INTERACTIONS BETWEEN PHOTOSYNTHETIC ELECTRON
TRANSPORT AND CARBON ASSIMILATION IN TRANSGENIC
TOBACCOS IMPAIRED IN PHOTOSYNTHESIS

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of Philosophy of the Australian National University

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CANDIDATE'S STATEMENT

Except where otherwise acknowledged, the work presented in this thesis is my own.

Sari Ruuska

[Signature]

Sari Ruuska
The essence of Chapter 2, entitled

*The Interplay between limiting processes in C₃ photosynthesis studied by rapid response gas exchange using transgenic tobacco impaired in photosynthesis*

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ABSTRACT

In nature the availability of the substrates for photosynthesis, light and CO₂, varies. As a consequence plants have evolved several mechanism to maximise the utilisation of these resources and to balance the capacities of light harvesting and carbohydrate biosynthesis. This thesis examines the coordination and poise between the photosynthetic electron transport and carbon assimilation in intact leaves. Material for the studies consisted of transgenic tobaccos with alterations in different parts of their photosynthetic machinery. The amounts of Rubisco (anti-SSu plants), chloroplast glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH plants) or cytochrome b₆f complex (anti-b₆f plants) have been reduced with antisense DNA technique.

In Chapter 2, a novel manner to examine the balance between RuBP regeneration capacity and Rubisco activity is described. Gas exchange system with a rapid response time allows fast changes in the light intensity and gas composition that the leaf is exposed to. It is therefore possible to record fast transients in the leaf's gas exchange rate. This gas exchange technique provided a direct means to measure potential electron transport rate (J) and Rubisco's maximal carboxylation capacity (V_{cmax}), two parameters important in photosynthetic modelling, and demonstrated how the balance between Rubisco activity and RuBP regeneration is altered in opposite directions in anti-SSu and anti-GAPDH plants. Combined measurements of post-illumination CO₂ uptake and RuBP concentration were also performed. The post-illumination CO₂ uptake underestimated true RuBP contents in the anti-SSu leaves, whereas the two corresponded well in wild-type leaves. It was found that Rubisco apparently inactivated rapidly after the light was turned off, leaving some RuBP unconsumed in the chloroplasts. However, the Rubisco carbamylation state and \textit{in vitro} activity remained high.

The interactions between electron transport and chloroplast metabolites in modulating the activity of Rubisco were examined in Chapter 4-6. Two contrasting plant types were used. Anti-b₆f plants have impaired electron transport capacity whereas carbon assimilation is impaired in anti-GAPDH plants. The CO₂ assimilation rates and RuBP contents decreased in both plant types. The low RuBP concentration
was not the only cause for the reduction in CO₂ assimilation rates observed in anti-\textit{bf} plants. The carbamylation level of Rubisco declined as well, suggesting that the activity of Rubisco activase was impaired. The whole-leaf ATP/ADP ratio in the anti-\textit{bf} plants was same as in the wild-type plants, but the activation level of chloroplast NADP-malate dehydrogenase (NADP-MDH) decreased significantly. The low NADP-MDH activity indicates that the electron transport rate, transthylakoid pH gradient (\(\Delta pH\)) and NADP⁺/NADPH ratio were severely reduced in anti-\textit{bf} plants, and these conditions may have reduced the activity of Rubisco activase. On the contrary, in anti-GAPDH plants high ATP/ADP ratio and high \(\Delta pH\) apparently enhanced the performance of the Rubisco activase. The carbamylation state of Rubisco remained high in these plants even when the RuBP content was reduced below the Rubisco site concentration; a condition that has been shown otherwise to cause decarbamylation.

In Chapters 7 and 8 the effects of decreased CO₂ assimilation capacity on the leaf pigments and the utilisation of light were examined. When the CO₂ assimilation rates were reduced in anti-SSu and anti-GAPDH plants, the dissipation of light energy as heat was enhanced. The de-epoxidation state of the of xanthophyll cycle pigments in light increased significantly in transgenic plants as well. Nevertheless, the ratio between xanthophyll cycle pigments and chlorophyll did not change, unless the CO₂ assimilation capacity was reduced to 10% of the wild-type. When the electron transport through PSII and electron transport required for CO₂ assimilation and photorespiration were measured simultaneously in anti-SSu leaves at several CO₂ and O₂ concentrations, the two estimates agreed well. No evidence was found for alternative electron sinks, such as O₂ photoreduction, that might be expected to be more active in transgenic plants. However, it was interesting to note that during the early phase of photosynthetic induction after a long dark period, chlorophyll fluorescence indicated extra electron transport through PSII which could not be accounted for gas exchange. This was observed in both wild-type and transgenic plants suggesting that alternative electron sinks could be important at transient conditions.
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<td>A</td>
<td>Antheraxanthin</td>
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<tr>
<td>activase</td>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase activase</td>
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<td>ADP</td>
<td>Adenosine dihosphate</td>
</tr>
<tr>
<td>AK</td>
<td>Adenylate kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CA1P</td>
<td>2'-carboxy D-arabinitol-1-phosphate</td>
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<td>Cc</td>
<td>Chloroplastic CO₂ concentration</td>
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</tr>
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<td>Jᵣ</td>
<td>Electron transport rate required to maintain CO₂ assimilation and photorespiration</td>
</tr>
<tr>
<td>Jₚₓₑᵪ</td>
<td>The maximal, light-saturated electron transport rate</td>
</tr>
<tr>
<td>Kc</td>
<td>Michaelis-Menten constant for carboxylation by Rubisco</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaels-Menten half-saturation parameter</td>
</tr>
<tr>
<td>$K_r$</td>
<td>The Michaelis-Menten constant for RuBP</td>
</tr>
<tr>
<td>$K_{r'}$</td>
<td>The effective Michaelis-Menten constant for RuBP</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Nicotinamide adenine dinucleotide (oxidised form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidised form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NPQ</td>
<td>Nonphotochemical quenching</td>
</tr>
<tr>
<td>NRD</td>
<td>Nonradiative dissipation</td>
</tr>
<tr>
<td>OAA</td>
<td>Oxalic acetic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Plastocyanin</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenol pyruvate</td>
</tr>
<tr>
<td>PFD</td>
<td>Photon flux density</td>
</tr>
<tr>
<td>2-PGA</td>
<td>2-phosphoglycolate</td>
</tr>
<tr>
<td>3-PGA</td>
<td>3-phosphoglyceric acid</td>
</tr>
<tr>
<td>PGM</td>
<td>3-phosphoglyceric acid mutase</td>
</tr>
<tr>
<td>$P_i$</td>
<td>Inorganic phosphate (ortophosphate)</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
</tr>
<tr>
<td>PQH</td>
<td>Plastohydroquinone</td>
</tr>
<tr>
<td>PRK</td>
<td>Phosphoribulokinase</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>QA</td>
<td>Primary quinone acceptor of PSII</td>
</tr>
<tr>
<td>$R_d$</td>
<td>Mitochondrial (dark) respiration</td>
</tr>
<tr>
<td>$R_t$</td>
<td>The total concentration of RuBP</td>
</tr>
<tr>
<td>Rubisco</td>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RuBP</td>
<td>Ribulose 1,5 bisphosphate</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation of the mean</td>
</tr>
<tr>
<td>S.E.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SBPase</td>
<td>Seduheptulose 1,7-bisphosphatase</td>
</tr>
<tr>
<td>SSu</td>
<td>Rubisco's small subunit</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-tris [hydroxymethyl] methylglycine; N-[2-hydroxy-1,1-bis (hydroxymethyl) ethyl] glycine</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-9hydroxymethyl-1,3-propanediol</td>
</tr>
<tr>
<td>T_x</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>V</td>
<td>Violaxanthin</td>
</tr>
<tr>
<td>V_{cmax}</td>
<td>The maximal carboxylation capacity of Rubisco</td>
</tr>
<tr>
<td>Z</td>
<td>Zeaxanthin</td>
</tr>
<tr>
<td>Γ*</td>
<td>CO₂ compensation point in the absence of mitochondrial respiration</td>
</tr>
<tr>
<td>ΔpH</td>
<td>Transthyakoid pH gradient</td>
</tr>
<tr>
<td>ΦPSII</td>
<td>Quantum yield of PSII</td>
</tr>
</tbody>
</table>
CHAPTER 1

GENERAL INTRODUCTION
1.1 A REVIEW OF THE PHOTOSYNTHETIC REACTIONS

In this Chapter I will give a brief introduction to the photosynthetic light and dark reactions, and the coordination between them. Each Chapter contains a more detailed introduction and literature review.

Essentially all free energy consumed by biological systems is derived from sunlight by the process of photosynthesis. In higher plants photosynthesis takes place in the chloroplasts. The inner space of chloroplast, stroma, is surrounded by two envelope membranes. The stroma contains an internal system of membranes called thylakoids, which are arranged as enclosed vesicles and often pack together to form grana stacks. Photosynthetic reactions of the chloroplast can be divided structurally and functionally into two parts: light and dark reactions (Fig. 1.1). The constituents of light reactions are associated with the thylakoid membranes, and produce the high-energy compounds ATP and NADPH. The enzymes which participate in the dark reactions are located in stroma, and use ATP and NADPH to synthesise carbohydrates from CO₂. The process of CO₂ fixation proceeds via the C₃ photosynthetic carbon reduction cycle (Calvin cycle), where the primary product is a 3-carbon acid. However, in some plants other photosynthetic pathways have evolved with different anatomical structures and metabolic pathways for carbon fixation. These are termed C₄ and CAM (crassulacean acid metabolism), where CO₂ is concentrated inside cells before fixation by C₃ cycle.

1.1.1 LIGHT REACTIONS CONVERT SOLAR ENERGY TO CHEMICAL FORM

During photosynthesis light is collected by chlorophyll pigments, which are bound to specific proteins. When a chlorophyll molecule absorbs a photon, it makes a transition to a high-energy state. Only specific chlorophylls, called reaction centres, are able to perform photochemistry powered by this transition. The other chlorophylls form the light-harvesting antenna and transfer their excitation energy to the reaction centres. Plants contain two types of reaction centres, photosystem II (PSII, absorption maximum at 680 nm) and photosystem I (PSI, absorption maximum...
Fig. 1.1 A simplified scheme of photosynthesis and photorespiration. Abbreviations: PSI and PSII, photosystem 1 and 2; PQ, plastoquinone; Cyt bf, cytochrome bf complex; PC, plastocyanin; Fd, ferredoxin; ATP-ase, ATP synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase 3-PGA, 3-phosphoglycerate; 2-PGA 2-phosphoglycolate; DiPGA, 1,3 bisphosphoglycerate; G3P, glyceraldehyde 3-phosphate; DHAP dihydroxyacetone phosphate; RuBP, ribulose 1,5 bisphosphate. The amounts of the highlighted enzymes (Rubisco, GAPDH, cytochrome bf) have been reduced with the antisense DNA technique in tobacco.
at 700 nm), each of which have their own antenna systems (Fig. 1.1). When the reaction centre of PSII is excited, it becomes an extremely strong oxidant and is able to extract electrons directly from a water molecule. The splitting of water liberates molecular oxygen well hydrogen ions whereas electrons are donated to a series of soluble and membrane-bound electron carriers.

The photosynthetic electron transport chain is composed of 4 integral membrane protein complexes (PSII, Cytochrome $b_6$ complex, PSI, ATP synthase) and mobile carriers (plastoquinone, plastocyanin) (Fig. 1.1). From the PSII complex electrons transferred to a plastoquinone (PQ) pool and then to the cytochrome $b_6$ complex and to the plastocyanin. Cytochrome $b_6$ complex also translocates protons from stroma to the intrathylakoid space, lumen. Plastocyanin is in turn oxidised by the excited PSI reaction centre in a manner analogous to PSII, and electrons derived from water are used to reduce ferredoxin (F$_4$). The two high-energy electrons of reduced F$_4$ are transferred to NADP$^+$ to synthesise NADPH. The protons originating from water, as well as the ones translocated by cytochrome $b_6$ complex form transthyakloid pH gradient. This electrochemical gradient is the driving force for the membrane-spanning ATP-synthase to combine ADP and P$_i$ to ATP.

1.1.2 DARK REACTIONS USE ATP AND NADPH IN CARBOHYDRATE SYNTHESIS

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the primary carbon fixing enzyme. Rubisco catalyses the reaction where atmospheric CO$_2$ is condensed with a 5-carbon phosphorylated sugar, ribulose 1,5-bisphosphate (RuBP), to form a transient 6-carbon intermediate which is rapidly hydrolysed to two 3-phosphoglyceric acid (3-PGA) molecules (Andrews and Lorimer 1987). 3-PGA is first phosphorylated using ATP, and then reduced to form triose phosphates using NADPH (Chapter 4, Fig. 4.2). Maintaining the CO$_2$ fixation requires that the acceptor molecule, RuBP, be constantly regenerated. As much as 5/6 of the newly formed triose phosphates is converted to RuBP in a series of enzymatic reactions of the Calvin cycle. The regeneration of RuBP consumes additional ATP (Fig. 1.1). The rest of the triose phosphates are used inside chloroplasts in starch synthesis, or are transported to cytosol to form sucrose.
Rubisco also catalyses oxygenation of RuBP, starting an energetically costly pathway of photorespiration (Fig. 1.1). The reason for this is that CO₂ and O₂ are competing substrates for the reaction with RuBP. Although Rubisco has a higher affinity for CO₂ than for O₂, the velocity of the oxygenase reaction in air is high because of the great difference between the concentrations of the two gases. In air, the concentration of O₂ is 21% and CO₂ only 0.035%, resulting to the ratio between carboxylation and oxygenation in leaves at +25 °C being around 3:1. The oxygenation of RuBP produces one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PGA). Photorespiratory pathway acts as a scavenger operation, metabolising 2-PGA and liberating CO₂, which can be refixed. It also consumes both ATP and reducing equivalents. This pathway involves a cooperation between 3 different organelles within a mesophyll cell: chloroplast, peroxisome and mitochondria. Briefly, 2-PGA is hydrolysed to glycolate, which can leave chloroplast to be further processed in peroxisomes and mitochondrion, such that eventually two 2-PGA molecules are converted into one 3-PGA which can re-enter the Calvin cycle and one CO₂ molecule is liberated (Lorimer 1981).

1.2 THE COORDINATION BETWEEN LIGHT AND DARK REACTIONS

Under natural conditions plants are subjected to varying environmental conditions. This variation results in changes in the supply of light or CO₂ for the photosynthesis. To ensure the coordination of the photosynthetic reactions, regulatory mechanisms exist in chloroplast which optimise the efficiency of light and dark reactions. Essentially, several reaction pathways ensure that the enzymes of Calvin cycle are turned off in darkness but, on the other hand, are able to quickly respond to a prevailing light intensity. Similarly, if the consumption of ATP and NADPH is suppressed, for example due to low intercellular CO₂, the efficiency of light harvesting can decrease. I will briefly outline some of the regulatory systems serving to balance the light utilisation and carbon metabolism in chloroplasts.
1.2.1 METABOLITES AS REGULATORS

Since the Calvin cycle (and photorespiration) consume ATP and NAD(P)H, the availability of these compounds determines the overall functioning of the two pathways. However, several photosynthetic enzymes are regulated by chloroplast metabolite levels. For example, fructose 1,6-bisphosphatase (FBPase) and seduheptulose 1,7- bisphosphatase (SBPase) are inhibited by their respective end-products, fructose 6-phosphate and seduheptulose 7-phosphate. This feedback inhibition prevents accumulation of Calvin cycle intermediates in stroma (Stitt 1996). Phosphoribulokinase (PRK) catalyses the formation of RuBP using ATP, and is inhibited by its product ADP. The product of carboxylation or oxygenation of RuBP, 3-PGA, is also a competitive inhibitor for PRK. These were just a few examples of the complicated metabolic regulation of the Calvin cycle, and the topic has recently been extensively reviewed (Stitt 1996).

1.2.2 STROMAL pH AND MAGNESIUM CONCENTRATION

During illumination protons are pumped from the stroma into the lumen (Fig. 1.1) in exchange for Mg\(^{2+}\) (Heldt 1979; Portis 1981). As a result, stromal pH rises from about pH 7 to 8 and simultaneously the concentration of free Mg\(^{2+}\) increases. Several Calvin cycle enzymes have a pH optimum around 8 and need Mg\(^{2+}\) as a cofactor, so these light-induced ionic movements promote their activation. Enzymes which are regulated in this manner include FBPase, SBPase (Stitt 1996) and Rubisco (Andrews and Lorimer 1987).

1.2.3 FERREDOXIN-THIOREDOXIN PATHWAY

Another way of signalling the illumination to chloroplast enzymes is via ferredoxin-thioredoxin pathway (Buchanan 1984). In light, PSI produces reduced ferredoxin (Fd\(_{\text{red}}\)) (Fig. 1.1). The reducing equivalents can be transferred from Fd\(_{\text{red}}\) to small proteins called thioredoxins (Tx), which have two cysteine groups in their active sites. The reduction of the cysteine groups opens the disulphide bridge to two separate SH-groups. Thioredoxins are able to reduce similar disulphide bonds in other enzymes like PRK, FBPase, and chloroplast glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Opening of the S-S bonds activates these enzymes.
1.2.4 RUBISCO - A SPECIAL CASE

The activity of Rubisco, as of many other stromal enzymes, responds to light. At high irradiances Rubisco is generally highly activated, but the activation decreases at low light levels. Rubisco is active only when a specific lysyl residue within its catalytic site is carbamylated (complexed with CO₂) and bound with Mg²⁺. This carbamylation process is considered to be enhanced by the light-dependent stromal alkalisation and increase in Mg²⁺ (Andrews and Lorimer 1987). However, the changes in carbamylation state in response to light are mediated by another stromal protein, Rubisco activase. Activase requires ATP to function, and is inhibited by ADP, and so presumably is sensitive to the stromal ATP/ADP ratio (Robinson and Portis 1989b). This could provide a means to link Rubisco carbamylation state to the activity of light reactions. A number of observations suggest though, that this may not be the only mechanism regulating Rubisco activase, but the activase may sense the transthylakoid proton gradient and/or electron transport rate directly (Campbell and Ogren 1990a; Campbell and Ogren 1992).

Rubisco activity is regulated also by chloroplast metabolites. Phosphorylated sugars, other than the substrate RuBP, can bind to Rubisco's catalytic sites and slow down catalysis or prevent carbamylation. Metabolites that are known to modulate Rubisco include 3-PGA and inorganic phosphate, Pᵢ. The substrate RuBP is an important regulator of Rubisco's activity as well, since it binds tightly to uncarbamylated sites (Andrews and Lorimer 1987).

1.2.5 THE EFFICIENCY OF LIGHT HARVESTING CAN CHANGE

The previous examples about the cooperation between light and dark reactions listed mechanisms that regulate the dark reactions, mainly in response to light stimulus. In addition, regulation of the initial light harvesting level can take place as well. If leaves receive light in excess to what can be used in CO₂ assimilation and photorespiration, the antenna pigments may become oxidised, or the PSII reaction centre itself can be inactivated by light exposure (Björkman 1981; Long et al. 1994; Trebst 1995). In order to avoid light-induced damage, the dissipation of excitation energy as heat in the antenna pigments increases (Björkman and Demmig-Adams 1995). This process is called nonradiative dissipation (NRD) (Krause et al. 1982).
The precise mechanism of NRD is not known, but the factor that triggers it appears to be the decrease in the lumenal pH. This is thought to result in structural changes in the light-harvesting chlorophyll protein complexes which in turn increase the rate-constant of heat dissipation in the pigment bed (Horton et al. 1994; Gilmore 1997). A group of carotenoids, xanthophyll cycle pigments, are thought to be involved in NRD as well. It has been suggested that the light-induced de-epoxidation of violaxanthin to zeaxanthin and antheraxanthin enhances NRD and so aids in preventing PSII damage (Demmig-Adams 1990; Demmig-Adams et al. 1996).

The absorption of light by PSII can be reduced also via state transitions, where the PSII light-harvesting chlorophyll protein complexes become phosphorylated and dissociate from the PSII reaction centres (Bennett et al. 1980; Cleland et al. 1990). Other mechanism that could potentially alleviate the overreduction of photosynthetic electron chain include the flow of electrons to alternative sinks and cyclic electron transport around PSII (Foyer et al. 1990). In this context alternative electron sinks mean other processes than CO₂ assimilation and photorespiration, which can utilise electrons. These cover nutrient assimilation, and O₂ photoreduction by PSI (Mehler reaction) followed by H₂O₂ reduction by ascorbate peroxidase pathway (Neubauer and Yamamoto 1992). The state transitions, O₂ photoreduction and PSII cycling have all been observed in vivo experiment, but their significance for photoprotection in leaves remains to be determined.
1.3 THE AIM OF MY THESIS

The aim of my thesis work was to study how the capacities of the light and dark reactions of photosynthesis are coordinated in intact leaves. I had three specific objectives. The first objective was to test whether a rapid-response gas exchange machinery could be used to study the balance between Rubisco activity and RuBP regeneration capacity in leaves. The second objective was to investigate how photosynthetic electron transport and chloroplast metabolites interact in determining the activity of Rubisco. The third objective was to examine how a restriction in carbon assimilation capacity feeds back to electron transport chain and the harvesting of light energy. My tools have been a variety of transgenic tobacco plants, which have alterations at different parts of their photosynthetic machinery. Combined gas-exchange measurements and biochemical assays were conducted on the leaves to elucidate the regulatory mechanisms in vivo.

1.4 THE OUTLINE OF THESIS

Section I: In vivo-studies of the limiting factors of CO₂ assimilation using rapid-response gas exchange measurements

In this Section (Chapter 2) I describe a novel approach to study the balance between RuBP regeneration capacity and Rubisco activity in leaves. I have used non-steady state gas exchange measurements conducted with a two-channel system, which has a short response time and enables rapid changes in the light intensity and gas composition in the leaf chamber. I also studied whether the post-illumination CO₂ uptake can be used as a reliable, nondestructive measure of leaf RuBP content.

Section II: The interplay between electron transport and chloroplast metabolites in regulating Rubisco activity

The second part (Chapters 4-6) examines photosynthetic and biochemical characteristics of two contrasting transgenic tobacco types with altered balance between light and dark reactions. One set of plants has impaired photosynthetic electron transport, whereas the capacity for RuBP regeneration is reduced in the other set. I study how photosynthetic electron transport, and chloroplast metabolites
interact in determining the activation of Rubisco at two different CO₂ concentrations. Because it was essential to measure several metabolites from a small amount of leaf material, a sensitive luminometric method for adenylate and metabolite analysis of tobacco leaf samples was developed (Chapter 3).

Section III: The effects of impaired carbon assimilation capacity on the leaf pigments and efficiency of light harvesting

The last Section examines how a reduction in the CO₂ assimilation capacity feeds back to the light harvesting and utilisation in transgenic plants. The extent of nonradiative dissipation of light energy was measured with chlorophyll fluorescence and the participation of the xanthophyll cycle pigments in photoprotection was assessed in Chapter 7. In Chapter 8 I compared two different methods in estimating the rate of photosynthetic electron transport in intact leaves, when the measurements were conducted either during the induction of photosynthesis or at steady-state. These comparisons were also used in assessing whether alternative electron sinks (other than CO₂ assimilation and photosynthesis) become more prevalent in transgenic plant with reduced photosynthetic capacity.
CHAPTER 2

THE INTERPLAY BETWEEN LIMITING PROCESSES IN $C_3$
PHOTOSYNTHESIS STUDIED BY RAPID-RESPONSE GAS EXCHANGE
2.1 INTRODUCTION

2.1.1 MODELLING C₃ PHOTOSYNTHESIS

In intact leaves, photosynthesis is an integral process which continuously responds to internal and external factors. The rate of photosynthesis is not determined only by the availability of substrates, CO₂ and light, but also by the activity of several metabolic pathways, such as photosynthetic electron transport (Heber et al. 1988), the Calvin cycle (Woodrow and Berry 1988) and carbohydrate biosynthesis (Stitt and Quick 1989; Neuhaus and Stitt 1990). These pathways, in turn, are regulated in a complex and interactive manner as briefly discussed in Chapter 1. The contributions of different biochemical factors in regulating photosynthesis under varying environmental conditions have been recently extensively studied by using transgenic plants (Stitt and Schulze 1994; Andrews et al. 1995a; Stitt 1995; Stitt and Sonnewald 1995).

The approach of the photosynthetic modellers has been to reduce the complexity of photosynthetic metabolism by identifying steps which may be important for optimal performance. Since the CO₂ fixation is catalysed by a single enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), it represents the key process in photosynthesis. The biochemical model of C₃ photosynthesis, which is based on the catalytic properties of Rubisco, was developed by Farquhar et al. (1980) and has since been widely used in photosynthesis research. I will give a short description of the basic equations of this model. More detailed discussion is found from for example Woodrow and Berry (1988).

Because Rubisco uses both CO₂ and O₂ as its substrate, the net rate of CO₂ assimilation in leaves is determined by the relative contributions of carboxylation and oxygenation of RuBP. When one mole of RuBP is oxygenated by Rubisco, the photorespiratory pathway releases 0.5 moles CO₂, which can be refixed (Fig. 1.1). Therefore the net rate of CO₂ assimilation (A) is described by the equation

\[ A = V_c + 0.5V_o - R_d \]
where \( V_c \) and \( V_o \) are the rates of RuBP carboxylation and oxygenation by Rubisco, respectively, and \( R_d \) is the rate of mitochondrial (dark) respiration.

The rate of RuBP oxygenation is dependent on the prevailing \( \text{CO}_2 \) and \( \text{O}_2 \) concentrations in the chloroplasts and on the relative specificity of Rubisco. The competition between \( \text{CO}_2 \) and \( \text{O}_2 \) can be summarised in the term \( \Gamma^* \) (Laisk 1977), which is the chloroplast \( \text{CO}_2 \) concentration at which the Rubisco's carboxylation rate equals photorespiratory \( \text{CO}_2 \) release. The value of \( \Gamma^* \) is dependent on the \( \text{O}_2 \) concentration (O) and the Rubisco's specificity for \( \text{CO}_2 \) as opposed to \( \text{O}_2 \) (\( S_{co}/O \)) as follows:

\[
\Gamma^* = \frac{0.5O}{S_{co}/O}
\]

When \( \Gamma^* \) is known, the rate of RuBP oxygenation can be related to the rate of RuBP carboxylation and chloroplast \( \text{CO}_2 \) concentration (\( C_c \)) as

\[
V_o = \frac{2\Gamma^*}{C_c} \cdot V_c
\]

and \( \text{CO}_2 \) assimilation rate can be expressed as

\[
A = (1 - \Gamma^*/C_c)V_c - R_d
\]

The rate of carboxylation of RuBP (\( V_c \)) depends on (1) the amount and activity of Rubisco, and (2) the rate of regeneration of the substrate RuBP, determined by the rate of electron transport and the activity of the Calvin cycle (Fig. 1.1). Farquhar (1979) presented a kinetic model about the relationship between Rubisco activity and RuBP concentration, which takes into account the high concentration of Rubisco in chloroplasts and its relatively low \( K_m \) for RuBP. This can lead to a situation where only a portion of the total RuBP is free and the rest is bound to Rubisco, and Michaelis-Menten type of enzyme kinetics is not valid. The \( \text{C}_3 \)-photosynthesis model is based on this observation on the special relationship between Rubisco and RuBP, which will be discussed in more detail in Chapter 6. According to the model of \( \text{C}_3 \) photosynthesis by Farquhar et al. (1980), at any given time, the \( \text{CO}_2 \)
assimilation rate can be described as being limited either by the RuBP-saturated rate of Rubisco, or by the rate of RuBP regeneration. The RuBP-saturated rate of carboxylation is given by

\[ V_c = \frac{C_c V_{c,\text{max}}}{C_c + K_c(1 + O/K_o)} \]

where \( V_{c,\text{max}} \) is the maximal carboxylation activity of Rubisco, and \( K_c \) and \( K_o \) are the Michaelis-Menten constants for \( CO_2 \) and \( O_2 \), respectively.

The RuBP-saturated rate of carboxylation \( (V_j) \) depends on the capacity of the electron transport and can be described as

\[ V_j = \frac{J}{4(1 + 2\Gamma^* / C_c)} \]

where \( J \) is the potential rate of electron transport needed for the Calvin cycle and photorespiratory pathways, and the factor 4 indicates that the transport of 4 electrons will produce enough ATP and NADPH required for RuBP regeneration in the Calvin cycle (Farquhar and von Caemmerer 1982). The potential rate of electron transport depends on absorbed irradiance \( (I_{ab}) \) and the maximal, light-saturated electron transport capacity \( (J_{\text{max}}) \). The relationship has been approximated by the empirical expression as

\[ J = \frac{J_{\text{max}} I_{\text{ab}}}{I_{\text{ab}} + 2.1J_{\text{max}}} \]

Subsequent modifications of the \( C_3 \) model have introduced a third limitation, the rate of triose phosphate utilisation (Sharkey 1985; Harley and Sharkey 1991), which is thought to become important when the consumption of triose phosphates by starch and sucrose synthesis cannot keep pace with the production rate of triose phosphates. This leads to a situation where the availability of free inorganic phosphate \( (P_i) \) becomes limiting for \( CO_2 \) assimilation. The limitation of triose phosphate utilisation is thought to occur for example under prolonged conditions of
high CO\(_2\) and high irradiance (Harley et al. 1992). Sharkey (1985) demonstrated that when the consumption and release of Pi of both Calvin cycle and photorespiratory pathways are combined, the carboxylation rate determined by the Pi availability (\(V_p\)) is expressed as

\[
V_p = 3\text{TPU} + 0.5V_o
\]

where TPU is the rate of triose phosphate limitation. In this Chapter I will limit the discussion of the limiting factors of C\(_3\) photosynthesis to \(V_c\) and \(V_j\) because the prolonged exposure of the leaves to high light and elevated CO\(_2\) were avoided.

The limitation by Rubisco capacity occurs generally at low intercellular partial pressure of CO\(_2\) (\(C_i\)), for example when the stomatal conductance is low. RuBP-regeneration limitation occurs at high \(C_i\) and high light, when the maximal capacity of the chloroplast’s electron transport chain or the Calvin cycle’s maximal capacity for RuBP regeneration is attained, or at low light, when the rate of electron transport is slow. Based on this, the CO\(_2\) assimilation rate of leaves can be described mathematically with two key rates: \(V_{cmax}\), the maximum Rubisco carboxylation rate, and \(J_{max}\), the maximum, light-saturated electron transport or RuBP regeneration rate. The ratio of \(J_{max}/V_{cmax}\) determines the \(C_c\) at which the transition from Rubisco carboxylation limitation to RuBP-regeneration limitation takes place at each set of environmental conditions. Consequently, these two parameters are important in simulations of carbon fluxes from leaf and plant to ecosystem levels (Wullschleger 1993; De Pury and Farquhar 1997; Walcroft et al. 1997). Generally, the values of \(V_{cmax}\) and \(J_{max}\) are derived from steady-state gas exchange measurements (Farquhar et al. 1980; Kirschbaum and Farquhar 1984; Wullschleger 1993), however, under some environmental conditions it is not always easy to determine the transition from RuBP saturation to RuBP limitation.

2.1.2 POST-ILLUMINATION CO\(_2\) UPTAKE

The amount of RuBP in the chloroplast depends on the balance between its consumption by Rubisco and the capacity for its regeneration and is thus a useful indicator of chloroplast metabolism. Studies with transgenic tobacco with impaired photosynthesis have shown large variations in RuBP pools (Quick et al. 1991; Paul et
However it is difficult to make these measurements under a variety of conditions, as the biochemical assay is destructive and requires rapid freeze clamping of leaves.

The concentration of RuBP can be measured after acid extraction of the photosynthetic material, by either via incorporation of $^{14}$C into 3-PGA or by spectrophotometric method. In addition, the pool of CO$_2$ acceptor compounds can support the fixation of CO$_2$ for some time after the illumination has ceased. This phenomenon, called post-illumination CO$_2$ uptake has been observed in isolated chloroplasts, algae and higher plant leaves (Miyachi 1979; Laisk et al. 1984; 1987; Chazdon and Pearcy 1986; Pons and Pearcy 1992). It has been concluded that, in C$_3$ plants, the post-illumination CO$_2$ uptake is mainly supported by RuBP accumulated during the preceding light period (Laisk et al. 1984). In addition, ATP and some Calvin cycle intermediates, like triose phosphates have been shown to participate the post-illumination CO$_2$ uptake in some cases as well (Sharkey et al. 1986b).

2.1.3 THE OUTLINE OF THE CHAPTER

In this Chapter, I explore the use of measurements of non-steady-state photosynthesis (Laisk and Oja 1974) to study the balance between Rubisco and RuBP regeneration capacities in wild-type tobacco (*Nicotiana tabacum* L. cv. W38) and transgenic tobaccos with an antisense DNA directed against Rubisco small subunit (anti-SSu plants) or chloroplast glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH plants) (Hudson et al. 1992; Price et al. 1995a). These three genotypes have different balances between Rubisco and RuBP regeneration capacities. A two-channel gas exchange system was used (Laisk and Oja 1998) which can record rapid transients in CO$_2$ exchange rates. Large RuBP pools were generated in leaves by exposing them briefly to very low CO$_2$, after which they were transferred to varying CO$_2$ concentrations, and momentarily maximal CO$_2$ assimilation rates were measured within the first 2-3 seconds. A comparison between the transient (RuBP-saturated) and steady-state rates was used to assess the RuBP-regeneration vs. Rubisco-limitation of CO$_2$ assimilation.
I also examined whether the post-illumination CO₂ uptake at low O₂ can be used as a rapid and nondestructive method to estimate leaf RuBP pool sizes. The post-illumination CO₂ uptake was measured using the two-channel gas exchange system, and later samples were collected from the same leaves under similar conditions with a rapid freeze clamping device for biochemical assays of RuBP content.

I would like to point out that in this study I used the transgenic plants to test the ideas about C₃ photosynthesis, and did not attempt to characterise them further than has already been done. In Chapters 4-8 I will study anti-GAPDH plants in more detail, and a comprehensive introduction to these plants is given there.
2.2 MATERIAL AND METHODS

2.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

Transgenic tobacco plants with reduced amounts of Rubisco were raised from the seeds collected from a selfed R₁ progeny of tobacco (*Nicotiana tabacum* cv. W38) transformant described by Hudson *et al.* (1992). Seedlings segregate in a 1 : 2 : 1 ratio into homozygous hemizygous or null type (wild-type). Homozygous plants (referred to as anti-SSu 2 plants) have two copies of the *rbcS* antisense gene and typically 10-15% of the wild-type Rubisco content. Hemizygous plants (referred to as anti-SSu 1 plants) have one copy of the antisense gene and 30-40% of the wild-type Rubisco content. The genotype of the anti-SSu plants was determined on the basis of growth rate and photosynthetic capacity. The anti-glyceraldehyde 3-phosphate dehydrogenase plants (referred to as anti-GAPDH plants) were the R₁ progeny of plant GAP-R. It had two active and independent inserts of the antisense gene, and R₁-seeds produce plants with a range of GAPDH activities (Price *et al.* 1995a). Untransformed cv. W38 tobaccos were used as controls. The plants were grown in 5 L pots in an air conditioned glasshouse with a peak irradiation of 700-900 μmol quanta m⁻² s⁻¹. Plants were given Hewitt's complete nutrient solution (Hewitt and Smith 1975) three times a week. Gas exchange measurements were made 6-10 weeks after germination on the youngest fully expanded leaves.

2.2.2 GAS EXCHANGE AND EXPERIMENTAL PROTOCOL

2.2.2.1 The rapid-response gas exchange system

The gas exchange system consists of two independent gas exchange systems ("channels") which can be connected to a sandwich-type leaf chamber (43×43×3 mm). System properties and detailed applications are described by Laisk and Oja (1998). The flow through the leaf chamber can be switched quickly from one system to another, which enables rapid changes in the gas environment of the leaf (less than 0.5 s). The upper surface of the leaf was sealed with starch paste to the water-thermostated leaf chamber window, which facilitates the heat-exchange coefficient between the leaf and the water bath. As a result, the leaf gas exchange through the lower epidermis only was measured. The leaf chamber was illuminated through a
fibre-optic light guide system connected to halogen lamps (Schott KL 1500). Gas-exchange parameters were calculated according to von Caemmerer and Farquhar (1981).

2.2.2.2 Steady-state and transient CO₂ assimilation rates.

Each series of measurements started by stabilising the leaf's CO₂ assimilation and transpiration rates at 300 μbar CO₂ in air. The PFD was 1000 μmol m⁻² s⁻¹ and the leaf temperature was maintained at 25 °C. The average water vapour mole fraction in the cuvette was 18 mmol/mol and leaf-to-air vapour-pressure difference around 15 mbar. When the stomatal opening was complete, the O₂ concentration was decreased to 1-2% to suppress photorespiration. A CO₂ response curve of steady-state CO₂ assimilation was then measured. The leaf was returned to 300 μbar CO₂ and subjected to rapid pulses of increasing CO₂ concentrations (Fig. 2.1) as described by Laisk (1985). Before each transient, a large leaf RuBP pool was generated by decreasing the incoming CO₂ partial pressure to zero for 15-20 s. After these measurements, dark transients were performed in similar conditions to measure CO₂ solubilisation. When leaf is subjected to large changes in CO₂ concentrations, either CO₂ uptake or outburst is observed as a result of equilibration between atmospheric CO₂ and CO₂ which is dissolved in the aqueous compartments (Fig. 2.1) (Laisk 1985). The pulses measured in dark were then subtracted from light pulses, and peak rates of CO₂ assimilation were measured 2-3 s after the leaf was transferred to the new CO₂ concentration from zero CO₂. For comparison, CO₂ uptake due to solubilisation was largely complete within 1-1.5 s after the transfer, so possible errors arising from varying stomatal conductances or different CO₂ solubilities in light and dark do not contribute greatly to the estimation of the maximal CO₂-assimilation rates (Fig. 2.1).

2.2.2.3 Post-illumination CO₂ uptake

The post-illumination CO₂ uptake of the leaves was measured with two different techniques. Either the post-illumination CO₂ uptake was measured always at 300 μbar CO₂ (method A, results in Fig. 2.4) or it was measured at the CO₂ concentration present in the preceding light period (method B, results in Figs 2.5 and
Fig. 2.1. The recorded CO$_2$ exchange rates during a typical measurement of transient assimilation rate in a wild-type tobacco. The CO$_2$ assimilation was brought to steady state at 300 µbar CO$_2$, 1% O$_2$ at PFD of 1000 µmol m$^{-2}$ s$^{-1}$. The CO$_2$ partial pressure was then decreased to zero for 15-20 s, and the leaf was rapidly transferred to the other gas channel with 1000 µbar CO$_2$ at time zero (dashed line). The transfer from zero to 1000 µbar CO$_2$ was repeated in darkness to estimate the CO$_2$ uptake due merely to solubilisation in the leaf (dotted line). The dark transient was subtracted from the light transient to measure the momentarily maximal rate of CO$_2$ assimilation (solid line).

When the two techniques were compared, the difference between the total post-illumination CO$_2$ uptake was in the order of 10%. All of the measurements were conducted at low O$_2$ (1-2%), after pre-equilibration at a PFD of 1000 µmol m$^{-2}$ s$^{-1}$ and the leaf temperature of 25 °C. An illustration of the post-illumination CO$_2$ uptake measurements is presented in Fig. 2.2.

**Method A:** CO$_2$ assimilation was allowed to reach a steady state at each CO$_2$ concentration. Then the light was switched off, the leaf was simultaneously transferred to 300 µbar CO$_2$, and the post-illumination CO$_2$ uptake was followed over
the next minute. Then the leaf was transferred to the previous CO₂ concentration, and the transient was repeated in darkness. This dark transient was measured to distinguish the CO₂ solubilisation uptake or outburst which occurs when the leaf is transferred between different CO₂ concentrations from the actual post-illumination CO₂ uptake (see also Fig. 2.1). The dark transient was then subtracted from the actual post-illumination CO₂ uptake.

**Method B** During measurements the CO₂ concentration was kept the same in the two gas exchange systems (“channels”). The leaf was transferred to the other gas exchange channel and darkened simultaneously. This was done to easily determine the accurate time when the light was turned off, since the hypothetical response curve of the gas exchange apparatus can vary between measurements (Laisk et al. 1984).

**2.2.2.4 Calculation of the post-illumination CO₂ uptake.**

When measured, the dark transients (CO₂ solubilisation or outburst, method A) were first subtracted from the post-illumination CO₂ uptake. Unlike Laisk et al. (1984), we assumed that the dark respiration was constant during the post-illumination measurement period and used the respiration rate recorded at 40-60 s after the light was switched off. The total post-illumination CO₂ uptake was then calculated as the integral between the actual CO₂ uptake and estimated respiration (Fig. 2.2). This method is likely to give us the maximal estimation of the total amount of CO₂ fixed during the dark period.
The light was turned off at time zero, and the CO₂ exchange in darkness was recorded. The rate of dark respiration was measured when the CO₂ evolution rate had stabilised after the leaf had been in darkness for about 1 min. The total post-illumination CO₂ uptake was calculated as the integral between the recorded CO₂ uptake and the rate of dark respiration (hatched area). In this case the leaf was kept in the same gas channel throughout the process.

2.2.2.5 Gas exchange measurements and freeze clamping of leaves

For comparison, the post-illumination CO₂ uptake and RuBP contents were measured on the same leaves. After the measurements of post-illumination CO₂ uptake, the leaf was stabilised in a different gas exchange chamber attached to a rapid-kill apparatus (Badger et al. 1984). After CO₂ assimilation rates had reached steady state, leaves were rapidly freeze clamped in situ. Gas exchange was measured with a portable open gas exchange system (LI-6400, Li-Cor) and conditions were the same as for the rapid-response gas exchange measurements. Rubisco activity and carbamylation level and metabolite pools (RuBP, 3-PGA) were measured from the leaf discs stored at -80 °C.
2.2.3 BIOCHEMICAL ASSAYS

2.2.3.1 Measurements of Rubisco carbamylation level and initial activity

Half of the freeze-clamped leaf disc (2.7 cm²) was homogenised in 1.4 ml of ice-cold CO₂-free extraction buffer containing 50 mM Heps-NaOH pH 7.8, 1 mM Na-EDTA, 5 mM MgCl₂, 10 mM DTT, 1 % (w/v) of polyvinylpolypyrrolidone and 1 mM phenylmethylsulfonylfluoride (PMSF). The extract was centrifuged for 15-20 s with a benchtop rotor and the supernatant was used for the assays immediately.

Rubisco initial activity was measured at 25 °C by adding a 10 μl aliquot of the supernatant into 262 μl of the reaction mixture containing 55 mM EPPS-KOH pH 8, 22 mM MgCl₂, 0.27 mM Na-EDTA, 13 mM NaH¹⁴CO₃ (specific radioactivity about 1500 cpm/nmol) and 0.4 mM RuBP. The reaction was terminated by adding 125 μl of formic acid after 60 s, samples were dried, and acid-stable ¹⁴C was measured by liquid scintillation.

Rubisco catalytic site concentration was determined by the stoichiometric binding of [¹⁴C]CPBP and the carbamylation level was measured by exchanging loosely bound [¹⁴C]CPBP at noncarbamylated sites with an excess of [¹²C]CPBP (Butz and Sharkey 1989). Total number of Rubisco sites were measured by incubating 100 μl of the supernatant with 29 μM [¹⁴C]CPBP (prepared as described by Collatz (1979), specific radioactivity about 17000 cpm/nmol), the total MgCl₂ concentration was increased to 20 mM and 15 mM NaHCO₃ was added. The reaction mixture was kept at room temperature for 45 min and then transferred to ice. The amount of initially carbamylated sites was measured by incubating a 100 μl aliquot of the supernatant with the similar 29 μM [¹⁴C]CPBP on ice for 30-45 min. Then [¹²C]CPBP was added to the final concentration of 1.5 mM, the mixture incubated at room temperature for 10-15 min and replaced on ice. Rubisco-CPBP-complexes were separated from unbound CPBP by gel filtration. The samples were applied to a Sephadex G-50 Fine columns (27 x 0.7 cm) equilibrated with 20 mM EPPS-NaOH pH 8, 75 mM NaCl₂. Samples were eluted with the same buffer with a flow rate of approximately 0.5 ml min⁻¹. Fractions containing Rubisco-CPBP
complexes were collected and the amount of \(^{14}\text{C}\)CPBP was determined by liquid scintillation.

2.2.3.2 GAPDH activity

Total GAPDH activity was determined a modification of a method described by Stitt \textit{et al} (1989). Leaf discs (1.6 cm\(^2\)) were extracted in 300 \(\mu\)l of ice cold extraction buffer containing 50 mM Hepes-KOH pH 7.4, 5 mM MgCl\(_2\), 2 mM EDTA, 5 mM DTT, 2 mM benzamidine, 2 mM \(\varepsilon\)-amino-n-caproic acid, 0.1% (v/v) Triton X-100 and 0.5 mM PMSF. Extracts were centrifuged at 4 °C for 2 min and 20-50 \(\mu\)l of supernatant was incubated at 25 °C for 10 min in the assay mixture (total volume 1.1 ml) containing 100 mM Hepes-KOH pH 8, 30 mM MgCl\(_2\), 20 mM KCl, 2 mM Na-EDTA, 4.5 mM DTT, 5.5 mM ATP, 0.2 mM NADPH and 60 U of phosphoglycerate kinase. The reaction was initiated by adding 5.5 mM 3-PGA. GAPDH activity was measured by following the initial rate of NADPH consumption.

2.2.3.3 Metabolite measurements

Half of the freeze-clamped leaf disc (2.7 cm\(^2\)) was ground in 200 \(\mu\)l of 5% (v/v) trifluoroacetic acid. Samples were processed and the contents of RuBP and 3-PGA were assayed by a spectrophotometric method (He \textit{et al}. 1997).

2.3 RESULTS

2.3.1 STEADY-STATE AND TRANSIENT CO\(_2\) ASSIMILATION RATES

I compared steady-state CO\(_2\) assimilation rates with transient rates measured at high RuBP-content/RuBP saturation at several CO\(_2\) concentrations. The rapid-response gas exchange system was used to generate large RuBP pools as described in Material and Methods-section. In Fig. 2.3 the steady-state and transient CO\(_2\) assimilation rates are shown as a function of \(C_i\). In wild-type tobacco the transient CO\(_2\) assimilation rates clearly exceed the maximum steady-state rates at high \(C_i\) (Fig. 2.3 A). The anti-Rubisco plants had overall lower rates of CO\(_2\) assimilation and there
was no difference between the transient and steady state rates at any $C_i$ (Fig. 2.3 B). Also shown are two different anti-GAPDH plants with 10\% (anti-GAPDH 1) and 2.5\% (anti-GAPDH 2) of GAPDH activity of the wild-type plants (Figs 2.3 C and D). At high $C_i$ both anti-GAPDH plants had higher transient CO$_2$ assimilation rates than steady-state rates. In addition, the difference between the steady-state and transient assimilation rates depended on the degree of the reduction in the GAPDH activity. Anti-GAPDH 1, which had only moderately reduced rates of CO$_2$ assimilation, more closely resembled the wild-type plant. Anti-GAPDH 2, with severely reduced rates of CO$_2$ assimilation, showed a greater differences between transient and steady-state rates. Furthermore, in this plant, the difference between transient and steady state rates was apparent at lower $C_i$.

![Graph showing CO$_2$ assimilation rates at different intercellular CO$_2$ partial pressures, $C_i$.](image)

**Fig. 2.3.** Comparison between steady-state (solid symbols) and transient (open symbols) rates of CO$_2$ assimilation at different intercellular CO$_2$ partial pressures, $C_i$. A. in wild-type tobacco, B. in two transgenic tobaccos with reduced amounts of Rubisco and C & D: in two transgenic tobaccos with reduced amounts of GAPDH. Measurements were made at PFD of 1000 $\mu$mol m$^{-2}$ s$^{-1}$, leaf temperature of 25 $^\circ$C and O$_2$ concentration of 1-2\%. The transient values were obtained after generating a large pool of RuBP by keeping the leaf at zero CO$_2$ for a few seconds, and then transferring it rapidly to each $C_i$. The dotted lines were fitted to the assimilation rates with the Michaelis-Menten equation: $(A+R_d)=(V_{\text{max}} \cdot C_i)/(K_m+ C_i)$.
The Michaelis-Menten equation was fitted to the steady-state rates at low CO₂ and all transient CO₂ assimilation rates to estimate the maximum carboxylation rate of Rubisco (V_{cmax}) (Table 2.1). The calculated V_{cmax} of the anti-Rubisco plants was 41% and 9% of wild-type, which is consistent with the previous measurements of Rubisco contents in these plants (Hudson et al. 1992). Even though steady-state CO₂ assimilation rates were decreased in anti-GAPDH plants, the calculated V_{cmax} was similar to wild-type value in both plants. The steady-state, RuBP limited CO₂ assimilation rates at high Cᵢ and low O₂ were impaired by reduction in GAPDH activity (Fig. 2.3 C&D). In wild-type plants the ratio between gross steady-state assimilation rate at high Cᵢ and V_{cmax} was 0.395, and in anti-GAPDH plants this ratio decreased as a result of the limitation to the capacity for RuBP regeneration by reduced amount of GAPDH activity (Price et al. 1995a).
Table 2.1. Photosynthetic characteristics of the wild-type and transgenic tobacco with an antisense gene against the small subunit of Rubisco (anti-SSu) or an antisense gene against GAPDH (anti-GAPDH). The maximal carboxylation rate of Rubisco ($V_{cmax}$) was estimated by fitting a Michalis-Menten equation through the transient assimilation values, and steady-state values measured at low $C_i$ (below 300 μbar CO$_2$) as presented in Fig. 2.1. Assimilation rates were measured at an irradiance of 1000 μmol quanta m$^{-2}$ s$^{-1}$, leaf temperature of +25 °C, and O$_2$ concentration of 1.5-2%. The steady-state gross CO$_2$ assimilation rate was calculated as $A+R_d$, where $R_d$ is the rate of dark respiration.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type (n=2)</th>
<th>Anti-SSu 1 (n=2)</th>
<th>Anti-SSu 2 (n=2)</th>
<th>Anti-GAPDH 1 (n=1)</th>
<th>Anti-GAPDH 2 (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assimilation rate at 300 μbar CO$_2$, 2% O$_2$ (μmol m$^{-2}$ s$^{-1}$)</td>
<td>17.0 (Ci=144 μbar)</td>
<td>8.1 (Ci=177 μbar)</td>
<td>1.9 (Ci=267 μbar)</td>
<td>13.5 (Ci=174 μbar)</td>
<td>4.78 (Ci=234 μbar)</td>
</tr>
<tr>
<td>Assimilation rate at high $C_i$ and 2% O$_2$ (μmol m$^{-2}$ s$^{-1}$)$^a$</td>
<td>27.7 ± 4.7</td>
<td>20.2 ± 3.0</td>
<td>3.48 ± 0.43</td>
<td>17.8 ± 1.08</td>
<td>5.18 ± 0.24</td>
</tr>
<tr>
<td>Estimated $V_{cmax}$ (μmol m$^{-2}$ s$^{-1}$)$^b$</td>
<td>72.5 ± 3.7</td>
<td>29.5 ± 1.8</td>
<td>6.35 ± 0.54</td>
<td>64.2 ± 2.5</td>
<td>89.2 ± 11.0</td>
</tr>
<tr>
<td>Steady-state gross CO$<em>2$ assimilation at high $C_i$ / estimated $V</em>{cmax}$</td>
<td>0.395</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.290</td>
<td>0.0636</td>
</tr>
</tbody>
</table>

$^a$ Average of assimilation rates measured between $C_i$ of 500 and 1500 μbar.

$^b$ $V_{cmax}$ was estimated using hyperbolic fit through an average of 20 (wild-type and anti-SSu plants) or 10 (anti-GAPDH plants) data points.
2.3.2 POST-ILLUMINATION CO₂ UPTAKE AT DIFFERENT CO₂ CONCENTRATIONS

It has been concluded that, in C₃ plants at low O₂, the observed post-illumination CO₂ uptake is mainly supported by RuBP that has accumulated in light (Miyachi 1979; Laisk et al. 1984). In addition, ATP and triose phosphates have been shown to participate the post-illumination CO₂ uptake as well (Sharkey et al. 1986b).

We were interested whether this gas-exchange technique could be used as a non-destructive means of determining RuBP contents of tobacco leaves. The post-illumination CO₂ uptake in wild-type and anti-Rubisco plants was measured at several CO₂ concentrations. In these experiments, 2-3 wild-type or anti-SSu plants from both genotypes were used, and one representative data set for each type is presented in Fig. 2.4.

In wild-type plants the integrated total post-illumination CO₂ uptake was highest at low Cᵢ (Fig. 2.4 B) and declined with increasing CO₂ concentration. The average (±S.E.) post-illumination CO₂ uptake at low Cᵢ (below 200 μbar) in wild-type plants was 148±14 μmol m⁻² but at the highest Cᵢ (around 1000 μbar) it decreased to an average of 59±16 μmol m⁻². In both antisense plants the post-illumination CO₂ uptake values were clearly smaller at low Cᵢ (below 300 μbar): the averages were 87±9 and 37±5 μmol m⁻² for anti-SSu 1 and anti-SSu 2 plants, respectively. When Cᵢ increased, the post-illumination CO₂ uptake decreased more gradually in the anti-SSu 1 plants than in the controls. At all CO₂ concentrations, the anti-SSu 2 plants had the lowest post-illumination CO₂ uptake, and it was independent of Cᵢ.

2.3.3 DARK RESPIRATION AT DIFFERENT CO₂ CONCENTRATIONS

The effect of atmospheric CO₂ on plant dark respiration has been widely studied, and it has been shown that, in many species, the respiration rate decreases when plants are subjected to either short or long-term increase in atmospheric CO₂, see Amthor (1991) for review of the topic. The leaf dark respiration rate at different CO₂ concentrations were measured, in connection with the post-illumination CO₂ uptake measurements. The respiration rate was determined after approximately 1 min in darkness. The wild-type plant had, on an average, a higher respiration rate than the
antisense plants, and the respiration rate was lower when Rubisco content was lower but there was no clear effect of CO₂ on dark respiration in any of the plant types (Fig. 2.4 C). The reason for the low respiration rates in antisense plants may be that they have accumulated less carbohydrates in their leaves due to suppressed CO₂ assimilation rates (Farrar 1985).

![Graph showing CO₂ assimilation rate, post-illumination CO₂ uptake and dark respiration rates in wild-type and anti-SSu tobacco plants.]

**Fig. 2.4.** CO₂ assimilation rate, post-illumination CO₂ uptake and dark respiration rates in wild-type and anti-SSu tobacco plants. A. Steady-state rates of CO₂ assimilation were measured at PFD of 1000 μmol m⁻² s⁻¹, leaf temperature of 25 °C, and O₂ concentration of 1-2%. B. After CO₂ assimilation rates had stabilised at each Ci light was switched off, the leaf was transferred simultaneously to 300 μbar CO₂ and the CO₂ exchange was followed in darkness for about 1 min. To account for CO₂ solubilisation or release resulting from CO₂ transfer the leaf was transferred back to the original CO₂ concentration, and the transfer to 300 μbar CO₂ was repeated in the dark. The resulting CO₂ solubilisation or outburst was subtracted from the postillumination trace. We assumed that the dark respiration was constant during the measurement period and the total post-illumination CO₂ uptake was calculated by integrating the area under the curve (see Materials and methods for further details). C. The dark respiration values were obtained from the dark transients for the postillumination CO₂ uptake determinations after the leaf had equilibrated in darkness for 1-2 min.
2.3.4 COMPARISON BETWEEN THE POST-ILLUMINATION CO₂ UPTAKE 
AND RuBP CONTENT

To date, there have been only a few direct comparisons between the post-illumination CO₂ uptake and leaf RuBP content, for example see Sharkey et al. (1986b). We measured both on the same leaves under similar conditions. Leaves were brought to steady-state at 160, 300 or 1000 μbar CO₂ at low O₂ to generate a range of RuBP contents, and either the post-illumination CO₂ uptake was measured or a leaf disc was rapidly freeze-clamped for metabolite assays. In Fig. 2.5 the post-illumination CO₂ uptake is plotted against RuBP content. While there was generally a good agreement between the two in wild-type plants, the post-illumination CO₂ uptake underestimated the actual RuBP content in the transgenic plants. On an average (±S.E.), the post-illumination CO₂ uptake was 75±4% of the RuBP pool in the anti-SSu 1 plants and only 51±6% in anti-SSu 2 plants, as compared to 103±7% in the control plants.

![Fig. 2.5. The comparison between the post-illumination CO₂ uptake and RuBP pools. Both were measured in the leaves of wild-type tobacco (●), anti-SSu 1 (□) and anti-SSu 2 plant (△) at different CO₂ concentrations. The anti-SSu 2 plant was grown in a growth cabinet at elevated CO₂ (1500 μbar) with a 14-h photoperiod (PFD about 400 μmol m⁻² s⁻¹). Measurements were conducted at PFD of 1000 μmol m⁻² s⁻¹, leaf temperature of 25 °C, O₂ concentration of 1-2% and different CO₂ concentrations. The post-illumination CO₂ uptake was measured at the same CO₂ concentration as the leaf had been in the light. RuBP pools were measured after acid extraction. The lines are not a fit to the data.](image-url)
2.3.5 TIME COURSES OF POST-ILLUMINATION CO₂ UPTAKE, RuBP AND 3-PGA CONTENT IN DARKNESS

From the previous results it seems that the discrepancy between the post-illumination CO₂ uptake and RuBP pools is larger in the antisense plants with reduced Rubisco contents, than in control plants. To examine this closer, the post-illumination CO₂ uptake was measured in a wild-type and an anti-SSu 1 plant at 300 μbar CO₂. The same leaves were then used for rapid freeze-clamping, where leaf discs were sampled under similar conditions directly from light, or 10 and 25 s after the light was turned off. The amounts of 3-PGA and RuBP as well as Rubisco initial activity and carbamylation level were assayed from the leaf discs.

The gas-exchange traces from the post-illumination CO₂ uptake are presented in Fig. 2.6 A. The arrows indicate the time points when either the remaining post-illumination CO₂ uptake was calculated or the biochemical parameters were measured. Although the initial dark CO₂ uptake rate was lower in the anti-SSu 1 plant than in wild-type, the shape of the CO₂ exchange curve was similar in both plants. The CO₂ uptake rate started to decline immediately after the light was switched off and thus the majority of the post-illumination CO₂ uptake took place during the first 20 s. In Figs 2.6 B&C we compare the time courses of the remaining post-illumination CO₂ uptake and RuBP pools. Again, the agreement between the two was good in control plant, and both post-illumination CO₂ uptake and RuBP pools declined concurrently in darkness. In anti-SSu 1 plant the situation was different (Fig. 2.6 C): the total post-illumination CO₂ uptake was about 20 % smaller than the RuBP content of the leaf. After 10 s in darkness, the CO₂ fixation rate was declining rapidly in the anti-SSu plant, as well as in the wild-type plant (Fig. 2.6 A); however, there was still a considerable amount of RuBP in the chloroplasts of anti-SSu plant at that time (Fig. 2.6 C). Even after 25 s in darkness, there was still 18 μmol RuBP m⁻² (twice the Rubisco site concentration) left in anti-SSu 1 plant, but the post-illumination CO₂ uptake had ceased.

The consumption of RuBP in darkness resulted in accumulation of 3-PGA in the leaves. The amount of 3-PGA increased to 250-280 μmol m⁻² after 10 s in darkness, so the 3-PGA/RuBP ratio increased about 35-fold in the control plant and
Fig. 2.6. The time courses of the CO₂ assimilation rate, post-illumination CO₂ uptake, RuBP and 3-PGA pools in the leaves of a wild-type and anti-SSu1. The post-illumination CO₂ uptake was measured as in Fig. 2.3. Measurements were made at PFD of 1000 μmol m⁻² s⁻¹, leaf temperature of 25 °C, CO₂ concentration of 300 μbar and O₂ concentration of 1-2%. 

A. The gas-exchange traces. The arrows show the time points when either the remaining post-illumination CO₂ uptake or metabolite pools were measured. B. and C. The dotted line show the Rubisco site concentration for each plant. Leaf discs for the RuBP and 3-PGA assays were sampled using the rapid-kill apparatus and assayed by spectrophotometric method. D. The total pool of esterified phosphates was calculated as 2 × RuBP + 3-PGA.
about 12-fold in the anti-SSu 1 plant (Fig. 2.6 D). Although the ratio between RuBP and 3-PGA changed dramatically during the dark period, the total amount of esterified phosphates (calculated as 2×RuBP + 3-PGA) remained approximately constant (Fig. 2.6 D).

The initial Rubisco activity and carbamylation level during the 25 seconds in darkness were also measured. The carbamylation level did not decrease in either of the plant types (Fig. 2.7 A), and the initial activity expressed on total Rubisco site basis remained constant as well (Fig. 2.7 B).

![Graph](attachment:image.png)

**Fig. 2.7.** The time course of Rubisco carbamylation level and initial activity. The Rubisco activity is expressed on a site basis. The measurements were made on the other half of the leaf discs collected for metabolite assays presented in Fig. 2.6.
2.3.6 POST-ILLUMINATION CO₂ UPTAKE AS A FUNCTION OF RuBP AND 3-PGA CONCENTRATIONS

It has been suggested that the conditions during the post-illumination CO₂ uptake measurement can inhibit the activity of Rubisco (Laisk et al. 1984), since the concentration of RuBP decreases and that of 3-PGA increases simultaneously. 3-PGA can compete with RuBP for Rubisco active sites. This causes an increase in the apparent Michaelis-Menten constant for RuBP (Kₐ), thus decreasing the Rubisco activity (Prinsley et al. 1986; Foyer et al. 1987; Laisk et al. 1987). We modelled the effects of RuBP and 3-PGA contents on the Kₐ, and consequently, the Rubisco carboxylation rate at different RuBP concentrations (Fig. 2.8) (von Caemmerer and Edmondson 1986). The predicted relationship between carboxylation rate and RuBP and 3-PGA contents was calculated in two different ways. Firstly, 3-PGA was assumed to be constant, 600 µmol m⁻², which is approximately twice the measured concentration of 3-PGA during the post-illumination CO₂ uptake (Fig. 2.6 D, detailed data not shown). Secondly, a dynamic response curve was created, where RuBP decreases and, simultaneously, 3-PGA increases. For the dynamic model, the measured RuBP and 3-PGA concentrations and post-illumination gas-exchange data of the wild-type tobacco were used (in light it had 107 µmol m⁻² RuBP and 89 µmol m⁻² 3-PGA). From the measured post-illumination CO₂ uptake curve (Fig. 2.6 A) it was calculated how much RuBP is consumed and 3-PGA produced at 1 s intervals, and these values were used for the model calculations.

In addition to the modelled responses of Rubisco carboxylation rate to the RuBP and 3-PGA contents, the actual CO₂ exchange rates are plotted in the Fig. 2.8 as a function of decreasing RuBP contents in both wild-type and anti-SSu plant. The decrease in RuBP contents are similarly calculated from the gas exchange data and measured RuBP pools (Figs 2.6 A-C) in 1 s intervals. The measured gross post-illumination CO₂ uptake values are normalised against the maximal CO₂ uptake rate early in the post-illumination period. The observed decline in Rubisco carboxylation rate in both plants is greater than either of the models predicts.
The modelled response of Rubisco carboxylation rate to 3-PGA and RuBP concentrations and measured post-illumination CO₂ uptake rates as a function of RuBP content. The effect of RuBP and 3-PGA contents on Rubisco activity was modeled in two ways. Firstly, we assumed that 3-PGA concentration was constant (600 μmol m⁻²) while RuBP concentration decreased (solid line). Secondly, we assumed that RuBP decreased from 107 μmol m⁻² to zero, while 3-PGA concentration increased from 89 to 303 μmol m⁻² (dashed line), (Fig. 2.6 A). The CO₂ assimilation rates, normalized against the maximal rate at the beginning of the dark period, are plotted as a function of the calculated RuBP content for wild-type (•) and anti-SSu (□) plant. The data points are calculated at 1 s intervals.

Model calculations

The effective Michaelis-Menten constant for RuBP (Kₑ) was calculated as

$$Kₑ = K_r(1+P)/K_p,$$

where P is 3-PGA concentration (mM), K_r is the K_m for RuBP (0.02 mM) and K_p is dissociation constant for the 3-PGA-active site complex (0.9 mM). The fractional activity of Rubisco ($V_c/W_c$) was then calculated from
\[ \frac{V_c}{W_c} = \frac{(E_t + R_t + K_r) - \sqrt{(E_t + R_t + K_r)^2 - 4E_t R_t}}{2E_t} \]

where \( E_t \) is total concentration of Rubisco sites (20 \( \mu \)mol m\(^{-2} \)), \( K_r \) the effective Michaelis-Menten constant for RuBP, and \( R_t \) the total concentration of RuBP (von Caemmerer and Edmondson 1986). The metabolite pools were converted to stromal concentrations by assuming that 10 \( \mu \)mol m\(^{-2} \) equals to 1 mM. In addition to the modelled responses of carboxylation rates, we calculated the decrease in RuBP content during the dark period, based on the gas exchange data shown in Fig. 2.6 A and RuBP content at the beginning of the post-illumination CO\(_2\) uptake (Fig. 2.6 B).

2.4 DISCUSSION

2.4.1 LIMITATION OF CO\(_2\) ASSIMILATION VIA RUBISCO VERSUS RuBP REGENERATION

Using the rapid-response gas exchange equipment, it is possible to create momentary RuBP-saturation of Rubisco also at high CO\(_2\) partial pressures. By comparing these transient CO\(_2\) assimilation rates with steady-state rates at several C\(_i\), it is possible to distinguish where the CO\(_2\) assimilation rate is limited by Rubisco carboxylation capacity or RuBP regeneration capacity. If the steady-state assimilation rate can be enhanced by pre-treating the leaf such that large, saturating RuBP pools are accumulated in the chloroplasts (ie. keeping them in very low CO\(_2\) for short while), then the CO\(_2\) assimilation is RuBP limited at this particular C\(_i\).

In wild-type tobaccos the transient (RuBP saturated) CO\(_2\) assimilation rates exceeded steady-state rates when C\(_i\) was above 300 \( \mu \)bar, indicating the transfer to RuBP-limitation of CO\(_2\) assimilation above this C\(_i\). Transient assimilation rates up to 2.5 times the maximum steady-state rate could be measured under the high-light conditions (Fig. 2.3 A). This demonstrates once more that the CO\(_2\) assimilation rates at high CO\(_2\) partial pressures and high light are limited by the supply of RuBP (Farquhar \textit{et al.} 1980; von Caemmerer and Farquhar 1981). In both hemi- and
homozygous anti-SSu plants there was no difference between transient and steady-state rates, confirming that Rubisco is RuBP-saturated even at high Cᵢ (Fig. 2.3 B). In plants with reduced activity of chloroplast GAPDH enzyme the RuBP regeneration capacity is decreased and as a consequence RuBP pools decrease dramatically, as shown by Price et al. (1995a). However, it was possible to build up substantial RuBP pools in these plants to induce high transient CO₂ assimilation rates. This was checked by measuring the post-illumination uptake in anti-GAPDH plants after 20 s low CO₂-treatment, (data not shown), and it was found to be close to the maximal post-illumination CO₂ uptake values in wild-type plants at low CO₂ (Fig. 2.4 B). The shifted balance between Rubisco and RuBP regeneration is demonstrated in Figs 2.3 C&D: anti-GAPDH 2-plant with severely reduced GAPDH-activity and steady-state CO₂ assimilation rate expressed the difference between transient and steady-state values at clearly lower Cᵢ (about 100 μbar) than anti-GAPDH 1 which was almost wild-type -like. The data support the binary model of Farquhar et al. (1980), which states that CO₂ assimilation can be described in terms of either Rubisco limitation or RuBP-regeneration limitation, and demonstrates that the balance between the two limiting factors can be changed by genetic manipulation of photosynthetic machinery.

Since the transient CO₂ assimilation values represent RuBP-saturation at each Cᵢ, they can be used to estimate Rubisco’s maximum capacity (Vᶜₘᵝₓ) (Laik 1985) (Table 2.1). The Vᶜₘᵝₓ in anti-SSu plants decreased in relation to wild-type, as the Rubisco content reduced. The fact that the Vᶜₘᵝₓ in anti-GAPDH plants was very similar to wild-type confirms the in vitro findings of Price et al. (1995a) that, although the rate of CO₂ assimilation is reduced in these plants, Rubisco is fully active. In addition the high Vᶜₘᵝₓ demonstrates that, although RuBP regeneration rate in anti-GAPDH plants is reduced, the low CO₂ treatment before transients was sufficient to generate high enough RuBP pools to momentarily saturate Rubisco.

Photosynthesis modellers are interested in measuring the two parameters that determine the photosynthetic capacity of C₃ plants, Vᶜₘᵝₓ (Rubisco’s maximum activity) and Jₘᵝₓ (the maximum rate of electron transport). Both can be calculated from steady-state photosynthesis measurements, and lately Wullschleger (1993) and Leuning (1997) have studied these parameters extensively. The steady-state electron transport rate (J) can be calculated from steady-state CO₂-assimilation rate (A) at high
C, and low O₂ as 4×(A+Rd), where Rd is the rate of dark respiration. V_{cmax} can be calculated from the transient CO₂ assimilation values (Table 2.1). Using values from Table 2.1, the average J/V_{cmax} ratio in wild-type tobacco is then 1.53 and decreases in anti-GAPDH plants.

2.4.2 POST-ILLUMINATION CO₂ UPTAKE UNDERESTIMATES RuBP-POOLS IN PLANTS WITH REDUCED RUBISCO CONTENTS.

The response of post-illumination CO₂ uptake to changing CO₂ in wild-type tobaccos was similar to reported CO₂ dependent changes in RuBP pools in C₃ plants at low O₂ (Badger et al. 1984; von Caemmerer and Edmondson 1986). At low and rate-limiting C, the post-illumination CO₂ uptake was high and decreased with increasing CO₂ (Fig. 2.4 B) in the same manner as also found by Laisk et al. (1984). CO₂ had less effect on the post-illumination CO₂ uptake in anti-SSu plants, which agrees with the notion that CO₂ assimilation in these plants is always RuBP-saturated. Altogether, the post-illumination CO₂ uptake in anti-SSu 1 plants was clearly lower than in control plants at low C, (below 300 μbar) (Fig. 2.4 B) and the anti-SSu 2 plants had the lowest values at all CO₂ concentrations. This raises the suspicion that the post-illumination CO₂ uptake does not directly reflect the RuBP content on cessation of illumination because Quick et al (1991) and Mate et al. (1996) showed that plants with reduced amounts of Rubisco had similar or higher RuBP-pools when compared to wild-type plants. The direct comparison between post-illumination CO₂ uptake and biochemically measured RuBP pools (Fig. 2.5) revealed that there was generally a good correlation between the two in wild-type plants, but the post-illumination CO₂ uptake underestimated the RuBP pools in anti-SSu plants. The discrepancy between the post-illumination CO₂ uptake and RuBP pools increased as the amount of Rubisco decreased in the leaves. In addition, the highest RuBP pools were found from the anti-SSu 2 plant. This indicates that there is no drastic decrease in the activity of the RuBP regeneration pathway in anti-SSu plants, as also shown by Hudson et al. (1992) who measured in vitro uncoupled electron transport rates and phosphoribulokinase activities in anti-SSu leaves which were comparable to wild-type rates.
Some part of the discrepancy between total post-illumination CO$_2$ uptake and RuBP content in anti-SSu plants might be attributed to uncertainty about the level of respiration in the early post-illumination period. The post-illumination CO$_2$ uptake continued for a more protracted period in the anti-SSu plants (Fig. 2.6 A) as a result of the lower Rubisco content. The slow tapering off of CO$_2$ uptake when Rubisco content was low made estimation of true respiration problematical and the lower apparent respiration rates in the anti-SSu plants (Fig. 2.4 C ) may be a reflection of this. Any underestimate of respiration will reduce the estimated post-illumination CO$_2$ uptake. For example, if the true respiration rate of the anti-SSu 2 plant was greater than that estimated for it (Fig. 2.2 C ) and was actually the same as that measured for the wild-type plants, then the ratio between post-illumination CO$_2$ uptake and RuBP content in these plants would rise from 0.51, as shown in Fig. 2.5, to 0.75. In this calculation, it was assumed that the post-illumination CO$_2$ uptake process continued for 2 min in the anti-SSu 2 plants.

2.4.3 THE APPARENT DEACTIVATION OF RUBISCO IN DARKNESS

The post-illumination gas-exchange traces (Fig. 2.6 A) show that CO$_2$ fixation started to decline immediately after the light was switched off in both plant types. This feature of the post-illumination CO$_2$ uptake process has been observed in several different species (Laisk 1985, Laisk et al. 1984; 1987; Ruuska et al. 1994). Knowing that the initial RuBP-pools exceeded the Rubisco active site concentration 6-fold in wild-type (Fig. 2.6 B) and 13-fold in anti-SSu 1 plant (Fig. 2.6 C), the post-illumination CO$_2$ uptake might be expected to persist undiminished for some time in darkness. However, even in the anti-SSu plant, the post-illumination CO$_2$ uptake was rapidly declining already after 10 s in darkness, despite the large remaining RuBP pool, which was almost 7 times the Rubisco site concentration (Fig. 2.6 C). From these data it can be concluded that the post-illumination CO$_2$ uptake underestimates RuBP pools in anti-SSu plants partially because Rubisco inactivates in darkness within 25 s. In wild-type plants, this period is sufficient for Rubisco to consume most of the RuBP, whereas a residual pool remains in anti-SSu plants or is metabolised subsequently quite slowly.
Rapid extraction of Rubisco from the leaf samples did not reveal any reasons for the inactivation. The carbamylation level did not decrease in either of the plants during the brief darkening (Fig. 2.7 A). This agrees with the previous work demonstrating that Rubisco requires several minutes to decarbamylate in darkness or at low light (Perchorowitz et al. 1981; Portis et al. 1986; Prinsley et al. 1986). The initial activity of Rubisco, expressed on a total Rubisco site basis, also remained high and constant during the whole post-illumination CO₂ uptake period (Fig. 2.7 B). The measured high initial activities suggest that there are no tightly binding inhibitors in the active sites, that could slow down the turnover rate of Rubisco in vivo.

2.4.4 POSSIBLE REASONS FOR THE RAPID DEACTIVATION OF RUBISCO IN VIVO

2.4.4.1 A drop in stromal pH

A sudden decrease in stromal pH could cause Rubisco to deactivate during the post-illumination CO₂ uptake. It has been estimated that the pH of the stroma in illuminated chloroplasts can approach 8 (Wu and Berkowitz 1992) but, when light is turned off, protons accumulated in the thylakoid lumen leak out immediately (Yamasaki and Nishimura 1988). In addition, the carboxylation of one RuBP molecule produces two 3-PGA molecules and two protons. The protons cannot be used in subsequent carbon reduction cycle reactions in darkness, because the supply of ATP and NADPH from the light reactions has ceased. Thus the consumption of RuBP during the post-illumination CO₂ uptake would exacerbate the drop in stromal pH. If we assume that there is approximately 10 mM RuBP in chloroplasts, the acid load from the carboxylation of all this pool alone would be 20 mM. It has been estimated from CO₂ solubilisation measurements that stromal buffering capacity is between 20 and 30 mM H⁺ per pH unit, and the capacity is higher in light than in dark (Hauser et al. 1995a; 1995b). In combination, these effects could cause the stromal pH to fall by 1 pH unit or more during post-illumination RuBP consumption, causing serious inhibition of Rubisco.
2.4.4.2 Inhibition by 3-PGA

Since 3-PGA can act as a competitive inhibitor in respect to RuBP, the changing RuBP/3-PGA ratio during the post-illumination CO₂ uptake could explain the apparent in vivo-deactivation of Rubisco. However, according the model originally presented by von Caemmerer and Edmonson (1986), the accumulation of 3-PGA during the post-illumination CO₂ uptake alone would not be sufficient to explain the observed inhibition of carboxylation (Fig. 2.8). Rather, the data resemble the results by Servaites and Geiger (1995) who showed that the decrease in Rubisco activity in response to increasing 3-PGA and Pᵢ and decreasing RuBP was almost linear, rather than curvilinear. Their interpretation was that "Rubisco shows negative co-operativity with respect to the binding of RuBP and related molecules". As a consequence, Rubisco would be able to respond rapidly to changes in RuBP/3-PGA ratio, which is shown to undergo large changes, for example in response to changing light (Badger et al. 1984; Servaites et al. 1989). Interestingly, there is some evidence that a small decrease in pH (0.2 pH units) can strengthen the inhibitory effect of 3-PGA on Rubisco activity considerably (Prinsley et al. 1986), which could also contribute to the lower observed Rubisco carboxylation rates than predicted from the effect of 3-PGA alone on Kᵣ (Fig. 2.8). In vitro, in the absence of 3-PGA, Rubisco has a broad pH optimum (Badger et al. 1974) but apparently 3-PGA can change this. It has been hypothesised that the different ionisation forms of 3-PGA have different inhibitory effects on Rubisco (Prinsley et al. 1986).

2.4.4.3 Inactivation of activase

A third possible explanation of the inability of Rubisco to carboxylate all RuBP in darkness is that Rubisco activase may inactivate rapidly when light is turned off. The activity of Rubisco activase is shown to be stimulated by ATP and inhibited by ADP in vitro, so presumably it is sensitive to stromal adenylate ratio (Robinson and Portis 1989a). It has been shown that the whole-leaf, as well as the chloroplast, ATP/ADP ratio falls instantly when the light is turned off (Hampp et al. 1982; Prinsley et al. 1986; Gilmore and Björkman 1994a), which could then affect activase. During the post-illumination CO₂ uptake, there is a rapid decrease in vivo-catalytic rate (Fig. 2.8) but no change in the extractable activity of Rubisco (Fig. 2.7 B).
Interestingly, this in vivo-deactivation of Rubisco, which vanishes upon extraction from the leaf, resembles the results obtained by He et al. (1997). They showed that the CO₂ assimilation rate per carbamylated Rubisco site decreased drastically in older leaves of plants with reduced amounts of Rubisco activase. However, the turnover rate of the carbamylated sites measured in vitro was only slightly lower than in wild-type plants. They suggested that, in vivo, activase has an effect on the rate of catalytic turnover of carbamylated sites, in addition to its role in promoting the carbamylation of Rubisco (Andrews et al. 1995a; He et al. 1997).

Singly or together, these factors could cause Rubisco activity to fall rapidly after darkening regardless of genotype. Nevertheless, in wild-type leaves, some Rubisco activity persisted long enough to carboxylate all of the RuBP present at the end of illumination. However, in the anti-SSu plants when the Rubisco content and activity was already low at the beginning of the dark period, dark-induced inactivation was complete before the RuBP had been consumed totally.

2.4.5 SUMMARY

It was shown that rapid CO₂ pulsing is a straightforward method for studying the balance between RuBP-regeneration limitation and Rubisco limitation of CO₂ assimilation in C₃ plants. In some cases, as in anti-GAPDH plants, the transition from Rubisco-limitation to RuBP regeneration limitation is difficult to determine from steady-state CO₂ assimilation rates alone. In addition, this approach provides a means to directly measure Rubisco's maximal carboxylation rate (V_{\text{max}}) as well as the rate of electron transport (J), thus enabling further comparative studies of the two parameters in different plant species, and their response to environmental factors such as temperature. It was also demonstrated that the post-illumination CO₂ uptake underestimates actual RuBP pools in tobacco plants with reduced amounts of Rubisco, apparently because Rubisco inactivates rapidly during the dark period, leaving some RuBP unconsumed. However, Rubisco carbamylation level or activity measured in vitro after rapid extraction did not decrease during the post-illumination CO₂ uptake. The changes in the chloroplast environment or lack of activase activity may be responsible for the rapid decline in Rubisco activity in vivo.
CHAPTER 3

A BIOLUMINESCENCE ASSAY FOR MEASURING PICOMOLAR AMOUNTS OF RuBP, 3-PGA, ATP AND ADP FROM TOBACCO LEAVES
3.1 INTRODUCTION

This Chapter describes the development of a firefly luciferase coupled assay for measuring picomolar amounts of ATP, ADP, 3-PGA and RuBP from tobacco leaf extracts. The luminometric approach was chosen to allow metabolite and adenylate, as well as enzymatic assays to be conducted from a single leaf disc sampled with a rapid freeze-clamping apparatus attached to a gas exchange measuring system (Badger et al. 1984). From the previous results it was known that the metabolite pools can be very low in the transgenic tobacco lines used in the experiments, and the detection limit of photometric assays would become a problem. The assay procedure was based on the method published by Lilley et al. (1985).

An improved acid extraction method for adenylates from tobacco leaf tissue is also presented. For most biological material treatment with 2-5% acid is enough to completely denaturate all proteins during adenylate extraction. However, some tissues, including tobacco leaves, contain acid-stable, nucleotide-hydrolysing enzymes which reactivate after the acid is neutralised, and destroy adenylates in the sample.

3.2 LUMINOMETRIC DETERMINATION OF ATP AND OTHER METABOLITES

The determination of ATP concentrations using bioluminescence is a well-established technique. Luciferase (from American firefly Photinus pyralis) catalyses the following reaction:

\[
\text{ATP + luciferin + O}_2 \xrightarrow{\text{LUCIFERASE, Mg}^{++}} \text{oxyluciferin + PP}_i + \text{AMP + CO}_2 + \text{light}
\]

The resulting light has an emission maximum at 562 nm. Under optimised conditions the intensity of the emitted light is directly proportional to the ATP concentration in the sample. The advantage of this assay system is that it is highly specific and very sensitive: the detection limit is as low as \(10^{-14}\) mol l\(^{-1}\) (Wulff and Doppen 1985).
There have been few published procedures for assaying other metabolites than ATP and ADP by coupling to luciferase reaction. McCoy and Doeg (1975), and later Lilley *et al.* (1985) utilised the glycolytic pathway to convert PEP, 2-PGA and 3-PGA stoichiometrically to ATP (for reaction sequence see Section 3.5). The key enzyme in this assay system is the phosphoglycerate mutase (PGM) which catalyses the isomerisation reaction between 3-PGA and 2-PGA. PGM is commercially available from animal sources. However, this preparation is not suitable for use as a coupling enzyme for the luciferase reaction because it requires glycerate-2,3-bisphosphate as a cofactor (Carreras *et al.* 1982). Some of this cofactor is hydrolysed to 2-PGA and 3-PGA by the animal PGMs, since they also exhibit bisphosphoglycerate phosphatase activity (Michal 1974). This causes increase in the background. Plant PGMs, on the other hand, do not manifest this cofactor requirement (Grisolia and Carreras 1975), thus being suitable as a coupling enzyme for the luminescence assays.

### 3.3 PURIFICATION OF PGA-MUTASE FROM WHEAT GERM

#### 3.3.1 PGA-MUTASE ACTIVITY ASSAY

Throughout the purification process the activity of PGM was assayed by coupling the formation of 2-PGA from 3-PGA to the enolase-catalysed formation of PEP (Grisolia and Carreras 1975) as follows:

\[
\begin{align*}
3\text{-PGA} \xrightarrow{\text{PGM}} & \ 2\text{-PGA} \\
2\text{-PGA} \xrightarrow{\text{ENO}} & \text{PEP}
\end{align*}
\]

The formation of PEP was measured as an increase in optical density at 240 nm at 25 °C. The conversion of 2-PGA to PEP is not strictly stoichiometric, but under these assay conditions 1.5 μmols of 2-PGA formed corresponds to 1 μmole of PEP measured. The standard assay mixture, in total volume of 1.5 mls, contained the following reagents: assay buffer (30 mM Tris-HCl pH 8.7, 0.3 mM MgSO₄), 10 mM 3-PGA, and 2 U enolase. The reaction was initiated by adding PGM.
3.3.2 ADENYLATE KINASE ASSAYS

Adenylate kinase (AK) catalyses the interconversion between ATP, ADP and AMP, and thus disturbs quantitative assays (for details see section 3.5.1). We monitored the AK contamination in the PGM preparation by measuring its activity by photometric assay using lactate dehydrogenase (LDH) and pyruvate kinase (PK) as the coupling enzymes (Kawai and Uchimiya 1995):

\[
\text{AMP} + \text{ATP} \xrightleftharpoons{\text{AK}} \xrightarrow{\text{PK}} \text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{Pyruvate} \\
\text{Pyruvate} + \text{NADH} \rightarrow \text{Lactate} + \text{NAD}^+
\]

The standard assay mixture (total volume 1 ml) contained 50 mM Hepes-KOH pH 7.8, 75 mM KCl, 2.5 mM MgCl₂, 1 mM ATP, 1mM AMP, 0.85 mM PEP, 0.2 mM NADH and 2.25 U of both PK and LDH. The reaction was started by adding the sample, and the production of ADP was measured through a decrease in A₃₄₀. The soluble protein concentration in all samples was determined by Coomassie Brilliant Blue method using bovine serum album as standard.

3.3.3 EXTRACTION AND PURIFICATION

We purified the PGM from wheat germ based on methods by Grisolia and Carreras (1975), Leadlay et al. (1977), Smith and Hass (1985) and Grana et al. (1989). All steps were carried out at 0-4 °C. PGM was extracted from fresh wheat germ (a gift from Young Roller Flour Mill Co LTD, Young, NSW, Australia) by homogenising with a Polytron on low setting on ice for 1 h in extraction buffer (20 mM Tris-HCl pH 8, 2 mM DTT, 2mM benzamidine, 2 mM ε-aminocapronic acid, 0.5 mM PMSF). The extract was filtered through Miracloth and centrifuged, and the crude extract (Fraction I) was fractionated with ammonium sulphate (pH 7, 40-60% saturation). The resulting pellet was resuspended in 20 mM Na-P0₄ buffer, pH 7.5, 1 mM DTT and dialysed against the same buffer overnight (Fraction II), snap-frozen in aliquots and stored at -80°C.
One third of the Fraction II was used for further purification. It was treated with bentonite to remove fatty substances (Grisolia and Carreras 1975) and batch-treated with ion exchange media. A slurry of DEAE-Sephasel in 20 mM Na-PO₄ buffer pH 7.5 (approximately 75 % settled media), was added to the sample, stirred for 20 minutes, and centrifuged for 5 mins at 700 g. The bound PGM was released from the resin by washing it with 160 mM Na-PO₄ buffer, pH 7.5. The sample was dialysed overnight against 20 mM Na-PO₄ buffer, pH 7.5, 1 mM DTT, and centrifuged, yielding Fraction III.

Fraction III (total protein content appr. 62 mg) was applied into a 60 ml Q-Sepharose Fast Flow column which had been equilibrated with 40 mM Na-PO₄ buffer pH 7.5. The charged column was rinsed with the same buffer for 3 column volumes, and eluted with a linear gradient of 40-160 mM Na-PO₄ buffer pH 7.5 at the flow rate of 4 ml min⁻¹. The fractions that contained the main PGM activity were pooled and reduced to a final volume of 12.5 mls with Amicon ultrafiltration system equipped with PM 30 membrane (MW 30 000 cutoff). The sample was dialysed overnight against 20 mM Na-PO₄ buffer, pH 7.5, 0.5 mM DTT (Fraction IV).

Fraction IV was reduced to about 1 ml using Millipore Ultrafree-15 filtering device (MW 30 000 cutoff) and was applied to a 5 ml HiTrap Blue affinity column equilibrated with 20 mM Na-PO₄ buffer pH 7.5. The sample was eluted with the same buffer at a flow rate of 0.5 ml min⁻¹, 1-ml fractions were collected and those containing PGM activity were combined. The preparation was diluted into total volume of 6 ml with 20 mM Na-PO₄ buffer pH 7.5, glycerol was added to a final concentration of 10 % (vol/vol), snap-frozen in small aliquots and stored in liquid nitrogen.

Fraction IV had a high enough PGM activity to be suitable for luciferase assays. However, it was evident that it also contained contaminating adenylate kinase (AK) activity, because PGM and AK co-eluted from the Q-Sepharose column (Fig. 3.1). In the luciferase coupled assay mixture, AK causes increase in the background (for full details of the assay conditions, see Section 3.5.1.). Therefore the sample was processed through Hi-Trap Blue affinity chromatography column (Pharmacia
Figure 3.1. Elution profiles of wheat germ phosphoglycerate mutase (PGM) and adenylate kinase (AK) from Q-Sepharose ion-exchange column. PGM (solid circles) and AK (open squares) activities were measured spectrophotometrically as described in section 3.3. AK activity was determined only from the fractions that were collected for further purification (fraction numbers 5-11).

Biotech). This affinity media specifically retains enzymes that have a binding site for nucleotides, thus freeing the preparation from AK.

The final preparation (Fraction V) had 0.36 mg protein ml\(^{-1}\), and PGM activity measured under standard assay conditions was 24 \(\mu\)mol 2-PGA min ml\(^{-1}\). This yields the specific activity of 70 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\)), and compared to the crude extract, the procedure produced more than 80-fold purification (Table 3.1). AK activity was not detectable in the final preparation. It is worth noting that because PGM has a high pH optimum and is inhibited by EDTA, the specific activity measured in the buffer used in the luciferase assays (10 mM Tris-KOH pH 7.75, 8.75 mM Mg-acetate, 0.5 mM EDTA, 20 mM KCl) was only 42 \(\mu\)mol 2-PGA min\(^{-1}\) (mg protein\(^{-1}\)), or 40% less than under optimal conditions at pH 8.7.
Table 3.1. Purification of 3-PGA-mutase from wheat germ. Details of the purification procedure steps are given in text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein content (mg ml(^{-1}))</th>
<th>Mutase activity (µmol 2-PGA min(^{-1}) ml(^{-1}))</th>
<th>Specific activity (µmol 2-PGA min(^{-1}) mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>550</td>
<td>11</td>
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<td>29.4</td>
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<tr>
<td>III*</td>
<td>33</td>
<td>1.88</td>
<td>13.2</td>
<td>7.02</td>
</tr>
<tr>
<td>IV</td>
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<td>6</td>
<td>0.36</td>
<td>25.2</td>
<td>70.0</td>
</tr>
</tbody>
</table>

* Only one third of the Fraction II was used in the subsequent purification.
3.4 PERCHLORIC ACID EXTRACTION FOR ADENYLATES AND METABOLITES FROM TOBACCO LEAVES

The turnover rate of the nucleotide phosphate groups in cells is very fast, so the half-life of ATP is short, a few seconds in whole plant cells and only about one second in chloroplasts (Pradet and Raymond 1983). Thus, in order to measure adenylate pools correctly it is important to stop metabolic activity of the tissue quickly under the particular conditions of interest. The rapid-kill gas exchange machinery which we used to collect the samples freezes the leaf tissue within 0.1 seconds of the interruption of the light beam (Badger et al. 1984).

The other critical factor in adenylate assays is the inactivation of the enzymes in the sample and extraction of the nucleotides. Typically, 2-10% trichloroacetic acid or perchloric acid dissolved in water is used in extraction of plant material (Pradet and Raymond 1983; Bergmeyer and Grassl 1985). However, adenylate assays can be compromised by enzymes that are not destroyed by the acid extraction, but reactivate after the extract is neutralised and hydrolyse the sample nucleotides. It has been reported that this kind of ATP-hydrolysing enzymes is found in several bacterial strains (Lundin and Thore 1975), as well as potato tubers (Biotto and Siegenthaler 1994) and tobacco (Nicotiana tabacum cv. W38) leaf and pith tissue (Ikuma and Tetley 1976). The acid-stable ATP-hydrolysing enzyme found in tobacco leaves is most probably similar to an apyrase (ATP diphosphohydrolase) from potato tubers. This enzyme could be inactivated by boiling the acid extracts, but this harsh procedure also destroyed the ATP in the sample (Ikuma and Tetley 1976). Later, it was shown that the apyrase in potato acid extracts could be inactivated by heating at 60°C for 5 minutes without a loss of ATP (Biotto and Siegenthaler 1994). It has been shown as well that nucleotide converting enzymes generally require Mg²⁺ or other divalent metal cation as a cofactor, and can be inactivated by including EDTA in the extraction acid (Lundin and Thore 1975).

Based on this information, a new extraction method for adenylates, 3-PGA and RuBP from tobacco leaves was developed. A half of the rapid-kill leaf disc (2.5 cm²) was quickly weighed (keeping it frozen), and ground into powder in a mortar.
cooled with liquid nitrogen. 500 μl of 5% perchloric acid containing 10 mM EDTA was pipetted on the top of the powder and the resulting acid-leaf tissue mixture was homogenised until it melted. The crude extract was transferred to a microcentrifuge tube and heated for 5 minutes in a 60 °C water bath. The extract was cooled on ice, and centrifuged at 4 °C for 10 minutes (10 000 g). An aliquot (350 μl) of the supernatant was neutralised to pH 6-7 using 3 M K₂CO₃ (about 55 μl) and kept on ice for at least 15 minutes before the precipitated KClO₄ was removed by centrifugation (10 mins, 10 000 g at 4 °C). The sample was then aliquoted, snap-frozen and stored at −80 °C before assays. The fresh weight of the leaf sample was taken into account when calculating the adenylate and metabolite contents.

3.5 LUMINOMETRIC DETERMINATION OF ATP, ADP, 3-PGA AND RUBP FROM LEAF EXTRACTS

3.5.1 ASSAY PRINCIPLE

The sequential assay method to convert the metabolites of interest to ATP and assay its amount with the sensitive bioluminescence method was utilised. In adenylate assays ATP was measured directly, and ADP was converted to ATP using pyruvate kinase (PK) in the following reaction:

\[
\text{ADP + PEP} \xrightarrow{\text{PK}} \text{ATP + pyruvate}
\]

The amount of ADP in the sample was obtained by subtracting the initial ATP concentration from the ADP+ATP concentration in the sample.

For metabolite contents, the method of Lilley et al. (1985) was expanded, by incorporating a third step, where Rubisco and NaHCO₃ were added to the assay mixture to convert RuBP into 3-PGA, which then was assayed according the original method. Thus, three separate assays were made for each sample (see reaction sequence below). First, the combined background amount of ATP, PEP and 2-PGA were assayed by including enolase (ENO) and PK in the assay mixture. In the second step, phosphoglycerate mutase (PGM) was added, and the total pool of ATP, PEP 2-
PGA and 3-PGA was determined. Finally, by including Rubisco and NaHCO₃, the pool of RuBP, 3-PGA, ATP, PEP and 2-PGA was measured. The actual amounts of 3-PGA and RuBP were then obtained by subtraction.

The complete reaction sequence is:

\[\text{Step 3: RuBP} + \text{CO}_{2} \xrightarrow{\text{Rubisco}} 2 \times 3 - \text{PGA}\]
\[\text{Step 2: } 3 - \text{PGA} \xrightarrow{\text{PGM}} 2 - \text{PGA}\]
\[\text{Step 1: } 2 - \text{PGA} \xrightarrow{\text{EN0}} \text{PEP} + \text{H}_2\text{O}\]
\[\text{PEP} + \text{ADP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}\]

There is a stoichiometric conversion of 3-PGA to ATP, and hence there is two ATPs formed for each RuBP molecule assayed.

It was noted by Lilley et al. (1985), and also by us, that there is a background formation of ATP in this assay system, due to a trace of AK present in the commercial PK preparation. AK then converts the substrate ADP in the metabolite assay mixture to ATP and AMP:

\[2\text{ADP} \xrightarrow{\text{AK}} \text{ATP} + \text{AMP}\]

In addition the tobacco Rubisco preparation, purified as described by Servaites (1985), also contained some AK activity. There are several ways to reduce the effect of this contaminating activity. One is to keep the substrate ADP concentration as low as possible. I used 3 μM ADP in the reaction mixture (ie. 240 pmol ADP in the total volume of 80 μl). Secondly, the conversion reaction was started by adding the coupling enzymes to the otherwise complete reaction mixture at 30-s intervals and incubating all the samples and their blanks exactly the same length of time. Thirdly, the concentrations of coupling enzymes were kept as low as possible. With these precautions the background ATP content in the metabolite measurements was between 0.5 and 1 pmols per assay after 20-25 minutes incubation period, and in ATP and ADP assays there was practically no background at all. We did not use inhibitors to overcome adenylate kinase contamination.
Because the concentrations of the coupling enzymes were very low, each set of assays included 10-20 pmol ADP, PEP, 3-PGA or RuBP-standard, whose conversion was checked. Although a very low ADP background and small amounts of coupling enzymes were used, a 100% conversion of at least up to 20 pmol of 3-PGA and 15 pmol of RuBP to ATP during 20 minutes incubation period at the assay conditions was obtained (Fig. 3.2).

![Figure 3.2. Conversion of 3-PGA (Δ) and RuBP to ATP in the luciferase-coupled assay system. The dotted lines represents the 1:1 ratio and the solid line represents 1:2 ratio. Assay conditions are described in text.](image-url)
3.5.2 ASSAY CONDITIONS

3.5.2.1 Chemicals

All standards, blanks and samples were diluted with 10 mM Tricine-KOH pH 7.75. Luciferin/luciferase preparations were diluted with 10 mM Tricine-KOH pH 7.75, 10 mM Mg-acetate, 1 mM EDTA, 0.5 mg BSA ml⁻¹, 0.5 M DTT. PEP solutions were treated with activated charcoal to remove possible nucleotide contamination.

PK and ENO were obtained from Boehringer Mannheim as ammonium sulphate suspensions, were centrifuged before use and the pelleted enzyme was redissolved in water. Tobacco Rubisco was prepared as described by Servaites (1985). Prior to use, Rubisco was exchanged to activating buffer (50 mM EPPS-NaOH pH 8.3, 25 mM MgCl₂, 1 mM EDTA, 10 mM NaHCO₃) using Amicon concentrator. Rubisco was activated before assays by incubating at +50 °C water bath for 10 minutes. ADP, PEP, and 3-PGA were obtained from Boehringer-Mannheim. RuBP was prepared and purified as described earlier (Kane et al. 1998). ATP used in internal standardisation was from Boehringer-Mannheim’s CLS II kit for luminometric assays and was diluted to a final concentration of 5 μM in 10 mM Tricine pH 7.75.

3.5.2.2 Internal standardisation technique

It is known that the luminescence yield is affected by factors such as temperature, ionic strength, sample color and pH. To avoid errors generated by varying luminescence yield between the samples, an internal standardisation method was used. Each sample was thus assayed twice: alone and a known amount (10 pmol) of ATP added into the sample. The amount of ATP in sample was then calculated as:

\[
ATP = \frac{R_U - R_B}{R_{IS} - R_U} \times Std
\]
In the equation, \( R_u \) is the relative light units (rlu) emitted by the sample, \( R_b \) is the rlu of the blank, \( R_{is} \) is the rlu of the sample with the added ATP and Std is the amount of added ATP. The neutralised perchloric acid extracts were diluted so that each sample contained approximately 10–30 pmol ATP after enzymatic conversions. Blanks contained similarly diluted, neutralised perchloric acid.

### 3.5.3.3 Adenylate assays

All adenylates and metabolites were assayed in a total volume of 80 µl in a transparent microcentrifuge tubes using TD 20/20 luminometer (Turner Designs, USA) fitted with an autoinjector. 100 µl of appropriately diluted luciferin/luciferase mixture was injected into the reaction mixture and total light signal emitted was integrated for 5 seconds after a delay of 3 seconds. From recordings of the light signal it was evident that this method provides a signal which is stable over a period of at least one minute. All assays were run in duplicate.

For ATP and ADP measurements the samples were diluted 1:20 with 10 mM Tricine-KOH pH 7.75. Blanks contained similarly diluted, neutralised perchloric acid. ATP assay mixture contained only the sample and the basic reaction buffer (10 mM Tricine-KOH pH 7.75, 8.75 mM Mg-acetate, 0.5 mM EDTA and 20 mM KCl). Internal standardisation was performed as described in Section 3.5.2.2.

For ADP determinations, the reaction buffer contained in addition 200 µM PEP. The enzymatic conversion of ADP to ATP was started by adding 0.2 U of PK to each sample mixture, and incubated for 25 minutes at room temperature. The internal standardisation was similar to ATP assays.

### 3.5.3.4 Metabolite assays

The samples were diluted 1:50 with 10 mM Tricine-KOH pH 7.75. The assay mix for determination of the metabolites was prepared by adding 3 µM ADP and 5 mM NaHCO₃ to the basic reaction buffer. The reaction for the measurement of the combined amounts of [ATP+PEP+ 2-PGA] was initiated by adding 0.4 U of PK and 0.04 U of ENO to the assay mixture, and the amount of ATP generated was measured after 25 minutes incubation. For assaying the total amount of 3-PGA and [ATP+2-
PGA+PEP], 0.003 U of PGM was added to each reaction mixture. RuBP assay mixtures contained additionally 0.008 U of tobacco Rubisco. Each measurement series contained several blanks and an external standard to check the enzymatic conversion. Internal standardisation was carried as described before.

3.6 RECOVERY TESTS

To test the recoveries of the adenylates and metabolites, a leaf disc was extracted as described before in perchloric acid. Two identical aliquots were taken from the crude extract, and approximately 20-30 nmol of ATP, ADP, 3-PGA and RuBP (together or separately) were added into the other aliquot. Both aliquots were processed and assayed as described before. The accurate concentrations of the added adenylates or metabolites were determined by spectrophotometric assays. ATP was determined by phosphoglycerate kinase- and ADP by pyruvate kinase-lactate dehydrogenase method (Bergmeyer and Grassl 1985). 3-PGA and RuBP were measured as described in Chapter 2. The recovery percent was calculated from the recovery samples minus the control samples. The results of several tests are presented in Table 3.2. The recoveries of ATP, ADP and RuBP were well above 90%. However, for some reason 3-PGA had the lowest and the most variable

Table 3.2. Recoveries of added adenylates and metabolites from acid extracts of tobacco leaves. Known amounts of adenylates and metabolites were added in tobacco leaf extracts which were further processed and assayed as described in the text. The recovery was calculated from the difference between recovery and control samples. The numbers in the brackets indicate the amount of replicates

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Recovery (%±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>99±2.2 (n=6)</td>
</tr>
<tr>
<td>ADP</td>
<td>92±3.5 (n=3)</td>
</tr>
<tr>
<td>3-PGA</td>
<td>86±6.8 (n=4)</td>
</tr>
<tr>
<td>RuBP</td>
<td>98±3.9 (n=5)</td>
</tr>
</tbody>
</table>
recoveries, although it should be both heat- and acid-stable. No corrections were made in the final contents of adenylates or metabolites in the samples.

3.7 SUMMARY

Tobacco leaf tissue contains a nucleotide hydrolysing enzyme which survives acid extraction even with 10% perchloric acid, and reactivates after the extract is neutralised. However, a brief heat-treatment (5 minutes, 60°C) in the presence of EDTA in the extraction acid is sufficient to completely denature this enzyme without a significant loss of sample adenylates or metabolites. The resulting neutralised extract is stable at least for several hours, when stored on ice.

The luciferase-coupled bioluminescence method is a very sensitive assay specific for ATP. The method by Lilley et al. (1985) was extended to sequentially convert RuBP and 3-PGA to ATP and measure the ATP content with luciferase reaction. The assay method requires that one of the coupling enzymes, phosphoglyceromutase, is purified from a plant source and treated with affinity chromatography to free it from a contaminating adenylate kinase activity, which otherwise causes a continuous increase in the assay background. The assay system is suitable for measuring picomolar amounts of ATP, ADP, 3-PGA and RuBP from leaf extracts. It is crucial, however, to carry adequate blanks, as well as standard assays, because in order to keep the background ATP formation in minimum the coupling enzyme contents are very low. An internal standardisation technique was used to avoid errors due to varying luminescence yields between different samples. The recovery tests confirmed that the assay system is accurately quantitative.
CHAPTER 4

LEAF GAS EXCHANGE, METABOLITE AND ADENYLATE POOLS AND NADP-MDH ACTIVATION IN ANTI-BF AND ANTI-GAPDH TOBACCOS
4.1 INTRODUCTION

Chapters 4-6 describe the photosynthetic and biochemical characteristics of two different, contrasting transgenic tobacco types: anti-\textit{bf} and anti-GAPDH plants. Anti-\textit{bf} plants have a defect in the photosynthetic light reactions, since the amount of chloroplast cytochrome \textit{bf}-complex has been reduced (Price \textit{et al.} 1995b). Anti-GAPDH plants have an impaired capacity for carbon assimilation, because the amount of one of the Calvin cycle enzymes, glyceraldehyde 3-phosphate dehydrogenase, has been reduced (Price \textit{et al.} 1995a). The antisense mRNA technique used to generate these plants has not affected other components of the photosynthetic reactions greatly, thus making them ideal to study the interactions between electron transport and carbon assimilation.

4.1.1 CYTOCHROME BF-COMPLEX

4.1.1.1 Structure of the complex

Cytochrome \textit{bf}-complex is one of the membrane-bound proteins of the photosynthetic electron transport chain. It functions as a plastohydroquinol-plastocyanin oxidoreductase, thus mediating electron flow between PSII and PSI. The cytochrome \textit{bf} complex consists of four polypeptides: cytochrome \textit{b}_{6}, cytochrome \textit{f}, Rieske iron-sulphur protein (Fe-S) and subunit IV (Fig. 4.1.). The functional groups of this complex are the three haems (two in cytochrome \textit{b}_{6}. subunit, one in cytochrome \textit{f}) and an iron-sulfur center in Rieske protein, which all contain bound iron atoms. These iron atoms can change their oxidation states, thus being able to function as redox-carriers (O'Keefe 1988; Marder and Barber 1989).

4.1.1.2 Function in photosynthesis

Cytochrome \textit{bf} complex is a unique part of the photosynthetic electron transport chain, because it also participates in proton translocation from stroma to lumen. In linear electron transport, cytochrome \textit{bf} complex accepts electrons from the membrane-embedded plastohydroquinol (PQH_{2}) pool, and passes them on to plastocyanin (PC), a luminal redox carrier. The oxidation of plastohydroquinol to plastoquinol (PQ) takes place near the luminal side of the cytochrome \textit{bf} complex,
with the released protons ending up in the lumen. This way, two protons per every two electrons are translocated. In addition, an auxiliary mechanism which increases the efficiency of proton translocation, Q-cycle, may function in chloroplasts (Cox and Olsen 1982; Rich 1988). In this scheme one electron from PQH₂ reduces PC, the other electron is recycled through the cytochrome f subunit to re-reduce PQ. This leads to the overall translocation of 4 protons per two electrons transported. The Q-cycle functions in the mitochondria (Trumpower 1990), but its role in photosynthetic electron transport is still controversial. Cytochrome bf complex also has a role in nonlinear electron transport. Under certain conditions cyclic electron flow from the reducing side of PSI through the cytochrome bf complex back to the ground state PSI is known to occur (Hauska et al. 1974; Hosier and Yocum 1985). As a consequence, only protons are translocated without NADPH synthesis.

Since cytochrome bf complex has a variety of functions and plays a central role in electron transport, changing its amount in chloroplasts should have marked consequences to photosynthesis. Manipulation of leaf cytochrome bf content has been achieved in transgenic tobacco plants. These plants have an antisense DNA construct against the nuclear-encoded Fe-S subunit (Price et al. 1995b), leading to a
decrease in the amount of the cytochrome $b_f$ complex (Price et al. 1998). The lowered content of this complex should have drastic effects on the energy status of the chloroplast since both ATP and NADPH synthesis are inhibited, altering the balance between electron transport and carbon assimilation capacity.

4.1.2 CHLOROPLAST GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

4.1.2.1 GAPDH catalyses the early steps in the Calvin cycle

NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is solely located in the chloroplasts. It functions in the photosynthetic carbon reduction cycle, participating in the reversible conversion of organic acid, 3-phosphoglyceric acid (3-PGA) to triose phosphates (Fig. 4.2). The majority of triose phosphates ($5/6$) are used for regeneration of RuBP, and the rest for starch or sucrose synthesis. It has been assumed that the conversion from 3-PGA to triose phosphates is close to the thermodynamic equilibrium (Dietz and Heber 1984) and the activities of phosphoglycerokinase and GAPDH are not limiting for photosynthesis (Price et al. 1995a). However, this view has been lately challenged by Fridlyand et al. (1998) who suggest that the 3-PGA reduction step could be a site for metabolic regulation.

![Fig. 4.2. Conversion of 3-phosphoglycerate to triose phosphates.](image)

The carboxylation of RuBP by Rubisco yields two molecules of 3-PGA, which are phosphorylated by phosphoglycerate kinase to 1,3-bisphosphoglycerate, utilizing ATP. Glyceraldehyde 3-phosphate dehydrogenase then uses NADPH to reduce 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate, which is isomerised by triose phosphate isomerase to dihydroacetone phosphate. Redrawn from Heldt (1997).
4.1.2.2 Activation and catalysis

GAPDH in higher plants consists of subunits A and B, which are quite similar in terms of DNA and amino-acid sequences (Cerff and Chambers 1979; Brinkmann et al. 1989). The subunits can have different aggregation states, mainly $A_8B_8$, $A_4B_4$ or $A_2B_2$. The largest oligomer is found predominantly from dark-adapted material and exhibits the lowest NADP-dependent activity, indicating that the activation of GAPDH includes a conversion from the high-molecular weight form to low molecular weight forms (Scagliarini et al. 1993). In addition, the well-documented light activation of GAPDH has been shown to involve redox regulation via ferredoxin thioredoxin pathway (Wolosiuk and Buchanan 1978). A recent model of light activation of GAPDH suggests that the thioredoxin mediated reduction of the $A_8B_8$ form of GAPDH does not alone cause dissociation of the complex, but somehow increases the susceptibility of the enzyme to effectors, such as 1,3 bisphosphoglycerate (substrate), NADPH and ATP. The presence of these effectors induce the formation of the lower molecular-weight forms of the heterodimer, and subsequently, activation of GAPDH (Baalmann et al. 1994; 1995).

While the activation of GAPDH has been studied to some detail, the catalytic mechanism, as well as molecular state of the enzyme in the chloroplast is unclear. Both A and B subunits carry an active site, which contains a sulfhydryl group of a cysteine residue as a functional redox-mediator group (Ferri et al. 1987). It has been suggested that GAPDH is a free stromal enzyme (Pupillo and Giuliano-Piccarri 1973), and more recently, that it may be a part of a larger multienzyme complex, which contains other Calvin cycle enzymes, like phosphoribulokinase or Rubisco, and these complexes could be associated with thylakoid membranes (Clasper et al. 1991; Süss et al. 1993).

Transgenic tobacco plants with reduced amounts of GAPDH (Price et al. 1995a) form an interesting contrast to the anti-$bf$ plants. In the two plant types the
coordination between photosynthetic light and dark reactions, or production and consumption of light-derived chemical energy has been altered in opposite directions. Comparison of the two plant types is undertaken in Chapters 4-6.

4.1.3 PREVIOUS RESEARCH WITH ANTI-GAPDH AND ANTI-BF PLANTS

Both anti-GAPDH and anti-cytochrome $bf$ tobaccos have been characterised earlier, in terms of for example gas exchange, Rubisco characteristics or metabolite contents. Price et al. (1995b) observed a strong correlation between CO$_2$ assimilation rate in air and the content of the cytochrome $bf$ complex. They also reported that the fluorescence parameters indicated a high reduction level of plastoquinone (PQ) pool, while transthylakoid pH gradient formation was impaired, consistent with the role of cytochrome $bf$ complex in electron transport. Photoinhibition and photoprotection in anti-$bf$ plants has been studied and it was found that although the anti-$bf$ plants had a higher degree of closed PSII reaction centres, they experienced much less photoinhibition than wild-type plants (Hurry et al. 1996). Recently, the carbon assimilation in anti-$bf$ plants was studied in more detail (Price et al. 1998), revealing that the decreased CO$_2$ assimilation rates were associated with severely reduced RuBP contents. In addition, Rubisco carbamylation level declined in anti-$bf$ plants, suggesting an additional mechanism which restricts CO$_2$ assimilation in these plants.

The engineering and characterisation anti-GAPDH plants was reported by Price et al. (1995a). A reduction in GAPDH activity lead to a decrease in RuBP pool sizes, however, the CO$_2$ assimilation was only affected when RuBP per Rubisco site-ratio fell below 2. As expected, in anti-GAPDH plants photosynthesis became RuBP-regeneration limited at lower CO$_2$ partial pressures than wild-type plants. Chlorophyll fluorescence measurements indicated that antisense plants had a high transthylakoid pH gradient, due to restriction in carbon assimilation. This large proton gradient apparently caused a drastic reduction in PSII efficiency and electron transport, since even plants with very low GAPDH activities were not photoinhibited.

4.1.4 THE OUTLINE OF THE CHAPTER

In this Chapter the previous studies of anti-GAPDH and anti-$bf$ plants are extended to examine the interactions between photosynthetic electron transport and
carbon assimilation in more detail. A range of transgenic plants were grown and the CO₂ assimilation rates were measured at 350 μbar CO₂ and high light. After the gas exchange measurements, leaf discs were rapidly freeze-clamped in situ. The amounts of chloroplast metabolites (RuBP, 3-PGA) were assayed from the leaf discs. Also the whole-leaf ATP and ADP levels were analysed, in order to examine the changes in energy balance due to chloroplast manipulation. The activation state of the chloroplast NADP-dependent malate dehydrogenase was measured and used as an indicator of stromal NADP⁺/NAPPH ratio (Scheibe and Jacquot 1983; Harbinson et al. 1990; Foyer 1993).

4.2 MATERIAL AND METHODS

4.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

4.2.1.1 Anti-bf plants

The transformation of tobacco (Nicotiana tabacum L. cv. W38) with an antisense construct directed against the Rieske FeS subunit of the chloroplast cytochrome bf complex has been described previously (Price et al. 1995b). Antisense plants used in this study were raised from selfed T₃ seeds of the line B6F-2.2-513-16, producing a range of phenotypes in respect to Rieske FeS protein content and CO₂ assimilation rates (Price et al. 1998). These plants show phenotypic differences already after germination, unlike the parent generation where the antisense effect is masked until the plants are about 5 weeks old (Hurry et al. 1996; Anderson et al. 1997). Both untransformed wild-type and antisense plants were grown in 5 L pots in sterilised garden soil in a growth cabinet, and fertilised with Hewitt’s complete nutrient solution (Hewitt and Smith 1975) three times a week. The light intensity was 100-120 μmol quanta m⁻² s⁻¹, light period 20 hours, and the temperature was kept constant at 25 °C. Since the CO₂ assimilation and growth rates are much slower in the transgenic plants, they were used at an older age than wild-type plants so that enough leaf material could be obtained for the experiments. Plants were used at 8 –
16 weeks after germination, when they had reached a height of approximately 30 cm, and the youngest full grown leaf was selected for the measurements.

### 4.2.1.2 Anti-GAPDH plants

The anti-glyceraldehyde 3-phosphate dehydrogenase plants were grown from T₁ seeds of plant GAP-R, and had a wide variety of GAPDH activities (Price et al. 1995a). Anti-GAPDH and control plants were grown in garden soil, in 5 L pots in an air-conditioned glasshouse where the peak irradiation was 700-900 μmol quanta m⁻² s⁻¹. These plants were fertilised in a similar manner to the anti-βf plants. Untransformed W38 plants were used as controls. Tobaccos were used between 6 and 10 weeks after germination, when they were a similar size to anti-βf plants and an equivalent, youngest fully expanded leaf was selected for the experimentation.

### 4.2.2 GAS EXCHANGE MEASUREMENTS AND RAPID FREEZE CLAMPING OF LEAVES

Leaf gas exchange was measured in a chamber attached to a rapid freeze-clamping apparatus (Badger et al. 1984) using a portable gas exchange system (LI 6400, Li-Cor Inc., NE, USA). CO₂ assimilation was measured at an irradiance of 1000 μmol quanta m⁻² s⁻¹, 350-380 μbar CO₂ in air and a leaf temperature of 25°C. The leaf was stabilised under these conditions for at least 40 minutes before it was rapidly freeze-clamped in situ from light. The apparatus produces two half-discs, 2.5 cm² each. One half was used for assays of Rubisco activity and carbamylation state, and either metabolite and adenylate content or NADP-dependent malate dehydrogenase activation level was measured from the other half of the leaf disc. The samples were stored at -80°C. Additional leaf discs were collected for chlorophyll determinations, cytochrome f-content measurements and GAPDH activity assays.

### 4.2.3 MEASUREMENTS OF RUBISCO SITE CONCENTRATION, CHLOROPHYLL, AND LEAF SOLUBLE PROTEINS.

One half of the freeze-clamped leaf disc was rapidly extracted and centrifuged for 15-20 seconds with a benchtop rotor for Rubisco assays as described in Chapter 2, Section 2.2.3.1. Rubisco site concentration was measured by [¹⁴C]CPBP-binding
method. For soluble protein assays, the extract was further centrifuged for 2 minutes at 10,000 g. Protein concentration of the supernatant was measured with the Coomassie Brilliant Blue method using BSA as a standard. Total chlorophyll and Chl \( a/b \) ratios were determined in 80% acetone buffered with 25 mM Hepes pH 7.5 (Porra et al. 1989).

### 4.2.4 METABOLITE AND ADENYLATE ASSAYS

The second half of the freeze-clamped leaf disc was extracted with 5% perchloric acid containing 10 mM Na-EDTA, and the extract was heat-treated, centrifuged and neutralised as described in Section 3.4. ATP, ADP, RuBP and 3-PGA concentrations were measured from the extracts with luminometric method as described in Section 3.5.

### 4.2.5 NADP-DEPENDENT MALATE DEHYDROGENASE ASSAY

The activation state of chloroplast NADP-malate dehydrogenase (MDH) was assayed according to the method of Scheibe et al. (1988) with minor modifications. One half of a freeze-clamped leaf disc (2.5 cm\(^2\)) was extracted with a glass homogeniser in 900 μl of ice-cold buffer containing 50 mM Na-acetate pH 6, 1% BSA, 4 mM DTT, 0.1% Triton X-100, 0.5 mM benzamidin and 0.5 mM \( \varepsilon \)-aminocaproic acid. The buffer had previously been sparged with humidified nitrogen, and was continuously flushed with nitrogen during the extractions to maintain \( \text{O}_2 \) concentration low. PMSF was added immediately prior to the extraction to the final concentration of 0.5 mM. The crude extract was centrifuged for 5 min at 10,000 g, at 4°C, and the supernatant was used for assays.

The initial activity of NADP-MDH was assayed immediately in a total volume of 1.5 ml, at 25°C, in a mixture containing 100 mM Tris-HCl pH 8 (sparged with humidified nitrogen), 1 mM Na-EDTA, 1 mM DTT, 0.2 mM NADPH and 100 μl of supernatant. The reaction was initiated by adding 2 mM oxaloacetic acid and the decline in \( A_{340} \) was monitored. Reductive activation of MDH was achieved by incubating an aliquot of the supernatant in 250 mM Tris-HCl pH 9 (sparged with nitrogen) and 125 mM DTT under nitrogen atmosphere at room temperature for 15 min. Time-course experiments were carried out to confirm that 15 min was sufficient.
to fully activate tobacco NADP-MDH. In addition, it was checked that the low DTT concentration in the extraction buffer (4 mM) did not cause activation of the enzyme. The activation state remained stable at least 15 min when the extract was kept at 0 °C.

NAD-dependent malate dehydrogenases in leaves can also utilise NADP (Vidal et al. 1977). Scheibe and Stitt (1988) measured both NADP and NAD dependent MDH activities from spinach leaves. They concluded that 0.2% of the total NAD-MDH activity is seen as a NADP-dependent activity, which is not related to chloroplast NADP-MDH activity. This spillover activity could become significant when the overall activity of NADP-MDH is low, because NAD-MDH has a high activity in leaves. On an average, tobacco leaves had a NAD-MDH activity of 530 μmol m⁻² s⁻¹. I corrected the NADP-MDH activities for NAD-dependent MDH activity similarly, assuming that the side activity is 0.2 % of the total NAD-MDH activity (Scheibe and Stitt 1988; Grace and Logan 1996). NAD-MDH activity was measured in a total volume of 1.5 ml in a mixture containing 100 mM Tris-HCl pH 8, 10 mM MgCl₂, 0.2 mM NADH and 10 µl of diluted supernatant. The reaction was started by adding 1 mM oxaloacetic acid (Scheibe and Stitt 1988). With this procedure, activation levels below 5% were obtained from dark-adapted or low light-grown tobaccos.

4.2.6 GAPDH ASSAYS

Total GAPDH activity was measured using a modified method by Stitt et al. (1989) as described in Section 2.2.3.2.

4.2.7 WESTERN BLOT QUANTITATION OF CYTOCHROME F CONTENT

Anti-bf plants have been characterised previously by measuring the amount of Rieske Fe-S polypeptide in leaves by Western blotting. In this study an antibody directed against the cytochrome f protein was used. A close correlation between Rieske Fe-S and cytochrome f proteins has been demonstrated (Anderson et al. 1997; Price et al. 1998), indicating that the content of the whole cytochrome bf-complex is reduced by the antisense construct.
Briefly, a leaf disc (1.6 cm²) was homogenised with a glass homogeniser into 500 µl of 2×Tricine-SDS sample buffer containing 5 % of β-mercaptoethanol. To ensure a complete solubilisation, the extracts were incubated at 50°C for 10 minutes, centrifuged (5 min, 10 000 g), aliquoted and snap-frozen in liquid nitrogen. The aliquots were stored at -80°C. Samples were boiled briefly prior to loading on 10-20 % Tricine gels (Novex). Proteins were resolved by electrophoresis, transferred to a nitrocellulose membrane and the amount of cytochrome f protein was measured using ECL peroxidase-luminol protocol (Amersham, UK) and Image-Quant software (Molecular Dynamics, CA, USA). For details of the ECL procedure see Mate et al. (1993).
4.3 RESULTS

4.3.1 THE EFFECT OF GROWTH CONDITIONS ON WILD-TYPE LEAF COMPOSITION

It has been noted before, that the phenotype of tobaccos with reduced amounts of cytochrome $b_{f}$ complex (anti-$b_{f}$ plants) changes with leaf age, as well as growth irradiance. The effect of growth light is significant, since the antisense effect is weakened or even lost if the plants are grown at high irradiance (Price et al. 1995b). Therefore it is necessary to grow these plants at low light to obtain a stable phenotype. For this experiment, the anti-$b_{f}$ plants were grown at 100-120 $\mu$mol quanta m$^{-2}$ s$^{-1}$. To partially overcome the low light environment and allow the plants to acquire a maximal carbon gain, the length of the photoperiod was 20 hours. On the contrary, plants with antisense gene targeted against glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH plants) show a stable phenotype and could be grown in glasshouse under natural illumination (peak illumination 700-900 $\mu$mol quanta m$^{-2}$ s$^{-1}$). As a consequence, there was a significant difference in the light environment between the two plant types.

Table 4.1 presents a comparison between the low-light and glasshouse grown wild-type plants (the controls). They were quite similar in many respects. The $\text{CO}_2$ assimilation rate, measured at 1000 $\mu$mol quanta m$^{-2}$ s$^{-1}$ and ambient (350 $\mu$bar) $\text{CO}_2$, as well as stomatal conductance were comparable. There was also little difference in leaf soluble protein content. However, the amount of Rubisco was about 30% less in low-light grown plants, leading to a lower proportion of Rubisco of total soluble protein. The percentage of Rubisco of the total soluble proteins were 28% and 37% for the low-light and glasshouse-grown plants, respectively. There was no difference in leaf chlorophyll content, and the low-light grown leaves had only a slightly lower chlorophyll $a/b$-ratio (Table 4.1).
Table 4.1. Physiological characteristics of low-light grown and glasshouse-grown wild-type tobacco plants. Means, standard errors of the means and the number of replicates (in brackets). The photosynthesis measurements were conducted at ambient CO₂ and light intensity of 1000 μmol quanta m⁻² s⁻¹. Leaf discs for the enzyme, metabolite and adenylate assays were rapidly freeze-clamped from the same conditions. Chlorophyll determinations were made on separate leaf discs.

<table>
<thead>
<tr>
<th></th>
<th>Low-light grown wild-type</th>
<th>Glasshouse-grown wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ assimilation rate at ambient CO₂ (μmol m⁻² s⁻¹)</td>
<td>12.8 ±1.1 (n=10)</td>
<td>13.7 ± 1.0 (n=5)</td>
</tr>
<tr>
<td>Stomatal conductance (mmol H₂O m⁻² s⁻¹)</td>
<td>0.289 ± 0.030 (n=8)</td>
<td>0.327 ± 0.037 (n=7)</td>
</tr>
<tr>
<td>Soluble proteins (g m⁻²)</td>
<td>3.68 ± 0.27 (n=7)</td>
<td>4.05 ± 0.25 (n=7)</td>
</tr>
<tr>
<td>Rubisco (g m⁻²)</td>
<td>1.05 ± 0.06 (n=7)</td>
<td>1.50 ± 0.09 (n=7)</td>
</tr>
<tr>
<td>Chlorophyll (mg m⁻²)</td>
<td>366 ± 14 (n=6)</td>
<td>343 ± 31 (n=6)</td>
</tr>
<tr>
<td>Chlorophyll a/b</td>
<td>2.88 ± 0.03 (n=6)</td>
<td>3.08 ± 0.03 (n=6)</td>
</tr>
<tr>
<td>Rubisco/Chlorophyll (mmol mol⁻¹)</td>
<td>4.24 ± 0.63 (n=4)</td>
<td>7.45 ± 0.69 (n=6)</td>
</tr>
<tr>
<td>RuBP (μmol m⁻²)</td>
<td>73 ± 8 (n=4)</td>
<td>130 ± 7 (n=4)</td>
</tr>
<tr>
<td>3-PGA (μmol m⁻²)</td>
<td>69 ± 8 (n=4)</td>
<td>78 ± 11 (n=4)</td>
</tr>
<tr>
<td>RuBP/ total Rubisco sites (mol mol⁻¹)</td>
<td>4.96 ± 0.47 (n=4)</td>
<td>6.44 ± 0.41 (n=4)</td>
</tr>
<tr>
<td>ATP+ADP (μmol m⁻²)</td>
<td>27 ± 3 (n=4)</td>
<td>42 ± 3 (n=4)</td>
</tr>
<tr>
<td>ATP/ADP (mol mol⁻¹)</td>
<td>1.40 ± 0.17 (n=4)</td>
<td>1.55 ± 0.34 (n=4)</td>
</tr>
<tr>
<td>NADP-MDH activation state (%)</td>
<td>77 ± 4 (n=5)</td>
<td>62 (n=2)</td>
</tr>
<tr>
<td>NADP-MDH total activity (μmol m⁻² s⁻¹)</td>
<td>10.4 ± 1.1 (n=5)</td>
<td>17.4 (n=2)</td>
</tr>
</tbody>
</table>
The growth light had apparently a strong impact on the RuBP-regeneration capacity of the leaves. When measured at high light, the low-light grown plants had about 40% smaller RuBP pools than glasshouse-grown plants. Because both RuBP and Rubisco contents were smaller in low-light grown plants, the ratio of RuBP to Rubisco sites was only slightly lower in the low light plants. Interestingly, low-light plants had also about 40% less ATP+ADP than glasshouse grown plants. When exposed to high light, the activation state of the chloroplast NADP-dependent malate dehydrogenase was about 15% higher in low-light grown plants.

4.3.2 GAS EXCHANGE CHARACTERISTICS

A set of plants with a range of cytochrome f contents was initially screened with the chlorophyll fluorescence technique (Price et al. 1995b). We obtained a range of plants with cytochrome f-contents between 60 and 5 % of the average wild-type level (Fig. 4.3 A). However, the variation in cytochrome f-content in wild-type plants was quite large (±20% of the average) as well. Similar variation in leaf Rieske Fe-S protein content has been observed earlier in wild-type tobacco (Price et al.1998). It is likely that the Western blotting is not the most accurate method for protein quantitation, but on the other hand, a large internal variation in biochemical characteristics seems to be typical for W38 tobaccos. For example see the variation in wild-type GAPDH activity in Fig. 4.4 and in Price et al. (1995a) and in Rubisco activity (Hudson et al. 1992). CO₂ assimilation rate, measured at ambient CO₂ and high irradiation, decreased when the amount of cytochrome f was reduced. The most severely affected anti-bf plants had an assimilation rates of 0.6-3 µmol m⁻² s⁻¹, when the average rate for the wild-type tobaccos was 13.9 µmol m⁻² s⁻¹. Anti-GAPDH plants had a range of GAPDH activities from about 50% to less than 5 % of wild-type level (Fig. 4.1 B.). There was no effect on the CO₂ assimilation rate until GAPDH activity was reduced below 30 % of wild-type level. Anti-GAPDH plants had a range of assimilation rates, to less than 10 % of the average wild-type rate.

The reduction of cytochrome f-content lead to a slight decrease in stomatal conductance in anti-bf plants (Fig. 4.4 A). However, the intercellular CO₂ concentration was still higher in antisense plants than in wild-type plants. On an average, the Cᵢ in wild-type plants was 240±9 µbar, and it increased up to 300 µbar in the most severe anti-bf plants (Fig. 4.4 C ). There was no difference in stomatal conductance between anti-GAPDH and wild-type tobaccos (Fig. 4.4 B), and the changes in Cᵢ were similar to what happened in anti-bf plants (Fig. 4.4 D).
Fig. 4.3. \(\text{CO}_2\) assimilation rates of anti-\(bf\) and anti-GAPDH tobacco plants measured at ambient \(\text{CO}_2\). The assimilation rates of A. low-light grown (100 \(\mu\text{mol quanta m}^{-2} \text{ s}^{-1}\)) wild-type tobacco plants (■) and transgenic tobacco plants with a variety of cytochrome \(bf\) contents (□), and B. glasshouse-grown wild-type (•) and transgenic tobaccos with different activities of GAPDH (○) were measured using a rapid-kill gas exchange machinery (Badger et al. 1984). Measurements were conducted at an irradiance of 1000 \(\mu\text{mol quanta m}^{-2} \text{ s}^{-1}\), \(\text{CO}_2\) partial pressure of 350-380 \(\mu\text{bar}\) in air and leaf temperature of 25°C. Leaves were kept under these conditions for at least 40 minutes before the \(\text{CO}_2\) assimilation rate was recorded. The data is presented as a function of cytochrome \(f\)-content as relative units on leaf area basis or total GAPDH activity. The curves were fitted by hand.
4.3.3 THE AMOUNT OF RUBISCO AND SOLUBLE PROTEINS

The amount of soluble proteins and Rubisco declined only when the amount of cytochrome \( bf \) content and GAPDH activity was reduced below 30% of the wild-type levels (Figs 4.5 A-D). The most severely affected transgenic plants had about 50% of the total soluble protein and Rubisco content of the wild-type plants. The percentage of Rubisco of the soluble proteins was not affected in the antisense plants, being on an average (±S.E.) 28±2% for all low-light grown plants and 31±3% for the all glasshouse-grown plants.
Fig. 4.5. Leaf soluble protein and Rubisco content of anti-bf and anti-GAPDH tobacco plants. Total amount of soluble proteins (A,B), Rubisco (C,D) and the relationship between Rubisco and soluble proteins (E,F) of low-light grown wild-type and anti-bf tobaccos and glasshouse grown wild-type and anti-GAPDH tobaccos. Symbols: low-light grown wild-type tobacco plants (■) anti-bf plants (□), glasshouse-grown wild-type (●) and anti-GAPDH plants (○). The measuring conditions are as in Fig. 4.3. The leaf discs used for these assays were sampled using the rapid freeze clamping device from the same leaves whose photosynthetic parameters are presented in Figs 4.3 and 4.4.
4.3.4 RuBP AND 3-PGA CONTENT

After the gas exchange measurements were performed, a leaf disc was rapidly freeze-clamped from light and used for metabolite, adenylate or enzyme assays. Metabolites and adenylates were assayed from the same leaf disc using a sensitive luminometric method, as described in Chapter 3, Section 3.6. The amount of RuBP decreased when either cytochrome bf-content or GAPDH activity was reduced (Fig. 4.6 A&B). In both plant types the RuBP contents declined very close to Rubisco site concentration, but not below it. There was no clear change in the amount of 3-PGA in either of the antisense types: only in the most severe anti-bf plants with more than 80% decline in the cytochrome bf content, the amount of 3-PGA decreased (Fig. 4.6 C&D) As a consequence, in both plant types the RuBP/3-PGA ratio fell significantly below wild-type levels (Fig. 4.6 E&F).

Fig. 4.6. Metabolite content of anti-bf and anti-GAPDH tobacco plants at ambient CO₂. RuBP (A, B) and 3-PGA (C, D) pool sizes and the ratio between RuBP and 3-PGA (E, F). Symbols: low-light grown wild-type tobacco plants (■), anti-bf plants (□), and glasshouse-grown wild-type (○) and anti-GAPDH tobaccos (○). The measuring conditions are as in Fig. 4.3. and the sampling of the leaves was as in Fig. 4.5.
4.3.5 WHOLE-LEAF ADENYLATE LEVELS

The amounts of ATP and ADP in the leaves were measured to assess changes in the adenylate pools due to the shifted balance between ATP production and consumption in the chloroplasts. The reduction in cytochrome \textit{b}f content did not change the whole-leaf adenylate levels: both the total amounts of ATP and ADP and the ratio between the two remained similar to the wild-type levels, even in the most severe antisense plants (Fig. 4.7 A,C,E,G). On an average (±S.E.), the low-light plants had 14.8±2 µmol ATP and 10.9±1 µmol ADP m\(^{-2}\) and the ratio of ATP/ADP was 1.43±0.1. On the contrary, when the activity of GAPDH was reduced below 50% of the wild-type level, the amount of ATP increased, and simultaneously ADP content decreased. This lead to an approximately two-fold rise in the ATP/ADP ratio when compared to wild-type plants (1.55±0.34 in wild-type, up to 3.1 in anti-GAPDH plants), whereas the sum of ATP+ADP remained constant, being 44±6 µmol m\(^{-2}\) in all glasshouse-grown tobbacos (wild-types and anti-GAPDH) (Fig. 4.7 B,D,F,H).
Fig. 4.7. Leaf adenylate content of anti-βf and anti-GAPDH tobacco plants at ambient CO₂. ATP (A, B) and ADP (C, D) concentrations, ATP/ADP ratio (E, F) and the sum of ATP+ADP (G, H). Symbols: low-light grown wild-type tobacco plants (■), anti-βf plants (○), glasshouse-grown wild-type (●), and anti-GAPDH plants (○). The measuring conditions and leaf sampling were as in Figs 4.3 and 4.5.
4.3.6 NADPH-MDH ACTIVATION LEVEL

The activation state of the chloroplast NADP-dependent malate dehydrogenase (NADP-MDH) decreased dramatically when the cytochrome bf content was reduced (Fig. 4.8. A). On an average (± S.E.), under the high light conditions the NADP-MDH activation in wild-type plants was 76±4 %, and it decreased to 5% in the most severely affected antisense plants. The reduction in the activation level was solely due to a decrease in the initial activity of the enzyme (data not shown). On the contrary, lowered GAPDH activities did not affect the NADP-MDH activation level when compared to the wild-type plants (Fig. 4.8.B). The activation level in wild-type and anti-GAPDH plants was 53±4%.

Fig. 4.8. NADP-dependent malate dehydrogenase activation levels of anti-bf and anti-GAPDH tobacco plants measured at ambient CO₂. MDH-activation in low-light grown wild-type and anti-bf tobaccos (A) and glasshouse grown wild-type and anti-GAPDH tobaccos (B). Symbols: low-light grown wild-type tobacco plants (■), anti-bf plants (□), glasshouse-grown wild-type (●), anti-GAPDH plants (○). The measuring conditions and leaf sampling were as in Figs 4.3 and 4.5.
The dependence of NADP-MDH activation state of the rate of electron transport is illustrated in Fig. 4.9. The rate of photosynthetic electron transport \( J_g \) in the anti-\( bf \) plants was calculated based on the gas exchange data as

\[
J_g = \frac{(A + R_d)(4C_i + 8\Gamma^*)}{(C_i - \Gamma^*)},
\]

where \( A \) is the \( CO_2 \) assimilation rate, \( R_d \) the dark respiration rate, \( C_i \) the intercellular \( CO_2 \) partial pressure and \( \Gamma^* \) the \( CO_2 \) compensation point in the absence of mitochondrial respiration (38.6 \( \mu \)bar) (von Caemmerer and Farquhar 1981, von Caemmerer et al. 1994). The relationship between the \( J_g \) and NADP-MDH activation level was strikingly linear.

![Fig. 4.9. The activation state of NADP-dependent malate dehydrogenase in low-light grown wild-type and anti-\( bf \) plants as a function of calculated electron transport rate at ambient \( CO_2 \). Measuring conditions, sampling and enzyme assays are as in Fig. 4.8. Symbols: low-light grown wild-type tobacco plants (■) anti-\( bf \) plants (□).](image)
4.4 DISCUSSION

4.4.1 LOW GROWTH LIGHT STABILISES THE PHENOTYPE IN ANTI-BF PLANTS WITHOUT GREATLY AFFECTING THE LEAF PHYSIOLOGY

Tobacco plants expressing an antisense DNA against the Rieske Fe-S protein of the chloroplast cytochrome bf complex will lose their phenotypes if grown at high irradiance (Price et al. 1995b). This effect of light intensity on the antisense gene expression is intriguing but little research has been conducted on this subject. It has been reported earlier, that even 90% reduction in the chloroplast Rieske Fe-S mRNA level does not decrease protein content in tobacco leaves (Palomares et al. 1993). This lack of correlation between mRNA and protein levels indicates that the synthesis of Fe-S protein is mainly under post-transcriptional regulation. The other polypeptides of the cytochrome bf complex, cytochromes f and b₆, respond strongly to light, so that the content of the whole bf complex increases significantly during high-light acclimation in leaves (Anderson et al. 1988). It is probable that the same strong light dependence of protein content also applies to the Rieske Fe-S protein, since in etiolated tobacco seedlings the levels of all polypeptides of cytochrome bf complex are very low (Palomares et al. 1993), which suggests a common control of expression for all subunits. It is then possible that the light-dependent post-transcriptional regulatory mechanism is accelerated by high light to the extent that the antisense phenotype will be lost anti-bf plants if they are grown at high light.

To maintain a stable genotype, anti-bf and wild-type plants were grown at 100-120 µmol quanta m⁻² s⁻¹ and 20-h photoperiod for my study. Typically low-light grown plants have distinctive features when compared to high-light grown plants. For example, leaves grown under high irradiation have a high light-saturated CO₂ assimilation rate and stomatal conductance. Low-light leaves tend to have less soluble proteins and Rubisco on an area basis, and the proportion of Rubisco of total soluble proteins decreases as well. Low-light leaves have decreased chlorophyll a/b ratio, but the amount of chlorophyll may be similar to high-light leaves. In general, the ratio of soluble proteins (and Rubisco) to chlorophyll declines considerably in low-light leaves (reviewed by Boardman 1977; Björkman 1981; Anderson et al. 1988; Evans 1988; Givnish 1988). However, the low-light grown plants which had 20-h
photoperiod did not exhibit these usual low-light features. This is illustrated in the comparison between low-light and glasshouse-grown wild-type tobaccos, presented in Table 4.1.

The gas exchange characteristics of wild-type tobaccos grown in the low light cabinet or in the glasshouse were similar, as reported earlier (Price et al. 1995a; 1998). There was no large difference in most biochemical parameters, either. The decrease in Rubisco content (30%) in low-light tobacco was modest compared to about 2–4 fold difference in Rubisco content in high-and low-light grown leaves of *Phaseolus vulgaris* and *Alocasia macrorrhiza* (Seemann 1989). The high-light tobacco leaves had only slightly higher ratio between Rubisco and chlorophyll (about 1.8 times the Rubisco/Chl ratio in low-light plants). This ratio can change as much as 4–5 fold as a result of light acclimation (Wild et al. 1975; Evans 1988). The chlorophyll $a/b$-ratio was 2.88 in low-light tobaccos, as opposed to 3.08 in high-light plants. Again the difference was small: for example, in pea leaves the $a/b$ ratio can vary from 3.2 (high light) to 2.4 (low light) see Fig. 1 in Evans (1988).

However, the low- and high-light grown tobaccos were not completely identical. RuBP pool sizes, measured at high light, were significantly smaller in low-light tobaccos in comparison to high-light tobaccos (about 55% of the RuBP in high-light leaves Table 4.1). This resembles the results obtained from high- and low-light grown *Phaseolus vulgaris* and *Alocasia macrorrhiza* (Seemann 1989). On a leaf area basis, the high-light leaves of both species had 1.4-2 times more RuBP under saturating light, irrespective of the $C_i$. However, if RuBP pools are normalised against Rubisco content in tobacco, this ratio is much less affected by the growth light (Table 4.1). This response was also noted by Seemann et al. (1989), and the authors concluded that different processes in light acclimation work to maintain stable ratios between Rubisco, its substrate RuBP and product, 3-PGA. This is obviously true for tobacco as well. An alternative explanation for the lower RuBP pools in low-light tobaccos is that the amounts of other Calvin cycle enzymes, in addition to Rubisco, may be smaller (Besford 1986), and this could restrict the RuBP regeneration rate. Nevertheless, although the amount of Rubisco and the RuBP/Rubisco sites-ratio decreased in low-light leaves, it did not affect the CO$_2$ assimilation rates.
Traditionally in light acclimation studies the length of the photoperiod is kept constant between different light treatments. In this study, the tobacco plants grown at low light had an extended photoperiod, 20 hours. This long daylength overcomes the usual effect of very low irradiance on the leaf composition, since the low-light grown leaves had only slight shade characteristics. A similar conclusion was made from acclimation studies with tomato: the leaves seem to respond to a total amount of photons absorbed, rather than to the intensity of the irradiance (Withers et al. 1991). This aspect of shade tolerance is very interesting regarding the signal transduction pathway that causes the changes leading to light acclimation in leaves. However, little research has been conducted on daylength effect on light acclimation.

4.4.2 CO₂ ASSIMILATION DECREASES IN ANTI-BF AND ANTI-GAPDH PLANTS AS RuBP CONTENTS DECLINE

The decrease in either cytochrome bf-content or GAPDH activity caused the CO₂ assimilation rates, measured at ambient (350 μbar) CO₂ and high light, to decline (Fig. 4.3) as demonstrated earlier (Price et al. 1995a; 1995b; 1998). The amount of Rubisco or soluble proteins did not change a lot in either of the antisense plant types, when compared to control plants, (Fig. 4.5) which is consistent with the earlier observations by the same authors. Only in the most severe antisense plants soluble proteins and Rubisco contents decreased, but not sufficiently to explain the reduction in CO₂ assimilation: at its greatest, the reduction in Rubisco content was about 50% in both plant lines, whereas the CO₂ assimilation rates decreased up to 90% of the wild-type rates (Fig. 4.3).

The reduction in CO₂ assimilation rates was not caused by a limitation of CO₂ availability either, since Cᵢ in fact increased in antisense plants. There was no marked effect on the functioning of stomata in anti-GAPDH plants. (Figs. 4.4 C&D). Interestingly, a decrease in the stomatal conductance was observed when the cytochrome bf-content was severely reduced (Fig. 4.4 A). This has been noted before (Price et al. 1998) and possibly implies that the antisense gene has decreased the electron transport capacity in guard cells as well. However, the decrease in stomatal conductance did not contribute the lower CO₂ assimilation rates: also in these antisense plants the Cᵢ was above the wild-type levels.
The reduction in CO₂ assimilation rates in both plant lines was associated with a decrease in RuBP pool sizes (Fig. 4.6). This resembles the effect of decreased phosphoribulokinase (PRK) content in tobacco leaves, which also reduced RuBP content and CO₂ assimilation rates (Paul et al. 1995b). I will discuss in more detail, to what extent low RuBP contents truly limit CO₂ assimilation in antisense plants in Chapter 5.

4.4.3 RuBP AND 3-PGA POOL SIZES

The concentration of any metabolite is the result of its production and consumption. The rate of RuBP consumption depends on the amount and activity of Rubisco, and the availability of Rubisco’s other substrate, CO₂. The production, or regeneration, of RuBP then depends on the availability of ATP and NADPH for the Calvin cycle, and on the activity of the Calvin cycle enzymes. In anti-GAPDH plants the reason for the lowered RuBP pools is straightforward: the reduced amount of GAPDH forms a bottleneck in the regeneration pathway. In anti-\(bf\) plants, however, there are two reasons. Firstly, the electron transport rate has decreased in these plants, supposedly leading to decreased ATP and NADPH synthesis. The lack of ATP and NADPH then restricts the rate of RuBP regeneration, because several steps in the Calvin cycle use them. Secondly, there is a possibility that in anti-\(bf\) plants the ferredoxin-thioredoxin-mediated activation of some of the Calvin cycle enzymes has also decreased, further impairing the RuBP regeneration capacity. This assumption is supported by the large decrease in NADP-MDH activation state, which indicates a severe limitation of the NADPH synthesis and ferredoxin reduction and is discussed in more detail in Section 4.4.5.

The decrease in either cytochrome \(bf\) content or GAPDH activity had little effect on 3-PGA (Fig. 4.6 C&D). In the case of anti-GAPDH plants, one could expect to see an increase in 3-PGA levels, since the consumption by the Calvin cycle has declined. It has been suggested, that some 3-PGA can be translocated from chloroplasts to cytosol, and this 3-PGA can then further be metabolised there, preventing the accumulation of 3-PGA in the chloroplast in anti-GAPDH plants (Price et al. 1995a). In anti-\(bf\) plants, the amount of 3-PGA did not decrease markedly even in the severely affected plants with very low CO₂ assimilation rates. This further
indicates that the turnover rate of the whole Calvin cycle may be decreased, due to lack of ATP and NADPH and/or inactivation of the thioredoxin-regulated enzymes.

4.4.4 DIMINISHED CONSUMPTION OF ATP IN CHLOROPLASTS 
INCREASES THE WHOLE-LEAF ATP/ADP RATIO IN ANTI-GAPDH 
PLANTS

The increase in the whole-leaf ATP/ADP ratio in anti-GAPDH plants (Fig. 4.7 F) is comparable to the observations of other transgenic tobaccos with impaired capacity for carbon assimilation, either due to the reduced amount of Rubisco (Quick et al. 1991), or PRK (Paul et al. 1995a). This effect of decreased carbon fixation capacity on the adenylate pools is analogous to the increase in ATP/ADP ratio when photosynthesis is limited by low CO₂ (Gardeström 1987; Gilmore and Björkman 1994a). An increase in ATP/ADP ratio is expected, since a majority of ATP which is generated in chloroplasts is used in carbon metabolism. Interestingly, the reduction state of the stromal NADP pool (estimated from NADP-MDH activation level, Fig. 4.8 B) did not increase in anti-GAPDH plants. This will be discussed more in Section 4.4.5.

4.4.5 ADENYLATE RATIOS DID NOT CHANGE IN ANTI-BF PLANTS

Unlike in anti-GAPDH plants, adenylate ratios in anti-bf plants did not change (Fig. 4.7 E). However, the severely decreased activation sate of NADP-MDH (Fig. 4.8 A) suggests that the rate of linear electron transport, and presumably also proton translocation have slowed down markedly. Chlorophyll fluorescence measurements also have indicated that the transthylakoid pH gradient is low in anti-bf plants (Price et al. 1995b). Since only the whole-leaf adenylates were measured, it is possible that the cytosolic and mitochondrial pools may have masked the changes in chloroplast ATP/ADP ratio. In a study conducted with protoplasts of wheat leaves, it was found that approximately 47% of the total adenylates were located in chloroplasts, 44% in cytosol and 9% in mitochondria (Stitt et al. 1982). The ATP/ADP ratio in cytosol is higher than in chloroplasts, whether measured from light or dark, because ATP is transported there from chloroplasts and mitochondria (Stitt et al. 1982; Gardeström and Wigge 1988; Heineke et al. 1991). In addition, it is possible that the chloroplast
adenylate ratio could have truly stayed at wild-type level in anti-\(bf\) plants. This may be because the rate of ATP consumption by the Calvin cycle has decreased, due to inactivation of redox-regulated Calvin cycle enzymes. To investigate this possibility, the activation state of chloroplast NADP-dependent malate dehydrogenase (MDH) was measured. The activation state can be used as an indicator of stromal NADP\(^+\)/NADPH ratio.

### 4.4.6 LINEAR ELECTRON TRANSPORT AND STROMAL REDUCTION STATE

Chloroplast NADP-dependent MDH catalyses the reaction where oxaloacetic acid (OAA) is reduced to malate. Malate can leave the chloroplast via a specific dicarboxylate carrier in the envelope, and can be re-oxidised in the cytocol to OAA by NAD-dependent MDH. OAA can then return to the chloroplast (Scheibe 1987). This metabolite shuttle, malate valve, can prevent the overreduction of the chloroplast NADP pool and thus facilitate the continuation of electron transport (Backhausen et al. 1994). NADP-MDH is normally inactive in darkness or at low light, and activates upon illumination. This light activation is mediated by the ferredoxin-thioredoxin system, which signals the onset of light to several chloroplast enzymes (Scheibe et al. 1986a). However, this reductive activation is inhibited by NADP\(^+\), making the enzyme sensitive to NADP\(^+\)/NADPH ratio (Scheibe and Jacquot 1983). Because of this additional allosteric regulation, the activation state of MDH can be used as a sensor for the stromal NADP-pool reduction state (Harbinson et al. 1990; Foyer 1993).

#### 4.4.6.1 NADPH synthesis is decreased in anti-\(bf\) plants

When the amount of cytochrome \(bf\) complex was reduced, the activation level of MDH decreased significantly (Fig. 4.8. A). The decrease in activation level was linearly correlated with the electron transport rate (calculated from CO\(_2\) assimilation, Fig. 4.9), resembling the results by Krall et al. (1995) and Harbinson et al. (1990). These authors modified the rate of linear electron transport by exposing leaves to different irradiances, and obtained a strong correlation between electron flux and NADP-MDH activation. The low activation state of MDH in anti-\(bf\) plants is clear
evidence of the decreased photosynthetic electron transport capacity, and indicates that the reduction state of the stromal NADP+ pool is significantly lowered. Decreased NADP+/NADPH ratio has two main implications on carbon fixation. Firstly, the lack of reducing equivalents limits the function of the Calvin cycle and RuBP regeneration. Secondly, the low stromal reduction status reduces the activity of the chloroplast ferredoxin-thioredoxin pathway, and thus the enzymes regulated by this system are likely to be less active. It has been shown that, of the redox-modulated chloroplast enzymes, fructose 1,6-bisphosphatase is especially sensitive to the stromal reduction state (Holfgrefe et al. 1997). The decreased activity of the Calvin cycle enzymes may also explain, why the ATP/ADP ratio did not change in anti-bf plants. The consumption of ATP by the Calvin cycle may be severely restricted, and the decreased rate of ATP synthesis is overruled (see Section 4.4.5).

The MDH activation level in the most severe anti-bf plants, 6%, (Figs. 4.8 A, 4.9) is equivalent to the values found in the wild-type leaves from darkness or low light growth conditions (data not shown). This demonstrates convincingly how the perception of light signal is prevented in anti-bf plants because the electron transport rate is restricted. When exposed to high light, MDH in the low-light grown wild-type leaves activated to over 80% of the total activity. This activation level is high, compared to for example 35% activation level in pea leaves measured in air and 1000 μmol quanta m⁻² s⁻¹ (Harbinson et al. 1990) and probably indicates the stress that the low-light grown plants experience at high light.

4.4.6.2 Stromal NADP⁺ pool is not overreduced in anti-GAPDH plants

MDH activation did not change in anti-GAPDH plants when compared to controls (Fig. 4.8 B). This is interesting, since one would expect it to rise in the antisense plants with decreased capacity to utilise reducing equivalents in carbon fixation. These results are comparable to data obtained by Lauerer et al. (1993) who measured the activation state of NADP-MDH from plants with reduced amounts of Rubisco. When measured at growth irradiance, the MDH activation of the anti-Rubisco plants was similar to the control plants (Lauerer et al. 1993). In my study, the anti-GAPDH plants and their controls were grown in a glasshouse. The plants experience light intensities up to 900 μmol quanta m⁻² s⁻¹, so they were well
acclimated to the gas exchange measuring conditions (1000 μmol quanta m$^{-2}$ s$^{-1}$) and avoided stromal overreduction. The maintenance of wild-type like NADP$^\ast$/NADPH ratio in anti-GAPDH plants requires drastic reduction of PSII efficiency, which is shown to happen (Price et al. 1995a). The chlorophyll fluorescence measurements indicated that the plastoquinone pool in anti-GAPDH plants stayed mainly oxidised, even under high light. The decrease in the quantum yield of PSII can be the result of either a direct effect of high transthylakoid pH gradient on PSII, or the enhancement of nonradiative dissipation of excitation energy in the pigment bed enhanced by xanthophyll cycle pigments (Demmig-Adams et al. 1989b; Horton and Hague 1991). In Chapter 7 it is shown that anti-GAPDH plants have significantly higher de-epoxidation state of xanthophylls at high light (Fig. 7.8) which is consistent with a greater need for nonradiative energy dissipation (Demmig-Adams et al. 1996). In addition, the high-light grown plants had overall about 1.5 times higher total activity of NADP-MDH than low-light plants (Table 4.1). The increased amount of NADP-MDH may then be another means to avoid excessive reduction of the NADP$^\ast$ pool, acting as an additional photoprotective mechanism.

**4.4.7 SUMMARY**

Two transgenic tobacco lines, whose balance between photosynthetic light and dark reactions had been altered to opposite directions were compared. The amount of one of the Calvin cycle enzymes, glyceraldehyde 3-phosphate dehydrogenase, was reduced in anti-GAPDH plants. The availability of NADPH, and presumably also ATP, for the Calvin cycle was reduced in anti-cytochrome $b$ $f$ plants with impaired electron transport. As a consequence, both plant types had low RuBP regeneration capacities, leading to decreased RuBP pool sizes. The whole-leaf ATP/ADP ratio increased in anti-GAPDH plants as a result of a restricted carbon assimilation. However, there was no change in the adenylate ratio in anti-$b$ $f$ plants, although the lowered activation state of chloroplast NADP-MDH confirmed that the electron transport rate was severely affected. This may be because cytosolic adenylate pools masked the drop in chloroplast ATP. However, the consumption of ATP in anti-$b$ $f$ chloroplasts may slow down as well, due to inactivation of redox-regulated Calvin cycle enzymes.
Although the ATP/ADP ratio increased in anti-GAPDH plants, the activation state of NADP-MDH did not change, indicating that they avoided overreduction of the stromal NADP⁺ pool via increased nonradiative dissipation of light energy and also possible via increased amount of chloroplast NADP-MDH.
CHAPTER 5

RUBISCO ACTIVATION IN ANTI-BF AND ANTI-GAPDH TOBACCOS
5.1 INTRODUCTION

In Chapter 4 I researched the effects of reduced electron transport capacity (anti-cytochrome *b* plants) or Calvin cycle activity (anti-GAPDH plants) on leaf CO$_2$ assimilation, metabolite and adenylate pools as well as energy status, as measured by the ATP/ADP ratios and NADP-MDH activation states. This Chapter will examine what effects these manipulations have had on Rubisco activation and catalysis.

5.1.1 THE STRUCTURE AND CATALYSIS OF RUBISCO

5.1.1.1 The structure of Rubisco

The primary carbon fixing enzyme in plants, Rubisco (ribulose 1,5-bisphosphate carboxylase-oxygenase) consists of eight large (LSu) and eight small subunits (SSu). The subunits are assembled such that four LSu dimers form the central part of the enzyme, and the SSus are located at the poles of the holoenzyme as two separate tetramers. The catalytic sites are situated at an interface between the two LSus of a dimer, and both subunits contribute to its conformation (Larimer *et al.* 1987; Knight *et al.* 1990; Curmi *et al.* 1991). The presence of SSus enhances the catalytic activity of Rubisco considerably, possibly by stabilising the structure of the holoenzyme or modifying the conformation of the active site itself. (Andrews 1988; Morell *et al.* 1997).

5.1.1.2 Carboxylation and oxygenation reactions

Rubisco is a bifunctional enzyme, which primarily catalyses either carboxylation or oxygenation of a five-carbon phosphorylated sugar, ribulose 1,5-bisphosphate (RuBP). The reaction mechanism of carboxylation has been studied in detail, while the oxygenation pathway is less well understood (Lorimer *et al.* 1986; Andrews and Lorimer 1987; Hartman and Harpel 1994). The carboxylation involves several sequential steps when the substrates and reaction intermediates remain bound to the active site. Briefly, bound RuBP first undergoes deprotonation and enolisation forming 2,3 enediol. Carboxylation of the 2,3 enediol yields a six-carbon intermediate (3-keto 2-carboxyarabinitol 1,5-bisphosphate). This intermediate is hydrated, and
subsequently cleaved yielding one molecule of 3-PGA and a carbanion form of PGA. The carbanion is protonated to form the second 3-PGA.

The oxygenation of RuBP is the starting point of photorespiration in C₃ plants. During oxygenation O₂ molecule, instead of CO₂, attacks the 2,3 enediol intermediate. The end products of this reaction are one molecule of 3-PGA, and one molecule of 2-phosphoglycolate (2-PGA), which is recycled to recover 75% of the carbon skeletons lost this way (Lorimer 1981). The photorespiratory pathway consumes reducing equivalents and ATP, thus decreasing the overall efficiency of photosynthesis (Chapter 1).

5.1.1.3 Side reactions

During in vitro-assays, the catalytic rate of purified, activated Rubisco gradually slows down. This phenomenon has been termed failover, and is the result of an accumulation of inhibitory by-products of Rubisco catalysis into the active sites. (Edmondson et al. 1990a; 1990b; 1990c; 1990d; Zhu and Jensen 1991a; 1991b) This is because the 2,3 enediol intermediate can undergo various side reactions. Incorrect reprotonation of the enediol produces xylulose 1,5-bisphosphate (XuBP), 3-ketoarabinitol 1,5-bisphosphate (KABP) or 3-ketoribitol 1,5-bisphosphate. In addition, in studies conducted with mutant Rubisco (Morell et al. 1994) and Synechococcus Rubisco LSu core in the absence of SSus (Morell et al. 1997) the formation of 1-deoxy-D-glycero-2,3-pentodiulose-5-phosphate was detected as a result of β-elimination of inorganic phosphate from the enediol intermediate.

5.1.2 REGULATION OF RUBISCO ACTIVITY

5.1.2.1 Carbamylation

The active site of all Rubiscos is catalytically competent only when it is complexed with CO₂ and Mg²⁺. This takes place when an activating CO₂ molecule attaches to the ε-amino group of Lysine 201 residue in LSu forming a carbamate. The carbamylation is followed by a rapid binding of Mg²⁺. The presence of a metal ion stabilise the carbamate and is required for binding the substrate. The activation is a
result of a subtle change in the conformation of the LSu (Andrews and Lorimer 1987).

5.1.2.2 Chloroplast CO$_2$ and sugar phosphates

The carbamylation of Rubisco occurs spontaneously in vitro if the enzyme is incubated in the presence of CO$_2$ and Mg$^{2+}$ in the absence of RuBP. However, the conditions in chloroplasts are such that spontaneous carbamylation cannot produce the high activation levels (80-90%) that are observed in illuminated leaves. Firstly, the $K_{act}(CO_2)$ (Michaels-Menten constant for activation of Rubisco by CO$_2$) required for activation at stromal pH and Mg-concentration is about 3 times higher than air-level chloroplast CO$_2$ ($K_{act}$ 25-30 μM, chloroplast CO$_2$ about 10 μM) (Lorimer et al. 1976). Secondly, numerous compounds can bind to Rubisco active sites and act as inhibitors.

Phosphorylated sugars have a strong tendency to bind either to uncarbamylated Rubisco sites preventing activation, or to carbamylated sites inhibiting catalysis. The substrate RuBP is an important regulator of Rubisco, since it binds tightly to uncarbamylated sites (Jordan and Chollet 1983) and its concentration in the chloroplasts in light can be very high (von Caemmerer and Edmondson 1986). Several Calvin cycle intermediates (fructose 1,6-bisphosphate, glucose 1-phosphate) bind to Rubisco as well (Jensen and Bahr 1977). Potent inhibitors are formed as catalytical by-products in Rubisco active sites, as discussed in Section 5.1.1.3 (failover products). Some plant species (soybean, tobacco, pineapple) produce a specific dark inhibitor of Rubisco, carboxyarabinitol 1-phosphate (CA1P), which binds to carbamylated sites and has to be removed from the active sites before catalysis can occur (Gutteridge et al. 1986).

5.1.2.3 Other effectors

It has been frequently suggested that 3-PGA may be an important modulator of Rubisco activity in vivo, since its concentration can be very high in chloroplasts and it acts as a competitive inhibitor with respect to RuBP (Prinsley et al. 1986; von Caemmerer and Edmondson 1986; Foyer et al. 1987; Servaites and Geiger 1995). The role of inorganic phosphate (P$_i$) in regulating Rubisco activity is more complex.
When chloroplastic P<sub>i</sub> is depleted, Rubisco activation decreases (Heldt et al. 1978; Sharkey et al. 1986a; Brooks et al. 1988b). It has been implied that this is due to a limitation for ATP synthesis, which then impairs Rubisco activase function (Sharkey 1990). On the other hand, it has been shown in vitro, that P<sub>i</sub> promotes Rubisco activity directly (Sawada et al. 1990; Sawada et al. 1992).

5.1.3 RUBISCO ACTIVASE

The carbamylation state of Rubisco changes in vivo in response to environmental conditions. The most common determinant for carbamylation is light: the activation state of Rubisco is low in darkness or low light, and it increases as irradiation increases (Mächler and Nösberger 1980; Robinson and Portis 1988). The changes in carbamylation levels are mediated by a soluble chloroplast protein, Rubisco activase. Activase was first identified in studies with Arabidopsis mutant which required high CO<sub>2</sub> to grow, and did not show the normal light activation of Rubisco (Salvucci et al. 1985).

5.1.3.1 Activase structure

Rubisco activase has been detected in all higher plants that have been studied (Salvucci et al. 1987). In many plant species there are two closely related activase isoforms, which result from a differential splicing of pre-mRNA species (Werneke et al. 1989). In spinach, the two polypeptides have molecular masses of 42 and 45 kDa (Werneke et al. 1988). The significance of the different isoforms is not fully understood, but a recent study reported that the 45 kDa form is more resistant towards thermal inactivation (Crafts-Brandner et al. 1997).

Tertiary structure of activase has not been determined yet. It is known though, that activase polypeptides form oligomers, and the size of the aggregations are affected by polyethyleneglycole (Salvucci 1992), Mg<sup>2+</sup>, ATP or ADP concentration (Wang et al. 1993). The aggregation increases the activity of Rubisco activase (Wang et al. 1993; Lilley and Portis 1997) and thus may be a regulatory mechanism.
5.1.3.2 *Mechanism of activase action*

The major function of Rubisco activase is to facilitate the detachment of RuBP or any other tight-binding sugar phosphate from Rubisco active sites (Wang and Portis 1992). In addition, it simultaneously lowers the $K_{act}(CO_2)$ such that high Rubisco carbamylation levels become possible under physiological conditions (Portis *et al.* 1986). An intrinsic property of activase is that it hydrolyses ATP, and this ATPase activity is inhibited by ADP (Streusand and Portis 1987; Robinson and Portis 1989a). The exact mechanism how activase binds to Rubisco and releases the bound ligand, simultaneously hydrolysing ATP, is not known. There seems to be no covalent modification of Rubisco, or cleavage of sugar phosphate, but rather, the interaction between Rubisco and activase involves a conformational change in Rubisco as a result of the physical interaction between the two proteins (Portis 1990; Salvucci and Ogren 1996).

Several distinct models have been developed to explain the function of activase. De Jiménez *et al.* (1995) suggested that activase belongs to a group of proteins called chaperones, that participate in the assembly and folding of other proteins. This was later challenged by Eckardt and Portis (1997), who observed that activase could not restore the activity of heat-denaturated Rubisco, which was the basis of the hypothesis by De Jiménez *et al.* Andrews *et al.* (1995b; 1996) proposed a model where activase binds to inactive Rubisco, and forces the opening of mobile loops in the active sites that are closed over the bound ligand. The hydrolysis of ATP is coupled to a conformational change in the activase itself, preceding the binding to Rubisco. A slightly different scheme was suggested by Salvucci and Ogren (1996). In their model the binding of ATP to activase facilitates the interaction between inactive Rubisco and activase. ATP hydrolysis takes place only after activase is complexed with Rubisco, and causes conformational changes that loosen the binding of sugar phosphate to the active site. Lately, a novel model was presented where activase functions similarly to actin, and the changes in the aggregation state are the basis of activase activation (Lilley and Portis 1997). The authors suggest that binding of ATP to activase monomers causes them to assemble into functional oligomers. The oligomers then interact with Rubisco holoenzyme and assist in the release of sugar phosphates in an event powered by ATP hydrolysis.
5.1.3.3 Activase regulation

Since activase requires ATP to function, and is inhibited by ADP, it has been suggested that activase's sensitivity to the stromal ATP/ADP ratio enables Rubisco activity to respond to irradiation (Streusand and Portis 1987; Robinson and Portis 1988; Robinson and Portis 1989a). However the ATPase activity of activase is not directly connected to the activation of Rubisco (Robinson and Portis 1989a; Shen and Ogren 1992). In addition, light activation of Rubisco in leaves can take place without noticeable changes in the ATP/ADP ratio (Brooks et al. 1988a). The same was seen in in vitro experiments, where light activation of Rubisco was observed under saturating ATP (Campbell and Ogren 1990b).

These and other observations lead to the conclusion that light activation of Rubisco is mediated by some unknown factor, in addition to the ATP requirement. (Campbell and Ogren 1992). This factor requires electron transport through PSI and formation of a transthylakoid pH gradient (Campbell and Ogren 1990a), and could be associated with thylakoid membranes (Portis 1995). Other details of this mechanism remain unknown. However, it can be hypothesized that Rubisco activase mediates the light signal to Rubisco by somehow sensing the transthylakoid pH gradient (Portis 1995). It has been suggested that activase itself may be associated with thylakoids (Byrd et al. 1995).

5.1.4 THE OUTLINE OF THE CHAPTER

In this Chapter the carbamylation state and in vitro-catalytic properties of Rubisco from anti-bf and anti-GAPDH plants are explored. The data presented is obtained from the same material (the other half of the freeze-clamped leaf disc) that was used for photosynthesis measurements, adenylate, metabolite and NADP-MDH assays, presented in Chapter 4. In this study the carbamylation state of Rubisco was measured using the CPBP-binding method, which utilises the different affinity of carbamylated and noncarbamylated Rubisco active sites to a tightly-binding 14C-labelled ligand (Butz and Sharkey 1989). In addition, the activation state of Rubisco was measured by comparing the initial and total activities, ie. the activity assayed immediately after rapid extraction and after an activating incubation in the presence of
Mg\(^{2+}\) and CO\(_2\). These two assays are seldom made from the same material and it was
of interest to have a close look at both carbamylation and activation ratios of Rubisco
in transgenic plants.

5.2 MATERIAL AND METHODS

5.2.1 PLANT MATERIAL, SAMPLING, METABOLITE AND ENZYME
ASSAYS

The antisense plant types and their growth conditions are described at Section
4.2.1. The measurements of gas exchange parameters and the subsequent rapid
freeze-clamping of leaves, as well as metabolite, GAPDH and cytochrome \(bf\) assays
are described in Sections 4.2.3. to 4.2.7.

5.2.2 RUBISCO CARBAMYLATION LEVEL AND ACTIVITY ASSAYS

Half of the freeze-clamped leaf disc (2.7 cm\(^2\)) was homogenised in 1.4 ml of
ice-cold CO\(_2\) free extraction buffer containing 50 mM Hepes-NaOH pH 7.8, 1 mM
Na-EDTA, 5 mM MgCl\(_2\), 10 mM DTT, 1 % (w/v) of polyvinylpolypyrrolidone and 1
mM phenylmethyl-sulphonylfluoride (PMSF). The extract was centrifuged for 15-20
seconds with a benchtop rotor and the supernatant was used for the assays
immediately. Rubisco initial activity was measured immediately at 25 °C by adding a
10 \(\mu\)l aliquot of the supernatant into 262 \(\mu\)l of the reaction mixture containing 55 mM
EPPS-KOH pH 8, 22 mM MgCl\(_2\), 0.27 mM Na-EDTA, 13 mM NaH\(^{14}\)CO\(_3\) (specific
radioactivity about 1500 cpm/nmol) and 0.4 mM RuBP. Total activity was assayed
after 10 \(\mu\)l of the supernatant was incubated at 25 °C for 5 minutes in 55 mM EPPS-
KOH pH 8, 22 mM MgCl\(_2\), 0.27 mM Na-EDTA, 14.5 mM NaH\(^{14}\)CO\(_3\). The assay was
initiated by adding 0.4 mM RuBP. In both assays the reaction was terminated after
60 seconds by adding 125 \(\mu\)l of concentrated formic acid, the samples were dried and
acid-stable \(^{14}\)C was measured by liquid scintillation. Rubisco catalytic site
concentration was determined by the stoichiometric binding of \([^{14}\text{C}]\)CPBP and the
carbamylation level was measured by exchanging loosely bound \([^{14}\text{C}]\)CPBP at
noncarbamylated sites with an excess of $[^{12}\text{C}]\text{CPBP}$ (Butz and Sharkey 1989). Rubisco-CPBP-complexes were separated from unbound CPBP by gel filtration as described in Section 2.2.

5.2.3 **RUBISCO ACTIVASE CONTENT**

Rubisco activase content was measured from leaf extracts as described by Mate et al. (1993). Briefly, a leaf disc was extracted quickly in ice-cold buffer (50 mM Hepes-KOH pH 7.8, 10 mM MgCl$_2$, 1 mM EDTA, 10 mM DTT, 1.5% PVPP and 1 mM PMSF) and centrifuged. Mercaptoethanol and SDS were added to the supernatant with the final concentrations being 5% and 2.3%, respectively. Extracts were denatured by boiling for about 5 minutes, aliquoted, snap-frozen and stored at -80 °C. Samples were boiled again briefly prior to loading on 10-20% Tricine gels (Novex). Proteins were resolved by electrophoresis, transferred to a nitrocellulose membrane and the amount of Rubisco activase protein was measured using ECL peroxidase-luminol protocol (Amersham, UK) and Image-Quant software (Molecular Dynamics, CA, USA), using purified tobacco activase as standard.
5.3 RESULTS

5.3.1 CO₂ ASSIMILATION RATE AND LEAF RuBP CONTENT

In Chapter 4 it was shown that the CO₂ assimilation rates in both transgenic tobacco plants were accompanied by reductions in the leaf RuBP contents (Fig. 4.6). In Fig. 5.1 the assimilation rates are presented as a function of leaf RuBP content. For comparison, this Figure contains results from previous experiments (Price et al. 1995a; 1998), where the assimilation rates and metabolite contents were measured from leaf material grown and sampled under conditions similar to my study (small symbols). The response of assimilation rate to RuBP content was different in the two transgenic plant types. In anti-GAPDH plants, the CO₂ uptake rate increased linearly when the RuBP concentration increased until the RuBP pool was about 50 μmol m⁻², beyond which the assimilation was independent on the RuBP. In anti-bf plants, the relationship between CO₂ assimilation rate and RuBP content was more linear.

5.3.2 RUBISCO CARBAMYLATION AND IN VITRO-ACTIVITIES

To examine more closely why the response of CO₂ uptake to RuBP content was different in the two plant types, the in vitro-characteristics of Rubisco were measured after rapid extraction. The leaves were sampled after they had been in high light (1000 μmol quanta m⁻² s⁻¹) for at least 40 minutes. When the amount of cytochrome bf content reduced, the Rubisco carbamylation status declined (Fig. 5.2 A). On an average, the carbamylation level in the low-light grown control plants, measured at high light, was 79%, and it decreased below 50% in the most severe anti-bf plants. There was no change in the carbamylation level in the anti-GAPDH plants (Fig. 5.2 B). The average Rubisco carbamylation status in all glasshouse grown plants was the same as in the low-light wild-types (77%).
Fig. 5.1. CO$_2$ assimilation rates of anti-GAPDH and anti-$bf$ and tobacco plants at ambient CO$_2$ as a function of leaf RuBP content. CO$_2$ assimilation rates of: A. glasshouse-grown wild-type (●) and transgenic tobaccos with different activities of GAPDH (○) and B. low-light wild-type tobacco plants (■) and transgenic tobacco plants with a variety of cytochrome $bf$ contents (□). The small symbols represent results from previous experiments: anti-$bf$ data is from Price et al. (1998) and anti-GAPDH data from Price et al. (1995a). The assimilation rates were measured at an irradiance of 1000-1500 μmol quanta m$^{-2}$ s$^{-1}$ at CO$_2$ partial pressure of 350-380 μbar in air and leaf temperature of 25°C. Leaves were kept under these conditions for at least 40 minutes before the CO$_2$ assimilation rate was recorded and a leaf disc was rapidly freeze-clamped. The lines were fitted by hand.
Fig. 5.2. Rubisco characteristics of anti-bf and anti-GAPDH tobacco plants at ambient CO₂. Carbamylation level (A, B), initial in vitro-activity of Rubisco/carbamylated Rubisco sites (C, D), and total in vitro-activity of Rubisco/total Rubisco sites (E, F). Symbols: glasshouse-grown wild-type (●) and transgenic tobaccos with different activities of GAPDH (○), low-light wild-type tobacco plants (■) and transgenic tobacco plants with a variety of cytochrome b/f contents (□). The leaves were kept at 350 μbar CO₂, air, at 1000 μmol quanta m⁻² s⁻¹ prior to the sampling. The activity results (C-F) are expressed as a percentage of the average wild-type activities. The means ±S.E. of the wild-type activities are given in the panels.
The carbamylation state assays were combined with the *in vitro*-assays of Rubisco activity from the same leaf extract. The initial activity of Rubisco (immediately after extraction) and total activity (after 5 minutes incubation at high Mg$^{2+}$ and CO$_2$) were measured. The catalytic turnover rates were calculated by dividing the initial or total *in vitro*-activities by the number of carbamylated or total Rubisco sites which were measured with the $[^{14}C]$CPBP method. In Fig. 5.2 C-F the catalytic turnover rates of Rubisco sites are presented as a proportion of the average rate in control plants. There was no difference in the turnover rate of the carbamylated sites (initial activity/carbamylated sites) in either of the transgenic plant types when compared to control plants. However, in anti-bf plants the total activity per total Rubisco sites decreased with the decreasing cytochrome $f$ content, such that the most severe anti-bf plants had about half the turnover rate of the control plants. The total turnover rates in anti-GAPDH plants were similar to controls.

5.3.3 THE CATALYTIC ACTIVITY OF RUBISCO IN VIVO

The carbamylation state of Rubisco decreased in anti-bf plants (Fig. 5.2 A), suggesting that the availability of RuBP may not be the only reason for low CO$_2$ assimilation rates in these plants. In order to assess whether the catalysis of Rubisco is affected in anti-bf plants *in vivo*, the CO$_2$ assimilation rate per carbamylated Rubisco sites is plotted as a function of the leaf RuBP content in Fig. 5.3. This figure also includes previous results from Price *et al.* (1995a; 1998), which are presented as small symbols

Since the turnover rate of carbamylated Rubisco sites decreases when RuBP becomes limiting, it is not easy to assess the *in vivo*-catalysis of Rubisco in these transgenic plants with reduced RuBP pool sizes. However, knowing that Rubisco functions optimally in anti-GAPDH plants, as judged by the high carbamylation state and *in vitro*-activities (Fig. 5.2 B,D&F), these plants were used as a control of the relationship between Rubisco and RuBP *in vivo*. In anti-GAPDH plants CO$_2$ assimilation per carbamylated site increased with increasing RuBP, until RuBP pool reached approximately twice the Rubisco site concentration (Fig. 5.3 A). Beyond this, the catalytic rate was independent of RuBP (Price *et al.* 1995a). This relationship between RuBP and Rubisco activity *in vivo* is illustrated by
Fig. 5.3 CO₂ assimilation rate per carbamylated Rubisco sites as a function of leaf RuBP content in anti-\(bf\) and anti-GAPDH tobacco plants at ambient CO₂. A. anti-GAPDH plants, B. anti-\(bf\) plants. The small symbols represent results from previous experiments: anti-\(bf\) data is from Price et al. (1998) and anti-GAPDH data from Price et al. (1995a). The assimilation rates were measured at an irradiance of 1000-1500 μmol quanta m⁻² s⁻¹ at CO₂ partial pressure of 350-380 μbar in air and leaf temperature of 25°C. The arrows indicate the average (±S.E.) Rubisco site concentration in both transgenic plants and their controls, which was 20±1 and 13±1 μmol m⁻² for all anti-GAPDH and all anti-\(bf\) plants, and their controls, respectively. The lines were fitted by hand, see text for details.
the dotted line. In Fig. 5.3 B a line depicting a similar relationship between the CO₂ assimilation rate per carbamylated sites and RuBP pool size for anti-bf plant is drawn, assuming that the catalytic activity of carbamylated sites saturates at RuBP/sites ratio of 2. Although this data set is scattered, there is no real evidence of any impairment of Rubisco catalytic activity in anti-bf plants which can not be explained by lowered RuBP pool sizes.

5.3.4 NADP-MDH ACTIVATION AND RUBISCO CARBAMYLATION IN ANTI-BF PLANTS

The decrease in Rubisco carbamylation in anti-bf plants is intriguing (Fig. 5.2 A) and it seems to correlate with a simultaneous decrease in the NADP-malate dehydrogenase activation level (Fig. 4.8). This is illustrated by Fig. 5.4 where the carbamylation state of Rubisco is presented as function of NADP-MDH activation state.

![Graph showing the comparison between the activation state of NADP-malate dehydrogenase and the carbamylation state of Rubisco in anti-bf plants. Symbols: low-light wild-type tobacco plants (■) and transgenic tobacco plants with a variety of cytochrome bf contents (□). The leaves were kept at 350 μbar CO₂, air, at 1000 μmol quanta m⁻² s⁻¹ prior to sampling.](image)

Fig.5.4. The comparison between the activation state of NADP-malate dehydrogenase and the carbamylation state of Rubisco in anti-bf plants. Symbols: low-light wild-type tobacco plants (■) and transgenic tobacco plants with a variety of cytochrome bf contents (□). The leaves were kept at 350 μbar CO₂, air, at 1000 μmol quanta m⁻² s⁻¹ prior to sampling.
5.4 DISCUSSION

5.4.1 RUBISCO CARBAMYLATION IN ANTI-BF AND ANTI-GAPDH PLANTS

5.4.1.1 Rubisco carbamylation is impaired in anti-bf plants

The Rubisco carbamylation state in all control and anti-GAPDH plants was on average 80% (Fig. 5.2 A&B). This high carbamylation state is typical for tobacco measured at high light (Mate et al. 1993; 1996; von Caemmerer et al. 1994). However, the carbamylation level of Rubisco decreased when the amount of cytochrome bf was reduced (Fig. 5.2 A), as reported before by Price et al. (1998). The reason for the reduction in Rubisco carbamylation in anti-bf plants could be that the restricted electron transport and proton translocation may result in a less alkaline stromal pH. The low pH may further decrease the free Mg$^{2+}$ concentration in stroma. Together the low pH and the lack of Mg$^{2+}$ could inhibit the carbamylation process (see discussion in Price et al. 1998). However, it is not certain that the stromal pH could be significantly less alkaline in the anti-bf plants. As discussed in Chapter 2, the stromal buffering capacity has been estimated being quite high (30 mM, Hauser et al. 1995a; 1995b) which should prevent large changes in pH.

The decrease in Rubisco carbamylation in anti-bf plants resembles the effect of reduced Rubisco activase content (Mate et al. 1993; 1996). However, when the amount of Rubisco activase was measured, it was similar in wild-type and anti-bf plants (data not shown). This raises the interesting possibility that the functioning of activase itself may be impaired in anti-bf plants. Rubisco activase needs ATP to perform and is inhibited by ADP (Robinson and Portis 1988). In anti-bf plants the electron transport rate is impaired, probably leading to a reduction in ATP synthesis. The lack of ATP, and low ATP/ADP ratio in chloroplasts would then be expected to limit activase activity. If this was the case, the decrease in carbamylation in anti-bf plants would be parallel to the suggested mechanism for Rubisco deactivation observed in phosphorus-depleted plants (Brooks et al. 1988b; Sharkey 1990). However, in Chapter 4 it was shown that in anti-bf plants the whole-leaf adenylate ratio was similar to wild-type ratios (Fig. 4.7 E). This lack of change in ATP/ADP ratio is a puzzle which we have no answer at the moment. Perhaps a decline in chloroplast ATP/ADP ratio was masked by high ATP-quotient of the cytosol. On the
other hand, it is possible that the inactivation of thioredoxin-regulated Calvin cycle enzymes could actually slow down the consumption of ATP in anti-\(\text{bf}\) chloroplasts such that there is no net change in ATP/ADP ratio (Section 4.4.5). The reduced activation level of NADP-MDH supports the latter possibility.

Subsequent studies have shown that the ATP/ADP ratio may not be the only mechanism regulating Rubisco activase, but that electron transport through PSI and transthylakoid pH gradient may have a role as well (Portis 1995). The drastic reduction in chloroplast NADP-MDH activation level (Fig.4.8 A) confirmed that the electron transport in anti-\(\text{bf}\) plants is severely impaired. Chlorophyll fluorescence measurements have established that the transthylakoid pH gradient is low in anti-\(\text{bf}\) plants (Price et al. 1995a; Hurry et al. 1996). Thus, it is likely that the conditions in chloroplasts are such that the functioning of Rubisco activase can be impaired. The correlation between the carbamylation state of Rubisco and the activation level of chloroplast NADP-MDH (Fig. 5.4) further strengthens the argument that Rubisco carbamylation is affected by the reduced electron transport rate in anti-\(\text{bf}\) plants.

5.4.1.2 Low RuBP does not influence Rubisco carbamylation in anti-GAPDH plants

The model of \(\text{C}_3\) photosynthesis states that at low light, the \(\text{CO}_2\) assimilation is limited by the RuBP regeneration capacity, which in turn is determined by the electron transport rate (Farquhar et al. 1980). However, when RuBP contents have been measured at low light intensities, they still have been above the Rubisco site concentration and so, apparently saturating for Rubisco (Badger et al. 1984; Krall et al. 1995). On the other hand, at low light the activation state of Rubisco decreases. The observations that when RuBP content is expected to be low Rubisco activation state tends to decrease, has lead to the suggestion that the carbamylation level of Rubisco is regulated such that the rate of RuBP consumption is matched with its regeneration rate (Mott et al. 1984, Sage et al. 1990). This regulatory system would then keep Rubisco always saturated in respect to RuBP. Thus, it could be expected that low RuBP pools could cause Rubisco to decarbamylate. This was not observed in my study, or in a previous work done with the anti-GAPDH plants (Price et al. 1995a). Although the RuBP contents decreased significantly in anti-GAPDH plants
(Fig. 4.6 B), the carbamylation state of Rubisco remained high (Fig. 5.2 B). However, it is worth noting that at ambient CO2, RuBP content did not decrease below Rubisco site concentration (see Fig. 5.3, detailed data not shown). The relationship between very low RuBP content and Rubisco carbamylation will be discussed further in Chapter 6.

Transgenic tobaccos with reduced amount of phosphoribulokinase have low RuBP contents as well (Paul et al. 1995b). Interestingly, it was reported that the activation state of Rubisco (measured as the ratio between initial and total activities) in these plants was actually higher than in wild-type tobaccos (Paul et al. 1996). The authors suggested that this increased Rubisco activation level was a result of low RuBP and 3-PGA pools that may increase the chloroplast pH, and high ATP, which increases the activity of Rubisco activase. Anti-GAPDH plants have low RuBP (Fig. 4.6 B) and high ATP (Fig. 4.7 B&F) contents as well. However, the activation state of Rubisco in anti-GAPDH plants was not higher than in control plants, whether measured as carbamylation state (Fig. 5.3 B) or as a ratio between initial and total activity of Rubisco (data not shown). The samples in the study by Paul et al. (1996) were collected from considerably lower light intensity than in my study (300 as opposed to 1000 μmol quanta m⁻² s⁻¹), which may explain the difference. It may be, that the 80% carbamylation state, which is routinely measured in tobacco leaves at high light, is the upper limit for carbamylation, and no matter how favourable conditions are in chloroplasts, this will not be exceeded.

5.4.2 IN VITRO-CATALYSIS OF RUBISCO

5.4.2.1 Total catalytic activity of Rubisco decreases in anti-bf plants

Rubisco can become inhibited by binding of phosphorylated sugars to the active site, and Rubisco activase facilitates the dissociation of these compounds promoting both carbamylation and catalysis (Section 5.1.2.2). The presence of tightly binding inhibitors in Rubisco active sites can be detected as a decrease in the catalytic rate of Rubisco measured under substrate saturation (Seemann et al. 1985; Keys et al. 1995; Parry et al. 1997). In my study both initial and total activities of Rubisco were

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measured and compared with the measurements of carbamylated and total active site concentrations.

The in vitro-turnover rates of the carbamylated sites in both anti-bf and anti-GAPDH plants were comparable to controls (Fig. 5.2 C&D), indicating that the carbamylated sites were free from inhibitory ligands after extraction. However, the total activity/total sites for the anti-bf plants decreased as the cytochrome bf content decreased (Fig. 5.2 E). The reason for this could be that the uncarbamylated Rubisco sites have bound inhibitory compounds, which do not dissociate during the 5 minutes incubation period. Nevertheless, during the carbamylation state assays these inhibitory ligands were displaced by \([^{14}\text{C}]\text{CPBP}\), which has very high affinity to Rubisco sites. The presence of the inhibitors in anti-bf plants could be another consequence of decreased activity of Rubisco activase, in addition to this low activity of activase causing the carbamylation state of Rubisco to decrease.

The identity of the inhibitor which binds specifically to uncarbamylated Rubisco sites in anti-bf plants is unknown. It is probably not CA1P, the dark inhibitor of Rubisco, because carbamylated sites were unaffected. The inhibitor in anti-bf plants would also seem to be different from the one observed in anti-activase tobacco leaves (He et al. 1997), because this unselective inhibitor bound equally well to carbamylated and uncarbamylated sites. One suggestion is that maybe there were several different inhibitors present in the anti-activase plants, and in anti-bf plants only one is present. One of the failover products, of Rubisco, XuBP, can bind tightly to uncarbamylated sites and also cause decarbamylation (Zhu and Jensen 1991a; 1991b). It is unlikely, though, that the inhibitor in anti-bf plants is XuBP. It would require that after XuBP is formed in Rubisco active sites during catalysis and supposedly removed by activase, it binds again specifically to uncarbamylated sites.

5.4.2.2 What factors govern Rubisco in vitro-activities?

Tightly-binding endogenous inhibitors are not the only factors that decrease Rubisco activity. After extraction Rubisco can become susceptible to several forms of inactivation. Rubisco extracted from tobacco may be partially degraded by endogenous proteases (Servaites 1985), or be inhibited by polyphenolics (Bahr et al.
1981) and polyphenol oxidase (Koivuniemi et al. 1980), both of which are abundant in tobacco leaves. It is therefore possible, that the decrease in the total catalytic activity of Rubisco in anti-βf plants is a result of direct inhibition. The leaves of the anti-βf plants could contain more harmful compounds that interact and inhibit Rubisco during the 5 minutes incubation period prior to total activity assays. Factors that may somehow induce formation of inhibitory compounds in chloroplasts could be the low stromal reduction state (Chapter 4, section 4.4.5), and highly reduced plastoquinone pool (Price et al. 1995b).

It is also worth considering that the Rubisco catalytic activities were altogether higher in glasshouse-grown wild-types (and all anti-GAPDH plants) than in low-light control plants, and the initial catalytic rates were higher than total rates (Fig. 5.2). These differences may reflect that the low light grown leaves generally have more inhibitory compounds. It has been shown that high Rubisco specific activities, which were measured from high-light grown tobaccos, correlated with lower polyphenol oxidase content (Koivuniemi et al. 1980). The extraction and assay buffers that I used (see Material and methods) did not contain casein or leupeptin which have shown to protect tobacco Rubisco against proteolytic degradation after extraction (Servaites 1985). Although PVPP removes phenolics during extraction, it does not remove the polyphenolic oxidase which can inactivate Rubisco (Koivuniemi et al. 1980). In addition, recent results from our laboratory suggest that the catalytic activity of tobacco Rubisco increases if the leaf extract is diluted substantially before assays (S. Whitney, personal communication). These observations point out that in vitro-assays of Rubisco activity have to be performed carefully, and highlights the importance of having adequate controls for the measurements.

5.4.3 TURNOVER RATE OF CARBAMYLATED RUBISCO SITES IN VIVO

The decrease in CO₂ assimilation rate in both anti-βf and anti-GAPDH plants was associated with decreasing RuBP contents (Chapter 4, Fig. 4.6). The Rubisco carbamylation state also decreased in the anti-βf plants (Fig. 5.2), suggesting that the function of Rubisco activase may be defective in these plants. Studies of transgenic tobaccos with severely reduced amount of Rubisco activase show that not only carbamylation, but also in vivo-tumover rates of carbamylated Rubisco sites are
impaired by the lack of activase (He et al. 1997). However, as both RuBP content and RuBP/3-PGA ratios are high in anti-activase plants (Table 1, He et al. 1997), the slow Rubisco turnover rate could be deduced resulting from the lack of activase. The RuBP concentrations are lowered in both anti-GAPDH and anti-bf plants, so it is more difficult to judge if there is any additional decrease in the catalysis of Rubisco, other than substrate limitation.

In an attempt to determine whether the CO₂ assimilation was limited solely by RuBP availability in anti-bf plants, the in vivo-catalytic rates (CO₂ assimilation/carbamylated sites) were assessed against RuBP pool sizes (Fig. 5.3). I used the data from anti-GAPDH plants as a control, since the in vitro-assays revealed that Rubisco functions optimally in these plants (Fig. 5.2). In anti-GAPDH plants the catalytic rate of carbamylated Rubisco sites was dependent on RuBP content until RuBP concentration exceeded approximately twice the average Rubisco site concentration, and then became independent on RuBP (Fig. 5.3 A). As already discussed by Price et al. (1995a), this finding differs from the model of C₃-photosynthesis by Farquhar et al. (1980). The model is based on the hypothesis that carboxylation rate increases linearly with increasing RuBP until the RuBP reaches site concentration, and is thereafter RuBP-saturated. The higher Kᵣ seen in vivo in anti-GAPDH plants is perhaps a result of the presence of other chloroplast compounds, like 3-PGA, that act as competitive inhibitors (Chapter 2). Alternatively, part of the RuBP may be chelated and unavailable as a substrate for carboxylation (von Caemmerer and Edmondson 1986; Price et al. 1995a).

When anti-GAPDH plants were used as controls, no further inhibition of Rubisco catalysis in anti-bf plants could be detected. The observed decrease in in vivo-catalysis of Rubisco in anti-bf plants was then due to RuBP limitation and the carbamylated Rubisco sites functioned normally in vivo. This is supported by in vitro-data, showing that the catalytic activity of carbamylated sites in anti-bf plants was comparable to wild-types (Fig.5.2 C). In this respect the anti-bf plants differ from anti-Rubisco activase plants, which had markedly slowed in vivo- and also slightly impaired in vitro-turnover rates of carbamylated sites (He et al. 1997). However, it should be noted that these two plant types have very different conditions in the chloroplasts: the RuBP-content in anti-activase plants is high and in anti-bf plants it is
very low. The redox state of chloroplast NADP pool in anti-βf plants is low, whereas in anti-activase plants it can be expected to be similar to (or higher than) in wild-type plants (based on the wild-type like MDH activation in anti-GAPDH plants, Fig. 4.8 B). In addition, the ΔpH is low in anti-βf plants and high in anti-activase plants. It is therefore possible that the difference in the conditions inside the chloroplasts has something to do with the difference in vitro-catalytic activities of Rubisco.

The anti-activase tobacco plants which had reduced in vivo-turnover rates of Rubisco, also had severely reduced activase contents and low carbamylation levels at high light (He et al. 1997). Judging by the carbamylation state, the reduction in Rubisco activase capacity in most of the anti-βf plants was not as strong as in these particular anti-activase plants. Nevertheless, when the amount of cytochrome βf complex was reduced to 20% or less of the average wild-type-level, the carbamylation decreased to below 50% (Fig. 5.2 A). This carbamylation state is not greatly different from what was observed in the anti-activase plants used in the study by He et al. (1997). Thus, it can be concluded that a severe reduction either in the amount of cytochrome βf complex or in the amount of Rubisco activase has equivalent effect on Rubisco carbamylation process. However, for some reason, the reduced cytochrome βf and activase content seems to affect in vivo- and in vitro catalysis differently.

5.4.4 SUMMARY

The lowered RuBP contents are not the only factor limiting the rate of CO₂ assimilation in anti-βf plants, but the decrease in the electron transport capacity has affected Rubisco as well. The carbamylation state of Rubisco reduced as the amount of cytochrome βf complex decreased. In addition, the activity assays indicated that the activation of uncarbamylated sites in vitro was impaired, possibly because tightly-binding inhibitors (XuBP, 3-KABP) or some other inhibitory substances (polyphenols, polyphenol oxidase) accumulated in the leaves or chloroplasts of anti-βf plants. However, the carbamylated sites functioned normally both in vivo and in vitro.
The results suggest that the functioning of Rubisco activase is impaired in anti-
bf plants. The possibility that the decrease in activase activity was due to a low chloroplast ATP and ATP/ADP ratio cannot be ruled out, because only the whole-leaf adenylates were measured. However, it is also possible that the ATP/ADP ratio in chloroplasts did not change greatly, because the capacity of the Calvin cycle to utilise ATP had decreased as well. It is therefore possible that the decrease in the activase function was caused by the low ΔpH, due to the decreased linear electron transport capacity in the anti-bf plants. These results are significant in the sense that this is the first occasion when a substantial inhibition of Rubisco activation in vivo has been reported as a result of manipulating the electron transport capacity by other means than changing light intensity, and highlights the complexity of mechanisms that balance the light and dark reactions of photosynthesis.
CHAPTER 6

THE RESPONSES OF GAS EXCHANGE, METABOLITE AND ADENYLATE POOL SIZES AND RUBISCO ACTIVITY TO SHORT-TERM ELEVATED CO₂ IN ANTI-BF AND ANTI-GAPDH TOBACCOS
6.1 INTRODUCTION

6.1.1 THE AIM OF THIS CHAPTER

In Chapters 4 and 5 I examined the interactions between CO₂ assimilation, metabolite and adenylate pools, as well as Rubisco activation in anti-cytochrome b₅f and anti-GAPDH tobaccos. This data was obtained from leaves that were photosynthesising at high light and 350 μbar CO₂ in air. This Chapter will study how these parameters changed when the same leaves were exposed to elevated CO₂ (700 μbar). For this experiment the leaves were kept under the measuring conditions for 30-40 minutes so that not only the rate of CO₂ assimilation but also metabolite and adenylate pools would reach a steady state.

There were three reasons why I chose to study the leaf performance at elevated CO₂. Firstly, I wanted to enhance the antisense effect with high CO₂. It was expected that the limitation to photosynthesis, posed by the reduced cytochrome b₅f content or GAPDH activity becomes more severe at elevated CO₂ due to increased carboxylation activity of Rubisco and demand for RuBP, ATP and NADPH. Secondly, it was necessary to study how the wild-type tobaccos respond to electron transport/RuBP-regeneration limitation. Thirdly, impaired RuBP regeneration capacity in these two different plant types offers a unique opportunity to study interactions between RuBP concentration and Rubisco activity.

6.1.2 THE EFFECT OF RuBP CONTENT ON RUBISCO ACTIVITY

The relationship between Rubisco activity and RuBP concentration is complicated by the high concentration of Rubisco sites in the chloroplasts (1-4 mM) (Jensen and Bahr 1977; Evans et al. 1994) and the relatively low Kₘ of Rubisco for RuBP (20 μM) (Badger and Collatz 1977; Yeoh et al. 1981). This may lead to a situation where only a portion of total RuBP is free, the rest being bound to Rubisco. Taking these considerations into account, Farquhar (1979) presented a kinetic model about how Rubisco activity responds to changing RuBP content. According to this model, once the amount of RuBP decreases below the Rubisco site concentration, the activity of Rubisco becomes linearly dependent on RuBP. For RuBP amounts which
are above the site concentration, Rubisco activity is saturated with respect to RuBP (Farquhar 1979). This was the basis for the subsequent model of C₃ photosynthesis, stating that at any given time CO₂ assimilation can be described as being limited either by Rubisco’s carboxylation capacity or by the RuBP regeneration rate (Farquhar et al. 1980).

Testing this model by measuring photosynthesis and RuBP concentrations under different conditions have produced controversial results. In a green algae *Chlamydomonas* and in isolated spinach leaf cells it was estimated that RuBP content decreased below Rubisco site concentration at low light, or at high light and elevated CO₂ (Collatz 1978). However, experiments conducted with intact leaves have often shown that, even in conditions where RuBP-limitation of photosynthesis is thought to occur, RuBP content still remains above Rubisco sites (Perchorowitz et al. 1981; Badger et al. 1984). Subsequent studies showed that at conditions where RuBP content is low, Rubisco carbamylation may decrease (Mott et al. 1984; Sharkey et al. 1986a; von Caemmerer and Edmondson 1986). This has lead to the suggestion that Rubisco carbamylation state is regulated in relation to RuBP consumption such that Rubisco always functions at RuBP saturation (Mott et al. 1984; Sage et al. 1990). Interestingly, my data (Fig. 4.6) as well as previous observations with transgenic plants with reduced RuBP regeneration capacity (Price et al. 1995a; 1998) indicate that RuBP/Rubisco sites-ratio stays above one at ambient CO₂ no matter how severely its regeneration is impaired. Therefore it is of interest to see if it is overall possible to decrease the steady-state RuBP pool sizes below Rubisco site concentration by taking the leaves to elevated CO₂, and if this happens, what consequences it has to the carbamylation state of Rubisco.
6.2 MATERIAL AND METHODS

6.2.1 PLANT MATERIAL AND MEASURING CONDITIONS

Gas exchange at elevated CO$_2$ was measured from the same leaves as were used for measurements at ambient CO$_2$, described in Chapters 4 and 5. A section of the leaf was enclosed in a rapid-kill chamber, and kept at 350 µbar CO$_2$ in air, 1000 µmol quanta m$^{-2}$ s$^{-1}$, 25 °C until stomatal opening was nearly complete. This took around 20 minutes, after which the incoming CO$_2$ partial pressure was increased to 700 µbar. The gas exchange characteristics were recorded after 30-40 minutes and a leaf disc was rapidly freeze-clamped in situ for subsequent biochemical assays (Badger et al. 1984). The average C$_i$ (±S.E) of all wild-type tobaccos was 490 ± 20 µbar, and for all antisense plants it was 563 ± 16 µbar (see Fig. 6.1).

6.2.2 BIOCHEMICAL ASSAYS

The amounts of metabolites and adenylates were measured from one half of the frozen leaf discs as described in Chapter 3. Rubisco carbamylation as well as initial and total activities were measured from the other half as described in section 5.2. Each leaf was characterised by measuring either the amount of cytochrome $f$ protein (Section 4.2.7) or total GAPDH activity (Section 2.2).
6.3 RESULTS

6.3.1 GAS EXCHANGE AT 350 AND 700 μbar CO₂

6.3.1.1 Anti-bf plants

The relationship between leaf cytochrome bf content and assimilation rate at high light became strikingly linear at elevated CO₂ (Fig. 6.1A). At 350 μbar CO₂ the relationship was more curvilinear (see Fig. 4.3 A). The rate of photosynthetic electron transport was calculated from the gas exchange data and the comparison between electron transport rates at 350 and 700 μbar CO₂ is presented in Fig. 6.1 B. There was no difference between the electron transport rates at the two CO₂ concentrations in the anti-bf plants and most of the wild-type plants.

6.3.1.2 Anti-GAPDH plants

The transfer to elevated CO₂ increased the assimilation rate in both wild-type and anti-GAPDH plants (Fig. 6.2 A and 4.3 B). The relationship between the assimilation rate and GAPDH activity was similar at 700 μbar CO₂ to what it was at 350 μbar, in terms that the photosynthetic rate did not decrease until GAPDH activity was reduced below 30% of the average wild-type level (Fig. 6.2 A). In the intermediate anti-GAPDH plants, with 50% of the average wild-type GAPDH activity, the assimilation rate measured at elevated CO₂ was actually slightly higher than in wild-type plants. In order to compare the total flux of carbon through the Calvin cycle at the two different CO₂ concentrations, the rate of 3-PGA production was calculated based on the CO₂ assimilation rates. In the case of anti-GAPDH plants, the measurements of 3-PGA production rate at different CO₂ concentrations were similar in the plants that had less than 30% of the wild-type GAPDH activity (Fig. 6.2 B). On the contrary, the 3-PGA production increased at elevated CO₂ in the wild-type and intermediate anti-GAPDH plants.
Fig. 6.1. Assimilation rate of wild-type anti-bf plants at elevated CO₂ and a comparison between electron transport rates at ambient and elevated CO₂. A. The CO₂ assimilation of wild-type (■) or transgenic tobaccos with reduced amounts of cytochrome bf complex (□) measured at 700 µbar CO₂, 21% O₂, 1000 µmol quanta m⁻² s⁻¹ and leaf temperature of 25 °C. B. The comparison between electron transport rates at 350 or 700 µbar CO₂ 21% O₂, and 1000 µmol quanta m⁻² s⁻¹. Electron transport was calculated from the gas-exchange data as \( \frac{(A+R_d)(4C_i+8\Gamma^*)}{(C_i-\Gamma^*)} \), where \( A \) is the CO₂ assimilation rate, \( R_d \) the dark respiration rate, \( C_i \) the intercellular CO₂ partial pressure and \( \Gamma^* \) the CO₂ compensation point in the absence of mitochondrial respiration (38.6 µbar) (von Caemmerer and Farquhar 1981, von Caemmerer et al. 1994). The dotted line represents 1:1 ratio.
Fig. 6.2. Assimilation rate of anti-GAPDH plants at elevated CO$_2$ and a comparison between 3-PGA production at ambient and elevated CO$_2$. A. The CO$_2$ assimilation of wild-type (●) and transgenic tobaccos with reduced amounts of chloroplast glyceraldehyde 3-phosphate dehydrogenase (○) measured at 700 μbar CO$_2$, 21% O$_2$, 1000 μmol quanta m$^{-2}$ s$^{-1}$ and leaf temperature of 25 °C). B. The comparison between rate of 3-PGA formation at 350 or 700 μbar CO$_2$ 21% O$_2$, and 1000 μmol quanta m$^{-2}$ s$^{-1}$. The rate of 3-PGA formation was calculated from the gas exchange data as $(2C_i+3Γ^*)/(C_i-Γ^*)(A+R_d)$, where $C_i$ is the intercellular CO$_2$ partial pressure, $Γ^*$ is the CO$_2$ compensation point in the absence of mitochondrial respiration (38.6 μbar), $A$ the CO$_2$ assimilation rate and $R_d$ the dark respiration rate (von Caemmerer and Farquhar 1981, Farquhar and von Caemmerer 1982, von Caemmerer et al. 1994). The symbols are as in Fig. A, and the dotted line represents 1:1 ratio.
6.3.1.3 STOMATAL CONDUCTANCE AND $C_t$

Stomatal conductance tends to decrease when leaves are exposed to elevated CO$_2$ (Willmer and Fricker 1996). When leaves were kept at 700 μbar CO$_2$ for 30-40 minutes, it caused a reduction in stomatal conductance in all transgenic and control plants, when compared to conductance at 350 μbar CO$_2$ (Figs 6.3 and 4.4). In both low light and glasshouse-grown wild-types, as well as in anti-$bf$ plants, the average stomatal conductance at 700 μbar CO$_2$ was only about half of the conductance measured at 350 μbar. However, the intercellular CO$_2$ increased in both transgenic plant types (Fig. 6.3 C&D).

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**Fig. 6.3.** Stomatal conductance and intercellular CO$_2$ partial pressure of anti-$bf$ and anti-GAPDH tobacco plants measured at elevated CO$_2$. Stomatal conductance to water vapour (A,B) and intercellular CO$_2$ (C-D) were measured from low-light grown control plants (■), tobaccos with reduced amounts of cytochrome $bf$ complex (□), glasshouse-grown control plants (●) and tobaccos with reduced activity of GAPDH (○) at 700 μbar CO$_2$, 21% O$_2$, 1000 μmol quanta m$^{-2}$ s$^{-1}$ and leaf temperature of 25 °C.
6.3.2 METABOLITE AND ADENYLATE CONTENTS AT ELEVATED CO₂

6.3.2.1 RuBP and 3-PGA

The contents of RuBP and 3-PGA measured at 350 and 700 µbar CO₂ are presented in Table 6.1. The RuBP content in all wild-type plants decreased 50-70% as CO₂ increased. This was associated with an increase in 3-PGA pool sizes. On an average, the elevated CO₂ had a stronger effect on the RuBP pools in anti-GAPDH plants than anti-bf plants. The average RuBP content in anti-GAPDH plants decreased more than 50% at high CO₂, whereas the decrease was less (30%) in anti-bf plants. The lowest measured RuBP/3-PGA ratios were found from anti-GAPDH plants at elevated CO₂.

Table 6.1. Rubisco site and metabolite contents of low-light grown wild-type and anti-bf tobacco plants and glasshouse-grown wild-type and anti-GAPDH tobaccos measured at 350 or 700 µbar CO₂. Measuring and sampling conditions were as in Figures 6.1 and 6.2. The values are averages ± S.E., with the number of replicates indicated in the brackets.

<table>
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<th>350 µbar CO₂</th>
<th>700 µbar CO₂</th>
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<td></td>
<td>WT (n=4) Anti-bf (n=13)</td>
<td>WT (n=4) Anti-bf (n=13)</td>
</tr>
<tr>
<td>RuBP (µmol m⁻²)</td>
<td>72 ± 8</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>3-PGA (µmol m⁻²)</td>
<td>68 ± 8</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>RuBP/3-PGA (mol mol⁻¹)</td>
<td>1.1 ± 0.2</td>
<td>0.44 ± 0.04</td>
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<td>Rubisco sites (µmol m⁻²)</td>
<td>15.4 ± 0.6</td>
<td>13.0 ± 1.3</td>
</tr>
<tr>
<td>RuBP/Rubisco sites (mol mol⁻¹)</td>
<td>4.7 ± 0.5</td>
<td>2.2 ± 0.3</td>
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<th></th>
<th>350 µbar CO₂</th>
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<td>Anti-GAPDH (n=9)</td>
<td>Anti-GAPDH (n=8)</td>
</tr>
<tr>
<td>RuBP (µmol m⁻²)</td>
<td>130 ± 7</td>
<td>42 ± 18</td>
</tr>
<tr>
<td>3-PGA (µmol m⁻²)</td>
<td>77 ± 11</td>
<td>92 ± 10</td>
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<td>RuBP/3-PGA (mol mol⁻¹)</td>
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<td>0.43 ± 0.12</td>
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<tr>
<td>Rubisco sites (µmol m⁻²)</td>
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<td>19.8 ± 1.7</td>
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<tr>
<td>RuBP/Rubisco sites (mol mol⁻¹)</td>
<td>6.4 ± 0.4</td>
<td>2.0 ± 0.6</td>
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</table>
6.3.2.2 ATP and ADP

The relationships between adenylate pools and cytochrome bₖ content or GAPDH activity were similar whether measurements were conducted at 350 or 700 μbar CO₂ (Figs 6.4 and 4.7). Generally, there was no difference in ATP/ADP ratio between anti-bₖ and low-light control plants (Fig. 6.4 E). The reduction in the activity of GAPDH caused an increase in the ATP/ADP ratio (Fig. 6.4 E), however, it was difficult to tell whether there was a significant difference in the adenylate ratio between wild-type and anti-GAPDH plants due to the scatter in the data.

6.3.3 RUBISCO

The carbamylation state of Rubisco was high in all wild-type plants, whether it was measured at 350 or 700 μbar CO₂ (Fig. 6.5). The carbamylation state was likewise high in all anti-GAPDH plants at both CO₂ concentrations. On an average, the carbamylation state in anti-bₖ plants was lower than in wild-type plants also when it was measured at high CO₂ (Figs. 6.5 A and 5.4 A). Interestingly, in some anti-bₖ plants the carbamylation state was slightly higher at 700 μbar CO₂ than it was at 350 μbar. The in vitro-turnover rates of carbamylated Rubisco sites in both antisense plant types were comparable to their respective controls at 700 μbar CO₂ (Figs 6.5 C&D), as was observed at 350 μbar CO₂ (Fig 5.4). In parallel to the difference in carbamylation state in anti-bₖ plants when measured at 350 or 700 μbar CO₂, the reduction in the total activity/total Rubisco sites was slightly reduced at elevated CO₂ (Fig. 6.5 E). At 350 μbar, the average total activity/total sites in anti-bₖ plants was 1.9 ± 0.1 s⁻¹ (75% of the average wild-type), whereas at 700 μbar it was 2.0 ± 0.1 s⁻¹ (81% of the wild-type).

6.3.4 RuBP/RUBISCO SITE RATIOS

The ratios between Rubisco sites and RuBP content at 350 and 700 μbar CO₂ are presented in Table 6.1. The RuBP pools sizes decreased when leaves were kept at elevated CO₂, but the ratio between RuBP and Rubisco sites remained above 1 in most of the wild type and anti-bₖ plants. At elevated CO₂, however, the RuBP contents in almost all anti-GAPDH plants decreased below the Rubisco site concentration, and the average RuBP/sites ratio was 0.72.
Fig. 6.4. Leaf adenylate content of anti-bf and anti-GAPDH tobacco plants at 700 μbar CO₂. ATP (A, B) and ADP (C, D) concentrations, ATP/ADP ratio (E, F) and the sum of ATP+ADP (G, H) were measured from low-light grown wild-type plants (■), transgenic tobaccos with reduced amount of cytochrome bf complex (□), glasshouse-grown wild-type plants (●) and transgenic tobaccos with reduced activity of GAPDH (○). The O₂ concentration was 21%, light intensity was 1000 μmol quanta m⁻¹ s⁻¹ and leaf temperature 25 °C.
Fig. 6.5. Rubisco characteristics of anti-bf and anti-GAPDH tobacco plants at elevated CO₂. Carbamylation level (A, B), initial in vitro-activity of Rubisco/carbamylated Rubisco sites (C, D), and total in vitro-activity of Rubisco/total Rubisco sites (E, F). Symbols: low-light wild-type (■), anti-bf plants (□), glasshouse-grown wild-type (●) and anti-GAPDH plants (○). Measuring conditions and leaf sampling are as in Fig. 6.4.
6.4 DISCUSSION

6.4.1 ELECTRON TRANSPORT AND 3-PGA PRODUCTION RATES

As discussed in Chapter 2, the rate of CO₂ assimilation in C₃ plants has been described as being limited either by the carboxylation capacity of Rubisco or by the rate of RuBP regeneration. Rubisco-limitation occurs generally at low intercellular CO₂ (Cᵢ), whereas RuBP-regeneration limitation occurs at high light and high Cᵢ when the maximal capacity of electron transport to produce ATP and NADPH is attained (Farquhar et al. 1980). In order to examine more closely the limiting factors of photosynthesis in anti-GAPDH and anti-bf tobaccos, the steady-state rates of CO₂ assimilation were measured at high light and two different CO₂ concentrations. From the gas-exchange data either the photosynthetic electron transport rate or the rate of 3-PGA production was calculated.

There was a strong linear relationship between cytochrome f content and the CO₂ assimilation rate at high light and elevated CO₂ (Fig. 6.1 A). This demonstrates that, at these conditions, electron transport capacity determines the photosynthetic rate in both anti-bf plants and low light grown wild-types. I used the comparison between calculated electron transport rates at 350 and 700 μbar CO₂ to determine whether the CO₂ assimilation is determined by Rubisco carboxylation or RuBP regeneration capacity at ambient conditions. In all anti-bf plants the total electron transport was similar at both CO₂ concentrations (Fig. 6.1 B). Thus, the transfer from 350 to 700 μbar CO₂ only decreased the rate of photorespiration in these plants, but could not increase the total flux of electrons through the photosystems. This was expected, considering that the amount of one electron transport chain component has been reduced in these plants. This comparison between electron transport rates at two CO₂ concentrations also suggest that all, except one, low-light grown wild-types, that were measured at two CO₂ concentrations, were electron transport-limited at 350 μbar CO₂ and high light. However, the curvilinear relationship between assimilation rate and cytochrome bf content at 350 μbar CO₂ and high light suggests that at ambient conditions some wild-type plants are limited by Rubisco capacity (Fig. 4.3). It is worth pointing out that the data presented in Fig. 4.3 is a combination of two
separate experiments, and the rate of CO₂ assimilation of the anti-\( b_f \) leaves was measured at two different CO₂ concentrations only in one experiment.

The activity of GAPDH does not appear to be limiting for photosynthesis even at elevated CO₂, because it could be reduced down to 30% of the wild-type level before the CO₂ assimilation rate decreased (Fig. 6.2 A). The capacity for 3-PGA reduction via GAPDH thus seems to be very high in wild-type tobaccos. As was the case in all anti-\( b_f \) plants, in severe anti-GAPDH plants (less than 30% of the wild-type GAPDH activity) transfer from 350 to 700 \( \mu \)bar CO₂ only decreased oxygenation reaction by Rubisco. The calculated rates of 3-PGA production, which account for both oxygenation and carboxylation of RuBP, were similar in these GAPDH-limited plants at both CO₂ concentrations (Fig. 6.2 B). This is indicative of a complete limitation of the CO₂ assimilation by RuBP availability. In the two intermediate anti-GAPDH plants (more than 30% of the wild-type GAPDH activity) the total rate of 3-PGA production increased at 700 \( \mu \)bar indicating that the CO₂ assimilation at 350 \( \mu \)bar was limited by Rubisco capacity.

Some glasshouse-grown wild-type plants seemed to be limited by Rubisco carboxylation capacity at 350 \( \mu \)bar CO₂ and some by RuBP regeneration capacity. This was similar to the situation found in low-light wild-type plants. It can be concluded that, at ambient CO₂ and high light, the wild-type tobaccos are close to the transition from Rubisco carboxylation-limitation to RuBP-regeneration limitation (von Caemmerer and Farquhar 1981).

6.4.2 METABOLITES AND ADENYLATES

6.4.2.1 RuBP contents decrease below Rubisco site concentration only in anti-GAPDH plants

Although the transfer to elevated CO₂ decreased the RuBP pool sizes in all plants, the RuBP content in wild-type plants remained well above the Rubisco site concentration (Table 6.1). This is similar to previous studies on the response of chloroplast metabolites to changes in CO₂. They show that even at high CO₂ RuBP contents tend to exceed Rubisco site concentration (Badger et al. 1984; Dietz and Heber 1984; von Caemmerer and Edmondson 1986; Seemann et al. 1987). By
increasing CO₂ it was possible to reduce the RuBP pool sizes in anti-GAPDH plants below the Rubisco site concentration. However, generally in anti-
 bf plants the RuBP/Rubisco sites-ratio remained between 1 and 2 (Table 6.1). This contrast in RuBP/sites-ratio between the two transgenic plant types probably reflects differences in Rubisco catalytic efficiency. Rubisco functions optimally in anti-GAPDH plants at both 350 and 700 µbar CO₂ since the carbamylation state remains high (Figs 5.2 B and 6.5). In anti-
 bf plants Rubisco carbamylation is impaired also at high CO₂ (Fig. 6.5 A.) As a consequence, in anti-
 bf plants the consumption of RuBP by Rubisco may not be as efficient. Another explanation could be that for some reason, a larger portion of RuBP is unavailable for carboxylation in anti-
 bf plants for example due to chelation to magnesium, see von Caemmerer and Edmondson (1986) for further discussion.

6.4.2.2 Whole-leaf adenylate ratios did not change at elevated CO₂

CO₂ concentration did not affect the whole-leaf adenylates in either of the antisense plant types and even the wild-type ATP/ADP remained between 1 and 2 despite the change in the measuring conditions (Figs 6.4 and 4.7). It could be expected that, as CO₂ assimilation rate increases at high CO₂, the ATP/ADP ratio decreases. However, similar to my findings, the adenylate charge (calculated as [2ATP+ADP]/ATP+ADP+AMP) remained at 1.3 in cotton leaves when CO₂ was varied between 200-500 µbar (Gilmore and Björkman 1994a). Likewise, there was virtually no change in the ATP/ADP ratio in spinach chloroplasts (increased from 2.2 to 2.3) when CO₂ was increased from 350 to 550 µbar, and it only decreased when the CO₂ was increased to above 1000 µbar (Dietz and Heber 1984) However, in both above mentioned studies the ATP/ADP ratio increased at low CO₂. Altogether, it seems that the whole-leaf (and chloroplast) ATP/ADP ratio is more sensitive to a decrease in CO₂ below ambient level than increase above it. The inhibition of assimilation by low CO₂ then causes accumulation of ATP, a situation which is comparable to restricted photosynthesis in transgenic plants with reduced carbon assimilation capacity (Quick et al. 1991; Paul et al. 1995b). Of course, as discussed in Chapter 4, it is possible that cytosolic pools may mask high CO₂-induced decrease in chloroplast ATP/ADP ratio.
6.4.3 ELEVATED CO$_2$ DID NOT HAVE A LARGE EFFECT ON RUBISCO

Rubisco carbamylation has been shown to decline at high CO$_2$ partial pressures, either at low or high O$_2$ (Sharkey et al. 1986a; von Caemmerer and Edmondson 1986). Under these conditions the RuBP pool sizes are small. It has been hypothesised that the Rubisco carbamylation state is regulated such that Rubisco always functions at RuBP-saturation (Mott et al. 1984; Sage et al. 1990). Lately, a model by Mate et al. (1996) has supported this by suggesting that RuBP bound to catalytic sites protects Rubisco from decarbamylation.

At high CO$_2$ the RuBP pools in anti-GAPDH plants decreased below the Rubisco site concentration (Table 6.1), however, the Rubisco carbamylation state remained high (Fig. 6.5 B). A recent in vitro study has confirmed that subsaturating CO$_2$ combined with subsaturating RuBP causes Rubisco to decarbamylate, but this decarbamylation can be prevented by Rubisco activase (Portis et al. 1995). It is therefore possible that the maintenance of the high Rubisco carbamylation state in the anti-GAPDH plants was due to an enhanced activity of Rubisco activase. This is supported by the high concentration of ATP, elevated ATP/ADP ratio (Fig. 6.4) and high transthyalakoid pH gradient (Price et al. 1995a), all of which are thought to promote the functioning of Rubisco activase (Portis 1992). An increase in activase performance was suggested to be the reason for elevated Rubisco activation levels in tobaccos with reduced amounts of phosphoribulokinase (Paul et al. 1996). These plants have high ATP/ADP ratios and reduced RuBP pool sizes as well. The high carbamylation state which is maintained in anti-GAPDH plants may also indicate that, in the absence of RuBP, binding of other chloroplast metabolites such as 3-PGA to Rubisco sites can prevent decarbamylation (Badger and Lorimer 1981).

In general, the carbamylation state of Rubisco in anti-$bf$ plants was lower than in wild-type plants at both 350 and 700 μbar CO$_2$. This was expected, since the conditions in the chloroplasts at 700 μbar CO$_2$ should be equally unfavourable for Rubisco activation as they were at 350 μbar. The electron transport rate was similar (Fig. 6.2), the ATP/ADP ratio did not change (Fig. 6.4), and the RuBP content decreased even further (Table 6.1). Interestingly, the observed decrease in Rubisco carbamylation seemed to be slightly relieved at elevated CO$_2$ in some anti-$bf$ plants (5
plants) (Figs 5.4 and 6.5). A possible explanation for the higher carbamylation state at 700 μbar CO₂ could be in the experimental setup. For the 350 μbar CO₂ measurements leaves were kept at high light for 40 minutes before freeze-clamping. During 700 μbar CO₂ measurements the leaves were kept at high light at least 20 minutes longer, because the stomatal opening was first induced at 350 μbar. It has been shown that in tobacco plants with moderately reduced activase content the rate of Rubisco carbamylation, following a transfer from low to high light, is slower than in wild-type plants. Eventually the carbamylation becomes similar to control plants (Hammond et al. 1998). Perhaps the rate of increase in carbamylation is slower also in anti-βf plants, with reduced activity of activase. All anti-βf plants were taken to the gas exchange measurements from the growth cabinet where the irradiance was only about 100 μmol quanta m⁻² s⁻¹. At those conditions the wild-type Rubisco carbamylation is around 50% and it increases to 80% when the leaves are exposed to high light.

6.4.4 RUBISCO ACTIVITY AND RuBP POOL SIZES IN ANTI-GAPDH PLANTS

The possibility to decrease RuBP pool sizes in anti-GAPDH plants without simultaneously affecting Rubisco carbamylation makes these plants ideal to test the mathematical model of Rubisco carboxylation rate in response to changing RuBP (Farquhar 1979). In Figure 6.6 the gross CO₂ assimilation (A+Rd) per carbamylated Rubisco sites in wild-type anti-GAPDH plants is presented as a function of RuBP concentration. This figure contains data from leaves that were sampled both at 350 and 700 μbar CO₂. For comparison, I have included the modelled dependence of carboxylation rate on RuBP and CO₂, based on the equations of Farquhar et al. (1980). This model takes into account the competitive inhibition of carboxylation by 3-PGA, which increases the Kᵣ.

At both CO₂ concentration the measured data points coincide well with the model predictions. RuBP-saturated carboxylation rate increases at elevated CO₂, as seen by the steeper slope of the modelled curve, and also from the data points measured at elevated CO₂. It seems that at 700 μbar CO₂ the wild-type plants are only at the verge of becoming RuBP-limited. This agrees with the RuBP-
measurements, which showed that even at elevated CO₂, the RuBP concentrations were still approximately twice the Rubisco site concentration (Table 6.1). Thus it can be concluded that in vivo RuBP acts similarly to a tight binding inhibitor, since the rate of catalysis is linearly dependent on the RuBP concentration until all the sites are filled, and thereafter an addition of RuBP does not affect the reaction rate. This observation does not support the suggestion that Rubisco exhibits significant negative anti-cooperativity with respect to binding of RuBP and 3-PGA (Servaites and Geiger 1995).

![Graph A](image1)

**Fig. 6.6.** CO₂ assimilation rate per carbamylated Rubisco sites as a function of RuBP content in anti-GAPDH tobacco plants at ambient and elevated CO₂. Symbols and measuring conditions are as in Fig. 6.3. The two lines illustrate the modelled responses of Rubisco carboxylation rate on RuBP and CO₂ concentrations, based on the equation of Farquhar (1979). Dotted and solid lines represent the ambient and elevated CO₂. Symbols: wild-type (●), anti-GAPDH plants (○). See text for the calculations.
Model calculations:

CO₂ assimilation rate was calculated from the ratio of the RuBP limited to RuBP saturated carboxylation rate (Vc/Wc) as

\[
\frac{V_c}{W_c} = \frac{(E_t + R_t + K_r) - \sqrt{(E_t + R_t + K_r)^2 - 4E_tR_t}}{2E_t}
\]

where \(E_t\) is total concentration of Rubisco sites (20 \(\mu\)mol m\(^{-2}\)), \(K_r\) the effective Michaelis-Menten constant for RuBP (2.9 \(\mu\)M, calculated on the basis of measured 3-PGA values in wild-type and anti-GAPDH plants, see Section 2.3.6) and \(R_t\) the total concentration of RuBP (von Caemmerer and Edmondson, 1986). The RuBP-saturated assimilation rate was estimated from the equation:

\[
(A + R_d) = (1 - \Gamma*/C_i)W_c
\]

The RuBP saturated carboxylation rate per carbamylated Rubisco sites was estimated as 0.92 and 1.44 s\(^{-1}\) at ambient and elevated CO₂ respectively, from the equation

\[
V_c = \frac{C_i \cdot k_{cat}}{C_i + K_c \cdot (1 + O/K_o)}
\]

where \(C_i\) is the intercellular CO₂ partial pressure (250 and 450 \(\mu\)bar at ambient and elevated CO₂ respectively), \(\Gamma^*\) is the CO₂ compensation point in the absence of mitochondrial respiration (38.6 \(\mu\)bar) and \(k_{cat}\) catalytic turnover rate of Rubisco (3.5 mol CO₂ (mol sites\(^{-1}\)) s\(^{-1}\)). \(K_c\) is Michaelis-Menten constant for carboxylation by Rubisco (260 \(\mu\)bar), \(O\) is \(O_2\) concentration (200 mbar) and \(K_o\) Michaelis-Menten constant for oxygenation by Rubisco (170 mbar) (von Caemmerer and Farquhar 1981; von Caemmerer et al. 1994).
6.4.5 SUMMARY

Gas-exchange measurements performed at elevated CO₂ (700 µbar) demonstrated that photosynthesis is completely limited by electron transport in all anti-\(bf\) plants, and RuBP regeneration capacity in those anti-GAPDH plants with less than 30% of wild-type GAPDH activity. For these plants we found that the increase in CO₂ concentration only decreased photorespiration, but did not affect the total electron transport rate of the rate of 3-PGA formation in these plants. Whole-leaf adenylate ratios were found to be similar whether measured at 350 or 700 µbar CO₂. As expected, elevated CO₂ the RuBP reduced pool sizes in all plants, and in anti-GAPDH plants RuBP/Rubisco sites-ratio decreased below 1. This subsaturating RuBP did not cause Rubisco decarbamylation in anti-GAPDH plants. The average carbamylation state of Rubisco in anti-\(bf\) plants was lower than in wild-type plants at 700 µbar CO₂, as it was at 350 µbar. In some anti-\(bf\) plants, however, the impaired Rubisco carbamylation, which was seen at 350 µbar CO₂ was slightly alleviated at high CO₂. This is surprising, and possibly implies that the rate of increase in Rubisco carbamylation, following the transfer from low to high light, is slower in anti-\(bf\) plants due to impaired activase function. Anti-GAPDH plants were also used to test the model of the response of Rubisco carboxylation rate to decreasing RuBP. The measured data fitted the model well, when the inhibitory effect of 3-PGA to carboxylation rate was accounted for. The data set showed no obvious signs of anti-cooperativity with respect to binding of RuBP and 3-PGA to Rubisco.
CHAPTER 7

XANTHOPHYLL CYCLE PIGMENTS AND FLUORESCENCE QUENCHING IN TRANSGENIC TOBACCOS WITH REDUCED CARBON ASSIMILATION CAPACITY
7.1 INTRODUCTION

In the three previous Chapters I have examined how the alteration in either electron transport or carbon assimilation capacity has affected the whole-leaf energy balance, and especially the activation of Rubisco. In this Chapter the focus is shifted to mechanisms of how carbon assimilation can regulate the efficiency of light harvesting and utilisation in chloroplasts. For this I will study how the reduced CO₂ fixation capacity affects the pigment composition and the capability for photoprotection in leaves of transgenic tobacco plants.

7.1.1 UTILISATION OF LIGHT ENERGY

When a chlorophyll molecule absorbs a photon it makes a transition to a high energy state. This excited state can be dissipated by three ways. The first mechanism, photochemical dissipation, is the utilisation of energy to power linear electron transport which produces chemical energy in the form of NADPH and ATP. The linear electron transport as a whole involves water oxidation in the PSII complex, subsequent electron transport to NADPH, proton translocation from stroma to lumen and the utilisation of transthylakoid pH gradient in ATP synthesis (Chapter 1). Photosynthetic carbon reduction and photorespiratory pathways then consume the majority of NADPH and ATP.

The second major excitation decay mechanism is termed nonradiative dissipation (NRD). It involves the release of energy as heat within the antenna pigments (Krause et al. 1982). The main trigger for NRD is probably the buildup of transthylakoid pH gradient (ΔpH), which is thought to affect the physical properties of the PSII antenna such that the rate constant of heat dissipation increases (Demmig-Adams 1990; Horton et al. 1994), however, the precise mechanism of this conversion is still unknown. It is widely considered that a group of carotenoid pigments, the xanthophyll cycle pigments, are also involved in NRD (see section 7.1.2). In general, NRD is greatly enhanced in conditions where leaves are exposed to light intensities which are in excess of the capacity for photochemistry and as such functions as a protective mechanism against possible damage of photosynthetic machinery (Björkman and Demmig-Adams 1995).
A third mechanism for dissipation of the excited state of the chlorophyll molecule is fluorescence. Although the proportion of absorbed light lost via fluorescence is small in comparison to photochemical and nonradiative dissipation (0.5-5% of absorbed photons), it provides a useful tool to estimate the other two components since the three processes are competitive (Bolhar-Nordenkampf and Oquist 1993). At room temperature the fluorescence is predominantly emitted by PSII and can be measured using pulse-modulated fluorimeters (Ogren and Baker 1985; Schreiber et al. 1986). The lowering, or quenching, of fluorescence caused by photochemical dissipation is termed photochemical quenching whereas all the other processes are summarised as nonphotochemical quenching.

7.1.2 XANTHOPHYLLS AND PHOTOPROTECTION IN LEAVES

The light-harvesting antennae of plants contain a large number of chlorophyll molecules and, in addition, they contain several different carotenoids. Higher plants have two types of carotenoids, carotenes and xanthophylls. The most ubiquitous carotene is β-carotene which is found in all photosynthetic organisms. Some species contain α-carotene as well (Demmig-Adams and Adams 1992). Common xanthophylls include lutein and neoxanthin as well as those participating in the xanthophyll cycle, violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z). The xanthophyll cycle pigments undergo light-dependent interconversions. Low lumenal pH activates a thylakoid membrane-bound de-epoxidase, which converts V to Z via A. This happens usually under excess light and the reverse reaction occurs when light is no longer excessive (Fig. 7.1). All xanthophyll pigments are derivatives of β-carotene and so possess a conjugated isoprenoid chain. The epoxidation/de-epoxidation reaction alters the length of this chain, which affects the physical properties of the molecule and its function in photoprotection (for reviews of structure and function of the xanthophyll pigments see Demmig-Adams et al. 1996; Yamamoto and Bassi 1996; and Gilmore 1997).

The importance of these accessory pigments is well demonstrated by the fact that carotenoids are found in all photosynthetic organisms, and they are closely associated with both antenna and reaction centre pigments (Siefermann-Harms 1985; Yamamoto and Bassi 1996). Furthermore, the ability of plant to regulate its
Fig. 7.1. The xanthophyll cycle. De-epoxidation of violaxanthin to zeaxanthin via antheraxanthin is favored under conditions of excess light absorption and high non-photochemical quenching. Epoxidation back to antheraxanthin occurs in the dark or partially under conditions of limiting light (Demmig-Adams and Adams 1996). Reproduced from Heldt (1997).

xanthophyll pool size seems to be an important mechanism in light adaptation. High-light grown leaves tend to contain more xanthophyll cycle pigments per unit chlorophyll than shade-leaves and the fraction of all carotenoids represented by V+A+Z also increases (Demmig et al. 1988; Thauer and Björkman 1990; Demmig-Adams et al. 1996) When the growth irradiance is changed, xanthophyll cycle pigment pool size adjusts to new conditions rapidly (Björkman and Demmig-Adams 1995).

Carotenoids have two functions in photosynthesis: they participate in light harvesting and photoprotection. Xanthophyll cycle pigments (V+A+Z) can act in photoprotection either directly or indirectly. Direct means include quenching of triplet chlorophyll and singlet oxygen (Krinsky 1979). Triplet chlorophyll can form if the excited state of chlorophyll (Chl*) is not dissipated rapidly through photochemistry or NRD. Chl* can then react with O₂ and highly reactive and
damaging singlet oxygen can be produced. Zeaxanthin may also act as a direct quencher of Chl* by accepting excitation energy from it, dissipating it thermally (Owens et al. 1992).

It is not known how the xanthophyll cycle pigments exactly perform in indirect photoprotection, ie. enhancement of NRD; however, there are several proposed mechanisms. Horton et al. (1991) have suggested that xanthophylls enhance the pH-dependent aggregation of PSII light-harvesting complexes. It has also been implied, that the rate of NRD can be influenced by protonation of the small chlorophyll binding proteins of the PSII inner antenna and subsequent binding of xanthophylls to them (Gilmore 1997). A separate view is presented by Havaux and Gruzecki (1993) who reported that Z decreases membrane fluidity. This can help to stabilise PSII complexes and may be important during temperature stress. Recently, it has been proposed that α-carotene-derived xanthophylls, such as lutein, may contribute to NRD as well (Niyogi et al. 1997)

7.1.3 THE OUTLINE OF THE CHAPTER

The pigment composition of the leaves and the functioning of xanthophyll cycle pigments were studied in transgenic tobacco plants with reduced carbon assimilation capacity. Two different transgenic plant types was used, plants with reduced amount of Rubisco (anti-SSu plants) or with reduced activity of chloroplast glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH plants). Both plant types were grown under similar conditions in glasshouse and the leaf pigment contents were analysed from them. In addition, the induction of photosynthesis was followed in anti-SSu plants after dark-adapted leaves were exposed to light, while xanthophyll conversion and fluorescence quenching parameters were measured simultaneously. After 1 hour the light was switched off and the dark recovery of fluorescence parameters and the reconversion of the xanthophyll pigments was followed.
7.2 MATERIAL AND METHODS

7.2.1 PLANT MATERIAL

Transgenic tobacco with reduced amounts Rubisco was germinated from the seeds collected from a selfed R1 progeny of tobacco (*Nicotiana tabacum* cv. W38) transformant (Hudson *et al.* 1992) with antisense construct against Rubisco’s small subunit (anti-SSu plants). Seedlings segregate in a 1 : 2 : 1 ratio into homozygous, hemizygous or null type (wild-type). Homozygous plants (referred to as anti-SSu 2 plants) have two copies of the *rbcS* antisense gene and typically 10-15% of the wild-type Rubisco content. Hemizygous plants (referred to as anti-SSu 1 plants) have one copy of the antisense gene and 30-40% of the wild-type Rubisco content. The anti-glyceraldehyde 3-phosphate dehydrogenase plants (anti-GAPDH plants) were the R1 progeny of plant GAP-R (Price *et al.* 1995a), and produce a range of GAPDH activities. Untransformed cv. W38 tobaccos were used as controls. All plants were grown in 5-L pots in garden soil and were given Hewitt's complete nutrient solution (Hewitt and Smith 1975) three times a week. Tobaccos were used 6-8 weeks after germination, depending on the growth rate of the plants. The detailed growth conditions are explained for each experimental set separately.

7.2.2 EXPERIMENT 1: LEAF PIGMENT CONTENTS IN ANTI-GAPDH PLANTS

Anti-GAPDH and wild-type tobaccos were grown in air-conditioned glasshouse where the peak light intensity was 900-700 μmol quanta m⁻² s⁻¹ (February-March 1997). The CO₂ assimilation rate of each plant used was measured on the youngest full-grown leaf (LI-6400 system, LiCor, Lincoln, NE), at 350 μbar CO₂ in air, irradiance of 1000 μmol quanta m⁻² s⁻¹ and leaf temperature of 25 °C. Discs for pigment and GAPDH-enzyme assays were collected later from the same leaf at noon on a sunny day. Discs were frozen rapidly in liquid nitrogen and stored at -80 °C.
7.2.3 EXPERIMENT 2: XANTHOPHYLL CYCLE AND FLUORESCENCE QUENCHING PARAMETERS IN ANTI-SSU PLANTS:

7.2.3.1 Plant material and experimental procedure

Anti-SSu and wild-type tobaccos were grown in air-conditioned glasshouse with a peak irradiation of about 700 μmol quanta m⁻² s⁻¹. All plants were kept in darkness overnight prior to the experiments. The youngest full-grown leaf was enclosed in a sandwich-type leaf chamber (43x43x3 mm) and the upper surface was sealed with starch paste to the water-thermostated leaf chamber window (Laisk and Oja 1998). The leaf chamber was illuminated through a fibre-optic light guide system connected to halogen lamps (Schott KL 1500) which provided the actinic white light, saturating pulses and far-red light. The chlorophyll fluorescence was measured by a PAM 101 fluorometer (H. Walz, Effeltrich, Germany) on a leaf area of (5x 15 mm) to one side of the leaf chamber.

The induction of photosynthesis and chlorophyll fluorescence quenching parameters were followed after the leaf was subjected to a step increase in light intensity from 10 to 800 μmol quanta m⁻² s⁻¹ at 340 μbar CO₂ in air. During the induction period the leaf gas exchange was measured continuously, and saturating light pulses (4000 μmol quanta m⁻² s⁻¹, duration 800 milliseconds) were applied every 30 seconds. Gas-exchange parameters were calculated according to von Caemmerer and Farquhar (1981). Chlorophyll fluorescence was recorded with a PC using FlashDisc-program (written by G. Seaton). Fₘ and F₀ (maximal and minimal fluorescence in dark) were determined before leaf was exposed to light and F₀' (minimal fluorescence in light) was measured several times during the illumination by darkening the leaf, and applying a 2-3 s. far-red light pulse. After 60 minutes the light was switched off, and the fluorescence parameters were recorded during a 60 minute recovery period. The saturating pulses were applied every 60 seconds during the first 30 minutes of the dark period, in order to measure the fast relaxation of the fluorescence quenching, and then the time between the flashes was changed to 180 seconds. The opposite leaf from each plant was subjected to similar light regime. Discs for pigment analysis were collected from this leaf prior to light treatment as well as after 5, 10, 15, 20, 30 and 60 minutes illumination, and at similar intervals during
the dark recovery period. The samples were immediately frozen in liquid nitrogen and stored in -80 °C.

7.2.3.2 Calculation of the fluorescence parameters.

Non-photochemical quenching (qN), was calculated as \((F_m-F_m')/(F_m-F_o)\) and photochemical quenching (qP) was calculated as \((F-F_o)/(F_m'-F_o)\) (van Kooten and Snel 1990). 1-qP is equivalent to the reduction status of QA, the primary acceptor of PSII. The extent of nonphotochemical quenching of \(F_m\) was also calculated according to Stern-Volmer equation, where \(NPQ=(F_m-F_m')/F_m\) (Bilger and Björkman 1994).

7.2.4 BIOCHEMICAL ASSAYS

Measurements of leaf pigments. Frozen leaf discs were extracted with 100% acetone as described in Watling et al. (1997). 2-3 leaf discs (0.75 cm²) were ground into powder with mortar and pestle in liquid nitrogen in a darkened room. Pigments were extracted with 100% acetone with approximately 1 mg of NaHCO₃ added to prevent acidification. The extract was made up to a known volume, incubated on ice for 30 minutes and centrifuged at 5000 g for 5 min at 1-2 °C. The supernatant was stored at 20 °C until analysis (no longer than 12 h). Pigments were separated and quantified using HPLC according to the method of Gilmore and Yamamoto (1991). A Spherisorb ODS1 column was used (5 μm particle size, 250 mm × 4.6 mm i.d. from Alltech Associates Inc., Deerfield IL., USA), and it was calibrated with standards of known concentrations. The Waters HPLC system was used, and it consisted of two model 510 pumps, a model 717 autosampler and programmable multiwavelength detector model 490. Two solvent gradient program was used. Solvent mixtures were: A, acetonitrile-methanol-0.1 M Tris-HCl buffer 0.1 M pH 8 (75:12:4.5); B, methanol-hexane (4:1). Solvent A was ran isocratically during 5.5 min with a flow rate of 2 ml min⁻¹ followed by a 2-min linear gradient to 100% solvent B, and the flow rate was simultaneously decreased to 1.2 ml min⁻¹. Solvent B was then ran isocratically 7.5 minutes. The columns were re-equilibrated between samples for 5 min with solvent A, flow rate 2 ml min⁻¹. Sample injections were 40-60 μl and absorptance at 440 nm was recorded.
Rubisco content and GAPDH activity. Leaf Rubisco content was measured by the $^{14}$C-carboxyarabinitol 1,5 bisphosphate (CPBP) binding to fully carbamylated Rubisco sites as described in Chapter 2. Total GAPDH activity was determined as described in Chapter 2.
7.3 RESULTS

7.3.1 LEAF PIGMENTS

The chlorophyll and carotenoid pigments were analysed from wild-type tobaccos as well as from a range of anti-SSu and anti-GAPDH plants. The anti-SSu 1 and anti-SSu 2 plants had, on an average, 30% and 10% of the wild-type Rubisco content, respectively. The range of GAPDH activities of the plants included in this study varied from 62% of the average wild-type activity to non-detectable (data not shown). The samples from anti-SSu plants were collected during the photosynthetic induction experiments as described in Section 7.2.3.1, and samples from anti-GAPDH plants were collected from the growth conditions. The photosynthetic rates were measured from the same plants as well.

Leaf pigment contents, as a function of CO$_2$ assimilation rate, are presented in Figs 7.2 and 7.3. The changes in the pigment content of the two transgenic plant types were similar: as the CO$_2$ assimilation capacity declined, the content of chlorophyll decreased (Fig. 7.2 A). The decrease was quite marked: the most severe anti-SSu and anti-GAPDH plants had only about half as much chlorophyll as the wild-type leaves. Despite the decline in total chlorophyll content, chlorophyll $a/b$ -ratio did not change and was on an average (±S.E) 3.15±0.05 in all leaves (data not shown). The total amount of carotenoids (β-carotene + lutein + neoxanthin +V+A+Z) decreased simultaneously with chlorophyll content (Fig. 7.2 B) so that the ratio between the two remained constant (Fig. 7.2 C). The amount of xanthophyll cycle pigments (V+A+Z) in wild-type and transgenic plants was relatively similar when expressed on a leaf area basis (Fig. 7.3 A). The ratio of V+A+Z to chlorophyll changed slightly differently in anti-SSu and anti-GAPDH plants. In anti-SSu plants the V+A+Z/Chl ratio increased so that in was, on an average, 1.5 times higher than in wild-type plants. This ratio increased only in the most severe anti-GAPDH plants with about 90% reduction in the CO$_2$ assimilation rate (Fig. 7.3 B). The changes in the proportion of xanthophylls of total carotenoids were alike in anti-GAPDH and anti-SSu plants: in wild-type V+A+Z constituted on an average 17% of the total xanthophyll pool, and the percentage approximately doubled in the most severe antisense plants (Fig. 7.3 C).
Fig. 7.2. Chlorophyll and total carotenoid contents in wild-type, anti-GAPDH and anti-SSu tobacco leaves. The pigment contents are presented as a function of steady-state CO₂ assimilation rates, measured at 800-1000 μmol quanta m⁻² s⁻¹, 350 μbar CO₂ in air and a leaf temperature of 25 °C. Symbols: anti-GAPDH plants (♦) and their controls (•), anti-SSu 1 (○), anti-SSu 2 (△) plants and their controls (○).
Fig. 7.3. Xanthophyll cycle pigments in wild-type, anti-GAPDH and anti-SSu plants. Symbols: anti-GAPDH plants (♦) and their controls (●), anti-SSu 1 (○), anti-SSu 2 (△) plants and their controls (○). The measuring conditions are as in Fig. 7.2.
7.3.2 PHOTOSYNTHESIS AND CHLOROPHYLL FLUORESCENCE IN ANTI-SSU PLANTS DURING PHOTOSYNTHETIC INDUCTION

The induction of photosynthesis was followed in wild-type and anti-SSu tobaccos after dark-adapted leaves were exposed to light. Leaf gas exchange and chlorophyll fluorescence were measured simultaneously, and samples were collected for pigment analysis. After 1 hour the light was turned off and the chlorophyll fluorescence measurements and pigment sampling were continued. 3-4 plants per genotype was analysed, and one representative of each is presented in the following figures.

7.3.1.1 Leaf gas exchange

The light induction of CO₂ assimilation and stomatal conductance of one representative wild type, anti-SSu 1 and anti-SSu 2 plant are presented in Fig. 7.4. The time course of CO₂ assimilation in the wild-type plants exhibited three phases. An initial slow phase (first 10 minutes), followed by a relatively rapid increase over the next 30-40 minutes until a final steady-state was reached. The rate of increase in CO₂ assimilation rate was determined mainly by stomatal opening. After the long dark adaptation (one night) the initial stomatal conductance was in most cases low. It then took about 10 minutes before stomata started to open in the light after which the opening was rapid. This pattern of stomatal opening and CO₂ assimilation is commonly observed in dark-adapted leaves (Kirschbaum and Pearcy 1988; Bro et al. 1996). In the anti-SSu plants the induction of CO₂ assimilation did not have equally distinct phases. The final, steady-state rates of CO₂ assimilation were 45% and 10% of the wild-type rates for anti-SSu 1 and 2 plants, respectively (Table 7.1). The rate of stomatal opening was similar in wild type and antisense plants, as was the final conductance. The intercellular CO₂ concentration (Cᵢ) was low (below 100 µbar) in the wild-type plants at the beginning of the induction (Fig. 7.4 C) and throughout the experiment it remained lower than the Cᵢ of the antisense plants.
Fig. 7.4. CO₂ assimilation, stomatal conductance and intercellular CO₂ concentration during the induction of photosynthesis. CO₂ assimilation rate (A) and stomatal conductance to water vapour (B) and intercellular CO₂ (C) were measured on attached leaves of wild-type (●), anti-SSu 1 (□) and anti-SSu 2 (△) tobaccos following an increase in light intensity from 10 to 800 µmol quanta m⁻² s⁻¹ at 350 µbar CO₂ in air. Leaf temperature was kept at 25 °C. Prior to experiments plants were kept in darkness overnight. One representative of each genotype is presented.
Table 7.1. Photosynthesis, chlorophyll fluorescence and xanthophyll pigments in wild-type and anti-SSu tobaccos during photosynthetic induction. Parameters were measured from attached leaves, following an increase in light intensity from 10 to 800 μmol quanta m$^{-2}$ s$^{-1}$ at 350 μbar CO$_2$ in air. Leaf temperature was kept at 25 °C. Prior to experiments plants were kept in darkness overnight.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=4)</th>
<th>SSu 1 (n=3)</th>
<th>SSu 2 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ assimilation (μmol m$^{-2}$ s$^{-1}$)</td>
<td>11.6±0.8</td>
<td>5.3±0.6</td>
<td>1.1±0.5</td>
</tr>
<tr>
<td>1-qP 60 min induction</td>
<td>0.44±0.04</td>
<td>0.67±0.04</td>
<td>0.75±0.03</td>
</tr>
<tr>
<td>NPQ 60 min induction</td>
<td>2.23±0.18</td>
<td>3.23±0.18</td>
<td>3.97±0.27</td>
</tr>
<tr>
<td>NPQ 60 min recovery</td>
<td>0.31±0.06</td>
<td>0.30±0.04</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>qN 60 min induction</td>
<td>0.80±0.02</td>
<td>0.88±0.01</td>
<td>0.91±0.01</td>
</tr>
<tr>
<td>qN 60 min recovery</td>
<td>0.27±0.04</td>
<td>0.25±0.06</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>A+Z/VAZ, % 0 minutes light</td>
<td>4.6±1.7</td>
<td>13.2±4.4</td>
<td>18.3±3.9</td>
</tr>
<tr>
<td>A+Z/VAZ, % 60 min induction</td>
<td>28.8±3.6</td>
<td>72.3±0.7</td>
<td>83.0±0.6</td>
</tr>
<tr>
<td>A+Z/VAZ, % 60 min recovery</td>
<td>7.8±1.6</td>
<td>29.3±6.7</td>
<td>36.0±8.5</td>
</tr>
<tr>
<td>ΦPSII (F$<em>{v}$/F$</em>{m}$) 0 minutes light</td>
<td>0.835 ± 0.007</td>
<td>0.824 ± 0.004</td>
<td>0.811 ± 0.004</td>
</tr>
<tr>
<td>ΦPSII (F$<em>{v}$/F$</em>{m}$) 60 minutes induction</td>
<td>0.298 ± 0.034</td>
<td>0.132 ± 0.011</td>
<td>0.085 ± 0.009</td>
</tr>
<tr>
<td>ΦPSII (F$<em>{v}$/F$</em>{m}$) 60 minutes recovery</td>
<td>0.759 ± 0.020</td>
<td>0.724 ± 0.011</td>
<td>0.673 ± 0.043</td>
</tr>
</tbody>
</table>
7.3.1.2 Fluorescence quenching parameters

On illumination the primary electron acceptor of PSII, QA, became highly reduced in all plant types, as seen in the rapid decline in 1-qP (Fig 7.5). However, in the wild-type plants QA became more oxidised as the CO₂ assimilation rate increased so that after 1 hour at light the reduction state was on an average 0.44. In both antisense plants QA remained highly reduced throughout the light period (Table 7.1). The nonphotochemical quenching (NPQ) increased rapidly in all plant types. In the wild-type plants NPQ (as well as qN, see Table 7.1) declined as the induction of the CO₂ assimilation proceeded. On the contrary, in the antisense plants NPQ remained high, or even increased, during the light treatment. At the end of the 1 hour light period NPQ in anti-SSu 1 and anti-SSu 2 plants was 1.5 and 1.8 times higher than in wild-type plants (Table 7.1). When the light was turned off, there was a rapid decline of NPQ in all plants during the first 3 minutes. The decrease continued so that after 10 minutes most of NPQ was relaxed in wild-type plants. In the anti-SSu plants the relaxation was slightly slower resulting in a more sustained NPQ during the first 30 minutes in dark. Nevertheless, the final level of NPQ after 1 h in darkness was similar in all plants (Fig. 7.3 and Table 7.1).

7.3.3 XANTHOPHYLL CYCLE PIGMENT CONVERSION

7.3.3.1 Anti-SSu plants during induction

A proportion of the xanthophyll cycle pigments in the anti-SSu plants remained in the de-epoxidated forms (A+Z) overnight. They had about 3 times more A+Z than wild-type plants at the beginning of the experiment (Fig. 7.6 and Table 7.1). Anti-SSu plants also converted significantly more V to A+Z in light: after 1 hour wild-type plants had approximately 30% of the total V+A+Z pool in A+Z-forms, whereas this was 72% and 83% in anti-SSu 1 and 2 plants, respectively. The conversion was fast, so that most of the Z was produced within the first 10 minutes in light. The anti-SSu plants also reconverted A+Z to V more slowly during the low-light recovery period, retaining up to 36% of the xanthophylls in the de-epoxidated forms, in comparison to 8% in the wild type.
Fig. 7.5. Chlorophyll fluorescence quenching parameters in wild-type and anti-SSu plants during photosynthetic induction and dark recovery. Fluorescence and gas exchange (Fig. 7.4) were measured from the same leaves. During the photosynthetic induction saturating pulses were given 30-s intervals. After 60 minutes light was turned off and the saturating pulses were applied every 60 seconds during the first 30 minutes of the dark period, after which the time between the flashes was changed to 180 seconds. 1-qP (V) is equivalent to the reduction state of QA, the primary electron acceptor of PSII. NPQ (♦) measures the extent of nonphotochemical quenching of chlorophyll fluorescence. One representative of each plant genotype is presented, the summary of the data is in Table 7.1.
Fig. 7.6. Xanthophyll cycle pigment conversion in wild-type and anti-SSu plants during photosynthetic induction and dark recovery. The opposite leaf to one used for gas exchange and fluorescence studies (Figs. 7.4 and 7.5) was subjected to similar light regime and samples were collected for pigment analysis. The contents of violaxanthin (V, ●), antheraxanthin (A, □) and zeaxanthin (Z, △) are given as a percentage of the total xanthophyll pool. One representative of each plant genotype is presented here, and the summary of the data is in Table 7.1.
7.3.3.2 Anti-GAPDH plants

Leaf discs from anti-GAPDH plants and their controls, growing in glasshouse, were collected at noon on a sunny day so that the xanthophyll de-epoxidation state under growth conditions could be determined. These midday conversion results are presented in Fig. 7.8. For comparison, the results of the xanthophyll measurements from anti-SSu plants, after 1-h light exposure, is included as well. The trend in the data is clear: as the capacity for CO₂ assimilation decreased, the de-epoxidation of the xanthophyll cycle pigments increased markedly. The wild type and wild-type-like anti-GAPDH plants converted only approximately 30% of their V pool to A+Z, whereas the conversion state increased to more than 80% in the most severe antisense plants.

Fig. 7.7. Xanthophyll pigment conversion in wild-type, anti-GAPDH and anti-SSu plants. The sum of zeaxanthin + antheraxanthin, as a percentage of the total xanthophyll pool, is presented as a function of steady-state CO₂ assimilation rates. Samples from anti-GAPDH plants and their controls were collected from growth conditions (glasshouse) at noon on a sunny day. For comparison, this figure also contains the pigment data of anti-SSu 1 and SSu 2 plants and their controls, measured after 1 h exposure to 800 µmol quanta m⁻² s⁻¹. Symbols and CO₂ assimilation measurement conditions are as in Fig. 7.2.
7.4 DISCUSSION

The present studies were conducted to examine the effects of decreasing CO₂ assimilation capacity on the composition and functioning of the leaf xanthophyll cycle pigments. It has been suggested that xanthophyll cycle pigments are important in dissipating the excess light energy, which cannot be used in photosynthesis. Therefore, it would be logical to expect that these pigments are more abundant in leaves which receive lot of light but whose photosynthetic capacity is low (Thauer and Björkman 1990). Due to the large interspecies variation in leaf pigment composition, comparisons between different species with high or low photosynthetic rates are complicated, even if they are grown under similar light conditions (Demmig-Adams and Adams 1992). Transgenic plants thus offer a good opportunity to study the direct effect of photosynthetic capacity on leaf pigments.

7.4.1 LIGHT ENERGY UTILISATION

As the photosynthetic capacity decreases in the transgenic plants, the proportion of absorbed light which can be used in photochemistry decreases. This situation is illustrated in Fig. 7.8. When leaves are exposed to high light (800 µmol quanta m² s⁻¹) wild-type tobaccos can utilise approximately 25% of the absorbed light in photosynthesis but this proportion declines to 5% in anti-SSu 2 plants with severely reduced CO₂ assimilation capacity. The aim of this study was to see how this increased need for nonradiative dissipation affects the leaf pigments and light harvesting efficiency.

7.4.2 LEAF PIGMENT COMPOSITION

7.4.2.1 Xanthophyll cycle pigments

Two different transgenic tobaccos were used in this study: plants with either reduced activity of chloroplast glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or reduced amount of Rubisco. The leaf pigment contents in both antisense tobaccos were comparable, when plants were grown under natural illumination in glasshouses (Figs 7.2 and 7.3). A large difference in the CO₂ assimilation capacity between wild-type and transgenic plants did not have a large effect on the ratio between
Fig. 7.8. The allocation of absorbed light to photosynthesis (P) and nonradiative dissipation (NRD) in a typical leaf of wild-type and anti-SSu tobaccos. The fraction of absorbed light that is utilised in photosynthesis was estimated from the steady-state CO₂ assimilation rates at the end of the induction period (see Fig. 7.4). The rate of electron transport was calculated as \((A+R_d)(4C_c + 8\Gamma^*)/(C_c \Gamma^*)\), where \(A\) is the CO₂ assimilation rate, \(R_d\) the dark respiration rate, \(C_c\) the chloroplast CO₂ partial pressure and \(\Gamma^*\) the CO₂ compensation point in the absence of mitochondrial respiration (38.6 µbar at 21% O₂). Chloroplast CO₂ \((C_c)\) was calculated as \(C_i - (A/g_a)\), where \(C_i\) is the intercellular CO₂ concentration, \(A\) the assimilation rate and \(g_a\) the transfer conductance for CO₂ (0.3 mol m⁻² s⁻¹ bar⁻¹ von Caemmerer and Farquhar 1981, von Caemmerer et al. 1994). Two absorbed photons per one electron was accounted for.

xanthophylls and chlorophyll. Only in the most severe antisense plants, with about 10% of the wild-type CO₂ assimilation rate, this ratio increased. This result agrees with previous observations from transgenic tobacco plants with reduced amount of the chloroplast fructose 1,6-bisphosphatase (FBPase) and subsequent 50% inhibition in the light-saturated electron transport capacity (Bilger et al. 1995). The \(V+A+Z/Chl\) ratio in these anti-FBPase tobaccos remained similar to wild-type plants, even if the plants were grown at several different light intensities.

The authors of the anti-FBPase tobacco-study repeated the experiments using anti-FBPase potato plants, which had 70% reduction in the light-saturated electron transport rate. The \(V+A+Z/Chl\) ratio increased in the anti-FBPase potatoes as opposed to the tobaccos (Bilger et al. 1995). The authors concluded that "regulatory mechanisms exist in potato that allow an adjustment of \(V+A+Z\) pool sizes according to photosynthetic capacity, and these mechanisms are absent in tobacco". My data agrees with this but also suggests that, in tobacco, the photosynthetic capacity needs to be reduced well over 50% before the ratio between \(V+A+Z\) and chlorophyll
increases, and even then the change in the ratio is due to a decrease in the chlorophyll content per leaf area. My results also raise the question, whether the difference in the response of pigment composition to reduction in electron transport rate, observed between the anti-FBPase tobaccos and potatoes, was due to a different degree of inhibition in the photosynthetic capacity. However, my study was conducted at only one irradiance level and under natural, fluctuating light as opposed to the study by Bilger et al. (1995).

7.4.2.2 Chlorophyll content

The amount of chlorophyll per leaf area decreased in both anti-SSu and anti-GAPDH plants in this study (Fig. 7.5 A). A decline in chlorophyll content is commonly seen in transgenic plants with reduced photosynthetic capacity. Anti-FBPase potato leaves had less chlorophyll than wild-type leaves, regardless of the growth irradiance (Bilger et al. 1995). In an earlier study it was reported that as the activity of GAPDH was reduced below 50% of the wild-type activity, the chlorophyll content started to decline (Price et al. 1995a). Similarly, in tobaccos with less than 50% of the wild-type Rubisco activity chlorophyll contents tend to be lower than in controls and less affected transgenic plants (Quick et al. 1991; Lauerer et al. 1993). In my study, the anti-SSu 1 plants (with 30% of the wild-type Rubisco content) had 30% less chlorophyll than wild-type plants, which seems to confirm the trend (Fig. 7.2 A). However, it is interesting to note that a previous study using the same anti-SSu 1 genotype found no difference in the amount of chlorophyll per leaf area (Evans et al. 1994). This discrepancy probably demonstrates that a 30-40% reduction in Rubisco content does not always lead to a decrease in chlorophyll content because the ratio between Rubisco and chlorophyll is also governed by factors like growth light and temperature.

It might be argued that the decrease in chlorophyll content itself is an adaptive mechanism to avoid excess light absorption, and the increase in V+A+Z/Chl ratio is merely a consequence of this. Bilger et al. (1995) concluded that no matter what the reason behind the reduction in chlorophyll content is (reduction in the chloroplast number, less thylakoid membranes per chloroplast or fewer pigment protein complexes per membrane area), the carotenoid content should follow these changes.
The ratio between the total amount of carotenoids and chlorophyll in my study indeed remained constant across the plant genotypes (Fig. 7.3 A), whereas the proportion of the total carotenoids represented by V+A+Z increased in the most severe transgenic plants (Fig. 7.3 C). The results suggest, that the severe reduction in the CO₂ assimilation capacity caused a specific increase in the xanthophyll pool pigments in the leaves, which was not just a consequence of the decreased chlorophyll content. It is also worth noting that the decrease in leaf chlorophyll content per se hardly has a significant photoprotective effect. The 50% reduction in chlorophyll content in anti-SSu 2 plants, compared to their controls, decreased the leaf absorptance only by about 10% (wild-type absorptance 77%, anti-SSu 1 75%, anti-SSu 2 66%, data not shown), see also Björkman and Demmig-Adams (1995). The lower chlorophyll content which was observed in the most severe antisense plants could be a consequence of the lower nitrogen and protein content of these leaves (Hudson et al. 1992; Price et al. 1995a)

The changes in the leaf pigments caused by a genetically reduced CO₂ assimilation capacity are to a some degree similar to changes occurring during high-light acclimation. In leaves transferred to higher growth light the V+A+Z/Chl ratio increases and there is also a pronounced increase in the proportion of all carotenoids represented by V+A+Z (Demmig et al. 1988; Demmig-Adams et al. 1989a; Thauer and Björkman 1990; Logan et al. 1997). These changes were seen in the severe transgenic tobaccos as well. The ratio between total carotenoids and chlorophyll is also typically greater in high-light grown leaves than in shade leaves (Demmig-Adams et al. 1996). This ratio remained constant in all tobaccos (Fig. 7.3 A).

7.4.3. NPQ AND XANTHOPHYLL DE-EPOXIDATION IN TRANSGENIC PLANTS

It is frequently suggested that nonphotochemical quenching (NPQ) involves the formation of a transthylakoid pH gradient (ΔpH) and also requires presence of Z and A. Together these factors cause changes in the light harvesting pigment-protein complexes which increase that rate constant of heat dissipation (Demmig et al. 1988; Gilmore and Yamamoto 1993; Horton et al. 1994; Demmig-Adams et al. 1996). The functioning of the xanthophyll cycle in anti-SSu plants was further studied by
subjecting dark-adapted leaves to high light, and following the induction of photosynthesis, xanthophyll conversion and fluorescence quenching parameters. In addition, xanthophyll conversion state at growth conditions was analysed from a range of anti-GAPDH plants growing in a glasshouse.

7.4.3.1 Fluorescence quenching

Upon illumination a high NPQ developed quickly (Fig. 7.5), indicating that the light exposure caused a rapid lumen acidification (Krause et al. 1982; Gilmore and Yamamoto 1992). The need for nonradiative dissipation is high at the beginning of the photosynthetic induction, because several components of the photosynthetic machinery are inactive. After long dark period, light is not only required to induce stomatal opening (Kirschbaum and Pearcy 1988; Pearcy 1988), but also to activate photosynthetic enzymes, including Rubisco (Pearcy and Seemann 1990; Lan et al. 1992; Woodrow and Mott 1992; Sassenrath-Cole and Pearcy 1994). The enzyme activation takes place in about 10 minutes following an increase in light intensity (Seemann et al. 1988; Woodrow and Mott 1989; Sassenrath-Cole and Pearcy 1994). The low capacity for photochemical dissipation at the beginning of the induction is demonstrated by the high reduction state of QA (Fig. 7.5).

As CO₂ assimilation and linear electron transport started to function in wild-type plants, the reduction state of QA decreased. However, in anti-SSu plants the reduction state of QA remained high. Anti-SSu plants also maintained significantly higher NPQ throughout the light period. Constitutive, high NPQ has been observed in several transgenic plants with impaired photosynthesis, for example anti-FBPase potato and tobacco plants (Bilger et al. 1995; Fisahn et al. 1995), transgenic tobaccos with reduced activity of phosphoribulokinase (Habash et al. 1996), Rubisco (Quick et al. 1991; Lauerer et al. 1993), nitrate reductase (Foyer et al. 1994) and GAPDH (Price et al. 1995a). Altogether these results demonstrate how lowered assimilation capacity leads to enhancement of NRD.
7.4.3.2 Xanthophyll conversion

The reduction in the CO₂ assimilation capacity affected the xanthophyll cycle pigment conversion. Even after 15 hours darkness, the anti-SSu plants retained xanthophyll cycle pigments in de-epoxidated forms (A+Z), unlike the wild-type plants (Fig. 7.6, Table 1). Similarly, leaves that experience significant light stress during day are known to retain A+Z overnight (Barker et al. 1998; Thiele et al. 1998). It has been suggested that a maintenance of low lumenal pH overnight is a prerequisite for A+Z retention (Verhoeven et al. 1996). Together the pre-existing low pH and A+Z would then offer photoprotection upon the first exposure to light.

When exposed to 800 μmol quanta m⁻² s⁻¹ light, all plants started converting V to A and Z. The response to the light was rapid, as most of the de-epoxidation took place within 10 minutes, probably following the light-driven lumen acidification. Low pH is required for A+Z formation for two reasons. Firstly, the pH-optimum of the lumenal xanthophyll de-epoxidase enzyme is around 5 (Yamamoto 1979), and secondly, the formation of its cofactor, ascorbate, is favoured in acidic conditions (Bratt et al. 1995). The fact that impaired photosynthetic capacity enhances violaxanthin de-epoxidation (A+Z formation) in transgenic tobaccos is well illustrated in Fig. 7.7 where A+Z content of both anti-SSu and anti-GAPDH plants are shown to increase even if the CO₂ assimilation rate is only moderately reduced. Similar results of enhanced violaxanthin de-epoxidation in transgenic plants have been obtained from anti-FBPase tobaccos and potatoes (Bilger et al. 1995; Fisahn et al. 1995), and transgenic tobaccos with reduced amount of nitrate reductase (Foyer et al. 1994). Accordingly, it has been observed that decreasing the CO₂ assimilation by lowering CO₂ increases Z+A formation in leaves (Gilmore and Björkman 1994a). This agrees with the notion that NPQ requires the presence of A+Z.

7.4.3.3 Transgenic plants showed no long-term signs of photoinhibition

Despite the high NPQ and elevated A+Z content in the anti-SSu tobacco leaves, the reduction state of QA remained high in light. This implies that the excitation pressure to the PSII was very high in the light, which commonly leads to photoinhibition, i.e. sustained reduction of PSII quantum efficiency (ΦPSII). Although the ΦPSII was markedly lower in anti-SSu plants during the light exposure,
in all plants it returned near (above 80%) the original dark values during the 60 minutes recovery period (Table 7.1). Thus antisense plants did not experience severe photoinhibition during this 1-h exposure to high light. This may be because the light intensity used in this experiment was comparable to what the plants would have encountered at growth conditions and, due to acclimation to these conditions, the photoprotective mechanisms were efficient enough to maintain PSII functionality despite a reduction in the CO₂ assimilation capacity.

7.4.4 NPQ RELAXES RAPIDLY IN DARKNESS WHILE XANTHOPHYLL RE-EPoxidATION PROCEEDS MORE SLOWLY

In light, the induction kinetics of NPQ and Z+A formation were alike in all leaves, which agrees with earlier findings (Björkman and Demmig-Adams 1995). As soon as the light was turned off, a majority of NPQ relaxed rapidly within a few minutes, whereas xanthophyll re-epoxidation proceeded more slowly (Fig. 7.5 and 7.6). This difference between A+Z and NPQ during dark relaxation is a commonly observed phenomenon, whereas the two tend to occur concomitantly in light (Gilmore and Yamamoto 1993; Bilger and Björkman 1994; Gilmore and Björkman 1994a; 1994b; Logan et al. 1997). It has been suggested, that this is an example of the interplay between ΔpH and A+Z in governing the extent of NPQ. The fast disengagement of NPQ results from rapid dissipation of ΔpH once the light intensity decreases (Bilger and Björkman 1994; Logan et al. 1997). Xanthophyll re-epoxidation, being an enzymatic process, proceeds slower.

We observed that NPQ relaxed to a same degree in all plants during 1 h in darkness (Fig. 7.5 and Table 7.1). This does not support the notion that ΔpH can be sustained overnight in anti-SSu plants, which then would explain why these plants have more A+Z after night (Table 7.1). The reason for the sustained A+Z may be simply that the NADPH required for the epoxidation of A+Z to V (Fig. 7.1) has been exhausted before the process was completed.
7.4.5 IMPAIRED ASSIMILATION RATE IS NOT ALWAYS ASSOCIATED WITH ELEVATED NPQ AND XANTHOPHYLL POOLS

My study, as well as several others, demonstrate how sink-limitation, i.e. decreased assimilation rate, generates greater need for NRD, which is seen as enhancement of NPQ and subsequent increase in xanthophyll cycle pigment pool size and/or de-epoxidation state. However, in transgenic tobaccos with reduced amount of chloroplast cytochrome $b$-complex (anti-$b$ plants) the decrease in photosynthetic capacity was accompanied by lower NPQ and xanthophyll de-epoxidation than in wild-type tobaccos (Hurry et al. 1996). Evidently this was because impaired electron transport prevented the buildup of transthylakoid pH gradient. Thus it can be concluded that it is the increased lumen acidification, associated with impaired capacity to use reducing equivalents, which signals the need for enhanced NPQ in transgenic plants (and partially is responsible for the actual NRD as well), and so probably also in plants that experience inhibition in CO$_2$ assimilation in field.

7.4.6 SUMMARY

Transgenic tobacco plants with reduced carbon assimilation capacity offer a unique opportunity to study how the photosynthetic capacity regulates the light harvesting. As the ability to use light for photosynthesis decreases, a greater need for nonradiative dissipation of energy is generated. In transgenic tobacco plants this is seen as constitutive, high nonphotochemical quenching of chlorophyll fluorescence (NPQ) when the leaves are exposed to light. It is widely suggested that the de-epoxidated forms of xanthophyll cycle pigments are important in the formation of NPQ. This is supported by the observation that the de-epoxidation state of the xanthophyll cycle pigments $(Z+A/V+A+Z)$ increases in transgenic tobaccos, even when the reduction in CO$_2$ assimilation capacity is moderate. Surprisingly, a reduction in the CO$_2$ assimilation capacity did not have a large impact on the leaf pigment composition in glasshouse-grown tobaccos. The ratio of the xanthophyll cycle pigments to chlorophyll and total carotenoids increased only in the most severe transgenic plants, with CO$_2$ assimilation capacity of 10% or less of the wild-type. These changes in the pigment ratios were accompanied with a decrease in the leaf chlorophyll content.
CHAPTER 8

ELECTRON TRANSPORT IN TRANSGENIC TOBACCOS WITH REDUCED CARBON ASSIMILATION CAPACITY
8.1 INTRODUCTION

8.1.1 ELECTRON TRANSPORT: REACTIONS AND RATE ESTIMATIONS

The linear electron transport chain in chloroplasts produces reduced ferredoxin (Fd_{red}). The reducing equivalents can be transferred from Fd_{red} to several acceptors. A large part of the electrons goes in reducing NADP^+ to NADPH (Fig. 8.1). The majority of NADPH is used to power either photosynthetic CO_2 assimilation or photorespiration. There are also additional pathways in chloroplasts that use either Fd_{red} or NADPH, and they are sometimes called alternative electron sinks (Genty and Harbinson 1996). One of the alternative sinks is oxaloacetic acid-malate shuttle, which can transport reducing equivalents out of the chloroplast (Chapter 4). PSI can also transfer electrons, either directly or via Fd_{red}, to O_2 in a process termed the Mehler reaction (Mehler 1957). The photoreduction of O_2 produces highly reactive superoxide radicals which are rapidly detoxified by the ascorbate-peroxidase pathway, which further consumes reducing equivalents in the form of NADPH or Fd_{red} (Badger 1985; Asada and Takahashi 1987). Other alternative sinks inside the chloroplasts include nutrient assimilation and biosynthetic activities (Fig. 8.1).

The occurrence and significance of Mehler reaction in vivo is unclear. It is thought to occur especially when ferredoxin pool is highly reduced, thus allowing linear electron flow to continue under limited NADPH consumption (Neubauer and Yamamoto 1992; Wiese et al. 1998). It has been suggested that, under these conditions, Mehler reaction can assist in development and maintenance of high ΔpH, which in turn enhances nonradiative dissipation of light energy and protects light reactions from photodamage (Neubauer and Yamamoto 1992; Björkman and Demmig-Adams 1995). The estimations of the rate of Mehler reaction vary: it has been reported being between 1-10% of total O_2-evolution in C_3 plants at steady-state conditions (Badger 1985; Robinson 1988) and 20-25% of the maximal electron transport rate (Laisk and Loreto 1996).
The rate of electron transport during photosynthesis in an intact leaf can be measured in two ways. Electron transport through PSII can be calculated on the basis of chlorophyll fluorescence measurements, provided that the incident light intensity and leaf absorptance are known (Genty et al. 1989). It is also possible to calculate the electron transport needed to support CO₂ fixation and photorespiratory pathway from gas-exchange measurements (von Caemmerer and Farquhar 1981). The latter calculations rely on knowing the CO₂/O₂ specificity of Rubisco, which determines the relative contributions of carboxylation and oxygenation of RuBP, as well as the rate of mitochondrial respiration in light and chloroplast CO₂ partial pressure. The comparison between these two methods can be used to detect the presence of alternative electron sinks in intact leaves (Loreto et al. 1994; Laisk and Loreto 1996) or the transfer conductance of CO₂ from the intercellular airspace to the site of carboxylation (Loreto et al. 1992; Evans and von Caemmerer 1996; Laisk and Loreto 1996; Maxwell et al. 1997).

![Nutrient assimilation and CO₂ assimilation and photorespiration](image)

**Fig. 8.1. A scheme for the usage of electrons in the chloroplasts.** Photosystem I produces reduced ferredoxin (Fd_red). The reducing equivalents can be transferred to NADPH, and further used in CO₂ assimilation, photorespiration, or transported to cytosol via oxaloacetate-malate (OAA-MAL) shuttle. Fd_red can also transfer electrons to thioredoxin proteins (Tx) which reduce disulphide bonds in their target enzymes. Electrons can be transferred to O₂, producing superoxide (O₂⁻) which is converted to hydrogen peroxide (H₂O₂) by superoxide dismutase (SOD). H₂O₂ is detoxified via ascorbate pathway which further uses either Fd_red or NADPH to regenerate ascorbate acid (AA) from monohydroascorbate (MDA). Sulphate (SO₄²⁻) and nitrite (NO₂⁻) assimilation take place in chloroplasts and consume reducing equivalents as well. Sulphate is incorporated into cysteine (Cys) and nitrite into glutamine (Glu).
8.1.2 THE OUTLINE OF THE CHAPTER

In this Chapter the photosynthetic electron transport rates are estimated via two different methods: gas exchange and chlorophyll fluorescence. The first objective was to see, how closely the two estimates of electron transport correlate during the photosynthetic induction. As presented in Chapter 7, both gas exchange and chlorophyll fluorescence were measured simultaneously from the leaves of wild-type and anti-SSu plants. The second objective was to examine whether alternative electron sinks are more prevalent in anti-SSu plants. It has been frequently suggested that the Mehler reaction becomes more active in situations where photosynthesis is inhibited and transthylakoid pH gradient increases. The anti-SSu plants, with their reduced photosynthetic capacity, are an excellent material to study this. Importantly, parameters that are used for electron transport rate calculations, such as the specificity factor of Rubisco and transfer conductance of CO₂ from the intercellular airspace to the site of carboxylation, have been previously determined for tobacco (von Caemmerer et al. 1994).

8.2 MATERIAL AND METHODS

8.2.1 EXPERIMENT 1: ELECTRON TRANSPORT RATE DURING PHOTOSYNTHETIC INDUCTION

8.2.1.1 Plant material and experimental setup

Plant material was described in section 7.2.1, and consisted of glasshouse-grown wild-type *Nicotiana tabacum* cv. W38 tobaccos and transgenic plants with 1 or 2 inserts of antisense DNA directed against Rubisco's small subunit. Plants with 1 insert will be referred as anti-SSu 1 plants, and those with 2 inserts will be referred as anti-SSu 2 plants. Growth conditions and experimental procedure was outlined in section 7.2.2. Chlorophyll fluorescence and gas exchange were recorded simultaneously following a light exposure of dark-adapted (about 15 hours) leaf material.
8.2.1.2 Electron transport rate calculations

The rate of electron transport required for the photosynthetic carbon reduction and photorespiration cycles ($J_g$) was calculated from gas exchange data according to von Caemmerer and Farquhar (1981) as

$$J_g = (A + R_d)(4 - C_c + 8T^*)/(C_c - T^*)$$

where $A$ is the net photosynthesis, $R_d$ is non-photorespiratory respiration and $C_c$ is the intercellular CO$_2$ partial pressure inside the chloroplast, assuming that the transfer conductance for CO$_2$ is 0.3 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$ (von Caemmerer et al. 1994) and cuticular conductance for H$_2$O is 2.5 mmol m$^{-2}$ s$^{-1}$ (Boyer et al. 1997). $R_d$ was assessed in the beginning of the experiment for each plant. The CO$_2$ partial pressure at which photorespiration equals photosynthesis in the absence of the day respiration ($T^*$) was 38.6 μbar, determined previously for tobacco (von Caemmerer et al. 1994).

The electron transport rate through PSII ($J_f$) was calculated from the fluorescence data according the following equation

$$J_f = (F_m - F)/F_m * Abs * PAR * 0.45$$

where $(F_m - F)/F_m$ is the quantum yield of electron flow to PSII (Genty et al. 1989). Leaf absorption (Abs) was measured for each tobacco genotype using an Ulbricht integrating sphere, and PAR is the incident light intensity. We assumed that PSII absorbs 45% of the total absorbed light (Laisk and Loreto 1996).

8.2.2 EXPERIMENT 2: STEADY-STATE ELECTRON TRANSPORT AT DIFFERENT OXYGEN CONCENTRATIONS

For a closer comparison of the electron transport rates calculated from the leaf gas exchange and chlorophyll fluorescence, I used wild-type, hemi- and homozygous anti-SSu tobaccos grown in an air-conditioned glasshouse, where the peak light intensity was 900-1000 μmol m$^{-2}$ s$^{-1}$ (October-November 1997). Leaf gas-exchange was measured with a LI 6400 portable system (LiCor, Lincoln, Nebraska) which had a LICOR CO$_2$ injector installed. Different O$_2$ concentrations of the carrier gas were obtained by mixing pure N$_2$ and O$_2$ using mass-flow meters (Mass-Flo type 1179,
MKS Instruments Inc., Andover, MA). A special leaf chamber was used, which had fibre optic bundles fitted into the window, and the fibres were connected to different light sources. Actinic light was provided by a tungsten halogen lamp. Chlorophyll fluorescence was measured using a pulse-modulated fluorometer (PAM 101, Walz, Effeltrich, Germany).

The CO₂ response curves of photosynthesis were measured at 2, 20 or 40% O₂ at an average irradiance of 830 μmol quanta m⁻² s⁻¹ at the leaf surface. The rate of non-photorespiratory respiration was determined in two ways: firstly, it was measured directly prior to experiments, when the leaves were in darkness. Secondly, the respiration rate at each O₂ concentration was found from the intersection of A vs. Cᵢ response curves and Γ*, assuming that Γ* is linearly related to the prevailing O₂ concentration. When gas exchange had reached steady-state at each CO₂ concentration (within about 10-15 minutes), 1-2 saturating flashes (800 ms) were given to determine the quantum efficiency of PSII. The traces for F and Fₘ' determinations were recorded using a computer-based program. The absorptance was measured for each leaf separately using an Ulbricht integrating sphere. The rate of electron transport was calculated on the basis of gas exchange and fluorescence as described earlier.
8.3 RESULTS

8.3.1 ELECTRON TRANSPORT DURING PHOTOSYNTHETIC INDUCTION

Since both the CO₂ assimilation rate and chlorophyll fluorescence were recorded simultaneously during the photosynthetic induction, it was possible to calculate the electron transport rate through PSII (J_f) and electron transport required for photosynthetic carbon assimilation and photorespiration (J_g). An interesting discrepancy between J_g and J_f was observed. As outlined in section 7.3.1.1, the initial stomatal conductance was generally low after the dark adaptation, and it took several minutes before stomata started responding to light (Fig. 7.3). Consequently, the rate of CO₂ assimilation remained low during the first 10 minutes and later followed the stomatal opening. In several plants with very low initial stomatal conductance (less than 5 mmol H₂O m⁻² s⁻¹) the quantum yield of PSII (ΦPSII) exhibited different kinetics from gas exchange. ΦPSII increased already during the first minutes, and continued to increase reaching steady-state approximately at the same time as gas exchange (Fig. 8.2 A). Consequently, in these cases only a fraction of the J_f could be explained by CO₂ assimilation and photorespiration during the first 10-15 minutes (Fig. 8.2 B). After about 20 minutes in light the J_f and J_g were alike.

The difference between J_f and J_g was dependent on the initial stomatal opening and was not affected by the plant genotype. This illustrated in Fig. 8.3, where the J_f /J_g-ratios of another wild-type and anti-SSu 1 plant are presented. Both of these plants had higher initial stomatal conductance (30 and 19 mmol H₂O m⁻² s⁻¹, wild-type and anti-SSu 1 respectively). The induction kinetics for J_f and J_g were similar, and there was only a small difference between J_f and J_g during the first 10 minutes.
Fig. 8.2. Electron transport rates calculated from gas exchange and fluorescence during photosynthetic induction in wild-type and anti-SSu tobaccos with low initial stomatal conductance. A. The time courses. B. The ratio between the two. Gas exchange and chlorophyll fluorescence were recorded simultaneously following a step increase in irradiance from 10 to 800 μmol quanta m$^{-2}$ s$^{-1}$, see Figs 7.2 and 7.3. The initial stomatal conductance of both plants was below 5 mmol H$_2$O m$^{-2}$ s$^{-1}$.  

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*Note:* The image contains graphs showing the electron transport rates (ETR) and the ratio between the two. The graphs illustrate the time courses and the ratio of ETR between wild-type (WT) and anti-SSu (SSu) tobaccos. The data are obtained from gas exchange and fluorescence measurements following a step increase in irradiance. The initial stomatal conductance of both plants was below 5 mmol H$_2$O m$^{-2}$ s$^{-1}$.
Fig. 8.3. Electron transport rates calculated from gas exchange and fluorescence during photosynthetic induction in wild-type and anti-SSu tobacco. A. The time courses. B. The ratio between the two. Gas exchange and chlorophyll fluorescence were recorded simultaneously following a step increase in irradiance from 10 to 800 μmol quanta m⁻² s⁻¹, see Figs 7.2 and 7.3. The initial stomatal conductance for this wild-type and anti-SSu 1 tobacco was 30 and 10 mmol H₂O m⁻² s⁻¹, respectively.
8.3.2 STEADY-STATE MEASUREMENTS

After discovering the intriguing discrepancy between \( J_g \) and \( J_f \) during the photosynthetic induction, we wanted to take a closer look on the two methods for electron transport rate estimations and to test the accuracy of calculations used. The \( \text{CO}_2 \) response of gas exchange and \( \Phi \text{PSII} \) were measured at three different \( \text{O}_2 \) concentrations and the corresponding electron transport rates were calculated. For this set of measurements a system which measured both the gas exchange and chlorophyll fluorescence over a leaf area of 4.15 cm\(^2\) was used, whereas the induction experiments were conducted using the rapid-response system which measures gas exchange over 18.5 cm\(^2\) and fluorescence from 0.75 cm\(^2\) area. The principle of the experiment is presented in Fig. 8.3. The \( \text{CO}_2 \) assimilation rate of a wild-type tobacco, as a function of chloroplast \( \text{CO}_2 \) concentration (\( \text{C}_c \)), becomes more inhibited at higher \( \text{O}_2 \) concentrations (panel A). However, the \( \Phi \text{PSII} \) at each \( \text{C}_c \) is similar regardless the \( \text{O}_2 \) concentration (panel B) showing that the reason for the different \( \text{CO}_2 \) assimilation rates at varying \( \text{O}_2 \) concentrations is the changing relationship between \( \text{CO}_2 \) uptake and photorespiration.
Fig. 8.4. CO₂ responses of assimilation rate and ΦPSII measured at different O₂ concentrations. The gas exchange and ΦPSII (Fm'-F/Fm') of a wild-type tobacco were measured simultaneously at three different O₂ concentrations (2, 20 and 40%). The measurements were conducted under steady-state conditions at irradiation of 830 µmol quanta m⁻² s⁻¹ and leaf temperature of 25 °C. Symbols: 2% O₂ ⋄, 20% O₂ ⋅, 40% O₂ ○.
8.3.2.1 Dark respiration

The rate of dark respiration in each plant was measured in two ways. First, it was measured prior to the experiments, when the leaf was kept in darkness in 350 μbar CO₂ and 20% O₂. Secondly, in order to see if the rate of dark respiration is affected by the O₂ concentration and light, it was estimated from the intersection of A vs. Cᵢ response curves and Γ*, assuming that Γ* is linearly related to the prevailing O₂ concentration. The difference between the two estimates of the dark respiration was small, indicating that the rate of mitochondrial respiration was similar in light and in dark, and that the O₂ concentration did not have any notable effect on it (Table 8.1).

Table 8.1. The rates of mitochondrial respiration and CO₂ assimilation in wild-type and anti-SSu tobaccos. The rate of respiration was estimated in two ways. It was measured directly prior to experiments, when the leaf was kept in darkness at 350 μbar CO₂, 20% O₂ (Rd dark). Secondly, the respiration rate was estimated from the intersection of A vs. Cᵢ-response curves and Γ* at 2, 20 and 40% O₂ (Rd Γ*, average±S.E. of the values measured at different O₂). The CO₂ assimilation rate was measured at 300 μbar CO₂, 20% O₂. Two plants per genotype were analysed.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Rd (dark) (μmol m⁻² s⁻¹)</th>
<th>Rd (Γ*) (μmol m⁻² s⁻¹)</th>
<th>A 300 μbar, 20% O₂ (μmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type A</td>
<td>3.5</td>
<td>3.2±0.3</td>
<td>10.1</td>
</tr>
<tr>
<td>Wild-type B</td>
<td>2.9</td>
<td>3.7±0.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Anti-SSu 1 A</td>
<td>1.6</td>
<td>1.9±0.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Anti-SSu 1 B</td>
<td>2.6</td>
<td>2.2±0.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Anti-SSu 2 A</td>
<td>1.6</td>
<td>1.2±0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Anti-SSu 2 B</td>
<td>1.2</td>
<td>1.0±0.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>
8.3.2.2 Electron transport

Two control, anti-SSu 1 and anti-SSu 2 plants were analysed, and the relationship between \( J_f \) and \( J_g \) is presented in Fig. 8.5 and Table 8.2. For the Fig. 8.4, the values of \( J_g \) and \( J_f \) were calculated as previously described, assuming that PSII absorbs 45% of the total absorbed photons (Laisk and Loreto 1996). Table 8.2 presents the linear regression parameters for all genotypes separately, calculated using both 45% and 50% PSII absorption coefficient. The relationship between \( J_f \) and \( J_g \) was similar at all \( O_2 \) concentrations. There was no difference between the plant genotypes, either (Fig. 8.5).

![Graph](image)

Fig. 8.5. Electron transport rate calculated from gas exchange and fluorescence during steady-state photosynthesis at different \( O_2 \) in wild-type and anti-SSu tobaccos.

Measuring conditions were as in Fig. 8.3. Symbols: Wild-type 2 % \( O_2 \) ●, 20 % \( O_2 \) ○, 40 % \( O_2 \); Anti-SSu 1, 2 % \( O_2 \) ■, 20 % \( O_2 \) □, 40 % \( O_2 \); Anti-SSu 2, 2 % \( O_2 \) ▲, 20 % \( O_2 \) ◆, 40 % \( O_2 \).
Table 8.2. Linear regression parameters for the comparison between electron transport rates calculated from fluorescence and gas exchange. The data was calculated assuming that PSII absorbs either 45 or 50% of the total absorbed light. The number of replicates for each plant genotype is given in the brackets, and the slope and intercept values are the mean±S.D. of the regression.

<table>
<thead>
<tr>
<th>Plant</th>
<th>PSII absorptance coefficient</th>
<th>Slope</th>
<th>Intercept</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (n=35)</td>
<td>0.45</td>
<td>1.01±0.06</td>
<td>16.5±6.3</td>
<td>0.88</td>
</tr>
<tr>
<td>Anti-SSu 1 (n=35)</td>
<td>0.87±0.04</td>
<td>13±3</td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>Anti-SSu 2 (n=36)</td>
<td>0.84±0.04</td>
<td>5.3±0.9</td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>All plants (n=106)</td>
<td></td>
<td>1.11±0.02</td>
<td>1.9±1.6</td>
<td>0.96</td>
</tr>
<tr>
<td>Wild-type (n=35)</td>
<td>0.50</td>
<td>1.13±0.07</td>
<td>17±7</td>
<td>0.90</td>
</tr>
<tr>
<td>Anti-SSu 1 (n=35)</td>
<td>1.09±0.04</td>
<td>6.0±1.9</td>
<td></td>
<td>0.96</td>
</tr>
<tr>
<td>Anti-SSu 2 (n=36)</td>
<td>0.93±0.05</td>
<td>5.9±0.9</td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>All plants (n=106)</td>
<td></td>
<td>1.25±0.02</td>
<td>1.7±1.5</td>
<td>0.97</td>
</tr>
</tbody>
</table>
8.4 DISCUSSION

8.4.1 IS THERE EXCESS ELECTRON TRANSPORT DURING THE INDUCTION OF PHOTOSYNTHESIS?

In these experiments two estimates of the photosynthetic electron transport were compared. The electron transport rate through PSII ($J_f$), was calculated from chlorophyll fluorescence. Electrons used for carboxylation and oxygenation reactions of RuBP ($J_g$) were estimated from the gas exchange measurements. When the two estimates were calculated during photosynthetic induction, it was observed that $J_f$ was 1.5-5 times higher than what could be contributed to CO$_2$ assimilation and photorespiration during the first 10-15 minutes of illumination (Fig. 8.2). This apparent excess electron transport, indicated by chlorophyll fluorescence can be attributed to two reasons: the assumptions used in the model or alternative (non-photosynthetic) electron sinks.

8.4.1.1 Experimental setup and model assumptions

I will first discuss the parameters that are needed for the electron transport rate calculations. For $J_f$ -calculations three factors have to be known accurately: incident light intensity, leaf absorptance and the excitation distribution between PSII and PSI. The rapid-response gas exchange system which was used in the induction experiments has a very uniform light field in the leaf chamber, and the incident light intensity can be measured accurately with a quantum sensor attached to fibre optics (Laisk and Oja 1998). The average leaf absorptance was determined for each plant genotype using an integrating sphere. This was done to correct for the decline in the chlorophyll content in the antisense plants (Fig. 7.2). The last factor, the excitation distribution between PSI and PSII, offers the least certainty. Generally, it is assumed to be equal (Loreto et al. 1992; 1994; Maxwell et al. 1997). However, studies by Laisk and Loreto (1996) showed that in C$_3$ plants PSII absorption coefficient varies. They determined the fraction of total absorbed light absorbed by PSII from the extrapolation of the dependence of the PSII quantum yield to $F/F_m$ to $F=0$. Since all our measurements were conducted at high light, i.e. at low PSII quantum yields, we could not reconstruct similar plots for PSII absorption determinations but chose the value to be 0.45. 173
The calculation of $J_g$ is somewhat more complicated. The equation (see section 8.2.1.2) requires a knowledge of: (1) the rate of mitochondrial respiration, (2) CO$_2$ concentration at the carboxylation sites ($C_c$), which is determined by stomatal conductance and internal conductance to CO$_2$, and (3) Rubisco specificity factor, which affects the relative contributions of carboxylation and oxygenation of RuBP. In these experiments the rate of mitochondrial respiration was measured before the leaf was exposed to light, and it was assumed to remain constant during the light period. For the estimation of $C_c$, CO$_2$ transfer conductance of 0.3 mol m$^{-2}$ bar$^{-1}$ was used, and this has been determined for glasshouse-grown wild-type and anti-SSu 1 tobaccos using combined gas exchange and carbon-isotope discrimination studies (Evans et al. 1994). Since the stomatal conductances were low at the beginning of the induction, a cuticular conductance for H$_2$O was included in the calculations as well (Boyer et al. 1997). Rubisco specificity factor has been determined for tobacco plants using gas-exchange technique (von Caemmerer et al. 1994).

A further problem that can distort the $J_g$ calculations is stomatal patchiness during the opening process. This is often difficult to distinguish. A recent study has clearly demonstrated a marked spatial heterogeneity of photosynthesis in leaves during photosynthetic induction, presumably due to uneven stomatal opening (Bro et al. 1996). If a leaf had areas of unequal photosynthetic activity, we would obtain an average of the CO$_2$ assimilation over the whole leaf area. If the areas of higher activities happened to be at the spot where the fluorescence was measured, perhaps induced by the frequent saturating flashes, a discrepancy between $J_f$ and $J_g$ could result. The fact that the disagreement between the two was greatest when the initial stomatal conductances were very low could indicate a heterogenous stomatal opening (Figs 8.2 and 8.3). However, the difference between the opening rate of the stomata in the spot where fluorescence was measured and the rest of the leaf would have to be considerable. If this was the reason for the discrepancy seen in Fig. 8.2, the stomata must have been widely open at the spot where the fluorescence was measured and closed everywhere else.
8.4.1.2 Possibility for extra sinks?

At steady-state, the two estimations of electron transport agree well. Therefore we are confident that the experimental setup itself was correct. It is possible that the discrepancy between $J_f$ and $J_g$ could be due to alternative electron sinks being involved during the early phase of photosynthetic induction. Since it is known that stomatal opening and many carbon metabolism enzymes need several minutes to activate in light (Kirschbaum and Pearcy 1988; Pearcy 1988; Woodrow and Mott 1992; Sassenrath-Cole and Pearcy 1994) the $CO_2$ assimilation and photorespiration proceed slowly immediately after exposure to light. Simultaneously, however, the light reactions are functioning so presumably the electron transport chain could become overreduced. In this situation the alternative electron sinks could become important in facilitating the linear electron transport. Similar results to ours has been recently reported in experiments with maize leaves (Earl and Tollenaar 1998). During the early stage of photosynthetic induction, chlorophyll fluorescence indicated electron transport which could not be accounted by $CO_2$ assimilation.

One might expect to find some extra electron transport at the beginning of induction, since many chloroplast enzymes need to be reductively activated through ferredoxin-thioredoxin system (Chapter 4). However this contribution is likely to be small. We indeed found that $J_f$ was in all plants higher than $J_g$ during the first minutes of induction (Fig. 8.3 B). Other extra electron sinks could include nitrate reduction, and $O_2$ photoreduction (Mehler reaction) accompanied by ascorbate peroxidase pathway (Fig. 8.1). There are some results, obtained using mass-spectrometric analysis, indicating that the Mehler reaction may occur at the beginning of light induction, ie. preceding the activation of $CO_2$ assimilation and photorespiration (Radmer and Kok 1976; Marsho et al. 1979; Behrens et al. 1982).

Oxaloacetate-malate shuttle can transport electrons from chloroplasts to cytosol. It has been shown that in a situation where NADPH accumulates upon illumination due to a lack of electron acceptors, NADP-malate dehydrogenase-enzyme is activated within 1-2 minutes from the onset of illumination (Scheibe et al. 1986b). This has lead to the hypothesis of this "malate valve" functioning as a safety mechanism to avoid overreduction of electron transport chain and subsequent
photoinhibition (Scheibe 1987; Backhausen et al. 1994). It could be expected that the malate valve is continuously active in anti-SSu plants, because the consumption of NADPH by carbon metabolism is impaired. However, at steady state, $J_f$ and $J_g$ corresponded well in the anti-SSu plants as well. This probably indicates that the transgenic plants were able to avoid overreduction of the stromal NADP$^+$ pool by engaging a high NPQ in light (Table 7.1). The activation level of NADP-MDH in anti-GAPDH was comparable to wild-type levels, when measured at high light (Fig. 4.8)

From this data it is not possible to distinguish the identity of the possible extra electron sinks active during the early phase of photosynthetic induction. Nevertheless, from the gas exchange and fluorescence data it is obvious that the induction of electron transport through PSII and gas exchange can exhibit different kinetics such that $J_f$ activates faster.

**8.4.2 STEADY-STATE MEASUREMENTS DO NOT INDICATE INCREASED CAPACITY OF ALTERNATIVE ELECTRON SINKS IN ANTI-SSU PLANTS**

To study in more detail the possible excess electron transport in tobacco, the steady-state $J_f$ and $J_g$ were measured at several $CO_2$ and $O_2$ concentrations. It has been estimated that the $K_m$ for $O_2$ of the Mehler reaction is between 25-60 μM, which equivalent to about 2-5% $O_2$ (Furbank et al. 1982; Wiese et al. 1998). We should then expect to see a difference in the relationship between $J_f$ and $J_g$ measured either at low or high $O_2$ if the excess electron sink was photoreduction of $O_2$.

A different experimental setup was now used, where both gas exchange and fluorescence were measured from the total leaf area enclosed in the chamber and the gas exchange was measured from both sides of the leaf. In addition, it was checked that the rate of mitochondrial respiration did not vary between different $O_2$ concentrations, and it was similar in light and dark (Table 8.1). As seen in Fig. 8.5 and Table 8.2, at steady-state and high light, the relationship between $J_f$ and $J_g$ was very similar at different $O_2$ and $CO_2$: $J_f$ was on an average 10% higher than $J_g$ when the PSII absorption coefficient was assumed to be 0.45. This can be interpreted as indicating that the capacity for possible extra electron transport is equal in all plants.
and, surprisingly, does not depend on $O_2$ or $CO_2$ concentrations. The results presented in Table 8.2 actually suggest, that the excess electron sinks might be most active in wild-type plants (the highest intercept).

The contribution of the alternative sinks has been estimated being quite small or negligible in other studies as well, when electron transport rates have been measured in intact leaves using combined gas exchange and fluorescence measurements (Edwards and Baker 1993; Laisk and Loreto 1996). In a study with wheat leaves, excess electron transport was only observed after photosynthesis was completely suppressed by inhibitor feeding (Loreto et al. 1994). On the other hand, measurements conducted during environmental stress conditions (drought, low temperature) have indicated that alternative sinks, especially the Mehler-peroxidase pathway, may actually function as a photoprotective mechanism in leaves (Biehler and Fock 1996; Cheeseman et al. 1997; Fryer et al. 1998). Since these stress factors are likely to inhibit carbon assimilation and photorespiratory pathways, it is surprising that we did not see excess electron transport in anti-SSu plants, even when they were subjected to low $CO_2$ and $O_2$ concentrations at high light. A study conducted with transgenic tobacco plants with reduced amount of chloroplast phosphoribulokinase and subsequently reduced $CO_2$ assimilation rates, found also no evidence of significant alternative electron sinks (Habash et al. 1996).

**8.4.3 SUMMARY**

In these experiments the photosynthetic electron transport rates were estimated in attached leaves of wild-type and anti-SSu tobaccos by measuring simultaneously gas exchange and chlorophyll $a$ fluorescence. During the early phase of the photosynthetic induction at high light in air, we observed the electron transport through PSII ($J_f$) was higher than what could be accounted for $CO_2$ assimilation and photorespiration ($J_g$) in both control and anti-SSu plants. The reason for the higher $J_f$ could be a heterogeneous stomatal opening in the leaf areas used for fluorescence and gas exchange measurements, since the difference between $J_f$ and $J_g$ was greater when the initial stomatal conductance was very low. On the other hand, since the $CO_2$ assimilation is inactive after a long dark period, alternative sinks may become important in facilitating linear electron transport under these conditions.
To investigate whether the alternative electron sinks become important when CO₂ assimilation is restricted, the steady-state $J_f$ and $J_e$ were measured from wild-type and anti-SSu plants at different CO₂ and O₂ concentrations. These measurements did not indicate any increased capacity for excess electron transport in anti-SSu plants, even when the leaves were exposed to low CO₂ and O₂ at high light. This was a surprising result and future work is planned on this subject.
CHAPTER 9

CONCLUDING REMARKS AND FUTURE RESEARCH DIRECTIONS
The aim of this thesis was to research the coordination between the photosynthetic electron transport and carbon assimilation in intact leaves. Material for the studies consisted of transgenic tobaccos engineered using antisense DNA technique. These plants have alterations in different parts of their photosynthetic machinery: the amounts of Rubisco (anti-SSu plants), chloroplast glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH plants) or cytochrome b\(f\) complex (anti-b\(f\) plants) have been reduced. The work provided new information about the photosynthetic regulation, and also created topics for further research.

9.1 RAPID-RESPONSE GAS EXCHANGE STUDIES

In Chapter 2 it was demonstrated that a rapid-response gas exchange system is an effective tool to study the balance between RuBP-regeneration limitation and Rubisco limitation of CO\(_2\) assimilation in C\(_3\) plants. Non-steady state gas exchange measurements can be used to directly assess Rubisco's capacity (\(V_{\text{cmax}}\)) and the potential rate of electron transport (\(J\)) in intact leaves, providing parameters important for photosynthesis modellers. This direct method makes further comparative studies of these two parameters relatively simple, for example in relation to temperature. The importance of temperature response studies of photosynthesis was recently highlighted by Leuning (1997), because the temperature dependency of \(J_{\text{max}}\) is often different to that of \(V_{\text{cmax}}\). In addition, the rapid-response technique is potentially an excellent way to probe the balance between RuBP regeneration and Rubisco in different plant species. It is generally found that \(V_{\text{cmax}}\) and \(J_{\text{max}}\) are higher in herbaceous annuals than in other plant types (Wullschleger 1993).

The rapid-response gas exchange system was found not to be particularly suited to estimate leaf RuBP pool sizes in transgenic plants. Nevertheless, the measurements revealed interesting aspects about Rubisco activity \textit{in vivo}. The post-illumination CO\(_2\) uptake method underestimated the true RuBP contents in anti-SSu plants because Rubisco seemed to inactivate quickly (within 30 s) after the light was turned off, leaving RuBP unconsumed. The reason for the rapid deactivation of Rubisco in dark could not be precisely determined. It is possible that Rubisco was inactivated by a sudden decline in stromal pH. Another possibility is that a rapid decrease in stromal ATP/ADP ratio and the disengagement of \(\Delta p\text{H}\) deactivated
Rubisco activase, and impaired the \textit{in vivo}-catalysis of Rubisco. However, this seems less likely, since the carbamylation state of Rubisco remained high during the post-illumination CO$_2$ uptake process.

Some observations suggest that activase may still somehow be involved in the rapid cessation of CO$_2$ uptake in darkness. It is worth noting here, that I initially attempted to study the post-illumination CO$_2$ uptake also in tobaccos with moderately reduced Rubisco activase content. This was unsuccessful, apparently because the combination of low CO$_2$ and low O$_2$ together with leaf darkening caused Rubisco to deactivate and it was impossible to measure the CO$_2$ response of assimilation and post-illumination CO$_2$ uptake (as in Fig. 2.4) for these plants (data not shown). It can be concluded that whatever the reason for the inactivation of Rubisco during the post-illumination CO$_2$ uptake was, activase was needed to restore the activity in light. Because activase was functioning optimally in wild-type and anti-SSu plants, this deactivation was not a problem during the steady-state CO$_2$ assimilation rate measurements, but prevented the accurate determination of RuBP content with the post-illumination CO$_2$ uptake method. It would be interesting to repeat the experiments with the anti-activase plants to determine whether the Rubisco inactivation was similar to what was seen in anti-SSu plants, ie. carbamylation level and \textit{in vitro}-activities did not change but \textit{in vivo}-catalysis was prevented. Perhaps some kind of tight-binding inhibitor accumulated in the Rubisco sites during the post-illumination CO$_2$ uptake or when the leaves were exposed to low CO$_2$ and O$_2$.

Due to the inactivation of Rubisco, the post-illumination CO$_2$ uptake at ambient CO$_2$ was dependent on the Rubisco content in tobacco leaves (Fig. 2.4). A positive correlation between mesophyll conductance and the post-illumination CO$_2$ uptake in leaves has been reported earlier (Laisk \textit{et al.} 1984). My results raise a question whether this correlation was also a result of Rubisco inactivation, and in general, the amount of CO$_2$ that can be fixed in darkness is not only a function of RuBP content, but is also determined by the amount of Rubisco.
9.2 HOW IS RUBISCO CARBAMYLATION REGULATED?

The roles of electron transport and chloroplast metabolites in determining the activity of Rubisco were studied in Chapters 4-6. Investigation was focused particularly on the reasons behind a decreased carbamylation level in anti-bf tobaccos. As the amount of cytochrome b6f was reduced, the carbamylation state of Rubisco decreased when measured from leaves which were kept at high light (Fig. 9.1 A, the same as Fig. 5.2 A). This finding was interesting as it is remarkably similar to the decrease in Rubisco carbamylation in wild-type leaves when the light intensity is

![Graph showing carbamylation state vs. Cytochrome b6f](image)

**Fig. 9.1.** Rubisco carbamylation state in wild-type and anti-bf tobaccos at high light and in wild-type tobaccos at different irradiances. **A.** The samples were collected from 350 µbar CO₂ in air, and the light intensity was 1000 µmol quanta m⁻² s⁻¹. **B.** The leaves were stabilised at 350 µbar CO₂ in air at different irradiances (von Caemmerer et al., unpublished data). The leaf temperature was 25 °C.
reduced (Fig. 9.1 B, von Caemmerer et al., unpublished data). It looks like the perception of light signal is interrupted in anti-bf plants, since the activation state of NADP-malate dehydrogenase, another light-regulated stromal enzyme, decreased to the levels equivalent to what is found in wild-type plants in dark.

I suggest that the decrease in Rubisco carbamylation in anti-bf plants is the consequence of Rubisco activase being unable to detect the light signal (for Rubisco, the lights are on but the curtains are closed). These results support previous studies (Brooks et al. 1989a; Campbell and Ogren 1990a; 1990b; 1992) implying that, although Rubisco activase needs ATP to function, the changes in the stromal ATP/ADP ratio are not the means how Rubisco carbamylation is adjusted to prevailing light by the activase. The ATP/ADP ratio, measured from whole leaves or chloroplasts, tends to remain stable over a range of light intensities (ie. electron transport rates) demonstrating how the photosynthetic regulation works to prevent large fluctuations in metabolite pool sizes (Brooks et al. 1989a; Dietz and Heber 1984).

It has been suggested that Rubisco activase could sense the light either via the electron transport through PSI or ΔpH (Campbell and Ogren 1990b). The results from my studies indicate that the light regulation of Rubisco activase is mediated via ΔpH. The electron transport rate was suppressed in anti-GAPDH plants as well, but the carbamylation state remained high. It is even possible that in anti-GAPDH plants the elevated ΔpH, aided by the high ATP/ADP ratio, increased the activity of Rubisco activase. The highly active Rubisco activase could then maintain high carbamylation levels in anti-GAPDH plants, even when RuBP pool sizes were reduced below Rubisco site concentration. It has been demonstrated that subsaturating CO₂ and RuBP can cause decarbamylation of Rubisco which can be prevented by Rubisco activase (Portis et al. 1995). An increase in activase activity is probably also the reason why transgenic tobaccos with reduced amount of Rubisco (Quick et al. 1991) and phosphoribulokinase (Paul et al. 1996) had higher Rubisco activation state than wild-type plants, when it was measured from leaves kept at low light (300 μmol quanta m⁻² s⁻¹). To follow this suggestion, it would be important to reconstruct a light-response curve of Rubisco carbamylation in anti-SSu and anti-GAPDH plants, similar to what was done for the wild-type tobacco (Fig. 9.1 B).
When the anti-\(hf\) plants were exposed to elevated \(CO_2\) (700 \(\mu\)bar), the carbamylation level of Rubisco actually increased in some plants, when compared to the carbamylation state in air. As this was found only in a few plants with severely reduced cytochrome \(hf\) content, further confirmation is needed. One possible explanation for this increase in carbamylation could be in the experimental setup. During the high \(CO_2\)-measurements, the low-light grown leaves were exposed to high light 20-30 minutes longer than during ambient-\(CO_2\) measurements, and this prolonged light exposure might be a reason for the higher carbamylation states. It would be essential to conduct a time-course experiment where the changes in the Rubisco carbamylation state in both wild-type and anti-\(hf\) tobaccos are followed after the low-light leaves are exposed to high light. The other explanation for the increase in carbamylation at high \(CO_2\) could be that in the severe anti-\(hf\) plants, with low RuBP content and inactive activase, Rubisco activation could be more dependent on the spontaneous carbamylation. Although the RuBP/Rubisco sites ratio remained above 1 in anti-\(hf\) plants, the amount of free RuBP may be smaller than the total pool. This situation would be equivalent to observations in \(Arabidopsis\) and wheat leaves in dark: as RuBP content is low and activase not functioning, Rubisco carbamylation is sensitive to \(CO_2\) (Mächler and Nösberger 1980; Salvucci \textit{et al.} 1986). To test this suggestion, the carbamylation state of Rubisco in wild-type anti-\(hf\) tobaccos should be measured as a function of \(CO_2\) in light and in dark. In severe anti-\(hf\) plants, the carbamylation should be expected to be equal in light and dark, and depend on the \(CO_2\). In wild-type the carbamylation should be \(CO_2\) dependent in dark but not in light. However, the \(CO_2\)-response of carbamylation could be more complicated in tobacco, because it synthesises CA1P in darkness. When carbamylation was measured in wild-type and anti-activase tobaccos growing either in air or at 0.5-0.3% \(CO_2\), it was found to be insensitive to \(CO_2\) both in light and dark (Mate \textit{et al.} 1993).

The next step in the activase research is to elucidate the mechanism how Rubisco activase senses \(\Delta p\text{H}\) \textit{in vivo}. Perhaps activase itself associates with the thylakoid membranes. The localisation of activase inside the chloroplasts could be studied by immunogold labelling. On the other hand, it is possible that another protein picks up the light signal and somehow transmits it to activase. This kind of partner protein could be a link between the thylakoid membranes and activase, and
identification of such a protein could change the whole perspective on activase function.

9.3 PROTECTION AGAINST EXCESS LIGHT

At high light, the probability for overexcitation of antenna pigments and subsequent photoinhibition of PSII centres increases in transgenic plants with reduced CO₂ assimilation capacity. This was counteracted by an enhanced nonradiative dissipation of light energy in the anti-SSu and anti-GAPDH plants. The de-epoxidation state of xanthophyll cycle pigments increased as well, demonstrating how the transgenic plants experienced excess light. For tobacco, it was interesting to find that the relationship between xanthophyll pool pigments and chlorophyll did not change until CO₂ assimilation was decreased at least 90% from the wild-type level, and even then the change in the ratio was due to a decrease in leaf chlorophyll content. This supports the suggestion, that in tobacco the xanthophyll pigment synthesis is not regulated by photosynthetic capacity (Bilger et al. 1995). Probably in tobacco, the carotenoid and chlorophyll synthesis are co-regulated and the link between the two is broken only when the CO₂ assimilation capacity is severely suppressed.

Based on studies of isolated chloroplasts and of plants at stress conditions, it has been suggested that electron transport to alternative sinks, especially to Mehler-peroxidase pathway, could serve as additional protection against photoinhibition (Foyer et al. 1987). It might be expected that the activity of these alternative sinks would increase in transgenic plants with reduced CO₂ assimilation capacity. However, combined chlorophyll fluorescence and gas exchange measurements at different CO₂/O₂ concentrations did not indicate any increased activity of alternative electron sinks in anti-SSu plants. Interestingly, during the early phase of the photosynthetic induction at high light in air, electron transport through PSII was higher than what could be accounted for CO₂ assimilation and photorespiration was observed in both control and anti-SSu plants. This discrepancy suggests that alternative electron sinks could be important at some transient conditions, but at steady-state and at irradiances close to the growth light nonradiative dissipation is sufficient in protecting PSII even in anti-SSu plants. We will follow up these
experiments by measuring CO₂ uptake and O₂ evolution at different conditions using mass spectrometer. This enables us to simultaneously measure chlorophyll fluorescence, CO₂ uptake, the oxygen evolution from PSII using ¹⁸O labelled oxygen, and the oxygen uptake by respiration, RuBP oxygenation and possible O₂ photoreduction.

9.4 FINAL WORDS

The engineering of transgenic plants has provided a unique opportunity to examine the regulation of photosynthesis in vivo. It has been possible to study Rubisco in its natural state in chloroplasts, and to elucidate the effects of impaired CO₂ assimilation capacity on the light harvesting and photosynthetic electron transport as well. The results of my studies suggest, that the transthylakoid pH gradient has a central role in photosynthetic control in chloroplasts. It is known, that ΔpH regulates the efficiency of light harvesting by triggering the nonradiative dissipation of light energy the antenna. In addition, ΔpH probably also is a starting point of a signal transduction chain from the light reactions to Rubisco activation.
REFERENCES


Eckardt, N. A. and Portis, A. R., Jr. (1997). Heat denaturation profiles of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and rubisco activase and the inability of


Lauerer, M., Saftic, D., Quick, W. P., Labate, C., Fichtner, K., Schulze, E.-D.,
carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' rbcS. VI. Effect on
photosynthesis in plants grown at different irradiance. Planta 190, 332-345.

Biochemistry 16, 3050-3053.

Leuning, R. (1997). Scaling to a common temperature improves the correlation between the

amounts of glycerate 3-phosphate, glycerate 2-phosphate and phosphoenol pyruvate by an
enzymic assay coupled to firefly luciferase/luciferin luminescence. Analytical Biochemistry
148, 282-287.

of ribulose-1,5- bisphosphate carboxylase oxygenase activase - Do the effects of Mg2+, K+,
and activase concentrations indicate a functional similarity to actin? Plant Physiology 114,
605-613.

of xanthophyll-cycle dependent energy dissipation in Alocasia brisbanensis to sunflecks in a


cconductance, photosynthetic electron transport and alternative electron sinks of field grown
wheat leaves. Photosynthesis Research 397-403.

conductance to CO2 flux by three different methods. Plant Physiology 98, 1437-1443.

Lorimer, G. H. (1981). The carboxylation and oxygenation of ribulose 1,5-bisphosphate: The
primary events in photosynthesis and photorespiration. Annual Review of Plant Physiology
and Plant Molecular Biology 32, 349-383.


Wang, Z. Y., Ramage, R. T. and Portis, A. R., Jr. (1993). Mg$^{2+}$ and ATP or adenosine 5'-[gamma-thio]-triphosphate (ATPgammaS) enhances intrinsic fluorescence and induces aggregation which increases the activity of spinach Rubisco activase. *Biochimica et Biophysica Acta* 1202, 47-55.


