Glycolate and glyoxylate metabolism in higher plants: How natural and artificial pathways contribute to plant metabolism

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Referent: Prof. Dr. Christoph Peterhänsel

Korreferent: Prof. Dr. Hans-Peter Braun

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Abstract

Photorespiration is the salvage reaction for 2-phosphoglycolate produced by the oxygenase function of Rubisco. The higher plant pathway has been defined by mutant screens in Arabidopsis thaliana. Although the biochemistry of this pathway is well accepted nowadays, data indicate a more complex mechanism of glycolate conversion with several branching points. The theory of a more complex mechanism is based on observations in higher plants that cannot be explained by following the major pathway. Additionally, the theory is supported by experiments with cyanobacteria, the evolutionary ancestors of chloroplasts. Cyanobacteria have three pathways for photorespiratory glyoxylate conversion that coexist within the same organism. Two pathways recycle glyoxylate to glycerate, which can be phosphorylated to reenter the Calvin Benson Bassham Cycle. One of them resembles the plant type photorespiratory cycle, the other resembles the bacterial glycerate pathway. A third pathway oxidizes glyoxylate to CO_2. In this work, I will present data on a glyoxylate conversion pathway in the chloroplast of higher plants that might represent a functional equivalent of the second cyanobacterial photorespiratory pathway. Beside investigations for a better understanding of the photosynthetic pathway in plants, I was also interested in transgenic approaches with the key aspect of either reducing or redirecting photorespiration. Regarding flux rates, photorespiration is the second most important pathway in plants. Photorespiration is often considered to be a wasteful process, because CO_2 and ammonia are released while at the same time energy in form of reducing power and ATP is consumed by this pathway. Consequently, transgenic approaches were engineered into Arabidopsis and tobacco plants to lower the losses through photorespiration. However, in the last decade, more and more data were published showing that photorespiration is an essential part of primary metabolism and plays an important role in energy dissipation instead of being a wasteful process only. Based on these results, the benefits and downsides of photorespiration and of the transgenic approaches are re-considered in this thesis.

Keywords: energy balance of photorespiration, photorespiratory bypasses, pyruvate dehydrogenase complex

Zusammenfassung

Die Photorespiration verwertet 2-Phosphoglycolat, das durch die Oxygenase4 Funktion von Rubisco gebildet wird. Der Stoffwechselweg wurde durch Mutantenanalyse in Arabidopsis thaliana aufgeschlüsselt. Obwohl die Reaktionsfolge des Stoffwechselweges mittlerweile anerkannt ist, gibt es ebenfalls Daten, die auf einen komplizierteren Mechanismus mit mehreren Gabelungen hinweisen. Die Theorie, die einen komplizierteren Verlauf vorhersagt, resultiert aus Beobachtungen, die sich nicht mit dem etablierten Model erklären lassen. Zudem wird die Theorie durch Experimente mit Cyanobakterien, den evolutionären Vorfahren heutiger Chloroplasten, gestützt. Cyanobakterien besitzen drei Stoffwechselwege zum Abbau von photorespiratorischem Glyoxylat, die parallel im gleichen Organismus vorkommen. Zwei dieser Stoffwechselwege produzieren Glycerat, welches phosphoryliert wird, um es dem Calvin Benson Bassham Zyklus zurückzuführen. Einer davon ähnelt dem pflanzen-typischen Stoffwechselweg, wohingegen der andere dem bakteriellen Glycerat-Stoffwechselweg entspricht. Ein dritter Stoffwechselweg oxidiert Glyoxylat komplett zu CO_2. In dieser Arbeit werde ich Daten präsentieren, die ein funktionelles Äquivalent eines zweiten cyanobakteriellen Stoffwechselweges der Photorespiration in höheren Pflanzen andeuten. Neben der Analyse mit dem Ziel, die einzelnen Reaktionen der Photorespiration besser zu verstehen, habe ich mich zusätzlich mit transgenen Ansätzen beschäftigt, die das Ziel haben, Photorespiration zu vermindern oder umzuleiten. Von der Durchflussmenge her gesehen ist die Photorespiration der zweit-wichtigste Stoffwechselweg in Pflanzen. Photorespiration wird häufig als verschwenderisch angesehen, da CO_2 und Ammonium freigesetzt werden und gleichzeitig Energie in Form von Reduktionsäquivalenten und ATP verbraucht wird. Als Konsequenz wurden transgene Ansätze in Arabidopsis und Tabak eingebracht, mit dem Ziel, Verluste durch Photorespiration zu vermindern. Allerdings wurden in der letzten Dekade immer mehr Ergebnisse veröffentlicht, die zeigen, welch starke Interaktion die Photorespiration mit dem Pflanzenmetabolismus verbindet. Zudem spielt die Photorespiration eine wichtige Rolle in der Ableitung von überschüssiger Energie als nur ein verschwenderischer Stoffwechselweg zu sein. Basierend auf diesen Resultaten wurden die positiven wie auch die negativen Seiten der Photorespiration erneut abgewogen.

Schlagwörter: Energiebilanz der Photorespiration, Alternativwege der Photorespiration, Pyruvat-Dehydrogensekomplex

1 General introduction

1.1 Photosynthesis

1.1.1 The core enzyme: Rubisco

Rubisco is the core enzyme of carbon fixation as it fixes anorganic carbon into an organic compound. The incorporated carbon is subsequently reduced in the Calvin Benson Bassham cycle (CBB cycle). On the molecular level, Rubisco catalyzes the ligation of carbon dioxide (CO_2) and ribulose 1,5 bisphosphate (RuBP) that produces two molecules 3-phosphoglycerate (3-PGA). This reaction can be considered as the most important process on earth as nearly all living organisms depend on this process. Rubisco had great evolutionary success and Raven (2009) estimated that 99.5 % of all autotrophic organisms use Rubisco for carbon fixation, whereas heterotrophic organisms are dependent on the uptake of the fixed carbon from autotrophs. Despite of its evolutionary success, Rubisco is regarded to be an inefficient enzyme. Rubisco is a very slow catalyst and carboxylates only 1-4 molecules RuBP per second per catalytic center in rice plants (Parry et al., 2007). The slow catalytic rate of Rubisco is one reason for the high abundance of this protein. Furthermore, Rubisco confuses CO_2 and O_2. Although it has a 100 times higher specificity for CO_2 than for O_2 (Jordan and Ogren, 1981), every forth reaction catalysed by Rubisco is actually an oxygenation (Sharkey, 2001). This observation can be explained by the current abundances of the two gases in the atmosphere, which are 0.04% for CO_2 and 21% for O_2. These two downsides contribute to the fact that Rubisco is the most abundant protein on earth (Ellis, 1979; Raven, 2013). Oxygenation of RuBP produces one molecule each of 3-PGA and 2-phosphoglycolate (2-PG) and is the initiation reaction of photorespiration, a process with high energy costs for the recycling of carbon to the CBB cycle. Although photorespiration has been described as light dependent decarboxylation of glycolate by Zelitch (1966) already in 1966 and glycolate has been observed as an early photosynthetic product already in 1948 (Benson and Calvin, 1950), the production of glycolate by the oxygenase function of Rubisco has first been postulated by Ogren in 1971 (Bowes et al., 1971).

Rubisco is activated in the light, which requires carbamylation of a lysine residue and subsequent binding of Mg^{2+} at the active site (Salvucci and Ogren, 1996). Under physiological conditions, carbamylation of lysine by CO_2 is limited by both, the low speed of the reaction and by inhibiting sugar phosphates bound to the active site of Rubisco. In both cases, light dependent action of Rubisco activase is assumed to activate Rubisco by an unidentified mechanism. Rubisco activase lowers the Km for carbamylation and most importantly helps to release the tightly bound sugar phosphates from the active site (Portis et al., 1986; Wang and Portis, 1992). In higher plants, Rubisco is a protein complex of approximately 550 kDa in size and consists of eight small and eight large subunits forming eight active sites (Spreitzer and Salvucci, 2002; Andersson, 2008).

In summary, the highly abundant protein Rubisco is a slow catalysts and has a dual function, which catalyzes the initiation reactions of two of the major pathways in plants, the CBB cycle and photorespiration.

1.1.2 The dark reaction of photosynthesis: The Calvin Benson Bassham cycle

The CBB cycle has been investigated by Calvin and coworkers in the 1950s by feeding radioactively labeled CO_2 to the unicellular green algae *Chlorella* and identification of the substances with incorporated label by two dimensional paper chromatography (Benson and Calvin, 1950). The CBB cycle is responsible for the primary production of carbohydrates used for storage and translocation of chemically bound energy. In higher plants, the CBB cycle is located in the chloroplast and constitutes of eleven enzymes catalyzing thirteen reaction steps (Raines, 2003). The CBB cycle can be categorized into three stages: carbon fixation, reduction of the integrated carbon and regeneration of the substrate for Rubisco. CO_2 enters the CBB cycle at the site of Rubisco, which produces two molecules 3-PGA. As already mentioned, another source of carbon for the CBB cycle is photorespiratory 3-PGA. After reduction of carbon at the expense of ATP and NADPH, the triosephosphate glyceraldehyde 3-phosphate (GAP) leaves the cycle. Three carboxylation reactions of Rubisco are necessary to fix the one atom molecule CO_2 into the three carbon atom compound GAP without any depletion of the CO_2 acceptor RuBP in the CBB cycle. By the fixation of three molecules CO_2 and the subsequent metabolism to recycle RuBP, nine molecules ATP and six molecules NADPH are consumed. The huge flux to provide reduced carbon for all anabolic pathways qualifies the CBB cycle to be the greatest energy sink in green tissue (Szecowka et al., 2013).

1.1.3 The light reaction of photosynthesis

Energy for CO_2 fixation is provided by the light reaction of photosynthesis. Plants are capable of converting light energy into chemically bound energy. In this process, light energy is used to induce electron shuffling by the oxidation of water. The electrons are transported via the electron transport chain (ETC) to ferredoxin, which can be used to reduce $NADP^+$. The light reaction is dominated by 4 protein complexes, which are located in the thylakoid membrane (Nelson and Ben-Shem, 2004), while only three of them form the ETC. At photosystem II (PSII), light energy is used to split water into molecular oxygen, protons and electrons. The oxygen is released and is the source of atmospheric oxygen (Igamberdiev and Lea, 2006). The protons produce an electrochemical gradient, the protonmotive force, over the thylakoid membrane, which is used for the production of ATP from ADP and anorganic phosphate (P_i) by the ATP synthase. ATP is a ubiquitous energy carrier throughout all living organisms. The electrons originally derived from water are transferred from PSII to plastoquinone, which additionally binds protons in the stroma forming plastoquinol. These protons are released to the lumen by reoxidation of plastoquinol to plastoquinone at the cytochrome b_6f complex. This enhances the protonmotive force. The electrons are further transferred to photosystem I (PSI) via

the cytochrome b_6f complex and plastocyanine. This complex is again using light to enhance the reducing power of the electrons before transferring them to ferredoxin. Ferredoxin is soluble in the stroma and is used for several reducing reactions in the chloroplast (Hanke and Mulo, 2012). One of the ferredoxin dependent enzymes is the ferredoxin-$NADP^+$ oxidoreductase (FNR). FNR generates the reducing equivalent NADPH. In summary, the products of the light reaction are molecular oxygen, NADPH and a proton gradient that is used for ATP synthesis.

In high light, the light reaction provides the cell with virtually unlimited amount of reducing power. Under CO_2 limitation, the capacity of the dark reaction cannot keep pace by regeneration of electron acceptors (Demmig-Adams and Adams, 1992), which results in production of reactive oxygen species (ROS). As a consequence, plants evolved strategies to dissipate excess energy and to cope with ROS.

1.2 Photoinhibition and photoprotection

1.2.1 Photoinhibition

Under high irradiance, the photosystems absorb more energy than can be used by assimilatory electron sinks. The effect is even more severe in drought or at high temperatures, when CO_2 availability is limiting. This can result in an overload of electrons in the electron transport chain, which can severely damage the photosystems by generation of ROS (Demmig-Adams and Adams, 1992). The lack of appropriate electron acceptors, mainly $NADP^+$, causes reduction of O_2 and induces oxidative stress. O_2 reduction at the photosystems produces superoxide (O_2^-). O_2^- is further converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (Asada, 2000). Additionally, high irradiance causes production of the ROS singlet oxygen ($^1O_2$) at PSII. This highly excited state is generated by triplet chlorophyll, which is produced at high photon flux. Singlet oxygen is highly reactive and damages lipids as well as proteins at the site of its formation. The D1 protein in the core of PSII is frequently damaged by $^1O_2$, a process called photodamage. As a consequence, the protein has to be removed and replaced. Actually, D1 has the lowest half life of all plastid encoded proteins, with a turnover time ($t_{1/2}$) of approximately 2 h (Sundby et al., 1993). A persisting period of high irradiance causes photoinhibition, when the rate of D1 translation cannot keep pace with its ongoing oxidation and degradation. Moreover, $^1O_2$ and H_2O_2 inhibit the translation of D1 protein, resulting in retarded replenishment of D1 to reach the full potential of PSII (Nishiyama et al., 2004). Thus, energy dissipation is an important process in plants exposed to high irradiances or drought. To avoid an over-reduced state, plants evolved various strategies that reduce the probability of photoinhibition, which are summarized as photoprotective mechanisms.

1.2.2 Photoprotective mechanisms

Plants evolved two major mechanisms avoiding photoinhibition. (i) They reduce the absorption or channeling of light energy and (ii) they provide multiple electron sinks that avoid overreduction of the ETC and/or that recover $NADP^+$.

High photon flux increases the turnover number of water splitting of photosystem II. This results in acidification of the thylakoid lumen. Decrease of luminal pH initiates several short-term processes that help to dissipate energy or to reduce light harvesting. Acidification is a signal to induce state transition of light harvesting complex II (LHCII) to PSI (Horton, 1983). Consequently, less energy is absorbed by PSII and fewer electrons are fed into the ETC. The higher capacity of PSI results in efficient transfer of electrons from the ETC to ferredoxin. Secondly, luminal acidification induces the production of violaxanthin, which is incorporated into to the thylakoid membrane. Violaxanthin quenches energy from chlorophyll by heat dissipation (Havaux and Niyogi, 1999). Thirdly, the orientation of the light harvesting complexes in the membrane towards the light source is modified in response to acidification of the lumen. This results in lower efficiency of light harvesting and increased heat dissipation (Foyer et al., 1990).

The reduction of oxygen at the photosystems is the initiation reaction of the water-water cycle. The photoprotective role of this pathway has been reviewed by Asada (2000). O_2^- is reduced to H_2O_2 by superoxide dismutase. H_2O_2 is discussed to be a messenger molecule, which might be involved in retrograde signaling for further adjustment of the redox state (Foyer and Noctor, 2005). However, hydrogen peroxide is also a highly reactive component that is further reduced to H_2O by ascorbate peroxidase. The electron donor for this reaction is ascorbate. Monodehydroascorbate reductase converts resulting monodehydroascorbate (MDA) to ascorbate by oxidation of ferredoxin. Alternatively, ascorbate can be recycled by oxidation of glutathione (GSH), which is subsequently reduced by glutathione reductase at the expense of NADPH (Hossain and Asada, 1984; Noctor and Foyer, 1998). Summarized, although reduction of oxygen at the photosystems is a necessary evil by the production of toxic ROS, plants also benefit from this pathway. The water-water cycle is an efficient way to eliminate high reducing power in the chloroplast as the electrons originating from water splitting at photosystem II are retransferred to oxygen to produce water (Park et al., 1996; Asada, 2000).

Regeneration of $NADP^+$ is crucial to avoid an over-reduced state of the ETC (Nogales et al., 2012). Endo et al could verify the role of cyclic electron flow via the NADPH dehydrogenase complex (NDH) in photoprotection (Endo et al., 1999). NDH is integrated in the thylakoid membrane and shows high homology to mitochondrial complex I (Battchikova et al., 2011). Again, the complex uses the reducing power of NADPH for building up a protonmotive force over the thylakoid membrane. Cyclic electron flow is also realized via ferredoxin and the cytochrome b_6f complex of the ETC (Iwai

et al., 2010). Both pathways feed electrons back into the ETC by the reduction of plastoquinone. The net result of cyclic electron flow is ATP synthesis at the expense of NADPH.

The malate valve is also involved in the prevention of oxidative stress (Scheibe et al., 2005). This process exports reducing power from the chloroplast. Plastidal malate dehydrogenase (MDH) reduces oxalacetate (OAA) to malate. Malate is exported from the chloroplast and is oxidized by the cytosolic, peroxisomal or mitochondrial isoenzyme (Raghavendra and Padmasree, 2003). The transport process is catalyzed by a dicarboxylate transporter that exchanges malate for OAA (Kinoshita et al., 2011). However, the importance of the malate valve for redox homeostasis has recently been doubted by Hebbelmann et al. (2012), because knockout mutants of the plastidal MDH did not show a chlorotic phenotype that would be expected in plants under oxidative stress.

In summary, plants evolved a number of strategies to prevent oxidative stress under conditions in which sink capacity of the CBB cycle is reduced due to limitation of the substrate CO_2. However, due to the oxygenase activity of the carbon fixing enzyme Rubisco, high rates of photorespiration are achieved even under ambient atmospheric CO_2 concentrations. Thus, the sink capacity of this pathway is also considered to play a major role in photoprotection (chapter 1.3.7).

1.3 Photorespiration

1.3.1 The major pathway in higher plants

The photorespiratory pathway of higher plants was resolved by an EMS mutant screen in *Arabidopsis thaliana* (Somerville and Ogren, 1979; Somerville, 2001). Basically, plants were grown under non-photorespiratory high CO_2 conditions before non-chlorotic plants were shifted to ambient air and thereby, chlorosis was induced in photorespiratory mutants. Chlorotic plants were shifted back to high CO_2 to rescue the severe photorespiratory phenotypes. Subsequently, photorespiratory mutants were screened for the mutated genes by analyzing changes in metabolites and enzyme activities. In total, the major photorespiratory cycle in higher plants is a complex pathway that requires the joined action of enzyme activities in three organelles (Figure 1). In the chloroplast, 2-PG is dephosphorylated by the plastidal 2-phosphoglycolate phosphatase (PGP). Glycolate formed by this function is transported into the peroxisome where it is oxidized to glyoxylate by glycolate oxidase (GO). Hydrogen peroxide (H_2O_2), a byproduct of this reaction, is detoxified by the highly abundant catalase (CAT). Glyoxylate is transaminated to glycine by either glutamate:glyoxylate transaminase (GGT) or serine:glyoxylate transaminase (SGT). Glycine is transported into the mitochondrion, where the joined functions of glycine decarboxylase (GDC) and serine hydroxymethyl transferase (SHMT) ligate two molecules glycine to serine thereby releasing CO_2 and ammonia (NH_3). Ammonia is refixed in the chloroplast by glutamine synthase (GS) and ferredoxin-dependent glutamine:oxoglutarate aminotransferase (Fd-GOGAT). Serine is exported back to the peroxisome and is transaminated by SGT to

hydroxypyruvate. Peroxisomal hydroxypyruvate reductase (HPR1) reduces hydroxypyruvate to glycerate, which is transported into the chloroplast. Finally, glycerate is phosphorylated by glycerate kinase (GLYK) to 3-PGA, which can reenter the CBB-cycle.

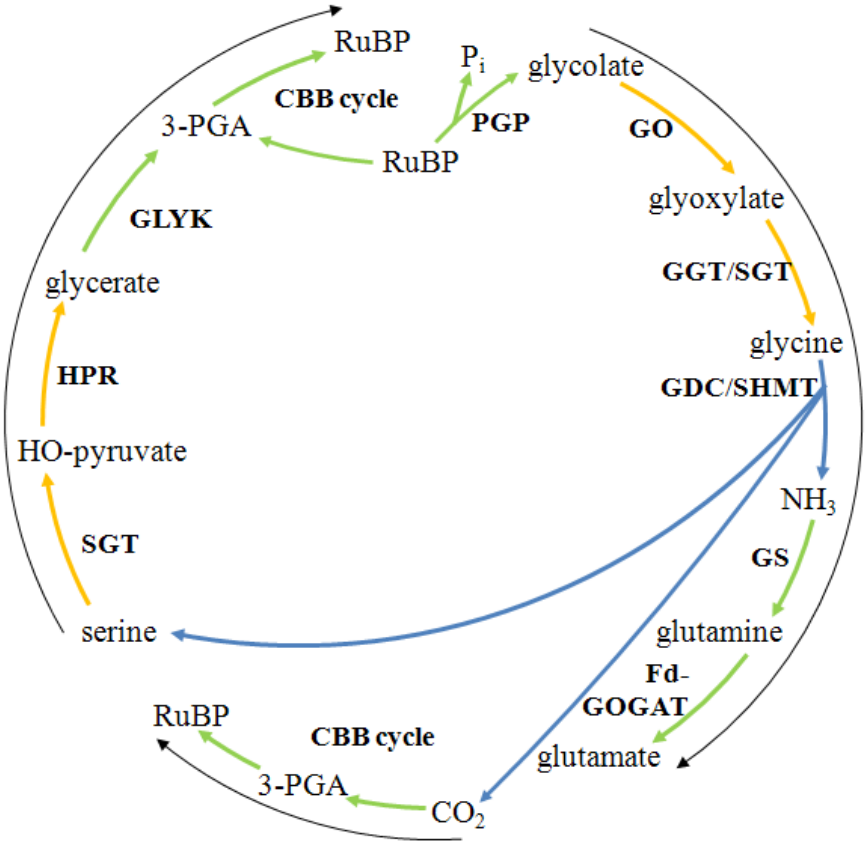

Figure 1: The major pathway of photorespiration in higher plants.

The figure is a schematic representation of the major photorespiratory pathway in higher plants. Green arrows represent plastidal reactions, yellow arrows peroxisomal, and blue arrows mitochondrial reactions. The refixation of photorespired CO_2 by the CBB cycle is included. The enzymes of each step are indicated in bold letters. CBB cycle, Calvin Benson Bassham cycle; PGP, phosphoglycolate phosphatase; GO, glycolate oxidase; GGT, glutamate:glyoxylate transaminase; SGT, serine:glyoxylate transaminase; SHMT, serine hydroxymethyl transferase; GDC, glycine decarboxylase; GS, glutamine synthase; Fd-GOGAT, ferredoxin-dependent glutamate:2-oxoglutarate amino-transferase; HPR, hydroxypyruvate reductase; GLYK, glycerate kinase; RuBP, ribulose 1,5 bisphosphate; Pi, inorganic phosphate; 3-PGA, 3-phosphoglycerate; HO-pyruvate, hydroxypyruvate

To date, only one of the transport steps described in here has been characterized (Eisenhut et al., 2012). Recently, Pick et al. (2013) described PLGG1, the plastidal glycolate/glycerate transporter. The photorespiratory pathway includes several enzymatic steps that consume energy. (i) Glycerate phosphorylation and ammonia fixation consume ATP. (ii) Hydroxypyruvate reduction and ammonia fixation consume reducing power in form of NADH or reduced ferredoxin, respectively. The investment of energy and the counterproductive CO_2 release during photorespiration are reasons for establishing transgenic approaches to plants with the aim of lowering photorespiratory flux (chapter 1.3.5).

1.3.2 Natural flexibility of photorespiration in higher plants

Although the major photorespiratory pathway is well established nowadays, there are open questions concerning observations made in several publications that cannot easily be explained by following the conventional photorespiratory cycle. Furthermore, there is some indication that intermediates in photorespiration can be converted in multiple reactions. Some of these reactions are already investigated in detail, while others remain to be elucidated.

Usually, photorespiratory CO_2 release by GDC/SHMT follows transamination by GGT1. However, mutants of these two enzymes, which were supposed to be essential elements of photorespiration (chapter 1.3.1) show photorespiratory CO_2 release. This indicates that there might be an alternative pathway for glyoxylate conversion. It is known that glyoxylate can be oxidized non-enzymatically by H_2O_2 (Igamberdiev and Lea, 2002), which is produced at high rates in the peroxisome. However, due to the vicinity and the excess of H_2O_2 scavenging catalase, this reaction seems not to be very probable. Grodzinsky (1978) discussed that catalase can take over the role of a peroxidase, which decarboxylates glyoxylate. Decarboxylation of glyoxylate would produce formate, which could be further oxidized to CO_2 by the action of mitochondrial localized formate dehydrogenase. Formate could also be metabolized by formyl-tetrahydrofolate synthetase, a reaction that would link photorespiration to C1 metabolism of the plant. The resulting formyltetrahydrofolate could be converted via two more enzymatic steps to methylene tetrahydrofolate. At this stage, it can reenter the photorespiratory cycle as a substrate for SHMT (Wingler et al., 1999). Another alternative of the major pathway is localized in the peroxisome. Initially, the peroxisomal HPR1 was described as hydroxypyruvate reducing enzyme (Tolbert et al., 1970). However, when reducing power is limiting in the peroxisome, hydroxypyruvate is exported from the peroxisome to be reduced to glycerate by either the cytosolic HPR2 (Timm et al., 2008) or by the plastidal HPR3 (Timm et al., 2011).

In mitochondria, another variant of the major pathway has been observed. In addition to the peroxisomal GO, Bari et al. (2004) identified a mitochondrial targeted glycolate dehydrogenase (GlcDH). Niessen et al (2007) showed that GlcDH is responsible for mitochondrial glycolate conversion and, moreover, that two alanine aminotransferases link mitochondrial glycolate oxidation to the major photorespiratory cycle by converting glyoxylate to glycine (Niessen et al., 2012). However, the preferred substrate of higher plant GlcDH is still discussed in the literature. Engqvist et al. (2009) could only detect low glycolate dependent enzyme activity of this enzyme *in vitro*. The electron acceptor of substrate oxidation remains to be elucidated, but electrons were transferred to cytochrome C *in vitro*.

Besides to glyoxylate decarboxylation in the peroxisome and mitochondrial glycine decarboxylation, a plastidal pathway for photorespiratory CO_2 release has been described. Kizaki and Tolbert (1969) observed glyoxylate decarboxylation activity in the chloroplast. Due to the light dependence of the CO_2 release, they presumed H_2O_2 formed in the Mehler reaction to react with glyoxylate non-

enzymatically. A non-enzymatic breakdown like this has been described (Tolbert et al., 1949; Igamberdiev and Lea, 2002) but even the authors stated that the decarboxylation rate is surprisingly high for a non specific reaction (Kisaki and Tolbert, 1969). Zelitch (1972) proposed a light driven enzymatic reaction of plastidal glyoxylate decarboxylation. He postulated a chlorophyll associated Mn^{2+} dependent protein that produces H_2O_2 only for the breakdown of glyoxylate. Additionally, Kebeish et al. (2007) transformed a glycolate conversion pathway in *A. thaliana* that includes a decarboxylation step. While measuring the activity, he observed that also wildtype plants are capable of glycolate decarboxylation by an unidentified mechanism. Summarized, although plastidal glyoxylate decarboxylation is observed multiple times, no specific enzyme could be identified (Kisaki and Tolbert, 1969; Zelitch, 1972; Oliver, 1981; Kebeish et al., 2007). Additionally, Goyal and Tolbert (1996) described a futile cycle of glycolate oxidation and reduction in the chloroplast. In analogy to mitochondrial complex II, they postulated a membrane bound glycolate-quinone oxidoreductase that feeds electrons into the electron transport chain to increase the protonmotive force across the thylakoid membrane. Formed glyoxylate could be reduced by plastidal NADPH-glyoxylate reductase to glycolate. Analogous to the NADPH dehydrogenase, the result of the cycle is ATP production at the expense of NADPH. However, this enzyme has not been detected hitherto and this reaction would not result in CO_2 release.

In summary, some of the alternative pathways just replace distinct reactions, while others lead to total oxidation of glycolate or connect photorespiration with C1-metabolism. In this thesis, the role of the pyruvate dehydrogenase complex in plastidal glyoxylate decarboxylation was investigated.

1.3.3 Evolution of photorespiration

1.5 billion years ago, the endosymbiosis between a eukaryotic cell and an ancestor of contemporary cyanobacteria resulted in the evolution of chloroplasts (Mereschkowsky, 1905; Hedges et al., 2004; Howe et al., 2008). Accordingly, cyanobacteria also use Rubisco for carbon fixation. Recent data suggest that photorespiration evolved already before the endosymbiotic event, although global accumulation of free atmospheric oxygen started only 0.6 billion years ago (Planavsky et al., 2012). This might be explained by local accumulation of oxygen and drain of CO_2 in dense mats of photosynthetic organisms. About 2.3 billion years ago, aggregates of cyanobacteria in stromatolites and microbial mats were more common than free floating cells (Giordano et al., 2005). Such a microcosmos could have accumulated oxygen, which promoted the oxygenase function of Rubisco. Photorespiration is still an integral part of cyanobacterial metabolism. Photorespiratory 2-PG in cyanobacteria is also dephosphorylated by a 2-phophoglycolate phosphatase (PGP). Cyanobacteria are prokaryotic cells without compartmentalization. As a consequence, oxygen is not the electron acceptor of glycolate oxidation like in the peroxisomes of higher plants as resulting H_2O_2 is toxic. Instead, cyanobacteria use glycolate dehydrogenases for glycolate oxidation. Their electron acceptors remain

to be determined, but *in vitro* activity could be measured with the cofactor NAD^+. Subsequently, three pathways for glyoxylate metabolism were described (Eisenhut et al., 2008, Figure 2).

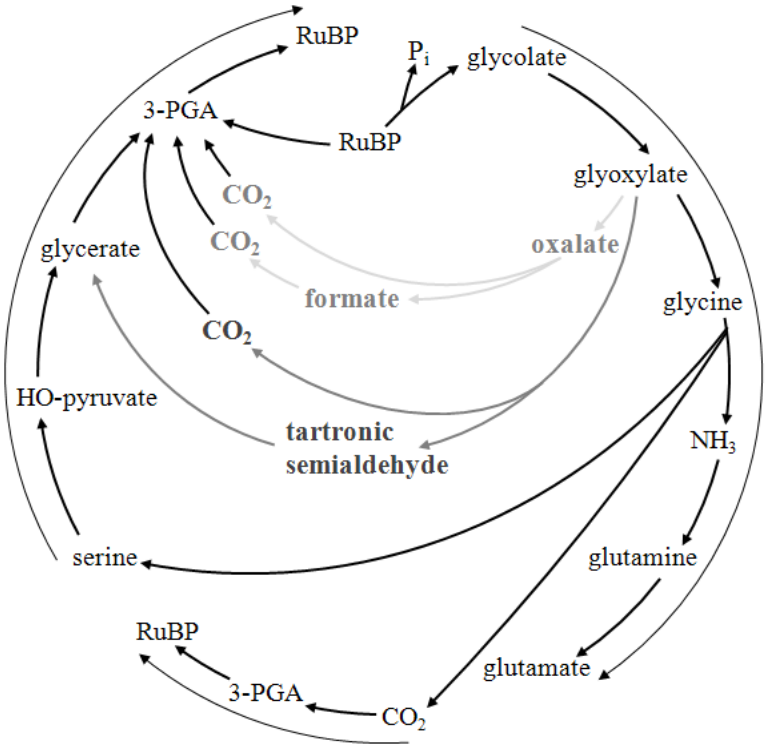

Figure 2: The photorespiratory pathways in cyanobacteria.

The figure is a schematic representation of the photorespiratory pathways in Synechocystis. The three pathways are kept in different greyscale. The refixation of photorespired CO_2 by the CBB cycle is included in the figure. RuBP, ribulose 1,5 bisphosphate; 3-PGA, 3-phosphoglycerate; HO-pyruvate, hydroxypyruvate

First, the plant-type photorespiratory C2 cycle and the bacteria-like glycerate pathway are described in cyanobacteria. Both pathways convert glyoxylate to glycerate. The bacterial like pathway has only two reaction steps, catalyzed by glyoxylate carboligase and tartronic semialdehyde reductase. In the first reaction, two molecules glyoxylate are ligated and CO_2 is released. In the second step, tartronic semialdehyde is reduced to glycerate under expense of reducing power (Eisenhut et al., 2006). Like in higher plants, the second pathway includes transamination of glyoxylate to glycine and ammonia release by GDC and SHMT. In this reaction, CO_2 is also released. Serine is subsequently deaminated and reduced to glycerate by serine:glyoxylate transaminase (SGT) and hydroxypyruvate reductase (HPR), respectively. To reenter the CBB cycle, glycerate is phosphorylated to 3-PGA in both pathways. Interestingly, phosphorylation takes two reaction steps. First, glycerate is phosphorylated at the C2 position by a class I glycerate kinase (GLYK), in contrast to the class III GLYK reaction in plants (Bartsch et al., 2008), which directly phosphorylates at the C3 position. In cyanobacteria, 3-PGA is then generated by a phosphoglyceromutase. Additionally, glyoxylate can be completely oxidized to CO_2. The first oxidation step is catalyzed by a hydroxyacid dehydrogenase followed by two decarboxylation events. An oxalate decarboxylase produces formate under CO_2 release followed by oxygenation of formate to CO_2 by formate dehydrogenase. This pathway could be observed in *in vitro* assays, but its relevance *in vivo* is not validated. Interestingly, the SHMT and the PGP from higher plants are not homologous to the cyanobacterial enzymes, although they catalyze the same reactions in the same pathway. This indicates a dual origin of the enzymes of the photorespiratory C2 cycle in higher plants (Kern et al., 2011; Bauwe et al., 2012).

1.3.4 Reduction of photorespiration in nature

Although photorespiration is well integrated into primary metabolism (chapter 1.3.6), photosynthetically active organisms evolved pathways and mechanisms to reduce flux through photorespiration by concentrating CO_2 at the site of Rubisco. This is interesting, as the transgenic approaches that reduce photorespiration are often criticized for the reduction of such an important pathway.

Although photorespiration probably evolved early in evolution (Giordano et al., 2005), carbon concentrating mechanisms (CCM) evolved several times in different clades, independently. The CCMs are based on the differential localization of Rubisco solely in specialized structures, in which CO_2 is enriched.

The CCM in cyanobacteria is probably the most effective mechanism to concentrate CO_2 (Meyer and Griffiths, 2013). The mechanism enables Rubisco of cyanobacteria, which has a Km of about 150 µM, to be saturated with substrate at an external CO_2 concentration of about 10-15µM (Price et al., 2007). The most important characteristics of the cyanobacterial CCM are a protein shell as diffusion barrier (carboxysome), the localization of carbonic anhydrases and Rubisco inside the carboxysome, and transporters that import inorganic carbon into the cell. The transporters are localized in the plasmamembrane and import both, CO_2 and bicarbonate (HCO_3^-). The energy supply for the import is variable as there are specialized proteins for the use of ATP, reducing power or an electrochemical gradient. The transporters work so efficient that internal CO_2 concentrations of 20-40mM are measured frequently. If CO_2 is imported, it is converted to HCO_3^- in the cytosol. HCO_3^- can diffuse into the carboxysome to the vicinity of Rubisco. Inside the carboxysome, carbonic anhydrases convert HCO_3^- to CO_2, which can then be fixed by Rubisco (Price et al., 2007).

Green algae like Chlamydomonas and a group of bryophytes, the hornworts, evolved a similar but cyanobacteria-unrelated CCM (Meyer and Griffiths, 2013). Rubisco is concentrated in a dense structure called pyrenoid (Holdsworth, 1971). Compared to cyanobacteria, the transport process of inorganic carbon is more complex as several membranes, each equipped with its own transporters, have to be crossed. Chlamydomonas possesses transporters for bicarbonate and it is discussed, whether CO_2 is also transported actively (Wang et al., 2011). Inside the chloroplast stroma, carbonic anhydrases strongly prefers production of HCO_3^- from CO_2. HCO_3^- is channeled by a yet unknown transporter into the thylakoid lumen. A carbonic anhydrase is located in pyrenoid spanning sections of the lumen, where it produces CO_2, which diffuses into the pyrenoid to be substrate for Rubisco (Sinetova et al., 2012).

Higher plants also evolved CO_2 concentrating mechanisms to enhance the CO_2 concentration at the site of Rubisco. The mechanism is quite different from cyanobacterial and algal CCMs, as only CO_2 but not HCO_3^- can be taken up from the environment. In C4 metabolism, carbon is primarily fixed by

the oxygen insensitive enzyme phosphoenolpyruvate carboxylase (PEPC) to produce the C4 compound oxaloacetate (OAA). In contrast, the first product of carbon fixation in C3 plants is the C3 compound 3-PGA. The C4 cycle evolved at least 66 times during evolution (Sage et al., 2011). As a consequence, there are multiple similar pathways, as different enzymes are recruited for the carbon concentrating mechanism. A few C4 organisms evolved C4 photosynthesis within a single cell (Edwards et al., 2004), but in most cases two types of specialized cells are required for C4 photosynthesis: mesophyll cells and bundle sheath cells (BSC). Several evolutionary steps were necessary for the establishment of C4 photosynthesis, as reviewed by Gowik and Westhoff (2011). Furthermore, several C3-C4 intermediate species were described. PEPC and, therefore, primary carbon fixation is located in the mesophyll cells while Rubisco is exclusively located in the chloroplast of BSC. The basic mechanism of C4 photosynthesis is the formation of a C4 acid in mesophyll cells by an enzyme that is not sensitive to O_2. After being transported to the BSC, the C4 acid is subsequently decarboxylated in bundle sheath chloroplasts to enhance the CO_2 concentration in close vicinity of Rubisco. The resulting C3 acid is transported back to a mesophyll cell to complete the cycle and to be available for another carboxylation reaction.

Another mechanism for lowering photorespiratory flux in nature is changing the specificity factor of Rubisco. Form II Rubisco is found in proteobacteria and consists of a dimeric homologue of the large subunit (Tabita et al., 2008). The evolution of the form I Rubisco with 8 small and 8 large subunits increased specificity of CO_2 over O_2 by a factor of 5-10 (Jordan and Ogren, 1981). C3 plants express Rubisco with the highest observed specificity factor. Organisms containing a Rubisco with a lower preference for CO_2 express a CCM to reduce oxygenation rates. Interestingly, lower specificity is accompanied by higher carboxylation velocity (Bainbridge et al., 1995; Tcherkez et al., 2006). Thus, less Rubisco is required for the fixation of CO_2 resulting in higher nitrogen use efficiency (Price et al., 2007).

1.3.5 Artificial photorespiratory bypasses

As photorespiration is a pathway with high energy costs, bypasses by biotechnological approaches were installed that aim in modulating glycolate metabolism and energy costs leading to advantages for these plants (Figure 3). The first bypass installed by Kebeish et al. (2007) into *Arabidopsis* simulates the cyanobacterial glycerate pathway (chapter 1.3.3) although the genes were cloned from E. coli. Glycolate is oxidized in the chloroplast by a glycolate dehydrogenase. Two molecules of the resulting glyoxylate are ligated to tartronic semialdehyde under the release of CO_2 by glyoxylate carboligase and reduced to glycerate by semialdehyde reductase. The second bypass engineered by Carvalho et al. (2011) into tobacco takes place in the peroxisome. Again, glyoxylate carboligase converts glyoxylate to tartronic seminadehyde followed by isomerization to hydroxypyruvate by hydroxypyruvate isomerase. In the third bypass described by Maier et al. (2012), glycolate is oxidized by glycolate oxidase in the chloroplast of *Arabidopsis*. The emerging H_2O_2 is scavenged by transgenic catalase.

Malate synthase produces malate from glyoxylate and acetyl CoA. Subsequently, malate is oxidized two times by the endogenous enzymes malic enzyme and pyruvate dehydrogenase complex, respectively, resulting in two molecules CO_2 and one molecule acetyl CoA. The latter can again be used as the substrate for the next malate synthase reaction. The first two bypasses produce the photorespiratory intermediates glycerate and hydroxypyruvate, respectively, which can be recycled to 3-PGA. In a strict sense, the third pathway is not a bypass, as it does not generate a photorespiratory intermediate but completely oxidizes glycolate to CO_2.

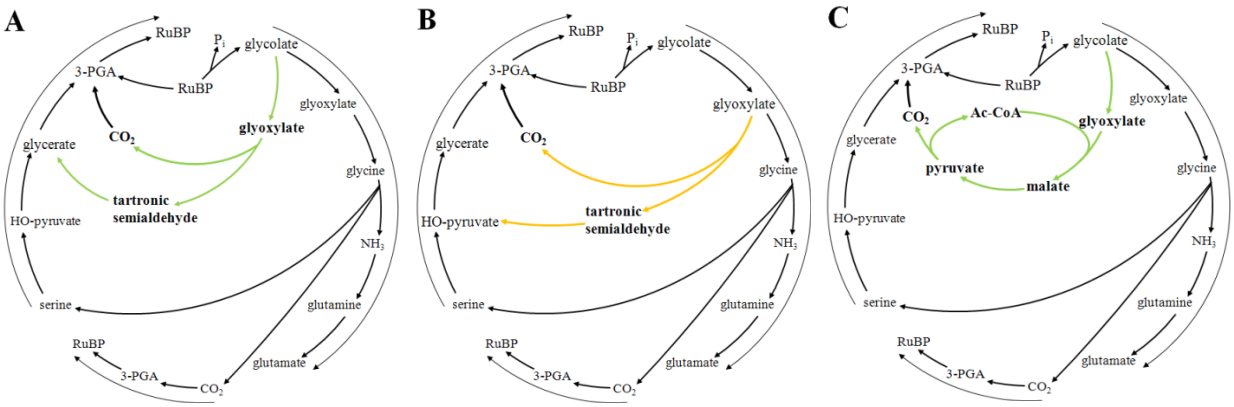

Figure 3: The photorespiratory bypasses of transgenic approaches

The figures are schematic representations of the photorespiratory bypasses. Black arrows represent the major pathway, green arrows plastidal, and yellow arrows peroxisomal reactions. The refixation of photorespired CO_2 by the CBB cycle is included in the figure. (A) Bypass 1 engineered by Kebeish et al, (B) Bypass 2 engineered by Carvalho et al, (C) Bypass 3 engineered by Carhalho et al. RuBP, ribulose 1,5 bisphosphate; 3-PGA, 3-phosphoglycerate; HO-pyruvate, hydroxypyruvate

This is a short overview about the transgenic approaches that alter photorespiration. As part of this thesis, the bypasses were reviewed and categorized for their energy costs in detail (Peterhansel et al., 2012), (chapter 2.22.1).

1.3.6 Integration of photorespiration into plant metabolism

Photorespiration is well integrated into plant metabolism. One important link is the dissipation of excess energy that will be discussed in the next paragraph. However, equally important is the recycling of 3-PGA for the CBB cycle. Moreover, photorespiration takes over a role in the removal of toxic intermediates and assimilation of plant metabolites. This chapter focuses on the integration of photorespiration into plant metabolism. How alternative pathways address these tasks was investigated and discussed within the thesis presented here.

Photorespiration is closely connected to the CBB cycle, not only by the dual function of Rubisco, but also by its products CO_2 and 3-PGA. The product of photorespiration and the primary intermediate of carbon fixation by Rubisco are both 3-PGA. The importance of photorespiration in the regeneration of the CBB cycle intermediate was summarized by Bauwe et al. (2012). They calculated that there is a

net loss of fixed carbon when oxygenation exceeds ½ of the carboxylation reaction which is a realistic scenario based on the specificity rates of Rubisco (Galmes et al., 2005). Due to photorespiration, three quarters of fixed carbon are rescued. Cegelski and Schaefer (2006) showed that flux through the CBB cycle is lowered under photorespiratory conditions even when RuBP is recycled in wildtype plants. While this can be attributed to lower Rubisco activity as a consequence to of reducing CO_2-availability, it is also shown that the reduction of assimilation in photorespiratory mutants can be compensated by supplementing the product of the knock-out reaction (Wingler et al., 2000). These experiments demonstrate that regeneration of 3-PGA through photorespiration is mandatory and alternative photorespiratory pathways have to address this trait.

Three toxic compounds are produced during photorespiration: 2-PG, glyoxylate and H_2O_2. 2-PG was shown to inhibit triose phosphate isomerase, an enzyme in the CBB cycle, and phosphofructokinase, an enzyme involved in starch degradation (Anderson, 1971; Kelly and Latzko, 1976). Glyoxylate was shown to reduce the activation state of Rubisco, but to date the mechanism remains unclear (Cook et al., 1985; Chastain and Ogren, 1989; Campbell and Ogren, 1990). The toxicity of H_2O_2 has multiple reasons. Its reactivity facilitates the unspecific oxidation of lipids and thiol groups of proteins (Apel and Hirt, 2004; Jang and Imlay, 2007). A regulatory role of H_2O_2 has been proposed recently (Foyer and Noctor, 2005), but photorespiratory H_2O_2 is efficiently scavenged by catalase, which excludes peroxisomal H_2O_2 from any regulation process under most conditions (Tolbert et al., 1968).

Photorespiration produces a set of amino acids. Interestingly, the three amino acids glycine, serine and glutamate are all linked to glutathione (GSH). GSH is a short molecule of only three amino acids, namely glutamate, cysteine and glycine. Glycine and glutamate are intermediates of the photorespiratory C2 cycle and of ammonia assimilation, respectively. Cysteine is produced from serine and the first step of this pathway, O-acetylation of serine, is mainly catalyzed in mitochondria (Wirtz et al., 2012). However, GSH synthesis was only shown to depend on photorespiratory glycine. Buwalda et al. (1990) proposed a role for photorespiration in glutathione synthesis, because GSH production was depending on light but could be restored in the dark by glycine supplementation. Moreover, the photorespiratory intermediates glycolate and glyoxylate could replace glycine. Additionally, Noctor et al. (1999) showed that suppression of photorespiration by increased CO_2 concentrations also diminished glutathione synthesis. This observation could be negated by supplementation of one of the photorespiratory intermediates glycolate or glycine. Glutathione plays an important role in redox regulation, because it can be oxidized to dimeric glutathione (GSSG) during the scavenging of reactive oxygen species (ROS). Interestingly, the same conditions favor photorespiration and demand of GSH (Noctor and Foyer, 1998), which makes photorespiration a valuable pathway for generation of amino acids for glutathione synthesis.

1.3.7 Photoprotection by photorespiration

Although 2-PG is a futile and toxic byproduct of the dual function of Rubisco and photorespiration is often seen as a wasteful process, photorespiration also evolved to be a valuable sink for reducing power (Figure 1). The sink capacity of photorespiration has been suggested to be of major importance in photoprotection. Kozaki and Takeba (1996) identified ammonia assimilation as a bottleneck of photorespiration. *Arabidopsis thaliana* plants overexpressing glutamine synthase (GS) were found to have enhanced tolerance to high light intensities. In contrast, plants with reduced levels of GS were more severely affected by high light. Additionally, Osmond and Grace (1995) measured the contribution of photorespiration to photon usage. They concluded that photorespiration has a protective role because the electrons, which are dissipated by photorespiration, would otherwise lead to inhibition or destruction of the PSII center. Thus, the sink capacity of photorespiration could be crucial for the installation of transgenic approaches that lower photorespiration. In this chapter, the reasons for the sink capacity of photorespiration are explained.

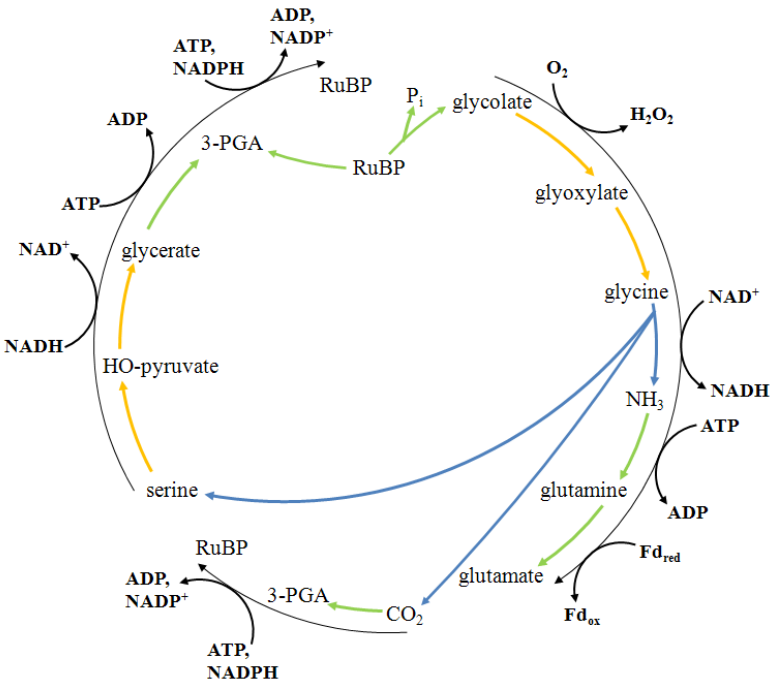

Figure 4: The major pathway of photorespiration in higher plants.

The figure is a schematic representation of the major photorespiratory pathway in higher plants including the energy balances of each reaction in bold letters. Green arrows represent plastidal reactions, yellow arrows peroxisomal, and blue arrows mitochondrial reactions. The refixation of photorespired CO_2 by the CBB cycle is included in the figure. RuBP, ribulose 1,5 bisphosphate; Pi, inorganic phosphate; 3-PGA, 3-phosphoglycerate; HO-pyruvate, hydroxypyruvate

As calculated by Peterhansel et al (2010) the oxygenase function of Rubisco and subsequent regeneration of RuBP from 3-PGA in the CBB cycle consumes statistically 3 molecules NADPH and 4.75 molecules ATP (chapter 2.1). Due to the dual function of Rubisco, the same conditions that promote accumulation of excess energy due to CO_2 limitation, also promote high flux through photorespiration (Ku and Edwards, 1977a, b; Lawlor and Fock, 1977; Jordan and Ogren, 1984; Brooks and Farquhar, 1985; Cornic and Briantais, 1991), thereby making an alternative sink distinct from carbon fixation accessible. The direct investment of energy for the photorespiratory C2 cycle has already been summarized (chapter 1.3.1, Figure 1) However, the major energy costs of photorespiration are due to CBB cycle activity. While 3-PGA can be metabolized in the CBB cycle the

second oxygenation product, 2-PG needs to be recycled at the expense of CO_2 release to reenter the CBB cycle. CO_2 again is a substrate for Rubisco and the reduction in the CBB cycle needs reducing power. Interruption of the CBB cycle at various reaction steps is shown to propagate photoinhibition by depletion of the CBB cycle and subsequent interruption of this electron sink (Takahashi et al., 2007). The net balance of energy usage of the CBB cycle as consequence of 2 oxygenations of RuBP is five molecules NADPH and 7.5 molecules ATP. Moreover, Haupt-Herting et al. (2001) calculated that in plants being exposed to severe drought stress, 60% of total CO_2 assimilation is due to re-assimilation of (photo)-respiratory CO_2 (Haupt-Herting et al., 2001). In line with these results Heber et al. (1996) showed that photosynthetic electron transfer is only reduced to 71% when CO_2-exchange is impaired due to stomatal closure. They identified photorespiration as major electron sink. Thus, under drought stress, the major activity of the CBB cycle can be directly linked to photorespiration. These data indicate the impressive role of photorespiration in energy dissipation.

Photorespiration can also be viewed as export mechanism for reducing power from chloroplasts (Igamberdiev et al., 2001). Glycolate that has been exported from chloroplasts undergoes two oxidation events. The first takes place in the peroxisome with oxygen as electron acceptor but here, the electrons are not used to generate reducing equivalents (Tolbert, 1981). The second takes place in the mitochondrion, in which NAD^+ is reduced to NADH. Igamberdiev et al. (2001) demonstrated that photorespiratory export of reducing power to mitochondria has high influence on their redox state. GDC deficient mutants have enhanced respiratory rates and induce redox transfer by activation of malate-oxaloacetate exchange to compensate for this loss. The export of photorespiratory reducing power to mitochondria can be such efficient that mitochondria accumulate excess reducing power. Reducing power is used by the mitochondrial electron transport chain to produce ATP by the generation of a protonmotive force that is used by the mitochondrial ATP synthase. Energy of excess NADH is dissipated in the mitochondrial electron transfer chain by uncoupling proteins (UCP) (Sweetlove et al., 2006), which enables the leakage of protons to lower the proton motive force.

Together, the capacity of photorespiration as electron sink strongly contributes to photoprotection. The thesis explains how different strategies of glycolate metabolism in photorespiration might contribute to energy dissipation and photoprotection.

1.4 Motivation

In the last years, several review articles were published about the topic photorespiration. However, each of them had its own focus on a special field of this topic. Bauwe et al. (2010, 2012) reviewed the evolution of photorespiration, Foyer et al. (2009) reported about enzymes and enzymatic regulation, Maurino and Peterhansel (2010) had their focus on metabolic engineering and some reviews show the connection between ammonia assimilation and photorespiration (Linka and Weber, 2005; Keys, 2006).

The review ‘Photorespiration’ integrates all topics into one article to get an insight into each field of photorespiration.

Photorespiration is one of the major pathways in plants (Szecowka et al., 2013). Photorespiration is the result of the oxygenase function of Rubisco and is the origin of a complex pathway that requires enzyme activities of three plant organelles: chloroplasts, peroxisomes and mitochondria. Photorespiration was first described as light dependent CO_2 release in photosynthetic tissue (Decker, 1954). First motivation to investigate this pathway was given by the observation that photorespiration is seemingly contra productive to photosynthesis. While photosynthesis generates oxygen and fixes carbon, photorespiration is a process of oxygen uptake and CO_2 release. Moreover, the oxygenation of ribulose 1,5 bisphosphate (RuBP) by Rubisco produces the toxic product 2-phosphoglycolate (2-PG). Recycling of 3-phosphoglycerate (3-PGA) by photorespiration produces two more toxic intermediates, H_2O_2 and glyoxylate. Additionally, photorespiration consumes energy in form of reducing power and ATP. Consequently, initial research on the pathway had the long-term objective to reduce flux through photorespiration. Approximately 60 years after photorespiration has been described as light enhanced respiration (Decker, 1954), this approach is still pursued. Three different pathways were engineered to minor losses through photorespiration (Peterhansel et al., 2008). Although two of them are successively transformed into the model plant *Arabidopsis thaliana* and the plants were shown to have enhanced growth and assimilation rates, the benefits of the pathways are discussed controversial by the community. This is mainly because the negative view of photorespiration is challenged by data that indicates an important role of photorespiration in plant metabolism (chapters 1.3.6, 1.3.7). Under conditions, when CO_2 availability is too low to keep pace with the conversion of light energy into ATP and NADPH, an alternative electron sink than CO_2 fixation in the CBB cycle is required. Photorespiration exports energy from chloroplasts to mitochondria, dissipates reducing power in the peroxisomes, and provides a futile cycle of ammonia release and subsequent refixation in chloroplasts. But the strongest sink is provided by the strong interconnection of photorespiration and the CBB cycle. Haupt Herting et al. (2001) calculated that 60% of CBB cycle activity in draught stressed plants can be deduced to photorespiration. Interestingly, the CBB cycle and photorespiration are directly linked by the dual function of Rubisco. Thus, as CO_2 becomes limiting, the oxygenase function is consequently upregulated to provide the CBB cycle with substrate for carbon reduction and energy consumption. A shortage of energy consuming pathways induces the generation of reactive oxygen species ROS (Voss et al., 2013). Photorespiration is shown to mitigate ROS production under various stress conditions like drought (Haupt-Herting et al., 2001), salinity (Yu et al., 2011) and chilling (Cheng et al., 2007) and a role for photorespiratory sink capacity and energy export mechanism in redox homeostasis has been postulated (Lepistö et al., 2009; Hebbelmann et al., 2012). In this respect, further evaluation is required, how and to which extend the transgenic approaches, which reduce photorespiration, could respond to these tasks of photorespiration.

The evolutionary origin of photorespiration can be tracked down to the beginning of oxygenic photosynthesis. Molecular oxygen could have accumulated locally in dense mats of photosynthesizing organisms. Thus, a basal photorespiratory cycle for metabolism of toxic 2-phosphoglycolate (2-PG) probably evolved even before the endosymbiotic event between a photosynthesizing prokaryote and a eukaryotic cell, which resulted in the evolution of contemporary chloroplasts. Synechocystis, one of the cyanobacterial descendants of these photosynthesizing prokaryotes, was shown to have three distinct pathways for glyoxylate metabolism. Interestingly, cyanobacteria and higher plants recruited different enzymes for catalysis of the same reactions of photorespiration, in especially 2-PG dephosphorylation and glycine decarboxylation (Kern et al., 2011). Both, the discovery of the three photorespiratory pathways in Synechocystis and the differential recruitment of enzymes for 2-PG metabolism demonstrate the variability of photorespiration in nature and indicate that there might be evolutionary remnants and alternative reactions. Interestingly, Niessen et al. (2007, 2012) could link a mitochondrial localized glycolate dehydrogenase to the major pathway by description of two alanine aminotransferases in Arabidopsis and rice. Timm et al. (2008, 2011) detected that there are also a cytosolic and a plastidal hydroxypyruvate reductase beneath the peroxisomal isoform in higher plants. Taking the metabolic flexibility of photorespiration and the cyanobacterial origin of photorespiration into account, a plastidal glycolate conversion pathway as evolutionary remnant seems promising. The plastidal decarboxylation of glycolate and glyoxylate has been observed several times in the last decades but the decarboxylation enzyme remained to be determined (Kisaki and Tolbert, 1969; Zelitch, 1972; Oliver, 1981; Kebeish et al., 2007).

To investigate the pathway, an established method for evaluation of the mitochondrial complexome was applied for the plastidal proteome of Arabidopsis thaliana (Klodmann et al., 2011) High resolution MS can be used to identify low abundance enzymes that might be responsible for specific reactions. 2D-BN/SDS-PAGE in combination with high resolution MS was shown to identify novel complexes in mitochondria (Klodmann et al., 2011). If the glyoxylate decarboxylase was attached to plastidal PGP to enable metabolic channeling, they could be found with this method. Additionally, contaminating organelles can be identified by aberrantly identified proteins. There is a mandatory demand to work with highly pure chloroplasts. Contamination could mask plastidal reactions by high rates of conversion by peroxisomal or mitochondrial enzymes. Contaminations with peroxisomal GO or mitochondrial GDC can be deleterious for all enzymatic activity based investigations in this topic. To achieve better annotation of the proteins, the proteome of *A. thaliana* was investigated.

The article “Photorespiration” gives a complete summary about this important pathway. The article is freely accessible online on http://thearabidopsisbook.org. The scope of the book is to ‘provide a scholarly and authoritative overview of the state of knowledge about the topic’ (http://thearabidopsisbook.org). Thus, the article does not present unpublished data, but shall provide the reader with state-of-the-art information about the pathway. The article describes several aspects of

photorespiration, provides detailed information about enzymes and enzymatic steps, the flexibility of the pathway in cyanobacteria and higher plants, the benefits of photorespiration and why there is a controversial dialogue about the importance of the pathway. Data is presented from more than 50 years of research, from the contradictory data mostly presented by Zelitch and Tolbert till the manipulation of the major pathway by various approaches.

The review “Photorespiratory bypasses: how can they work?” has its focus on 3 pathways that aim to enhance plant productivity by biotechnical installation of photorespiratory bypasses. By these, the flux through the photorespiratory C2 cycle shall be lowered with the consequence of lower energetic costs. These bypasses were never put into relation with each other and there is little understanding, how they cope with the new view of photorespiration as a mandatory pathway with an important role in energy dissipation. The core of the article lists the energy balances of the pathways calculated on a ‘status quo ante’ basis. This means that the energy costs for the reestablishement of the situation before the oxygenase event, in especially regeneration of RuBP, are integrated into the calculation. The energy balance is used to anticipate the influence of the pathways on primary metabolism and to estimate beneficial conditions.

The manuscript “A role for the chloroplast pyruvate dehydrogenase complex in plant glycolate and glyoxylate metabolism” has its focus on an open question of photorespiratory glycolate metabolism. Plastidal glycolate and glyoxylate conversion to CO_2 has been observed several times in the last decades but previous studies about plastidal glycolate or glyoxylate conversion failed to identify the responsible glycolate decarboxylase or glyoxylate decarboxylase, respectively. The natural pathway of plastidal glyoxylate decarboxylation became the focus of interest as two artificial photorespiratory pathways were engineered that base on plastidal glycolate conversion and decarboxylation and show enhanced phenotypes. The natural pathway was investigated if it could be a functional equivalent of one of these pathways.

The manuscript ‘The protein complex proteome of chloroplasts in *Arabidopsis thaliana*’ is a resource paper for plant physiologists. It was shown, that a similar technology could identify novel protein complexes in mitochondria of Arabidopsis thaliana. The proceedings in resolution of mass spectrometric data enable identification of low abundance proteins, which might be assigned to novel complexes. The manuscript is associated with a Gelmap software to enable protein search and spot identification of all identified proteins. Moreover, the high purity of the chloroplast in combination with high resolution mass spectrometry enabled identification and localization of proteins, which had no functional annotation before.

2 Publications and manuscripts

The review entitled ‘Photorespiration’ by Christoph Peterhänsel, Ina Horst, Markus Niessen, Christian Blume, Rashad Kebeish, Sophia Kürkcüoglu, and Fritz Kreuzaler is published in The Arabidopsis Book, 8 (2010), e0123. doi:0110.1199/tab.0130. All authors took part in writing the article while each author had his own chapter. Christoph Peterhänsel wrote the introduction and the conclusion and supervised the contributions of the other authors. Ina Horst wrote the chapter ‘The major photorespiratory pathway in Arabidopsis’, Christian Blume the chapter ‘The light side and the dark side of photorespiration’, Markus Niessen the chapter ‘Metabolic complexity: Photorespiratory pathways in Arabidopsis’, Sophia Kürkcüoglu the chapter ‘Metabolic complexity: Photorespiration is necessary for all organisms performing oxygenic photosynthesis, Rashad Kebeish the chapter ‘Metabolic Engineering: Manipulating photorespiration’. The figures were prepared by Christoph Peterhänsel, Ina Horst and Markus Niessen.

The review entitled ‘Photorespiratory bypasses: how can they work?’ by Christoph Peterhänsel, Christian Blume, and Sascha Offermann is published in the Journal of Experimental Botany, 64 (2012), 709-715. All authors took part in writing while Christoph Peterhänsel was the major contributor. Figure 1 and table 1 were prepared by Christian Blume while table 2 was prepared by Christoph Peterhänsel.

The manuscript entitled ‘A role for the plastidal pyruvate dehydrogenase complex for glycolate and glyoxylate metabolism’ by Christian Blume, Christof Behrens, Holger Eubel, Hans-Peter Braun, and Christoph Peterhänsel has been submitted to Phytochemistry and is currently under revision. Christof Behrens performed 2D BN/SDS PAGE and mass spectrometry analysis as well as identifying the proteins from the obtained spectra under supervision of Hans-Peter Braun. Evaluation of the protein data was performed by Christof Behrens and Christian Blume. Except from the data obtained by Christof Behrens, all laboratory work as well as data evaluation was performed and optimized by Christian Blume. All authors took part in writing the manuscript while Christian Blume and Christoph Peterhänsel were the major contributors. All figures were prepared by Christian Blume, except for figure 5 concerning 2D BN/SDS PAGE, which was prepared by Hans-Peter Braun.

The manuscriped entitled ‘The protein complex proteome of chloroplasts in Arabidopsis thaliana’ by Christof Behrens, Christian Blume, Michael Senkler, Holger Eubel, Christoph Peterhänsel, and Hans-Peter Braun has been submitted to Journal of Proteomics and is currently under revision. Christian Blume optimized the chloroplast purification and quality control of the chloroplasts. Christof Behrens performed 2D-BN/SDS-PAGE as well as mass spectrometry analysis. Protein data was evaluated by Christof Behrens and Hans-Peter Braun, who also prepared the annotation for the Gelmap3.0 software

developed by Michael Senkler. Christof Behrens annotated the proteins for subcellular localization based on SUBA3 database. All authors took part in writing the manuscript with Christof Behrens and Hans-Peter Braun as major contributors. The figures were prepared by Christof Behrens and Hans-Peter Braun except for Figure 1 concerning the chloroplast intactness assay, which was prepared by Christian Blume.

2.1 Photorespiration

The Arabidopsis Book

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Photorespiration

Christoph Peterhansel,[a,1] Ina Horst,[a] Markus Niessen,[a] Christian Blume,[a] Rashad Kebeish,[a] Sophia Kürkcüoglu,[a] and Fritz Kreuzaler[b]

[a] Leibniz University Hannover, Institute of Botany, Herrenhaeuser Strasse 2, 30419 Hannover, Germany
[b] RWTH Aachen University, Institute of Botany, Worringer Weg 1, 52056 Aachen, Germany
[1] Address correspondence to cp@botanik.uni-hannover.de

Photorespiration is initiated by the oxygenase activity of ribulose-1,5-bisphosphate-carboxylase/oxygenase (RUBISCO), the same enzyme that is also responsible for CO_2 fixation in almost all photosynthetic organisms. Phosphoglycolate formed by oxygen fixation is recycled to the Calvin cycle intermediate phosphoglycerate in the photorespiratory pathway. This reaction cascade consumes energy and reducing equivalents and part of the afore fixed carbon is again released as CO_2. Because of this, photorespiration was often viewed as a wasteful process. Here, we review the current knowledge on the components of the photorespiratory pathway that has been mainly achieved through genetic and biochemical studies in Arabidopsis. Based on this knowledge, the energy costs of photorespiration are calculated, but the numerous positive aspects that challenge the traditional view of photorespiration as a wasteful pathway are also discussed. An outline of possible alternative pathways beside the major pathway is provided. We summarize recent results about photorespiration in photosynthetic organisms expressing a carbon concentrating mechanism and the implications of these results for understanding Arabidopsis photorespiration. Finally, metabolic engineering approaches aiming to improve plant productivity by reducing photorespiratory losses are evaluated.

1. INTRODUCTION

1.1. The Origin and Significance of Photorespiration

Photorespiration is the process of light-dependent uptake of molecular oxygen (O_2) concomitant with release of carbon dioxide (CO_2) from organic compounds. The gas exchange resembles respiration and is the reverse of photosynthesis where CO_2 is fixed and O_2 released. As shown in Figure 1, the entrance reactions to both photosynthesis and photorespiration are catalyzed by the same enzyme: Ribulose-1,5-bisphosphate-carboxylase/oxygenase (RUBISCO, EC 4.1.1.39). Carbon fixation results in the formation of two molecules 3-phosphoglycerate (PGA) that are integrated into the Calvin cycle ultimately to form sugars. During oxygen fixation, one molecule of PGA and one molecule of 2-phosphoglycolate are formed. The latter is converted back to PGA in the photorespiratory cycle. The pathway requires energy (ATP) and reducing (NAD(P)H) equivalents. This is in great part due to the release of CO_2 and ammonia (NH_3) that have to be refixed (for details see chapter 2).

RUBISCO evolved already early in biotic evolution about 3 billion years ago probably from enzymes involved in sulfur metabolism (Tabita et al., 2007), but is until today the enzyme accounting for the vast amount of net CO_2 fixation from the atmosphere. At the time of RUBISCO evolution, the only source of molecular oxygen in the atmosphere was probably photolysis of water by UV light and concentrations were 10^{-14} below present concentrations (Buick, 2008). At the same time, CO_2 concentrations were at least 100-fold higher than today (Kasting and Howard, 2006; Kasting and Ono, 2006). Initial RUBISCO enzymes were probably extremely bad in discriminating CO_2 and O_2 (Tabita et al., 2007; Badger and Bek, 2008) due to the absence of evolutionary pressure. With the advent of oxygenic photosynthesis in cyanobacteria, huge amounts of CO_2 were fixed into biomass that in part sedimented and did not return into the global carbon cycle. Concomitantly, equimolar amounts of O_2 were released into the atmosphere, because water was used as the reductant for the photosynthetic electron transport chain (Xiong and Bauer, 2002). Cyanobacteria, the subsequently evolving algae and particularly land plants (Igamberdiev and Lea, 2006) were so successful in doing this that O_2 became the second most prominent gas in today's atmosphere and CO_2 is extremely scarce. Selection pressure induced some improvement in RUBISCO's specificity for CO_2 that might not be further optimized without slowing down catalytic rates (Tcherkez et al., 2006): The more the structure of the bound CO_2 molecule resembles a carboxylate group, the better it will be discriminated from O_2, but the worse the first intermediate of the condensation can be cleaved into the end products of the

reaction. Therefore, O_2 uptake by RUBISCO and the subsequent metabolism of the reaction product in the photorespiratory pathway is the second highest metabolite flux in plants behind photosynthesis with flux rates around 25 % of the photosynthetic rate at 25 °C (Sharkey, 1988). Photorespiratory fluxes are even higher under hot and dry growth conditions mainly for three reasons:

1. The specificity of RUBISCO for CO_2 relative to O_2 decreases with temperature (Ku and Edwards, 1977a; Jordan and Ogren, 1984; Brooks and Farquhar, 1985).
2. The solubility of O_2 in aqueous solutions such as the cytoplasm and the chloroplast stroma is less affected by higher temperatures than the solubility of CO_2 (Ku and Edwards, 1977b).
3. Plants exposed to limited water supply reduce gas exchange in order to minimize evaporation. CO_2 concentrations around RUBISCO rapidly decrease whereas O_2 is available in excess (Lawlor and Fock, 1977; Cornic and Briantais, 1991). This favors the oxygenase over the carboxylase reaction.

Under such conditions, the leaf internal CO_2 concentration may approach the apparent CO_2 compensation point where CO_2 uptake by photosynthesis equals CO_2 release by photorespiration and respiration. This finally results in an inability of the plant to grow autotrophically (Sage, 2004).

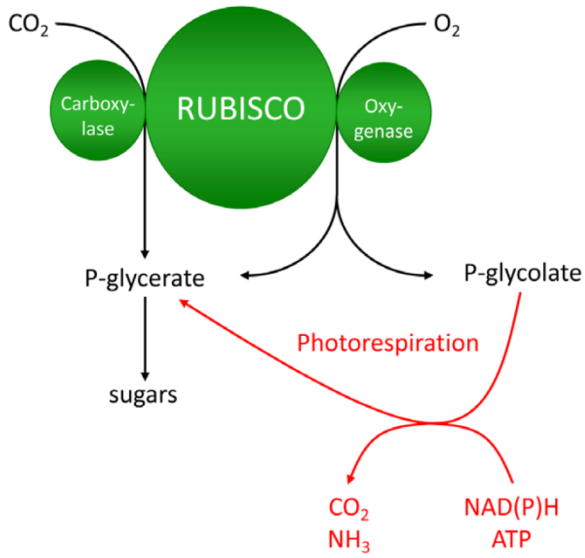


Figure 1: Schematic overview of photosynthesis and photorespiration.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) catalyzes both CO_2 and O_2 fixation. The product of CO_2 fixation is phosphoglycerate (P-glycerate) that enters the Calvin cycle. During oxygenation, equimolar amounts of P-glycerate and phosphoglycolate (P-glycolate) are formed. P-glycolate is recycled to P-glycerate in the photorespiratory pathway. In this reaction cascade, reducing equivalents (NAD(P)H) and energy equivalents are consumed. Ammonia (NH_3) and CO_2 are released and have to be refixed. Under current atmospheric gas concentrations and moderate environmental conditions, approximately each fourth reaction catalyzed by RUBISCO is an oxygenase reaction.

1.2. Photorespiration: An Early Case Study for Arabidopsis Genetics

In the early 1970s, Arabidopsis was introduced as a plant model for genetic analyses. Before, the genetics of other plants such as barley or maize had been studied extensively, but a plant with features of a model organism was required: These features included a small genome size, efficient inbreeding, a small space required for growth and a short generation time (Redei, 1975). The necessity of a genetic approach in photorespiration research and the benefits of the Arabidopsis system were early recognized by Chris and Shauna Somerville in William Ogren's laboratory. At that time, there were still ongoing debates whether the substrate for photorespiration was produced by the oxygenase activity of RUBISCO and which biochemical reaction resulted in the release of CO_2. The conflicting scientific hypotheses are excellently reviewed in Ogren (2003). The Somervilles' approach was to chemically mutagenize Arabidopsis seeds and to grow the M2 generation derived from this mutagenesis at 1 % CO_2 (see Figure 2). The rate of photorespiration was very low under these conditions, thus, mutants in the pathway were expected to grow normally. At seedling stage, all plants showing growth defects or chlorosis were discarded to remove mutants with developmental defects apart from photorespiration. The remaining plants were shifted to normal air with a CO_2 concentration of approximately 0.03 %. Plants that developed chlorosis under these conditions were identified as potential mutants in components of the photorespiratory pathway. This was again verified by shifting plants back to 1 % CO_2 where photorespiratory mutants should recover from growth inhibition. Using this strategy, they identified "dozens" of plants with an inheritable conditional chlorotic phenotype at ambient CO_2 concentrations (Somerville, 2001). Genetic mapping and map-based isolation of the mutated gene (Jander et al., 2002; Peters et al., 2003) was not straightforward in Arabidopsis at these times, thus, enzymatic assays, metabolite profiles and gas exchange measurements were used to characterize the nature of the genetic lesion causing oxygen sensitivity. The first published mutant carried a mutation in phosphoglycolate phosphatase (PGLP, Somerville and Ogren, 1979) which indicated that phosphoglycolate is indeed the source substance for photorespiration. Together with earlier results that molecular oxygen is a competitive inhibitor of CO_2 fixation and that RUBISCO can form phosphoglycolate from ribulose-1,5 bisphosphate and O_2 (Bowes et al., 1971; Bowes and Ogren, 1972), this result unequivocally linked photorespiration to the oxygenase activity of RUBISCO. Shortly after, analysis of a mutant in mitochondrial serine hydroxymethyltransferase (SHM) defined the major biochemical reaction resulting in photorespiratory CO_2 release (Somerville and Ogren, 1981). Mutants deficient in chloroplastic glutamate-oxoglutarate aminotransferase (GLU, Somerville and Ogren, 1980b) and a chloroplast dicarboxylate transporter (DIT, Somerville and Ogren, 1983) confirmed the earlier observations by Keys et al. (1978) that ammonia released during photorespiration is refixed in the chloroplast. These and other mutants are described in more detail in chapter 2. This screen is one of the first examples where the power of Arabidopsis as a tool for plant genetic studies was proven. However, a similar screen performed shortly later in barley identified beside mutants known from Arabidopsis additional mutants in catalase (CAT, Kendall et al., 1983) and glutamine synthetase (GS, Wallsgrove et al., 1987)

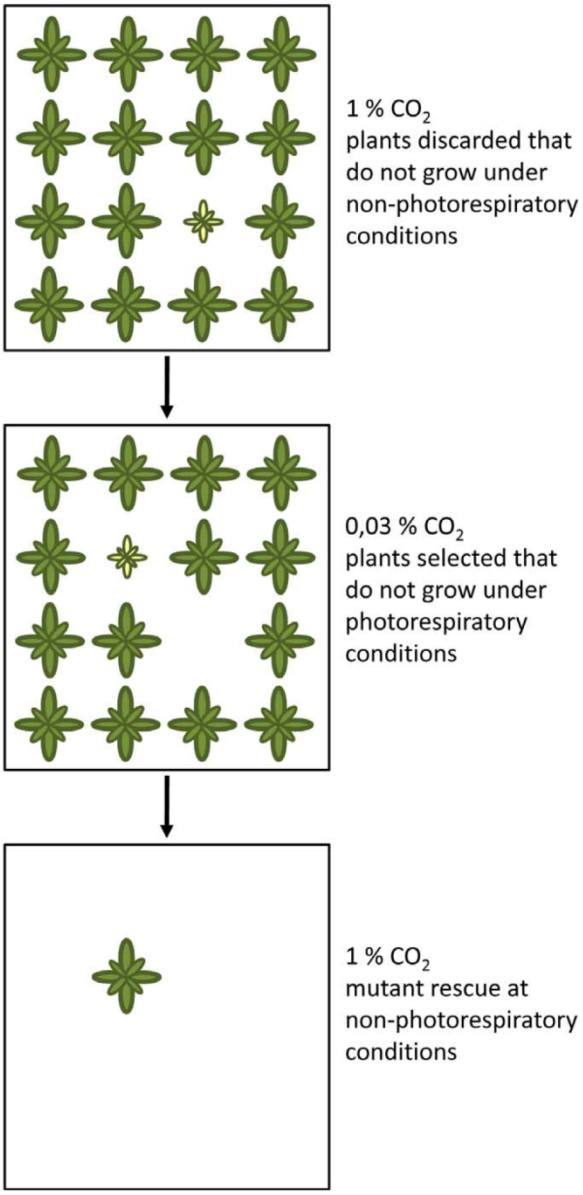


Figure 2: Screening for photorespiratory mutants of Arabidopsis**.**

A segregating M2 population is grown at a high CO_2 concentration that suppresses photorespiration. Stunted or chlorotic plants at this stage probably carry defects in genes unrelated to photorespiration and are discarded. Healthy plants that become chlorotic after the population was shifted to atmospheric CO_2 concentrations are candidates for photorespiratory mutants. These mutants are rescued by shifting plants back to a high CO_2 concentration.

that have not been recovered in the Arabidopsis screens. Mutants for some of the major photorespiratory genes were never identified. Today, we know that this was in part due to genetic redundancy, e.g. glycolate oxidase (GO) is encoded by multiple genes (Reumann, 2004). Other genes are also required for growth under non-photorespiratory conditions (Engel et al., 2007) or alternative pathways can partially replace the deleted activity (Niessen et al., 2007; Timm et al., 2008). The latter case is discussed in more detail in chapter 4. In summary, the mutant approach clarified many open questions about the major photorespiratory pathway in Arabidopsis. It is now evident that most of the components are shared and that photorespiration is required by all oxygenic photosynthetic organisms (see chapter 5).

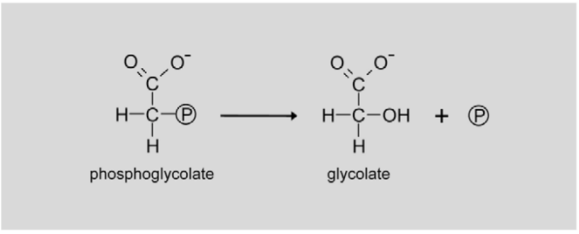
The work on photorespiration during these years was motivated by the expectation that a reduction of photorespiration could significantly improve plant productivity (Zelitch and Day, 1973; Kelly and Latzko, 1976). However, attempts to identify second-site mutations that could alleviate the deleterious effects of a disrupted photorespiratory pathway were never successful (Somerville and Ogren, 1982b). Metabolic engineering approaches now indicate that deviation of some of the glycolate from photorespiration into novel pathways might indeed enhance biomass production and possibly yield (see chapter 6). Moreover, numerous studies describe photorespiration as a beneficial pathway that supports growth under stress conditions (Wingler et al., 2000) or that provides metabolites for other basal metabolic pathways of the plant (Novitskaya et al., 2002). These positive aspects of photorespiration are exemplified in chapter 3.

2. THE MAJOR PHOTORESPIRATORY PATHWAY IN ARABIDOPSIS

In this chapter, we describe the biochemical pathway that converts the vast amount of 2-phosphoglycolate (PG) to 3-phosphoglycerate (PGA). The pathway components were mainly identified by molecular and biochemical studies in Arabidopsis. Figure 3 provides an overview of the major photorespiratory pathway. Each specific reaction is discussed below.

2.1. Enzymatic Reactions in the Major Photorespiratory Pathway

2.1.1. Dephosphorylation of 2-Phosphoglycolate



2-Phosphoglycolate phosphatase (PGLP, EC 3.1.3.18) catalyzes the entry reaction of photorespiration, dephosphorylation of phosphoglycolate formed by the oxygenase activity of RUBISCO to glycolate. Knock-outs of this gene requiring high CO_2 for survival have been identified in both the Arabidopsis and barley mutant

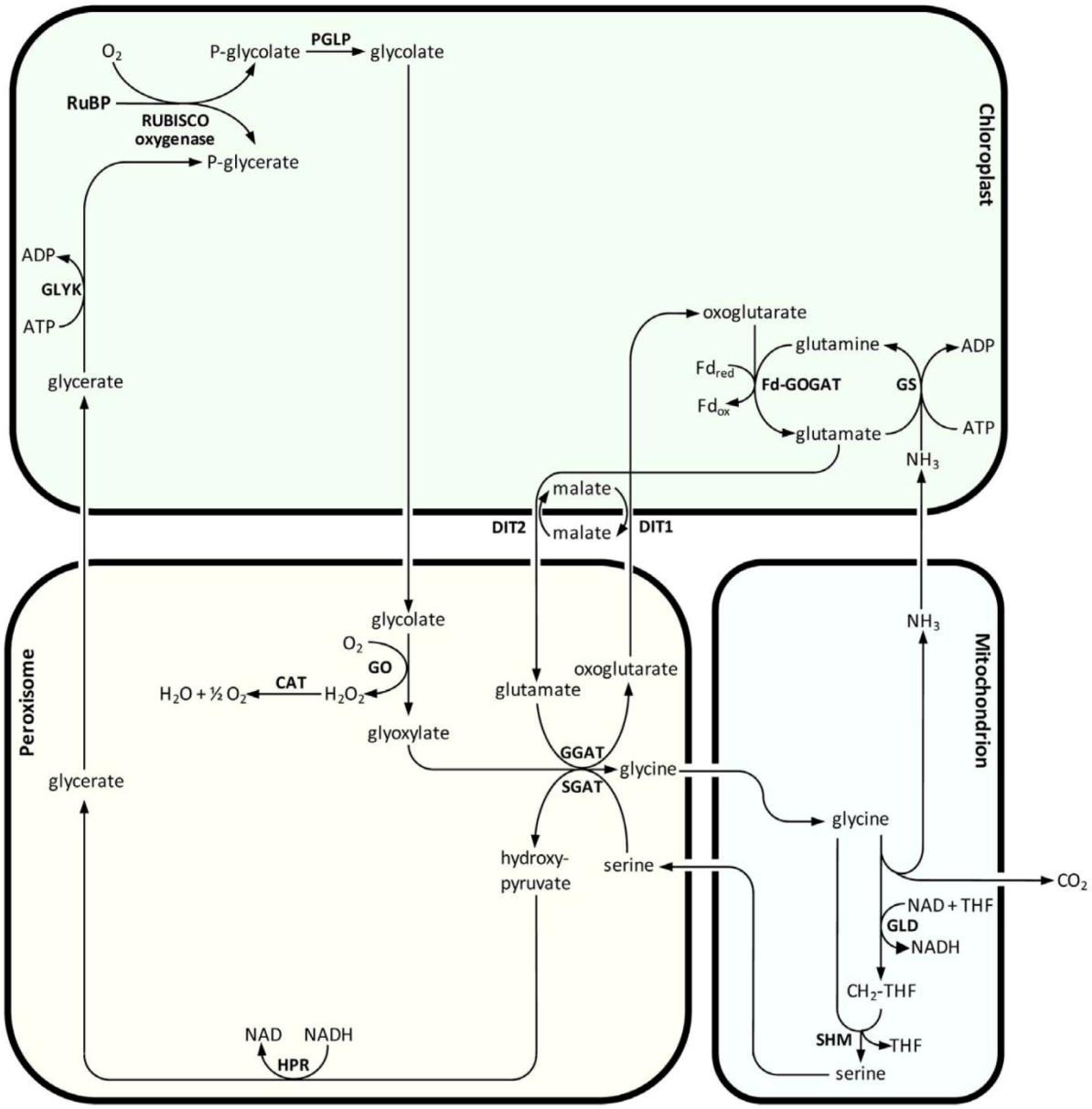


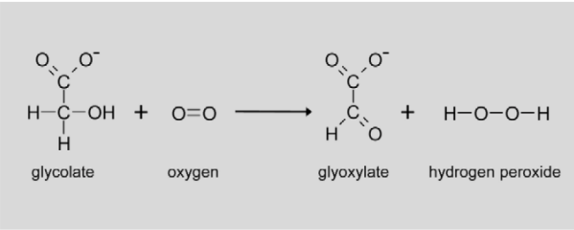
Figure 3. The major photorespiratory pathway in Arabidopsis.

Overview of the major photorespiratory pathway in Arabidopsis. For details and chemical structures, see text. RuBP, ribulose-1,5-bisphosphate; RUBISCO, RuBP carboxylase/oxygenase; PGLP, phosphoglycolate phosphatase; GO, glycolate oxidase; CAT, catalase; GGAT, glyoxylate:glutamate aminotransferase; SGAT, serine:glyoxylate aminotransferase; DIT1, dicarboxylate transporter 1; DIT2, dicarboxylate transporter 2; GLD, glycine decarboxylase; SHM, serine hydroxymethyltransferase; HPR, hydroxypyruvate reductase; GLYK, glycerate kinase; GS, glutamine synthetase; Fd-GOGAT, glutamine:oxoglutarate aminotransferase; THF, tetrahydrofolate; CH_2-THF, 5,10-methylene-THF. The stoichiometry of the reactions is not included.

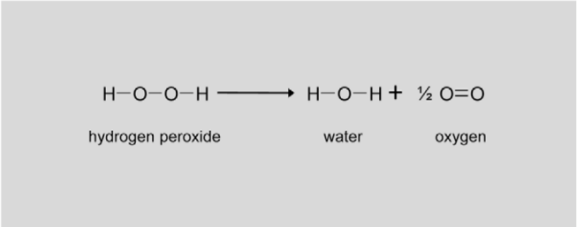
screens (see chapter 1) indicating that the functional PGLP is encoded by a single gene. The Arabidopsis mutant CS119 revealed oxygen-sensitive photosynthesis, strong accumulation of phosphoglycolate and absence of PGLP activity (Somerville and Ogren, 1979). The responsible gene has been recently identified by Schwarte and Bauwe (2007) using a candidate gene approach. T-DNA insertion lines for two (At5g36700 encoding AtPGLP1 and At5g47760 encoding AtPGLP2) of a total of 13 annotated potential *Pglp* genes in Arabidopsis were selected based on homology of the deduced protein to the afore identified *Chlamydomonas* PGLP. Only one of the two lines showed the typical conditional lethal phenotype. When this gene was sequenced in mutant CS119 lacking PGLP activity, a splice site mutation was identified that resulted in the formation of non-functional transcripts. Thus, At5g36700 encodes the photorespiratory PGLP in Arabidopsis.

There is relatively little known about the regulation of PGLP in Arabidopsis. The gene is expressed in leaves and green parts of flowers. Transcription of the gene is induced by light and repressed by osmotic stress or low nitrate availability (Foyer et al., 2009). Biochemical analyses have been performed with purified enzyme form spinach, tobacco, *Chlamydomonas* and cyanobacteria. These analyses revealed an usual requirement for both cations such as Mg^{2+} and anions such as Cl^- for enzymatic activity (Husic and Tolbert, 1984; Norman and Colman, 1991). It was suggested that the phosphate moiety of phosphoglycolate is transferred to the protein and that the Cl^- ion is required for hydrolysis of the reaction intermediate (Seal and Rose, 1987). Whereas the spinach enzyme was allosterically inhibited by ribose-5-phosphate (Husic and Tolbert, 1984), activation by the same compound was described for the *Chlamydomonas* enzyme in another study (Mamedov et al., 2001).

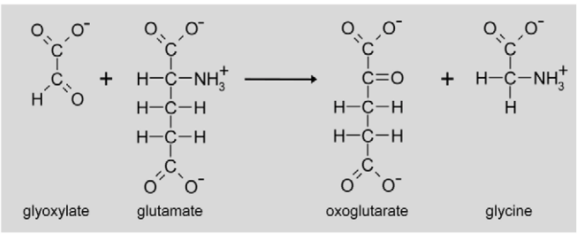
2.1.2. Glycolate metabolism in the peroxisome



Glycolate oxidase (GO, EC 1.1.3.15) belongs to the class of flavin-dependent 2-hydroxy acid oxidases and catalyzes the oxidation of glycolate to glyoxylate. Molecular oxygen is used as a co-substrate and, thus, H_2O_2 is produced by the enzyme. The *Go* gene family in Arabidopsis contains 5 members of which three are potentially localized in the peroxisome (Reumann, 2004; Reumann et al., 2007). Two of these genes (At3g14415 and At3g14420) are transcriptionally co-regulated with other photorespiratory genes with regard to tissue-specific expression as well as positive regulation by light and nitrogen (Reumann and Weber, 2006; Foyer et al., 2009). Probably because of this genetic redundancy, no mutants were identified in the classical Arabidopsis and barley screens (Somerville and Ogren, 1982b; Blackwell et al., 1988). In tobacco and rice, lines with reduced GO activity were generated by co-suppression (Yamaguchi and Nishimura, 2000) and inducible antisense expression (Xu et al., 2009). These studies revealed a positive and linear correlation between GO-activity and the net photosynthetic rate. Once photosynthetic rates fell below 40% of control plants, photoinhibition occurred indicating that GO can exert a strong regulatory effect on photosynthesis, possibly through a reduction in RUBISCO activation state (Xu et al., 2009). Beside metabolic regulation, GO activity is also induced by pathogen attacks. This is probably due to the formation of H_2O_2 and its importance as a second messenger in plant defense reactions (Taler et al., 2004, see also chapter 3).



H_2O_2 produced by the glycolate oxidase reaction is disproportionated to H_2O and O_2 by catalase (CAT, EC 1.11.1.6) in order to prevent accumulation of reactive oxygen species. In Arabidopsis, three catalase genes are known, but only one gene (*Cat2*, At4g35090) encodes the major enzyme involved in photorespiration. In ambient air, a mutation in *Cat2* caused severely decreased rosette biomass, intracellular redox perturbation and activation of oxidative signaling pathways. These effects were absent when mutant plants were grown at high CO_2 concentrations (Queval et al., 2007). *Cat2* gene expression is light-stimulated and clock-regulated with an induction in the early morning (Zhong et al., 1997; Queval et al., 2007).

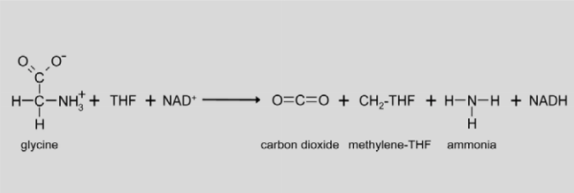


The next reaction step in the photorespiratory pathway is the transamination of glyoxylate to glycine by glutamate:glyoxylate aminotransferase (GGAT, EC 2.6.1.4). In Arabidopsis, two loci encode peroxisomal glutamate:glyoxylate aminotransferases (At1g23310 encoding GGAT1 and At1g70580 encoding GGAT2) (Igarashi et al., 2003; Liepman and Olsen, 2003; Igarashi et al., 2006). *Ggat*1 knock-outs show a weak photorespiratory phenotype and survive in air at moderate light intensities. Residual GGAT activity can be detected in mutant leaf extracts (Igarashi et al., 2003). Furthermore, both genes exhibit a differential diurnal expression pattern. Whereas *Ggat*2 is induced at the beginning of the photoperiod, *Ggat*1 is rather repressed. However, GGAT activity in leaves remains constant during the day (Igarashi et al., 2006) suggesting that both loci contribute to the total leaf GGAT content.

*Ggat*1 is not only highly expressed in leaves but also in roots, indicating that its function is not restricted to photorespiration.

2.1.3. Mitochondrial conversion of glycine to serine

The combined reaction of glycine decarboxylase (GLD) and serine hydroxymethyltransferase (SHM) represents the mitochondrial part of the major photorespiratory pathway. Glycine is decarboxylated and deaminated by the multienzyme complex glycine decarboxylase yielding CO_2, NH_3, NADH and 5,10-methylene-tetrahydrofolate. The latter is used by SHM as a substrate for the transfer of an activated C1 moiety to another molecule of glycine resulting in the formation of serine.



The GLD reaction involves four different proteins (Figure 4). P-protein (EC 1.4.4.2) is a pyridoxal-5-phosphate-containing homodimer, that catalyzes the actual glycine decarboxylation reaction (Bauwe and Kolukisaoglu, 2003). The remaining amino methylene part of glycine is transferred to the distal sulphur atom of the oxidized lipoamide arm of H-protein (Douce et al., 2001). H-protein is not an enzyme component of the complex but a co-factor that interacts with all three enzymes of the complex. The amino methylene group bound to the H-protein is further metabolized by the monomeric aminomethyltransferase T-protein (EC 2.1.2.10) (Douce and Neuburger, 1999). During this reaction, NH_3 is released and a methylene group is transferred to tetrahydrofolate resulting in the formation of 5,10-methylene tetrahydrofolate (CH_2-THF) (Bauwe and Kolukisaoglu, 2003). Concomitantly, the lipoamide arm of H-protein becomes fully reduced and needs to be re-oxidized for the next reaction cycle. This reaction is catalyzed by L-protein, a homodimeric dihydrolipoamide dehydrogenase (EC 1.8.1.4). Electrons are first transferred to the co-factor FAD that is re-oxidized by NAD^+ resulting in the formation of NADH (Bauwe and Kolukisaoglu, 2003). The four proteins are present in unequal molar amounts in the complex. For pea, a subunit stoichiometry of 4P:27H:9T:2L was observed (Oliver et al., 1990), but the functional relevance of this composition of the complex is still elusive. In Arabidopsis, the four proteins are encoded by eight genes, two genes each for P-protein (At4g33010 and At2g26080) and L-protein (At3g17240 and At1g48030), three genes for H-protein (At2g35370, At2g35120 and At1g32470) and one gene for T-protein (At1g11860) (Bauwe and Kolukisaoglu, 2003). Most of these genes are strongly light regulated and some are repressed by low nitrogen concentrations, like most other genes contributing to the photorespiratory pathway (Foyer et al., 2009 and http://www.genevestigator.com).

The classical Arabidopsis mutant *gld*1 showing a photorespiratory phenotype related to loss of function of the GLD complex (Somerville and Ogren, 1982a) was characterized on the

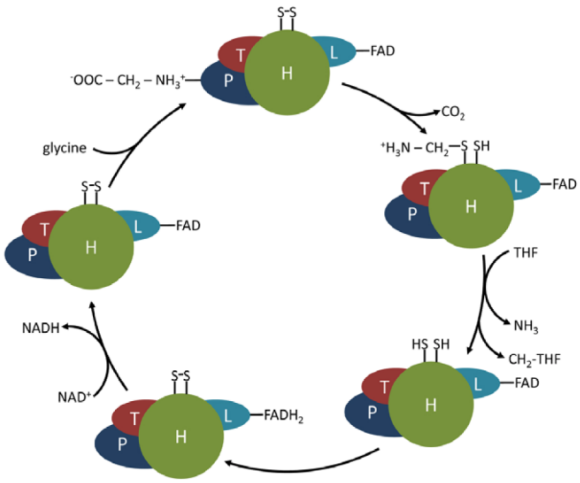
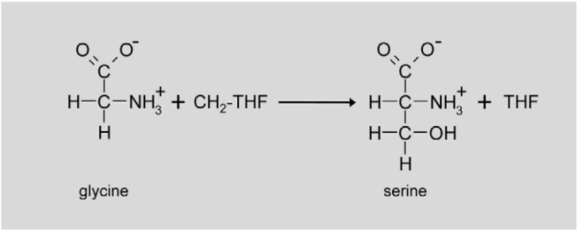


Figure 4: The glycine decarboxylase complex in Arabidopsis.

Glycine cleavage by the glycine decarboxylase (GLD) complex. GLD consists of P-, H-, T- and L-protein. THF = tetrahydrofolate; CH_2-THF = 5,10-methylene THF. Glycine binds to the pyridoxalphosphate co-factor (not shown) of P-protein, is decarboxylated and transferred to H-protein. T-protein releases NH_3 and transfers the methylene group to THF. L-protein catalyzes the re-oxidation of H-protein. The subunit stoichiometry is not shown and the size of the symbols does not imply the size of the proteins. For further details, see main text.

molecular level by Ewald et al. (2007). The mutant is deficient in a 3-oxoacyl-(acyl-carrier-protein) synthase (At2g04540, *Mt-kas*1) that is required for lipoylation of H-protein. Some residual lipoylation by an independent second pathway might be sufficient to rescue the phenotype under non-photorespiratory conditions. In contrast, the combined knock-out of the two genes encoding P-protein is lethal under all growth conditions (Engel et al., 2007) suggesting that the GLD reaction is not only essential for photorespiration, but also for the synthesis of activated C1 moieties required for nucleic acid and amino acid metabolism (Cossins and Chen, 1997)

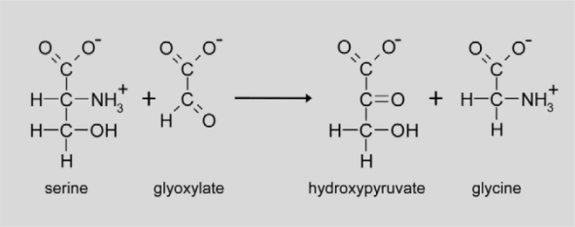


The photorespiratory SHM enzyme (EC. 2.1.2.1) is encoded by a single gene (At4g37930) in Arabidopsis. A knock-out of this gene is lethal at ambient CO_2 levels, but mutants grow normally at high CO_2 (Somerville and Ogren, 1981; Voll et al., 2006). The mRNA abundance is positively regulated by light and the circadian clock, and the gene is not expressed in roots (McClung et al., 2000). Recently, a second class of mutants deficient in mito-

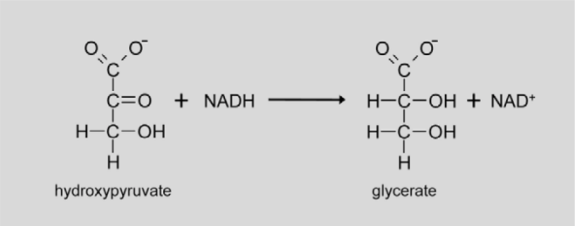
chondrial SHM activity was identified (Jamai et al., 2009). A mutation in *Glu*1 (At5g04140) which encodes ferredoxin-dependent glutamine:oxoglutarate aminotransferase (Fd-GOGAT (TAIR abbreviation: GLU); EC 1.4.7.1) reduced mitochondrial SHM activity. It was known before that this protein is involved in ammonia re-assimilation in the chloroplast during photorespiration (Coschigano et al., 1998, see also below), but the new results indicate that Fd-GOGAT is targeted not only to the chloroplast but also to the mitochondria where it physically interacts with SHM. This interaction is necessary for photorespiratory SHM activity (Jamai et al., 2009).

An additional level of regulation of the GLD/SHM complex was recently identified by characterization of plants that were unable to convert 10-formyl-THF, an intermediate of C1 metabolism (Hanson and Roje, 2001), to formate and THF (Collakova et al., 2008). In these plants, the isomer 5-formyl-THF accumulated and inhibited photorespiration resulting in retarded growth and glycine accumulation. Thus, 5-formyl-THF might act as a physiological regulator of GLD/SHM activity *in vivo*.

2.1.4. Formation of glycerate in the peroxisome

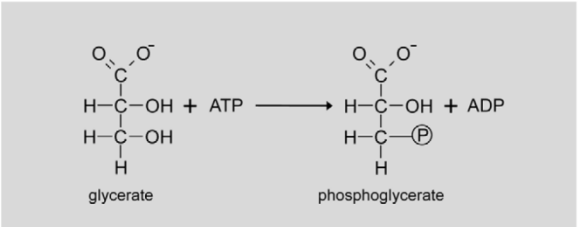


Back in the peroxisome, serine is deaminated to hydroxypyruvate by serine:glyoxylate aminotransferase (SGAT (TAIR abbreviation: AGT), EC 2.6.1.45). The amino group is used for the concomitant amination of glyoxylate to glycine. This is important for the stoichiometry of photorespiration, because only one amino donor for the GGAT reaction is recycled during NH_3 re-assimilation (see below), but two glycine are required for the mitochondrial formation of serine. In Arabidopsis, SGAT is encoded by a single gene (At2g13360) and its deletion results in plants that are unviable under normal atmospheric conditions (Somerville and Ogren, 1980a; Liepman and Olsen, 2001). A similar phenotype was also described for a barley SGAT mutant (Murray et al., 1987). The Arabidopsis gene is induced by light and repressed by low nitrogen like most of the other photorespiratory genes (Foyer et al., 2009), but transcripts are also detected in flowers and siliques apart from leaves (Liepman and Olsen, 2001).



Hydroxypyruvate resulting from the SGAT reaction is reduced to glycerate by hydroxypyruvate reductase (HPR, EC 1.1.1.29). In Arabidopsis, the peroxisomal reaction is catalyzed by HPR1 (At1g68010) using NADH as a co-factor (Givan and Kleczkowski, 1992). The *Hpr*1 gene shows similar regulatory properties like *Sgat* with positive light regulation and repression under low nitrogen conditions (Foyer et al., 2009). Reducing equivalents for the reaction are probably imported into the peroxisome by a malate/oxaloacetate shuttle and released by a peroxisomal malate dehydrogenase (pMDH, Cousins et al., 2008). However, Arabidopsis mutants with deletions in the genes encoding HPR1 (Timm et al., 2008) and pMDH (Pracharoenwattana et al., 2007) as well as barley HPR mutants (Murray et al., 1989) do not show drastic photorespiratory phenotypes. These data suggest the existence of alternative pathways for the conversion of hydroxypyruvate to glycerate or other metabolites that are further discussed in chapter 4.

2.1.5. Regeneration of phosphoglycerate

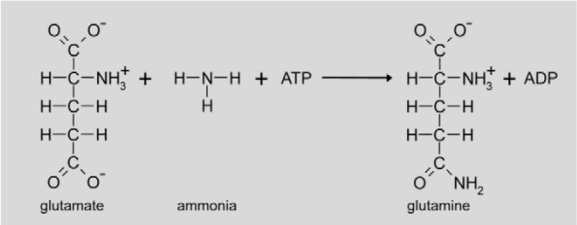


The chloroplastic D-glycerate 3-kinase (GLYK, EC 2.7.1.31) catalyzes the phosphorylation of glycerate to 3-phosphoglycerate with ATP as a co-substrate. As no mutants were identified in the classical screens, the gene was instead isolated by purification of the enzymatic activity from Arabidopsis leaf extracts and partial sequencing of the purified protein (Boldt et al., 2005). GLYK is encoded by a single copy gene (At1g80380). Knock-out of this gene results in a conditionally lethal photorespiratory phenotype. The enzyme is the founder of a structurally and phylogenetically novel protein family that is different from known glycerate kinases of non-photosynthetic organisms (Boldt et al., 2005), albeit sequence homologues are also found in yeast (Bartsch et al., 2008). Transcriptional regulation of the *Glyk* gene is different from most of the other photorespiratory genes. Particularly, *Glyk* gene expression is not activated by light (Foyer et al., 2009).

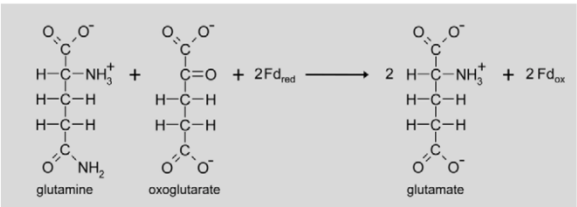
2.1.6. Ammonia re-assimilation

During the GLD reaction, one-quarter of the carbon that entered the photorespiratory cycle and an equimolar amount of NH_3 are released. Re-assimilation of NH_3 takes place in the chloroplast through the sequential action of two enzymes, glutamine synthetase (GS; EC 6.1.3.2) and ferredoxin-dependent glutamine:oxoglutarate aminotransferase (Fd-GOGAT (TAIR abbreviation: GLU); EC 1.4.7.1) (Keys, 2006). NADH-GOGAT (EC 1.4.1.14), that is additionally found in chloroplasts, does not play a significant role in photorespiration (Coschigano et al., 1998).

The net reaction of the GS/Fd-GOGAT cycle is the amination of oxoglutarate to glutamate consuming one ATP and two reduced ferredoxin.



First, GS catalyses the amination of glutamate to glutamine which requires one ATP. GS mutants have not been identified in Arabidopsis, but in barley (Wallsgrove et al., 1987). The barley mutant showed the typical requirement for elevated CO_2 concentrations emphasizing the major contribution of photorespiration to plant NH_3 production and re-assimilation (Keys, 2006). Six Arabidopsis genes encode GS enzymes, but only GS2 (encoded by At5g35630) is targeted to the chloroplast (Peterman and Goodman, 1991). Transcriptional regulation of the gene for GS2 is similar to most photorespiratory genes including light induction and repression by low nitrogen (Peterman and Goodman, 1991; Foyer et al., 2009).



Fd-GOGAT uses the glutamine formed by GS as an amino donor for the transamination of oxoglutarate. Two molecules of glutamate are the product of this reaction. Whereas one glutamate is used as a substrate for the next NH_3 assimilation reaction by GS, the second is transferred to the peroxisome for transamination of glyoxylate to glycine. Arabidopsis contains two expressed genes for Fd-GOGAT (*Glu*1 (At5g04140) and *Glu*2 (At2g41220)), but only mutation of the *Glu*1 gene resulted in a photorespiratory phenotype (Coschigano et al., 1998). *Glu*1 mRNA shows highest abundance in leaves and is specifically induced by light and sucrose. In contrast, *Glu*2 is only slightly expressed in leaves, but the mRNA preferentially accumulates in roots (Coschigano et al., 1998). Although the photorespiratory nitrogen cycle is closed in theory as all the re-fixed NH_3 is required for transamination of glyoxylate, some excess NH_3 might escape fixation in the GS/Fd-GOGAT cycle. There is physiological evidence that formation of carbamoyl phosphate and ultimately arginine provides an alternative route for NH_3 re-assimilation (Potel et al., 2009).

As described above, Fd-GOGAT has been shown to be dual targeted to the chloroplast and the mitochondrion (Jamai et al., 2009). Mitochondrial localization is seemingly not important for NH_3 re-assimilation, but rather for regulation of SHM activity. However, dual targeting has also been suggested for GS2 based on the localization of overexpressed fusions of the protein to green fluorescent protein (Taira et al., 2004). The presence of both enzymes would imply that photorespiratory NH_3 could also be directly re-assimilated in the mitochondrion. On the other hand, neither Fd-GOGAT nor GS have been identified in proteome analyses of the mitochondrial matrix (Eubel et al., 2007; Jamai et al., 2009). This at least implies that the proteins are scarce in the mitochondrion which would again support a regulatory instead of a catalytic role. Further research is necessary to clarify the role of these dual targeting events in photorespiration.

2.2. Transport of Photorespiratory Intermediates

Due to the complex biochemistry of photorespiration and the involvement of three different compartments, numerous transport steps over membranes are necessary for function of the pathway. However, most transporters have not been identified or even biochemically characterized in detail. The available information on the transporters for photorespiratory intermediates is summarized below. Data on the oxaloacetate/malate shuttles in the membranes of all organelles that are indirectly involved in photorespiration by transferring reducing equivalents during photorespiration (Reumann and Weber, 2006) are not included.

2.2.1. Chloroplast transport

The first transmembrane transport process necessary during the photorespiratory pathway is the transport of glycolate out of the chloroplast into the cytosol. This transporter has not been characterized in Arabidopsis, but was studied before in pea and spinach. Substrate specificity of the chloroplast glycolate transporter was analyzed in isolated pea chloroplasts. The uptake of radiolabeled glycolate into isolated chloroplasts was strongly inhibited by glycerate (Howitz and McCarty, 1983). Based on this, it was concluded that the same transporter is responsible for both glycolate and glycerate transport. This was further supported by reconstitution of transport activity into liposomes. The transporter exchanged glycolate for glycerate (Howitz and McCarty, 1991). The gene encoding this transporter is unknown to date.

During the re-assimilation of ammonia, oxoglutarate enters the chloroplast whereas glutamate leaves the chloroplast. A two-translocator model exists for these transport processes (Weber and Flügge, 2002). The model includes the oxoglutarate/malate translocator (At5g12860 encoding DIT1) and the glutamate/malate translocator (At5g64290 encoding DIT2.1) (Woo et al., 1987). During screening for photorespiratory mutants, Somerville and Ogren (1983) identified a mutant in a potential dicarboxylate translocator that has later been shown to carry a mutation in the gene for DIT2.1 (Renne et al., 2003). In contrast, a knockout of the gene for DIT2.2 (At5g64280) did not result in an apparent phenotype (Renne et al., 2003) and the encoded protein did not show dicarboxylate transport activity (Taniguchi et al., 2002). Hence, its function remains unknown and an involvement in photorespiration is unlikely. *Dit*1 mutants were not identified in Arabidopsis, but antisense analyses in tobacco verified a role for this protein in photorespiration (Schneidereit et al., 2006). Whereas *Dit*1 clusters with most other photorespiration genes in transcrip-

tional profile analyses, *Dit*2.1 shows a broader expression profile especially with regard to tissue specificity and light regulation (Renne et al., 2003; Foyer et al., 2009).

2.2.2. Peroxisomal transport

The high fluxes of photorespiratory intermediates, such as glycolate, glycerate, glutamate, oxoglutarate, glycine, and serine across the membrane of leaf peroxisomes require efficient transport systems (Reumann and Weber, 2006). The presence of an anion-selective porin-like channel was observed in electrophysiological experiments with purified peroxisome membranes in spinach and *Ricinus* (Reumann et al., 1995; Reumann et al., 1997). Further experiments indicated that the channel is permeable for most photorespiratory intermediates (Reumann et al., 1998). However, such peroxisomal porins have not been unequivocally identified in proteomic studies in Arabidopsis, perhaps because they were misclassified as mitochondrial contaminants, but are in fact dual-targeted to both organelles (Kaur et al., 2009).

2.2.3. Mitochondrial transport

During photorespiration, two molecules of glycine are imported into mitochondria for each serine that is exported. Whereas the transporter has not been identified in Arabidopsis, biochemical data are available from spinach leaf mitochondria (Yu et al., 1983). Based on the biphasic transport kinetics observed, an active transport system is postulated only for low substrate concentrations. Both glycine and serine can inhibit transport of the other compound, respectively, suggesting the presence of a single antiport system for the two amino acids. Passive diffusion might dominate over active transport at higher concentrations of glycine and serine (Yu et al., 1983). This could explain why the transporter has never been identified in screens for photorespiratory mutants.

2.3. Energy Balance Sheet

Photorespiration imposes costs on plant energy metabolism. The oxygenase reaction of RUBISCO does not result in energy or carbon gain for the plant. The ribulose-1,5-bisphosphate molecule for this reaction is therefore “wasted”. Our calculation of the energy costs is based on the assumption that this molecule of ribulose-1,5-bisphosphate has to be completely regenerated to reconstitute the “*status quo ante*”, i.e. the metabolic state of the Calvin cycle before oxygen fixation. Table 1 summarizes these costs. Possible energetic costs of transport processes are unknown.

1. The glycine decarboxylase reaction releases 0.5 CO_2 and 0.5 NH_3 per phosphoglycolate formed. The carbon atom in CO_2 was oxidized from oxidation state +I to +IV during oxygen fixation and glycine decarboxylation and needs to be re-reduced by the carboxylase activity of RUBISCO and the subsequent reactions of the Calvin cycle. This requires 3 ATP and 2 NADPH per CO_2 molecule (Benson and Calvin, 1950), thus half of the costs for 0.5 CO_2. NH_3 has to be re-fixed in the plastidal GS/Fd-GOGAT cycle (see above). For the calculation, 1 reduced ferredoxin is assumed to be equivalent to 0.5 NAD(P)H, because ferredoxin reduction requires one electron, whereas NAD(P)H reduction requires two electrons.
2. The balance sheet for reducing equivalents is neutral for the subsequent reactions of photorespiration, because one NADH is formed during the re-oxidation of the H protein of GLD and one NADH is used for the oxidation of hydroxypyruvate to glycerate.
3. Per phosphoglycolate, 0.5 glycerate are formed that are phosphorylated by glycerate kinase for re-integration into the Calvin cycle. This requires 0.5 ATP. Both these 0.5 phosphoglycerate (PGA) as well as 1 PGA formed by the oxygenase reaction of RUBISCO are again metabolized in the Calvin cycle for the regeneration of ribulose-1,5 bisphosphate. The Calvin cycle consumes 1 NADPH and 1.5 ATP per PGA. Total costs for undoing oxygen fixation therefore sum up to 4.75 ATP and 3 reducing equivalents.
4. Reducing equivalents (NAD(P)H) can be converted to energy equivalents (ATP) in the mitochondrial electron transport chain. Exact ratios vary with conditions, but 2.5 ATP are produced from one NADH in average (Ferguson, 1986; Hinkle, 2005). This calculation results in total energy costs of 12.25 ATP.

Table 1. The energy balance sheet of the oxygenase reaction of RUBISCO and photorespiration.

	Red. eq.	**ATP**
Reduction of 0.5 CO_2	1	1.5
Re-fixation of 0.5 NH_3	0.5	0.5
Phosphorylation of 0.5 glycerate	0	0.5
Reduction of 1 PGA formed by RUBISCO	1	1.5
Reduction of 0.5 PGA formed by photorespiration	0.5	0.75
	3	**4.75**
Assuming 2.5 ATP/NAD(P)H	7.5	4.75
		12.25

Based on these calculations, what is the energy burden of the oxygenase reaction on carbon fixation? RUBISCO highly prefers CO_2 to O_2 as a substrate with a specificity factor of approximately 80 for land plants (Jordan and Ogren, 1981) although this varies between species and is dependent on temperature (Ku and Edwards, 1977a; Jordan and Ogren, 1984). However, atmospheric concentrations of O_2 are much higher than CO_2 concentrations (250 µM and 8 µM, respectively, at 25 °C) resulting in an oxygen surplus of 31-fold. Therefore, approximately each fourth reaction of RUBISCO is an oxygenase instead of a carboxylase reaction under moderate growth conditions (Ehleringer et al., 1991). Costs for each carbon fixation reaction are 3 ATP and 2 NADPH, or 8 ATP in total if reducing equivalents are converted to energy equivalents as described above. Statistically, the costs for one oxygenation reaction have to be allocated to 3 carboxylations, i.e. 4.1 ATP per carboxylation. This results in extra energy costs of about 50 % for photosynthesis caused by the oxygenase activity of RUBISCO. This calculated number is comparable to the measured about 40-50 % increase of photosynthetic rates in Arabidopsis and other C3 plants in atmospheres containing low O_2 concentrations (Hesketh, 1967; Kebeish et al., 2007).

3. THE LIGHT SIDE AND THE DARK SIDE OF PHOTORESPIRATION

As detailed in the last chapter, photorespiration imposes significant extra energy costs on carbon fixation. From this, the view of photorespiration as a wasteful process originated. On the other hand, the pathway rescues ¾ of the carbon in phosphoglycolate that would be otherwise inaccessible for further metabolism. Thus, photorespiration can also be regarded as an important pathway that makes the best of a bad situation caused by RUBISCO's seemingly inevitable oxygenase activity. Beside this, there are several additional arguments for a positive function of photorespiration in plant metabolism:

3.1. Photorespiration Removes Toxic Metabolic Intermediates

Photorespiration is the only pathway in the plant for phosphoglycolate metabolism that would otherwise accumulate. Phosphoglycolate has been shown to strongly inhibit triose phosphate isomerase from pea leaves at concentrations in the low micromolar range (Anderson, 1971). This would interfere with the regeneration of ribulose-1,5-bisphosphate in the Calvin cycle. In addition, phosphoglycolate inhibits the chloroplast phosphofructokinase (Kelly and Latzko, 1976) and, by this, the replenishment of Calvin cycle intermediates from the breakdown of transitory starch. This dual inhibitory function is probably responsible for the low photosynthetic rate of mutants accumulating phosphoglycolate (Somerville and Ogren, 1979).

To our knowledge, no direct inhibitory function has been described for glycolate, the product of phosphoglycolate dephosphorylation, albeit glycolate accumulation has been observed in many photorespiratory mutants. Glycolate is further converted to glyoxylate and this compound is again a strong inhibitor of photosynthesis. Campbell and Ogren (1990) and Chastain and Ogren (1989) reported that micromolar concentrations of glyoxylate inhibited activation of RUBISCO in isolated organelles and *in vivo*. However, inhibition of the activase enzyme (Campbell and Ogren, 1990) or purified RUBISCO (Cook et al., 1985) required much higher concentrations. Furthermore, glyoxylate was inefficient in rendering the chloroplast envelope permeable to protons as observed for other weak acids before (Flügge et al., 1980). Thus, glyoxylate negatively impacts on the activation state of RUBISCO by a yet unidentified mechanism. The importance of this mechanism is exemplified by the presence of glyoxylate reductase in the cytosol and the chloroplast that might function in protecting the photosynthetic machinery from excess glyoxylate leaking from the peroxisomes (Givan and Kleczkowski, 1992; Allan et al., 2009). Conversely, glyoxylate may be instrumental in reducing RUBISCO activity and, by this, also phosphoglycolate formation under conditions where photorespiration cannot cope with the rates of RUBISCO oxygenase activity. This might be of physiological importance under low nitrogen supply when the transamination reactions converting glyoxylate to glycine are limiting photorespiratory flux. The glyoxylate overflow would simply stop both photosynthesis and photorespiration under such emergency conditions. Alternative mechanisms to metabolize glyoxylate are also discussed explicitly in chapter 4. Taken together, photorespiration is important for the removal of toxic intermediates, but surprisingly little is known about the regulatory function of these intermediates in coordinating photorespiration with other pathways of plant basal metabolism.

3.2. Photorespiration Protects from Photoinhibition

Under stress conditions, such as drought, cold, or high light, NADPH production in the light reactions of photosynthesis often exceeds the demand of the Calvin cycle for reducing power. In that case, energy is dissipated as heat or electrons are transferred from different complexes in the electron transport chain to other acceptor molecules than NADPH. If the acceptor molecule is O_2, the reaction results in the formation of reactive oxygen species (ROS, reviewed in Murata et al., 2007). Because of their high reactivity, ROS unspecifically oxidize proteins and lipids. Moreover, ROS also interfere with recycling of specific components of the electron transport chain by inhibiting translation of new proteins (Nishiyama et al., 2004). The chloroplast contains several mechanisms to detoxify ROS. There are compounds that scavenge ROS non-enzymatically like carotenoids and flavonoids, and enzymes, like ascorbate peroxidase, superoxide dismutase and catalase, that detoxify ROS in partly complex pathways (reviewed in Apel and Hirt, 2004). A welcome effect of these pathways is that they ultimately consume reducing equivalents and by this contribute to the provision of fresh $NADP^+$, the terminal electron acceptor of the photosynthetic electron transport chain. Alternatively, excess light energy can be mitigated by the xanthophyll cycle that dissipates the energy of absorbed photons into heat (Yamamoto et al., 1962; Davison et al., 2002). Also photorespiration can act as an electron sink especially under stress conditions (Wingler et al., 2000) by consuming reducing equivalents during the refixation of released ammonia and by exporting reduced components from the chloroplast to the mitochondrion (Igamberdiev and Lea, 2002). This might even result in situations where the mitochondrion becomes over-reduced instead of the chloroplast because of the huge amounts of NADH generated by glycine decarboxylation. To allow for high fluxes through photorespiration under such conditions, mitochondrial electron transport might be uncoupled from ATP synthesis by uncoupling proteins (UCPs) that catalyse proton conductance through the inner mitochondrial membrane and dissipate the energy of the mitochondrial proton gradient as heat (Sweetlove et al., 2006). The importance of photorespiration in protection from photoinhibition was directly tested by Kozaki and Takeba (1996) by measuring the rates of photoinhibition in plants with enhanced or diminished capacities for photorespiration caused by manipulation of the levels of glutamine synthetase (GS). Plants with an improved capacity for photorespiration because of increased GS levels were more tolerant to high light stress. Accordingly, photorespiratory mutants of Arabidopsis showed enhanced photoinhibition (Takahashi et al., 2007). In this study, the authors provide evidence that photoinhibition was rather caused by suppression of the repair of photosystem II (PSII) than by an increase in PSII damage.

Any mechanism that uses photorespiration as a pressure relief valve for reducing power from the chloroplast would ultimately run out of the O_2 acceptor molecule ribulose-1,5-bisphosphate because of the net carbon loss associated with photorespiration.

Replenishment of O_2 acceptor molecules is probably brought about by starch breakdown (Weise et al., 2006). As a consequence, the chloroplast stroma is seemingly less reduced under conditions of high photorespiration than during maximum photosynthesis (Weise et al., 2006). Still, the relative importance of photorespiratory export of reducing equivalents among the multitude of different processes that protect plants from photoinhibition is a matter of ongoing debates and might strongly depend on the conditions (cf. Osmond et al., 1997). Beside its function as an export mechanism for reducing power, photorespiration is also directly involved in the synthesis of sufficient amounts of glycine that is required to cover the strongly enhanced demands for glutathione during stress (Noctor et al., 1998; Noctor et al., 1999).

3.3. Photorespiration Supports Plant Defense Reactions

H_2O_2 is known to be a signaling molecule in plants involved in both biotic and abiotic stress responses. Because of its participation in multiple pathways, H_2O_2 is especially suitable for mediating crosstalk between the different resistance mechanisms (reviewed in Neill et al., 2002). During pathogen attack, H_2O_2 has multiple roles in the so called oxidative burst. On the one hand, it triggers the plant defense response system, including cell wall strengthening and activation of phytoalexin biosynthesis (Wu et al., 1997). On the other hand, H_2O_2 can damage the pathogen by its reactive potential. Ultimately, H_2O_2 triggers the hypersensitive response and the attacked cell eventually undergoes programmed cell death (Heath, 2000). As photorespiration is a major source of H_2O_2 in plants, an involvement in resistance reactions was often discussed (Chamnongpol et al., 1998). This was recently confirmed by the identification of “enzymatic resistance” genes in melon (Taler et al., 2004). Efficient resistance of a wild melon line to the oomycete *Pseudoperonospora cubensis* was shown to be caused by constitutively enhanced expression of two genes encoding peroxisomal glyoxylate aminotransferases. A mechanism was suggested, in which the elevated activity of these enzymes positively feeds back on glycolate oxidase, thereby enhancing the capacity for H_2O_2 release.

3.4. Photorespiration is Intimately Integrated into Primary Metabolism

Photorespiration is obviously tightly integrated into plant primary metabolism. Most photorespiratory intermediates are also part of other metabolic pathways and photorespiration significantly contributes to the synthesis of several amino acids (Novitskaya et al., 2002). Photorespiration connects the metabolic compartments of the cell and is therefore ideally suited to transport information about the energetic state between these compartments (Nunes-Nesi et al., 2008). The conversion of glycine to serine in the mitochondrion is probably essential to supply serine for the cytosolic production of C1-compounds that are in turn required for biosynthetic processes such as methionine synthesis (Mouillon et al., 1999; Engel et al., 2007). Beside this, specific roles have been suggested for photorespiration under certain growth conditions:

Sucrose and starch are the major sinks for photosynthetic products in leaves. Under conditions of high light and carbon availability, synthesis of these sink compounds might not keep pace with production of phosphorylated intermediates in the Calvin cycle. This results in an imbalance of phosphate release during synthesis of sucrose or starch and phosphate use during the synthesis of Calvin cycle intermediates that may ultimately limit photosynthesis (Harley and Sharkey, 1991). Under such conditions, fixation of oxygen by RUBISCO and the subsequent reactions of photorespiration might provide the cell with an additional method for the synthesis of sink products such as glycine and serine instead of sucrose and starch (Harley and Sharkey, 1991). This is consistent with the finding that source leaves start to export glycine and serine instead of sucrose to sink leaves under conditions of high photorespiration (Madore and Grodzinski, 1984).

Another connection of photorespiration and nitrogen metabolism was observed by Rachmilevitch et al. (2004). They found that nitrate assimilation was inhibited in an atmosphere containing 2% O_2 where photorespiration is repressed. They proposed a mechanism how photorespiration provides reducing equivalents for cytoplasmic nitrate reductase: The reducing equivalents are exported from the chloroplast into the cytoplasm via the malate/oxaloacetate shuttle to provide reducing power for peroxisomal hydroxypyruvate reduction. Part of these reducing equivalents might then be diverted for nitrate reduction. Reducing equivalents for peroxisomal reactions can alternatively be provided by mitochondria that synthesize the required amounts of NADH during photorespiration (Raghavendra et al., 1998). However, there is evidence that part of the mitochondrial reducing equivalents may rather be used for ATP synthesis (Krömer and Heldt, 1991). The relative importance of these processes requires detailed investigation.

4. METABOLIC COMPLEXITY: ALTERNATIVE PHOTORESPIRATORY PATHWAYS IN ARABIDOPSIS

Conflicts about the major routes in phosphoglycolate metabolism were decided by the mutant screens in Arabidopsis and barley (see chapters 1 and 2). Indications for alternative routes were mostly not followed afterwards. However, significant new information about such alternative pathways accumulated during the last few years indicating a metabolic flexibility of photorespiration that is much higher than predicted from the earlier biochemical studies. An overview of possible alternative pathways is given in Figure 5.

4.1. Possible Alternative Pathways in the Chloroplast

An endogenous pathway for glycolate metabolism in the chloroplast was proposed by Goyal and Tolbert (1996). Using biochemical analyses, they demonstrated the existence of a salicylhydroxamic acid (SHAM)-inhibited light-dependent glycolate-quinone oxidoreductase system that is associated with thylakoid membranes of the chloroplasts of both algae and spinach. By this system, electrons from glycolate oxidation could be transferred to the photosynthetic electron transport chain and used for ATP synthesis. In this scenario, glyoxylate would be reduced back to glycolate by the plastidal glyoxylate reductase (Givan and Kleczkowski, 1992). This would provide an alternative sink for ex-

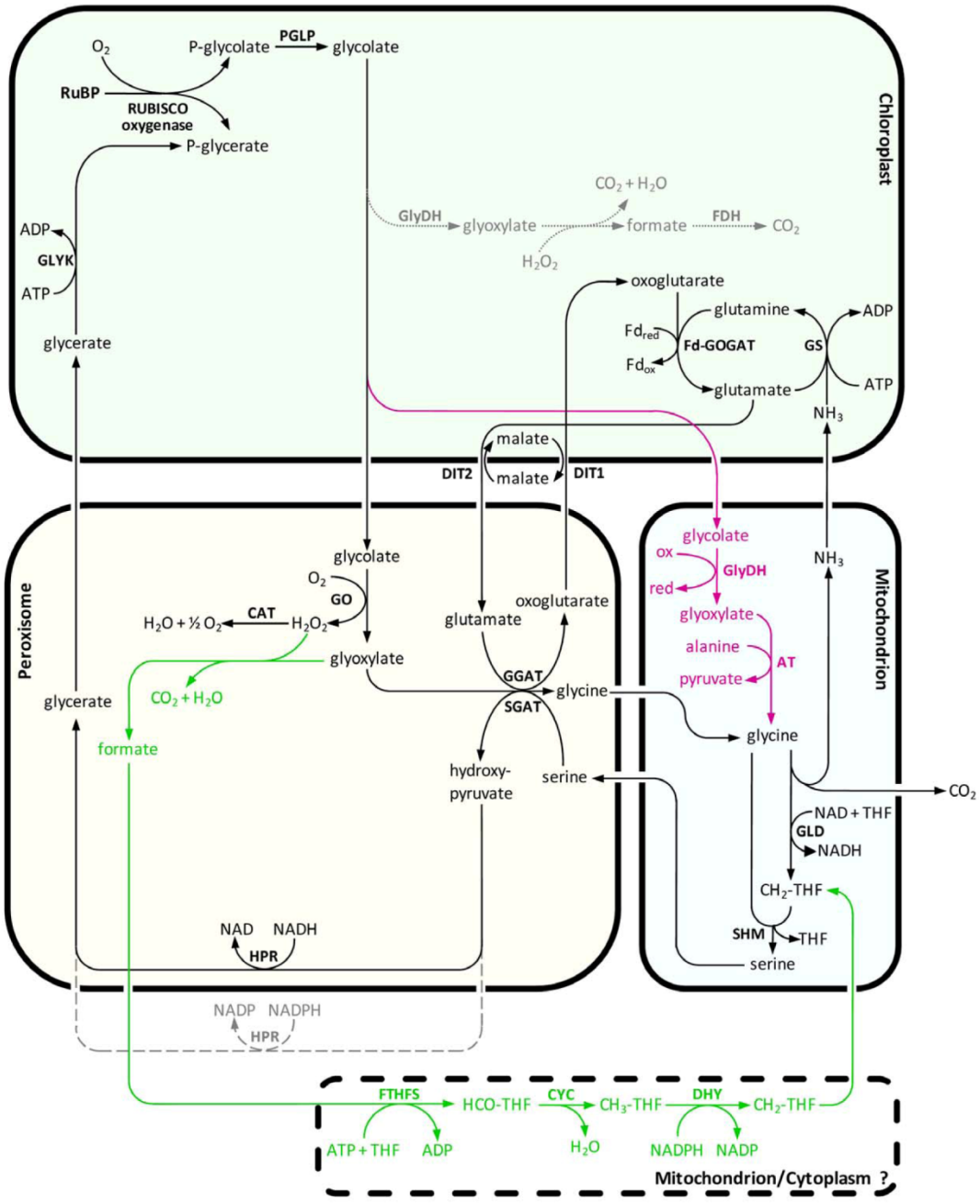


Figure 5: Possible alternative photorespiratory pathways in Arabidopsis.

Overview of the major photorespiratory pathway (black) and four possible alternative photorespiratory pathways (dotted grey, green, red, and dashed grey) in Arabidopsis that are described in the main text. RuBP, ribulose-1,5-bisphosphate; RUBISCO, RuBP carboxylase/oxygenase; PGLP, phosphoglycolate phosphatase; GO, glycolate oxidase; CAT, catalase; GGAT, glyoxylate:glutamate aminotransferase; SGAT, serine:glyoxylate aminotransferase; DIT1, dicarboxylate transporter 1; DIT2, dicarboxylate transporter 2; GLD, glycine decarboxylase; SHM, serine hydroxymethyltransferase; HPR, hydroxypyruvate reductase; GLYK, glycerate kinase; GS, glutamine synthetase; Fd-GOGAT, glutamine:oxoglutarate aminotransferase; GlyDH, glycolate dehydrogenase; AT, aminotransferase; FDH, formate dehydrogenase; FTHFS, 10-formyl-THF-synthetase; CYC, 5,10-methenyl-THF-cyclohydrolase; DHY, 5,10-methenyl-THF-dehydrogenase; THF, tetrahydrofolate; HCO-THF, formyl-THF; CH_3-THF, methenyl-THF; CH_2-THF, methylene-THF. The stoichiometry of the reactions is not included.

cess electrons under stress conditions (Allan et al., 2009, see also chapter 3). The presence of a glycolate-oxidizing enzyme in chloroplasts of Arabidopsis was recently confirmed by Kebeish et al. (2007). However, the cycle of glycolate oxidation and glyoxylate reduction does not consume glycolate and is therefore not suitable as an alternative pathway for the conversion of the products of RUBISCO's oxygenase activity. Instead, glyoxylate coming from plastidal glycolate oxidation might be further oxidized to CO_2 in the chloroplast (Kisaki and Tolbert, 1969; Zelitch, 1972; Oliver, 1981, dotted grey pathway in Figure 5). Slow CO_2 release from glyoxylate in chloroplasts from a set of plant species was shown to be stimulated by light and oxygen (Kisaki and Tolbert, 1969; Zelitch, 1972). Although glycolate was not converted by chloroplasts to CO_2 in these studies, later analysis in Arabidopsis also revealed coupling of glycolate oxidation and CO_2 release (Kebeish et al., 2007). Conversion of glyoxylate to CO_2 might be catalyzed by plastidal pyruvate decarboxylase that also accepts glyoxylate as a substrate (Davies and Corbett, 1969). Alternatively, glyoxylate can react non-enzymatically with H_2O_2 or O_2^- to formate (Elstner and Heupel, 1973; Halliwell and Butt, 1974, see also chapter 4.2). Such reactive oxygen species are produced in the chloroplast especially under stress conditions (see chapter 3). Formate could be further oxidized to CO_2 by a formate dehydrogenase that is dual-targeted to mitochondria and chloroplasts in Arabidopsis (Olson et al., 2000; Herman et al., 2002). Together, this would result in a low-capacity pathway for glycolate oxidation in the chloroplast that is more energy-efficient than the major photorespiratory pathway and that enhances plastidal CO_2 concentrations. However, ribulose-1,5-bisphosphate is not recycled in these reactions which may result in a depletion of CO_2 acceptor molecules at higher fluxes.

4.2. Possible Alternative Pathways in the Peroxisome

The presence of an alternative pathway for glyoxylate conversion in the peroxisome that does not involve transamination to glycine was suggested by the characterization of Arabidopsis mutants deficient in SHM (Somerville and Ogren, 1981). Although accumulating high amounts of glycine, these mutants still released CO_2 from photorespiratory intermediates. The responsible pathway might involve the non-enzymatic decarboxylation of glyoxylate to formate using H_2O_2 as an oxidizing agent (Oliver, 1981; Igamberdiev and Lea, 2002). Alternatively, catalase can also act as a peroxidase in the oxidation of glyoxylate (Grodzinski, 1978). Peroxisomal glyoxylate decarboxylation could play a physiological role especially under conditions of low nitrogen supply where transamination is inhibited (Somerville and Ogren, 1982b; Singh et al., 1986; Wingler et al., 1999).

Formate resulting from glyoxylate decarboxylation might act a C1 donor in the formation of serine. Based on studies of barley and *Amaranthus* mutants lacking GLD activity, Wingler et al. (1999) suggested that the C1-tetrahydrofolate (THF) synthase pathway converts photorespiratory formate to 5,10-methylene-THF (green pathway in Figure 5). The enzymes involved in plants are a monofunctional 10-formyl-THF-synthetase (FTHF synthetase, EC 6.3.4.3) and a bifunctional 5,10-methenyl-THF-cyclohydrolase:5,10-methylene-THF dehydrogenase (CYC-DHY, EC 3.5.4.9 and EC 1.5.1.5). FTHF synthetase catalyses the synthesis of 10-formyl-THF, which is converted into 5,10-methenyl-THF by CYC and reduced to 5,10-methylene-THF by DHY. 5,10-methylene-THF is used by SHM to synthesize serine from glycine (Prabhu et al., 1996; Hanson and Roje, 2001). In the barley and *Amaranthus* GLD mutants, evidence for function of this pathway in photorespiration was provided by the increase in FTHF-synthetase, the accumulation of glyoxylate and formate under photorespiratory conditions, and the ability of the mutants to utilize formate and glycolate for the formation of serine (Wingler et al., 1999). Similar flux analyses were later on also performed with Arabidopsis mutants deficient in GLD activity that confirmed the described results (Li et al., 2003). However, the enzymes catalyzing the conversion of formate to 5,10-methylene-THF have been poorly characterized in Arabidopsis and the subcellular localization is unknown. Enzyme activities have been shown to be mainly associated with the cytosolic fraction in pea leaves (Chen et al., 1997), but isoenzymes are also present in the mitochondrion and the chloroplast (Hanson and Roje, 2001; Christensen and MacKenzie, 2006). In animals and yeast, partially redundant pathways exist in the cytoplasm and the mitochondrion (Piper et al., 2000; Christensen and MacKenzie, 2006). Folate transporters have been identified in the Arabidopsis chloroplast membrane (Bedhomme et al., 2005) and animal mitochondria (McCarthy et al., 2004). The pathway for the conversion of formate resulting from peroxisomal glyoxylate decarboxylation was therefore placed by default in the cytoplasm in Figure 5 (green pathway).

4.3. Possible Alternative Pathways in the Mitochondrion

Metabolic profiling of *Go* antisense lines in rice (see chapter 2) revealed the expected accumulation of glycolate, but downstream metabolites such as glycine and serine were not reduced in concentration. This confirmed that GO-catalyzed glycolate oxidation is the dominant pathway for glycolate conversion, but also implied the existence of alternative pathways for glyoxylate production (Xu et al., 2009). Moreover, *Ggat*1 knockout lines (Igarashi et al., 2003, see also chapter 2) showed only weak photorespiratory phenotypes that might not be fully explained by the residual low activity of GGAT2 suggesting the existence of alternative pathways for the conversion of glycolate to glycine.

Leaf-type peroxisomes are absent in chlorophytes, the second big lineage of green algae, that subsumes the classes *Chlorophyceae*, *Ulvophyceae*, and *Trebouxiophyceae* (Frederick et al., 1973). Beside secreting glycolate from the cell, this group uses a mitochondrial glycolate dehydrogenase (GlyDH, EC 1.1.99.14) for the production of glyoxylate that most probably transfers electrons to the respiratory electron transport chain (Paul and Volcani, 1976). Analyses in the trebouxiophycean species *Eremosphaera viridis* revealed that the subsequent metabolism is similar to photorespiration in higher plants, but that all reactions are located in mitochondria and that the capacity of this pathway for glycolate conversion is lower compared to the major pathway in higher plants (Stabenau and Winkler, 2005). The importance of the mitochondrial GlyDH in chlorophytes has been demonstrated by the characterization of a *Chlamydomonas* strain carrying a plasmid insertion in the corresponding gene. The mutation is conditionally lethal and the strain is only capable of growing at elevated CO_2 concentrations (Nakamura et al., 2005, see also chapter 5).

In charophytes, the nearest relatives to land plants, glycolate oxidation was shifted from the mitochondrion to the peroxisome (Stabenau and Winkler, 2005), most probably because the specific activity of GlyDH is low and allows for low flux rates only. The peroxisomal GO does not show significant homology to GlyDH on the protein level and differs in important enzymatic properties: GlyDH enzymes use D-lactate as an alternative substrate, whereas GO preferentially oxidizes L-lactate. This allows for a precise discrimination of both activities.

Studies in Arabidopsis questioned the clear differentiation of glycolate metabolism in higher plants and chlorophytes (Bari et al., 2004). An enzyme with homology to bacterial and algal GlyDH enzymes (At5g06580) was shown to be targeted to the mitochondrion. The recombinant enzyme complemented *Escherichia coli* mutants deficient in GlyDH activity and oxidized glycolate to glyoxylate *in vitro*. Knock-out of At5g06580 drastically reduced the capacity of isolated Arabidopsis mitochondria to convert glycolate to CO_2 and reduced metabolite flux through photorespiration (Niessen et al., 2007). Inhibitor studies suggested that a mitochondrial alanine:glyoxylate aminotransferase might further convert glyoxylate resulting from the GlyDH reaction to glycine (Niessen et al., 2007, red pathway in Figure 5). Alternatively, this reaction might also be catalyzed by mitochondrial γ-aminobutyrate transaminase that has been shown to accept glyoxylate as a substrate. The accumulation ofγ-aminobutyrate during stress is a well documented phenomenon and would provide enough amino donor for the transamination of glyoxylate (Clark et al., 2009). By any of the aminotransferase reactions discussed, mitochondrial glycolate oxidation can be linked to the GLD/SHM complex of the major pathway.

The main advantage of the mitochondrial pathway is the direct coupling of glycolate oxidation to the electron transport chain that allows to recover energy equivalents (Paul and Volcani, 1976; Stabenau and Winkler, 2005). The combination with the high capacity major pathway in a single cell provides the plant with an optimized adaptability to changes in environmental conditions. However, neither knock-out of GlyDH nor γ-aminobutyrate transaminase resulted in an obvious phenotype under normal growth conditions (Niessen et al., 2007; Clark et al., 2009). In addition, the view that Arabidopsis GlyDH is involved in photorespiration was recently challenged by characterization of the enzymatic properties (Engqvist et al., 2009). *In vitro* data revealed a 2000-fold higher preference for D-lactate compared to glycolate due to a very low catalytic rate with the latter substrate. The enzyme was necessary for growth of Arabidopsis on media containing high concentrations of D-lactate, but not of glycolate. However, glycolate might have been detoxified by the peroxisomal glycolate oxidase in such a setup and this would mask a possible supplementary function of GlyDH activity. Further research is necessary to explain the conflicting data sets. As At5g06580 is expressed in all plant organs, the function of the enzyme might also differ between roots and leaves.

4.4. Possible Alternative Pathway in the Cytosol

As specified in chapter 2, *Hpr*1 encodes the peroxisomal hydroxypyruvate reductase involved in the major photorespiratory pathway. However, knock-out in Arabidopsis (Timm et al., 2008) or barley (Murray et al., 1989) did not result in severe photorespiratory phenotypes. It was therefore speculated that cytosolic HPR2 (Kleczkowski et al., 1991) could provide a bypass for the peroxisomal reaction (dashed grey pathway in Figure 5). A double mutant with T-DNA insertions in the genes encoding both HPR1 and HPR2 was recently characterized by Timm et al. (2008). The double knock-out showed the typical air-sensitivity and drastically reduced photosynthetic performance of photorespiratory mutants. Thus, the cytosolic pathway seemed to have the capacity to fully replace the peroxisomal pathway at least under moderate growth conditions. However, metabolic profiling and gas exchange revealed clearly stronger perturbances of photosynthesis and basal metabolism in the HPR1 compared to the HPR2 mutant (Timm et al., 2008). This suggests that peroxisomal HPR1 plays a dominant role under moderate growth conditions whereas HPR2 provides an overflow mechanism for the utilization of excess hydroxypyruvate leaking from peroxisomes under conditions of very high photorespiratory flow. Such a function in protection and/or improvement of the energy balance under non-standard growth conditions might be a joint feature of all alternative pathways and explain why these pathways have been conserved in evolution.

5. METABOLIC COMPLEXITY: PHOTORESPIRATION IS NECESSARY FOR ALL ORGANISMS PERFORMING OXYGENIC PHOTOSYNTHESIS

This chapter is not about photorespiration in Arabidopsis. However, recent results on the significance of photorespiration in other photosynthetic organisms shed new light on the evolution and the major function of photorespiration and are therefore discussed here.

The use of RUBISCO for CO_2 fixation is common to all organisms with oxygenic photosynthesis: cyanobacteria, algae, lower and higher plants. Despite billion years of evolution, all RUBISCO enzymes also share oxygenase activity and, therefore, produce phosphoglycolate when exposed to oxygen as a substrate (Andersson, 2008, see also chapter 1). However, several lineages independently evolved mechanisms to reduce the probability of oxygen fixation:

1. Cyanobacteria and many algae concentrate CO_2 in the ultimate vicinity of RUBISCO (Giordano et al., 2005; Price et al., 2007; Spalding, 2007). Both mechanisms are based on a tight association of RUBISCO with carbonic anhydrase and a shell made from proteins or starch, respectively, around these complexes. CO_2 that enters the cell is converted to bicarbonate, diffuses into the vicinity of RUBISCO and is reconverted to CO_2 immediately before fixation by RUBISCO. It has been argued that the carbon concentrating mechanism (CCM) mainly serves the saturation of RUBISCO at low availability of gaseous CO_2 in an aqueous environment (Giordano et al., 2005; Raven et al., 2008). Nevertheless, suppression of oxygen fixation is at least a welcome side effect.
2. In higher plants, evolutionary pressure resulted in an enhancement of the specificity factor of RUBISCO. This parameter indicating the relative preference of RUBISCO for CO_2 over O_2 increased in evolution of the green lineage from cyanobacteria over green algae and is highest for RUBISCOs from higher

plants (Jordan and Ogren, 1981), although some even more specific RUBISCO enzymes evolved in non-green algae perhaps as an adaptation to specific ecological niches (Badger and Bek, 2008). However, there is a negative correlation between specificity and the catalytic rate of RUBISCO (Zhu et al., 2004): Highly specific enzymes are mostly slow catalysts (see also chapter 1) although there is some variation around this general rule also in higher plants (Galmes et al., 2005). This might have limited the further evolution of RUBISCOs with even higher specificities.

3. Some land plants that were exposed to conditions of high photorespiratory pressure, evolved biochemical pumps that concentrate CO_2 at the site of fixation. These mechanisms are all based on a primary fixation of bicarbonate into organic acids by phosphoenolpyruvate carboxylase (PEPC). In a second step, decarboxylation of these organic acids enhances the CO_2 concentration around RUBISCO resulting in a strong decrease in the probability of oxygen fixation. Primary and secondary CO_2 fixation can be either spatially (C4 metabolism) or temporally (crassulacean acid metabolism (CAM)) separated. For informative reviews on these mechanisms, see Sage (2004) and Dodd et al. (2002), respectively.

Despite of the presence of mechanisms that reduce oxygen fixation, there is evidence for functional photorespiratory pathways in all these organisms. Mutant analyses analogous to Somerville's studies on Arabidopsis in the 1970s (see chapter 1) imply that these pathways are not only an evolutionary relict, but required for survival:

Despite knowledge about the presence and activity of photorespiratory enzymes (Frederick et al., 1973) and the description of oxygen-dependent CO_2 release in green algae (Birmingham et al., 1982), metabolic flux through the photorespiratory pathway has only been shown recently (Stabenau and Winkler, 2005). In screens for *Chlamydomonas* mutants that require high CO_2 for growth, mostly mutations in components of the CCM were identified (Van et al., 2001). However, Suzuki et al. (1990) described a mutant that accumulated high concentrations of phosphoglycolate and showed a high CO_2-requiring phenotype although the CCM was fully functional. A second mutant with a transposon insertion in the gene encoding glycolate dehydrogenase (GlyDH) showed a similar phenotype and excreted large amounts of glycolate under conditions that favor phosphoglycolate formation (Nakamura et al., 2005). Thus, at least the species *Chlamydomonas* requires photorespiration for growth at normal CO_2 concentrations.

In cyanobacteria, knowledge about photorespiration was very limited until recently. Some photorespiratory enzymes were shown to be present by biochemical methods (Grodzinski and Colman, 1976; Norman and Colman, 1991, 1992) and glycolate excretion was observed under photorespiratory conditions (Renström and Bergman, 1989). A survey of the *Synechocystis* genome sequence revealed that genes for enzymes of both the higher plant photorespiratory pathway and a bacterial pathway for conversion of glycolate to glycerate were present (Eisenhut et al., 2006, Figure 6). The higher plant pathway plays the major role in phosphoglycolate metabolism, but can be partially replaced by the bacterial pathway. Nevertheless, even double mutants, where both pathways were disrupted, were able to grow under ambient CO_2 concentrations, which was attributed to the activity of the

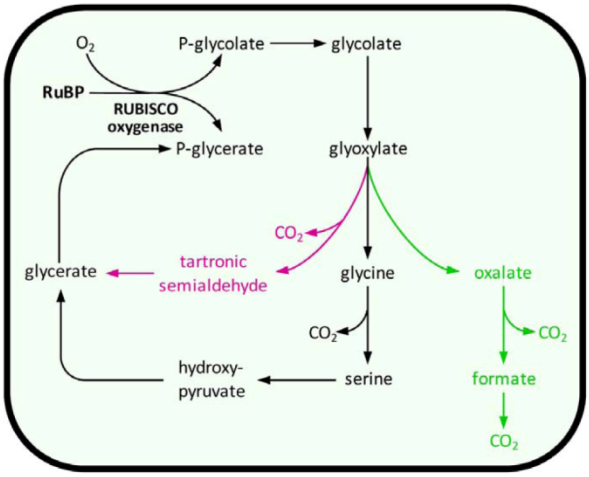


Figure 6. The three pathways for glyoxylate conversion in *Synechocystis*.

Overview of the metabolism of photorespiratory glyoxylate in *Synechocystis*. The pathways are homologous to the major pathway in Arabidopsis (black), the bacterial glycerate pathway (red), or involve the complete oxidation of glyoxylate to CO_2 (green), respectively. RuBP, ribulose-1,5-bisphosphate; RUBISCO, RuBP carboxylase/oxygenase. The stoichiometry of the reactions is not included.

CCM (Eisenhut et al., 2006). Instead, the same group identified a third pathway for glycolate metabolism that includes a series of decarboxylations: Glyoxylate is first converted to oxalate, then to formate and finally to CO_2 (Eisenhut et al., 2008, Figure 6). This pathway actually resembles possible alternative routes for glycolate metabolism in Arabidopsis chloroplasts and/or peroxisomes (see chapter 4). Simultaneous knock-out of all the three routes for glycolate conversion in a triple mutant finally caused a high CO_2-requiring phenotype. Beside indicating the importance of photorespiration in cyanobacteria, these data also imply that not only photosynthesis, but also photorespiration was transferred from cyanobacteria to plants during endosymbiosis (Eisenhut et al., 2008).

C4 metabolism evolved on top of C3 photosynthesis. Relocation of photorespiratory CO_2 release to the bundle sheath was probably one major step during the evolution of C4 plants (Ohnishi et al., 1985; Sage, 2004). Photorespiratory enzymes were detected in C4 plants (e.g. Popov et al., 2003; Majeran et al., 2005) and inhibition of C4 photosynthesis by O_2 could be observed under certain conditions (Chollet and Ogren, 1975; Dai et al., 1993; Martinelli et al., 2007). In isolated bundle sheath strands of both maize and *Panicum*, operation of the photorespiratory cycle could be shown by metabolic flux labeling (Farineau et al., 1984). All this argued for presence and function of photorespiration in C4 plants, however, flux rates were mostly believed to be very low to insignificant. Again, identification of a mutant confirmed that photorespiration is also essential for C4 plants (Zelitch et al., 2008). A maize line with a transposon insertion in the *Go*1 gene, that encodes the major bundle sheath glycolate oxidase, required

elevated CO_2 for growth and accumulated glycolate to high concentrations when shifted from permissive CO_2 concentrations to ambient air. This was accompanied by a clear reduction in photosynthetic rates.

Based on these results, it can now be concluded that photorespiration is essential for growth of most if not all organisms that perform oxygenic photosynthesis. As phosphoglycolate formation is low in species with efficient CO_2 concentrating mechanisms, carbon loss in the absence of a recycling mechanism is probably not problematic. It rather seems that the accumulation of intermediates that are inhibiting photosynthesis (see chapter 3) is causal for the severe phenotype of photorespiratory mutants in these species. These findings are also important for understanding the role of photorespiration in C3 plants such as Arabidopsis.

METABOLIC ENGINEERING: MANIPULATING PHOTORESPIRATION

Early during the identification of the photorespiratory cycle, researchers realized that a reduction in photorespiration would show considerable promise to enhance plant carbon fixation, growth and yield. This was one major motivation to invest significant research efforts into the elucidation of the photorespiratory pathway (Ogren, 2003). Indeed, the biochemical inhibition of glycolate synthesis (and consequently photorespiration) by glycidate in tobacco leaf disks resulted in a strong enhancement of photosynthesis (Zelitch, 1974) although some of these results were heavily discussed and could not be reproduced by others in later experiments (Chollet, 1977). In a complementary setup, experiments with tobacco varieties suggested that lines with lower rates of photorespiration had enhanced photosynthesis (Zelitch and Day, 1973). However, as described in chapter 1, mutants with reduced photorespiration and enhanced photosynthesis were never recovered from extensive mutant screens in Arabidopsis. Chris Somerville concluded that changing the specificity of RUBISCO for oxygen relative to carbon dioxide would be the only way to reduce photorespiratory losses (Somerville and Ogren, 1982b). Since then, many scientific efforts have been pursued in this direction and major progress has been obtained in understanding the dual function of RUBISCO and in manipulating the enzyme (Zhu et al., 2004; Mueller-Cajar and Whitney, 2008). However, the final breakthrough of engineering a plant with a better RUBISCO and higher photosynthesis has still not been accomplished.

In parallel, several groups tried to establish components of the C4 CO_2 concentrating mechanism in Arabidopsis and other C3 plants in order to reduce photorespiration (Haeusler et al., 2002). As described in chapter 5, C4 plants invest energy to concentrate CO_2 around RUBISCO. By this, they reduce oxygen fixation and the subsequent photorespiratory losses. The trade-off between energy investment and the reduction of photorespiratory losses is positive when photorespiratory rates are high, i.e. under hot and dry growth conditions. Enzymes involved in C4 metabolism were well-characterized and, thus, researchers started to overexpress the encoding genes in C3 plants (reviewed in Matsuoka et al., 2001; Leegood, 2002). This type of approach based on C4-like pathways that were not separated into two different tissues for primary and secondary CO_2 fixation showed very limited success in reducing photorespiration so far. Most scientists now agree that it will be essential to understand the genetic control of the establishment of C4 leaf anatomy if an efficient CO_2 pump should be set up in C3 plants. Efforts in this direction have recently been reinitiated with the long-term aim to convert rice into a C4 plant and, by this, to cover the ever growing demands for food in developing countries (Hibberd et al., 2008).

Beside the improvement of RUBISCO's specificity and the transfer of a C4-like mechanism, a third different approach has been tested recently. The idea is based on redirecting photorespiration instead of reducing it. The novel pathways should provide alternatives for the metabolism of photorespiratory intermediates that result in an improved energy balance. Chapter 4 described a considerable biochemical plasticity of photorespiration that received little attention after the identification of the major pathway components. The presence of alternative photorespiratory pathways indicates that a deviation of metabolites from the major pathway might be acceptable or even beneficial for plants under certain environmental conditions. Templates for new metabolic pathways are available from non-photosynthetic microorganisms that are capable of growing on glycolate as a sole carbon source by converting it to glycerate (Lord, 1972) or malate (Kornberg and Sadler, 1961), respectively. As shown in Figure 7 (red pathway), installation of the glycerate pathway from *Escherichia coli* in the plant chloroplast establishes a novel pathway that starts and ends with intermediates of photorespiration. By this, a shortcircuit is created that re-directs photorespiratory metabolism. The pathway is made up from three different enzymes: The first reaction resembles the peroxisomal glycolate oxidase reaction in producing glyoxylate from glycolate, but the bacterial glycolate dehydrogenase (GlyDH) uses organic compounds as co-factors instead of molecular oxygen. Unfortunately, the GlyDH enzyme from *E. coli* is made up from several subunits from which the three subunits D, E, and F are necessary for function (Pellicer et al., 1996). Thus, installation of this enzymatic activity requires the simultaneous overexpression of three genes. In step 2 of the pathway, two molecules of glyoxylate are ligated by glyoxylate carboligase (GCL) forming tartronic semialdehyde. This reaction produces CO_2 in the chloroplast similar to the mitochondrial CO_2 release in the major photorespiratory pathway. Tartronic semialdehyde is reduced to glycerate by tartronic semialdehyde reductase (TSR) and the glycerate kinase reaction of the endogenous pathway is used to recycle phosphoglycerate. Installation of this shortcircuit in Arabidopsis by repeated transformation and genetic crossing resulted in an improvement of biomass production of about 30 % under short-day growth conditions (Kebeish et al., 2007). As shown in Figure 8, the effect was even stronger at longer growth periods and additional nutrient supply. Three possible explanations are available why the shortcircuit is superior to the endogenous pathway:

1. The endogenous pathway combusts reducing power when forming H_2O_2 instead of organic reducing equivalents during the conversion of glycolate to glyoxylate. Dependent on the availability of reducing equivalents, this may limit growth.
2. The shortcircuit does not include NH_3 loss and refixation, again saving energy both in the form of ATP and reducing equivalents. In sum, 1. and 2. have the potential of reducing photorespiratory energy requirements from 4.75 ATP and 3 reducing equivalents to 4.25 ATP and only 2 reducing equivalents. Thus, the photorespiratory "burden" on CO_2 fixation

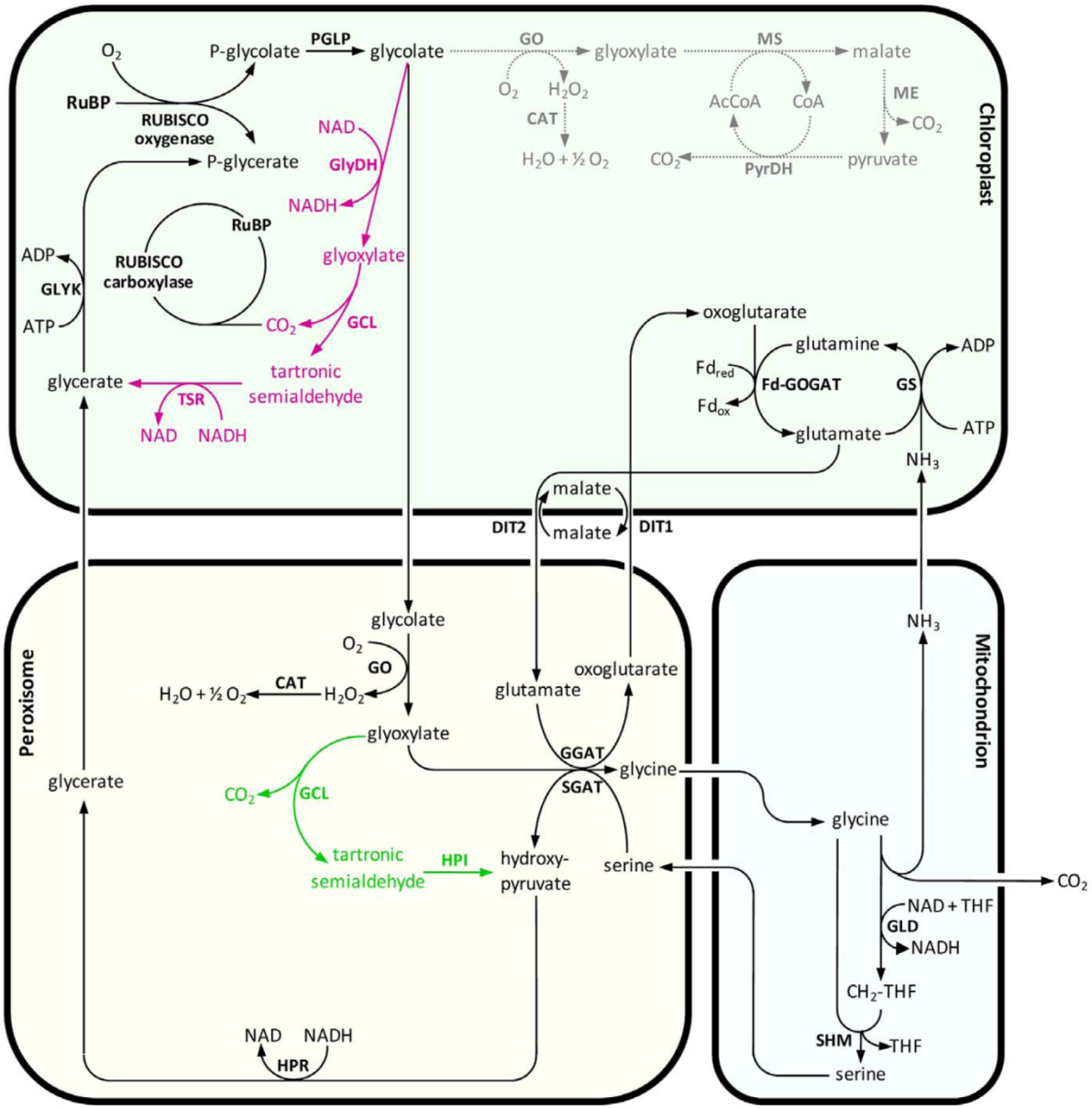


Figure 7. Transgenic pathways for the reduction of photorespiratory losses in Arabidopsis.

Overview of the major photorespiratory pathway (black) and different transgenic approaches for the reduction of photorespiratory losses (red, dotted grey, and green). The red pathway shows the glycerate pathway from *E. coli* integrated into the chloroplast, the dotted grey pathway shows the alternative complete oxidation of glycolate inside the chloroplast, and the green pathway shows a shortcircuit inside the peroxisome. RuBP, ribulose-1,5-bisphosphate; RUBISCO, RuBP carboxylase/oxygenase; PGLP, phosphoglycolate phosphatase; GO, glycolate oxidase; CAT, catalase; GGAT, glyoxylate:glutamate aminotransferase; SGAT, serine:glyoxylate aminotransferase; DIT1, dicarboxylate transporter 1; DIT2, dicarboxylate transporter 2; GLD, glycine decarboxylase; SHM, serine hydroxymethyltransferase; HPR, hydroxypyruvate reductase; GLYK, glycerate kinase; GS, glutamine synthetase; Fd-GOGAT, glutamine:oxoglutarate aminotransferase; GlyDH, glycolate dehydrogenase; GCL, glyoxylate carboligase; TSR, tartronic semialdehyde reductase; MS, malate synthase; ME, malic enzyme; PyrDH, pyruvate dehydrogenase; AcCoA, acetylated Coenzyme A; CoA, Coenzyme A; HYI, hydroxypyruvate isomerase; THF, tetrahydrofolate; CH_2-THF, methylene-THF. The stoichiometry of the reactions is not included.

would be reduced from 50 % to 37 % (cf. chapter 2) assuming the all photorespiratory glycolate would be redirected into the shortcircuit.

3. CO_2 release is shifted from the mitochondrion to the chloroplast. Based on physiological measurements, this enhances the CO_2 concentration in the chloroplast, improves the probability of refixation of the released CO_2 molecule, and reduces the probability of the next oxygenase reaction (Kebeish et al., 2007).

Interestingly, overexpression of GlyDH alone was already sufficient to induce the growth phenotype in large part (Kebeish et al., 2007). This provides independent evidence that chloroplasts are capable of further metabolizing glyoxylate as already suggested in chapter 4.

The alternative conversion of glycolate to malate instead of glycerate (dotted grey pathway in Figure 7) has also been tested (Maurino and Flügge, 2009). In this approach, glycolate is oxidized by glycolate oxidase and the resulting H_2O_2 is detoxified by catalase. Malate synthase forms malate from glyoxylate and Acetyl-CoA present in the chloroplast. Through two additional endogenous reactions, Acetyl-CoA is recycled and glyoxylate fully converted to CO_2. Also installation of this pathway in Arabidopsis resulted in an enhanced growth phenotype (Maurino and Flügge, 2009).

A third possible synthetic pathway to improve the energy balance of photorespiration was suggested by Parry et al. (2007) (green pathway in Figure 7). The authors aimed at bypassing photorespiratory NH_3 release by installation of a shortcircuit in the peroxisome. This pathway contained two enzymes, glyoxylate carboligase and hydroxypyruvate isomerase. The former enzyme ligates two molecules of glyoxylate to form tartronic semialdehyde as described above and the latter converts tartronic semialdehyde to hydroxypyruvate that is again integrated into the endogenous pathway. Overexpression of this pathway in tobacco resulted in plants with chlorotic lesions for unknown reasons.

Results from these studies suggest that manipulation of photorespiration can in fact improve plant photosynthesis as hoped during the early days of photorespiration research. It is fascinating to see that two of the recently developed engineering approaches resemble those pathways that nature established in *Synechocystis* as alternatives to the major pathway. This knowledge was not available when the synthetic pathways were designed, but the coincidence hopefully further motivates scientists to use natural variation as a template for genetic engineering. However, the results from the third study also sound a note of caution that any synthetic interference with basal metabolism might result in unexpected phenotypes as long as the metabolic integration of photorespiration is not fully understood.

7. CONCLUSIONS

In this review, we summarized the current knowledge about photorespiration in Arabidopsis. Although the initial identification of the process dates to the pre-Arabidopsis era of plant research, work in Arabidopsis allowed for the detailed characterization of most of the pathway components. Future work in this direction will hopefully lead to the identification of the multiple metabolite transporters necessary for photorespiration. More ecophysiological work is also necessary to understand regulation and metabolic integration of the pathway and the significance of alternative detours under certain growth conditions.

Figure 8. Photorespiratory bypass increases biomass production in Arabidopsis.

Arabidopsis plants were grown at 100 µE light and a 8h light/16 h dark light regime for 12 weeks. After 8 weeks, plants were repotted from 8 cm pots to 15 cm pots. The upper right and lower left plants express the photorespiratory bypass (red pathway in Figure 7), the upper left and lower right plants are wildtype controls.

The ease of Arabidopsis transformation allowed for testing synthetic pathways that deviate some of the phosphoglycolate from photorespiration. Promising initial results currently motivate researchers to test the applicability of these and related approaches in crop improvement (Hibberd et al., 2008; Peterhansel et al., 2008; Sage and Sage, 2009). But what is the significance of photorespiration in a future earth atmosphere with high CO_2 concentrations? Current projections expect CO_2 concentrations to rise by more than 40 % compared to today until 2050. An upper limit of perhaps double the current CO_2 concentration may be reached until 2100 (Intergovernmental panel on climate change, 2007). At first sight, doubling the atmosphere's CO_2 content would reduce photorespiration to one half (Sharkey, 1988) which would, in turn, diminish the importance of photorespiration in the control of plant performance. However, the situation is much more complex. An expected increase in average atmospheric temperatures by 3 °C (Intergovernmental panel on climate change, 2007) alone will reduce the specificity of RUBISCO for CO_2 by approximately 10 % (Jordan and Ogren, 1984). Actual leaf temperatures might increase much stronger because higher CO_2 concentrations tend to induce stomatal closure and by this reduce transpirational leaf

cooling (Ainsworth and Rogers, 2007; Bernacchi et al., 2007). Furthermore, the frequency and intensity of droughts is predicted to increase (Li et al., 2009), growth conditions at which photorespiration is high. Independent of climate change, the ever growing world population will demand food production on less favourable grounds, in particular in hot and dry areas (Bruinsma, 2009; Eckardt et al., 2009). Thus, beside the fascination provided by the intricate complexity of plant carbon metabolism, there are very good reasons for plant researchers to continue studying photorespiration.

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2.2 Photorespiratory bypasses: how can they work?

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OPINION PAPER

Photorespiratory bypasses: how can they work?

Christoph Peterhansel*, Christian Blume and Sascha Offermann

Leibniz University Hannover, Institute of Botany, Herrenhaeuser Straße 2, 30419 Hannover, Germany

* To whom correspondence should be addressed: E-mail: cp@botanik.uni-hannover.de

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Abstract

Photorespiration has been suggested as a target for increasing photosynthesis for decades. Within the last few years, three bypass pathways or reactions have been designed and tested in plants. The three reactions bypass photorespiration either in the chloroplast or in the peroxisome, or oxidize glycolate completely to CO_2 in the chloroplast. The reactions differ in their demand for energy and reducing power as well as in the catabolic fate of glycolate. The design, energy balance, and reported benefits of the three bypasses are compared here, and an outlook on further optimization is given.

Key words: CO_2 refixation, bypass, energy balance, glycolate oxidase, photorespiration, synthetic biology.

Introduction

The dual function of ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) and the associated carbon and nitrogen losses in the course of the photorespiratory cycle were discovered in the 1970s and 1980s (Ogren, 1984). Much of this research was motivated by the hope that a reduction of photorespiration could improve plant photosynthesis and yield (Zelitch and Day, 1973). This idea was first challenged by the conditionally lethal phenotypes of photorespiratory mutants and by the unavailability of second-site suppressor mutants that would identify alternative catabolic routes for photorespiratory metabolites (Somerville and Ogren, 1982; Somerville, 2001). The prevailing view on photorespiration has completely changed since the pathway is recognized today as an integral element of primary carbon metabolism that interacts with many other pathways (Maurino and Peterhansel, 2010; Bauwe *et al*., 2012). One important step towards this change in perspective was the unexpected finding that photorespiration is essential for the survival of all organisms that use Rubisco for CO_2 fixation (Nakamura *et al*., 2005; Eisenhut *et al*., 2008; Zelitch *et al*., 2009). Previous to these studies, the low rates of photorespiration observed in photosynthetic organisms with carbon-concentrating mechanisms such as C_4 plants and most green algae and cyanobacteria were assumed to be insignificant. Moreover, important functions were assigned to photorespiration in the protection of plants from excess light (Kozaki and Takeba, 1996) or in supplying reducing power for nitrate assimilation (Rachmilevitch *et al*., 2004; Bloom *et al*., 2010).

Independently of this new positive reputation of photorespiration, three different principles for the reduction of photorespiratory losses by genetic engineering were suggested and two of them proved to be successful, at least in the model system *Arabidopsis* (Kebeish *et al*., 2007; Carvalho *et al*., 2012; Maier *et al*., 2012). In this review, we discuss the strengths and weaknesses of the different approaches and speculate about the physiological basis of the observed growth improvement.

Three bypasses have been suggested

In 2007, our group proposed a bypass to photorespiration in the chloroplast with the objective of reducing photorespiratory losses (Kebeish *et al*., 2007). The strategy is shown as bypass 1 on the background of photorespiration in Fig. 1. Key enzymatic reactions are explained in Table 1. The pathway starts with glycolate and ends with glycerate. Thus, it diverts metabolites from photorespiration, but also feeds back into this cycle. Glycolate is oxidized by a glycolate dehydrogenase (GlycolateDH) from *E. coli* that uses organic co-factors instead of oxygen as electron

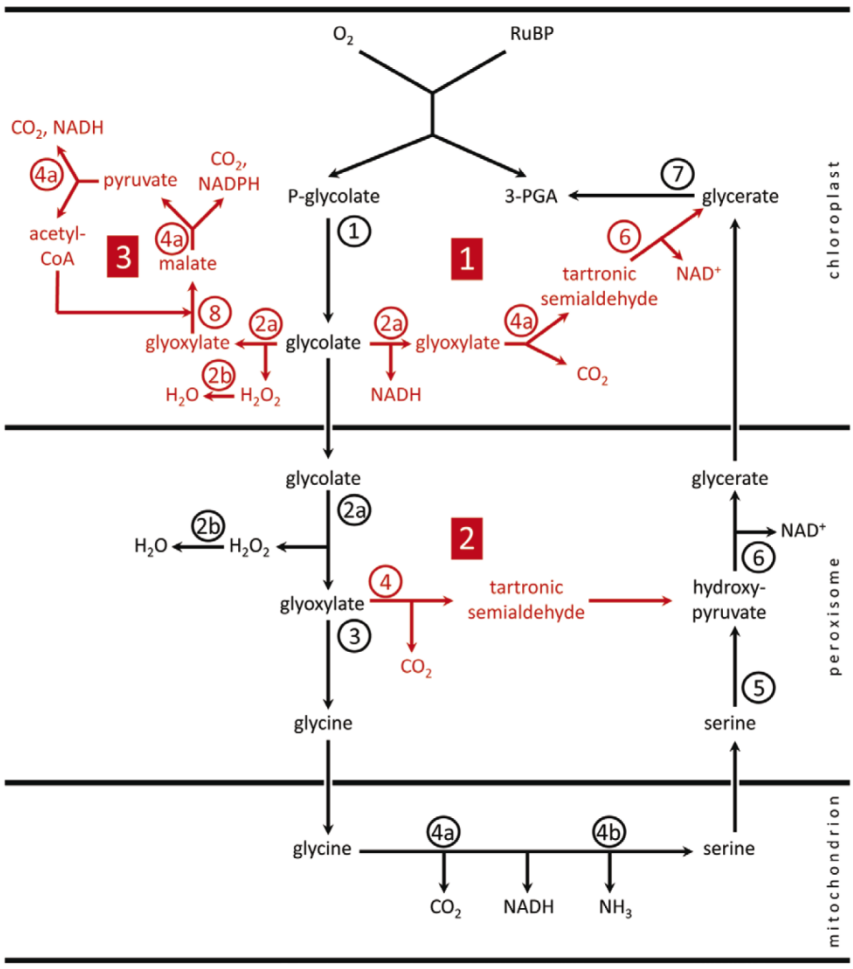

Fig. 1. Scheme of photorespiration (black) and the three bypasses for the reduction of photorespiratory losses (red, numbers in boxes). The arrows indicate enzymatic reactions or transport steps. Enzymatic reactions (numbers in circles) are explained in Table 1. The stoichiometry of the reactions is not included. 3-PGA, 3-phosphoglycerate.

Table 1. Comparison of the enzymatic reactions of photorespiration and the three bypasses

	Reaction	Photorespiration	Bypass 1	Bypass 2	Bypass 3
1	Phosphoglycolate dephosphorylation	PGLP	PGLP	PGLP	PGLP
2a	Glycolate oxidation	GO	GlycolateDH	GO	GO
2b	H_2O_2 detoxification	CAT	–	CAT	CAT
3	Transamination	GGAT	–	–	–
4a	Decarboxylation	GDC, SHMT	GCL	GCL	ME, PDH
4b	NH_3-release	GDC, SHMT	–	–	–
5	Transamination	SGAT	–	–	–
6	Reduction	HPR	TSR	HPR	–
7	Glycerate phosphorylation	GK	GK	GK	–
8	Other				MS

PGLP, phosphoglycolate phosphatase; GO, glycolate oxidase; CAT, catalase; GGAT, glutamate:glyoxylate aminotransferase; GDC, glycine decarboxylase; SHMT, serine hydroxymethyltransferase; SGAT, serine:glyoxylate aminotransferase; HPR, hydroxypyruvate reductase; GK, glycerate kinase; GlycolateDH, glycolate dehydrogenase; GCL, glyoxylate carboxylyase; TSR, tartronic semialdehyde reductase; ME, malic enzyme; PDH, pyruvate dehydrogenase; HPI, hydoxypyruvate isomerase; MS, malate synthase.

acceptors (Lord, 1972). This allows for the conservation of reducing power associated with this reaction as reducing equivalents. Similar to photorespiration, two C2 compounds (glyoxylate) are merged to one C3 compound [here tartronic semialdehyde (TS)] with the release of CO_2 (note that stoichiometries are not included in the figure). CO_2 release is shifted from mitochondria to chloroplasts and catalysed by a glyoxylate carboligase (GCL) also derived from *E. coli* (Chang *et al*., 1993) in plants over-expressing this bypass. The product of the decarboxylation reaction is then reduced to glycerate by tartronic semialdehyde reductase (TSR). Similar to the major pathway, three-quarters of the glycolate fed into this bypass are re-converted into Calvin cycle intermediates. This reduces carbon outflow from the cycle caused by RuBP oxygenation. A major difference from photorespiration is that ammonia release is avoided and therefore refixation is not required. Concomitantly with the first publication introducing this bypass, it became evident that the photosynthetic cyanobacterium *Synechocystis* can use the same pathway as an alternative to photorespiration (Eisenhut *et al*., 2006).

Bypass 2 (Fig. 1) resembles bypass 1 in that it starts with a photorespiratory intermediate and produces another photorespiratory intermediate (Carvalho *et al*., 2012). The decarboxylation reaction is catalysed by the same enzyme as in bypass 1, but targeted here to the peroxisome. The resulting tartronic semialdehyde is fed back into photorespiration by an isomerase reaction (HYI). In this bypass, ammonia release is abolished and three-quarters of the carbon from glycolate is converted to 3-PGA. This balance is identical to the balance of bypass 1. Organisms using this pathway for photorespiration have not yet been identified, but the reactions of bypass 2 might be involved in glycolate or glyoxylate metabolism of *E. coli* (Ashiuchi and Misono, 1999).

Bypass 3 is not a true bypass as there is no reconnection to the major pathway (Fahnenstich *et al*., 2008; Maier *et al*., 2012). Instead, this pathway completely oxidizes glycolate to CO_2 by a combination of endogenous and newly introduced enzymes. Glycolate oxidation in this pathway is catalysed by a plant glycolate oxidase (GO) that was relocated from the peroxisome to the chloroplast. As this enzyme produces equimolar amounts of H_2O_2 during glycolate oxidation, an additional catalase (CAT, also from the peroxisome) is required for detoxification. Together with acetyl-SCoA, glyoxylate resulting from glycolate oxidation is converted to malate by malate synthase (MS), thus, two C2 compounds are metabolized to one C4 compound here. The malate synthase reaction is normally part of storage lipid mobilization in glyoxysomes of young plants (Kindl, 1993; Cornah *et al*., 2004). In *E. coli*, the gene for malate synthase is part of the operon encoding the subunits of GlycolateDH (Pellicer *et al*., 1996) suggesting an additional role in glycolate metabolism in prokaryotes. The remainder of the pathway is supposed to be catalysed by endogenous enzymes. Decarboxylation of malate to pyruvate by malic enzyme (ME) is the first CO_2 release reaction. Further oxidation of pyruvate to CO_2 by pyruvate dehydrogenase (PDH) produces acetyl-SCoA as a by-product that can be used for the next formation of malate from glyoxylate. Both endogenous reactions of bypass 3 result in the formation of reducing equivalents and shift CO_2 release reactions from the mitochondrion to the chloroplast. Experimental evidence for the presence of the required enzyme activities in chloroplasts is available (Johnston *et al*., 1997; Maier *et al*., 2011). In total, the net reaction balance of bypass 3 is the conversion of glycolate and O_2 to CO_2 and H_2O accompanied by the formation of reducing equivalents. Noteworthy, the reaction depletes the Calvin cycle from intermediates when compared with photorespiration or the two other bypasses as no 3-PGA is recycled.

Potential benefits of the three bypasses

From an engineer's point of view, architecture of the bypasses is much simpler than the circuitous photorespiratory pathway. Particularly, bypasses 1 and 3 avoid any transmembrane transport whereas photorespiration is associated with at least 12 transport processes (Reumann and Weber, 2006). Bypass 2 would also clearly reduce the number of necessary transport steps compared with photorespiration. However, an answer to the question whether reduction of transport steps would improve energetics of the pathway has to await the molecular identification of the transporters involved.

Two potential benefits of the bypasses on plant carbon assimilation are conceivable. Bypass 1 and bypass 3 release CO_2 in the chloroplast. This might increase the chloroplastic CO_2 concentration and reduce the probability of further oxygenation reactions. From the physiological characterization of bypass 1 plants, there is evidence that such an increase in chloroplastic CO_2 indeed takes place based on the reduction of the CO_2 compensation point compared with the controls (Kebeish *et al*., 2007, see also below). For bypass 3, no change in compensation point was reported. From the stoichiometry of the reaction, it would have been expected that the extra release of CO_2 (2 CO_2/glycolate instead of 0.5 CO_2/glycolate in photorespiration) would increase the CO_2 compensation point. This has, for instance, been described for *Arabidopsis* mutants that convert part of the glycolate to CO_2 in the peroxisome (Cousins *et al*., 2008). The apparent absence of such an increased compensation point in bypass 3 over-expressors (Maier *et al*., 2012) already indicates an improved availability of CO_2 in the chloroplast. Efficacy of a chloroplastic CO_2 release system would strongly depend on the degree of refixation of CO_2 released in the mitochondrion by photorespiration (or in the peroxisome by bypass 2). If the CO_2 released outside the chloroplast would be refixed anyway, the location of CO_2 release should not matter. It has been suggested that such refixation can be significant if chloroplasts cover the outer surface of cells so that any CO_2 released somewhere in the cell has to diffuse through a chloroplast before reaching the intercellular space (Sage and Sage, 2009). Even an active channelling mechanism for CO_2 from the mitochondrion to the chloroplast in higher plants has been suggested (Braun and Zabaleta, 2007; Zabaleta *et al*., 2012). In C_4 plants, optimization of CO_2 refixation by the restriction of photorespiratory CO_2 release to the mitochondria of bundle sheath cells might have been the first dedicated evolutionary step towards an efficient CO_2 concentration system (Sage *et al*., 2012). Thus, there is ample evidence for evolutionary pressure towards the optimization of CO_2 fixation. As the efficiency of the endogenous refixation system is still a matter of ongoing debate, the benefit of chloroplastic CO_2 release cannot be included in the energy balance of the various pathways, although significant impact can readily be imagined.

The second pontential benefit relates to the energy balance of the bypasses. Table 2 shows a calculation of the demand of the different bypasses for reducing equivalents (red. eq) and ATP. This is based on a balance sheet for photorespiration that has recently been published (Peterhansel *et al.*, 2010) and that calculated the energy demand to reestablish the 'status quo ante', i.e. completely to resynthesize the RuBP that has been oxygenated by Rubisco. Here, the energy balances of the bypasses are calculated compared with photorespiration using the same assumptions. Note that this calculation is different from the calculation used by Maier *et al.* (2012), because the costs for the reduction of released CO_2 are integrated here. All numbers in the calculation are per molecule glycolate synthesized and therefore per oxygenation event. Several aspects have to be taken into consideration.

(i) All bypasses avoid ammonia release and, thus, do not require ammonia refixation.
(ii) All bypasses circumvent the mitochondrial GDC/SHMT reaction. Absence of NADH synthesis in this reaction has to be accounted for as a penalty in terms of energy balance.
(iii) Bypass 1 uses a glycolate dehydrogenase for glycolate oxidation that synthesizes reducing equivalents instead of transferring electrons to oxygen. This reduces the net consumption of reducing equivalents in this pathway.
(iv) Bypass 3 liberates two CO_2 from glycolate that have to be refixed instead of 0.5 CO_2 for photorespiration and bypasses 1 and 2. Based on Calvin cycle stoichiometry, we calculate three ATP and two reducing equivalents per CO_2 refixation. This makes 4.5 ATP and three reducing equivalents for the extra CO_2 released in bypass 3.
(v) Bypass 3 does not produce 3-phosphoglycerate (3-PGA) as part of the glycolate recycling process. Therefore, no costs for the re-reduction of 3-PGA in the Calvin cycle have to be calculated. Moreover, the bypass avoids NADH consumption for hydroxypyruvate (HO-Pyr) or tartronic semialdehyde (TS) reduction, which is integrated in the calculation as an additional advantage.
(vi) Bypass 3 synthesizes NAD(P)H in both the malic enzyme reaction and the pyruvate dehydrogenase reaction. This results in an additional bonus of two reducing equivalents per glycolate.
(vii) When reducing equivalents are used for ATP synthesis in the mitochondrial electron transport chain, an average of 2.5 ATP/NAD(P)H are synthesized (Ferguson, 1986; Hinkle, 2005). This rough estimate is used here to convert reducing power into energy equivalents.

In summary, bypass 1 shows an improved energy balance compared with photorespiration. This is mostly due to the energy gain during glycolate oxidation. The energy consumption of bypass 2 is only slightly lower than the photorespiratory energy demand. Bypass 3 ends up with an increased energy demand relative to photorespiration. Thus, the potential benefit of bypass 3 strongly depends on whether chloroplastic CO_2 release improves CO_2 refixation and/or reduces the probability of oxygen fixation.

Importantly, it is questionable whether an improved energy balance of photorespiration is always desirable for the plant. Oxygen fixation by Rubisco is highest in hot and dry climates (Ku and Edwards, 1977*a*, *b*). Under these conditions, energy supply from the light reactions is often not limiting photosynthesis. Instead, strong electron sinks are required to avoid overreduction

Table 2. The energy balance sheet of photorespiration and the three bypasses in comparison

	Photorespiration		**Bypass 1**		**Bypass 2**		**Bypass 3**	
	Red.eq.	**ATP**	**Red.eq.**	**ATP**	**Red.eq.**	**ATP**	**Red.eq.**	**ATP**
Reduction of 0.5 CO_2	1	1.5	1	1.5	1	1.5	1	1.5
Re-fixation of 0.5 NH_3	0.5	0.5	0	0	0	0	0	0
Phosphorylation of 0.5 glycerate	0	0.5	0	0.5	0	0.5	0	0
Reduction of 1 PGA	1	1.5	1	1.5	1	1.5	1	1.5
formed by RubisCO								
Reduction of 0.5 PGA formed	0.5	0.75	0.5	0.75	0.5	0.75	0	0
by photorespiration								
Bonus for GlycolateDH			–1					
Penalty for missing GDC			0.5		0.5		0.5	
NADH synthesis								
Penalty for complete glycolate							3	4.5
oxidation (1.5 CO_2 extra)								
Bonus for no HO-Pyr/TS reduction							0.5	
Bonus for ME and PDH							–2	
Total units	**3**	**4.75**	**2**	**4.25**	**3**	**4.25**	**4**	**7.5**
Assuming 2.5 ATP/NAD(P)H	7.5		5		7.5		10	
Total ATP units		**12.25**		**9.25**		**11.75**		**17.5**

Red. eq., reducing equivalents; PGA, 3-phosphoglycerate; GlycolateDH, glycolate dehydrogenase; GDC, glycine decarboxylase; HO-pyr, hydroxypyruvate; TS, tartronic semialdehyde; ME, malic enzyme; PDH, pyruvate dehydrogenase. Positive numbers refer to requirements and negative numbers to produced red. eq. or ATP.

of the chloroplastic electron transport chain and generation of reactive oxygen species (ROS) (Demmig-Adams and Adams, 1992; Murata *et al*., 2007). Perhaps it is one of the benefits of the peroxisomal glycolate oxidase that ROS are generated under controlled conditions in the peroxisome allowing for efficient detoxification. In line with this argument, there is evidence that both glycolate dehydrogenases and glycolate oxidases, respectively, were used for glycolate oxidation in different evolutionary lineages of cyanobacteria and unicellular photosynthetic eukaryotes (Kern *et al*., 2011). It seems reasonable that higher plants preferred a glycolate oxidase rather than a glycolate dehydrogenase for oxygen fixation because this enzyme burns excess reducing power instead of producing even more. This effect would even be amplified if excess glyoxylate produced by glycolate oxidase would leak out of the peroxisome into the cytoplasm where it could be reduced back to glycolate by a cytosolic glyoxylate/hydroxypyruvate reductase (Givan and Kleczkowski, 1992; Timm *et al*., 2008). This could establish a cycle that efficiently oxidizes excess reducing equivalents. Similar to the CO_2 refixation argument above, the actual benefit will strongly depend on environmental and metabolic parameters.

Reported benefits of the three bypasses

Data on the characterization of transgenic plants over-expressing one of the three bypasses have now been published. Bypass 2 was partially established in tobacco (Carvalho *et al*., 2012). Whereas the first introduced enzyme, GCL, was highly expressed in peroxisomes, HYI expression was undetectable probably due to co-suppression of RNA accumulation (Eamens *et al*., 2008). GCL over-expressors exhibited chlorosis and stunted growth when grown in ambient air, but not at elevated CO_2 levels. This phenotype was reminiscent of classical photorespiratory mutants and indicated that conversion of glyoxylate to tartronic semialdehyde in the peroxisome without further metabolism to hydroxypyruvate might be deleterious for the plant, perhaps because it provides a metabolic dead-end. This observation already suggests caution when manipulating photorespiratory fluxes, probably because of the above-mentioned intimate integration of photorespiration in primary plant metabolism (Bauwe *et al*., 2012).

Bypass 1 and bypass 3 have been established in *Arabidopsis*. Besides over-expression of the complete pathway, transgenics over-expressing components of the pathways were also studied (Kebeish *et al*., 2007; Fahnenstich *et al*., 2008; Maier *et al*., 2012). Both studies indicated that the bypasses led to enhanced growth under the test conditions (8 h days and approximately 100 µE light) with reported maximum biomass increases of 30–50% at the end of the growth period. Even in *Arabidopsis*, photosynthesis is definitely light-limited under these conditions suggesting an energy advantage as a basis of the growth effect. However, as our calculation does not reveal any energy advantage for bypass 3, the more probable interpretation is that enhancement of chloroplastic CO_2 concentrations might control enhanced growth. Both studies also mention that enhanced biomass accumulation was absent under long-day conditions. This could be interpreted as another argument for improved energy balance as the major factor controlling enhanced growth. However, it should be taken into consideration that the vegetative growth period of *Arabidopsis* is very short in long days. A small increase in daily photosynthesis by over-expression of a bypass might result in a small increase in leaf area compared with the wild-type at the end of the day. This would again increase the leaf area that is available for photosynthesis during the next day and would result in an exponential relationship between daily carbon gain and final biomass (assuming that no other factors are limiting). In this scenario, the short vegetative period might not allow for the detection of growth increases although photosynthesis might be enhanced.

Interestingly, both studies also report that leaves of transgenic plants had a tendency for flatter and thinner growth. These results might indicate links between physiological and anatomical adaptations that have to be followed up in further studies.

Both papers used glycine levels and/or glycine/serine ratios (Novitskaya *et al*., 2002) to estimate the flux through photorespiration in the presence and absence of the respective bypass. Reported reductions range from 2-fold to 5-fold under conditions of high photorespiratory flux indicating that a significant part of glycolate is catabolized in chloroplasts in transgenic plants. This is also consistent with post-illumination CO_2 burst values (Atkin *et al*., 1998) that are reduced in plants over-expressing bypass 1. Moreover, plants over-expressing an incomplete bypass 3 without a catalase for H_2O_2 detoxification showed stunted growth (Maier *et al*., 2012). This also indicated that appreciable amounts of glycolate were oxidized in the chloroplast and produced damaging concentrations of H_2O_2. Although these estimates all point into the same direction, final accurate numbers about metabolic flow through the bypasses probably require the use of metabolic flux labelling with heavy isotopes of CO_2, O_2, or glycolate (Fernie *et al*., 2005; Hasunuma *et al*., 2010).

Gas exchange measurements indicated that both bypasses enhance the photosynthetic rate when calculated per leaf area (for bypass 1, Kebeish *et al*., 2007) or per mol chlorophyll (for bypass 3, Maier *et al*., 2012), respectively. Only for bypass 1, a reduction of the CO_2 compensation point was reported (Kebeish *et al*., 2007), suggesting better CO_2 refixation. As stated above, even an unchanged compensation point in bypass 3 over-expressors argues for better refixation because of the different stoichiometry of CO_2 release.

Outlook

Our theoretical calculations and the experimental results indicate that photorespiratory bypasses can enhance plant productivity. Whether this is due to better CO_2 availability for refixation as compared with photorespiration or caused by improvements in the energy balance of the pathway still remains to be shown. It is therefore important to learn more about the degree of refixation of photorespiratory CO_2 in wild-type plants. Moreover, growth studies with bypass plants under different combinations of varying CO_2 supply and limited irradiation (Poorter and Pérez-Soba, 2001) will help to elucidate the bottleneck that is overcome by bypass installation.

Bypass 1 still suffers from unequal transgene expression and the multiple proteins necessary for establishment of the pathway.

This is exemplified by the linear dependence of the growth effect on the expression of subunit F of *E. coli* GlycolateDH that is much lower than the expression of the other genes. However, physiological measurements and growth assays with plants over-expressing GlycolateDH alone suggested that glyoxylate synthesis in the chloroplast is already sufficient to induce the growth effect and partly to reduce flux through photorespiration (Kebeish *et al*., 2007; Peterhansel and Maurino, 2011). It was speculated that endogenous enzymes are capable of oxidizing glyoxylate to CO_2 at low rates. This is consistent with experimental results describing CO_2 release from glycolate or glyoxylate in chloroplast extracts (Kisaki and Tolbert, 1969; Zelitch, 1972; Kebeish *et al*., 2007). The carbon and energy balance of such a pathway would be similar to what has been described here for bypass 3. More knowledge about the endogenous enzymes involved in downstream reactions is necessary for improving the efficiency of this pathway by genetic engineering.

The combination of glycolate oxidase and catalase enzymes for glycolate oxidation in the chloroplast requires sophisticated balancing of both activities to avoid deleterious side-effects. GlycolateDH enzymes might be better suited for this function, but the *E. coli* enzyme used for bypass 1 is made up from three subunits complicating the genetic approach. A single-subunit GlycolateDH enzyme has been identified in a mutational screen in *Chlamydomonas* (Nakamura *et al*., 2005). Also the *Synechocystis* homologue to subunit D of *E. coli* GlycolateDH seems to exhibit GlycolateDH activity (Eisenhut *et al*., 2006). Such enzymes may also provide superior starting points for the efficient establishment and characterization of photorespiratory bypasses in crop plants. However, as both green algae and cyanobacteria express efficient CO_2 concentrating mechanisms (Price *et al*., 2007; Spalding, 2007) and photorespiratory flux rates are low, the enzymatic proficiency of these enzymes will have to be studied in more detail.

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2.3 A role for the chloroplast pyruvate dehydrogenase complex for glycolate and glyoxylate metabolism

A role for the chloroplast pyruvate dehydrogenase complex in plant glycolate and glyoxylate metabolism

Authors

Christian Blume[1], Christof Behrens[2], Holger Eubel[2], Hans-Peter Braun[2], Christoph Peterhansel[1,*]

Affiliations

[1]Leibniz University Hannover, Institute of Botany, D 30419 Hannover, Germany

[2]Leibniz University Hannover, Institute of Plant Genetics, D 30419 Hannover, Germany

[*]Corresponding author: Christoph Peterhansel, +49-511-7622632, cp@botanik.uni-hannover.de

Abstract

Glyoxylate is a peroxisomal intermediate of photorespiration, the recycling pathway for 2-phosphoglycolate (2-PG) produced by the oxygenase activity of Rubisco. Under hot and dry growth conditions, photorespiratory intermediates can accumulate and must be detoxified by alternative pathways, including plastidal reactions. Moreover, there is evidence that chloroplasts are capable of actively producing glyoxylate from glycolate. Further metabolic steps are unknown, but probably include a CO_2 release step. Here, we report that CO_2 production from glycolate and glyoxylate in isolated tobacco chloroplasts can be inhibited by pyruvate, but not related compounds. We isolated a protein fraction that was enriched for the chloroplast pyruvate dehydrogenase complex (PDC). The fraction contained a protein complex of several MDa in size that included all predicted subunits of the chloroplast PDC and a so far unidentified HSP93-V/ClpC1 heat shock protein. Glyoxylate competitively inhibited NADH formation from pyruvate in this fraction. The Km for pyruvate and the

Ki for glyoxylate were 330 µM and 270 µM, respectively. Glyoxylate decarboxylation was also enriched in this fraction and could be in turn inhibited by pyruvate. Based on these data, we suggest that the chloroplast PDC is part of a pathway for glycolate and/or glyoxylate oxidation in chloroplasts.

Keywords: photorespiration, heat shock protein Hsp93-V, two-dimensional BN/SDS-PAGE

Abbreviations: PDC, pyruvate dehydrogenase complex; TPP, thiamine pyrophosphate; 2D-BN/SDS-PAGE, two dimensional blue native SDS-PAGE

1. Introduction

Glyoxylate is a central intermediate of photorespiration, by mass flux the second most important biochemical pathway behind photosynthesis (Sharkey, 1988). Photorespiration is the salvage reaction for 2-phosphoglycolate formed during oxygenation of ribulose-1,5-bisphosphate by Rubisco (Bowes et al., 1971). All Rubisco enzymes catalyze this reaction beside their carboxylation function (Andersson, 2008). Photorespiration starts with dephosphorylation of 2-PG in chloroplasts. Resulting glycolate is exported to the peroxisome, where it is oxidized to glyoxylate and transaminated to glycine. Glycine is transported to mitochondria where two molecules of glycine are converted to one molecule of serine. This reaction includes the release of CO_2 and NH_3 that have to be refixed. Refixation requires energy and reducing power. Thus, photorespiration is an energy-consuming pathway that can limit plant productivity when high amounts of 2-PG are formed. Serine is transported back to the peroxisome, where it is deaminated and reduced to glycerate. The pathway ends with phosphorylation of glycerate in the chloroplast. The product 3-phosphoglycerate can be integrated into the Calvin cycle and used for carbohydrate production (Boldt et al., 2005).

The amount of 2-PG synthesized by Rubisco varies dependent on conditions. High temperatures reduce the specificity of Rubisco for CO_2 over O_2 and increase the relative solubility of O_2 in the aqueous cytoplasm (Brooks and Farquhar, 1985; Jordan and Ogren, 1984; Ku and Edwards, 1977). Furthermore, high temperatures often coincide with drought. Closure of stomata under these conditions limits gas exchange and CO_2 availability in the leaf intracellular space. All this can result in high rates of 2-PG formation (Cornic and Briantais, 1991; Lawlor and Fock, 1977). Under these conditions, intermediates of photorespiration can accumulate (Campbell and Ogren, 1990; Srivastava et al., 1099) and can be further metabolized by alternative pathways. It has been suggested that glyoxylate can be directly decarboxylated in peroxisomes to formate if capacities of photorespiration are exhausted (Grodzinski, 1978). If this mechanism is also not efficient enough, glyoxylate can diffuse back into the chloroplast and inhibit Calvin cycle enzymes (Campbell and Ogren, 1990; Chastain and Ogren, 1989). Probably for this reason, chloroplasts contain protective enzymes that can reduce glyoxylate back to glycolate (Allan et al., 2009). However, there is also evidence that chloroplasts contain a pathway that oxidizes glycolate to glyoxylate and further to CO_2 (Frederick et

al., 1973; Kebeish et al., 2007). When glyoxylate is fed to isolated chloroplasts, CO_2 release from this compound can be observed (Zelitch, 1972). Enzymes catalyzing the reaction steps of this pathway have not been identified so far.

In this paper, we report evidence that the chloroplast pyruvate dehydrogenase complex (PDC) from tobacco decarboxylates glyoxylate and participates in the plastidal conversion of glycolate to CO_2. In plants, PDCs are present in both mitochondria and chloroplasts. Both complexes consist of several copies each of at least four enzymes, which together convert pyruvate to Acetyl-CoA under release of CO_2 and production of NADH (Reid et al., 1977). Whereas the mitochondrial complex mainly provides substrates for the citric acid cycle (Randall et al., 1977), the chloroplast homologue produces precursors for fatty acid biosynthesis (Camp and Randall, 1985). Subunits of the chloroplast PDC were previously predicted based on their homology to their mitochondrial counterparts (Johnston et al., 1997). As part of this study, chloroplast PDC and its subunit composition were analysed by combination of 2D-BN/SDS-PAGE (Schagger et al., 1994) and mass-spectrometry resulting in the identification of novel associated proteins. Furthermore, an estimation of the molecular weight of the native PDC is given.

2. **Material and methods**

2.1 Plant material and growth conditions

Leaves from 28-32 days old *Nicotiana tabacum* (*cv PDB6*) plants grown under long day conditions (14h illumination, 10h darkness) in the greenhouse were used for chloroplast isolation.

2.2 Chloroplast isolation

15 g of leaf material were homogenized 2 times in three 5s -intervals in a pre-chilled 500 ml Warring blender and chilled grinding buffer containing 50 mM Hepes-KOH, pH 8.0; 2 mM EDTA; 1 mM $MgCl_2$; 5 mM sodium ascorbate; 0.5% w/v BSA. The sap was filtered through a 30µm nylon mesh and the filtrate was centrifuged at 300 x g for 4 minutes. The pellet was resuspended in a minimal volume of grinding buffer and 1 ml was loaded on a 10 ml 35% v/v self-forming Percoll gradient, containing 50 mM Hepes-KOH, pH 8.0 and 330 mM sorbitol. After centrifugation for 20 minutes at 19.000 rpm in a swing-out rotor (SW40Ti, Beckman Coulter, Inc.), intact chloroplasts were isolated from the lower green fraction near to the bottom of the gradient. The supernatant was discarded and the chloroplast fraction was washed with 5 volumes SH-buffer (50 mM Hepes-KOH, pH 8.0; 330 mM sorbitol; 1 mM $MgCl_2$). For disruption of chloroplasts, the pellet was resuspended in extraction buffer (50 mM Hepes-KOH, pH 8.0; 5 mM $MgCl_2$; 0.1% v/v Triton X-100). All steps were conducted at 4°C.

2.3 SDS-PAGE and Western blotting

All electrophoretic steps were conducted in a Mini Protean II cell from Bio-Rad (Hercules, USA). Standard protocols were used for SDS-PAGE and Western blot. The primary antibodies against catalase and cytochrome C oxidase subunit II were purchased from Agrisera (Vännäs, Sweden) and diluted 1/1000 in TTBS (10mM Tris-HCl, pH 7.5; 150mM NaCl, 0.05% v/v Tween-20) supplemented with 1% milk powder for final use. After washing, the blots were incubated with the secondary antibody against rabbit IgG, alkaline phosphatase conjugated from Fermentas (St. Leon-Rot, Germany). Bands were detected with NBT/BCIP solution from Roche (Mannheim, Germany).

2.4 Enrichment of the pyruvate dehydrogenase complex

The PDC purification protocol was adapted from Camp & Randall (Camp and Randall, 1985). Chloroplasts were frozen for 5 minutes to facilitate breakage before they were osmotically shocked by resuspension in RS-buffer (50 mM Hepes-KOH, pH 8.0; 5 mM $MgCl_2$; 100 mM NaCl). The precipitation steps were conducted with 3 and 6% PEG8000, respectively. The pellet was resuspended in a minimal volume RS-buffer and used in enzymatic assays and for 2D-BN/SDS-PAGE.

2.5 Enzymatic assays

CO_2-release from [1,2-^{14}C]glycolate or [1-^{14}C]glyoxylate (Hartmann Analytics, Braunschweig, Germany) was measured in disrupted chloroplasts corresponding to 40 µg of plastidal protein or 5 µg enriched PDC, respectively. Specific radioactivities were 1850 MBq $mmol^{-1}$ and final concentrations were 0.1 mM for each substrate. Released CO_2 was absorbed in 0.5 M NaOH. Samples were incubated overnight and NaOH was constantly mixed to absorb all released CO_2. For inhibition experiments, 1 mM of either glyoxylate, pyruvate, formate, oxalate or malate, pH 8.0, was added at the start of the assay.

The pyruvate dehydrogenase assay was adapted from Lernmark and Gardestrom (Lernmark and Gardestrom, 1994). Pyruvate dependent NAD^+ reduction was recorded photometrically at 340 nm in a plate reader (Synergy Mx, Biotek, Winooski (Vt), USA) at 25°C. The assay-medium contained 50 mM Hepes-KOH, pH 8.0; 100 mM NaCl; 5 mM $MgCl_2$; 2 mM NAD^+; 0.2 mM CoA; 0.4 mM TPP (thiamine pyrophosphate); 0.2% w/v BSA and 20 µg disrupted chloroplasts or 3 µg enriched PDC, respectively.

2.6 2D Blue native (BN) / SDS PAGE

200 µg of precipitated protein resuspended in 100 µl of RS-buffer or isolated chloroplasts in 100 µl SH-buffer corresponding to 400 µg protein (see above) were supplemented with 100 µl solubilization buffer BN (1.5 M aminocaproic acid; 100 mM Bis-tris; 1 mM EDTA, pH 7.0) containing n-

dodecylmaltoside (Sigma Aldrich, St. Louis, USA) with a final detergent/protein ratio of 2 g/g and incubated on ice for 10 minutes. 10 µl of Coomassie blue solution (750 mM aminocaproic acid; 5% w/v Coomassie blue 250 G) were added to each sample prior gel-loading. High-range BN-PAGE was carried out according to (Wittig et al., 2006) using a gradient-gel (2.5-16% acrylamide from top to bottom). Tricine SDS PAGE (Schagger and von Jagow, 1987) for the second gel dimension was carried out in a 3-step gel (16.5% polyacrylamide separation gel; 10% polyacrylamide spacer gel; 2.5% polyacrylamide sample gel; acrylamide-bisacrylamide ratio 16:1) (Jansch et al., 1996). 2D gels had dimensions of 16 cm × 16 cm x 0.1 cm and all electrophoretic separations were carried out in the Protean II electrophoresis chamber from Bio-Rad (Hercules, USA). Proteins were stained by the Coomassie blue-colloidal procedure (Neuhoff et al., 1988). Images of the stained gels were acquired using Umax Powerlook III Scanner (Umax Data System, Taipei, Taiwan) with a resolution of 400 dpi. Protein spots of interest were selected by visual inspection of the gel.

2.7 Protein identification by mass spectrometry

Protein spots were excised from the gel using a manual spotpicker (Genetix, New Milton, UK) and subjected to MS analysis according to (Klodmann et al., 2011) with the settings outlined in (Behrens et al., 2012). Data analysis was carried out using ProteinScape2.1 software (Bruker, Bremen, Germany) and the Mascot search engine (Matrix Science, London, UK). The TAIR10 database (Arabidopsis.org) was searched for matching proteins because of the lack of sufficient protein data available for *Nicotiana*. Search parameters were: enzyme, trypsin/P (up to one missed cleavage allowed); global modification, carbamidomethylation (C), variable modifications, acetyl (N), oxidation (M); precursor ion mass tolerance, 30 ppm; fragment ion mass tolerance, 0.05 Da; peptide charge, 1+, 2+, and 3+; instrument type, electrospray ionization quadrupole time of flight. Minimum peptide length was 4 amino acids, and protein and peptide assessments were carried out if the Mascot Score was greater than 50 for proteins and 15 for peptides.

3. Results

We had previously reported that disrupted chloroplasts are capable of converting [^{14}C]glycolate to $^{14}CO_2$ (Kebeish et al., 2007). We now aimed at identifying possible intermediates of this pathway by adding unlabeled putative competitors to the radioactive assay. In order to avoid any contamination by other organelles, we optimized the chloroplast purification procedure from young tobacco leaves. Figure 1 shows the result of the purification. In Coomassie stained gels, we detected the large subunit of Rubisco as a chloroplast marker. Catalase (Cat) as a peroxisome marker and cytochrome c oxidase (Cox) as a mitochondrial marker were detected by Western Blot. Whereas the total leaf extract contained all three proteins, Cat and Cox were not detectable in the purified chloroplast fraction. Moreover, purified chloroplasts were intact according to the Hill intactness assay (Walker, 1990) (data not shown). Before feeding metabolites, chloroplasts were disrupted with low amounts of detergent (see Material and methods) to allow for access of the various test compounds to the chloroplast stroma. Intact chloroplasts did not efficiently take up these substances. As shown in Figure 2a, CO_2 release from glycolate was dependent on protein activity and absent from the denatured fraction. Figure 2b shows the CO_2 release in the presence of unlabeled putative intermediates of the oxidation reaction. Addition of unlabeled glyoxylate reduced CO_2 release suggesting that glyoxylate is an intermediate of the pathway. A similar degree of reduction was also obtained with pyruvate, but not with oxalate, formate, or malate. These data suggest that pyruvate inhibits a step in plastidal glycolate or glyoxylate oxidation.

In order to directly test whether pyruvate can also inhibit glyoxylate oxidation, we used the same chloroplast fractions, fed [^{14}C]glyoxylate, and measured $^{14}CO_2$ release (Figure 2c). Chloroplasts released CO_2 from glyoxylate with similar efficiency as from glycolate (cf. Figure 2a). As expected, unlabeled glyoxylate was capable of inhibiting radioactive CO_2 release, but a similar degree of inhibition was also obtained with pyruvate.

Pyruvate and glyoxylate are two chemically similar short chain α-keto carbonic acids. We therefore hypothesized that both substances compete for binding to the same enzyme. Pyruvate is decarboxylated in chloroplasts by the plastidal pyruvate dehydrogenase complex (PDC). This reaction

is linked to NADH formation (see Introduction). We were able to detect pyruvate-dependent NADH formation in disrupted chloroplasts (Figure 3). Glyoxylate as an alternative substrate did not result in significant NADH formation, but glyoxylate inhibited pyruvate-dependent NADH formation in this assay.

In order to provide further evidence that the PDC is catalyzing both pyruvate and glyoxylate decarboxylation, we enriched the chloroplast PDC using differential PEG precipitation (Camp and Randall, 1985). To resolve the subunit composition of the complex, 2D-BN/SDS-PAGE was applied. This approach combines separation of native protein complexes up to 45 MDa in the first dimension (Strecker et al., 2012) with analysis of subunit composition of complexes in the denaturating second dimension. Beside residual contamination with Rubisco, three versions of a complex of roughly 2-7 MDa in size (highlighted box) were found in the PDC-enriched fraction. At least two of these complexes were not detectable in the crude chloroplast extract (Figure 4). The complexes were composed each of five to six subunits of 90, 53, 50/49, 45 and 42 kDa. Proteins in the largest complex were identified by mass spectrometry (Table 1). All known enzymatic subunits of chloroplast PDC were detected (E1-alpha, E1-beta, E2, and E3). A gene encoding a homologue of E2, At1g34430, was also identified in the same complex but we were not able to discriminate between the two homologous genes for E3 subunits (At3g16950 and At4g16155). Furthermore, we detected the Hsp93-V/ClpC1 protein, a heat shock protein 100 (HSP100) homologue that is also associated with the Clp protease (Schirmer et al., 1996). Two additional weak bands above band 2 were identified as Rubisco and ATP synthase subunit α contamination. The sum of the molecular masses of all detected subunits is much lower than the apparent molecular mass of the complex. This suggests that multiple copies of the subunits are present in the complex like reported previously for other PDCs (Reid et al., 1977).

In the PDC-enriched fraction, pyruvate-dependent NADH formation was 13.5-fold increased compared to crude chloroplast extracts and could again be inhibited by glyoxylate (Figure 5). Using this assay, we determined the Ki for glyoxylate in the enriched PDC fraction (Figure 6). Graphical representation using the Dixon plot (Dixon, 1953) resulted in an estimated Ki value of 270 µM. This was verified using the Km,app method (Ki = 283 µM, data not shown). Both methods were reviewed by (Kakkar et al., 1999). Based on the reaction velocities at 0 mM inhibitor concentration from the

same experiment, we determined the Km for pyruvate to 330 µM. Thus, both compounds are bound by the enzyme complex with similar affinities.

We also measured glyoxylate-dependent CO_2 release activity of the PDC-enriched fraction (Figure 7). Compared to the crude extract, the PDC-enriched fraction showed an increase in glyoxylate decarboxylation activity by a factor of 9.7. Addition of unlabeled pyruvate inhibited CO_2 release efficiently.

Together, we conclude that the chloroplast PDC exists as a multimeric protein complex which includes the ClpC1 heat shock protein. The complex can catalyze glyoxylate decarboxylation, but this reaction is not linked to NADH formation.

4. Discussion

In this paper, we provide evidence that tobacco chloroplast PDC can catalyze glyoxylate decarboxylation (Figure 7). However, glyoxylate decarboxylation did not result in NADH formation (Figure 3) whereas electrons from decarboxylation of pyruvate were used for NADH formation by the complex (Figures 3 & 5) (Reid et al., 1977). To explain this observation, the series of biochemical reactions catalyzed by the PDC has to be considered: CO_2 release is the first step in the reaction series. With pyruvate as a substrate, a hydroxyethyl moiety remains attached to thiamine pyrophosphate (TPP), a cofactor of the PDC. This moiety is moved on to lipoic acid and subsequently released as Acetyl-CoA. Electrons from a concomitantly reduced disulfide bond in lipoic acid are transferred to FAD and finally to NAD^+ for NADH production (Reid et al., 1977). In analogy, CO_2 release from glyoxylate results in attachment of a hydroxymethyl moiety to TPP (Walsh et al., 1976). There is evidence that the transfer of this smaller moiety to lipoic acid or the subsequent release do not function efficiently. Therefore, electrons are not transferred to NAD^+. Consistent with this idea, Walsh et al (Walsh et al., 1976) observed that pyruvate and glyoxylate can be decarboxylated by mitochondrial PDC from pig heart even when no NAD^+ as electron acceptor is provided. The question of whether TPP or lipoic acid are regenerated or rather exchanged after carbon transfer in this assay has not been investigated so far. TPP is only loosely attached to the PDC complex (Reid et al., 1977) and our assays contained excess amounts of TPP (see Material and methods). Therefore, regeneration of TPP was probably not necessary *in vitro*. The *in vivo* situation is less clear. Bacteria contain enzymes such as glyoxylate carboligase (GCL) that use hydroxymethyl-TPP as a substrate and regenerate TPP during this reaction (Koch and Jaenicke, 1963; Kohlhaw et al., 1965). A homologue to GCL in plant chloroplasts is acetolactate synthase (At3g48560) (Chipman et al., 2009). However, it is unknown whether this enzyme can further metabolize hydroxymethyl-TPP. In case regeneration of TPP from hydroxymethyl-TPP would be impossible *in vivo*, the drain of TPP by glyoxylate decarboxylation, dependent on the amount of glyoxylate present in the chloroplast, could also provide an explanation for the observed toxic effects of glyoxylate on chloroplast metabolism (Campbell and Ogren, 1990; Chastain and Ogren, 1989). In this line, it has been shown that glyoxylate in mitochondria can irreversibly inhibit the TPP-containing enzyme oxoglutarate dehydrogenase (Adinolfi et al., 1969). On

the other hand, the competitive nature of the inhibition of NADH formation from pyruvate by glyoxylate (Figure 6) does not argue for an irreversible inhibition of PDC under our test conditions.

In order to analyze composition of the chloroplast PDC, we identified the proteins present in a complex that was highly enriched in this fraction (Figure 4, Table 1). We found three versions of this complex in the 2-7 MDa region. This molecular mass estimation is based on the position of the PDC in the first gel dimension and the acrylamide concentration in the BN-PAGE ranging from 2.5-16 % which is close to the concentration of 3-13 % suggested by Strecker et al. (Strecker et al., 2012) for a separation range of 50 kDa to 10 MDa. However, apparent molecular masses of protein complexes determined by BN-PAGE might not correspond exactly to the calculated masses due to unequal dye binding, attachment of lipids and potential internal charges resulting in an varying migration behavior (Wittig et al., 2010). Our estimation is in accordance with the result of Olinares et al. (Olinares et al., 2010) who suggested more than 5 MDa for plastidal PDC according to size exclusion chromatography. Thus, chloroplast PDC is probably a multimeric complex made up from several copies of each its constituents. Within the complex, we identified all PDC subunits with predicted chloroplast localization, but none of the mitochondrial subunits (Table 1). This indicates that the fraction was not contaminated with mitochondrial PDC. In addition to the established subunits of the complex, we were able to show that the protein encoded by At1g34430, a homologue of the E2 subunit of chloroplast PDC, forms part of the chloroplast PDC. Colocalization of the two homologues within the same complex indicates divergent function for the proteins. In line with this argument, Mooney et al (Mooney et al., 2002) hypothesized that the protein encoded by At1g34430 might resemble the E3 binding protein of mammalian and yeast PDC. In addition to the E2 homologue, we identified the ClpC1 subunit of the chloroplast clp protease complex as a constituent of the PDC (Table 1). This protein is present in all three versions of the PDC. A protein band of similar size had been shown before to co-purify with chloroplast PDC activity (Camp and Randall, 1985), but had not been identified on the molecular level so far. The ClpC1 protein is a HSP100-like chaperone that helps chloroplast Clp protease with unfolding of substrate proteins (Gottesman, 1996). In addition, a more general role of related HSP100 proteins in stress response pathways has been implicated (Schirmer et al., 1996). ClpC1 belongs to a class of chaperone-like AAA^{+}-ATPases with a function in the assembly

of protein complexes (Burton and Baker, 2005; Neuwald et al., 1999). Interestingly, exogenous ATP supplied during the purification enhanced the amounts of PDC purified from chloroplasts, suggesting a stabilizing effect of ClpC1 for the complex (Blume and Peterhansel, unpublished data). Knockout mutants of ClpC1 in Arabidopsis show a severe chlorotic phenotype (Sjögren et al., 2004). The presence of ClpC1 in the PDC together with the essential function of the PDC in fatty acid biosynthesis (see Introduction) might help to explain this observation.

When comparing glyoxylate metabolism in higher plants and cyanobacteria, the evolutionary ancestors of chloroplasts (Gupta and Brian Golding, 1996), there are obvious differences: Higher plants only express a single photorespiratory pathway, perhaps with a few minor detours (Niessen et al., 2012; Peterhansel et al., 2010). Cyanobacteria also express the photorespiratory pathway known from higher plants (Eisenhut et al., 2006). However, they contain two alternative pathways that are both capable of replacing the photorespiratory pathway. One of these pathways is based on the complete oxidation of glyoxylate to CO_2 via oxalate and formate (Eisenhut et al., 2008). Whereas neither oxalate nor formate inhibited release of CO_2 from radioactive glycolate in disrupted tobacco chloroplasts, glyoxylate and pyruvate inhibited the reaction (Figure 2). Thus, glyoxylate oxidation by the PDC is an analogous reaction, but not an evolutionary remnant, of the complete oxidation pathway from cyanobacteria.

Acknowledgements

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Tables

Table1: List of identified proteins in the largest PDC complex as named in Figure 4C.

Spot[1]	SC [%][2]	No of pept.[3]	Accession[4]	MW [kDa][5]	Score[6]	Name
1	40.7	37	At5g50920.1	103.4	1715	Clp protease ATP-binding subunit
	7.5	9	At1g34430.1	48.3	204	E2 subunit of cp PDC (dihydrolipoamide S-AC)
	2.1	1	At3g16950.1/	60.7	56	E3 subunit of cp PDC (dihydrolipoamide DH)
			At5g16155.1			E3 subunit of cp PDC (dihydrolipoamide DH)
2	8.8	4	AtCg00490.1	52.9	212	Rubisco, large subunit
	2.1	1	At3g16950.1/	60.7	56	E3 subunit of cp PDC (dihydrolipoamide DH)
			At5g16155.1			E3 subunit of cp PDC (dihydrolipoamide DH)
3	13.8	13	At1g34430.1	48.3	476	E2 subunit of cp PDC (dihydrolipoyllysine-AC)
	12.1	2	At3g25860.1	50.1	247	E2 subunit of cp PDC (dihydrolipoamide S-AC)
	4.2	2	AtCg00490.1	52.9	67	Rubisco, large subunit
	1.4	1	At3g49030.1	50.8	52	F-box and Leu-rich repeat domains cont. protein
4	7.1	7	At3g25860.1	50.1	204	E2 subunit of cp PDC (dihydrolipoyllysine-AC)
	6.9	1	At1g34430.1	48.3	189	E2 subunit of cp PDC (dihydrolipoamide S-AC)
	4.2	2	AtCg00490.1	52.9	83	Rubisco, large subunit
5	11.6	5	At2g39730.1	52.0	242	Rubisco activase
	10.1	4	At1g01090.1	47.1	204	E1α subunit of cp PDC (pyruvate DH)
	4.4	1	At1g73110.1	48.3	82	P-loop cont. NTP hydrolases superfamily protein
6	19.3	5	At1g12900.1	42.8	323	glyceraldehyde-3-P DH A, subunit 2
	9.6	3	At1g30120.1	44.2	134	E1β subunit of cp PDC (pyruvate DH)

Abreviations: DH – dehydrogenase, AC – acetyl transferase

[1] Spot identification number according to Figure 5C

[2] Sequence coverage of protein based on found unique peptides

[3] Number of unique peptides found by mass-spectrometry

[4] Accession number of identified protein homologue from *Arabidopsis thaliana* in the TAIR10 database

[5] Molecular weight of identified protein homologue from *Arabidopsis thaliana* including target peptide

[6] Mascot probability based scoring

Figure legends

Figure 1: Purification of chloroplasts. Indicated amounts of plastidal and crude leaf protein were separated on a 12% SDS-PAGE. (A) Coomassie Brilliant Blue staining. The picture shows the gel region around Rubisco (55 kDa). (B) Western blot with an antibody against the peroxisomal marker Catalase (52 kDa) (C) Western Blot with an antibody against the mitochondrial marker Cytochrome C oxidase II (30 kDa).

Figure 2: $^{14}CO_2$ release from 100 µM [^{14}C]glycolate (A, B) or 100 µM [^{14}C]glyoxylate (C), repectively, in purified and disrupted chloroplasts. Purified chloroplasts (cp) were disrupted and incubated with 100 µM [^{14}C]glycolate or [^{14}C]glyoxylate, respectively. Radioactivity captured in NaOH was determined by scintillation counting. (A) Comparison of CO_2 release in crude chloroplast extract and denatured extract. (B, C) Compounds as indicated in the figure were added at a final concentration of 1 mM to the reaction mix at the start of the reaction. Data are the average of at least 3 independent experiments ± standard error. Cp, chloroplast fraction; denat, denatured by boiling for 10 min; pyr, pyruvate; glx, glyoxylate; form, formate; oxal, oxalate.

Figure 3: Pyruvate-dependent NADH production in disrupted chloroplasts (cp). 1 mM glyoxylate and/or 1 mM pyruvate were added to the crude extracts and NADH formation was recorded photometrically. Data are the average of at least 3 independent experiments ± standard error.

Figure 4: Electrophoretic spearation of (A) the plastidal proteome or (B) the enriched pyruvate dehydrogenase complex fraction. 400 µg (A) and 200 µg (B) of protein were subjected to 2D-BN-SDS-PAGE. Gels were stained with Coomassie Blue-Colloidal. Positions of Rubisco small and large subunits are indicated. A protein complex that was enriched after PEG precipitation is enlarged (C). Proteins present in the complex are numbered. The corresponding protein identities are listed in Table 1.

Figure 5: Pyruvate-dependent NADH production in disrupted chloroplasts (cp) and a protein fraction enriched for the pyruvate dehydrogenase complex (PDC). 1 mM glyoxylate was added as an inhibitor. Data are the average of 4 independent experiments ± standard error.

Figure 6: Pyruvate-dependent NADH formation in a protein fraction enriched for the pyruvate dehydrogenase complex at different concentrations of pyruvate and glyoxylate. The reciprocal value of the reaction rate was drawn against the glyoxylate concentration. Ki = Negative glyoxylate concentration at the intercept of the linear regression lines. Data are the average of 6 independent experiments. Standard errors are excluded for clarity, but never exceeded 11% of the average.

Figure 7: $^{14}CO_2$ release from 100 µM [^{14}C]glyoxylate in crude chloroplast extracts (cp) and a protein fraction enriched for the pyruvate dehydrogenase complex (PDC). Radioactivity captured in NaOH was determined by scintillation counting. 1 mM pyruvate was added as an inhibitor. Data are the average of 6 independent experiments ± standard error.

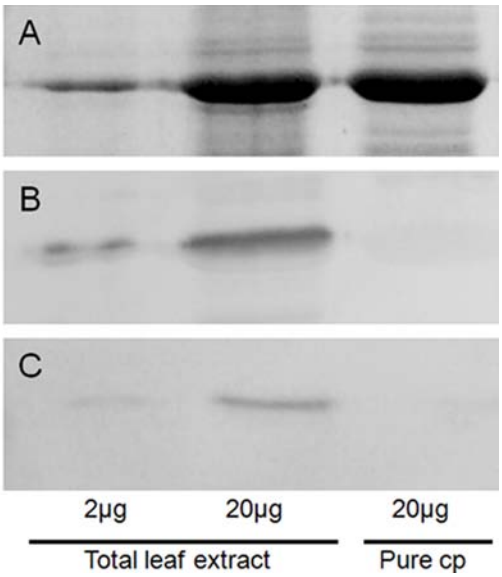

Figure 1

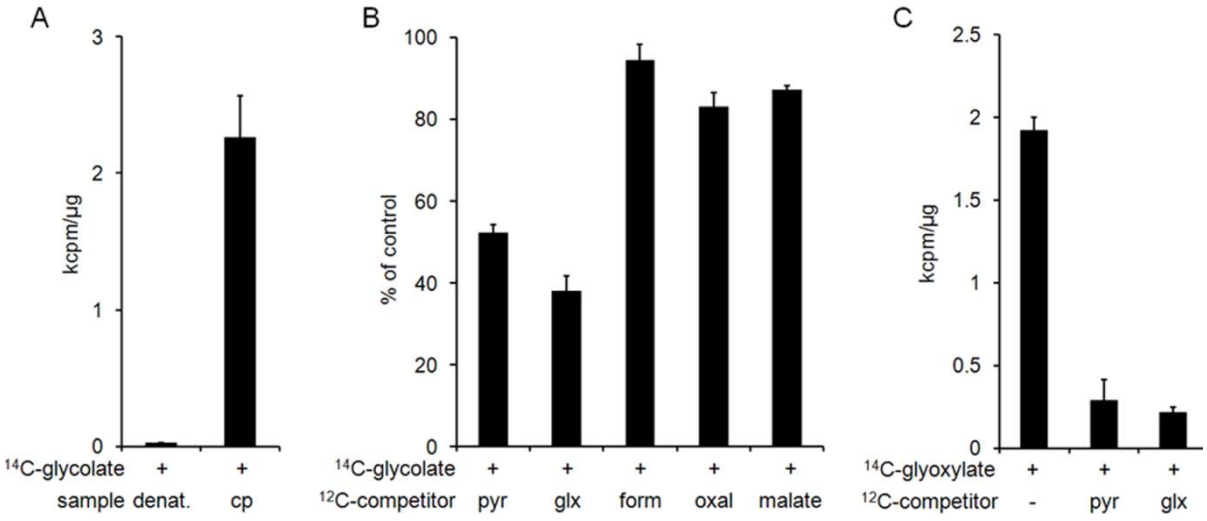

Figure 2

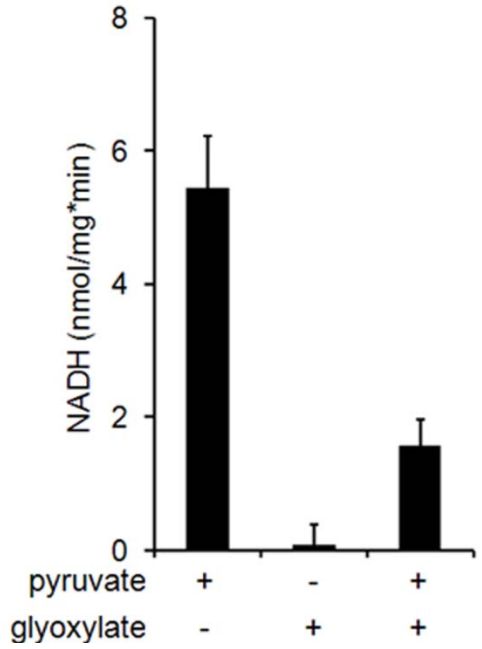

Figure 3

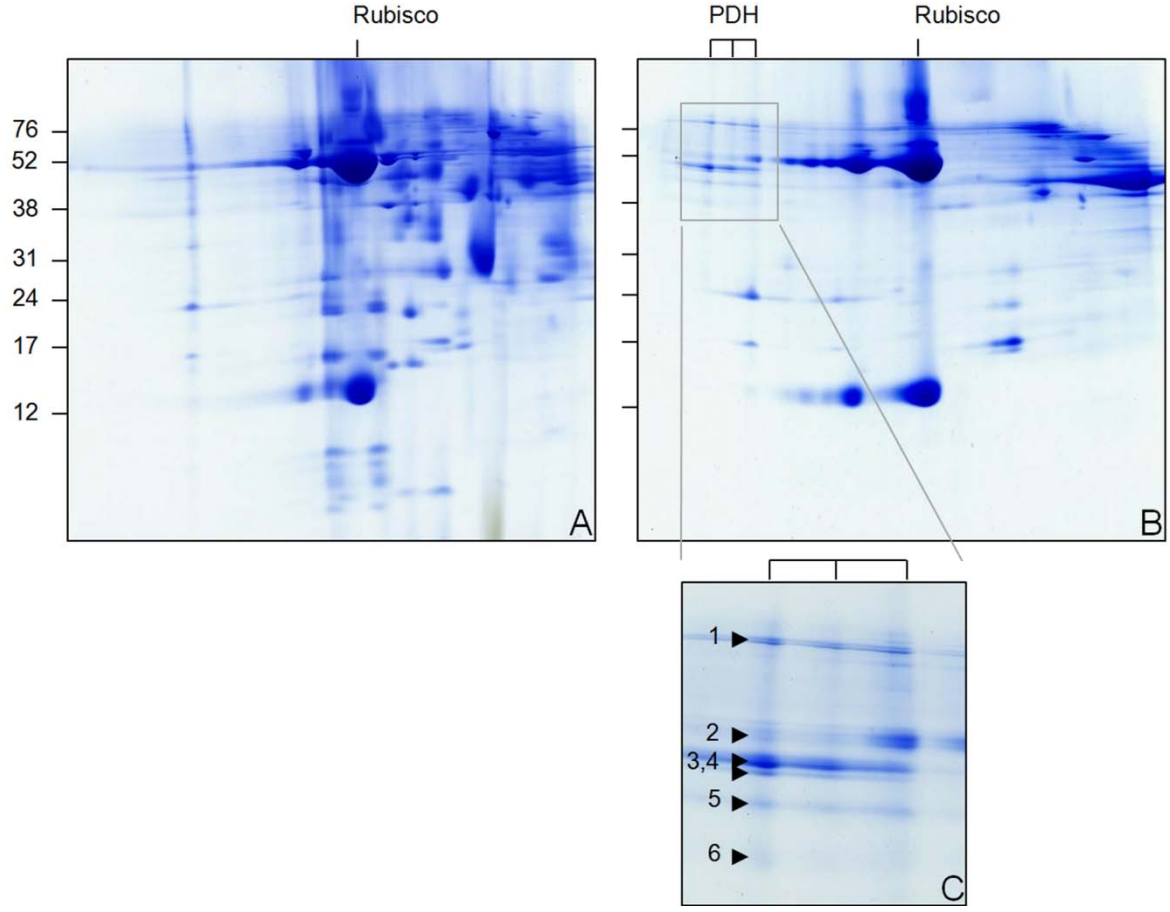

Figure 4

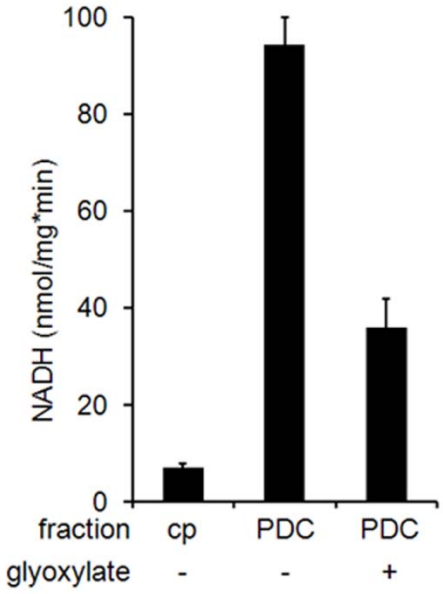

Figure 5

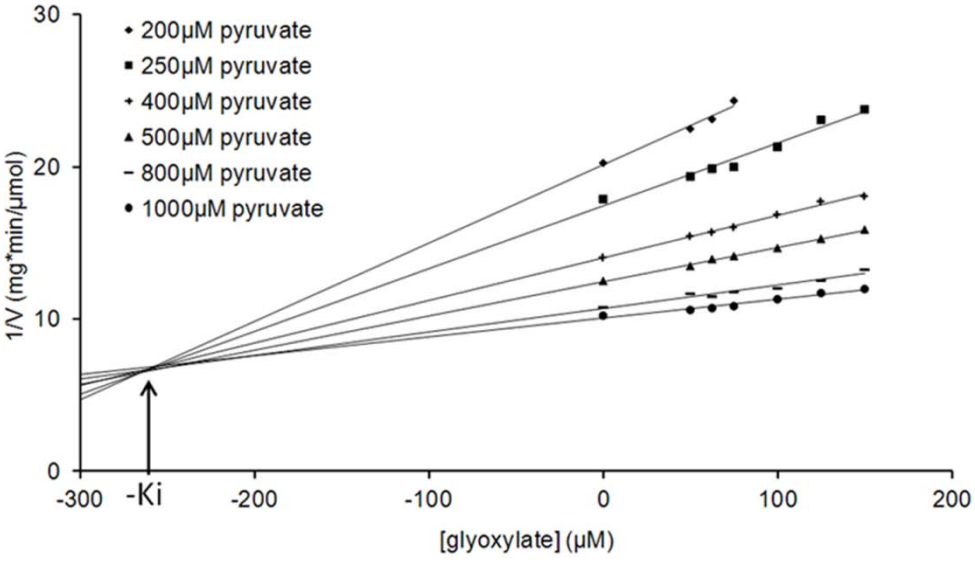

Figure 5

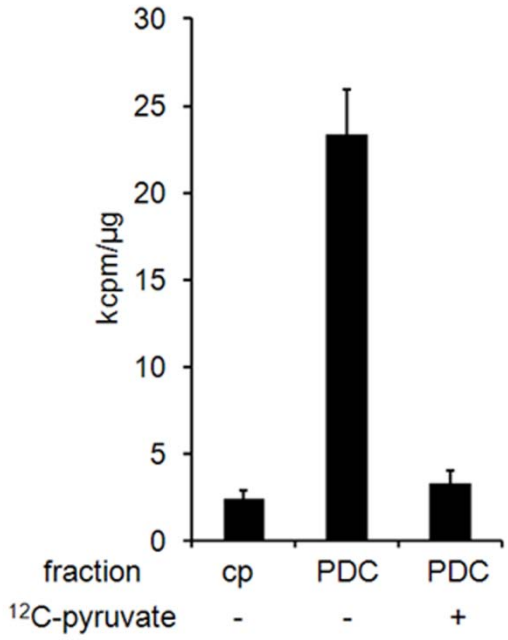

Figure 7

2.4 The ‘protein complex proteome’ of chloroplasts in Arabidopsis thaliana

The ‘protein complex proteome’ of chloroplasts in *Arabidopsis thaliana*

Christof Behrens[1], Christian Blume[2], Michael Senkler[1], Holger Eubel[1], Christoph Peterhänsel[2], and Hans-Peter Braun[1*]

[1] Institute of Plant Genetics, Faculty of Natural Sciences, Leibniz Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany

[2] Institute of Botany, Faculty of Natural Sciences, Leibniz Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany

* Author for correspondence
Hans-Peter Braun, Institute of Plant Genetics, Faculty of Natural Sciences, Leibniz Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany
Tel: +49511 7622674, Fax: +49511 7623608, e-mail: braun@genetik.uni-hannover.de

Running title: The GelMap of Arabidopsis chloroplasts

Key words: GelMap, chloroplast, protein complex, blue native, *Arabidopsis thaliana*

Abbreviations: MS, mass spectrometry; BN, blue native; DH, dehydrogenase

Abstract

Here, a first GelMap of the chloroplast “protein complex proteome” of *Arabidopsis thaliana* is presented. The GelMap software tool allows assigning multiple proteins to gel spots, thereby taking advantage of the high sensitivity of state-of-the-art mass spectrometry systems. Furthermore, the software allows functional annotation of all identified proteins. If applied to a 2D Blue native (BN) / SDS gel, GelMap can selectively display protein complexes of low abundance. For the chloroplast GelMap, highly purified organelles were separated by 2D BN / SDS PAGE and spots were automatically detected using Delta 2D software. From 293 spots, a total of 2,103 proteins were identified (on average 7.2 proteins per spot). The dataset includes more than 500 different types of proteins, most of which form part of protein complexes. The quality of the map is reflected by its inclusion of a more or less complete set of protein complexes described for chloroplasts in the literature. The potential of GelMap to define new protein-protein interactions is illustrated by presenting data on so far unknown protein complexes, e.g. complexes containing Deg proteases or antioxidant proteins like thioredoxin and peroxiredoxin. The GelMap is publically available at www.gelmap.de/arabidopsis-chloro and may be used as a resource for identifying novel protein complexes within any of its functional categories.

Introduction

The entirety of chloroplasts is by far the largest metabolically active compartment of the mature leaf cell. On a volume base, only the vacuole is larger but comparatively inactive with respect to biochemical reactions. Photosynthesis and a very broad range of other metabolic pathways take place in chloroplasts. It is estimated that 2000 – 3500 different types of proteins are present in chloroplasts [1]. Most of these proteins are nuclear encoded, synthesized in the cytoplasm and post-translationally transported into the organelle. Only about 120 proteins are synthesized within the chloroplast [2].

For more than a decade, proteomics has been successfully employed to systematically characterize the protein inventory of the chloroplast, especially in the model plant *Arabidopsis thaliana*. To date, 1200 – 1300 distinct types of proteins were identified in this cellular compartment (reviewed in van [1,3,4]). Based on the careful proteomic analyses of chloroplast subfractions, many of these proteins could be assigned to suborganellar locations, like the two chloroplast envelopes, the stroma, the thylakoid membranes and the thylakoid lumen [5-12]. Available data from these proteome projects are publically accessible via databases like PPDB, AT_Chloro or plprot ([13-15], reviewed in [1,16]). Additional proteins are predicted to be localized in chloroplasts by bioinformatics tools. The SUBA database integrates experimental and theoretical data on subcellular protein localization [17,18]. Various databases dedicated to protein localization in Arabidopsis are integrated via the “GATOR” aggregation portal [19].

So far, only few proteome projects aiming to investigate protein-protein interactions in chloroplasts have been conducted. In the frame of these studies, many chloroplast proteins were found to form part of more or less stable protein complexes. The oligomeric state of a stromal fraction of Arabidopsis was systematically analysed by “clear native” gel electrophoresis and the very large stromal complexes by chromatography in combination with mass spectrometry and hierarchical clustering [11,12].

Blue native PAGE combined with different protein identification technologies was employed to characterize protein complexes of the thylakoid and envelope membranes (e.g. [20-29]).

Protein complexes separated by native PAGE are routinely resolved into their subunits upon combination of the native gel dimension with a second gel dimension in the presence of SDS. On the resulting 2D gels, the subunits of protein complexes form vertical rows of spots. However, due to extensive spot overlapping, many protein complexes of low abundance are masked by complexes of high abundance. Indeed, using the high sensitivity of modern MS systems, analyses of gel spots nearly always lead to the identification of entire sets of proteins.

Recently, the “GelMap” software tool was developed to allow extensive and functional annotation of complex MS datasets obtained for 2D protein gels [30-31]. All proteins within each spot are annotated. Furthermore, all proteins within all spots are functionally annotated. Filter options permit selecting proteins of predefined functional categories. By evaluating the vertical positioning of proteins on 2D Blue native / SDS gels, the software allows the visualization of protein complexes of low abundance. Recently, characterization of a mitochondrial fraction of Arabidopsis by GelMap-based saturation analyses of a 2D Blue native / SDS gel allowed defining more than 30 distinct protein complexes, several of which are of low abundance and were not described before [32].

Here, we present a first GelMap on the chloroplast proteome. 315 spots resolved on a Coomassie stained 2D Blue native / SDS gel were analysed by high sensitivity MS, allowing the identification of 2,103 proteins (on average 7.2 proteins per spots). Evaluation of the dataset by GelMap provided new insights into the oligomeric state of the chloroplast proteome. The dataset is publically available at www.gelmap.de/arabidopsis-chloro and can be used for the search of novel protein complexes.

Materials and Methods

Preparation of chloroplasts

Arabidopsis thaliana Col-0 plants were grown in the greenhouse under a long day light regime for 5 weeks. All steps of chloroplast preparation were carried out either at 4℃ or on ice. In detail, 30 g of leaf material were ground twice for 5 seconds in 400 ml chilled homogenization buffer containing 50 mM HEPES-KOH, pH 8.0, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM sodium ascorbate and 0.5% w/v BSA using a blender (Waring, Torrington, USA). The homogenate was filtered through a 30µm nylon mesh and the filtrate was spun down at 300 x g for 4 minutes. The pellet was gently resuspended in a minimal volume of chilled grinding buffer and 10-ml 35% v/v self-forming Percoll gradients (containing 50 mM HEPES-KOH, pH 8.0, and 330 mM sorbitol) were loaded with 1 ml of the organelle suspension each. After centrifugation for 20 min at 19,000 rpm in a swing out rotor (SW40Ti, Beckman Coulter, Inc., Brea, USA), intact chloroplasts were recovered from the lower green fraction. The supernatant was discarded and the chloroplast fraction was washed with 10 volumes of chilled SH-buffer (50 mM HEPES-KOH, pH 8.0; 330 mM sorbitol). Chloroplasts were again spun down at 1,000 x g for 5 minutes and resuspended in chilled SH-buffer at a final concentration of 100 mg chloroplast protein/ml. Aliquots of 150µl were frozen in liquid nitrogen and stored at -80 ℃.

Estimation of chloroplast intactness by oxygen evolution

Oxygen evolution of chloroplasts was recorded in a clark type oxygen electrode (Hansatech, King's Lynn, UK) at 25℃ according to Walker 1990 [33]. Chloroplasts, either freshly prepared or disrupted by osmotic shock (corresponding to 25 µg chlorophyll as estimated according to Arnon 1949 [34]), were mixed with 1 ml of assay medium containing 50 mM HEPES-KOH, pH 7.6, 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 0.5 mM $K_2HPO_4^-$. The artificial electron acceptor $K_3Fe(CN)_6$

(FeCy) was used at a concentration of 2 mM. All measurements were carried out at 800µE illumination.

2D Blue native (BN) / SDS PAGE

One aliquot of a chloroplast suspension (see above) was supplemented with 150 µl solubilisation buffer BN (1.5 M aminocaproic acid, 100 mM Bis-tris, 1 mM EDTA, pH 7.0) containing 2 % w/v n-dodecylmaltoside (Sigma Aldrich, St. Louis, USA) and incubated on ice for 10 minutes. Afterwards, 15 µl of Coomassie blue solution (750 mM aminocaproic acid, 5% w/v Coomassie blue 250 G) were added and the samples were directly loaded on to a BN-Gel. BN-PAGE was carried out according to Wittig et al. 2006 [35] using a gradient-gel (6-15 % acrylamide from top to bottom). Tricine SDS PAGE [36] for the second gel dimension was carried out in a 3-step gel (16.5% polyacrylamide separation gel, 10% polyacrylamide spacer gel, 10% polyacrylamide sample gel; acrylamide-bisacrylamide ratio 16:1, respectively; [37]). All electrophoretic separations were carried out in the Protean II electrophoresis chamber from Bio-Rad (Hercules, USA). 2D gels had dimensions of 16 cm × 16 cm x 0.1 cm. Proteins were stained by the Coomassie blue-colloidal procedure [38].

Image acquisition and spot detection

Images of the stained gels were aquired using Umax Powerlook III Scanner (Umax Data System, Taipei, Taiwan) and stored in JPEG data format with a resolution of 400 dpi. Protein spots were detected automatically on the images using the Delta2D software (version 4.3, Decodon, Greifswald, Germany) and manual correction. Coordinates of all detected spots were recorded automatically.

Protein identification by mass spectrometry

Detected spots were excised from the gel using a manual spotpicker (Genetix, New Milton, UK) and subjected to MS analysis according to Klodmann et al. 2011 [32] with

the settings outlined in Behrens et al. 2013 [39]. Data analysis was carried out using ProteinScape2.1 software (Bruker, Bremen, Germany), the Mascot search engine (Matrix Science, London, UK) and the TAIR10 database (Arabidopsis.org). Search parameters were: enzyme, trypsin/P (up to one missed cleavage allowed); global modification, carbamidomethylation (C), variable modifications, acetyl (N), oxidation (M); precursor ion mass tolerance, 30 ppm; fragment ion mass tolerance, 0.05 Da; peptide charge, 1+, 2+, and 3+; instrument type, electrospray ionization quadrupole time of flight. Minimum ion score was 15, minimum peptide length was 4 amino acids, significance threshold was set to 0.05 and protein and peptide assessments were carried out if the Mascot Score was greater than 25 for proteins and 20 for peptides. In case of ambiguous assignments peptides were accepted based on the protein score. By using a randomized decoy database strategy [40] for the TAIR10 database a false discovery rate of 4.28 % was calculated based on the unique peptides.
Subcellular localization of all identified proteins was extracted from the SUBA3 database [18] using the SUBAcon Bayes approach. In case of contradictions between experimental and prediction data, the experimental data were favoured. Proteins with no experimentally derived localization data were designated “new plastid”, if plastids were a valid compartment according to prediction algorithms included in SUBA3.

Database and reference map generation using GelMap 3.0

The web-based GelMap 3.0 software tool (www.gelmap.de), was used to generate an interactive reference map of the chloroplast ‘protein complex proteome’. For this approach, an image file of the 2D gel, the coordinates of all spots and all related protein and peptide data were combined according to the requirements of the software tool (www.gelmap.de/howto).

Results and Discussion

Isolation of intact chloroplasts from Arabidopsis

Chloroplasts easily break during isolation, causing partial loss of the stromal fraction. The intactness of chloroplasts isolated form Arabidopsis was tested by O_2 evolution assays in the presence / absence of the artificial electron acceptor potassium ferrocyanide [33]. Since membranes are impermeable for the acceptor, intact chloroplasts are expected to have low O_2 evolution rates. In contrast, if organelles lose their structural integrity, O_2 evolution rates increase. A fraction including a high proportion of intact chloroplasts was used for the generation of the GelMap (Fig. 1).

Separation of chloroplast proteins by 2D Blue native / SDS PAGE

Different detergents were tested for membrane solubilization prior to BN / SDS PAGE. Since digitonin resulted in comparatively stronger smearing effects on 2D gels (not shown), solubilization was carried out using dodecyl maltoside, which is a classical detergent in chloroplast research [40]. Separation of a dodecyl maltoside-solubilized chloroplast fraction by 2D BN / SDS PAGE and protein staining by Coomassie-blue allowed visualizing chloroplast protein complexes of known identity [20,22]: The two photosystems, the RubisCO complex, the F_1 part of ATP synthase, the b_6f complex and trimeric LHCII (Fig. 2). The gel was calibrated by the molecular masses of well-defined mitochondrial protein complexes (calibration of the first gel dimension) and a commercial molecular mass standard (second gel dimension; Fig. 3, Supplementary Figure 1).

Spot Detection and MS

Automated spot detection by Delta 2D revealed 315 spots (Fig. 4). Analyses of the spots by LC-ESI tandem mass spectrometry were carried out using the MicrOTOF Q

II system, set to maximal sensitivity. For 293 out of 315 spots, proteins could be identified. This represents a success rate of >93%. Within the 293 spots, our analyses revealed 2,103 proteins in total (from now on termed “all proteins”), which were identified from 11,664 unique peptides. This on average corresponds to 7.2 different proteins per spot. However, several proteins were identified in more than one spot. Our analysis revealed 529 distinct proteins (“unique proteins”). Because most proteins showed different apparent molecular masses in the 1st and 2nd dimension, they probably form part of protein complexes.

Evaluation of organelle purity based on MS results

The subcellular identity of all proteins was evaluated using the SUBA3 database [18]. This database summarizes information on subcellular protein localization for *Arabidopsis thaliana*. The following results were obtained (Fig. 5, Supp. Tables I and II): (i) Of the 297 “first hit” proteins (the identified proteins with the highest MASCOT score within each spot, respectively), 284 are classified “plastid” in SUBA3 (97%). Only 9 proteins (3 %) are assigned to other cellular compartments (Fig. 5A). (ii) Of the 529 “unique proteins” identified in our study, 412 proteins are classified “plastid” (78%), 87 proteins (16 %) are assigned to other cellular compartments and 30 proteins (6 %) are of unknown localization (Fig. 5B). This lower percentage of plastid proteins within this dataset is expected because the analysis not only includes “first hit” proteins but additionally proteins with comparatively low MASCOT scores. (iii) If all 2,103 proteins identified in all spots are included in the evaluation, 89% of the proteins are classified “plastid” in SUBA3, 9% are classified as other cellular compartments and 2% of the proteins could not be assigned to any subcellular compartment (Fig. 5C). This slightly higher percentage of chloroplast proteins again is expected because several proteins are included in this dataset more than one time, which is more likely for chloroplast than for non-chloroplast proteins due to their enrichment within our biochemical fraction. (iv) Finally, if all 11,664 unique peptides identified in

the course our analysis are included in the evaluation, more than 95% match to proteins classified “plastid” in SUBA3 (Fig. 5D). This number might most realistically reflect the purity of our biochemical fraction. We conclude that the fraction used for the generation of the chloroplast GelMap was of high purity.

Of the 9% proteins not assigned to “plastids” according to SUBA3 (dataset “all proteins”, Fig. 5C), 4.5% are classified “cytosolic”. It remains to be established whether these proteins represent “contaminants” in our fraction or rather are attached to the chloroplast surface for biological reasons. Defined “surface proteomes” were reported previously for plant organelles [42]. Only 1% of the identified proteins were classified “mitochondrial” or “peroxisomal” according to SUBA3. These organelles might have been co-purified during chloroplast preparation due to similar size or density properties. However, since organelles physically interact in the plant cell their co-purification also might reflect biological interaction. Interestingly, 7 of the overall 22 mitochondrial proteins identified in our study are involved in photorespiration (dataset “all proteins”, Fig. 5C), a metabolic process which is based on organelle cooperation.

SUBA3 evaluation of our dataset revealed 29 proteins which so far have unknown subcellular localization (Supp. Table II). Since our biochemical fraction is highly enriched in chloroplasts, these proteins are candidates to genuinely be present within this organelle and therefore are termed “new plastid”. However, further biochemical investigations are necessary to confirm this conclusion.

Annotation of the "protein complex proteome" of Arabidopsis chloroplasts using GelMap 3.0

An underlying principle of GelMap is the annotation of not only “first hit” proteins within gel spots, but the presentation of the complete MS data sets related to all spots on a 2D gel [30,31]. Furthermore, all proteins within all spots are assigned to functional groups. If applied for the evaluation of 2D BN / SDS gels, the filter options of GelMap

allow to selectively display proteins which may represent subunits of protein complexes of low abundance. This approach was successfully used to systematically define mitochondrial protein complexes in Arabidopsis [32]. To facilitate the build of future GelMaps, a new version (GelMap 3.0) was developed, which includes the following improvements: (i) A new upload wizard facilitates the creation of a GelMap project in two steps: The upload of a Microsoft Excel spreadsheet (.xsl or .xlsx) or OpenOffice .ods file and the selection of special columns such as coordinates and IDs. (ii) All gel images can now be uploaded in high resolution and result in high resolution zoomable maps. No data is lost due to compression. (iii) An improved search option allows the creation of custom filters: By entering a string that is searched not only in the title but in all supplied information columns, GelMap 3.0 offers a new clickable filter. (iv) All proteins are now directly linked to three external protein databases (SUBA, GATOR, and NCBI).

The GelMap of Arabidopsis chloroplasts

Using GelMap 3.0, a GelMap was generated for the ‘protein complex proteome’ of chloroplasts (www.gelmap.de/arabidopsis-chloro; Figure 6). All proteins are assigned to one of eight categories (photophosphorylation, carbon fixation, transport, nucleic acid biosynthesis & processing, protein folding and processing, other metabolic pathways, proteins not assigned to a functional category, uncharacterized). Within these categories, proteins are classified according to functional subcategories like metabolic pathways, protein complexes etc. Proteins can be found on the map using one of the following three options: (i) selecting functional categories and subcategories offered by the menu to the right of the 2D gel, (ii) using the “search” option below the menu (either enter an accession number or the name of a protein) or (iii) by directly clicking a spot. In the latter case, a frame opens which lists all proteins identified in this spot. If the map is used by clicking on a functional category in the menu, all proteins assigned to the category are selectively displayed on the map. Vertical

positioning of the spots allows identifying protein complexes. Many of the complexes in the chloroplast GelMap are known protein complexes, such as the photosystems. However, some other protein complexes were not described so far (see examples below). In fact, GelMap is a tool for the discovery of new protein complexes and the scientific community is invited to use the map for this purpose.

A guideline for the interpretation of GelMap data

The following remarks should be considered when using the map:

- Within each spot, proteins are sorted according to MASCOT scores. Proteins with high scores normally are of high abundance. However, scores also depend on the biochemistry of the proteins and therefore only reflect semi-quantitative information.
- On average, each protein is detected four times on the map (some proteins more than 20 times, some proteins only once). This might reflect presence of a protein in different protein complexes (for instance a holo-complex and its assembly intermediates). However, detection of a protein at several sites can also be caused by “smearing” effects of the 2D BN / SDS PAGE system. If there are indications for smearing, the MASCOT scores for a protein of interest can be compared and used to identify the position of its highest abundance.
- Proteins are included in the map if the MASCOT score is >30. Proteins identified by only one peptide are included in the dataset for providing maximal information.
- Native apparent molecular masses of proteins below 100 kDa are slightly uncertain, because correlation between molecular mass and migration on the blue native gel might be reduced in this gel region. Above 100 kDa, the correlation is very good [43].

- Proteins are assigned to only one functional category. In several cases, proteins could be assigned to more than one category, e.g. a protein kinase phosphorylating a component of photosystem II to the category “Photophosphorylation: auxiliary proteins” or “Protein folding and processing: protein modification”. If a protein of interest cannot be found in an expected category, it may still be found by entering its accession number or name in the “search” field at the bottom of the menu.
- More than 170 proteins are currently assigned to the category “proteins not assigned to a functional category”. This publication cannot present a complete interpretation of the ‘protein complex proteome’ of chloroplasts. Usage of the chloroplast GelMap by the scientific community should allow identifying several further protein complexes.

The ‘protein complex proteome’ of Arabidopsis chloroplasts

To illustrate the capacity of the chloroplast GelMap, information on several protein complexes is provided in the following sections (Table 1, Supp. Figure 2). The obtained data nicely confirm the composition of many known protein complexes. Furthermore, examples for the potential of GelMap to define novel protein complexes are given.

1. Photosystem I

This protein complex is present in several different forms on the map. The most abundant form (560 kDa) is the photosystem I supercomplex and includes the subunits of the photo reaction center I and the Lhca1-4 proteins [44,45]. Smaller forms of photosystem I are of comparatively low abundance (470, 340, 320 kDa), lack the Lhca1-4 proteins and most likely are assembly intermediates of photosystem I. Photosystem I forms a supercomplex with the NAD(P)H dehydrogenase complex and the Lhca5-6 proteins, which migrates at 1500-1700 kDa [46]. Other large assemblies in-

cluding photosystem I subunits run at 700-1150 kDa but are of very low abundance. Their exact compositions are currently not known. The Lhca1-4 proteins also are present as monomers in the 25 kDa range but additionally run at ~75 kDa in the native gel dimension. The latter forms are possibly Lhca heterodimers [47] which either are assembly intermediates or dissociation products of the photosystem I supercomplex [48].

Using mass spectrometry, subunits A, B, C, D1, D2, E1, E2, F, G, H2, K, L, N, and P of the photo reaction center I were identified. However, subunits P and N were not found as constituents of photosystem I complexes but only as monomers. The I, J, and O subunits were not detected, probably due to their very low molecular mass.

2. NAD(P)H dehydrogenase complex

This protein complex plays a role in cyclic photophosphorylation and was exclusively found in association with photosystem I and the Lhca5-6 subunits (1500-1700 kDa). Chloroplast NAD(P)H complex consists of four subcomplexes [46,49]. Subunits of all subcomplexes were identified by mass spectrometry (nomenclature according to [50]): subunits Ndh A, E and F (membrane subcomplex), Ndh H, I, K and M (subcomplex A), subunits PnsB1 and PnsB2 (subcomplex B) and subunits PnsL1, PnsL4 and PnsL5 (luminal subcomplex). Compared to photosystems, the abundance of the NAD(P)H dehydrogenase complex is low, which possibly explains that some of its known subunits were not detected by MS. At the same time, other proteins identified in the 1500-1700 kDa range on our 2D gel might represent so far unknown constituents of this protein complex. However, the possible presence of these proteins in the NAD(P)H complex awaits further evaluation.

3. Photosystem II

On our GelMap, photosystem II is present in seven forms with apparent molecular masses of 210, 260, 560, 700, 840, 1000 and 1150 kDa. It previously was reported that distinct thylakoid subfractions include differing forms of the Photosystem II [23].

The main form on our map is the photo reaction center II dimer (560 kDa) which does not include light harvesting (Lhcb) proteins. In contrast, the larger photosystem II supercomplexes include Lhcb proteins and their abundances increase with mass of the supercomplexes. In accordance with published data [21,51,52], the 700, 840, 1000 and 1150 kDa complexes probably include 1, 2, 3 and 4 light harvesting trimers (each composed of Lhcb1, Lhcb2 and Lhcb3) and additionally the monomeric light harvesting proteins CP29 (Lhcb4), CP26 (Lhcb5) and CP24 (Lhcb6). Molecular masses of the supercomplexes are slightly smaller than reported previously, which most likely is due to differing conditions during protein solubilization. The 260 kDa form of photosystem II is the photo reaction center II monomer and the 220 kDa complex a monomer lacking the CP43 subunit.

Overall, 36 distinct proteins forming part of photosystem II complexes and supercomplexes were identified by mass spectrometry: all 6 types of Lhcb proteins (13 isoforms), the photo reaction center II subunits PsbA, B, C, D, E, F, H, L, O, P, Q, R, S, CP27, CP29 (some of them in several isoforms) and others. Photosystem II includes a large number of small and extremely small subunits [53,54], some of which were not identified in the frame of our current study.

Lhcb proteins were not identified in the dimeric photo reaction center II but only in the supercomplexes. Also, the 33 kDa subunit (PsbO, also termed OE33) of the “oxygen evolution” module of photosystem II was only present in the supercomplexes. The Lhcb1, Lhcb2 and Lhcb3 proteins form LHCII trimers (120 kDa). Furthermore, the LHCII trimers form a 210 kDa complex together with the monomeric CP29, CP26 and CP24 proteins, which was reported previously [22]. Hence, LHCII trimers seem to pre-assemble with the CP29, CP26 and CP24 proteins before binding to the photo reaction center II dimer.

4. Cytochrome b_6f complex

On our map, the main form of the b_6f complex runs at 260 kDa in the native gel dimension. It represents the dimeric state of the complex [55]. It is composed of 8 subunits (PetA, B, C, D, G, L, N, M), six of which are included in the map.

5. ATP synthase complex

The F_0F_1 holo-complex runs at 540 kDa, the F_1 part of the enzyme at 300 kDa. The latter subcomplex either is a break down product of the holo enzyme or an assembly intermediate, as recently shown for the mitochondrial F_1 complex [56]. Eight subunits of the F_0F_1 complex were found in the course of this study (Table 1).

6. Protein complexes of the Calvin cycle

All enzymes of the Calvin cycle are included in the map. At least three enzymes constitute protein complexes. The RubisCO complex (430 kDa, 8x the “large” and 8x the “small” subunit), the glyceraldehyde 3-phosphate dehydrogenase (80 kDa; heterodimer), and the fructose 1,6-bisphosphate aldolase (110 kDa, homodimer). The RubisCO complex has a calculated molecular mass >500 kDa but migrates slightly faster in the BN gel dimension. This was already described previously [20] and most likely is caused by its very compact structure.

7. The TIC and TOC preprotein translocases

Only few of the TIC and TOC subunits were found in the spots analysed by MS. The Tic110 and Tic55 subunits form a complex of 170 kDa. In contrast, previous analyses have revealed a TIC complex of 230 kDa [57]. Interestingly, Toc75 was identified in the 1500 kDa mass range, possibly indicating its presence in a high molecular mass translocon complex.

8. Ribosomal complexes

Numerous ribosomal subcomplexes are present in different regions of the map. Overall, 28 ribosomal proteins were detected by mass spectrometry. According to the SUBA database, 26 of these proteins are located in plastids, one in mitochondria and one in the cytosol. Considering that the overwhelming majority of the identified ribosomal proteins is located in plastids, the intracellular whereabouts of the latter two proteins deserve to be reinvestigated. Overall, 18 proteins of the large ribosomal

subunit were identified (Rpl 1, 2, 3, 4, 5, 6, 10, 11, 12A, 12B, 15, 19, 20, 21, 22, 27, 28, 30) and 10 of the small subunit (Rps 1, 2, 3, 4, 5, 7, 8, 11, 16, 30). Several ribosomal subcomplexes are in the 700 – 1700 kDa region and include the subunits Rps 3, 4, 5, 8, 11 and Rpl 12B, 19, 20, 21, 22. The presence of ribosomal subcomplexes of > 1000 kDa on native gels was reported previously [12].

9. Nucleoid complexes

Chloroplast DNA-dependent RNA polymerase B subunit (RpoB) was detected in the 1500 kDa range. Furthermore the “plastid transcriptionally active chromosome proteins” (pTAC proteins) pTAC3, pTAC4, pTAC14 and pTAC16 were identified in this molecular mass range. It was previously reported that pTAC proteins and RpoB co-localize to the chloroplast nucleoid [58-59].

10. Protein complexes involved in protein folding and processing

Three chaperonin complexes are included in our map: The Cpn60 complex (790 kDa, 7 α-subunits and 7 β-subunits, or 14 β-subunits, respectively; [60]), the Cpn20 complex (70 kDa; homotetramer; [61]) and the Cpn10 complex (heptamer).

11. FtsH complexes

The Arabidopsis genome encodes 12 FtsH proteases, 6 of which have been shown to be present in chloroplasts (FtsH1, 2, 5, 8, 11 and 12, [62]). While FtsH 1, 2, 5 and 8 are monomers on our map, the FstH 11 and 12 proteins form complexes with apparent molecular masses of 1500 kDa. A 1500 kDa complex including FtsH 11 was described previously [63]. Furthermore, a >1000 kDa complex composed of Fts10 and Fts12 was reported for yeast mitochondria [64].

12. Clp protease complexes

Chloroplasts include Clp protease complexes of 350 and 200 kDa [65]. The map includes clp proteins at these and other positions. Clp P3 and P5 proteins form part of

a previously characterized clp ring complex migrating at 220 kDa in the native gel dimension.

13. Deg complexes

Deg proteases are responsible for specific degradation of photosystem components, e.g. PsbA (D1) and PsbO (OEC33) [66]. Deg1 (41 kDa) and Deg5 (33 kDa) proteins co-migrate at about 80 kDa on the blue native gel dimension. This might indicate physical interaction.

14. Peroxiredoxin complexes

A 23 kDa Peroxiredoxin (PrxB; At5G06290) runs at 210-260 kDa on the native gel dimension. *In vivo* oligomerization of this protein to dodecamers was recently demonstrated [67]. This is in line with the apparent molecular mass detected on our map.

15. Thioredoxin complex

ATHM2 (AT4G03520), a small thioredoxin of 13 kDa, runs at about 40 kDa on the native gel dimension. This indicates formation of a homotrimeric or homotetrameric complex.

16. Acetyl CoA carboxylase complex 1500 kDa

A subunit of chloroplast acetyl-CoA carboxylase (ACC) was found at 1500 kDa on our map. It was reported previously that ACC forms a large oligomeric complex [12].

Besides the 32 complexes described above (Table 1, Supp. Figure 2), numerous further potential protein complexes are included in our data set. GelMap should be instrumental in defining new protein complexes in chloroplasts.

Outlook

Many proteomic investigations are carried out on the basis of 2D gels. Usually, gel-based proteome projects offer information on the identity of most abundant ("first hit") proteins within analyzed gel spots. However, due to a dramatic increase in MS sensitivity, today's MS analyses of gel spots normally lead to identification of entire protein sets. This is expected because protein spots overlap in 2D gels. Unfortunately, proteins of low abundance were often overseen in previous projects. GelMap offers complete annotation of MS data related to 2D gels. If used for the evaluation of blue native / SDS gels, it allows to systematically displaying protein complexes. Recently, 35 mitochondrial protein complexes were described for Arabidopsis by this approach. Here, we describe more than 30 chloroplast protein complexes (Table 1, Supp. Figure 2). The GelMaps for mitochondria and chloroplasts of Arabidopsis are/will be integrated into the GATOR aggregation portal (http://gator.masc-proteomics.org/). GelMaps for other plant organelles are planned, eventually covering the entire plant cell. In the future, this envisioned database may represent a valuable tool for detecting protein-protein interactions in plant cells.

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Supplementary Material

Supp. Figure 1:	Scaling of the 2D gel used for the generation of the chloroplast GelMap
Supp. Figure 2:	Selected complexes identified on the chloroplast GelMap of *Arabidopsis thaliana*
Supp. Table I.	Summary: Subcellular localization of identified proteins
Supp. Table II:	Subcellular localization of “unique” and “first hit” proteins
Supp. Table III:	Protein table of the chloroplast GelMap (www.gelmap.de/arabidopsis-chloro)
Supp. Table IV:	Peptide table of the chloroplast GelMap (www.gelmap.de/arabidopsis-chloro)

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Table 1: The 'protein complex proteome' of chloroplasts in *Arabidopsis thaliana*

apparent molecular mass (kDa)[1]	protein complex	identified subunits[2]
1700-1500	FTSH complex	FTSH11, FTSH12
1700-1500	transcription complex	RpoB, pTAC3, pTAC4, pTAC14 and pTAC16
1700-1500	supercomplex of the NAD(P)H dehydrogenase complex + photosystem I + Lhca5, Lhca6	Ndh A, E, F, H, I, K, M, PnsB1, PnsB2, PnsL1, PnsL4, PnsL5, subunits of photosystem I, Lhca5, Lhca6
1500	acetyl CoA carboxylase complex	α-subunit
1150	photosystem II supercomplex ($C_2S_2M_2$)	photosystem II subunits + Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6 + OE33
1000	photosystem II supercomplex ($C_2S_2M_1$ / $C_2S_1M_2$)	photosystem II subunits + Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6 + OE33
840	photosystem II supercomplex (C_2S_2 / $C_2S_1M_1$)	photosystem II subunits + Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6 + OE33
790	chaperonin-60 complex	7 α-subunits + 7 β-subunits or 14 β-subunits
700-1700	ribosomal subcomplexes	Rps 3, 4, 5, 8, 11 and Rpl 12B, 19, 20, 21, 22
700	photosystem II supercomplex (C_2S_1 / C_2M_1)	photosystem II subunits + Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6 + OE33
560	photosystem II dimer (C_2)	PsbA, B, C, D, E, F, H, L, S a.o.
560	photosystem I	PsbA, B, C, D1, D2, E1, E2, F, G, H2, K, L, Lhca1, Lhca2, Lhca3, Lhca4
540	F_1F_0 ATP synthase	α, β, χ, δ, ε, A, B, B'
470	photosystem I subcomplex	photosystem I lacking the Lhca proteins
430	RubisCO	8 large subunits + 8 small subunits
340, 320	photosystem I subcomplexes	photosystem I lacking the Lhca proteins and further subunits of unknown identity
300	F_1 ATP synthase	α, β, χ, δ, ε,
260	photosystem II monomer (C)	PsbA, B, C, D, E, F, H, L, S a.o.
260	cytochrome b_6f complex (dimeric)	PetA, B, C, D, G, L,
220	Cpl ring complex	Clp-P3, Clp-P5
210	peroxiredoxin B	dodecameric complex
210	subcomplex of the photosystem II monomer	photo reaction center (monomer) lacking CP43
210	LHC assembly complex	Lhca1, Lhca2, Lhca3, Lhca4, Lhca5, Lhca6,
165	Tic translocon	Tic110, Tic55
120	LHCII-trimer	Lhca1, Lhca2, Lhca3
110	fructose-1,6 bisphosphate aldolase	homodimer
85	glyceraldehyde 3-phosphate DH	homo / heterodimer
80	chaperonin-20 complex	tetramer
75	Lhca complex	heterooligomer, Lhca1, Lhca3, Lhca4
75	Deg1-Deg5 complex	Deg1, Deg5
70	chaperonin-10 complex	homoheptamer
50	thioredoxin m2	homotrimer / homotetramer (?)

[1] apparent molecular mass as determined in this study
[2] Further subunits might form part of the complexes but were not detected in the frame of our study

Figure legends

Figure 1: Assessment of structural integrity of isolated chloroplasts. Light-dependent (800 µE) oxygen evolution of freshly prepared and osmotically disrupted chloroplasts was measured in presence of the membrane impermeable electron acceptor potassium ferrocyanide according to Walker 1990 [33]. Oxygen evolution was standardized to chlorophyll content of samples as estimated by the method of Arnon et al. 1949 [34]. Means and standard errors were derived from six replicates. The intactness of the organelle fractions was approximately 85%.

Figure 2: Chloroplast protein complexes of Arabidopsis as resolved by 2D blue native / SDS PAGE. Chloroplasts were solubilized with dodecyl maltoside as described in the Materials and Methods section. Proteins of 15 mg chloroplasts were loaded onto the 2D gel. Proteins were visualized by Coomassie-colloidal staining. Identities of known chloroplast protein complexes are given on top the gel. PSII-sc: photosystem II supercomplexes; PSI: photosystem I; $PSII_2$: dimeric photosystem II; RubisCO: Ribulose bisphosphate Carboxylase/Oxygenase; cF_1: F_1 part of the chloroplast ATP synthase complex; PSII: photosystem II; Cyt b_6f: cytochrome b_6f complex; $LHCII_3$; trimeric light harvesting complex II; $LHCII_1$: monomeric LHCII proteins (Lhcb4, Lhcb5, Lhcb6, the CP29, 26 and 24 proteins).

Figure 3: Molecular mass calibration of a 2D BN / SDS gel for the chloroplast GelMap. 2D BN / SDS PAGE was carried out as described in the Materials and Methods section. The calibration of the BN gel dimension is based on co-electrophoresis of OXPHOS complexes from Arabidopsis (1500 kDa: supercomplex formed of respiratory complexes I and III_2; 1000 kDa: complex I; 500 kDa: dimeric complex III_2; 300 kDa: F_1 part of the mitochondrial ATP synthase complex; for details see Supp. Fig. 1). Further masses were set according to the size of well-defined chloroplast protein complexes (120 kDa: native LHCII trimer; 30 kDa: native LHCII monomers). Calibration of the SDS gel dimension is based on the masses of a commercial molecular mass standard except for the 15 and 4 kDa masses which were set according to well defined chloroplast proteins from Arabidopsis (15 kDa: small subunit of RubisCO, 4 kDa: PETG, PSBF, PSBL subunits). Masses of protein complexes / proteins in-between the calibration lines were calculated using Eureqa software (version 0.97 beta, http://creativemachines.cornell.edu/eureqa). For further details see Supp. Figure 1.

Figure 4: Automatic spot detection on the 2D BN / SDS gel shown in Figure 2 using the DELTA 2D software package (version 4.3.2). By using this tool, 315 distinct protein spots were defined on the 2D gel (encircled in pink).

Figure 5: Subcellular localization of (A) the proteins with the highest Mascot scores in all 293 spots (“first hit“ proteins), (B) all unique proteins, (C) all identified proteins and (D) all unique peptides according to the SUBA3 database. Percentage of proteins with either plastidial localization according to SUBA3 (plastid) or proteins found in a chloroplast preparation for the first time in the course of this study (plastid new) are indicated. For details see Supplementary Tables I and II.

Figure 6: The GelMap for chloroplasts of *Arabidopsis thaliana* (www.gelmap.de/arabidopsis-chloro).

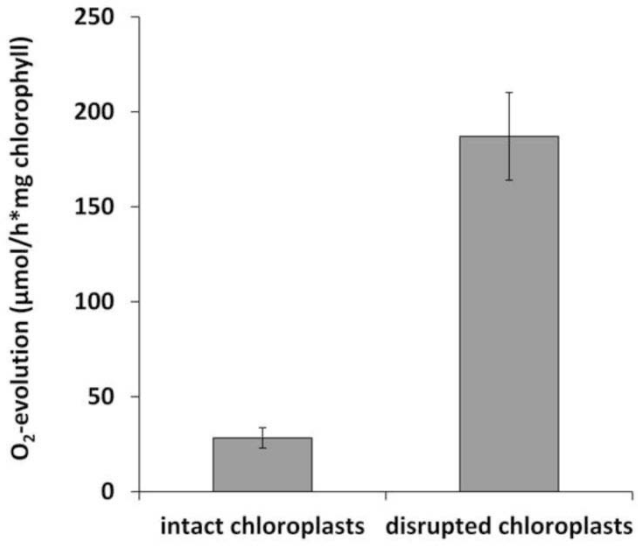
250
200
150
100
50
0
O2-evolution (µmol/h*mg chlorophyll)
intact chloroplasts
disrupted chloroplasts

Figure 1

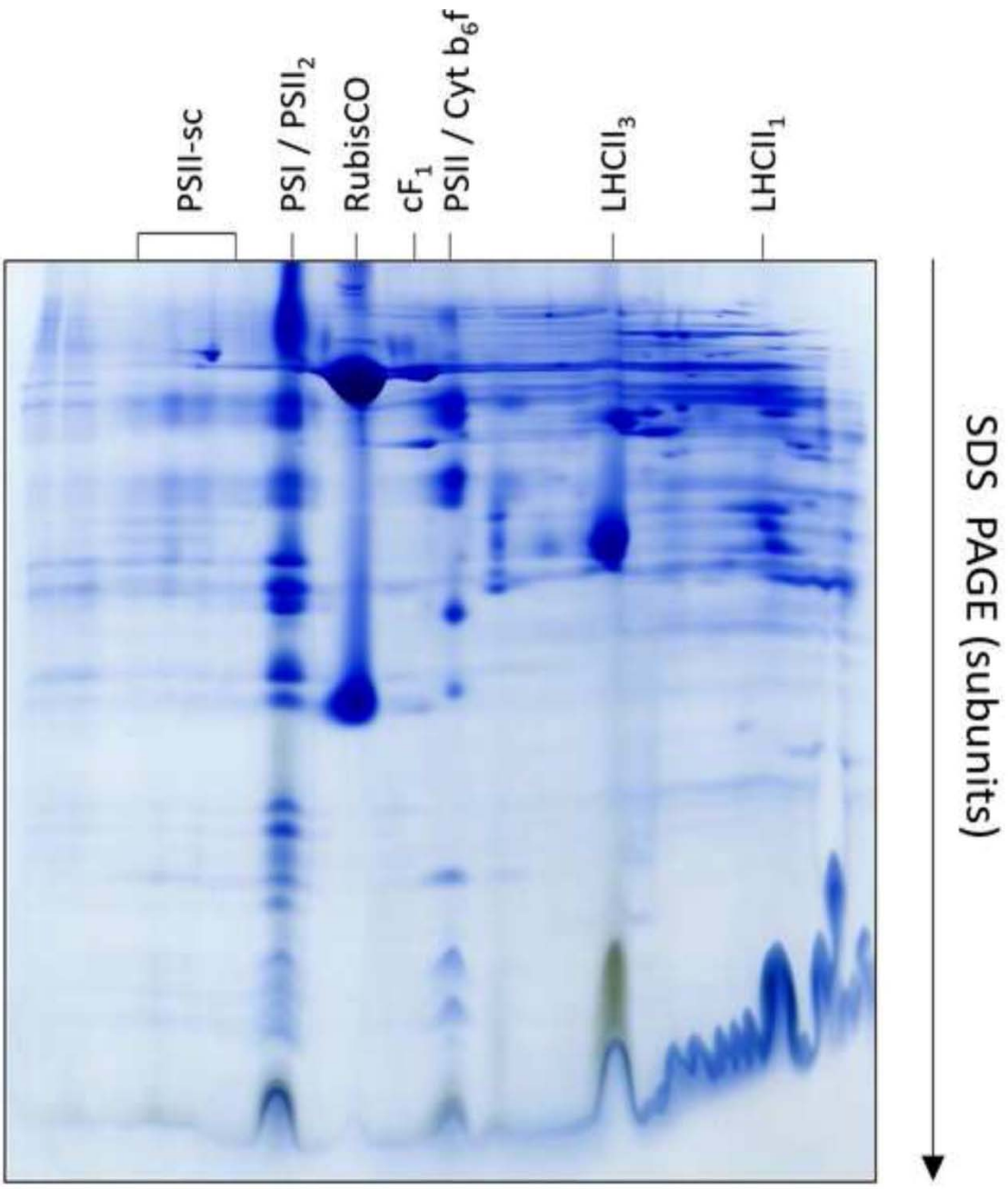
PSII-sc
PSI / PSII2
RubisCO
cF1
PSII / Cyt b6f
LHCII3
LHCII1
SDS PAGE (subunits)

Figure 2

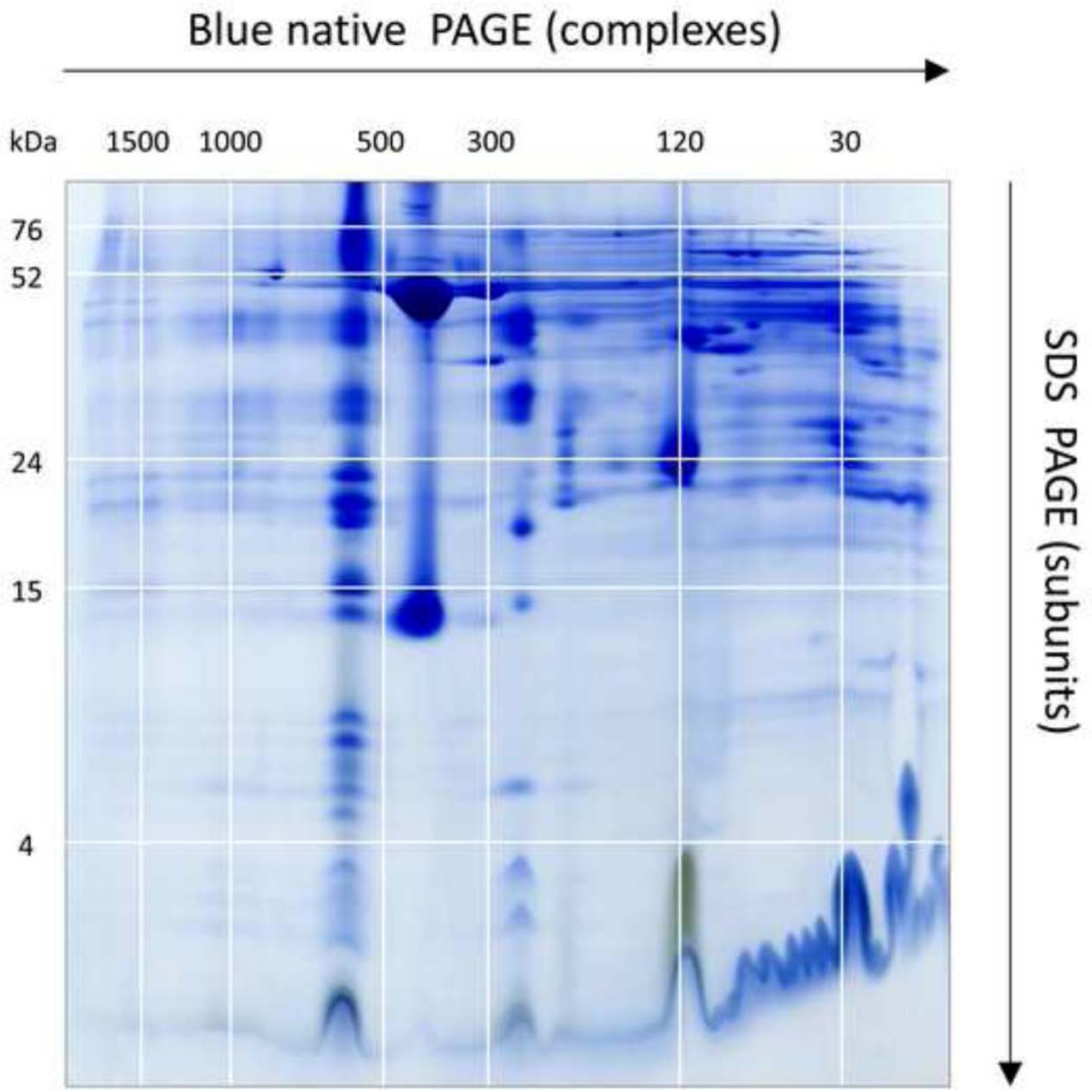
Blue native PAGE (complexes)
kDa
1500
1000
500
300
120
30
76
52
24
15
4
SDS PAGE (subunits)

Figure 3

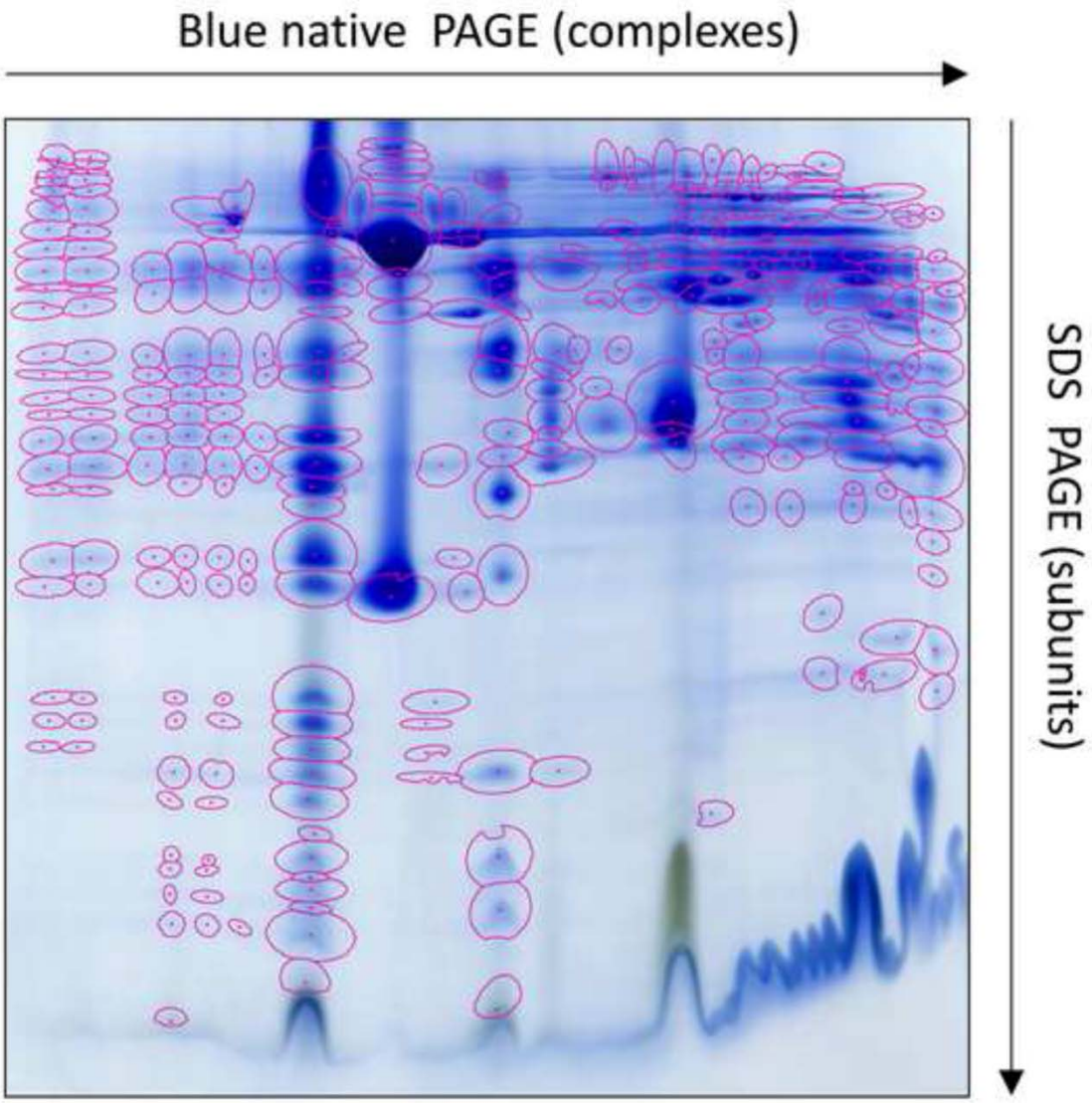

Figure 4

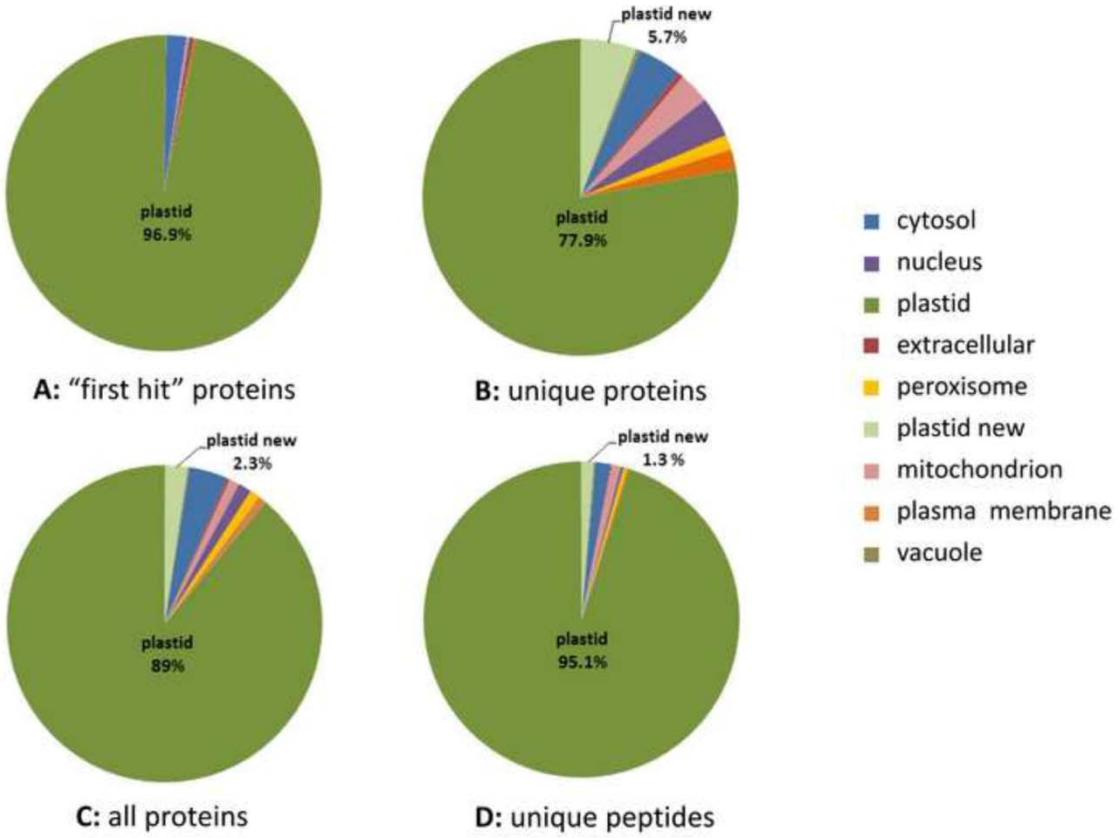

Figure 5

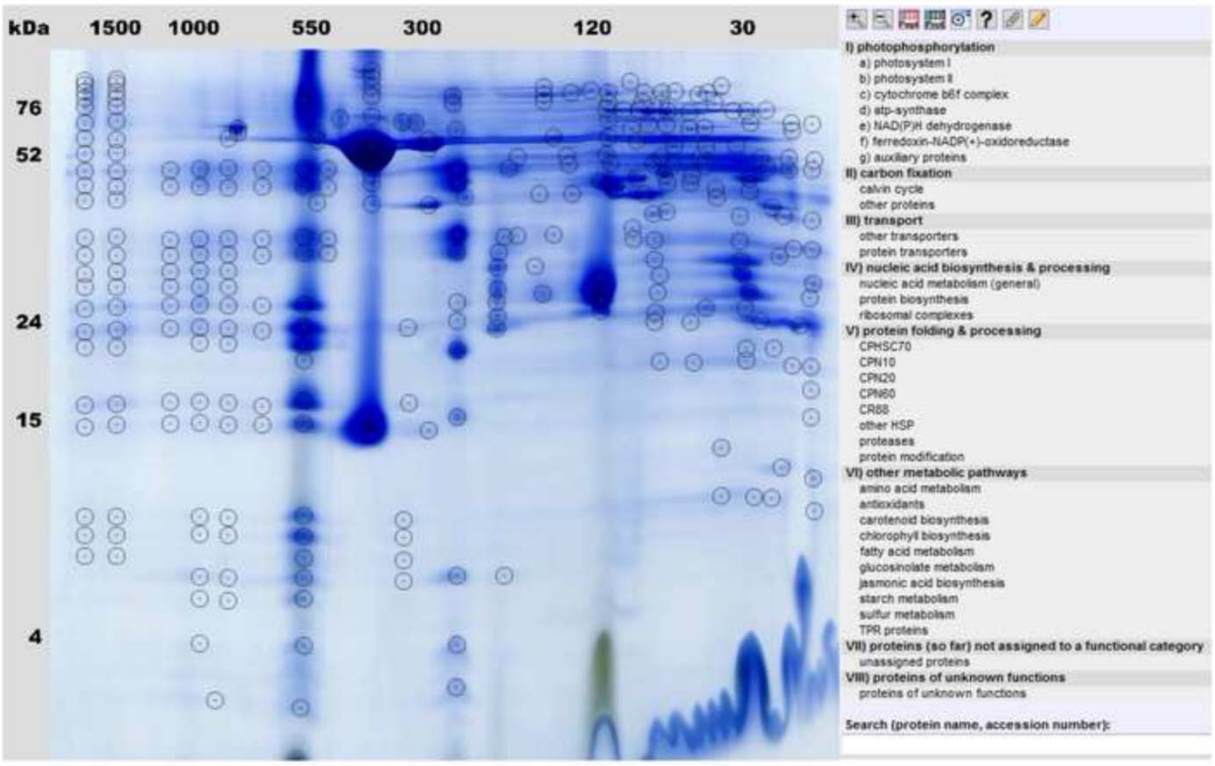

Figure 6

3 General discussion

Current studies on photorespiration are aiming in two different directions. On the one hand, basic research uncovers new relevance of the pathway, evolutionary traits and further interactions with primary metabolism. Recent data suggest that photorespiration developed early in evolution. The coevolution of oxygenic photosynthesis and photorespiration supports the strong integration of photorespiration into primary metabolism. Additionally, alternative routes and enzymes of photorespiration are investigated. This research was strengthened by the discovery that cyanobacteria contain three different photorespiratory pathways (Eisenhut et al., 2008). As part of this thesis, a role for the pyruvate dehydrogenase complex in photorespiratory glyoxylate decarboxylation was described. The second direction of research on photorespiration is the establishment of transgenic approaches to reduce photorespiratory flux. First, some focus is put on the optimization of Rubisco to a higher specificity for CO_2 (Andrews and Whitney, 2003; Peterhansel et al., 2008) but Bainbridge et al. (1995) among others showed that higher specificity is mostly accompanied by lower catalytic rates, which complicates screening for a better performing Rubisco (Whitney et al., 2011). Second, much effort is invested in enhancement of the CO_2 concentration in the vicinity of Rubisco of C3 plants and the installation of the C4 cycle or a cyanobacterial CCM in C3 species are long term objectives (Peterhansel, 2011; Covshoff and Hibberd, 2012; Price et al., 2013). The third approach to minimize photorespiratory losses begins after oxygen fixation by Rubisco (Figure 3, Kebeish et al., 2007; Carvalho et al., 2011; Maier et al., 2012). The three transgenic approaches or bypasses for reduction of photorespiration were shortly described in the introduction. A detailed characterization of the bypasses with a focus on their energy balances is part of this thesis (Peterhansel et al., 2012, chapter 2.2). Evidence was provided that altering the photorespiratory C2 cycle can be advantageous for plants under controlled conditions. In the introduction, the importance of photorespiration and its close connection to plant metabolism is presented. Taking this into consideration, the observation of enhanced growth of the transgenic plants expressing one of the bypasses is surprising. Based on current knowledge of photorespiration summarized in the introduction and on the calculation of the energy balances described in chapter 2.2, I will re-discuss here the benefits and drawbacks of the bypasses in comparison to natural pathways. In addition, the energy balance of the newly identified route via the plastidal pyruvate dehydrogenase complex (PDC) is discussed.

3.1 Energy balance contribution of the photorespiratory bypasses

In chapter 2.22.1 the energy balances for the photorespiratory bypasses are calculated. In this review, we had the main focus on the beneficial side of reducing energy costs of photorespiration. But as it was stated in chapter 1.3.7, the capacity of photorespiration as sink for excess energy plays an important role in plant metabolism. In this paragraph, I want to evaluate the pathways for benefits and

disadvantages of energy consumption. The three bypasses have different energy costs as given in table 2 of chapter 2.2. Bypass 3 dissipates more energy than any other photorespiratory cycle, when the energy costs for RuBP regeneration are integrated into the calculation. The main reason for the high energy costs of this pathway is the complete decarboxylation of photorespiratory glycolate. Reduction of this CO_2 in the CBB cycle consumes high amounts of energy. This is a disadvantage of the pathway under energy limiting conditions. However, as described in the introduction, high rates of photorespiration are promoted by high light and drought (Lawlor and Fock, 1977; Brown and Morgan, 1980; Cornic and Briantais, 1991). Under these conditions, CO_2 availability for reduction in the CBB cycle is too low to keep pace with light harvesting and generation of NADPH and ATP by the light reaction of photosynthesis. Therefore, excess reducing power is available that needs to be dissipated. By Bypass 3, a huge electron sink for energy dissipation is made accessible, which could be advantageous under the indicated conditions. On the contrary, the bypasses 1 and 2 both have lower energetic costs than the major pathway. This can be attributed to bypassing of the transamination step, by which the energy consuming refixation of released ammonia is avoided. Additionally, bypass 1 uses a glycolate dehydrogenase for glycolate oxidation, by which reducing power is made accessible. The reduced energy needs of these two bypasses are beneficial when energy is limiting. Under adverse conditions, the reduced sink capacity can be deleterious for the plants. As described in the introduction, reduced sink capacity can induce oxidative stress in the chloroplasts by production of reactive oxygen species (ROS) at the photosystems, which promote photodamage. The question is, if alternative photoprotective mechanisms of the chloroplast, which are described in the introduction, can effort the need for energy dissipation. Interestingly, the energetic costs of photorespiration vary in nature. Cyanobacteria evolved three different photorespiratory pathways, each with different energy costs. Energy consuming ammonia refixation is only required in the plant type photorespiratory pathway while complete oxidation of glyoxylate has even higher energy costs. In higher plants, glycolate oxidation by mitochondrial glycolate dehydrogenase reduces the energy costs of photorespiration by 20%. In this concern, it would be interesting to know the fluxes of the pathways and if they adapt in response to environmental changes. As described in the introduction, the dependence of photoprotection on photorespiration has been shown with mutants of the photorespiratory glutamate synthase (GS). Overexpressors as well as knock-down mutants were generated to modulate flux through photorespiration (Kozaki and Takeba, 1996). However, GS fixes ammonia and the reduced capacity of nitrogen fixation can limit production of the D1 protein of the photosystem II, which has a low halftime in high light (Li and Sherman, 2002). The reduced ability of D1 regeneration could be the reason for the observed sensitivity of the mutants to high light. Moreover, ammonia is reported to have a toxic effect on photosystem II (Drath et al., 2008). However, the foremost energy sink of photorespiration that contributes to photoprotection is CBB cycle activity (Takahashi et al., 2007). Calculated on the basis of the energy balances by Peterhansel et al. (2010, chapter 2.1), this makes 80% of the total energy costs of photorespiration in higher plants. As

described in the introduction, Haupt-Herting et al (2001) as well as Heber et al (1996) reported that under drought stress, the main activity of the CBB cycle can be deduced to photorespiration. However, none of the transgenic approaches reduces the capacity of the CBB cycle as all products of the bypasses, which are 3-phosphoglycerate (3-PGA) and CO_2, can be metabolized in the CBB cycle.

Together, it can be concluded that sink capacity of photorespiration is variable in nature. Thus, also the variation by the transgenic approaches should not be disadvantageous. The greatest energy sink of photorespiration is CBB cycle activity in response to photorespiration. This capacity is not reduced by any bypass. On the contrary, CBB cycle activity might be even increased in transgenic lines, as stated in the next paragraph.

3.2 Contributions of photorespiratory CO_2 release in the chloroplast

The bypasses 1 and 3 by Kebeish et al. (2007) and Maier et al. (2012), respectively, shift photorespiratory CO_2 release from mitochondria to chloroplasts. In bypass 1, decarboxylation is catalyzed by glyoxylate carboligase (GCL). In bypass 3, two decarboxylation reactions by malic enzyme and the pyruvate dehydrogenase complex take place in the chloroplast. The authors propose, that translocation of photorespiratory CO_2 release shall increase its refixation probability. In this context, it would be interesting to know, to which extend mitochondrial photorespired CO_2 in C3 plants is refixed by chloroplasts under natural conditions. Braun and Zabaleta (2007) proposed a mechanism for efficient shuttling of CO_2 via carbonic anhydrases and bicarbonate transporters (see also Zabaleta et al., 2012). Additionally, Sage and Sage (2009) proposed that the refixation of photorespiratory CO_2 in rice is very efficient as chloroplasts cover the whole cell surface. As a consequence, CO_2 released by mitochondria must pass through a chloroplast before diffusing out of the cell (Busch et al., 2013). The authors assume that this anatomy enhances refixation probability and only low amounts of CO_2 are released. In contrast, Haupt-Herting et al (2001) could measure release of photorespiratory CO_2 from leaves of tomato plants, although they do not provide absolute numbers. It would be interesting to measure photorespiratory CO_2 release of transgenic plants expressing one of the two bypasses 1 or 3 with the methodology established by Haupt-Herting. This could verify the postulation that the location of release of photorespired CO_2 has an impact on refixation probability. Thus, less CO_2 would escape from leaves of the transgenic plants compared to the natural condition. Interestingly, shift of photorespiratory CO_2 release is also suggested to be one of the first steps in the evolution of C4 plants (Gowik and Westhoff, 2011). In C3-C4 intermediate species, photorespiratory glycine decarboxylation is limited to an inner cell layer, which forces CO_2 to pass through a number of cells before being released from the leaf (Rawsthorne et al., 1998; Engelmann et al., 2008). The passage through multiple cells increases refixation probability of photorespired CO_2. The evolution of a natural pathway with the same purpose supports the postulations by Kebeish et al. (2007) and Maier et al. (2012) that refixation of photorespiratory CO_2 can be improved.

In the introduction, CCMs of various photosynthesizing organisms were described. They are based on the evolution of Rubisco containing compartements, in which CO_2 is actively concentrated. CCMs evolved independently in cyanobacteria, green algae and higher plants in order to reduce photorespiration and to enhance photosynthesis, nitrogen use efficiency and, in higher plants, water use efficiency. The shift of photorespiratory CO_2 release to the chloroplasts by the bypasses 1 and 3 can be viewed as basal CCMs as they increase the CO_2 concentration in the vicinity of Rubisco. An open question is whether the bypass reactions can be as efficient as a true CCM. The naturally evolved CCMs are very effective. The cyanobacterial CCM enhances the CO_2 concentration by three orders of magnitude (Price et al., 2007). The natural CCMs nearly completely suppress the oxygenase function of Rubisco by active uptake of carbon from the surrounding. On the contrary, the photorespiratory bypasses depend on an internal carbon source, photorespiratory glycolate. Thus, reduction of the oxygenase function of Rubisco subsequently reduces CO_2 release by the bypasses due to substrate limitation, which in turn promotes again the oxygenase function. However, at the equilibrium, the carbon concentration in the vicinity of Rubisco should be increased in the transgenic plants although the photorespiratory bypasses cannot be as efficient as natural CCMs.

What does a possible CCM tell us about the energy contribution of photorespiration to plant redox-homeostasis? To answer this question, the energy balances of photorespiration and photosynthesis are calculated on basis of the values given by Peterhansel et al (2010), chapter 2.12.1 (Table 1). For the calculation, the production of NADH by GDC is set off against its consumption by HPR. The energy costs are calculated in each case for 3 catalyses by Rubisco: 2 oxygenations promote release of one molecule CO_2 and the refixation by Rubisco is integrated into the calculation. Thus, two oxygenations count for three carboxylations. Conclusively, the oxygenase function of Rubisco and subsequent photorespiration consumes only about 2% more energy than the dark reaction of photosynthesis would consume with the same number of reactions. Moreover, the three carboxylation reactions, on which the calculation is based, produce one molecule GAP that can leave the CBB cycle without depleting regeneration of RuBP. The calculation does not include anabolic pathways that the released GAP will enter. Thus, there is little difference on the energy level if a carboxylation or an oxygenation takes place and as a consequence, a CCM has little influence on the redox state.

Table 1: The energy balance sheet of photorespiration and the CBB cycle calculated for 2 oxygenations with subsequent CO_2 refixation in the CBB cycle and 3 carboxylations, respectively.

	2 oxygenations (+1 carboxylation)		3 carboxylations	
	Red. equivalents	ATP	Red. equivalents	ATP
Reduction of CO_2 formed by PR	2	3	-	-
Refixation of NH_3	1	1	-	-
Phosphorylation of glycerate	-	1	-	-
Reduction of 3-PGA formed by Rubisco	2	3	6	9
Reduction of 3-PGA formed by PR	1	1.5	-	-
	6	**9.5**	**6**	**9**
Assuming 2.5 ATP/NADPH	15	9.5	6	9
		24.5		**24**

3.3 Product contributions of the photorespiratory bypasses

One of the main tasks of photorespiration is the recycling of 3-PGA for the CBB cycle (chapter 1.3.6). The bypasses 1 and 2 produce photorespiratory intermediates, which are converted to 3-PGA. Bypass 3 does not recycle 3-PGA. Instead, photorespiratory glycolate is completely oxidized to CO_2. In the introduction, experiments were described, which demonstrated the necessity of recycling the CBB cycle intermediate by photorespiration (Blackwell et al., 1987; Blackwell et al., 1990). However, the carbon released by bypass 3 is not lost for the CBB cycle. Refixation by Rubisco redirects the carbon to the CBB cycle. Under conditions, where two carboxylation reactions compensate for each oxygenase function of Rubisco, the CBB cycle does not deplete (Table 2). All carbon that is released by decarboxylation of glycolate is refixed and can be used for formation of RuBP. This regeneration is on the expense of glyceraldehyde 3-phosphate use for anabolic processes. In contrast, the major photorespiratory pathway and the bypasses 1 and 2 already avoid depletion of the CBB cycle, even if the rate of oxygenation doubles the rate of carboxylation. One carboxylase reaction is sufficient to maintain the carbon pool in CBB cycle intermediates. The higher requirement for CO_2 fixation per oxygenase reaction of bypass 3 should be measureable in an enhanced CO_2 compensation point. Interestingly, this is not observed by Maier et al. (2012). The authors explain this observation by low flux through the bypass and to the shift of photorespiratory CO_2 release into the chloroplast. The benefits of this shift were already described in this discussion.

Table 2: Compensating stochiometry of carboxylation and oxygenation of different photorespiratory pathways to avoid carbon depletion of the CBB cycle.

	Major pathway / bypasses 1 & 2			Bypass 3		
	Number of reactions	Carbon invested by RuBP-conversion	Carbon recycling by fixation in 3-PGA	Number of reactions	Carbon invested by RuBP-conversion	Carbon recycling by fixation in 3-PGA
RuBP-oxygenation	2	10	9	1	5	3
RuBP-carboxylation	1	5	6	2	10	12
Sum of investment / regeneration		**15**	**15**		**15**	**15**

Photorespiration has another role in the assimilation of metabolites for different purposes than 3-PGA regeneration. In the introduction, a possible role of photorespiration in glutathione biosynthesis was discussed. It has been shown that photorespiratory glycine is crucial for glutathione synthesis (Buwalda et al., 1990; Noctor et al., 1999). One of the roles of glutathione is implied in the scavenging of reactive oxygen species (ROS) in the chloroplast (Hossain and Asada, 1984). Thus, photorespiration might have another role in the mitigation of oxidative stress in the chloroplast by production of glycine for glutathione synthesis. The export of glycine from photorespiration is at the cost of regeneration of CBB cycle intermediates. However, none of the bypasses addresses this problem. Interestingly, the two established pathways both show an enhanced phenotype mainly under low light intensities of 100 µmol m^{-2} s^{-1}. Under these conditions, few ROS are produced at the

photosystems and glutathione demand is low. Increase of the light intensity to 400 µmol $m^{-2}s^{-1}$ reduced the effect of the bypass 1. This is explained by the reduced impact of low energy consumption by this bypass under these conditions. In plants expressing bypass 3, photon flux of 600 µmol m^{-2} s^{-1} even induced a chlorotic phenotype. The authors deduce this to insufficient catalase activity but do not discuss glutathione concentrations. However, all bypasses have limited capacity. Photorespiration is estimated to have the 2nd greatest flux rate in plant metabolism (Szecowka et al., 2013). No author claims that their transgenic approach can fully substitute the photorespiratory C2 cycle. The limited capacities of the transgenic approaches imply that the impact of the bypasses decreases as photorespiratory flux increases. Thus, high light induces the major pathway and enables glycine und thus glutathione synthesis.

It would be interesting to measure glutathione level in plants overexpressing one of the photorespiratory bypasses. Nevertheless, conditions that require enhanced levels of glutathione also induce increased flux through the major pathway in the transgenic plants. This should meet the requirement of photorespiratory glycine for glutathione synthesis.

3.4 Balance of a possible plastidal glycolate mechanism via the pyruvate dehydrogenase complex

Bypass 1 and 3 catalyze the decarboxylation of photorespiratory glyoxylate inside the chloroplast. However, this reaction has also been observed several times in chloroplasts of wildtype plants of different species (chapter 1.3.2). The underlying mechanism has been studied as part of this thesis (chapter 2.3). Enrichment of plastidal pyruvate dehydrogenase showed co-purification of glyoxylate decarboxylation activity. The success of the enrichment could be verified by 2D-BN/SDS-PAGE, by which a complex of approximately 5-7 MDa could be identified, which contains all relevant subunits of PDC and that could not be seen on gels with the non-enriched protein fraction. Moreover, glyoxylate could be shown to be a competitive inhibitor of pyruvate oxidation by PDC. The products of glyoxylate decarboxylation by PDC are CO_2 and a hydroxymethyl-residue that is linked to the coenzyme of PDC, which is TPP. There is evidence that the hydroxymethyl-TPP cannot be converted by PDC and that the cofactor has to be exchanged. The fate of the hydroxymethyl-residue has not been resolved hitherto. Comparable with the already discussed bypasses 1 and 3, the naturally occurring decarboxylation of photorespiratory glyoxylate in the chloroplast enhances the CO_2 concentration in the chloroplast as well as refixation probability of photorespiratory CO_2. Thus, the pathway might be beneficial for the plant (chapter 3.2). Another positive aspect of plastidal glyoxylate decarboxylation is detoxification of glyoxylate, which is known to reduce Rubisco activation (Cook et al., 1985; Chastain and Ogren, 1989; Campbell and Ogren, 1990). However, in other terms, the proposed mechanism is difficult to classify in means of energy balance and relation to primary metabolism. There are too many uncertainties about the pathway beyond glyoxylate decarboxylation. The first uncertainty of the pathway is that the origin of plastidal glyoxylate could not be resolved. There are several candidate

genes for glycolate oxidation, although all of them are unlikely due to various concerns. The plastidal enzyme glyoxylate reductase (GR2) is shown to catalyze glycolate oxidation only at a poor rate (Simpson et al., 2008). For the second candidate, GlcDH, there is evidence that it is solitarily directed to the mitochondria although *in silico* analysis using the TargetP algorithm (Emanuelsson et al., 2000) also suggested a plastidal localization (Bari et al., 2004). A different glycolate oxidizing enzyme in the chloroplast was postulated by Goyal and Tolbert (1996) but subunits of the anticipated glycolate-quinone oxidoreductase failed to be detected hitherto. We also failed to identify this protein with the gelmap tool for plastidal protein complexes in Arabidopsis thaliana (chapter 2.4) when searching for PGP interacting proteins or complexes with similar size and composition as mitochondrial complex II. Moreover, the toxicity of glyoxylate to Rubisco activation state probably prevents major production of this compound in the chloroplast. Thus, the most likely source for plastidal glyoxylate is minor efflux from the peroxisomes. The second uncertainty of the pathway is the fate of the decarboxylation product, hydroxymethyl-TPP. There are several possibilities for conversion of this product. The hydroxymethyl-residue can be released from TPP either by a nucleophilic or by an electrophilic attack, resulting in formate or formaldehyde, respectively, which could be further oxidized to CO_2. On the other hand, hydroxymethyl-TPP might be the substrate for a hitherto unidentified enzyme equivalent to glyoxylate carboligase from bacteria. In that case, tartronic semiadelyhde would be formed, which is also part of the photorespiratory bypass 1 and of the photorespiratory glycerate pathway of cyanobacteria. Additionally, there might be a link to C1 metabolism. Hydroxymethyl-TPP is activated formaldehyde and analogous to TPP-activated acetaldehyde, which is known to be involved in several pathways (Holzer and Beaucamp, 1961). Similar reactions can be assumed for this compound. Consequently, with the current knowledge it is impossible to integrate the pathway into plant metabolism. A lower energy demand than the major pathway by recycling glycerate without ammonia release is possible as well as much higher energy costs due to complete oxidation of glyoxylate. The same reasons do not allow the classification of the pathway in terms of plant metabolism. If the hydroxymethyl-TPP is not recycled to a CBB cycle intermediate or CO_2, decarboxylation of glyoxylate by PDC would result in depletion of the CBB cycle. If TPP would not be regenerated by release of the active formaldehyde, this pathway might be even toxic due to depletion of TPP. Further experiments are necessary to elucidate the reactions beyond glyoxylate decarboxylation and a possible role of the pathway *in vivo*.

3.5 Concluding remarks

There is growing evidence that photorespiration is not just the wasteful process it has long been considered. Independent data suggests that photorespiration plays an important role as electron sink and in the regeneration of CBB cycle intermediates. However, the evolution of pathways that strongly reduce the oxygenase function of Rubisco shows that this reaction is not mandatory for energy dissipation as long as the CBB cycle has enough substrate. In this respect, the role of the CCM-like

strategies of glyoxylate decarboxylation in the chloroplast is interesting. Additionally, three different pathways for glyoxylate metabolism evolved in cyanobacteria and metabolic flexibility is also observed in higher plants. This indicates that the metabolic identity of the major pathway is not mandatory and that alternative pathways can take over its role in the degradation of toxic intermediates and/or the regeneration of CBB cycle intermediates. Bypass 1 directly resembles one of the cyanobacterial photorespiratory pathways and a functional equivalent of complete oxidation of glyoxylate as realized by bypass 3 is also observed in cyanobacteria. The energy costs differ from the costs of the major pathway, which might even be advantageous under special conditions. Summarized, the recently assigned roles of photorespiration do not exclude that the pathways can be applied. Further experiments are required that should focus on a variety of conditions. The same can be assigned to glyoxylate decarboxylation by the pyruvate dehydrogenase complex. Further experiments shall show, if the observations can be verified *in vivo* and which conditions may induce this pathway.

4 Affix

4.1 Abbreviations

$^1O_2$	singlet oxygen
2D	two-dimensionsal
2-PG	2-phophoglycolate
3-PGA	3-phosphoglycerate
acetyl-CoA	acetyl-coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BN	blue-native
BSC	bundle sheath cell
CAT	catalase
CBB cycle	Calvin Benson Bassham cycle
CCM	carbon concentratin mechanism
CO_2	carbone dioxide
complex I	NADH:ubiquinone oxidoreductase
complex II	succinate dehydrogenase
Cyt b_6f	cytochrome b_6f complex
DHA	dehydroascorbate
DIT	dicarboxylate transporter
e^-	electron
EMS	Ethyl methanesulfonate
ETC	electron transport chain

Fd	ferredoxin
Fd-GOGAT	ferredoxin-dependent glutamine:oxoglutarate aminotransferase
FNR	ferredoxin-NADP oxidoreductase
GAP	glyceraldehyde 3-phosphate
GDC	glycine decarboxylase
GGT	glutamate:glyoxylate transaminase
GLYK	glycerate kinase
GlcDH	glycolate dehydrogenase
GO	glycolate oxidase
GR2	glycolate reductase
GS	glutamine synthase
GSH	glutathione, reduced
GSSG	dimeric glutathione, oxidized
h	hour(s)
H^+	proton
H_2O	water
H_2O_2	hydrogen peroxide
HCO_3^-	biscarbonate
HPR	hydroxypyruvate reductase
kDa	kilo Dalton
Km	Michaelis Menten constant
LHC	light harvesting complex
m	meter
MDa	mega Dalton
MDHA	monodehydroascorbate

MDH	malate dehydrogenase
Mg^{2+}	magnesium ion
mM	millimolar
Mn^{2+}	manganese ion
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NDH	NADPH dehydrogenase
NH_3	ammonia
O_2	molecular oxygen
O_2^-	superoide
OAA	oxaloacetate
PC	plastocyanine
PDC	plastidal pyruvate dehydrogenase complex
PEPC	phosphoenolpyruvate carboxylase
PGP	phosphoglycolate phosphatase
P_i	inorganic phosphate
PLGG1	plastidal glycolate/glycerate transporter
PQ	plastoquinone
PR	photorespiration
PSI	photosystem I
PSII	photosystem II
redox	reduction-oxidation

ROS	reactive oxygen species
Rubisco	ribulose 1,5 bisphosphate carboxylase/oxigenase
RuBP	ribulose 1,5 bisphosphate
s	second
SDS	sodium dodecylsulfate
SGT	serine:glyoxylate transaminase
SHMT	serine hydroxymethyl transferase
TPP	thiamine pyrophosphate
UCP	uncoupling protein
µM	micromolar
µmol	micromole

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Curriculum vitae

Personal information

Name:	Christian Blume
Date of birth:	07.03.1983
Place of birth:	Kiel
Nationality:	German
Marital status:	married

Education:

School:

1989 – 1993

Elementary School, Claus-Rixen-Schule Altenholz

1993 – 2002

Secondary School, Gymnasium Altenholz; Qualification: Abitur

Military service:

Oct. 2002 – Jun. 2003:

3 months basic training in Bremerhaven, thereafter signaler on naval ship ‚Alster'

Academic education:

Oct. 2003 – Nov. 2008

Student at the Reihnisch-Westfälische Technische Hochschule (RWTH) Aachen

Nov 2008

Diploma degree.in Biology,

Thesis: „Gain and loss of chromosomes in *Candida glabrata*" - at Lunds University, Sweden, under supervision of Prof. Jure Piskur and Prof. Ulrich Klinner

Since Feb. 2009

PhD student at the Leibniz Universität Hannover, Institute of Botany, Christoph Peterhänsel;

Research associate at the Leibniz Universität Hannover, Institute of Botany, Prof. Dr. Christoph Peterhänsel

4.3 Publications

4.3.1 Research papers, reviews and manuscripts

Polakova, S., **Blume, C.**, Zarate, J.A., Mentel, M., Jorck-Ramberg, D., Stenderup, J., and Piskur, J. (2009). Formation of new chromosomes as a virulence mechanism in yeast Candida glabrata. Proc Natl Acad Sci U S A **106,** 2688-2693.

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Peterhansel, C., **Blume, C.**, and Offermann, S. (2012). Photorespiratory bypasses: how can they work? J Exp Bot **64,** 709-715.

Behrens, C., **Blume, C.**, Senkler, M., Eubel, H., Peterhansel, C., Braun, H.P. The protein complex proteome' of chloroplasts in *Arabidopsis thaliana*. *In revision for Journal of Proteomics*.

Blume, C., Behrens, C., Eubel, H., Braun, H.P., Peterhansel, C. A role for the chloroplast pyruvate dehydrogenase complex in glycolate and glyoxylate metabolism. *In revision for Phytochemistry.*

4.3.2 Conference contributions

Blume, C., van de Steene, N., Kebeish, R., Niessen, M., van Rie, J.,Metzlaff, M.,Peterhänsel, C., Bypassing photorespiration by a single enzyme, GABI Statusseminar, 2010, Potsdam

Blume, C., Kebeish, R., Niessen, M., Peterhänsel, C., Overexpression of a single enzyme reduces photorespiration, Tagung Molekularbiologie der Pflanzen, 2011, Dabringhausen

Blume, C., Peterhänsel, C., Development of a method for direct determination of plastidal glycolate conversion, Photorespiration – Key to Better Crops, 2012, Warnemünde

4.4 Eidesstattliche Erklärung

Hierdurch erkläre ich, Christian Blume, dass ich meine Dissertation mit dem Titel „Glycolate and glyoxylate metabolism in higher plants: How natural and artificial pathways contribute to plant metabolism“ selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den

4.5 Danksagung

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