question was recently given by detailed analyses of sulfate assimilation in *Flaveria* species with a gradient of C_4 photosynthetic characteristics. Overall, across a range of *Flaveria* species, cysteine and GSH levels roughly followed the C_3–C_4 gradient, with C_4 lines having the highest foliar thiol levels, C_3 species having the lowest, and C_2 intermediates showing increasing foliar cysteine and GSH levels with increasing C_4 characteristics (Gerlich *et al.*, 2018). However, Gerlich *et al.* (2018) not only measured sulfur metabolites in these species, but also performed reciprocal grafting experiments to evaluate the contribution of roots to sulfur nutrition in *Flaveria*. Surprisingly, they found that in C_4 but not C_3 *Flaveria*, both sulfate assimilation and GSH biosynthesis occur predominantly in the roots (Gerlich *et al.*, 2018). This suggests that compartmentalization of sulfur assimilation also occurs in eudicot C_4 plants; however, the separation does not take place between M and BS cells as observed in C_4 monocots, but rather between roots and shoots. Interestingly, the study also suggested that the localization of sulfate assimilation and GSH synthesis may be driven by serine synthesis, proposing a new avenue for exploration of the control of the spatial localization (Gerlich *et al.*, 2018). This important discovery shows that during the transition from C_3 to C_4 metabolism, multiple evolutionary solutions leading to alteration in C_4 sulfur metabolism may exist (Williams *et al.*, 2013). Furthermore, this study highlights the importance of evaluating the contributions of root nitrogen and sulfur metabolism in the evolution of C_4 metabolism, which in the case of sulfur metabolism has been poorly appreciated in monocots to date. From an engineering perspective, characterizing these natural evolutionary solutions may help us better understand how to overcome genetic obstacles and transfer C_4 characteristics into C_3 crops. Regarding the ancestral state or ancestral characteristics of sulfate assimilation, this question is more difficult to address at this time as cell-specific characterization of sulfur metabolism has been performed in very few C_3 species.

The differential spatial localization of sulfate and nitrate assimilation in C_4 plants indicates that cysteine may play an additional role in metabolic coordination. Indeed, experiments performed in maize suggest that, unlike in C_3 plants, cysteine but not GSH triggers the demand-driven control of sulfate assimilation (Bolchi *et al.*, 1999). In this experiment, sulfur-starved maize seedlings were treated with buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, and subsequently fed either L-cysteine or D-cysteine. Only seedlings fed with L-cysteine showed a decreased sulfur deficiency response, suggesting that the effect was not due to thiol feeding alone, but was specific for the biologically relevant form of cysteine and not dependent on GSH biosynthesis, as in C_3 plants (Bolchi *et al.*, 1999; Lappartient *et al.*, 1999). This experiment has suggested that cysteine is the major regulator of sulfate assimilation in maize—a role typically associated with GSH.

Knowledge gaps and new directions

Perhaps the most pressing and difficult to answer questions surrounding sulfate (and nitrate) assimilation in C_4 plants are centered on the consequences of confining sulfate assimilation to the BS (and nitrate assimilation to the M). Given the number of independent evolutionary origins of C_4 photosynthesis, why is this spatial configuration so often recruited by C_4 plants and, importantly, why are there exceptions, as

seems to be the case for *Flaveria*? Additionally, why is nitrate reduction confined to M and sulfate reduction confined to BS in some species? Both of these processes are metabolically expensive in terms of energy and redox equivalents. Thus, if energy or redox status were providing the selective force to drive this metabolic rewiring, we might expect that sulfate and nitrate reduction would be confined to the same cell type. Despite the surprising metabolic differences recently identified in sulfur metabolism of C_3 and C_4 *Flaveria* roots, it remains unknown if these metabolic alterations were accompanied by changes in root nitrogen metabolism. Considering the well-documented alterations in *Flaveria* leaf nitrogen metabolism between C_3 and C_4 species and the potential role these metabolic changes played in driving the transition toward C_4 metabolism, additional studies to unravel root nitrogen metabolism in *Flaveria* are warranted (Mallmann *et al.*, 2014). It also needs to be tested whether M-specific localization of nitrate reduction might be a consequence of the C_2 cycle. The confinement of GDC to the BS results in a net transfer of ammonium from the M to BS (Mallmann *et al.*, 2014), which might be counteracted by an increase in nitrate reduction rate in M and a decrease in BS. A more thorough investigation of nitrogen metabolism in C_2 and C_3–C_4 plants will help to test this hypothesis. Finally, it is unclear if partitioning of sulfate assimilation is a consequence of C_4 metabolism, or if it is a prerequisite for C_4 metabolism. Answers to these questions are likely to be connected to one another and may be revealed through focused genetic and biochemical studies aided by systems biology and evolutionary insights, particularly as such analyses are now feasible in non-model organisms. Thus, we would like to highlight a few promising directions that might help identify key regulatory nodes of sulfur metabolism in C_4 plants and pave the way to deeper mechanistic understanding.

Transcriptional regulators

Despite the realization that sulfate assimilation undergoes spatial reconfiguration in C_4 plants and to some extent also in C_3 eudicots, little work in understanding the molecular basis for this restructuring has been performed. Generally, very little is known about transcriptional regulators of sulfate assimilation beyond Arabidopsis. For example, homologs of the main transcriptional regulator of sulfur deficiency response in Arabidopsis, SLIM1, have been identified in maize and other monocots; however, to date, none of these has been linked with sulfur nutrition in these organisms (Gallie and Young, 2004; Mao *et al.*, 2006). However, two rice homologs of SLIM1 were able to complement the Arabidopsis mutant, suggesting that they are functionally conserved in C_3 monocots. It remains an open question as to whether SLIM1 homologs from C_4 plants are also involved in sulfur responses and if they have been recruited to orchestrate some of the changes observed in C_4 sulfur metabolism. However, a function for SLIM1 in the spatial distribution of the pathway is unlikely as *SLIM1* transcript does not show such a localization in Arabidopsis (Aubry *et al.*, 2014). Answers to these questions will probably require cell-specific investigations.

Transporters

The differential spatial distribution of the metabolic pathways can function only if the substrates, intermediates, and products can be easily transported between the cells. However, very little is known regarding the cell to cell transport of sulfur metabolites, including cysteine and GSH. Our current understanding of C_4 sulfate assimilation suggests that primary cysteine synthesis occurs exclusively in the BS. Thus, cysteine transport to the M and other cell types is necessary (Burgener *et al.*, 1998; Kopriva and Koprivova, 2005). Plants are known to possess amino acid transporters, and many of them are capable of transporting cysteine (Miranda *et al.*, 2001; Tegeder, 2012); however, specific genes involved in establishing or maintaining this spatial gradient have not been identified to date. It is currently unclear if specific cysteine transporters exist in plants or if cysteine transport occurs via permeases or some unidentified mechanism. It is also unknown whether sulfate is transported into M cells to contribute to anion homeostasis. Similarly, nothing is known about the oxidized pathway of sulfate assimilation. For example, is APS kinase also differently expressed between M and BS? Are sulfotransferases catalyzing biological sulfations present in the M of C_4 plants? Is there a specific PAPS transporter between BS and M similar to those found in plastid membranes in Arabidopsis (Ashykhmina *et al.*, 2018)? Judging from Arabidopsis, which can be considered to have a C_4-like localization of sulfate assimilation, this might be the case. APS kinase, several sulfotransferases, and the PAPST1 transporter are highly enriched in the BS, together with genes for sulfate assimilation and glucosinolate synthesis (Aubry *et al.*, 2014). However, the significance of sulfated metabolites other than glucosinolates is poorly understood, and this aspect of sulfur metabolism in C_4 plants awaits initial studies.

In contrast, GSH transporters in plants have been identified. Transport of both the reduced and oxidized forms of GSH is very important for C_4 monocots as oxidized glutathione (GSSG) cannot be reduced to GSH in the BS. This is because glutathione reductase (GR), a key enzyme in the glutathione–ascorbate cycle, is not expressed in these cells. Thus, GSSG must be transported to M cells for recycling (Kingston-Smith and Foyer, 2000). Furthermore, recent work in *Flaveria* suggests that GSH biosynthesis largely occurs in the roots of this C_4 eudicot. Thus, GSH must be transported to the shoots and unloaded from the vasculature into the BS and from there to the M, suggesting that a number of GSH transporters are required in *Flaveria* (Gerlich *et al.*, 2018).

Thus far, all of the confirmed plant GSH transporters belong to the oligopeptide family of transporters and are homologs of the high-affinity yeast GSH transporter ScOPT1. The first plant GSH transporter, BjGT1, was cloned from *Brassica juncea*; however, functional homologs have been identified in several other species, including maize (Bogs *et al.*, 2003; Pang *et al.*, 2010). Most recently, mutants in two previously characterized GSH transporters from Arabidopsis, AtOPT4 and AtOPT6, were identified as having GSH-related phenotypes, including decreased GSH in floral organs and disrupted ionomic profiles when exposed to Cd (Zhang *et al.*, 2016; Wongkaew *et al.*, 2018). These studies suggest that OPTs do indeed play a role

in GSH or sulfur metabolism. Unfortunately, the affinity and specificity of these transporters are much lower than observed for ScOPT1 and they are probably only minor contributors to the high GSH flux observed within most plant cells, suggesting that additional high-affinity transporters may exist. Clearly, the molecular nature of cysteine and GSH transport between cells and tissues remains an intriguing question for the future not only in connection with C_4 plants.

Sensors and signaling molecules

The sequestration of sulfate assimilation into the BS of C_4 plants sets up an intriguing metabolic scenario where sulfate is imported into the BS and a reduced sulfur compound (cysteine or possibly GSH) is exported to the rest of the plant to satisfy its sulfur needs. Thus, sulfur assimilation within the BS could be regulated by the supply (availability of sulfate through import) or by the demand (rate of utilization of the exported reduced sulfur). At a whole-plant level, it is thought that plants sense and respond to lowered internal sulfate content (i.e. lack of sulfate availability); however, the exact sensor(s) have not been identified (Lee *et al.*, 2012). In the case of C_4 BS, the regulatory mechanisms and signaling molecules are likely to be even more complex, as fine-tuning of sulfate assimilation requires tight control over sulfate import as well as cysteine and/or GSH export to maintain appropriate levels of reduced sulfur throughout the plant. One intriguing possibility is that cysteine itself acts as a signaling molecule in C_4 plants. As discussed previously, cysteine is thought to undergo long-distance transport in maize, and supplying exogenous cysteine to maize ameliorated sulfur deficiency responses while exogenous GSH did not. However, this response has not been demonstrated in other C_4 plants.

Sulfated peptides have been identified in C_3 plants as signaling molecules, including phytosulfokines, root meristem growth factors (RGFs), or Casparian strip integrity factors (CIF1 and CIF2) (Matsuzaki *et al.*, 2010; Sauter, 2015; Doblas *et al.*, 2017). These peptides are sulfated by a tyrosylprotein sulfotransferase in the *trans*-Golgi, and then secreted into the apoplast, where they are proteolyticaly cleaved. Once in the apoplast, the secreted peptides can be perceived by membrane-bound receptor kinases belonging to the leucine-rich repeat family. These important signaling molecules have been implicated in many processes. Phytosulfokines have been shown to promote plant growth, act in funicular pollen tube guidance, affect ethylene production, and alter immune responses (Sauter, 2015). RGFs are important for maintenance of the root meristem and control cell proliferation (Matsuzaki *et al.*, 2010), while CIFs control formation of the root Casparian strip barrier (Doblas *et al.*, 2017). These target activities, cell proliferation and diffusion barriers, might also be important for establishment of C_4 photosynthesis, making sulfated peptides attractive candidates for new signals. Because their activity is regulated by sulfation, they are ultimately regulated by sulfur metabolism, potentially linking these processes with sulfur. Thus, it is possible that multiple sulfur sensors and signaling molecules exist in C_4 plants, and these molecules may or may not be broadly conserved across C_4 species due to multiple evolutionary origins of C_4 metabolism. However, as is the case in C_3 plants, the identity of sulfur sensors remains elusive.

Temporal regulation

One final and speculative area for future studies is the open question of temporal regulation of sulfur metabolism. While C_4 metabolism seeks to increase carbon assimilation efficiency by spatially separating the site of carbon capture and carbon assimilation, some plants living in extremely arid regions instead separate carbon fixation and carbon assimilation in a temporal manner to conserve water. In crassulacean acid metabolism (CAM), carbon is only captured at night while temperatures are low and the CO_2 is stored in the vacuole as the four-carbon acid malic acid (Borland and Taybi, 2004). This minimizes water loss through stomata. During the daytime, the stomata close and the malic acid is metabolized back into CO_2 and pyruvate to support the Calvin cycle. Similar to C_4 metabolism, this metabolic strategy has multiple evolutionary origins having arisen independently at least 35 times (Heyduk *et al.*, 2016). While it is unclear if these plants have also developed novel temporal regulation of sulfur assimilation, it is easy to speculate that the demands for reduced sulfur compounds in these plants are dramatically different from those of C_3 and C_4 plants. The temporal differences in carbon metabolism can be expected to pose a challenge for the coordination with nitrogen and sulfur assimilation for cysteine synthesis. Thus, the potential for novel temporal regulation of sulfur (and nitrogen) assimilation in CAM plants remains a tantalizing possibility for future research.

Conclusions

The key role cysteine synthesis plays in linking together sulfur, carbon, and nitrogen assimilation is obvious, and the importance of understanding how these pathways are coordinated cannot be understated. While this is true for both C_3 and C_4 organisms, C_4 plants pose an additional challenge due to the spatial organization of carbon, nitrogen, and sulfur metabolisms. We presume that the spatial rewiring of sulfur assimilation confers some advantage to C_4 plants or, alternatively, failure to do so results in some penalty. Yet, because it is more difficult to assess the cost–benefit relationships associated with sulfur compounds than for simple carbon compounds, correlative relationships have thus far been the only measure available. However, these correlative measures fail to assess the order in which these adaptations occur, namely did changes in sulfur metabolism occur before the development of C_4 metabolism, after the development of C_4 metabolism, or did they co-evolve alongside C_4 metabolism. Answers to these questions could have major impacts on efforts to engineer C_4 metabolism into C_3 crops.

While differences in sulfur assimilation between C_3 and C_4 species are known to exist, it is still unclear if these differences apply broadly to all C_4 species or if major differences exist between monocot and eudicot C_4 species. Furthermore, because C_4 metabolism has evolved independently >60 times, even if

many of the mechanisms underlying sulfur (and nitrogen) metabolism are conserved between monocot and eudicot C_4 species, it is likely that there are numerous subtle differences in regulation. While we are beginning to appreciate the spatial redistribution of sulfate assimilation in C_4 plants, the underlying regulatory networks, including transcriptional regulators, transporters, and sensors, have yet to be explored. Thus, despite our growing knowledge of plant sulfur assimilation, it is clear that many major discoveries have yet to be made.

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