Chapter 6

Photorespiration: The Bridge to C_4 Photosynthesis

Hermann Bauwe*
Department of Plant Physiology, University of Rostock, Albert-Einstein-Straße 3, D-18051 Rostock, Germany

Summary

Photorespiration is one of the major highways of carbon metabolism in C_3 plants and hence in the biogeosphere. By mass flow, excelled only by photosynthesis, it actually constitutes the second-most important process in the land-based biosphere. The underlying biochemical pathway, the photorespiratory carbon oxidation or C_2 cycle, compensates for the oxygenation of ribulose 1,5-bisphosphate by serving as a carbon-recovery system reconverting 2-phosphoglycolate to 3-phosphoglycerate. While this ancient ancillary metabolic process enables C_3 plants to thrive in an oxygen-containing environment, it also

*Author for Correspondence, e-mail: hermann.bauwe@uni-rostock.de

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sacrifices a significant part of the freshly assimilated carbon to the atmosphere. Biochemically, this sacrifice is made by the decarboxylation of the C_2 cycle intermediate Gly.

C_3 plants lose much photorespiratory CO_2 to the atmosphere. In contrast, photorespiration is very low in C_4 plants. C_3-C_4 intermediate plants, the phylogenetic predecessors of C_4 plants, use Gly as a vehicle to transport freshly assimilated carbon from the mesophyll to the bundle sheath where it is released as photorespiratory CO_2. Possibly, this extra CO_2 supply was a pacemaker for the subsequent substantial accumulation of chloroplasts in the bundle sheath cells of C_3-C_4 plants. Eventually, this photorespiration-driven CO_2 pump was first superimposed and then replaced by the C_4 cycle, another auxiliary pathway to the Calvin cycle, which creates even more favorable photosynthetic conditions within the bundle sheath. It thus appears as if photorespiration triggered C_4 plant evolution not only indirectly by exerting selective pressure in favor of low-photorespiration carbon assimilation, but primarily by providing the first strategy on how to improve the intercellular CO_2 distribution in leaves.

This chapter will review molecular aspects of photorespiration and introduce some measurement techniques. It will then briefly describe current knowledge about C_3-C_4 photosynthesis and discuss the workings of a photorespiration-driven CO_2 concentration mechanism as one of the first steps in the evolution of C_4 photosynthesis.

I. Introduction

Photorespiration is the light- and O_2-dependent release of CO_2 by plants, but the term also denotes the underlying biochemical processes in plants and microorganisms. It occurs concurrently with photosynthetic CO_2 uptake. Photorespiration is therefore normally concealed and becomes apparent only under specific experimental conditions. In fact, one of the first indications of photorespiratory processes came with Decker's observation (1955) in the mid-1950s that respiratory rates of leaves immediately after the end of illumination were substantially higher than those after a couple of minutes in darkness. This phenomenon was later called the post-illumination CO_2 outburst (PIB).

Photorespiratory CO_2 evolution is not restricted to these artificial conditions. For instance, when a leaf is placed in a closed measuring chamber and illuminated, the CO_2 concentration will decrease until the rate of photosynthetic CO_2 uptake equals the combined rates of photorespiratory and respiratory CO_2 release. This final steady-state equilibrium concentration of CO_2, the CO_2 compensation point (Γ), is strongly determined by the photosynthetic type of the respective plant. In C_3 plants, Γ is approximately 40–60 $\mu l\, l^{-1}$ whereas C_4 plants usually show values below 10 $\mu l\, l^{-1}$. Historically, many C_4 plants have been identified in Γ-based screening programs.

Photorespiratory CO_2 release and photosynthetic CO_2 assimilation are tightly linked on the biochemical level. This is because they are both initiated by the dual activities of Rubisco. The relative rates of these two reactions, carboxylation versus oxygenation of RubP, are solely determined by intrinsic catalytic properties of Rubisco and by the CO_2/O_2 concentration ratio. Carboxylation of RubP yields two molecules of glycerate 3-phosphate (3PGA), which become reduced to triosephosphate in subsequent reactions of the Calvin cycle. In contrast, oxygenation of RubP leads to the synthesis of equimolar amounts of 3PGA and glycolate 2-phosphate

Abbreviations: BSC – Bundle sheath cells; CAT – Catalase; CCM – CO_2-concentrating mechanism; GGT – Glu:glyoxylate aminotransferase; GS – Gln synthetase; GOGAT – Gln:2-oxoglutarate amidotransferase (Glu synthase); GDC – Gly decarboxylase; GLYK – Glycerate 3-kinase; 3PGA – Glycerate 3-phosphate; GOX – Glycolate oxidase; 2PG – Glycolate 2-phosphate; PGLP – Glycolate 2-phosphate phosphatase; 3HP – Hydroxypyruvate; IRGA – Infrared gas analyzer; MC – Mesophyll cell; HPR1 – NADH-dependent hydroxypyruvate reductase; HPR2 – NADPH-dependent hydroxypyruvate reductase; 2OG – 2-Oxoglutarate; PIB – Post-illumination CO_2 burst; RubP – Ribulose 1,5-bisphosphate; Rubisco – RubP carboxylase/oxygenase; SHMT – Ser hydroxymethyltransferase; SGT (AGT) – Ser:glyoxylate aminotransferase; S – Specificity factor of Rubisco; THF – Tetrahydrofolate

(2PG). 2PG cannot enter the Calvin cycle but is converted to 3PGA in a series of enzymatic reactions that collectively comprise the C_2 oxidative photosynthetic carbon cycle, or shorter, the C_2 cycle (historical overview in Lorimer, 1981). Decarboxylation of Gly, an intermediate compound of the C_2 cycle, by the mitochondrial enzyme Gly decarboxylase (GDC) results in the release of photorespiratory CO_2 at rates which are much higher than those of other respiratory processes.

In rough accordance with earlier estimates, recent in vivo ^{13}C labeling experiments indicate that photorespiration is about 17% of photosynthesis for a well-watered and fertilized plant under temperate conditions (Cegelski and Schaefer, 2006). This fraction can be significantly higher in warmer and drier environments. Photorespiration hence represents a major metabolic process in C_3 plants, which can occur with rates comparable to those of photosynthetic CO_2 fixation. Due to the unproductive re-release of 20–50% of the freshly assimilated CO_2, photorespiration considerably slows down the rates of net CO_2 fixation in C_3 plants. These negative effects triggered the search for mutations or inhibitors that specifically block photorespiration, but leave photosynthesis unimpaired. Subsequent to some early enthusiastic reports, however, it was realized that such attempts do not increase photosynthetic efficiency, but rather exacerbate the problems of photorespiration. This conclusion received strong support from the analysis of Arabidopsis mutants with defects in C_2 cycle enzymes (historical perspective in Somerville, 2001).

What is the biological function of photorespiratory processes? The answer, in essence, is quite simple: the synthesis of 2PG by RubP oxygenase as the primary reaction of the C_2 cycle appears to have no 'function' at all! It is an inevitable consequence of the chemistry of the RubP carboxylase reaction and reflects the evolutionary origin of plant photosynthesis in the anaerobic environment of the Precambrian ocean (Lorimer and Andrews, 1973). The C_2 cycle, as an important ancient auxiliary metabolic process, compensates for the oxygenation of RubP by serving as a carbon-recovery system reconverting 2PG carbon to 3PGA. During this process, a significant part of the freshly assimilated carbon is sacrificed to survive in an otherwise toxic environment (Osmond, 1981). It is the C_2 cycle, at least for plants, which makes autotrophic CO_2 fixation possible in an environment containing high oxygen concentrations. This is the central and primary function of the C_2 cycle.

In addition to this central and indispensable function of the C_2 cycle, however, there exist several secondary adaptation benefits for the life of plants under certain environmental conditions. The most prominent example is the possible contribution of the C_2 cycle to the protection of the photosynthetic apparatus from photoinhibition (pros and cons discussed in Osmond et al., 1997). Overexpression of the C_2 cycle enzyme hydroxypyruvate reductase led to an improved tolerance of tobacco to warm temperatures (Oliver et al., 1995). It is also related to thermotolerance that photorespiratory metabolism, in a not understood manner, affects the biosynthesis of monoterpenes (Penuelas and Llusia, 2003). More recently, linkages have been revealed between photorespiration and oxidative stress-related repair processes within photosystem II (Takahashi et al., 2007), and light-stress protection of cyanobacteria (Hackenberg et al., 2009).

How, then, can photorespiration be 'a bridge to C_4 photosynthesis'? It is a widely accepted notion that the negative effects of photorespiratory CO_2 losses on plant growth exerted one of the selective pressures that led to the evolution of C_4 traits. However, there exists a second relation between C_4 photosynthesis and photorespiration. This relation becomes apparent from specific features of C_3-C_4 intermediate plants, which use photorespiratory Gly as a vehicle for shuffling CO_2 from the mesophyll to the bundle sheath (Rawsthorne, 1992). Molecular phylogenetic studies place this photosynthetic type between C_3 and C_4 in the evolution of C_4 photosynthesis (Kopriva et al., 1996b; McKown et al., 2005). It thus appears that the evolution of the C_4 syndrome was triggered by and possibly even required the preceding presence of a much simpler CO_2 transport system, which was based on relatively small alterations to photorespiratory Gly metabolism. In fact, fully developed C_4 plants did not lose but in contrast retained a fully functional C_2 cycle.

This chapter will give a survey on the biochemistry and genetics of the photorespiratory C_2

cycle and a short introduction into the measurement of photorespiration. Then, specific features of photorespiratory metabolism in C_3-C_4 intermediate plants will be discussed in their relation to the evolution of C_4 photosynthesis.

II. Biochemistry and Genetics of the C_2 Cycle

The biochemistry and cellular biology of the C_2 cycle of higher plants is very complex. It involves a core of nine individual enzymatic reactions and at least three auxiliary enzymes distributed over three different organelles and the cytosol (Fig. 1). Rubisco produces 2PG by oxygenation of RubP in the chloroplast, several C_2 cycle enzymes are localized within the peroxisomes and the actual release of photorespiratory CO_2 from Gly occurs in the mitochondria. Gly decarboxylation also produces NH_3, which becomes reassimilated in the chloroplasts. The flow of reactions implies a fixed stoichiometry of RubP oxygenation, Gly decarboxylation, and photorespiratory 3PGA synthesis. It should be noted, however, that in vivo ^{13}C and ^{15}N labeling experiments recently substantiated earlier suggestions that this stoichiometry may not be that strictly fixed (Hanson and Peterson, 1985; Cegelski and Schaefer, 2006).

Knowledge about the transmembrane transport of the core intermediates of the highly compartmentalized C_2 cycle is very much incomplete (Reumann and Weber, 2006; Weber and Fischer, 2007). Transport of glycolate, hydroxypyruvate (3HP), and several other compounds related to

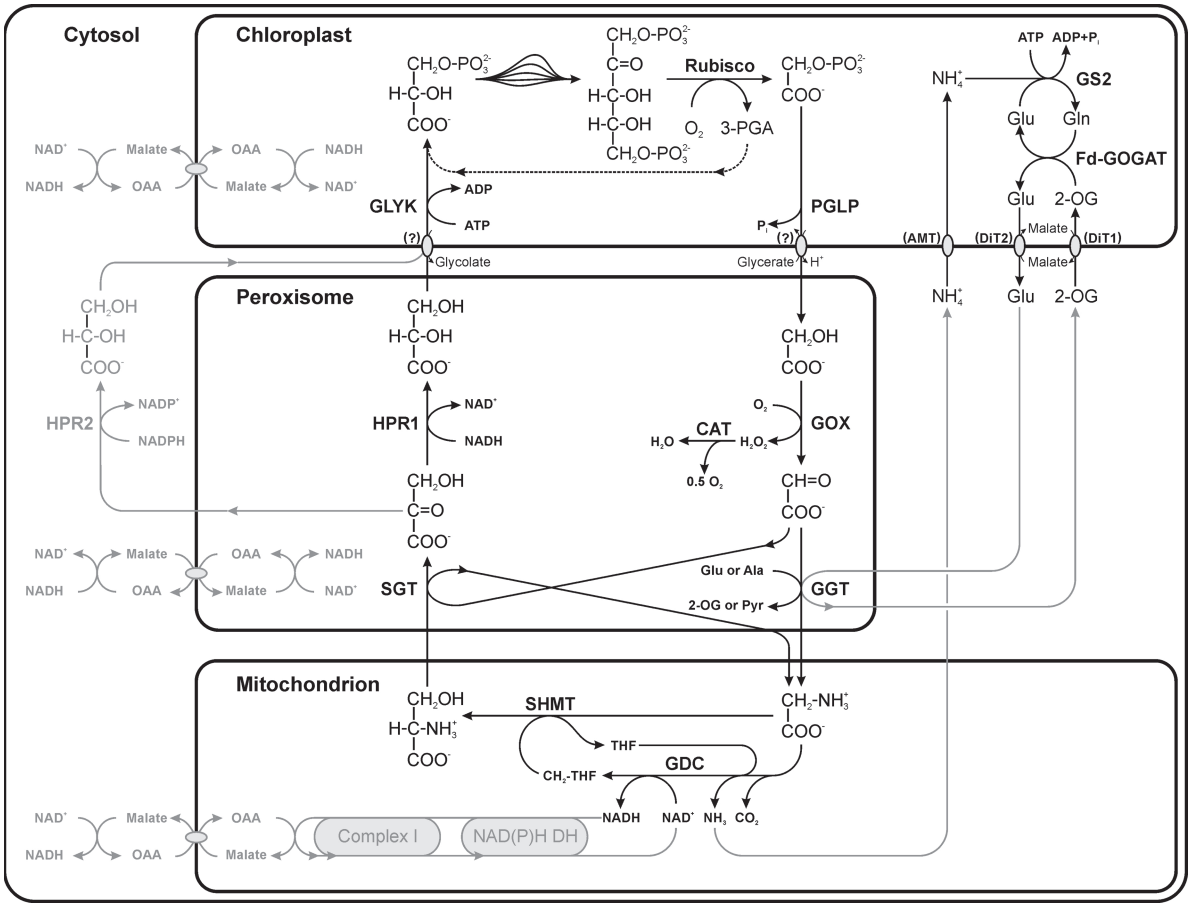

Fig. 1. Flow-chart of reactions constituting the core of the C_2 cycle and several auxiliary reactions including those of the photorespiratory nitrogen cycle.

photorespiratory metabolism across the peroxisomal membrane occurs via porin-like channels (Reumann, 2000). A chloroplastic glycolate/glycerate transporter has been described (Howitz and McCarty, 1991), but was not yet identified on the molecular level. Even less is known about the passage of Gly and Ser through the inner mitochondrial membrane. While the presence of Gly/Ser antiporters in the inner mitochondrial membrane was suggested already more than 20 years ago (Walker et al., 1982) and significant progress exists in the identification of human Gly transporters (e.g. Betz et al., 2006), the respective transporters in plants are not yet known.

A. Chloroplasts Produce 2-Phosphoglycolate by Oxygenation of RubP

Rubisco initiates the C_2 cycle by the synthesis of 2PG (Bowes et al., 1971; historical perspective in Portis and Parry, 2007). The multimeric enzyme is present in the chloroplast stroma in the very high concentrations of 200–250 mg ml^{-1} and constitutes about 30–50% of the soluble protein in leaves of C_3 plants. With an estimated amount per capita of about 10 kg, the enzyme is the most abundant protein on Earth. In plants as in all chlorophyll a-containing organisms, Rubisco comprises two types of polypeptide subunits, a plastome-encoded ~55 kDa large subunit carrying the catalytic site and a nuclear-encoded ~15 kDa small subunit. The subunits are organized as hexadecamers (L_8S_8, so-called form I Rubisco) to form a 560 kDa holoenzyme, but other forms exist in prokaryotes and are thought to have evolved from a primordial archaeal Rubisco-like protein (Tabita et al., 2008; Saito et al., 2009). These ancestor enzymes have no Rubisco activity but are enolases involved in methionine salvage pathways (Ashida et al., 2005).

Very crucial for Rubisco catalysis is the preceding activation via carbamylation of an active site lysyl residue. Subsequent stabilization of the lysyl carbamate by binding of Mg^{2+} then provides the cofactor necessary for both carboxylation and oxygenation of RubP (Cleland et al., 1998). Before this activation can occur, bound RubP or other sugar phosphates need to be removed from the non-carbamylated site by Rubisco activase (Portis et al., 2008).

The relative rates of carboxylation and oxygenation are determined by the kinetic properties of Rubisco and by the CO_2/O_2 concentration ratio. To conveniently describe this situation, the V_{max} and K_m values are often combined in the so-called specificity factor S ($V_c \cdot K_o/V_o \cdot K_c$, where V and K represent V_{max} and K_m of the respective reactions) of Rubisco. This parameter, in addition to the absolute k_{cat} values for the carboxylation and oxygenation reaction, respectively, is an important diagnostic factor for the overall efficiency of the enzyme and shows significant natural variability. Computational simulations suggest that substantial increases in crop carbon gain could result if Rubiscos with higher specificity factors were expressed in C_3 plants. Consequently, there is much interest in identifying 'better' natural or mutant Rubisco variants and integrating them into plants. Such experiments have not yet been very successful and some skepticism is also expressed (Tcherkez et al., 2006). Latest strategies attempt to produce such hypermorphs by directed evolution and genetic selection in engineered *E. coli* (Mueller-Cajar and Whitney, 2008).

B. 2-Phosphoglycolate Becomes Dephosphorylated to Glycolate

2PG is hydrolyzed by a chloroplastic phosphatase (PGLP) with high specificity for 2PG. The enzymatic properties of the enzyme from different eukaryotic organisms are very similar suggesting a common evolutionary origin. During catalysis, PGLP becomes phosphorylated. It requires Cl^- and Mg^{2+} ions for full activity and is reversibly inhibited by Ca^{2+} ions.

$$\text{PGLP}: 2-\text{Phosphoglycolate} + H_2O \rightarrow \text{Glycolate} + \text{Pi}$$

Two orthologs are present in the genomes of both Arabidopsis and rice, the photorespiratory PGLP1 (all relevant C_2 cycle enzymes are listed in Table 1) and the cytosolic PGLP2. PGLP1-deficient mutants cannot grow under ambient conditions and are viable only at 0.5–1% CO_2 (Schwarte and Bauwe, 2007). Similar to all other C_2 cycle enzymes, PGLP activity and *PGLP1* transcript levels increase rapidly during illumination of etiolated seedlings. PGLP2 does not contribute to photorespiratory metabolism but could be involved in the degradation of minor

Table 1. Enzymes of the photorespiratory C_2 cycle and their encoding genes in Arabidopsis. Related genes with unidentified functions or functions outside of the core C_2 cycle are not listed. Where the knockout results in a low CO_2-sensitive phenotype, which indicates a major function in photorespiration, the respective gene is printed in bold. References: PGLP (Schwarte and Bauwe, 2007); GOX nomenclature (Reumann et al., 2004); SGT (Liepman and Olsen, 2001); GGT (Igarashi et al., 2003); GLDP (Engel et al., 2007); LPD (Lutziger and Oliver, 2000); SHMT (Voll et al., 2006); HPR (Mano et al., 1997); GLYK (Boldt et al., 2005).

	Arabidopsis	TAIR Code	Localization
2PG phosphatase	AtPGLP1	**At5g36790**	Chloroplasts
Glycolate oxidase	AtGOX1	At3g14420	Peroxisomes
	AtGOX2	At3g14415	Peroxisomes
	AtGOX3	At4g18360	Peroxisomes
	AtHAOX1	At3g14130	Peroxisomes
	AtHAOX2	At3g14150	Peroxisomes
Ser:glyoxylate aminotransferase	AtAGT1	**At2g13360**	Peroxisomes
Glu:glyoxylate aminotransferase	AtGGT1	**At1g23310**	Peroxisomes
	AtGGT2	At1g70580	Peroxisomes
Gly decarboxylase P-protein	AtGLDP1	At4g33010	Mitochondria
	AtGLDP2	At2g26080	Mitochondria
Gly decarboxylase H-protein	AtGLDH1	At2g35370	Mitochondria
	AtGLDH2	At2g35120	Mitochondria
	AtGLDH3	At1g32470	Mitochondria
Gly decarboxylase T-protein	AtGLDT1	**At1g11860**	Mitochondria
Gly decarboxylase L-protein	AtmLPD1	At3g17240	Mitochondria
	AtmLPD2	At1g48030	Mitochondria
Ser hydroxymethyltransferase	AtSHM1	**At4g37930**	Mitochondria
	AtSHM2	At5g26780	Mitochondria
Hydroxypyruvate reductases	AtHPR1	At1g68010	Peroxisomes
	AtHPR2	At1g79870	Cytosol
Glycerate kinase	AtGLYK	**At1g80380**	Chloroplasts

amounts of 2PG as they are produced in all cells during DNA repair processes.

C. Glycolate Becomes Oxidized to Glyoxylate and H_2O_2 in the Peroxisomes

Peroxisomes are closely associated with both chloroplasts and mitochondria which appears to be related to the cooperation of these three organelle types in photorespiration. This physical association with other organelles reflects that peroxisomes, the 'organelles at the crossroad', metabolically link different cell compartments (Igamberdiev and Lea, 2002).

Glycolate oxidase (GOX) is the first peroxisomal enzyme of the C_2 cycle. The flavinmononucleotide (FMN)-dependent enzyme transfers electrons from glycolate to molecular O_2 to produce glyoxylate and H_2O_2. In the first part of this two-step reaction, glycolate is oxidized by the flavin and in the second part the reduced FMN becomes re-oxidized by oxygen to produce H_2O_2 in an irreversible reaction.

$$\text{GOX}: \text{Glycolate} + O_2 \rightarrow \text{Glyoxylate} + H_2O_2$$

GOX is usually isolated as a tetra- or octamer composed of identical subunits of ~43 kDa and

its primary structure had initially been determined by peptide sequencing (Cederlund et al., 1988). The enzyme was crystallized many years ago and represents one of the few peroxisomal proteins for which high resolution crystal structures were obtained.

In an evolutionary context, it is interesting to note that GOX only occurs in the charophycean algae (which contain peroxisomes), the most likely predecessors of land plants, and the other members of the Streptophyta. The sister group of chlorophycean algae uses mitochondrial or plastidial glycolate dehydrogenases instead (e.g. Stabenau and Winkler, 2005). In Arabidopsis, GOX is encoded by five redundant genes, which explains the lack of mutants with a high-CO_2-requiring phenotype. Notably, four of these genes are arranged as two pairs in close vicinity on chromosome 3. The two GOX genes which are most likely involved in photorespiration, *AtGOX1* and *AtGOX2*, form a tandem, while *AtHAOX1* and *AtHAOX2* are separated by just one gene. The distance between the two gene pairs is as low as ~130 kb indicating one ancestral locus for all four genes. The localization of *AtGOX3* on another chromosome might be due to a segmental duplication similar to the events that led to the formation of the majority of Arabidopsis genes. Very similar to Arabidopsis, five probably functional *GOX* genes exist in the rice genome, but none of these genes is located in close vicinity to its homologs. In contrast, only one GOX gene is present in the C_4 plant maize. Knockout of this gene resulted in a high-CO_2 requiring phenotype, which demonstrates the need of an intact photorespiratory metabolism not only in C_3 but also in C_4 plants (Zelitch et al., 2009). In tobacco, RNAi-induced GOX deficiency resulted in a distinctly higher susceptibility to photoinhibition (Yamaguchi and Nishimura, 2000).

The large amount of H_2O_2 produced during photorespiration is degraded by the peroxisomal enzyme catalase (CAT). This reaction, although not covered in this chapter, is a very important side reaction of the photorespiratory C_2 cycle, and CAT-deficient mutants show severe leaf bleaching under normal environmental conditions (Blackwell et al., 1988; Queval et al., 2007). Photorespiratory H_2O_2 production and CAT activity gain further importance because H_2O_2 is a key player in several cellular processes including systemic acquired acclimation to excess light and other stress and programmed cell death responses.

D. At Least Two Peroxisomal Transaminases Convert Glyoxylate to Gly

Rates of non-enzymatic decarboxylation of glyoxylate are normally very low, and glyoxylate becomes more or less completely converted to Gly by at least two aminotransferases. In addition to their function in the C_2 cycle, these enzymes also regulate leaf amino acid content (Igarashi et al., 2006). Because of their multiple substrate specificities, there is still some confusion with respect to the identity and naming of these enzymes.

$$\text{SGT: Ser} + \text{Glyoxylate} \rightarrow \text{3-Hydroxypyruvate} + \text{Gly}$$

The homo-dimeric Ser:glyoxylate aminotransferase (SGT) uses Ser as the amino donor and is encoded by single genes in Arabidopsis (AGT1, Liepman and Olsen, 2001) and in rice. The enzyme catalyzes transamination reactions in the combinations Ala:glyoxylate, Ser:glyoxylate, and Ser:pyruvate. Kinetic data suggest that the quasi-irreversible Ser:glyoxylate transamination is the preferred in vivo reaction (Nakamura and Tolbert, 1983).

Mutation of *AGT1* in the conditionally lethal *Arabidopsis sat* mutant leads to a loss of SGT activity, but AGT and GGT activities were maintained at nearly wild-type levels (Somerville and Ogren, 1982; Liepman and Olsen, 2001). The mechanism of a reported contribution of SGT to pathogen defense in some species is not yet understood (Taler et al., 2004).

$$\text{GGT: Glu}\,(\text{Ala}) + \text{Glyoxylate} \rightarrow 2\text{-Oxoglutarate}\,(\text{Pyruvate}) + \text{Gly}$$

The re-import of amino groups from Glu into the C_2 cycle is catalyzed by Glu:glyoxylate aminotransferase (GGT). In Arabidopsis, GGT is encoded by a pair of nearly identical paralogous genes, *GGT1* and *GGT2* (Liepman and Olsen, 2003; Igarashi et al., 2003). The respective recombinant enzymes displayed biochemical characteristics very similar to one another and to the Arabidopsis protein purified from leaves. Confirming earlier

reports, they catalyze amino group exchanges in the combinations Glu:glyoxylate, Ala:glyoxylate, Glu:pyruvate, and Ala:2-oxoglutarate (2OG), but not with Ser as the amino-group donor.

GGT1 represents the major GGT form in leaves. This follows from the analysis of an Arabidopsis *ggt1* knockout-mutant, which showed by about 80% reduced activities with the donor:acceptor combinations Glu:glyoxylate and Ala:glyoxylate. In contrast, activities with the combinations Ala:2OG and pyruvate:Glu (the reverse reaction) were reduced only by some 30–40%. Glu levels slightly increased and the levels of both Gly and Ser decreased when the mutant was grown in normal air conditions (Igarashi et al., 2003).

Schemes of the C_2 cycle usually neglect that Ala might well be an additional amino donor for photorespiratory Gly formation. Early observations in that direction now receive increasing support, e.g. by the capability of GGT to use Glu and Ala as the amino donor at equal rates (Igarashi et al., 2003). Such high activities with Ala are remarkable because the equilibrium of the reaction with glyoxylate lays toward the complete conversion of Ala to pyruvate. Moreover, the pool of Ala in the photosynthesizing cell is very large and Ala is rapidly labeled in the carboxyl group during $^{14}CO_2$ fixation (Nishimura and Akazawa, 1975). Similarly, both Ser and Ala can equally serve as the amino donor for SGT (Liepman and Olsen, 2001). This possibility of an open flux of nitrogen by import and export of amino acids into and from the photorespiratory nitrogen cycle is also reflected in the ^{15}N-labeling patterns of Glu, Gly, Ser, and Ala (Masclaux-Daubresse et al., 2006). All these data strongly indicate the position of Ala as another important amino group donor within or to the C_2 cycle.

E. Mitochondrial Reactions of Gly Yield Ser, CO_2, NH_3, and NADH

In illuminated C_3 plants grown in ambient air, photorespiratory Gly is synthesized at very high rates. While some Gly can be directed into other pathways, most of it becomes rapidly converted to Ser in the mitochondrial matrix. This NAD^+- and tetrahydrofolate (THF)-dependent process requires two enzymes, the multienzyme complex Gly decarboxylase (GDC) and Ser hydroxymethyltransferase (SHMT). Notably, both GDC and SHMT are highly susceptible to oxidation in vivo (Taylor et al., 2004) and possible targets for regulation by thioredoxin (Marti et al., 2009). As side products of the GDC reaction, photorespiratory CO_2 and NH_3 are released and NADH is generated.

$$\text{GDC: Gly} + \text{THF} + \text{NAD}^+ \rightarrow \text{CH}_2 - \text{THF} + \text{CO}_2 + \text{NH}_3 + \text{NADH}$$

$$\text{SHMT: Gly} + \text{CH}_2 - \text{THF} \rightarrow \text{Ser} + \text{THF}$$

$$\text{Sum: 2 Gly} + \text{NAD+} \rightarrow \text{Ser} + \text{CO}_2 + \text{NH}_3 + \text{NADH}$$

1. Gly decarboxylase

Gly decarboxylation was first discovered in non-plant organisms and the enzymology and function of GDC in plants has been frequently reviewed (e.g. Douce et al., 2001). In addition to its crucial role in photorespiration, GDC is also indispensable for general one-carbon metabolism with its multitude of biosynthetic reactions. This dual function of GDC is mirrored in the finding that deletion of GDC is fatal to plants and corresponding null mutants cannot be recovered even under non-photorespiratory conditions (Engel et al., 2007).

GDC is an atypical multienzyme complex comprising the four not very tightly associated protein components P-, H-, T-, and L-protein. All four proteins are nuclear-encoded and their combined presence within the mitochondrial matrix is necessary for catalytic activity of the complex. Although the individual proteins were purified earlier from non-plant organisms, the GDC complex has been isolated for the first time from pea leaf mitochondria, where it occurs in very high concentrations of up to 200 mg ml^{-1}. The GDC subunits assemble spontaneously within the mitochondrial matrix with an approximate subunit ratio of two P-protein dimers, 27 H-protein monomers, and nine T-protein monomers per one L-protein dimer. In analogy to the E2 dihydrolipoyl acyltransferase core of α-ketoacid dehydrogenases, H-protein supposedly could provide a central association core in this process. In comparison with other multienzyme complexes, however, the interaction between the individual GDC protein components is very fragile and

stable only at high concentration. The structure of the holoenzyme has not yet been resolved; however, some structure-function relationships between the individual subunits are known from crystallographic data and from nuclear magnetic resonance studies.

A. P-Protein

The actual binding and decarboxylation of Gly is catalyzed by the pyridoxal 5-phosphate-containing P-protein (GLDP), which is a homodimer of two ~105 kDa polypeptides. P-protein needs two substrates, Gly and H-protein, and catalyzes a reversible Gly:Lipoyl-H-protein oxidoreductase reaction.

$$\text{GLDP: Gly} + \text{Lipoyl-H-Protein} \rightarrow \text{S - aminomethyldihydrolipoyl-H-Protein} + CO_2$$

First, a Schiff base between the carbonyl group of pyridoxal-5-phosphate (PLP) and a lysyl residue of P-protein is exchanged for a Schiff base between PLP and the amino group of Gly. Then, α-elimination results in the release of CO_2, not bicarbonate (Sarojini and Oliver, 1983), and the remaining aminomethylene group becomes conveyed to the oxidized lipoyl group of H-protein. P-protein and H-protein alone can catalyze a reversible Gly-bicarbonate exchange reaction where exogenous CO_2 can replace the carboxyl carbon of Gly. Although resulting in very low catalytic efficiency, even H-protein can be replaced by lipoate in both the Gly decarboxylation and the Gly synthesis reaction.

P-protein is encoded by two genes both in Arabidopsis and in rice. The individual knockout of either of these two genes in Arabidopsis does not significantly alter metabolism and photosynthetic performance indicating functional redundancy. In contrast, as already mentioned, a double-mutant with two inactivated *GLDP* genes does not develop beyond the cotyledon stage in air enriched with 0.9% CO_2 and the seedlings do not survive for longer than about 3–4 weeks under these non-photorespiratory conditions (Engel et al., 2007). This feature distinguishes the GDC-lacking double-mutant from all other known photorespiratory mutants. An earlier reported GDC-deficient Arabidopsis mutant, *gld1* (Somerville and Ogren, 1982) is defective in mitochondrial lipoate synthesis and contains only minor amounts of functional H-protein (Ewald et al., 2007).

B. H-Protein

The ~14 kDa H-protein (GLDH, also known as aminomethyl carrier protein) carries covalently bound lipoic acid as its prosthetic group and represents the dominant lipoylated protein in photosynthesizing plant cells (Gueguen et al., 2000). Lipoylation of H-protein occurs via a two-step mechanism and nearly fully depends on the intra-mitochondrial biosynthesis of octanoyl-ACP (where ACP stands for acyl-carrier protein) by octanoyl-ACP synthase. First, lipoyltransferase conveys the octanoyl residue from octanoyl-ACP to the H-apoprotein. Next, S-adenosylmethionine-dependent lipoate synthase attaches the two sulphur atoms from S-adenosylmethionine on-site (reviewed in Rébeillé et al., 2007).

H-protein has no catalytic activity itself, but interacts as a mobile substrate via its lipoamide arm one after the other with P-, T-, and L-protein. In light of this central function for catalysis and the suspected role in the assembly of the holoenzyme, it was named the ‘structural and mechanistic heart’ of the GDC complex (Douce et al., 2001). One of the major functions of H-protein is the stabilization of the methylamine moiety remaining after decarboxylation of Gly by P-protein thus preventing its spontaneous degradation. Once aminomethylated by P-protein, the lipoate arm becomes locked within a cleft at the surface of the H-protein and released only by interaction with T-protein. This differs from structural models for other α-ketoacid dehydrogenase complexes where the lipoyl domain of the E2 subunit, which is structurally and functionally analogous to the H-protein, can freely rotate in the complex (e.g. Guilhaudis et al., 1999).

Plant H-proteins are often encoded by small multigene families and the individual H-proteins supposedly fulfil different functions in plant metabolism (Kopriva and Bauwe, 1995; Rajinikanth et al., 2007). In Arabidopsis, H-protein is encoded by a small gene family comprising three members. Two of the encoded proteins are very similar to each other but share only limited (about 60%) identity with the third homologue protein.

Notably, the lipoamidyl residue of H-protein is very sensitive to modification by 4-hydroxy-2-nonenal, which is a cytotoxic product of lipid peroxidation under environmental stress. This has been taken as an indication that GDC and hence the proper functioning of the C_2 cycle are among the first casualties of oxidative damage to leaves (Taylor et al., 2004). A corresponding 'lipoate lyase' able to remove damaged lipoic acid, regenerating the H-apoprotein and allowing reattachment of an unmodified lipoate remains to be discovered.

C. T-Protein

The ~41 kDa monomeric T-protein (GLDT) acts as an aminomethyl transferase and needs both THF and aminomethylated H-protein as substrates. In the absence of THF, formaldehyde is produced instead of CH_2-THF. T-protein is the only GDC subunit that is most likely encoded by single-copy genes both in Arabidopsis and in rice. This singular occurrence could indicate a central role of T-protein in the regulation of GDC biosynthesis and might explain the yet unsuccessful search for knockout mutants for this gene.

$$\begin{aligned}\text{GLDT: S-aminomethyldihydrolipoyl-H-Protein} \\ + \text{THF} \rightarrow \text{Dihydrolipoyl-H-Protein} \\ + \text{CH}_2 - \text{THF} + \text{NH}_3\end{aligned}$$

The folate binding site is formed via the interaction with H-protein in a 1:1 molar ratio and comprises several lysyl residues that interact with the glutamyl α-carboxyl groups of THF. The current model implies that the polyglutamyl tail of the folate substrate is inserted into a formed cavity leaving the pteridine ring near the entrance of the cavity in the context of the catalytic reaction (Okamura-Ikeda et al., 2003). As already mentioned, interaction results in a change of the conformation of the H-protein, leading to the release of the aminomethylated lipoate arm from the protecting cleft followed by nucleophilic attack on the methylene carbon by the N-5 atom of the THF's pterin ring (Guilhaudis et al., 2000). Products of this reaction are photorespiratory NH_3, CH_2-THF, and H-protein with a fully reduced dihydrolipoyl group.

D. L-Protein

The re-oxidation of the H-protein's dihydrolipoyl group closes the H-protein reaction cycle. This final reaction is catalyzed by L-protein (LPD, dihydrolipoamide dehydrogenase), which is a homo-dimer of ~50 kDa polypeptides and needs both FAD and NAD^+ for catalysis. Except the catalytic interaction with the lipoyl arm, there is no apparent molecular recognition and interaction between L-protein and the reduced H-protein (Douce et al., 2001). During oxidation of the dihydrolipoyl-H-protein, FAD is reduced to $FADH_2$ which, in turn, becomes re-oxidized by NAD^+ resulting in the synthesis of one NADH per decarboxylated Gly.

$$\begin{aligned}\text{LPD: Dihydrolipoyl-H-Protein} + \text{NAD}^+ \leftrightarrows \\ + \text{Lipoyl-H-Protein} + \text{NADH}\end{aligned}$$

The massive carbon flux through the GDC reaction requires high rates of NADH re-oxidation, which are substantially higher than the predicted rates of mitochondrial electron transport and oxidative phosphorylation (e.g. Noguchi and Yoshida, 2008). As much as 25–50% of the produced reducing equivalents is thought to be exported to the cytosol via a malate/oxaloacetate shuttle and contributes to the peroxisomal reduction of 3HP to glycerate. The responsible transporter (DCT, At5g19760), as the first dicarboxylate/tricarboxylate carrier in plants, has been cloned and functionally characterized (reviewed in Picault et al., 2004). The mitochondrial malate shuttle system is seemingly complemented by a glycerol 3-phosphate shuttle operating in the opposite direction to ensure proper malate/OAA ratios in the cytosol (Shen et al., 2006). It is not yet clear how much of the remaining GDC-generated NADH actually drives oxidative phosphorylation to provide ATP to the cytosol, but this fraction may vary depending on many circumstances. Most of it is probably recycled to NAD^+ via a non-phosphorylating by-pass comprising one of the mitochondrial non-proton pumping NAD(P)H dehydrogenases (NDA1, At1g07180) and alternative oxidase which thus closely interact with photorespiration. The mitochondrial uncoupling protein UCP1, which bypasses ATP synthesis by facilitating the re-entry of H^+ into the mitochondrial matrix

(Sweetlove et al., 2006), also helps in maintaining adequate redox poise of the mitochondrial electron transport chain.

L-protein is a component not only of GDC but also, as the so-called E3 subunit, a component of the three 2-oxoacid dehydrogenase complexes oxidizing pyruvate, 2OG, and the branched-chain 2-oxoacids (Mooney et al., 2002). The mitochondrial L-protein is encoded by two genes in Arabidopsis, *mtLPD1* and *mtLPD2*. The knockout of *mtLPD2* did not result in an apparent phenotype and the two enzymes hence appear as entirely interchangeable among the different multienzyme complexes (Lutziger and Oliver, 2001).

2. Ser hydroxymethyltransferase

SHMT consists of four pyridoxal 5-phosphate-containing ~53 kDa subunits and catalyzes the CH_2-THF-dependent conversion of Gly to Ser. SHMT, in addition to T-protein represents the second of two major folate-dependent mitochondrial proteins. The two enzymes bind a large fraction of the mitochondrial folate pool thereby also preventing oxidative degradation of this very labile and oxidizable compound.

$$\text{SHMT: Gly} + \text{CH}_2 - \text{THF} \leftrightarrows \text{Ser} + \text{THF}$$

As a second result of the SHMT reaction, CH_2-THF is recycled to THF for its re-use in the GDC reaction. The more than twofold higher activity of GDC relative to SHMT ensures a high CH_2-THF/THF ratio in the mitochondrial matrix, which drives the SHMT reaction in the thermodynamically not favoured direction towards the synthesis of Ser (Douce et al., 2001). In a second catalytic activity, SHMT can produce considerable amounts of 5-formyl-THF. This compound is a strong inhibitor of SHMT and becomes recycled to THF in a series of recently identified reactions including 10-formyl-THF deformylase. Deletion of this enzyme results in a remarkable increase of leaf Gly levels, indicating the essential function of this pathway for photorespiration (Goyer et al., 2005; Collakova et al., 2008).

Intriguingly, AtSHMT1 appears to require the presence of a second enzyme, ferredoxin-dependent glutamate synthase (Fd-GOGAT). Fd-GOGAT is responsible for NH_3 assimilation in chloroplasts, but also targeted to the mitochondria, where it has been shown to physically interact with SHMT1. This interaction appears to be necessary for full catalytic activity of SHMT1 in vivo, but the underlying mechanism remains speculative (Jamai et al., 2009).

SHMT, other than GDC, is not exclusively restricted to the mitochondria. Specific isoforms are present in the cytosol and in plastids, where they catalyze the Ser-to-Gly reaction to provide CH_2-THF for a large number of biosynthetic reactions in both photosynthesizing and non-photosynthesizing tissues. The resulting Gly is then re-converted to Ser by the mitochondrial GDC-SHMT system. Accordingly, the Arabidopsis genome contains at least five and perhaps even seven SHMT genes, *AtSHM1* to *7*. Only *AtSHM1* and *AtSHM2* encode mitochondrial proteins. Genetic mapping and knockout studies have identified AtSHM1 as the photorespiratory SHMT, whereas functional deletion of AtSHM2 does not lead to any apparent phenotypic alterations and cannot complement the *shm1* mutation (Voll et al., 2006). The rice genome probably contains five SHMT genes. Only *OsSHM1*, an ortholog of *AtSHM1*, encodes a protein with a mitochondrial targeting peptide.

Disruption of *AtSHM1* not only impairs photorespiratory metabolism, but also leads to a constitutive expression of salicylic acid-inducible genes and genes involved in H_2O_2 detoxification. Notably, *shm1* mutants were also more susceptible than control plants to infection with biotrophic and necrotrophic pathogens (Moreno et al., 2005). This once more indicates a contribution of photorespiratory processes to dissipatory mechanisms that minimize the level of reactive oxygen species. Excessive accumulation of reactive oxygen species impairs cell death containment and could counteract the effectiveness of the plant defenses to restrict pathogen infection.

F. Hydroxypyruvate Is Produced from Ser and Becomes Reduced to Glycerate

After export from the mitochondria, Ser becomes converted to 3HP by the aforementioned peroxisomal enzyme SGT (AGT1), which transfers the

amino group of Ser to glyoxylate, yielding 3HP and Gly. In the penultimate step of the C_2 cycle, most 3HP becomes reduced to D-glycerate by the peroxisomal NADH:3HP reductase (HPR1) using NADH imported from the cytosol via malate shuttles.

$$\text{HPR1: Hydroxypyruvate} + \text{NADH} \leftrightarrows \text{Glycerate} + \text{NAD}^+$$

HPR1 consists of two identical ~42 kDa polypeptides. Together with catalase, glycolate oxidase, and NAD^+:malate dehydrogenase, HPR1 is one of the four major proteins in peroxisomes. In vivo, the enzyme functions primarily as a NADH:3HP reductase (Tolbert et al., 1970). Although with much lower affinity, however, HPR1 also accepts glyoxylate as substrate and was therefore originally described as NADH-specific glyoxylate reductase.

Intriguingly, two *HPR1* genes are present in soybean with one of the corresponding enzymes being a primary target for the P34 syringolide elicitor. After binding of the elicitor, a hypersensitive response is induced by inhibiting one or more yet unknown HPR1 functions in soybean (Okinaka et al., 2002). In many other plants, however, HPR1 is encoded by single genes. Functional deletion of HPR1 in Arabidopsis results in an only minor impairment of plant growth in normal air, very much resembling the low-CO_2 response of an earlier reported HPR-deficient barley mutant (Murray et al., 1989) and that of a recently described double-knockout mutant without peroxisomal malate dehydrogenase (Cousins et al., 2008). The missing low-CO_2 response is very atypical for photorespiratory mutants and indicates the operation of an HPR1-independent route of 3HP-to-glycerate conversion. While a candidate enzyme for this alternative reaction had been suggested (Givan and Kleczkowski, 1992), it was only recently that the responsible enzyme was properly identified as a cytosolic NADPH-dependent 3HP reductase, HPR2, and its metabolic function analyzed by a reverse genetics approach (Timm et al., 2008).

The current model of cooperation between HPR1 and HPR2 is based on the well established fact that NADH cannot permeate through the peroxisomal membrane. Instead, reducing equivalents are imported into peroxisomes in the form of malate provided from chloroplasts and mitochondria via malate/oxaloacetate shuttles, which can be limiting in specific environmental conditions (Yu and Huang, 1986; Reumann and Weber, 2006). Hence, likely determined by the rate of NADH supply to the peroxisomes, a variable fraction of 3HP exits the peroxisomes and becomes reduced in the cytosol. Flux analyses indeed indicate that the cytosolic route is operating not only under the condition of abnormally high photorespiration, but also in moderate environments (Timm et al., 2008). This new model implies that the C_2 cycle, by the cooperation of peroxisomal and cytosolic oxidoreductase reactions, gains additional flexibility in the adaptation to different environmental and possibly developmental conditions. Notably, some plants produce peroxisomal and cytosolic isoforms of HPR1 by alternative splicing of a single pre-mRNA, and the transcript level of the cytosolic form is greatly enhanced under photorespiratory conditions (Mano et al., 1999).

G. Glycerate Becomes Phosphorylated and 3PGA Re-enters the Calvin Cycle

Glycerate enters the chloroplast via a yet unidentified glycolate/glycerate transporter (Howitz and McCarty, 1991). As the ultimate step of the C_2 cycle, glycerate 3-kinase (GLYK) completes the C_2 cycle by returning three fourth of the initially 'misdirected' carbon atoms back to the Calvin cycle in the form of 3PGA.

$$\text{GLYK: D-Glycerate} + \text{ATP} \rightarrow \text{3-Phosphoglycerate} + \text{ADP}$$

The molecular structure and the encoding gene of the ~41 kDa monomeric enzyme were identified only recently (Boldt et al., 2005). GLYK is encoded by a single gene in Arabidopsis. T-DNA insertion knockout mutants show no GLYK activity and are not viable in normal air; however, they grow under elevated CO_2 providing direct evidence for the obligatory nature of the ultimate step of the C_2 cycle under photorespiratory conditions. The enzyme is phylogenetically unrelated to known glycerate kinases from bacteria and animals, which produce 2-phosphoglycerate

(Bartsch et al., 2008), but orthologous proteins are present in other plants, fungi, and some cyanobacteria.

H. Transcriptional Regulation of Photorespiratory C_2 Cycle Genes

The C_2 cycle is a subset of the reactions of photosynthetic carbon assimilation. It is therefore not surprising that C_2 cycle enzymes and their transcripts are present in heterotrophic tissue in only low amounts and that their biosynthesis is induced by light or responsive to cytokinin. Light induction is probably mediated via phytochrome A and cryptochromes. Transcriptional regulation of C_2 cycle enzymes has been examined by the analysis of individual genes or enzymes, organellar proteomes (e.g. mitochondria, Bardel et al., 2002), and expressed sequence tag (EST) arrays. In these latter studies, a light-dependent increase of nearly all genes encoding C_2 cycle enzymes in Arabidopsis was observed after illumination with blue, red, and far-red light. The induction by far-red or blue light was observed not only in wild-type plants but also in *phyA* and cryptochrome null (*cry1*/*cry2*) mutants. On the other hand, *phyB* mutants did not show induction by red light (Ma et al., 2001). Related *in silico* analyses of Arabidopsis genes encoding peroxisomal proteins revealed distinct co-expression patterns for photorespiration-related genes (Reumann and Weber, 2006). This latter finding may prove very helpful for future work because it opens a new strategy to identify yet unknown components of the photorespiratory pathway, such as transporters.

III. Related Reactions and Interactions with Other Metabolic Pathways

A. Reassimilation of Photorespiratory NH_3

Photorespiratory CO_2 evolution is intrinsically associated with the generation of equimolar amounts of NH_3, which are recaptured via the 'photorespiratory nitrogen cycle' (Keys et al., 1978; Keys, 2006). This pathway is also responsible for the entry of NH_3 derived from other sources into general metabolism, and a close interaction of photorespiration and nitrate assimilation has been proposed (Rachmilevitch et al., 2004). NH_3 recovery occurs with very high efficiency, and only ~0.01% of photorespiratory NH_3 is lost (Mattsson et al., 1997).

Biochemically, this process occurs in the chloroplastic glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. The chloroplastic isoform of GS, GS2, combines Glu and NH_3 to form Gln, and the NH_3 acceptor molecule Glu is re-synthesized by the ferredoxin-dependent isoform of GOGAT.

$$\text{GS: L-Glutamate} + NH_4^+ + \text{ATP} \leftrightarrows \text{L-Glutamine} + \text{ADP} + P_i$$

$$\text{GOGAT: L-Glutamine} + \text{2-oxoglutarate} + Fd_{red} \leftrightarrows 2\ \text{L-Glutamate} + Fd_{ox}$$

Arabidopsis like all higher plants studied so far contains a single gene (*GLN2*) for GS2 and multiple genes for cytosolic GS1. Mutation of *GLN2* results in a conditional lethal phenotype under photorespiratory conditions (Blackwell et al., 1988). One out of two genes encoding Fd-GOGAT isoforms in Arabidopsis, *GLU1*, is dominantly expressed in leaves and was mapped to the same location as an earlier Fd-GOGAT mutant. Leaves of this *gls* mutant contain less than 5% of the wild-type levels of Fd-GOGAT activity (Somerville and Ogren, 1982). A second, NADH-dependent isoform accounts for only about 5% of total leaf GOGAT (*GLT1*) activity and is involved in the non-photorespiratory ammonium assimilatory pathway in roots (Coschigano et al., 1998; Suzuki and Knaff, 2005). *GLN2* and *GLU1* hence play the major roles in photorespiration and primary nitrogen assimilation in leaves.

At least in Arabidopsis, GS2 is dual targeted to chloroplasts and mitochondria indicating that some photorespiratory NH_3 could be directly fixed within the mitochondria (Taira et al., 2004). In course of these reactions, Gln produced in the mitochondria is supposedly used for the synthesis of carbamoylphosphate which combines with ornithine to yield citrulline. Citrulline is supposedly exported from the mitochondria via a hypothetical and not yet identified ornithine-citrulline transporter. Considering that NH_3 refixation is a

two-step process requiring glutamate synthase, which is exclusively located in plastids, this hypothesis was recently extended by the suggestion of two alternative NH_3 shuttles between the mitochondria and the chloroplast, an ornithine-citrulline shuttle and a Gln-Glu shuttle (Linka and Weber, 2005).

It is not known whether NH_3 leaves the mitochondria and enters the chloroplasts by diffusion or via specific transporters. Ammonium transport across the plasma membrane is facilitated by ammonium transporters and, in animal systems, specific aquaporins. Such transport systems were not yet identified in mitochondrial or plastidial membranes. Much more is known about the uptake of 2OG into chloroplasts and the export of Glu for peroxisomal transamination reactions. This process is mediated by a two-translocator system. Both translocators, DiT1 for 2OG and DiT2 for Glu, facilitate transport of the respective compounds by counter-exchange with malate, resulting in no net malate transport. Antisense α-DiT1 plants and *dit2* mutant Arabidopsis and barley plants show a heavily disturbed phenotype (e.g. Weber and Fischer, 2007).

B. Regulatory Interaction with Respiration

Regulatory interactions between photorespiration and respiration in the light are not very well understood, but received increasing attention in recent years. Many observations point to an important role for TCA cycle function in the regulation of photosynthesis and photorespiration well beyond the suggested functions of mitochondria in providing ATP for sucrose synthesis and carbon skeletons necessary for nitrate reduction in the cytosol (e.g. Nunes-Nesi et al., 2007; Noguchi and Yoshida, 2008).

Day respiration, i.e. the processes by which non-photorespiratory CO_2 is produced by illuminated leaves, is only about 20–50% of the night respiration rate (Atkin et al., 2000). Some recent data suggest an even higher inhibition of TCA cycle decarboxylations in combination with an opposed partial upregulation under conditions of elevated photorespiration to meet the increased demands of NH_3 reassimilation (Tcherkez et al., 2008). Mechanistically, the downregulation of the TCA cycle activity in the light is not fully understood; however, it is inhibited by its products, NADH and ATP, and activated by its substrates, NAD and ADP. The relative levels of these compound affect the supply of other substrates to the cycle and determine the activity of several internal enzymes including, for example, isocitrate dehydrogenase, which is inhibited by high NADH/NAD (Igamberdiev and Gardeström, 2003). Mitochondrial pyruvate dehydrogenase is inhibited by NADH and controlled by regulatory phosphorylation. The inactivating kinase is activated by NH_4^+, which is present in large amount in illuminated leaf mitochondria (Tovar-Mendez et al., 2003). In the dark, NH_4^+ and NADH levels drop and a phosphatase reactivates PDH, which corresponds well to the lower light suppression of TCA cycle reactions observed in GDC-deficient transgenic plants (Bykova et al., 2005).

C. One-Carbon Metabolism

With GDC and the mitochondrial SHMT, the C_2 cycle shares two major enzymatic reactions with cellular one-carbon (C_1) metabolism. CH_2-THF as the central C_1 carrier compound, directly or after conversion to methyl-, methenyl- and formyl-THF or the synthesis of S-adenosylmethionine, provides C1 units to very many biosynthetic pathways not only in plants but also in all other prokaryotic and eukaryotic organisms (e.g. Hanson and Roje, 2001).

Although plant mitochondria contain fivefold more folate compounds than chloroplasts, this high CH_2-THF/THF pool does not equilibrate with the cytosolic or chloroplastic pools and cannot be a major direct source of one-carbon units for biosynthetic reactions outside the mitochondria (e.g. Orsomando et al., 2005). Instead, all the non-mitochondrial subcellular compartments seem to rely on their own SHMT-catalyzed synthesis of C1 units, which occurs in cooperation with GDC via a Ser-Gly cycle (Mouillon et al., 1999). That is, GDC and SHMT interconnect the metabolism of one-, two-, and three-carbon compounds in most if not all organisms, and it appears likely that both enzymes were recruited for their novel role in the C_2 cycle from C_1 metabolism.

It is a yet unresolved question whether C_1 metabolism, in comparison with the C_2 cycle, has different regulatory requirements to GDC and SHMT. Different enzymatic properties are

apparently not required for T-protein, the only GDC component that is encoded by single-copy genes in Arabidopsis, rice and other plants. In contrast to T-protein, however, all other GDC components are encoded by multiple genes. It is remarkable that the number of GDC subunit genes is the same in Arabidopsis and in rice. Any specific functions or, alternatively, the degree of functional redundancy of the multi-copy paralogous genes and their products are yet to be examined. Some data suggest that specific forms of H-protein are involved in C_1 metabolism in developing xylem rather than in photorespiration of photosynthesizing tissue (Wang et al., 2004). Likewise, the observation that alternative H-proteins, originating by light-regulated alternative splicing of a single mRNA in some species, are present in leaves and roots in different ratios may also indicate different requirements for enzymatic regulation in the C_2 cycle vs. C_1 metabolism of heterotrophic tissue (Kopriva et al., 1996a).

D. Alternative Sources and Destinies of C_2 Cycle Metabolites

The aforementioned findings (e.g. Cegelski and Schaefer, 2005) of a not very strictly fixed stoichiometry indicate that the C_2 cycle could be a more flexible metabolic pathway than it is usually thought. Accordingly, some earlier experiments, performed mainly in the 1960–1970s, suggest the presence of alternative routes of glycolate synthesis (e.g. Eickenbusch and Beck, 1973). Although by far most of the 2PG in photosynthesizing cells originates from RubP oxygenation, it is also continuously generated in small amounts by other processes, for instance those related to DNA repair. While contributions of such processes to 2PG synthesis may be negligible, bypassing reactions to the core C_2 cycle or export to other pathways could be of higher significance. Due to the complex biochemistry of these compounds, the presence of such reactions is most likely for glycolate and 3HP.

Unicellular algae lacking peroxisomes, in contrast to higher plants and many multicellular algae, have no GOX activity and oxidize glycolate by a mitochondrial glycolate dehydrogenase (Stabenau and Winkler, 2005). An equivalent enzyme has recently been identified in Arabidopsis mitochondria, indicating the conservation of the algal photorespiratory pathway (Bari et al., 2004).

In unicellular algae and in higher plants, glycolate can also be oxidized directly within the chloroplasts by a glycolate:plastoquinone oxidoreductase (also known as glycolate dehydrogenase). This enzyme is associated with the thylakoid membranes and supposedly acts as an electron donor in cyclic photophosphorylation providing additional ATP without NADPH accumulation. The generated glyoxylate is probably recycled to glycolate by a chloroplastic NADPH-specific glyoxylate reductase (Goyal and Tolbert, 1996).

IV. Measurement of Photorespiration

The exact determination of photorespiration rates is difficult for two reasons. Firstly, photorespiratory CO_2 evolution is concealed by CO_2 uptake and re-fixation in the Calvin cycle as well as by CO_2 release from mitochondrial 'dark' respiration continuing in the light. Secondly, photorespiratory O_2 uptake occurs concurrently with photosynthetic O_2 evolution and with O_2 consumption during mitochondrial respiration and other metabolic processes including the direct reduction of O_2 at photosystem I, the so-called Mehler reaction.

There are several methods available for the more or less accurate determination of photorespiration rates. Each of these methods has some technical or theoretical problems, which compromise the results, and sizeable differences can be obtained using different methods. Three of the more common methods will be shortly described below but more are available. For instance, photorespiration can also be assessed by the combination of gas exchange measurements with the determination of electron transport rates by fluorescence, mass spectrometry (Haupt-Herting et al., 2001), and nuclear magnetic resonance techniques (Cegelski and Schaefer, 2006). More detailed treatises on how to measure photorespiration can be found in former reviews (Sharkey, 1988; Laisk and Oja, 1998; von Caemmerer, 2000; Long and Bernacchi, 2003).

A. Post-illumination Burst of CO_2 (PIB)

One possible way to rapidly terminate photosynthesis is switching off the light. Thereafter, C_2 cycle metabolism continues for much longer than

CO_2 assimilation, and an approximate measure of the efflux of CO_2 can be obtained using an open infrared gas analyzer system (IRGA). Two peaks are commonly observed during the PIB, but only the first one is associated with photorespiration while the second peak rises during equilibration of mitochondrial respiration. With well-designed equipment allowing rapid-response measurements, CO_2 uptake usually approaches 0 after 5 s and the rate of CO_2 evolution peaks approximately 13 s from darkening (Laisk and Oja, 1998). Due to the masking of the initial slope by continuing CO_2 assimilation that is supported by decreasing but for a while still existing pools of RubP and its precursors, however, this method fails to give quantitative measures of photorespiration.

B. Measurement of $^{14}CO_2$ Evolution

A much more reliable technique, developed by Olav Keerberg and colleagues (Pärnik and Keerberg, 2007), distinguishes between the two opposite fluxes of photosynthetic CO_2 fixation and (photo)respiratory CO_2 evolution, but requires a more complex measuring system. In short, metabolically active leaf carbon pools are labeled with $^{14}CO_2$, and $^{14}CO_2$ evolution is then monitored in the presence of very high (30 ml l^{-1}) concentrations $^{12}CO_2$. Refixation of $^{14}CO_2$ evolved inside the leaf is close to zero under this condition. Photorespiration and respiration are distinguished on the basis of data obtained from measurements of $^{14}CO_2$ evolution under normal (210 ml l^{-1}) and low (15 ml l^{-1}) concentrations of oxygen, while different time periods during the initial $^{14}CO_2$ labeling allow differentiation between CO_2 release from primary versus stored photosynthates. This approach not only yields accurate estimates of photorespiration rates, but can also quantitatively assess internal refixation of photorespiratory CO_2.

C. Extrapolation from CO_2 Response (A/c_i) Curves

This method does not require equipment for the on-line analysis of $^{14}CO_2$, but is somewhat less accurate. Apparent CO_2 exchange rates (A) are measured in an open IRGA system at several CO_2 concentrations. Intracellular CO_2 concentrations (c_i), which are indirectly determined from the simultaneous measurement of transpiration, are used to eliminate effects of the boundary layer and stomata aperture. The preferred CO_2 concentration range spans from below the CO_2 compensation point Γ (i.e., the atmospheric CO_2 concentration where total CO_2 uptake equals total CO_2 efflux) up to 200–300 µl l^{-1} CO_2. The CO_2 response curve is nearly linear in this range. Extrapolation to zero CO_2 yields the rate of CO_2 release from the leaf, i.e., the sum of photorespiration and respiration in the light.

CO_2 exchange rates, of course, are minimal under such conditions resulting in experimental errors. Moreover, at CO_2 concentrations below twofold Γ the CO_2 response curve is commonly curvilinear, bending toward lower values of apparent photorespiration. This effect is due to smaller RubP pools and can be avoided in very rapid measurements (Laisk and Oja, 1998). Therefore, although convenient for comparisons between species, this method also gives only rough estimates of photorespiration rates and is restricted to the conditions of unphysiologically low CO_2/O_2 ratios.

D. Estimation from Rubisco Kinetics and Gas Exchange Measurements

An important feature of Rubisco is the competition of CO_2 and O_2 for the same catalytic site. This and other features have been integrated into mathematical models of photosynthesis and combined with the analysis of whole leaf gas-exchange parameters. The most broadly used 'Farquhar model' (Farquhar et al., 1980) allows the determination of the in vivo RubP oxygenation rate from gas exchange measurements thus separating contributions from respiration in the light to total CO_2 efflux. This approach requires the determination of the chloroplastic CO_2 concentration where the rate of RubP carboxylation equals the rate of photorespiratory CO_2 release. This CO_2 concentration is defined as the photosynthetic CO_2 compensation point in the absence of respiration in the light, Γ*, and can be determined from the interception point of CO_2 curves measured at different light intensities. The same data set gives an estimate for R_d, the respiration in the light. As an experimentally sometimes more convenient alternative, Γ* can also be obtained from the (in C_3 plants) linear O_2 dependence of Γ between 1.5% and 21% O_2 (e.g. Laisk and Oja, 1998).

The mathematical modeling approach, in short, is as follows (von Caemmerer, 2000). The ratio of RubP oxygenation to carboxylation, v_o/v_c, is determined solely by the kinetic constants of Rubisco as shown in Eq. 6.1 where V_o, V_c, K_o, and K_c are the in vivo maximal rates and the Michaelis-Menten constants for oxygenation and carboxylation, respectively. O and C represent the chloroplastic O_2 and CO_2 concentrations. S is the so-called specificity factor of Rubisco.

$$\Phi = \frac{v_O}{v_C} = \left(\frac{V_O K_C}{K_O V_C}\right) \cdot \frac{O}{C} = \frac{1}{S} \cdot \frac{O}{C} \quad (1)$$

Net flux of CO_2 will be zero if exactly two oxygenations of RubP occur for every carboxylation. This happens at the chloroplastic CO_2 concentration Γ*.

$$\frac{v_o}{v_c} = 2 = \frac{1}{S} \cdot \frac{O}{\Gamma *} \quad (2)$$

Re-arranging Eq. 6.2 yields an expression for the specificity factor.

$$S = \frac{O}{2\,\Gamma *} = \frac{\Delta O}{2\,\Delta \Gamma} \quad (3)$$

By using Eq. 6.3 in combination with Eq. 6.1, one can now calculate the oxygenation to carboxylation ratio in vivo at any reasonably given chloroplastic CO_2 and O_2 concentration. The rate of photorespiratory CO_2 production can then be determined with the help of two more experimental parameters to be determined by gas exchange measurements, the net CO_2 assimilation rate A and the rate of respiration in the light Rd (Eq. 6.4).

$$A = v_c - 0.5\, v_o - R_d \quad (4)$$

Using Eq. 6.4 and keeping in mind that the stoichiometry of the C_2 cycle requires two molecules 2PG to produce one molecule of CO_2, primary rates of photorespiratory CO_2 evolution can be calculated according to Eq. 6.5.

$$0.5\, v_o = \frac{A + R_d}{\frac{2}{\Phi} - 1} \quad (5)$$

V. The Role of Photorespiration for the Evolution of C_4 Photosynthesis

As already indicated, photorespiration reflects the evolutionary origin of oxygenic photosynthesis about 2.5 billion years ago in the anaerobic environment of the Precambrian ocean (Canfield, 2005). Due to the lack of free oxygen, RubP oxygenation could not occur and photorespiratory glycolate recycling was unnecessary. Much more recently, about 1.1–0.54 Ga ago (e.g. Kennedy et al., 2006), the level of oxygen in the atmosphere rose to relatively high levels, resulting in increasingly significant competition of O_2 with CO_2 for the active site of Rubisco.

This had severe consequences for the further evolution of photosynthetic bacteria, algae, and later land plants including those of the C_4 photosynthetic type. While details of the evolution of C_2 cycle are not well known, the pathway is not restricted to the Chlorobionta, but already present in cyanobacteria. In these ancestors of plastids, it overlaps and cooperates with the bacterial glycerate pathway (Eisenhut et al., 2006). Cyanobacteria improve their photosynthetic efficiency very much by active uptake of CO_2 and bicarbonate via CO_2-concentrating mechanisms (CCM; Kaplan et al., 2007). Despite this, the artificial obstruction of cyanobacterial glycolate metabolism leads to toxic Gly accumulation (Eisenhut et al., 2007). This suggests that photorespiratory glycolate metabolism is not just present but essential for cyanobacteria, where it employs multiple routes, one of which might have been conveyed endosymbiontically to plants (Eisenhut et al., 2008).

Land plants evolved from ancestral freshwater algae from the Charophyceae group that were closely related to the unicellular flagellate *Mesostigma viride* (Petersen et al., 2006; Simon et al., 2006) and to the possibly even more ancient unicellular green alga *Ostreococcus tauri* (Palenik et al., 2007). While the respective analyses still need to be extended, first data suggest that major changes in photorespiratory metabolism took place already very early during the evolution of unicellular flagellates, i.e., before the transition of plants to land approximately 480–700 million years ago. Once on land, plants had easier access to CO_2 than bacteria and algae, but also faced periods of limiting water supply or elevated temperatures and hence decreased CO_2/O_2 ratios. The resulting problems for photosynthetic

efficiency were further exacerbated by the significant decline of atmospheric CO_2 levels during the last 500 million years in combination with at least one intermediate rise of atmospheric oxygen to about 35% (Beerling and Berner, 2000; Pearson and Palmer, 2000). The most effective option to deal with the elevated potential for RubP oxygenation was the addition of a CCM to the Calvin cycle, the C_4 cycle. C_4 plants usually show only small or even no apparent photorespiratory CO_2 release. This is because of the C_4 cycle-mediated CO_2 enrichment within the bundle sheath, which out-competes oxygen and efficiently reduces 2PG synthesis by Rubisco. Hence, smaller amounts of photorespiratory CO_2 are produced and more or less completely recaptured by the entry enzyme into the CO_2 concentration cycle of C_4 plants, phosphoenolpyruvate carboxylase.

Details of the individual steps leading from C_3 to C_4 photosynthesis in the last 15–30 million years are still far from being well understood. C_4 photosynthesis differs from the original C_3 photosynthesis by quite a number of molecular and morphological features, which implies the likeliness of transitional, C_3-C_4 intermediate, species. *Mollugo verticillata* (Aizoaceae) was the first plant species reported which has a number of features, for example leaf anatomy, photorespiration, and primary photosynthetic products, that can neither be easily associated with C_3 nor with C_4 photosynthesis (Kennedy and Laetsch, 1974). Since then, C_3-C_4 intermediate and C_4-like species have been identified in about 30 species of 11 genera representing nine different families (Table 2). These plants represent a broad variety of transitions between C_3 and C_4. Phylogenetic analyses demonstrate that C_3-C_4 intermediate plants represent transient forms between C_3 and C_4 *Flaveria* species (Powell, 1978; Kopriva et al., 1996b; McKown et al., 2005) and have likewise suggested that they may be derived from C_3 plants in *Moricandia* (Bauwe, 1983).

Photosynthetic rates of C_3 and C_3-C_4 intermediate species are comparable in a range of environmental conditions, but the responses of gas-exchange parameters that provide a measure of photorespiratory activity differ widely between these two groups. For instance, leaves of C_3-C_4 intermediate species show a higher CO_2 affinity than related C_3 species (e.g. Ku and Edwards, 1978; Bauwe and Apel, 1979). The higher CO_2 affinity of leaves from C_3-C_4 intermediate species is mirrored in lower values for Γ. At atmospheric O_2 concentration, the respective Γ values are generally between 10 and 30 μl l^{-1} and hence much lower than the typical range for Γ of C_3 plants. Historically, it is interesting to note that several C_3-C_4 intermediate plants, such as *Moricandia arvensis* (L.) DC and *Panicum milioides* Nees ex Trin., have been identified in the aforementioned Γ-based screening programs set up to identify further C_4 plants.

At low photon flux densities, close to the light compensation point, Γ of a C_3-C_4 intermediate species can be almost as high as that of a C_3 species; however, in contrast to C_3 plants where Γ is essentially unaffected by light intensity, Γ of C_3-C_4 intermediate species is strongly light dependent and declines steeply as the light intensity increases (e.g. Brown and Morgan, 1980). This response was an early indication that the mechanism which lowers Γ (i.e., reduces apparent photorespiration) in C_3-C_4 intermediate species is dependent on the rate of photosynthesis.

Rather than the linear response of Γ generally observed with C_3 plants (compare Eq. 6.2), C_3-C_3 intermediate plants show a biphasic response of Γ to increasing O_2 concentration (e.g. von Caemmerer, 1989), which is another very indicative feature of C_3-C_4 intermediate photosynthesis. As the O_2 concentration is raised to 10–15%, relatively low increases of Γ occur, followed by a steeper and linear response above 20%. Typically, the slope of the Γ/O_2 response curve (γ) at higher O_2 concentrations is lower than corresponding values for C_3 plants. In C_3 plant photosynthesis models, γ is proportional to the specificity factor S of Rubisco; however, no corresponding differences exist in the kinetic properties of Rubisco of C_3 and C_3-C_4 intermediate plants (Bauwe, 1984). In combination with the presence of Rubisco in both mesophyll cells (MC) and bundle sheath cells (BSC), and in accordance with the higher CO_2 affinity of leaves, lower values for γ indicate that a significant fraction of total Rubisco operates at an elevated CO_2/O_2 concentration ratio in C_3-C_4 intermediate species.

C_3-C_4 intermediate species are usually native to warm environments like Mexico and Florida (*Flaveria*) or Mediterranean countries (*Moricandia*).

Table 2. C_3-C_4 intermediate land plants.

Family	Species	References
Aizoaceae	*Mollugo verticillata*	(Kennedy and Laetsch, 1974)
	M. lotoides	(Kennedy et al., 1980)
	M. nudicaulis	(Raghavendra et al., 1978)
	M. pentaphylla	(cf. Sage et al., 2004)
Poaceae	*Panicum decipiens*	(cf. Sage et al., 2004)
	P. milioides	(cf. Sage et al., 2004)
	P. schenckii	(cf. Sage et al., 2004)
Boraginaceae	*Heliotropium convulvolaceum*	(Vogan et al., 2007)
	H. greggii	(Vogan et al., 2007)
	H. racemosum	(Vogan et al., 2007)
Poaceae	*Neurachne minor*	(cf. Sage et al., 2004)
Brassicaceae	*Moricandia arvensis*	(cf. Sage et al., 2004)
	M. nitens	(cf. Sage et al., 2004)
	M. sinaica	(cf. Sage et al., 2004)
	M. spinosa	(cf. Sage et al., 2004)
	M. suffruticosa	(cf. Sage et al., 2004)
	Diplotaxis tenuifolia	(cf. Sage et al., 2004)
Asteraceae	*Flaveria angustifolia*	(cf. Sage et al., 2004)
	F. anomala	(cf. Sage et al., 2004)
	F. chloraefolia	(Holaday et al., 1984)
	F. floridana	(Holaday et al., 1984)
	F. linearis	(cf. Sage et al., 2004)
	F. pubescens	(cf. Sage et al., 2004)
	F. ramosissima	(cf. Sage et al., 2004)
Asteraceae	*Parthenium hysterophorus*	(cf. Sage et al., 2004)
	Bougainvillea cv. Mary Palmer	(Sabale and Bhosale, 1984)
Amaranthaceae	*Alternanthera ficoides*	(cf. Sage et al., 2004)
	A. tenella	(cf. Sage et al., 2004)
Chenopodiaceae	*Salsola arbusculiformis*	(Voznesenskaya et al., 2001)
Cleomaceae	*Cleome paradoxa*	(Voznesenskaya et al., 2007)

While most of the described gas exchange data have been obtained under laboratory conditions, some data are available from field measurements. For *F. floridana*, in its natural habit, it was demonstrated that Γ was still low at leaf temperatures between 35°C and 40°C, but dramatically increased in a sympatric C_3 species, and photosynthetic rates were 2.4–4 times higher in the C_3-C_4 intermediate species at the same conditions (Monson and Jaeger, 1991). These data show that the improved gas-exchange characteristics discussed above indeed translate into improved fitness and so provide the basis for the evolution of more C_4-like traits.

With few possible exceptions, significant levels of C_4 cycle activity were not observed in most C_3-C_4 intermediate species. While there is usually some enhanced incorporation of $^{14}CO_2$-carbon into C_4 compounds, this carbon is not conveyed to the C_3 cycle. Hence (the sometimes called 'type I') C_3-C_4 intermediate species typically do not have a functional C_4 cycle. This is different in the so-called C_4-like ('type II' C_3-C_4 intermediate) *Flaveria* species *F. brownii*, *F. palmeri* or *F. vaginata*, where a limited C_4 cycle operates in parallel with direct CO_2 fixation by Rubisco (Bassüner et al., 1984; Edwards and Ku, 1987; Moore et al., 1989).

Despite the absence of a C_4 cycle, however, leaves of C_3-C_4 intermediate plants have a distinctive anatomy which is clearly different from C_3 plant and similar to C_4 plants. In comparison with C_3 plants, e.g. the vascular bundles show increased vein density in leaves of C_3-C_4 intermediate plants and are surrounded by BSC containing a remarkably high number of organelles reminiscent of the Kranz anatomy of C_4 plants. In addition, mitochondria and peroxisomes are found in close association with chloroplasts. A recent re-examination of anatomical features for a broad range of *Flaveria* species demonstrated that key anatomical features of C_4 plants are required for C_3-C_4 intermediacy and Kranz anatomy is fully developed before complete C_4 biochemistry is achieved (McKown and Dengler, 2007).

It has been early suggested that these specific ultrastructural properties could facilitate reassimilation of photorespiratory CO_2 via the Calvin cycle (Brown, 1980), and C_3-C_4 intermediate plants are indeed capable of a much more efficient internal recycling of photorespiratory CO_2 than C_3 plants (Holbrook et al., 1985). Quantitative estimates showed that between 70% and 90% of photorespiratory CO_2 is recaptured before it escapes from the leaf of a C_3-C_4 intermediate plant, which is much higher than the about 30–50% refixation typically observed with C_3 plant leaves (Bauwe et al., 1987).

Following up reports on some atypical features of Gly catabolism, it was hypothesized that a relatively large portion of the GDC activity may take place in the BSC resulting in higher CO_2 recycling and low photorespiration rates in leaves of C_3-C_4 intermediate species. Because of the similarity of this model to the metabolic situation in C_4 plants, where GDC is confined to the BSC, it was also suggested that C_3-C_4 intermediate species may have partially developed this biochemical feature of C_4 photosynthesis (e.g. Edwards and Ku, 1987).

This hypothesis was validated by extensive immunolocalisation studies. Where it has been subsequently examined, at least the P-protein of GDC is not uniformly distributed in the leaves of C_3-C_4 intermediate species, but confined to the BSC. In the leaves of C_3-C_4 intermediate *Panicum* and *Flaveria* species, e.g. all four GDC subunits are missing in the MC, whereas significant levels of H-, T-, and L-protein are present in the mesophyll of C_3-C_4 intermediate *Moricandia* species (e.g. Hylton et al., 1988; Morgan et al., 1993; Devi et al., 1995; Ueno et al., 2003; Voznesenskaya et al., 2007). Because the presence of all GDC subunits is necessary for GDC activity, the deletion of only one subunit will unavoidably cause GDC deficiency in the mesophyll. It shall therefore be noted that an *absolute* confinement of the respective GDC subunits to the bundle sheath neither has been proven nor is likely, because of the un-replaceable function of GDC in C_1 metabolism in all cells (Engel et al., 2007). C_1 metabolism, however, requires much less GDC activity in comparison with the high fluxes through photorespiratory metabolism.

Based on these localization studies and following on from earlier models, a model for photorespiratory metabolism in leaves of C_3-C_4 intermediate plants has been proposed which can satisfactorily explain the high degree of light-dependent recapture and the low apparent rate of photorespiration (Rawsthorne, 1992). Both MC and BSC of C_3-C_4 intermediate species contain a fully functional Calvin cycle leading to oxygenation of RubP and hence photorespiratory Gly production in both cell types. Due to the lack of GDC in the mesophyll, Gly accumulates to a considerable steady-state level driving its passive transport into the GDC-containing bundle sheath where decarboxylation results in photorespiratory CO_2 release. This requires high activities of both GDC and SHMT in the bundle sheath, and an about fivefold elevated SHMT activity was indeed detected in the bundle sheath of C_3-C_4 intermediate plants (Rawsthorne et al., 1988). It should be mentioned that SHMT is also required for the rapid recycling of CH_2-THF to THF. In contrast to Gly and Ser, neither of these two compounds can equilibrate between different cells. The distinctly elevated levels of Ser in the leaves of C_3-C_4 intermediate plants suggest that a considerable fraction of this amino acid diffuses back into the mesophyll to ensure continued operation of the mesophyll-located Calvin cycle.

The physiological consequences of this ‘Gly shuttle’ are usually discussed with respect to the high refixation of photorespiratory CO_2 achieved by C_3-C_4 intermediate plants. In contrast, the inherent potential for elevating bundle sheath CO_2 levels is less often considered (e.g. von Caemmerer, 1989; Bauwe and Kolukisaoglu, 2003).

This latter aspect, however, is possibly of much higher importance for the subsequent evolution of C_4 plants than the pure refixation aspect. In leaves of C_3-C_4 intermediate plants, the C_2 cycle uses Gly as vehicle to move a substantial fraction of freshly assimilated carbon from the mesophyll (the photorespiratory CO_2 'pump's' inlet) to the bundle sheath (the 'pump's' outlet) where it is released as photorespiratory CO_2. This must have led to an elevated CO_2 concentration in the bundle sheath, as it also follows from model calculations (von Caemmerer, 1989). C_3 plants typically invest very little in chloroplasts in the bundle sheath, which appears economical because of the probably low CO_2 concentration in this location (Fig. 2). The operation of the C_2 pathway, for the time being stabilized by improved refixation, would provide more favorable photosynthetic conditions in BSC, making the accumulation of chloroplasts an advantageous consecutive step. Correspondingly, relocation of the photorespiratory Gly-to-Ser conversion could have been a universal starting point for the subsequent evolution of C_4 photosynthetic features. Further support for this hypothesis will certainly require the identification of C_3-like plants that have GDC already confined to BSC, but still contain only few chloroplasts in these cells. Promising genera to search for such plants could be the well analyzed genus *Flaveria* (McKown and Dengler, 2007) and the more recent additions to the list of C_3-C_4-containing genera, *Cleome* (Marshall et al., 2007) and *Heliotropium* (Vogan et al., 2007).

All known C_3-C_4 intermediate species already partition a significant number of chloroplasts to the BSC and hence possess, roughly speaking, two fractions of Rubisco operating under different CO_2/O_2 ratios. Most likely, the BSC fraction of Rubisco operates under (at least 'somewhat') more favorable conditions than the MC fraction. This hypothesis has been tested by quantitative $^{14}CO_2$ labeling experiments with leaves of several *Flaveria* species designed to allow the determination of in-vivo RubP carboxylation/oxygenation ratios (B. Bassüner, T. Pärnik, O. Keerberg and H. Bauwe, unpublished). These data indicate that the C_3-C_4 intermediate species *F. pubescens* has an about twofold increased carboxylation/oxygenation ratio of RubP relative to the C_3 plant *F. cronquistii*. As mentioned above, Rubiscos of these two species show identical in vitro properties and the enzyme of the C_3-C_4 intermediate plant should hence operate under an approximately doubled mean CO_2 concentration in comparison with that of the C_3 plant.

VI. Future Prospects

Photorespiration is a major and indispensable, although costly, part of the network of C_3 plant primary metabolism. It's often disputed function is very clear: recycling of the (in C_3 plants) more or (in C_4 plants) less massive amounts of 2PG produced by Rubisco. In light of the fact that many essentials of the C_2 cycle where already discovered in the 1960–1980s, it is surprising that a significant share of the components of the pathway, such as metabolite transporters, probably metabolic enzymes, and perhaps regulatory proteins are not yet known. Likewise, our understanding of interactions with other major metabolic pathways is still very limited, and most of the accumulated knowledge is restricted to the C_3 type of land plant photosynthesis.

Photorespiration is not only a multifaceted and high-throughput biological process of its own importance. It is also a determinant factor of C_3 crop biomass production. In nature, plant growth is probably not very often limited by photosynthetic carbon fixation, but by the availability of nutrients and water, attack of pathogens, and other stresses. In an agricultural environment where those limitations are managed, productivity is co-determined by multiple factors as well, but it fundamentally depends on the photorespiration-affected efficiency of crop plants to convert atmospheric CO_2 into biomass by the process of photosynthesis. Therefore, the negative impacts of photorespiratory CO_2 losses on agricultural productivity have challenged researchers for many decades. It was only very recently that an artificial redirection of photorespiratory C fluxes indeed led to improved growth rates and biomass production of a C_3 plant (Kebeish et al., 2007). In contrast to earlier attempts to modify photorespiratory metabolism, which all failed to improve plant growth, this new result eventually proved that photorespiratory metabolism can be optimized by molecular plant breeding.

Photorespiration occurs in aquatic microorganisms, too, but land plants are in a different

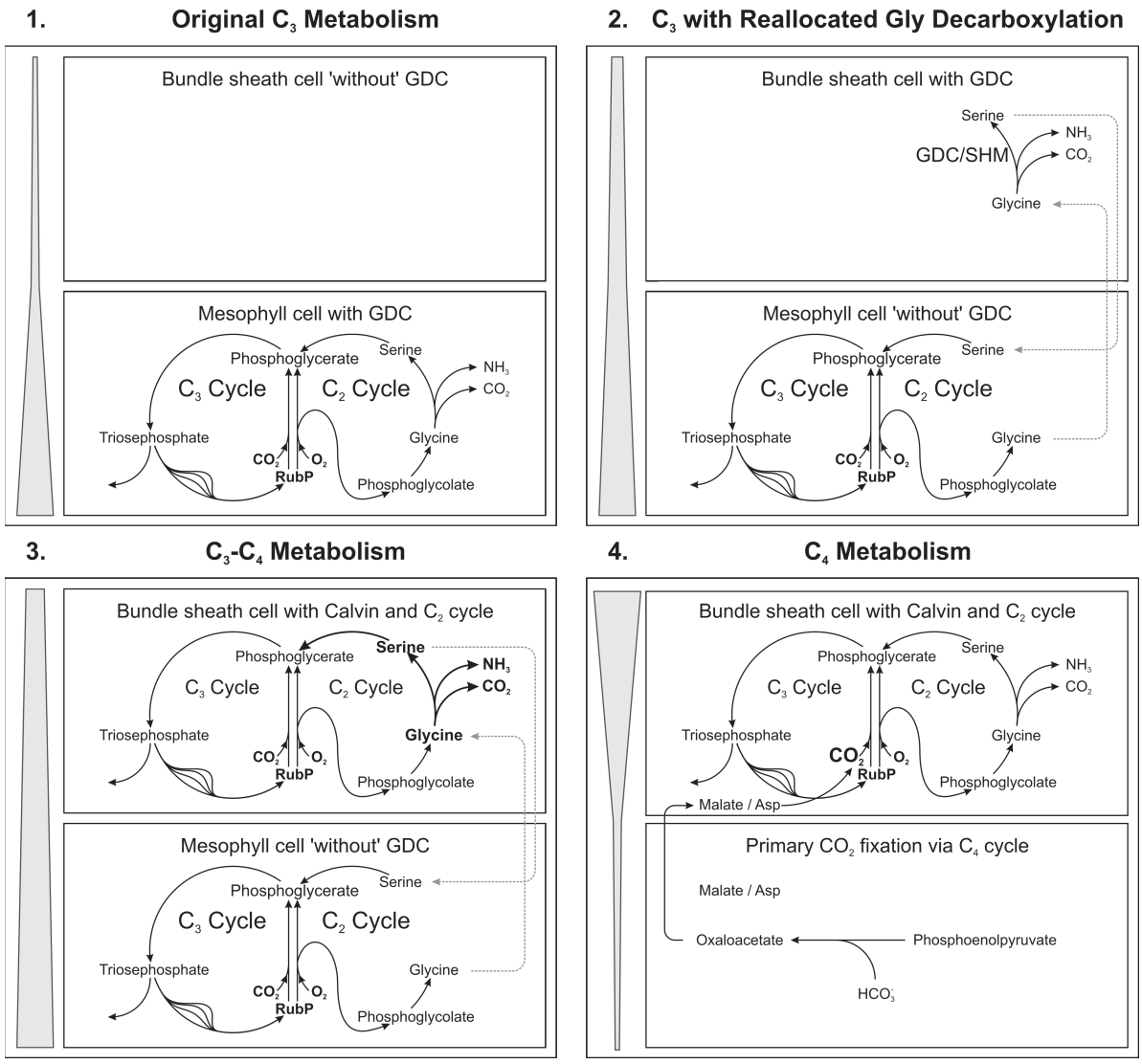

Fig. 2. A plausible sequence of major steps towards C_4 photosynthesis. By reallocation of GDC from the mesophyll (**1.**) to the bundle sheath, Gly starts to act as a vehicle for transferring a significant fraction of freshly assimilated carbon to the mesophyll (**2.**) This makes metabolic investment in more bundle sheath chloroplasts beneficial (**3.**) In addition to the extra CO_2 from Gly decarboxylation, enhanced Calvin cycle activity in the bundle sheath is also pushed by the additional 3PGA, while RubP synthesis in the mesophyll may be discriminated. Once this key anatomical component of C_4 photosynthesis exists, the C_4 cycle gradually first superimposes (in the C_4-like species) and finally replaces (in C_4 species) the photorespiratory CO_2 pump. Thereafter, smaller investments in mesophyll chloroplast numbers in the mesophyll become feasible (**4.**). The left-handed grey areas indicate approximate CO2 concentration gradients between mesophyll and bundle sheath in the four panels.

situation. While they have easier access to CO_2 than bacteria and algae, stomata close under conditions of limiting water supply, which reduces transpiration and enhances photorespiration. Elevated temperatures increase photorespiratory CO_2 losses to even higher levels. These two stressors, limiting water supply and high temperatures, led to the evolution of C_4 photosynthesis. Photorespiration is hence closely related to the evolution of C_4 plants including highly productive crops. These plants, however, do not merely 'avoid' photorespiratory CO_2 losses. Paradoxically, their

evolution started with a photorespiration-driven primary CCM, which used the large daily amounts of glycine produced during photorespiration instead of a C_4 acid as a transport vehicle for CO_2. It thus appears as if photorespiration contributed to the evolution of C_4 photosynthesis in two ways, by exerting selective pressure in favor of more efficient photosynthesis and by providing the first plan on how to improve the intercellular CO_2 gradients in leaves.

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Corrections

1. The Sage et al. 2004 ref should be Sage et al. 1999, both in the table and the references.

2. The species Mollugo lotoides and Mollugo pentaphylla are not C3-C4, but are C3.

3. The Panicum intermediates are now called Steinchisma. The genus can be shown as Panicum (=Steinchisma).