Regulation of sulfate metabolism in C4 plants

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General introduction

The challenge of the coming decades is to strongly enhance agricultural yield in a sustainable manner. A strategy that holds great potential to sustainably boost agricultural yield is to cultivate more C_4 plants. However, C_4 plants can only be grown optimally, if we understand how these plants metabolize the macronutrient sulfur, because sulfur availability is suboptimal in many regions across the globe and hence limiting crop yield and quality. The current chapter provides background information to the above rationale, for this dissertation analyzes how C_4 photosynthesis affects the regulation of sulfate uptake and reduction.

The Green Revolution

In the past decades, agriculture has undergone significant changes that enhanced crop productivity (Khush 2001; Pingali 2012). These changes are collectively termed the Green Revolution and included the introduction of synthetic fertilizers, new irrigation methods, high-tech mechanization, and high-yielding crop cultivars (Khush 2001; Pingali 2012). The Green Revolution was associated with the intensification of farming systems, which transformed the countryside (Khush 2001; Pingali 2012).

The Green Revolution solved the problem of food shortages in developed countries, but it simultaneously generated environmental problems (Khush 2001; Pingali 2012). For instance, irrigation has resulted in the salinization of agricultural lands, rendering these unsuitable for most staple crops (Munikumar *et al.* 2021; Wang *et al.* 2021). Moreover, fertilizer application has resulted in the eutrophication of natural ecosystems, which negatively affects wild plants and species at other trophic levels (Govers *et al.* 2014; Clark *et al.* 2017). Accordingly, the intensification of farming systems negatively affected biodiversity, both below and aboveground (Kentie *et al.* 2013; Banerjee *et al.* 2019; Onrust *et al.* 2019; Raven and Wagner 2021).

Despite the environmental problems that are associated with the Green Revolution, agricultural productivity should further increase, because a significant part of the world population still suffers from starvation (van Dijk *et al.* 2021). Therefore, we require a new Green Revolution that takes sustainability problems into account. Agricultural practices should be aimed at the sustainable cultivation of highly productive crops.

The increased cultivation of C_4 plants, which include maize (*Zea mays*) and

sugarcane (*Saccharum officinarum*), may significantly contribute to a new Green Revolution (Hibberd *et al.* 2008; Ort *et al.* 2015; Jobe *et al.* 2020). The engineering of non-C_4 plants into C_4 plants may increase agricultural productivity and sustainability, because C_4 plants can be highly productive, while using water and nitrogen efficiently (Majeran and van Wijk 2009; Covshoff and Hibberd 2012; Atkinson *et al.* 2018). These agriculturally beneficial traits arise from how C_4 plants operate photosynthesis. The next sections outline the basics of photosynthesis, the distinctive photosynthesis of C_4 plants, and how this distinctive photosynthesis resulted in the beneficial traits of C_4 plants.

The basics of photosynthesis

Photosynthesis consists of two reaction series: the light reactions and the Calvin-Benson(-Bassham) cycle (Sage and Zhu 2011; Sage 2016). The light reactions are catalyzed by photosystems that are located in the chloroplast's thylakoid membranes (Sage and Zhu 2011; Sage 2016). During these reactions, light energy is converted to chemical energy (*viz.* ATP and NADPH; Sage and Zhu 2011; Sage 2016). This chemical energy is subsequently used in the Calvin-Benson cycle, which is located in the chloroplast's stroma (Sage and Zhu 2011; Sage 2016). Initially, the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylates ribulose 1,5-bisphosphate to produce two 3-phosphoglycerate molecules (PGA; Fig. 1). PGA is next phosphorylated to 1,3-bisphosphoglycerate. The latter compound is subsequently reduced to glyceraldehyde 3-phosphate (G3P) with the reducing energy of NADPH. G3P is either used to synthesize sugars or to regenerate ribulose 1,5-bisphosphate. This basic form of photosynthesis is termed C_3 photosynthesis, since the first carbon compound produced in this photosynthesis (i.e., PGA) contains three carbon atoms (Sage and Zhu 2011; Sage 2016). The majority of staple crops perform C_3 photosynthesis, including rice (*Oryza sativa*), wheat (*Triticum aestivum*), and potato (*Solanum tuberosum*; Sage 2016).

The photosynthetic efficiency of C_3 photosynthesis is low, partly because C_3 photosynthesis suffers from Rubisco being a dual-specific enzyme (Erb and Zarzycki 2017). Apart from fixing atmospheric carbon dioxide (CO_2), Rubisco can fix oxygen (O_2; Erb and Zarzycki 2017). At ambient CO_2 concentrations (400 ppm) and temperatures (20 °C), Rubisco fixes O_2 instead of CO_2 in approximately 20% of its reactions (Sharkey 1988; Sage 2004). The oxygenation of ribulose 1,5-bisphosphate produces one PGA and one 2-phosphoglycolate (PG) molecule

(Sage 2004). Carbon fixed into PG is lost from the Calvin-Benson cycle (*viz.* PG cannot be metabolized to G3P; Sage 2004). Additionally, PG is phytotoxic, because it inhibits the activity of enzymes involved in e.g., glycolysis (Kelly and Latzko 1976). Therefore, in a process termed photorespiration, C_3 plants convert PG to PGA (Sage and Zhu 2011; Sage 2016). Photorespiration not only consumes two ATP molecules and two reducing equivalents, but it also releases one molecule of previously fixed CO_2 (Sharkey 1988; Sage 2004). Consequently, photorespiration is an energy costly process (Sharkey 1988; Sage 2004).

Beyond the basics: C_4 photosynthesis

C_4 plants possess a distinctive photosynthesis to prevent Rubisco's oxygenation reaction and hence photorespiration (Sage 2004). These plants evolved under dry, saline and warm conditions (Sage 2004). Such conditions can profoundly increase the incidence of Rubisco's oxygenation reaction by lowering CO_2 levels in the foliage (Sage 2004). The conditions can induce stomatal closure, which can impair the uptake of atmospheric CO_2. The evolutionary answer to preventing largescale oxygenation and photorespiration was not a Rubisco enzyme that is unable to catalyze oxygenation reactions, because such enzyme would have a low catalytic turnover rate (K_{cat}; Sage 2002; Sage *et al.* 2012). Instead, the answer was C_4 photosynthesis (Sage 2016). C_4 photosynthesis convergently evolved over 60 times in plant families across the Angiosperm phylogeny and it relies on concentrating CO_2 around Rubisco (Hatch 1987; Lundgren *et al.* 2014). High CO_2 levels in the vicinity of Rubisco virtually eliminate Rubisco's oxygenation reaction (Hatch 1987; Lundgren *et al.* 2014).

C_4 photosynthesis is characterized by a spatial separation of photosynthetic processes between the leaf's mesophyll (M) and bundle sheath (BS) cells (Fig. 1; Hatch and Slack 1966; Hatch 1987; Lundgren *et al.* 2014). Atmospheric CO_2 is initially converted into carbonic acid in M cells. The enzyme phosphoenolpyruvate carboxylase (PEPC) subsequently metabolizes this acid into oxaloacetate (OAA). OAA contains four carbon atoms, which explains the name C_4 photosynthesis. OAA is next reduced and the resulting compounds are transported to the leaf's BS cells. In the BS, the compounds are decarboxylated, resulting in high CO_2 levels. These high CO_2 levels cause Rubisco to only catalyze carboxylation reactions, since in C_4 plants Rubisco and the other enzymes of the Calvin-Benson cycle are exclusively located in the BS. Consequently, C_4 photosynthesis virtually eliminates photorespiration.

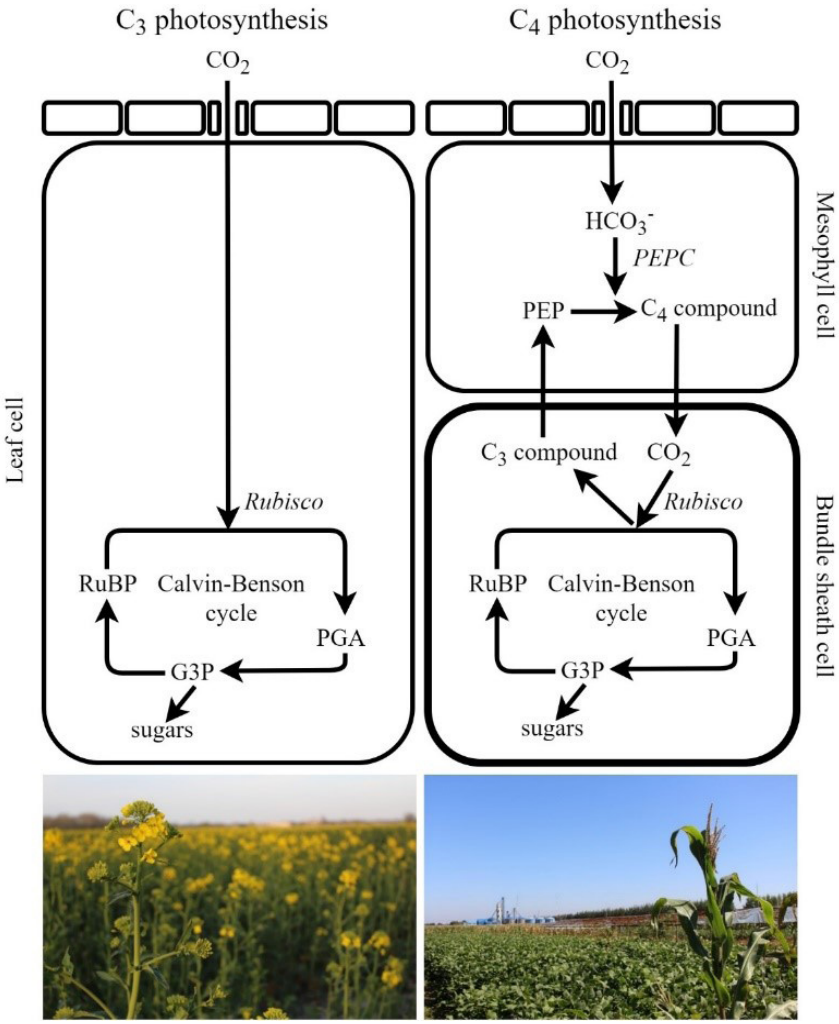

Fig. 1 Schematic representation of C_3 and C_4 photosynthesis and pictures of a C_3 (oilseed rape; *Brassica napus*) and C_4 plant (maize; *Zea mays*). For details, see the main text.

Several leaf anatomical features, collected termed the Kranz anatomy, facilitate the optimal operation of C_4 photosynthesis (Lundgren *et al.* 2014). Firstly, the BS cells of C_4 plants are larger than those in C_3 plants (Lundgren *et al.* 2014). This enlargement allows the BS cells of C_4 plants to contain a high number of chloroplasts (Lundgren *et al.* 2014). Secondly, in C_4 plants, the BS is usually densely packed with M cells and the cell walls of the BS are often thickened. These features prevent CO_2 leakage from the BS (Lundgren *et al.* 2014). Finally, the M and BS cells are well-connected for an efficient transport of metabolites

(Lundgren *et al.* 2014). Whereas lowering the distance between adjacent bundle sheaths (e.g., by arranging M cells in only one or two cell layers around veins) is one strategy to achieve this, increasing the number of plasmodesmata is another (Weiner *et al.* 1988; Danila *et al.* 2016).

The virtual absence of Rubisco's oxygenation reaction and photorespiration in C_4 plants are associated with agriculturally beneficial traits (Majeran and van Wijk 2009; Covshoff and Hibberd 2012; Atkinson *et al.* 2018). The absence of these metabolic processes may substantially increase photosynthetic efficiency. Consequently, C_4 plants can have higher growth rates than C_3 plants. Additionally, due to the C_3-C_4 photosynthetic efficiency difference, C_4 plants generally require lower Rubisco levels than C_3 plants. Since a significant fraction of nitrogen is present in Rubisco, C_4 plants consequently have a higher nitrogen use efficiency than C_3 plants. Furthermore, since C_4 plants concentrate CO_2 in BS cells, C_4 plants can more efficiently absorb atmospheric CO_2 than C_3 plants (*viz.* the CO_2 gradient between the inside and outside of a leaf is steeper in C_4 than C_3 plants). Thus, C_4 plants generally require a lower stomatal conductance compared to C_3 plants, which results in less water evaporation and therefore a higher water use efficiency. The combination of high growth rates and an efficient nitrogen and water usage explains why cultivating more C_4 crops can sustainably enhance agricultural yields.

Sulfur metabolism in plants

To optimally grow C_4 crops, it is crucial to understand how C_4 plants metabolize the macronutrient sulfur, since the suboptimal availability of this nutrient hampers crop growth and quality across the world (Box 1; Pasricha and Fox 1993; Schnug and Haneklaus 1994, 2005; Zhao *et al.* 2007).

Sulfur is required for the synthesis of various molecules (Hawkesford and De Kok 2006). The element is present in cysteine and methionine, which are constituents of proteins (Hawkesford and De Kok 2006). Cysteine contains a thiol group, which is important for the correct folding of proteins via the formation of disulfide bonds (Hawkesford and De Kok, 2006). Sulfur is also present in (1) the tripeptide glutathione, which functions as an overflow of excessively absorbed sulfur, (2) vitamins, which function in photosynthesis, respiration, and other redox processes, (3) sulfolipids, which affect chloroplast ultrastructure, and (4) sulfated compounds, which determine a plant's taste and smell (De Kok *et al.*

1997; Rausch and Wachter 2005; Hawkesford and De Kok 2006; Shimojima 2011; Calderwood and Kopriva 2014).

Plants mainly acquire sulfur as sulfate (SO_4^{2-}), which is taken up via the roots (Hawkesford and De Kok 2006; De Kok *et al.* 2007). The uptake and *in planta* distribution of sulfate are facilitated by distinct transporters (Takahashi *et al.* 2000; Hawkesford 2003; Buchner *et al.* 2004). Based on sequence similarity, these transporters are classified into four groups (Takahashi *et al.* 2000; Buchner *et al.* 2004). Group 1 contains high-affinity transporters (K_m: 5-10 µM sulfate) with SULTR1;1 and SULTR1;2 functioning in the primary uptake of sulfate from the rhizosphere (Yoshimoto *et al.* 2002, 2007). Group 2 consists of low-affinity transporters (K_m > 100 µM sulfate; Takahashi *et al.* 2000; Buchner *et al.* 2004). These transporters are located in xylem cells and phloem companion cells and are involved in the transport of sulfate between the shoot and root (Buchner *et al.* 2004). Group 3 and Group 4 transporters presumably function in the distribution of sulfate within cells, with the Group 4 transporters mediating sulfate efflux from vacuoles (Buchner *et al.* 2004).

Sulfate is metabolized into cysteine (Fig. 2; Bick and Leustek 1998; Kopriva and Rennenberg 2004; Hawkesford and De Kok 2006; Rennenberg and Herschbach 2014). The enzymes of this metabolism are located in plastids of both the root and shoot. Sulfate is initially activated by ATP sulfurylase (ATPS) to produce adenosine 5'-phosphosulfate (APS), which is subsequently reduced to sulfite by APS reductase (APR). Sulfite is further reduced to sulfide by sulfite reductase (SIR). Sulfide is incorporated by the enzyme OAS(thiol)lyase into cysteine via a reaction with *O*-acetylserine (OAS), a serine derivative produced by the enzyme serine acetyltransferase (SAT). Cysteine forms a central molecule in sulfur metabolism from which other sulfur-containing compounds are synthesized. Apart from the synthesis of methionine and subsequently proteins, cysteine can be used to synthesize glutathione. After the enzyme γ-glutamylcysteine synthethase conjugates cysteine to glutamate to produce γ-glutamylcysteine, glutathione synthethase couples γ-glutamylcysteine to glycine to produce glutathione.

Box 1: The need for plant sulfur research

Until the 1980s, plants in developed countries largely acquired sulfur from the atmosphere. Sulfur dioxide (SO_2) emissions were high and crops used this gaseous air pollutant as a sulfur source (De Kok *et al.* 1989; Bloem *et al.* 2014). The impact of SO_2 is, however, paradoxical, since above a threshold concentration SO_2 harms plants and animals, including humans (Schnug *et al.* 1995; Schnug 1998). The detrimental effects of SO_2 caused policymakers to enforce clean air acts, which resulted in a strong decrease in atmospheric SO_2 levels (Schnug *et al.* 1995; Schnug 1998). However, simultaneously, crops started to feature sulfur deficiency symptoms, including reductions in yield and quality (e.g., color, taste, and smell; Schnug *et al.* 1995; Schnug 1998). Apparently, soils did not supply sufficient sulfur to these crops. In agriculture, sulfur deficiency problems were aggravated by (1) the increased use of sulfur-free fertilizers, including urea, diammonium phosphate, and potassium chloride, (2) the intensification of cropping systems, and (3) the application of crop irrigation (Schnug and Haneklaus 1994, 2005; Zhao *et al.* 2007). Studying the regulation of sulfur metabolism yields knowledge that can be used to improve sulfur fertilizer levels and to identify breeding targets for a more efficient sulfur uptake and use.

Sulfur-sufficient (+S; left) and sulfur-deficient (-S; right) maize plants

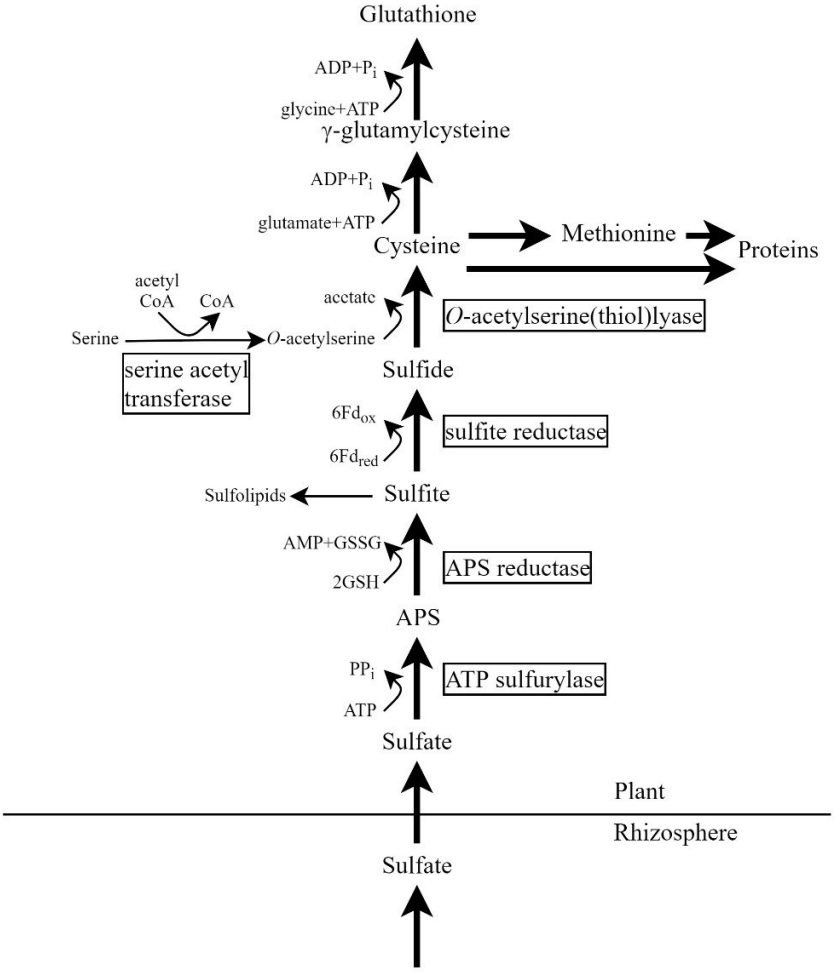

Fig. 2 The metabolism of sulfate in cysteine and subsequently other compounds. For details, see the main text.

Regulation of sulfate metabolism

It is only sketchily understood how the metabolism of sulfate in cysteine is regulated in C_4 plants. Research into this regulation has focused on C_3 plants. Specifically, research focused on *Brassica*, because these plants have high agricultural relevance and are prone to sulfur deficiency due to their high sulfur demand for growth (Schnug and Haneklaus 2005). Briefly, in *Brassica*, the rate of sulfate uptake and reduction are adjusted to the plant's sulfur demand for growth (Lappartient *et al.* 1999; Hawkesford and De Kok 2006; Koralewska *et al.* 2007). If

Brassica was deprived of sulfate, the activity of the sulfate uptake transporters and APR were upregulated (Buchner *et al.* 2004; Koralewska *et al.* 2007, 2008 2009a,b). Upon a more prolonged sulfate deprivation, the shoot-to-root ratio was altered, in favor of the root, to optimize access to sulfur. In *Brassica*, signals from both the shoot and root regulated these alterations and the involved regulatory signal pathways have partly been elucidated (Buchner *et al.* 2004; Koralewska *et al.* 2007, 2008 2009a,b; Aarabi *et al.* 2020).

Findings on the regulation of sulfate metabolism in *Brassica* cannot simply be generalized to C_4 plants. Firstly, the physiology of C_4 plants differs significantly from that of C_3 *Brassica*. Secondly, C_4 plants evolved under specific environmental conditions (e.g., warm and dry conditions), and these conditions are known to affect the regulation of sulfate metabolism (De Kok *et al.* 1991; Ahmad *et al.* 2016; Batool *et al.* 2018).

Accordingly, observations suggest a different regulation of this metabolism in C_3 and C_4 plants. In the genus *Flaveria*, which contains C_3 and C_4 species, as well as C_3-C_4-intermediate species (*viz.* species that are in an evolutionary transition stage to become C_4), cysteine and glutathione levels, as well as APR activity, were higher in C_4 than C_3 plants (Koprivova *et al.* 2001; Weckopp and Kopriva 2014; Gerlich *et al.* 2018). It has further been speculated that C_3 and C_4 plants feature a distinctive location of sulfate metabolism in the leaf (Koprivova *et al.* 2011). In monocot C_4 plants, the leaf's BS cells play a central role in sulfur metabolism. Across these plants, the first enzyme of sulfate metabolism, ATPS, was almost exclusively located in the BS (Gerwick and Black 1979; Gerwick *et al.* 1980; Schmutz and Brunold 1984). This causes the highly reactive substrates for the next steps in sulfate metabolism, and thus the complete metabolism, to be restricted to the BS cells. Additional research with the C_4 monocot maize showed that the enzyme APR was indeed also confined to BS cells and that the end-product of sulfate metabolism, cysteine, is exported from BS cells (Schmutz and Brunold 1984; Burgener *et al.* 1998; Kopriva *et al.* 2001). However, in dicot C_4 *Flaveria* species, APR transcripts, proteins, and activity were detected in both BS and M cells (Koprivova *et al.* 2001). Therefore, the BS location of sulfate metabolism is not universally associated with C_4 photosynthesis. In this context, the C_3 plants thale cress (*Arabidopsis thaliana*) and rice preferentially expressed sulfate metabolism genes in BS cells, which suggests that factors other than photosynthesis system determine the cellular location of sulfate metabolism (Aubry *et al.* 2014; Hua *et al.* 2021).

Outline of the present dissertation

In this dissertation, the focus lies on elucidating how C_4 photosynthesis affects the regulation of sulfate uptake and reduction at the whole plant level. However, chapter 2 first emphasizes the importance of sulfur for plants. It indicates that sulfur deprivation not only deteriorates plant growth, but also the production of floral displays that are crucial for plant and pollinator fitness, and hence the proper functioning of (agro)ecosystems.

H_2S fumigation has clarified the whole plant regulation of sulfate uptake and reduction in C_3 *Brassica* and therefore chapter 3 reviews the impact of atmospheric hydrogen sulfide (H_2S) on plants.

In chapter 4, the C_3 plant barley (*Hordeum vulgare*) was fumigated with H_2S to assess if the whole plant regulation of sulfate metabolism differs between C_3 barley and *Brassica*. The chapter shows that the regulation of sulfate metabolism profoundly varies among C_3 plants.

Chapters 5 and 6 outline the impact of H_2S on the C_4 plant maize. Chapter 5 indicates that, in contrast to general assumptions, H_2S does not function as a signal molecule in the regulation of maize's stomatal dynamics. If H_2S had altered these dynamics, the interpretation of the impacts of H_2S fumigation on enzyme activities could have been affected, for these dynamics may, hypothetically, affect the rate of H_2S uptake. Chapter 6 shows that the whole plant regulation of sulfate uptake and reduction is not distinctive in C_3 and C_4 plants.

In chapter 7, sulfate metabolism was compared between C_3 and C_4 *Panicum*, *Cleome*, and *Atriplex* species. The first findings from this comparison suggest that C_4 plants have higher cysteine and glutathione levels as well as a higher APR activity than C_3 plants. Thus, although sulfate reduction is not distinctively differently regulated in C_3 and C_4 plants, the capacity for sulfate reduction may be higher in C_4 plants. The comparison also indicated that C_4 plants may have higher phosphate levels than C_3 plants and thus that C_4 photosynthesis may have broad implications for the mineral nutrition of plants.

The conclusion from this dissertation is that plants profoundly differ in the whole plant regulation of sulfate uptake and reduction, but that C_3 and C_4 photosynthesis are not associated with distinctive forms of this regulation. To further

appreciate the whole plant regulation of sulfate metabolism and variation therein, the nature of the pathways that govern sulfate uptake and reduction should be analyzed. Physiological approaches should occupy a prominent position in such analyses, since chapter 8 synthesizes that the whole plant regulation of sulfate uptake and reduction does not directly result from the molecular mechanisms that control the expression of these metabolic processes.