CO-REGULATION OF THE ELECTRON TRANSPORT AND CARBON ASSIMILATION IN C_3 AND C_4 PLANTS: THE ROLE OF CF_O-CF_1 ATP SYNTHASE

By

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To the Faculty of Washington State University:

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Abstract

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Photosynthetic electron transport and Calvin cycle reactions need to be co-regulated in order to provide optimal flux into end product and minimize the formation of reactive oxygen species leading to photo-inhibition. An important means of dissipating excess energy is mediated by an increased acidification in the lumen of thylakoid membranes of the chloroplast which has been proposed to occur through increased photochemistry through cyclic electron flow (CEF) via photosystem (PS) I, or by linear electron flow in the Mehler reaction. We have shown that decreases in the thylakoid membrane ATP synthase conductance to protons is an important component in this dissipation of excess energy and photoprotection. It is universal and it takes place in C_3 plants, as well as in all three biochemical subtypes of C_4 plants. C_4 plants showed a similar pattern of ATP synthase regulation to C_3 plants despite the differences in photosynthetic carbon metabolism. Down-regulation of ATP synthase proton conductivity at low CO_2 and high light increases intrathylakoid H^+ concentration which activates the energy dissipation mechanism, thus protecting PS II. Three mutants were tested which provided support for this hypothesis: one in photochemistry (related to CEF), one in CO_2 fixation (Rubisco = ribulose 1,5-bisphosphate carboxylase oxygenase), and one in carbohydrate biosynthesis (starch-less). A

possible mechanism of regulating ATP-synthase conductance to protons is through the levels of inorganic phosphate (P_i) in the chloroplast stroma, since this is a substrate for the enzyme. We tested this hypothesis using a starch-less mutant. This mutant is limited in utilizing the products of photosynthesis and is considered to cause a build-up of organic-P, a depletion of P_i and feedback inhibition of photosynthesis. ATP synthase conductivity closely followed the change in activity of carbon fixation reactions, which supports the hypothesis that P_i is a regulator of ATP synthase. The low Rubisco mutant of tobacco and a mutant of *Arabidopsis* which affects CEF also provided support for regulation of ATP synthase conductance having a key role in photoprotection and dissipation of excess energy.

TABLE OF CONTENTS

Page

Dedication

This dissertation is dedicated to my mother Aili who provided moral support for my education and who helped me financially during my undergraduate years at Tartu University.

LIST OF FIGURES

LIST OF ABBREVIATIONS

A net rate of CO_2 fixation (μmol $m^{-2}s^{-1}$)

AC assimilatory charge (post-illumination uptake of CO_2), (μmol m^{-2})

AGP ADP-glucose pyrophosphorylase

APFD absorbed photosynthetic quantum flux density (μmol $m^{-2}s^{-1}$)

CET cyclic electron transport

CF_oCF_1 ATP synthase thylakoid membrane ATP synthase

ECS electrochromic shift

ECS_t total electrochromic shift ~ pmf, (ΔA_{520})

Φ_{PSII} PS II quantum yield (F_v/F_M')

Φ_{PSI} PS I quantum yield

Fd ferredoxin

g_{H^+} ATP synthase proton conductance (s^{-1})

J_{O2} gross rate of O_2 evolution from PSII (μmol $m^{-2}s^{-1}$)

J_{PS1} PS I electron transport rate (μmol electrons $m^{-2}s^{-1}/4$)

LED light emitting diode

LEF linear electron flow (μmol $m^{-2}s^{-1}$)

LHC light harvesting complex

NDH NAD(P)H dehydrogenase

NPQ non-photochemical quenching

qE non-photochemical energy dependent quenching (heat dissipation)

1-qL reductive state of plastoquinone pool

P_i inorganic phosphate, ortho-phosphate

PFD	incident photosynthetic quantum flux density (μmol $m^{-2}s^{-1}$)
PS I	photosystem I
PS II	photosystem II
pmf	proton motive force
$\mathbf{R_D}$	rate of dark-type mitochondrial respiration (μmol $m^{-2}s^{-1}$)
Rubisco	ribulose 1,5-bisphosphate carboxylase oxygenase
TPU	triose-phosphate utilization
$\mathbf{v_c}$	velocity of (Rubisco) carboxylase (μmol $m^{-2}s^{-1}$)
$\mathbf{v_o}$	velocity of (Rubisco) oxygenase (μmol $m^{-2}s^{-1}$)
$\mathbf{v_c + v_o}$	rate of RuBP utilization by Rubisco (μmol $m^{-2}s^{-1}$)
$\mathbf{v_H}^+$	proton flux

GENERAL INTRODUCTION

Photosynthetic metabolism is highly regulated to ensure its kinetic stability and optimal functioning under variable environmental conditions. The objective of this thesis is to improve our understanding of the regulation that coordinates the activities of biochemical processes that constitute photosynthetic metabolism. The component processes that must be coordinated in C_3 photosynthesis are: (1) the light capture/water splitting reactions; (2) the electron transport reactions in the thylakoids; (3) the photosynthetic carbon reduction (PCR) cycle (also known as the reductive pentose phosphate or the Calvin cycle) in the chloroplast stroma; (4) the photorespiratory carbon oxidation (PCO) cycle which is attached to it and operates across the chloroplast, the peroxisome and the mitochondrion; (5) the sucrose synthesis pathway in the cytosol; and, (6) the starch synthesis pathway in the chloroplast stroma. In C_4 species the tight coordination between the activity of the C_4 pump and CO_2 fixation in the PCR cycle in the bundle sheath chloroplasts must take place in addition to previously mentioned processes related to C_3 species. Wide variation in environmental conditions (particularly light intensity, temperature, and CO_2 concentration within the leaf) and in the metabolic consumption of product within the plant through the course of a day makes coordination between different processes a complicated task. Electron transport reactions must be regulated to match the NADPH and ATP demand by the PCR cycle and excess light energy must be harmlessly dissipated; CO_2 assimilation must be balanced by the export of triose phosphates into cytosol for sucrose synthesis and to starch synthesis in the chloroplast stroma maintaining at the same time stable concentrations of PCR cycle

intermediates. In this work we focus on coordination between carbon assimilation reactions and regulation of light harvesting and electron transport.

General overview of the regulation of light reactions

During photosynthesis light is absorbed by light harvesting complexes and light energy is harnessed by photochemical reactions to generate NADPH and proton motive force (*pmf*). The latter includes the proton concentration difference across the thylakoid membrane (ΔpH) and electrical potential difference ($\Delta\Psi$), both of which are used to drive synthesis of ATP catalyzed by the proton translocating CF_oCF_1 ATP synthase of the thylakoid membrane. The excess light is dissipated by means of non-photochemical processes in order to avoid over-reduction of the electron transport chain and formation of highly reactive oxygen radicals. Initiation of energy dependent quenching (qE) involves activation (via protonation) of violaxanthin de-epoxidase and PsbS, a component of the photosystem II (PS II) antenna complex, as a result of lumen acidification driven by photosynthetic electron transfer (reviewed by Miller et al., 2001; Horton et al., 1996; Niyogi et al. 2005).

Electron transport chain in thylakoid membranes is sometimes described as a molecular conveyer belt for electrons (Critchley, 1988). Electrons are pulled from water and transferred from the PS II reaction center (P_{680}) to a downhill energy gradient through pheophytin, Q_A and Q_B to plastoquinone. Plastoquinone diffuses in the thylakoid membrane. It picks up two protons from chloroplast stroma and reacts with the cytochrome b_6f complex passing one electron through the Rieske protein and cytochrome *f* to plastocyanin and the other into the Q cycle. Plastocyanin passes electrons to PS I ($P700^+$) which eventually reduces ferredoxin. Electrons from ferredoxin can be used in

multiple ways but the main path is to NADPH that is used mostly as a reductant in Calvin cycle. The rate-limiting reaction in the thylakoid linear electron transport is the oxidation of plastoquinol by cytochrome b_6f complex and it is controlled by the concentration of plastoquinol and by the pH of the thylakoid lumen (Stiehl and Witt, 1969; Joliot and Joliot, 1992).

It has been recognized that for high rates of carbon assimilation NADPH and ATP need to be produced in correct stoichiometry, ATP/ADP, NADPH/NADP and ΔpH needs to be kept within a certain range. The latter is achieved through regulation on many levels including cyclic and psudocyclic photophosphorylation, feed-forward regulation of Calvin cycle via the thioredoxin system, regulation of the NADPH/ATP ratio with NADP malate dehydrogenase ("malate valve"), and regulation of Calvin cycle by metabolite levels and allosteric interactions (reviewed Baker *et al*., 2007). Many details and quantitative relationships of the regulation still need to be discovered. One of the questions studied in this work is how coordination between PCR/PCO cycles and light reactions is established when CO_2 assimilation is restricted by lack of substrate or by stressful conditions at high light?

Limiting reactions and regulation of photosynthetic carbon metabolism

Response of the photosynthesis rate to CO_2 usually reveals a so called Blackman type curve (Blackman, 1905): the initial increase of the CO_2 assimilation rate levels off and becomes insensitive to further increase of the substrate (Fig. 1). The reason for such kinetic behavior is the presence of several potentially limiting reactions in the system that limit the flux of carbon under different conditions. With increasing CO_2 under light saturation the initial rise in CO_2 assimilation is limited by Rubisco activity in C_3 plants

(PEPC in C_4 species) up to a certain point (usually around 500 µbar) and then photosynthesis becomes limited by other internal reactions that varies dependent on conditions and on the physiological status of the leaf.

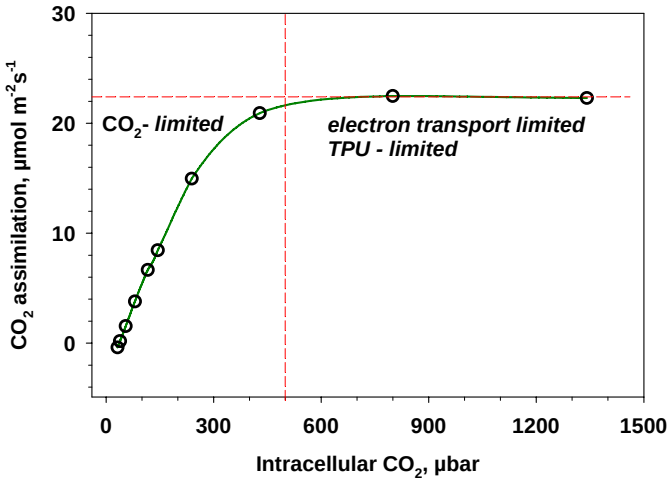

Figure 1. Typical steady state CO_2 response curve. CO_2 limited region and electron transport limited (RuBP regeneration limited), or triose phosphate utilization (feedback) limited, regions are shown. The response is illustrated with tobacco (*N. sylvestris*) at a leaf temperature of 24°C, light intensity of 800 µmol $m^{-2}s^{-1}$ with a Rubisco V_{max} for this plant of 60 µmol $m^{-2}s^{-1}$ (unpublished).

The Rubisco in the leaf is assumed to be RuBP-saturated and CO_2 limited on the initial slope of the CO_2 response curve (Fig. 1). There, the model asserts that the appropriate RuBP-saturated carboxylation and oxygenation kinetics for the quantity of Rubisco within the leaf are the only kinetics needed to represent the integrated system (von Cammerer and Farquhar, 1981). Taking a cue from the intuitively obvious fact that deviation from RuBP saturated kinetics at low light levels is connected to limited light

capture/electron transport activity, Farquhar *et al*. (1980) presumed that the deviation from RuBP-saturated Rubisco kinetics at high light and CO_2 was a result of a limited capacity for electron transport. There is experimental evidence that the capacity of the light reactions may exceed that required to support maximum rates of CO_2 assimilation (Dietz and Heber, 1984; Stitt, 1986). A more recent view considers that CO_2 saturated rates of photosynthesis is a compromise between the light reactions, Calvin cycle activity (RuBP regeneration) and feedback influences from limited capacity for starch and sucrose synthesis (TPU, triose-phosphate utilization) (Woodrow and Berry, 1988). The details of how the potential for the CO_2 saturated, and the light-saturated, rate of photosynthesis is determined and regulated still needs to be discovered.

Feedback regulation of photosynthesis

High levels of CO_2 or low temperatures at ambient conditions sometimes lead to so called "feedback limitation" where capacity for end product synthesis, sucrose and starch, becomes limiting. Considerable evidence has accumulated that feedback limitations represent the situation in which the fraction of total chloroplast ortho-phosphate (P_i) free to enter new fixation reactions becomes limiting. As the rate of end product synthesis determines the rate at which P_i recycles back to Calvin cycle and becomes available for ATP synthesis reactions in chloroplast stroma triose phosphate utilization can effectively limit photosynthesis. Specifically, under feedback conditions the availability of P_i is believed to limit the production of ATP (Sharkey, 1985).

Feedback conditions can occur at elevated CO_2 and saturating light due to a limitation on utilization of triose-P, the product of CO_2 assimilation. This is characterized by oxygen insensitivity of CO_2 assimilation (Cornic and Louason, 1980),

or by an increase in the rate of CO_2 assimilation by oxygen (McVetty and Canvin, 1981; Viil *et al*., 1972), as opposed to oxygen inhibition at low CO_2 due to photorespiration caused by oxygen competition with CO_2 for reaction with RuBP. Since the availability of P_i has been suggested to be limiting under feedback conditions, the inhibition of photosynthesis by low O_2 has been explained by reduction in P_i availability (Sharkey, 1985; Sharkey and Vassey, 1989).

When photosynthesis is limited under high light by either the availability of CO_2, or by feedback due to limited utilization of triose-P, electron transport is considered to be limited from the carbon side of photosynthesis, resulting in an increased need to harmlessly dissipate excess absorbed light energy. The question is how this requirement to dissipate energy is signaled to light reactions and how the protective mechanisms are activated? One possible answer emphasizes on activation of cyclic electron flow or electron flow to O_2 (Mehler reaction) under these conditions signaled by an increase in the reduction state of the system (Heber and Walker, 1992). Cyclic electron flow and Mehler reaction activity are relevant to our project as they both pump protons but do not produce reducing equivalents for the Calvin cycle. Recent work with the *Arabidopsis* PGR5 mutant, which possibly blocks cyclic electron transport (around PS I) (Munekage *et al*., 2004; Munekage *et al*., 2002), suggests that cyclic electron flow needs to be present for efficient photoprotection.

Kramer and coworkers (Kanazawa and Kramer, 2002; Kramer *et al*., 2003; Kramer *et al*., 2004) have demonstrated a strong decrease in ATP synthase conductivity and increased modulation of qE when the CO_2 and O_2 concentrations are lowered. This has led to a hypothesis that ATP synthase activity modulation is the major regulatory

point, and that it is controlled at the substrate level by the availability of P_i in the stroma of the chloroplast.

P_i hypothesis for regulation of ATP synthase and intrathylakoid pH

The important property of the photosynthesis system is the constancy of stromal inorganic and esterified phosphate, at least in the short term. The movement of phosphate between cytosol and chloroplast stroma via the phosphate translocator does not have a net phosphate flux (Fleige *et al.,* 1978; Flügge, 1991). This property not only ties free P_i concentration in chloroplast stroma to the rate of P_i recycling from starch and sucrose synthesis; but, also to P_i partitioning between free P_i and esterification in pools of Calvin cycle intermediates. The other property of photosynthesis is the relatively high K_M for P_i for (~0.9 mM) for ATP synthase (Grotjohann and Gräber, 2002). Taking together these phenomena makes P_i a good candidate as a regulator of ATP synthesis. Whether or not our results are consistent with, or support, this hypothesis were evaluated in this thesis.

ATP synthase may be allosterically regulated to modulate non-photochemical quenching in changing environmental conditions. There is evidence for thiol modulation of the enzyme by reductive conditions (Bald *et al*., 2001), recently the regulatory protein (14-3-3 protein) has been described as having an effect of ATP synthase activity (Bunney *et al*., 2001) and multiple phosphorylation sites have been reported (del Riego *et al*., 2006).

Chapter 1 of the thesis is a study of changes in photosynthetic parameters and regulation under feedback inhibition utilizing a wild-type and starch deficient mutant of tobacco. Chapter 2 is a study of the regulation of photosynthesis during C_4 photosynthesis utilizing representative species for the three types of C_4 cycle. C_4 plants,

in comparison to C_3 plants, have low rates of photorespiration, photosynthetic coordination of photosynthesis between two photosynthetic cell types, and differences in the requirement for ATP/NADPH per CO_2 fixed. Chapter 3 is a study to evaluate different possible mechanisms of regulation of light harvesting and electron transport by ATP synthase using photosynthetic mutants.

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CHAPTER ONE

Feedback limitation of leaf photosynthesis induces non-photochemical quenching and down-regulates electron transport activity by decreasing proton conductivity of CF_o-CF_1 ATP synthase

ABSTRACT

The transthylakoid proton motive force (*pmf*) is an essential intermediate in photosynthesis, driving the synthesis of ATP at the thylakoid ATP synthase as well as acting as a central feedback regulatory signal, activating the photoprotection of chloroplasts from excess light via the qE-response, and down-regulating electron transfer through the cytochrome b_6f complex. The extent of *pmf* is regulated in part by the activity of the ATP synthase, resulting in changes to the proton conductance of the thylakoid membrane (g_{H^+}). A low g_{H^+} retards proton efflux from the lumen increasing *pmf.* It has been proposed that the ATP synthase, and thus g_{H^+}, may be controlled by the availability of inorganic phosphate (P_i) in the stroma. In particular, P_i is considered to be limiting when photosynthesis is controlled by feedback due to limited capacity to utilize triose-P. We studied changes in g_{H^+}, CO_2 assimilation, antenna regulation and cytochrome b_6f activity under feedback conditions in wild type and, a starchless mutant of *Nicotiana sylvestris* (NS 458) defective of plastid phosphoglucomutase. When feedback occurs in the starchless mutant there is a marked reversal of O_2 sensitivity, a phenomenon which we exploited to modulate feedback limitation under high CO_2. Measurements of the magnitude of g_{H^+} and *pmf* were made *in vivo*, while Rubisco

oxygenation (v_o) and carboxylation (v_c) rates, and O_2 evolution from PSII (J_{O2}), were calculated from simultaneous measurements of leaf CO_2 exchange and fluorescence yield. It was shown that g_{H^+} increases proportionally with an increase in v_c+v_o, and with an increase in J_{O2}, when O_2 was increased from 0-40% under CO_2-saturated photosynthesis. Increasing O_2 under high levels of CO_2 also caused an increase in the RuBP and ATP pool size. The data support the hypothesis that feedback-limited conditions control photosynthesis via effects on the ATP synthase, which regulates the light reactions via its control of the proton gradient. Because previous experiments have linked P_i levels to feedback limitations, our results are also consistent with a role for P_i, either directly or indirectly, at the ATP synthase in the co-regulation of the light and dark reactions.

INTRODUCTION

Higher plant photosynthesis is finely regulated to prevent photodamage when light absorption exceeds the capacity of the plant to use it. A key intermediate in this regulation is the thylakoid proton motive force (*pmf*) which is generated by light-driven electron transfer and used to drive ATP synthesis at the thylakoid ATP synthase (Boyer, 1997). The *pmf* also regulates both light capture, by activating the qE-response (Horton *et al.*, 1996; Niyogi, 2000), and the cytochrome b_6f complex, thus preventing the accumulation of highly-reducing intermediates in the electron transfer chain (Hope *et al*., 1994; Kramer *et al*. 1999; Takizawa *et al*., 2008). Photosynthesis can be limited by different factors depending on conditions. Under low light there is limited capacity for generating assimilatory power and regenerating RuBP. Under low CO_2, Rubisco exerts a

high degree of control with limited carboxylase activity and enhanced oxygenase activity and photorespiration. With ample CO_2 and high light, the flux through the Benson-Calvin cycle is controlled by the capacity for RuBP regeneration (e.g. capacity of photochemistry to provide energy for regeneration), by the capacity to utilize triose-P for synthesis of end products (e.g. starch and sucrose), or export of sucrose which if limited can result in "feedback regulation" (Woodrow and Berry, 1988).

With feedback inhibition of photosynthesis, the capacity of Rubisco to fix CO_2 exceeds the capacity of triose-P to be converted into carbohydrates, or the capacity to utilize carbohydrates (Sharkey *et al*., 1986; Sage and Sharkey, 1987). Feedback is more likely to occur under high light, high leaf conductance for CO_2 from the atmosphere to Rubisco, and moderate to low temperatures where the capacity for Rubisco to function as a carboxylase can exceed capacity for synthesis of carbohydrates (starch and sucrose) (Sage and Sharkey, 1987; Sharkey *et al*., 1995; Leegood and Edwards, 1996; Sun *et al*., 1999a). The potential for feedback is increased under high light with CO_2 enrichment which decreases photorespiration and increases capacity for carboxylation over a wider temperature range. Feedback caused by limitations on triose-P utilization becomes especially evident when starch synthesis is impaired by mutations in ADPG pyrophosphorylase (Neuhaus and Stitt, 1990; Sun *et al*., 1999b), and plastid phosphoglucoisomerase activity (Hanson and McHale, 1988; Peterson and Hanson, 1991; Hanson, 1990; Hanson, 1992; Huber and Hanson, 1992; Eichelmann and Laisk, 1994; Sharkey *et al*., 1995) or sucrose synthesis by mutants of cytosolic fructose-1,6-biphosphatase activity (Sharkey *et al*., 1988).

It has been proposed that the light reactions of photosynthesis are regulated by modulating the activity of the ATP synthase, possible by altering the concentration of P_i in the stroma (Woodrow and Berry, 1988; Kanazawa and Kramer, 2002).

It is generally accepted that the concentration of P_i in the chloroplast stroma is an important regulator of processes in photosynthesis. The total chloroplast stromal phosphate pool (which includes P_i and the organic phosphates of the Benson-Calvin cycle intermediates) is conserved, at least over the short term. The uptake of P_i by the chloroplast in exchange for export of triose-P, on the phosphate translocator, for sucrose synthesis is necessary to maintain synthesis of ATP and carbon fixation (Flügge and Heldt, 1984).

Under feedback conditions, limitation on utilizing triose-P for sucrose synthesis can result in a decrease in P_i and an increase in the 3-phosphoglycerate/P_i ratio in the chloroplasts which up-regulates ADPG pyrophosphorylase and starch synthesis (Preiss, 1991). In this case, P_i is not thought to be recycled at sufficient rates by triose-P metabolism to carbohydrate so that organic-P turnover and P_i become limiting for ATP synthesis and the regeneration of RuBP (Sharkey, 1990). Thus, under feedback conditions the rate of photosynthesis is considered limited by reactions which regenerate P_i from organic-P. Under photosynthesis without feedback, P_i is continuously being regenerated by metabolism of triose-P to starch and sucrose, by metabolism in the Benson-Calvin cycle through fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and GAP-dehydrogenase, and in photorespiration by glycolate phosphatase.

In the current work, we test the involvement of ATP synthase as a key component of feedback regulation of the light reactions. It is known that limiting CO_2 causes a decrease in the proton conductivity of the chloroplast ATP synthase (g_H^+) (Kanazawa and Kramer, 2002) which and the resulting retardation of proton efflux increases the steady-state proton motive force , activating qE-type non-photochemical dissipation of excitation energy. This *in vivo* regulation of ATP synthase has been documented using non-invasive optical methods (Kramer *et al*., 2004); but, the mechanism controlling g_H^+ is not clear. One possibility is that ATP synthase activity is regulated by limitations in stromal inorganic phosphate (P_i) (Robinson and Giersch, 1987; Sharkey, 1990; Kanazawa and Kramer, 2002; Takizawa *et al*., 2008).

METHODS

Plants of *Nicotiana sylvestris* were grown in Econair (Winnipeg, Canada) growth chambers in fertilized potting soil in 8 L pots (one plant per pot), with a 14/10 h day/night cycle at 28/22 °C, 50% relative humidity, 380 µbar CO_2, and an incident photosynthetic quantum flux density (PFD) of 800 µmol m^{-2} s^{-1}. The wild type, and the plastid phosphoglucomutase deficient mutant of *N. sylvestris*, NS 458, created by Hanson (1988) were used. The NS 458 mutant tested negative for iodine staining for starch.

Measurments of electrochromic shift (ECS_t) and proton conductance (g_H^+) of thylakoid membranes.

Steady state light-driven *pmf* and g_H^+ were estimated by following the absorbance

changes attributable to *ECS*, at 520 nm, with rapid light to dark transitions using a leaf spectrophotometer based on those constructed earlier (Kramer and Sacksteder, 1998; Sacksteder *et al*., 2001), but adapted with a 1 cm^2 area gas-tight leaf chamber. A small air space (3 mm thickness) was left on the lower side of the leaf, and gas with known humidity, O_2 and CO_2 content (mixed with the FastEst system, Tartu, Estonia; Laisk *et al*., 2002) was constantly passed over the lower leaf surface at a rate of 10 cm^3 s^{-1}. The window was illuminated by actinic diffuse red LED light. The modulated measurement light at 520 nm was provided by another set of LED-s. A similar system, described in Kohzuma *et al*. (2008), was used for measurement cyt_f reduction kinetics under essentially identical conditions. The device lacks temperature control and leaf temperature was dependent on room temperature (~24^{o}C).

Leaf gas exchange and fluorescence yield measurements

For each experiment in which measurement of ECS, gas exchange and chlorophyll fluorescence analyses were measured under the same conditions on equivalent leaf material using the FastEst gas system (Tartu, Estonia, described in detail in (Laisk et al., 2002). The system was equipped with a Li-Cor 6251 (Lincoln, Nebraska) CO_2 analyzer, and Applied Electrochemistry Inc. (Sunnyvale, California) S-3A O_2 analyzer. Leaf gas exchange characteristics, net rates of CO_2 fixation (A), C_i, APFD and leaf temperature were determined as in (Laisk and Loreto, 1996). The photosynthetic parameters: Rubisco oxygenation (v_o) and carboxylation (v_c) rates, and PS II electron transport (J_{O2}), were calculated from simultaneous measurements of leaf CO_2 exchange and fluorescence yield as in (Kiirats *et al*., 2002).

Rubisco v_c and v_o were calculated according to Farquhar, Berry and van Caemmerer model (Farquhar *et al*., 1980) using the measured CO_2 assimilation rate (A), measured dark respiration rate (R_d) and the following equations:

$$A = v_c - 0.5v_o - R_d$$

$$v_c = \frac{V_c \cdot C_c}{C_c + K_c(1 + O_c/K_o)}$$

$$v_o = \frac{V_o \cdot O_c}{O_c + K_o(1 + C_c/K_c)}$$

$$V_c = \frac{(A - R_d) \cdot (C_c + K_c(1 + O_c/K_o)}{C_c - 0.5 \cdot O_c/S}$$

$$V_o = \frac{V_C \cdot K_o}{K_c \cdot S}$$

where V_c , V_o = maximum carboxylation and oxygenation velocities, respectively, K_c , K_o = carboxylation and oxygenation Michaelis constants, respectively, C_c, O_c = CO_2 and O_2 concentrations at Rubisco active sites, respectively, and S = Rubisco specificity for CO_2 relative to O_2. The mitochondrial CO_2 evolution rate at light (R_d) was taken as 5% of CO_2 and light saturated CO_2 net assimilation rate (A_{max}), K_c, K_o and S at 25^{o}C 11μM, 300μM and 88 corrected to temperature as in (Woodrow, Berry, 1988) were used.

The yield of PSII was measured by chlorophyll fluorescence using a Walz PAM 101 fluorometer (Effeltrich, Germany). The gross rate of O_2 evolution from PSII (J_{O2}) was calculated as:

$$J_{O2} = APFD \cdot Y_{II} \cdot (F_m'-F_s)/F_m')/4$$

where $(F_m'-F_s)/F_m'$ is the yield of PS II, F_s is fluorescence yield of steady state photosynthesis, F_m' is maximal fluorescence yield by exposure to a 1 s pulse of 15,000 μmol $m^{-2}s^{-1}$ light, Y_{II} is the relative optical cross-section of PSII (the fraction of light absorbed by PSII) as determined in (Laisk *et al*., 2002) from light response curves for leaf O_2 evolution under a low O_2 background. *APFD* is the absorbed photosynthetic photon flux density at steady state which was calculated with absorption coefficient of 0.85 (Genty *et al*., 1989; Krall and Edwards, 1992). NPQ and 1-qL were calculated as in Kramer *et al.* (2004). NPQ for all experiments represents the energy dependent component, qE, where F_m was measured after 15 min of darkness. The assimilatory charge (AC) during photosynthesis in the light was determined at a given time as described in (Laisk *et al*., 2002) by measuring the magnitude of the post-illumination uptake of CO_2.

Measurements of RuBP and ATP pool sizes

FasEst leaf gas exchange system was used to record leaf net CO_2 uptake and fluorescence yield. After reaching steady-state photosynthesis under defined conditions, leaves were killed by fast-filling the leaf chamber with cooled 95% ethanol (~ -80 °C). The frozen leaf piece (5 cm^2) was ground to a fine powder in liquid N_2 in a mortar (previously cooled with liquid N_2) followed by extraction for 15 min in 3 ml 1N $HClO_4$

in a small beaker. The extract was centrifuged 2 min at 5000 g. The supernatant was neutralized with 5 M KOH. Insoluble $KHClO_4$ was removed by centrifugation and RuBP and ATP contents were determined (either immediately, or the extract was stored in liquid N_2 for later analysis).

RuBP content was measured as in (Wirtz *et al*., 1980) by measuring ^{14}C incorporation into acid stable product with purified Rubisco. ATP was assayed using the luciferin/luciferase assay method (Strehler and McElroy, 1957); 50 µL of the leaf extract, diluted 1 to 10 with assay buffer, was injected into a 100 µL assay medium in luminometer chamber (Aminco Chemo-Glow Photometer, American Instrument Co., USA). The assay medium contained 75 mM Hepes/KOH at pH 7.5, 5 mM $MgSO_4$, 1 mM Na_2HAsO_4, 1 mM mercaptoethanol, 0.2 mM Luciferin (Sigma), and 13 x 10^3 Light Units of Luciferase (Sigma). The initial peak of the luminescence signal was proportional to ATP content.

RESULTS

1. *ATP synthase proton conductivity under variable O_2 and CO_2 conditions at light saturation in wild type and starchless mutant of Nicotiana sylvestris.*

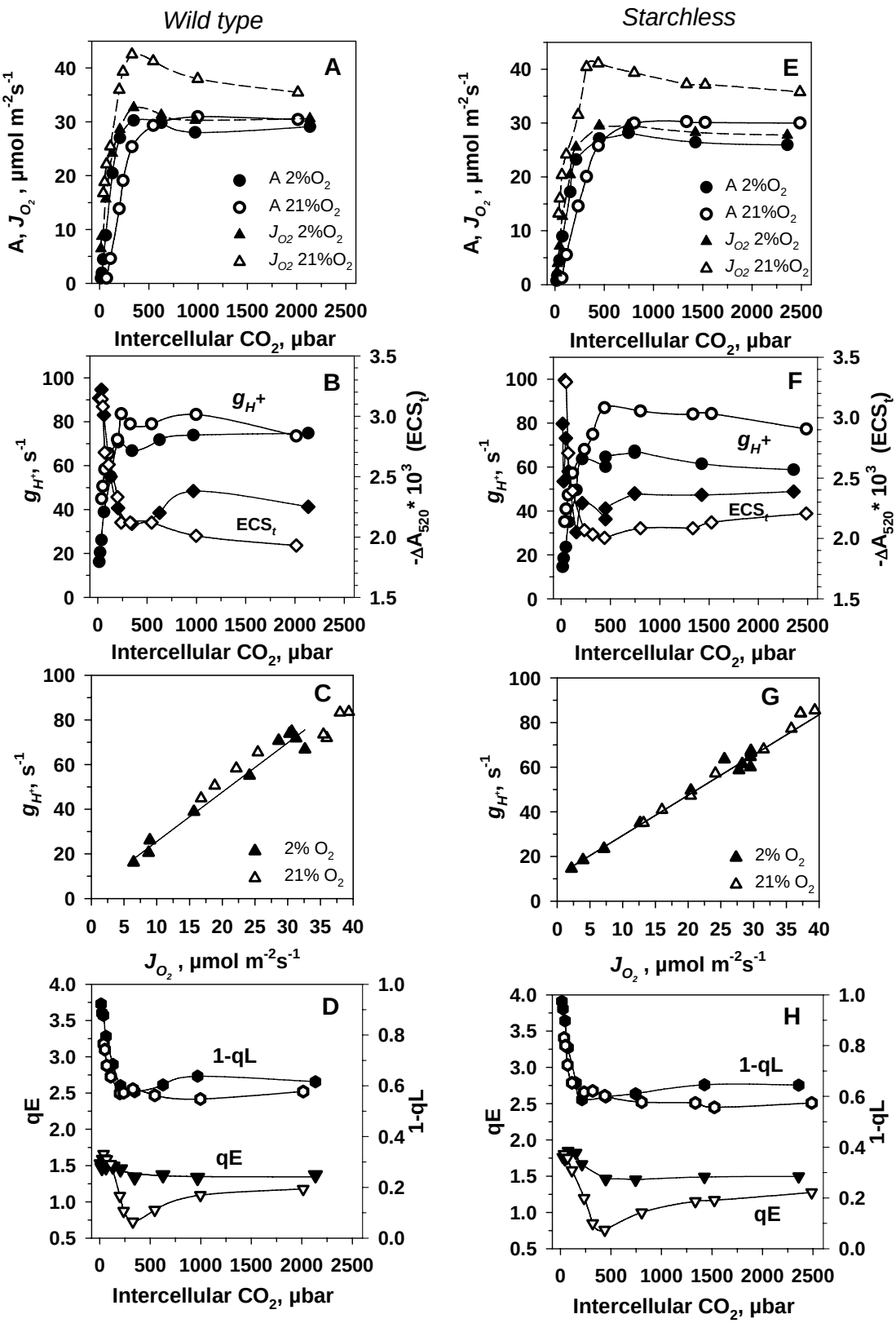
Wild type
Starchless
A
E
A 2%O2
A 21%O2
JO2 2%O2
JO2 21%O2
A, JO2 , µmol m-2s-1
Intercellular CO2, µbar
B
F
gH+
ECSt
gH+, s-1
-ΔA520 * 10^3 (ECSt)
C
G
2% O2
21% O2
JO2 , µmol m-2s-1
D
H
1-qL
qE

Figure 1.1. Measurements in wild type and starchless mutant *N. sylvestris* of the CO_2 assimilation rates (*A*), gross rates of O_2 evolution (J_{O2}), electrochromic shift (ECS_t), ATP synthase proton conductivity (g_{H^+}) and fluorescence parameters (qE and 1-qL) with changes in intercellular levels of CO_2. Leaf temperature was 24°C and absorbed PFD 970 μmol m^{-2} s^{-1}. Black symbols represent 2% O_2, white symbols represent 21% O_2. In panel A. symbols for J_{O2} are Δ = 21% O_2, ▲ = 2% O_2 and symbols for *A* are ○ = 21% O_2, ● = 2% O_2. J_{O2} was calculated from the measured fluorescence yields (see Methods). Measurements were made first under varying CO_2 with 21% O_2, and then on the same leaf under varying CO_2 at 2% O_2. The data points represent steady state values that was defined as relatively constant A and fluorescence yield under given conditions. The data represent measurements on one leaf but the experiments were repeated 3 times with the same results.

Fig. 1.1 shows results from measuring electrochromic shift parameters (g_{H^+} and ECS_t) (see Avenson *et al*. 2005; Cruz *et al*. 2005; Baker 2008, on the methods) in parallel with leaf CO_2 assimilation rates (*A*) and gross rates of O_2 evolution (J_{O2}) at saturating PFD (970 μmol m^{-2} s^{-1}) and 2% versus 21% O_2 in wild type *N. sylvestris*. Fig. 1.1A and B show the response of *A* and J_{O2} versus intercellular level of CO_2 (C_i). Under limiting [CO_2] we observed an inhibition of *A* by 21% O_2, whereas under CO_2 saturated conditions O_2 sensitivity was reversed with a slight stimulation of *A* by 21% O_2. Under 2% O_2 we observed a close relationship between *A* and J_{O2}; whereas under 21% O_2, J_{O2} was much higher than *A* under limiting CO_2 (Fig. 1.1A, B). From measurements of CO_2

fixation (*A*) in panels A and E in Fig. 1, the activity of Rubisco (the sum of carboxylase plus oxygenase, $v_c + v_o$) was calculated (see Methods). Then Rubisco activity was plotted against J_{O2}, showing a linear relationship (Fig. 1.2). J_{O2} (Fig. 1.2) tended to be higher at 21% O_2 than calculated v_c+v_o indicating some flow of electrons to other processes than CO_2 assimilation or photorespiration. With increasing levels of CO_2 up to ~300 µbar CO_2 under both 2 and 21% O_2 led to a substantial increase in g_H^+, from about 45 to 85 s^{-1} concomitant with large decreases in ECS_t (Fig. 1.1B,F).

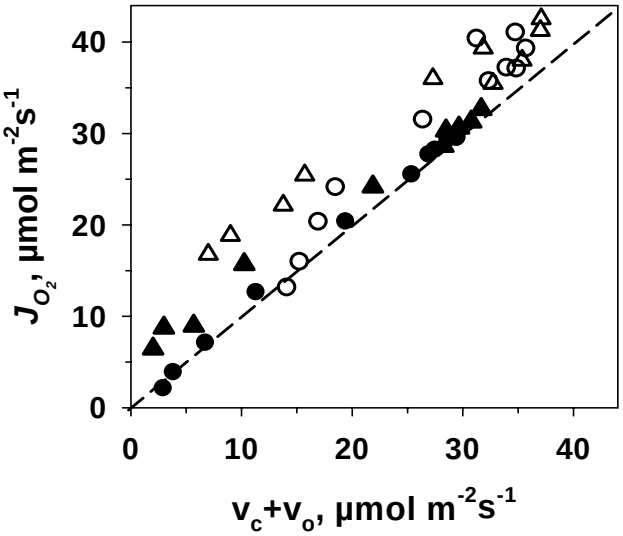

Figure 1.2. Relationship between v_c+v_o and J_{O2}. The data from experiment fig. 1.1 were used.

The conductance of ATP synthase (g_{H^+}) in wild type was higher under 21% than 2% O_2 at varying levels of C_i (except at 2000 µbar CO_2) (Fig. 1.1C). A similar trend was seen in the starchless mutant (Fig. 1.1F), except that g_{H^+} was substantially lower than Wt at 2% O_2, resulting in a much larger O_2-induced increase.

Increasing CO_2 up to ~300 µbar CO_2 in both 2 and 21% O_2 resulted in a drop in the fraction of reduced PSII centers, estimated by 1-qL (Kramer *et al*. 2004) (Fig. 1.1D, H).

While qE was relatively constant over varying CO_2 under 2% O_2, there was a large drop in qE under 21% O_2 with minimal value around 300 µbar CO_2 (Fig. 1.1D, H) which coincided with maximum J_{O2} (Fig. 1.1A, E). There was also strong relaxation of qE under 21% O_2 at the shoulder region of CO_2 response curve (C_i ~300-500 µbar CO_2). There was a strong, nearly linear relationship between J_{O2} (reflects linear electron transport) and g_{H^+} (Fig. 1.1C, G), and between Rubisco activity ($v_c + v_o$) and g_{H^+} (not shown).

The changes in photosynthetic parameters in the starchless mutant of *N. sylvestris*, which is defective in plastid phosphoglucose mutase (Hanson and McHale, 1988) were similar to that of the wild type particularly under limiting CO_2 (Fig. 1.1). However, the lack of starch synthesis in the mutant resulted in a more distinct reversal of sensitivity to O_2 under saturating levels of CO_2 with an enhancement of A by 21% (Fig. 1.1E). The mutant also showed strong enhancement of g_{H^+} by 21% O_2 under feedback conditions (Fig. 1.1F). The patterns of changes in 1-qL and qE in the mutant (Fig. 1.1H) were similar to those in the wild type (Fig. 1.1D). A plot of J_{O2} versus g_{H^+} under varying

CO_2 and O_2 under both CO_2 limited and CO_2 saturated conditions with 2 versus 21% O_2 shows there is a linear relationship in both mutant and wild type (Fig. 1.1D). Also, the relationship reveals RuBP oxygenation and carboxylation affect g_{H^+} equally.

2. *ATP synthase proton conductivity changes under feedback conditions lead to the regulation of qE, and Cyt b_6f complex in Nicotiana sylvestris*

The hypothesis that feedback limitation of CO_2 assimilation slow electron transfer reactions of photosynthesis via effects on the ATP synthase was tested by comparing parameters of photochemistry, including *Cyt b_6f* complex, at 2 and 21% O_2 and 2000 µbar CO_2 and 25°C in *N. sylvestris* wild type and starchless mutant. Compared to the wild type, the starchless mutant showed reduced turnover rates for the *Cyt b_6f* (Fig. 1.3A), as well as decreased g_{H^+} (Fig. 1.3B) and J_{O2} (Fig. 1.3C), while having higher ECS_t (Fig. 1.3D) and qE (Fig. 1.3D) under 2% O_2 and 2000 µbar CO_2.When we switched from 2 to 21% O_2 caused a substantial stimulation of the rate constant for *Cyt b_6f* (Fig. 1.3A), in g_{H^+} (Fig. 1.3B) and J_{O2} (Fig. 1.3C) in the mutant, with less effect in the wild type.

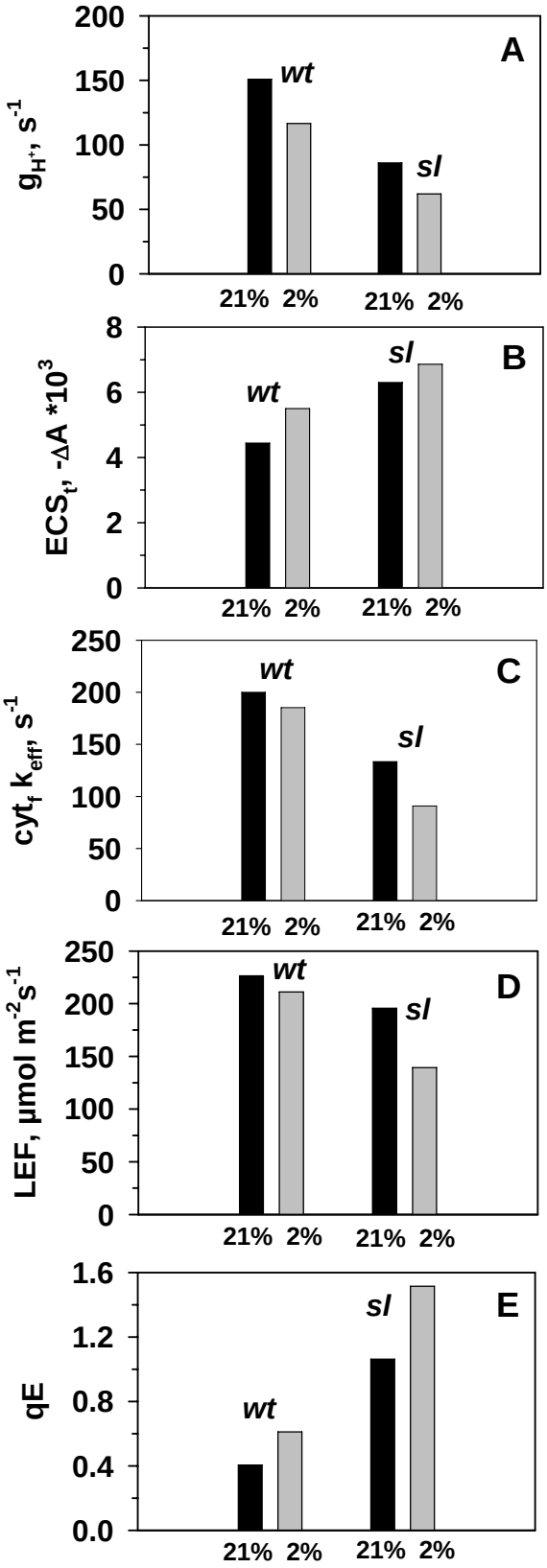
A
B
C
D
E
wt
sl
21%
2%
g_{H^+}, s^{-1}
ECS_t, $-\Delta A *10^3$
cyt_f k_{eff}, s^{-1}
LEF, µmol $m^{-2}s^{-1}$
qE

Figure 1.3 Steady state electron transport parameters at 2 vs. 21% O_2 in wild type (wt) and starchless (sl) mutant of *N. sylvestris* at 2000 μbar CO_2 and 800 μmol $m^{-2}s^{-1}$ PFD, and 25°C. Measurements include ATP synthase proton conductivity (g_{H^+}), electrochromic shift (ECS_t), *cyt f* effective kinetic constant, and gross rates of O_2 evolution (J_{O2}). Measurements were made first at 21% O_2, then ~15 min after switching to 2% O_2. Results are representative of two separate experiments.

3. Effect of varying O_2 levels, under saturating CO_2 and light, on ATP synthase conductivity, photosynthesis and assimilatory charge in the starchless mutant of Nicotiana sylvestris.

Changes in photosynthetic parameters over a range of O_2 levels were studied in the starch-less mutant. Figure 1.4 shows the effects on the kinetics of inhibition of photosynthesis by switching from 21% to 2% O_2 under high CO_2 (2000 μbar). Following a reduction in O_2 concentration to 2%, *A* decreased rapidly to a low level (much more than in wild type, not shown), after which there was a relatively flat minimum which lasted for about 400 s, followed by a transitory increase (Fig. 1.4). There was some rise in *A*; but, after reaching steady-state rates remained lower than under 21% O_2 (not shown).

The effects of increasing O_2 under feedback conditions in the starchless mutant (1800 μbar CO_2, 750 PFD and 32°C) on CO_2 assimilation rate, leaf fluorescence parameters and parameters derived from electrochromic shift measurement (g_{H^+} and ECS_t) were studied (Fig. 1.5). The O_2 concentration was changed from steady state photosynthesis at 1800 μbar CO_2 and 21% O_2 to different O_2 concentrations between 0

and 40%. Based on results of Fig. 1.4, measurements were made ~5 min after changing O_2 concentrations to obtain the initial maximum effect of O_2 on photosynthesis

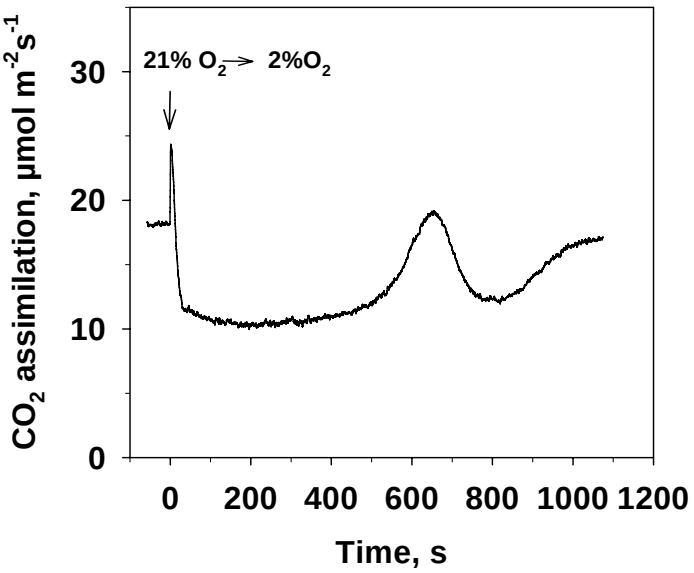

Figure 1.4. Illustration of the transient change, and inhibition of the rate of CO_2 assimilation, when the O_2 concentration was changed from 21% to 2% in the starchless mutant of *N. sylvestris* at 2000 µbar CO_2, 800 µmol $m^{-2}s^{-1}$ absorbed PFD, and 32 °C.

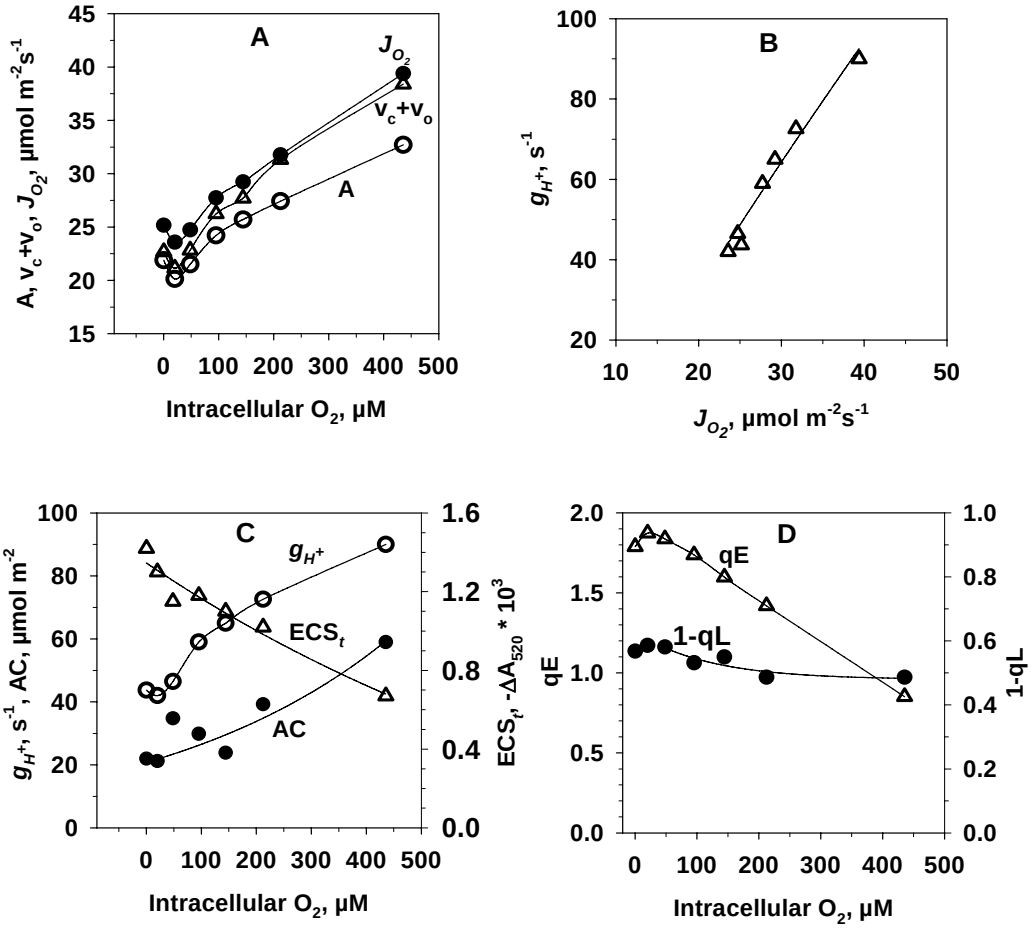

Figure 1.5. The effect of varying levels of O_2 on photosynthetic parameters in the starchless mutant of *N. sylvestris*. Measurements were made at 1800 µbar CO_2, 750 µmol m^{-2} s^{-1} absorbed PPFD, and leaf temperature of 32 °C. CO_2 assimilation rates (*A*), gross rates of O_2 evolution (J_{O2}), electrochromic shift (ECS_t), ATP synthase proton conductivity (g_{H^+}) and fluorescence parameters (qE and 1-qL). Measurements were made at 21% O_2 (reference point), 5 min after switching to another O_2 level, at 21% O_2, 5 min after switching to another O_2 level, etc. The results represent one experiment, three replicate experiments gave the same result. It suggests that the factor regulating

Sun *et al.* (1999a) have shown that this immediate effect of O_2 is linearly related to the effect of O_2 after reaching steady-state (which is presumably caused by effects of the availability of free P_i).

Under feedback conditions in the starchless mutant, there was a near linear increase in PSII activity (J_{O2}) and *A* with increasing O_2 concentration (Fig. 1.5A). Also, the calculated values of v_c+v_o that represent Rubisco activity and J_{O2} were very close at all O_2 concentrations (Fig. 1.5A). The enhancement of gross rates of O_2 evolution (J_{O2}) with increasing O_2 was linearly related to changes in g_{H^+} (Fig. 1.5B).

ECS_t and qE decreased with increasing O_2 concentration, while g_{H^+} increased (Fig. 1.5C, D). There was little change in the fraction of closed centers (1-qL) with increasing O_2 (Fig. 1.5D).

An indirect estimate of the RuBP pool size, which exists during photosynthesis under a given condition, can be made by measuring the post-illumination CO_2 uptake under low O_2, called the "assimilatory charge" (AC) (Laisk *et al.*, 2002). With increasing O_2 under feedback conditions there was a parallel increase in AC (Fig. 1.5B) with enhancement of *A* which indicates O_2 enhancement of *A* is associated with an increase in the RuBP pool.

5. Effect of O_2 on RUBP and ATP pools under feedback conditions in the starchless mutant of Nicotiana sylvestris.

Table 1.1. Measurements of the pool sizes of ATP and RuBP during steady state photosynthesis in the starchless mutant of *N. sylvestris* under feedback conditions.

Measurements were made after reaching steady-state under 2% O_2, and then 5 min after switching to 21% O_2. The conditions were 1800 µbar CO_2, 800 µmol $m^{-2}s^{-1}$ absorbed PPFD, 32 °C, and 2 versus 21% O_2, n=3.

O_2 level	A µmol m^{-2} s^{-1}	RuBP µmol m^{-2}	ATP µmol m^{-2}
2 % O_2	8.5 ± 2.0	55.5 ± 6.8	21.1 ± 3.2
21% O_2	14.3 ± 1.9	81.2 ± 5.8	39.5 ± 7.5

The pool sizes of RuBP and ATP were estimated in the starchless mutant under feedback conditions by freeze-fixing the leaf under 2% versus 21% O_2 (Table 1.1). Measurements under 2% O_2 were made 5 min after switching from 21 to 2%. Rates of *A* and pool sizes were much higher under 21% than under 2% O_2, RuBP (~1.5 fold), ATP (1.9 fold) and *A* (1.7 fold).

DISCUSSION

The ATP synthase is down-regulated under feedback limiting conditions.

The first goal of this work was to test a key postulate of the P_i feedback model: that regulation of the ATP synthase activity is involved in regulation of the light reactions under feedback limiting conditions. Our results show this to be true. Strikingly, the extent of feedback limitation, as seen during progressive release upon gradually increased

O_2 levels, was linearly related to ATP synthase g_{H^+} activity *in vivo*. The inverse sensitivity of A to O_2 is diagnostic for feedback-limiting conditions. Moreover the suppression of ATP synthase by low O_2 effects were substantially higher in the starchless mutant, where feedback limitations are expected to be more pronounced.

Down-regulation of the ATP synthase under feedback-limiting conditions increases lumen acidification thus down-regulating the light reactions.

The decrease in g_{H^+} restricted proton efflux, leading to an increase in light-induced *pmf* for a given electron or proton flux (Fig. 1.5), as previously seen under low CO_2 conditions. In turn, the increased *pmf* resulted in acidification of the lumen as evidenced by the increased activation of qE (Figure 5), and down-regulation of the cytochrome b_6f complex (Figure 5). Indeed, as shown in Fig. 3, the slowdown in A under varying levels of feedback limitation, could be explained by control of LEF at the level of *cyt* b_6f complex turnover.

Thus, our results are broadly in agreement with the proposal of Sharkey and coworkers (Sharkey, 1990), that feedback limitation of photosynthesis controls the light reactions by limiting proton efflux through the ATP synthase, leading to acidification of the lumen pH and subsequent slowing of the *cyt* b_6f complex. The same lumen acidification also activates the qE response, protecting the photosynthetic apparatus from photodamage (review in Li *et al*., 2005).

Some authors propose that *pmf* generated by cyclic electron flow makes a significant contribution to regulation of NPQ (Miyake *et al*., 2005). Our results do not support significant involvement of cyclic electron flow or water-water cycle in regulation of electron transport and energy quenching under the conditions studied. Apparently

some water-water cycle activity occurs at 21% O_2 (Fig. 2); but, it appears to be a rather constant proportion of electron flow without regulatory significance. Joliot and Joliot (2006) showed involvement of PSI cyclic electron flow during induction of photosynthesis in dark to light transitions. Free P_i concentration in dark adapted leaves increases due to decrease in organic pools. The Calvin cycle phosphorylated intermediate pools start building up slowly after onset of illumination. ATP-synthase conductance is expected to be high during this period due to increased P_i and cyclic photophosphorylation may be needed to build up organic-P pools, rather than to generate high ΔpH and NPQ.

ATP synthase regulation is consistent with the P_i-regulatory hypothesis. The P_i-feedback regulation hypothesis states that, under feedback-limiting conditions, stromal P_i drops below its K_M at the ATP synthase, slowing its turnover. We have confirmed the slowing of the ATP synthase, and subsequent consequences for control of LEF, as described in the previous section. In this section we discuss the possibility that this alteration in ATP synthase activity can be ascribed to decreased stromal P_i levels.

In principle, the ATP synthase can be regulated at several levels, both allosterically e.g. via thioredoxin-modulation (Scheibe, 1991) or phosphorylation (Bunney *et al*., 2001; del Riego *et al*., 2006), or by latering substrate (ADP or P_i) levels. The K_M for ADP at the ATP synthase is about 30 μM while that for P_i is 0.5-0.9 mM (Grotjohann and Graber, 2002; Pänke and Rumberg 1999). Stromal ADP content was measured at ~0.5 mmol/L in the light (Stitt *et al*., 1982). If this ADP level reflects free concentrations, it will be well above its K_M at the ATP synthase, and thus not limiting.

In order to explain our results solely by P_i limitation, the stromal P_i in intact leaves must be in the range of the K_M for P_i for ATP synthase (~0.5 – 1 mM). The P_i concentration in the chloroplast stroma in leaves is difficult to measure due to relatively high concentration of P_i (~20 times) in vacuoles (Wirtz *et al*., 1980). Photosynthesis was found to be saturated in the stroma of isolated chloroplasts at a concentration of the active form of P_i at 2 – 2.5 mM (Robinson and Giersch, 1987). These authors also concluded that the stromal concentration of P_i in isolated chloroplast is 1.5 – 2.0 mM during photosynthesis with optimal concentrations of P_i in the reaction medium. It has also been demonstrated that limitation of P_i supply to chloroplasts leads to a decrease in stromal P_i to a point where ATP synthesis may become P_i limited (Giersch and Robinson, 1987). It is proposed the same occurs under feedback limitation in leaves (Sharkey and Vanderveer, 1989; Paul and Foyer, 2001). Mannose feeding experiments into the leaves showed strong inhibition of photosynthesis explained by mimicking feedback by sequestering cytosolic free P_i (Harris et al., 1983; Morison and Batten, 1986). This effect was shown to be related to slowing of the ATP synthase (Takizawa *et al*., 2008).

It is possible that chloroplasts *in vitro* at high light do not fully recover the levels of Calvin cycle phosphorylated intermediates seen in intact leaves which would result in a higher P_i pool size in isolated chloroplasts. For example RuBP concentrations in leaves can be as high as 10 mM (Woodrow and Berry, 1988) such that only small changes in RuBP concentration could have a major effect on P_i concentration. Also, the active concentration of P_i is likely much lower than the measured total P_i due to binding to cell structures.

Possibly at low CO_2 that P_i is partitioned strongly into organic phosphates, mainly hexose phosphates, RuBP and PGA. The free P_i pool size increases in parallel to photosynthesis rate due to a balance in P_i fluxes.

Regarding oxygen enhancement effect on the rate of photosynthesis at elevated CO_2 (see Figs. 1.4, 1.5), our results are consistent with multiple hypotheses for the basis of sink limitation (triose phosphate utilization limited) and P_i fluxes (Sharkey, 1985; Sharkey and Vassey, 1989; Harley and Sharkey, 1991). The thylakoid ATP synthase proton conductivity (g_H^+) increases with increasing O_2 in parallel with increases in photosynthesis (Fig. 1.5) suggesting an increase in the rate of synthesis of ATP by increased rate of supply of P_i which leads to an increase in assimilatory charge, RuBP pool size and carboxylation rate (Fig. 1.5, Table 1.1).

The rate of increase of photorespiration in changing from 2 to 21% O_2 as an additional carbon sink at 1800 μbar CO_2 is not sufficient to explain enhancement of photosynthesis in starchless mutant (Fig. 1.4, 1.5). Rather it is expected that the increase in photosynthesis by increasing O_2 should be linked to an increase in synthesis of carbohydrates or rate of translocation of sucrose through some mechanism if its accumulation causes feedback. Some flux of carbon through the glycolate pathway may increase mitochondrial production of ATP (using NADH generated from glycine decarboxylase), which could support increased biosynthesis of sucrose in the cytosol. Hanson (1992) found that light saturated photosynthesis under low O_2 and high CO_2 in the starchless mutant of N. sylvestris was especially sensitive to oligomycin, an inhibitor of mitochondrial respiration.

Chloroplast stroma P_i is involved in regulating multiple processes: stabilizing pool sizes of metabolites in the Calvin cycle, regulating carbon partitioning and NPQ. In starch synthesis the PGA/P_i ratio is an important regulator of ADP-glucose pyrophosphorylase (AGP) activity: P_i is an inhibitor, PGA an activator (Preiss, 1991). It could be asked whether the requirement for low P_i (~0.5 - 1 mM) in our regulatory scheme is consistent with the suppression of carbon flux into starch at low CO_2 which is regulated by P_i and PGA? Interpreting the data of Kleczkowski *et al.*, (1993) and Kleczkowski (1999), for P_i to have an inhibitory effect on starch biosynthesis the PGA concentration in chloroplast stroma should be in the range of 1 mM or less. The estimated PGA concentration in sunflower leaf chloroplasts at 90 μbar CO_2 and 2% O_2 was ~0.5 mM (unpublished data of O. Kiirats) which would allow strong inhibition of starch synthesis by low P_i. At high CO_2, the PGA level increases to about 4 mM (Wirz *et al*, 1980) such that P_i would be ineffective as an inhibitor for AGP. Also, the $I_{0.5}$ value for P_i for inhibition of AGP activity [45 μM (Copeland and Preiss, 1981)] is strongly influenced by the level of chloroplast phosphorylated metabolites which alters the effect.

ATP synthase activity had a direct effect on thylakoid lumen pH which in turn regulates qE and the *cyt* b_6f complex (see Fig. 1.3). The following sequence of events is suggested to control Rubisco activity and down regulation of PSII. Limited capacity to utilize triose-P for synthesis of carbohydrates, or to export sucrose to sink tissue, causes an accumulation of organic-P and a decrease in P_i in the cytosol. Low P_i in the cytosol limits its uptake by chloroplasts in exchange for triose-P, which lowers chloroplast P_i and its availability for synthesis of ATP (Sharkey and Vanderveer, 1989; Sharkey, 1990).

In conclusion, our observations can be explained with a simple model (Fig. 1.6).

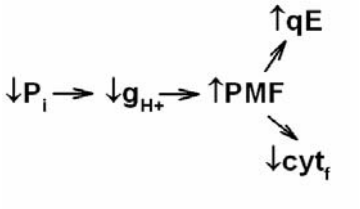

Figure 1.6. The scheme of P_i effects on electron transport and energy dependent exiton quenching.

Under feedback conditions, a decrease in P_i has a direct effect on the kinetics of the ATP synthase causing a decrease in its conductivity to protons, which in turn increases the *pmf*; the build-up of *pmf* increases excition quenching (qE), and decreases the rate constant for *cyt* b_6f.

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CHAPTER TWO

Co-regulation of dark and light reactions in three biochemical subtypes of C_4 species

ABSTRACT

Regulation of light harvesting in response to changes in light intensity, CO_2 and O_2 concentration was studied in C_4 species representing three different metabolic subtypes: *Amaranthus edulis* (NAD-malic enzyme), *Sorghum bicolor* (NADP-malic enzyme) and *Panicum texanum* (PEP-carboxykinase). Several photosynthetic parameters were measured on an intact leaf in order to describe PS I and PS II activities, thylakoid proton circuit, O_2 evolution and CO_2 assimilation rates. The C_4 subtypes had similar energy requirements for photosynthesis since there were no significant differences in maximal quantum efficiencies for gross rates of O_2 evolution (average value = 0.072 O_2/quanta absorbed, ~14 quanta per O_2 evolved). The PS I activity (calculated from A_{830} nm signal) was slightly more than twice the PS II electron flux in the malic enzyme species, suggesting that the extra ATP required to support C_4 photosynthesis can be produced by cyclic electron flow with a ratio of $2H^+/e^-$. Generally a linear relationship was observed between PS I and PS II rates, as well as between PS II flux and proton flux. At excess light, ATP synthase conductance responded strongly to changes in electron flow, decreasing almost linearly when photosynthesis was suppressed with low CO_2. It is proposed that ATP synthase conductance is controlled at the substrate level by P_i

availability. The results suggest that NPQ development is controlled by ATP synthase conductance rather than by generation of *pmf* by cyclic or pseudocyclic electron flow.

INTRODUCTION

C_4 plants effectively concentrate CO_2 at the site of RuBP carboxylation, thus inhibiting photorespiration, but it comes with a significant energy cost through the C_4 cycle. Beyond the requirement for 3 ATP and 2 NADPH per CO_2 fixed by Rubisco, the additional energy requirements depend on assimilatory power used in the 3 types of C_4 pathways found among C_4 species. The calculated theoretical minimum energy requirements per CO_2 fixed in NADP-malic enzyme (NADP-ME) and NAD-malic enzyme (NAD-ME) type plants is 5 ATP and 2 NADPH, while in PEP-carboxykinase (PEP-CK) type species it is 3.6 ATP and 2.3 NADPH (Kanai and Edwards, 1999). In C_4 plants extra ATP may be generated through cyclic or pseudocyclic photophosphorylation; in NADP-ME type C_4 plants additional ATP is provided by cyclic photophosphorylation in bundle sheath chloroplasts (Kanai and Edwards, 1999). In C_3 plants the need for cyclic electron transport and the cyclic pathways are currently being extensively debated and experimentally evaluated (Kramer *et al*. 2004; Johnson 2005; Joliot and Joliot, 2006).

In C_4 plants there are two suggested pathways of cyclic electron flow around photosystem I: ferredoxin:plastoquinone oxidoreductase (Fd/PQ) dependent and NAD(P)H dehydrogenase (NAD(P)H -plastoquinone reductase) (NDH) dependent flow (Takabayashi *et al*., 2005; Ivanov *et al*. 2007; Majeran and van Wijk, 2009).

An additional possible contributor to ATP generation in C_4 and C_3 plants is the photoreduction of oxygen in photosystem I by the Mehler reaction, water-water cycle (Asada, 1999). The contribution of the water-water cycle to ATP production is likely to be relatively small in C_4 plants (Laisk and Edwards, 1998; Badger *et al*., 2000).

In C_4 plants, photosynthetic electron transport, Calvin cycle reactions, and the C_4 cycle, need to be co-regulated in order to provide optimal flux into end products, and minimize the formation of reactive oxygen species which can lead to photo-inhibition. In C_3 plants various processes have been proposed to function to dissipate excess energy including the chloroplast membrane malate/oxaloacetate shuttle, cyclic electron flow, the Mehler reaction, and activation of nonphotochemical quenching (NPQ) by increased thylakoid lumen acidification. We have shown in studies on C_3 plants that the intrathylakoid proton concentration is sensitive to ATP synthase conductance and this is considered to play a key role in controlling thylakoid lumen pH and NPQ (Kanazawa and Kramer, 2002; Kramer *et al*., 2004). Down-regulation of ATP synthase proton conductivity under high light and limiting CO_2 increases the intrathylakoid H^+ concentration, which in turn activates the NPQ mechanism and protects PS II.

It has been proposed that cyclic electron flow can increase the concentration of protons in the lumen and induce NPQ. In a putative mutant having altered cyclic electron flow (*Arabidopsis* mutant PGR5), leaves were unable to build up a high proton gradient even under low CO_2, and the plant suffered from photo-inhibition under increasing light (Munekage *et al*., 2005). The inability of PGR5 mutants to build up a proton gradient was shown to be at least partially a result of very high thylakoid proton conductivity in these mutants (Avenson *et al*., 2005), but there was no increase in ATP synthase content in

PGR5 leaves compared to wild type.

It is not clear what controls induction of NPQ in C_4 plants through increased acidification of the lumen, e.g. proton pumping by cyclic electron flow and/or regulation of ATP synthase proton conductance, and whether there are significant differences in comparison to C_3 plants. Since cyclic photophosphorylation is considered to be more active in C_4 plants to meet needs for additional ATP, it might also be involved in induction of NPQ under excess light. There are also differences in ATP and NADPH consumption per CO_2 fixed between the C_4 subtypes due to different ATP demands and differences in ratios of PS I and II between mesophyll and bundle sheath chloroplasts (Edwards and Walker, 1983; Edwards and Voznesenskaya, in press). Also, the CO_2 pump gives C_4 plants some advantage over C_3 species allowing higher electron transport at high light and current ambient CO_2 concentrations which results in less over-reduction of the electron transport chain.

A proposed mechanism for regulation of ATP-synthase activity is through changes in the level of inorganic phosphate (P_i) in the chloroplast stroma; i.e. limiting P_i as one of the substrates would directly lower conductance of ATP synthase (Kramer *et al*., 2004). In addition, changes in free energy (ΔG) of ATP synthesis reaction may affect the ATP synthase activity. The increase of some phosphorylated intermediates with increased assimilation rate to support the required metabolite gradients for transport between mesophyll and bundle sheath cells in C_4 plants may also have an effect on the P_i status in chloroplasts (Leegood and Walker, 1999; Laisk and Edwards, 2000).

In this work we measure optical parameters of PSI and PSII, leaf gas-exchange and leaf absorbance change at 520 nm upon light-dark transition (electrochromic shift

signal) of species representing the three C_4 subtypes in order to evaluate the possible regulatory mechanisms controlling NPQ.

METHODS

Amaranthus edulis [NAD-ME], *Panicum texanum* [PEP-CK] and *Sorghum bicolor* [NADP-ME]), were grown in Econair (Winnipeg, Canada) growth chambers in fertilized potting soil in 8 L pots (one plant per pot), with a 14/10 h day/night cycle at 28/22 ^{o}C, 50% relative humidity, ~380 µbar CO_2, and an incident photosynthetic quantum flux density (PFD) of 800 µmol m^{-2} s^{-1}.

Leaf gas exchange fluorescence yield and ΔA_{830} measurements

Leaf gas exchange and fluorescence yield were measured with the FastEst gas system (Tartu, Estonia, Laisk and Oja, 1998). The system was equipped with a LiCor 6251 (Lincoln, Nebraska) CO_2 analyzer, Walz PAM101 fluorometer (Effeltrich, Germany) and Applied Electrochemistry Inc. (Sunnyvale, California) S-3A oxygen analyzer. Leaf gas exchange characteristics *A, C_i*, PFD and leaf temperature were calculated as in Laisk and Loreto (1996). The true O_2 evolution rate from PSII was calculated as

$$J_{O_2} = APFD \cdot Y_{II} \cdot (F_m' - F_s)/F_m')/4$$

and PS I rate per four electrons as

$$J_{PSI} = APFD \cdot Y_I \cdot (P/P_m)/4$$

Where F_s is fluorescence yield of steady state photosynthesis, F_m' is maximal fluorescence yield by exposure to a 1 s pulse of 15,000 µmol $m^{-2}s^{-1}$ light, *APFD* is the

absorbed photosynthetic quantum flux density at steady state. The absorption coefficient of leaves was measured using integrating sphere (Labsphere, North Sutton, NH). Y_{II} and Y_I = fractions of light absorption by PSII and PSI, $(F_m'-F_s)/F_m'$ = quantum yield of PSII (Genty *et al*., 1989) and P/P_m quantum yield of PSI according to the saturating pulse method (Klughammer and Schreiber, 1994). P_m represents leaf 830 nm maximum absorbance change (from dark level to the level of Far red combined with saturating flash) and P represents maximum absorbance change during illumination with saturating flash. 50 ms 10 000 μmol m^{-2} s^{-1} saturating flash was used.

For the estimate of Y_{II}, the method proposed by Laisk and Loreto (1996) was used. The relative proportion of absorbed light associated with linear electron transport (Y) was found as the slope of the plot F_v/F_m' $(1-F_s/F_m')$ versus 4 times quantum yield of O_2 evolution at low oxygen background (0.025 %) and high CO_2 (3%) measured at different light intensities. 5% of light was considered absorbed by other leaf structures, Mehler reaction and other possible losses. The remaining proportion of light was taken as absorbed by PS I. NPQ and qL were calculated as in Kramer *et al.* (2004). NPQ (F_m/F_m'-1) for all experiments represents the energy dependent component of NPQ (= qE) where F_m was measured after 15 min of darkness.

Measurments of electrochromic shift (ECS_t) and proton conductance (g_H^+) of thylakoid membranes.

Steady state light-driven *pmf* and g_H^+ were estimated by following the absorbance changes attributable to *ECS*, at 520 nm, with rapid light to dark transitions. The diffused-optics spectrophotometer, which was constructed in-house (Kramer and Sacksteder, 1998; Sacksteder *et al*., 2001) has a 1 cm^2 window that was clamped on the leaf. A small

air space (3 mm thickness) was left on the lower side of the leaf and gas with known humidity, O_2 and CO_2 content (mixed with the FastEst system) was constantly passed over the lower leaf surface at a rate of 10 cm^3 s^{-1}. The window was illuminated by actinic diffuse red LED light. The modulated measurement light at 520 nm was provided by another set of LED-s. Most experiments were done at room temperature 24 ^oC.

RESULTS

Light response.

Light response curves were measured under near atmospheric levels of CO_2 (360 μbar) under 21% O_2, as well as under high CO_2 (0.15% and 3%) conditions to ensure both CO_2 cycles (C_4 pump and Calvin cycle in bundle sheath chloroplasts) are CO_2 saturated. The results under 0.15% and 3% CO_2 were very similar to results under 360 μbar CO_2 (not shown), indicating very high CO_2 had no special effects on photochemistry and CO_2 fixation.

From optical measurements of PSI and PSII, rates of flux per 4 electrons through each photosystem were estimated, J_{PS1} and J_{O2}, respectively. There was a continuous rise in rates of photochemistry in all three C_4 subtypes with increasing PPFD up to the equivalent of full sunlight (~2000 PFD). The maximum quantum efficiency of O_2 evolution (the initial slope of the light response curve) was similar in all three species. The average maximum quantum efficiencies for O_2 evolution obtained from multiple experiments were: 0.072 ± 0.0066 (SD, n = 13) for *S. bicolor*, 0.073 ± 0.005 (SD, n = 20) for *A. edulis* and 0.075 ± 0.01 (SD, n = 14) for *P. texanum*. In the NADP-ME type species *S. bicolor* and the NAD-ME type species *A. edulis*, the estimated flux through

PSI was about two-fold higher than through PSII, while in the PEP-CK type species *P. texanum* the estimated flux through PSI was about 1.6 fold higher than through PSII. From analyses of electrochromic shift measurements with increasing PFD, there was little change in conductance of ATP synthase (g_H^+) in *S. bicolor* and *A. edulis* while in *P. texanum* there was some initial rise in g_H^+ with increasing PFD. In all 3 species

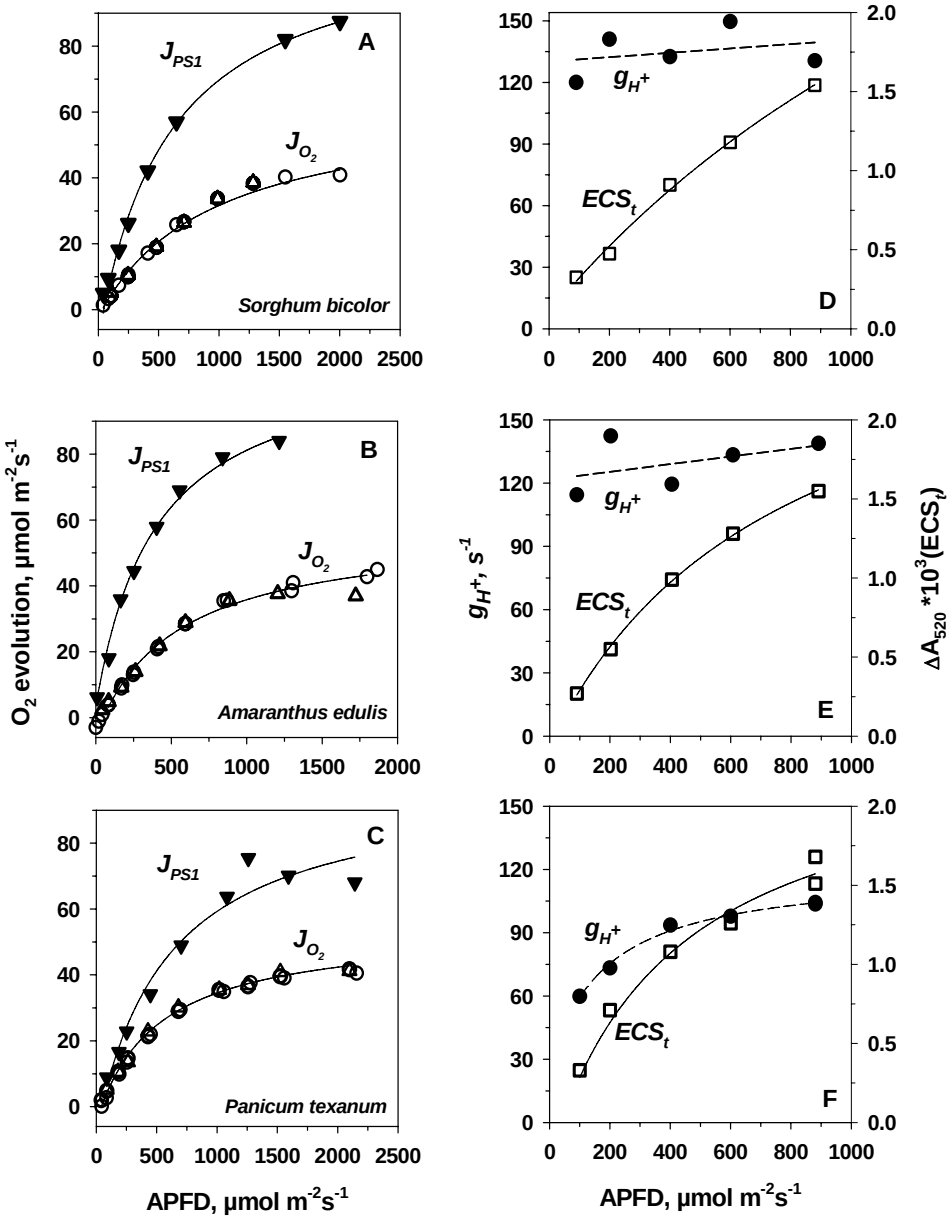

Figure 2.1. Light response of photosynthesis parameters for three C_4 subtypes: *Sorghum bicolor* (NADP-ME), *Amaranthus edulis* (NAD-ME) and *Panicum texanum* (PEP-CK). For panels A, B, C light response of O_2 evolution was measured at 3% CO_2 (30 mbar) CO_2 and low oxygen (0.02%) (white circles). J_{O2} at 3% CO_2 (white triangles) was matched with O_2 evolution rate (white circles) by adjusting Y_{II}. This Y_{II} was used to calculate relative light absorption by PS I and J_{PS1} (black triangles). Left panels (C,D,E) describe light response of thylakoid membrane proton conductivity (g_H^+, black circles) and leaf light to dark transient absorbance change at 520 nm (ECS_t, white circles). Leaf temperature was 29 ^oC.

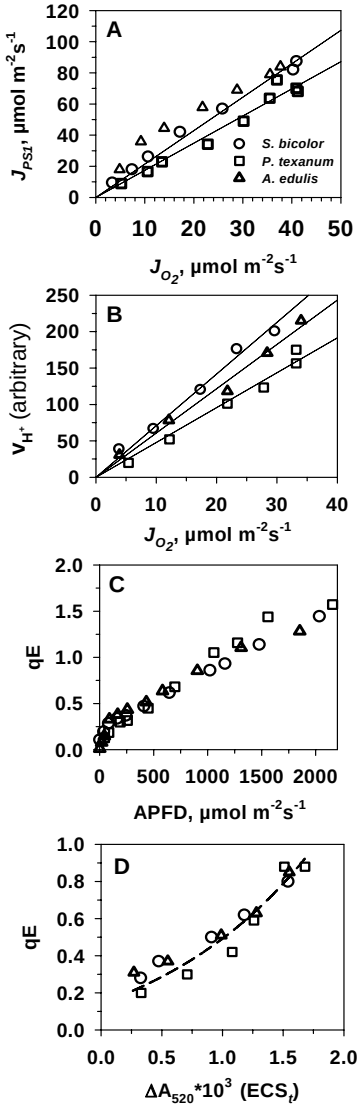

Figure 2.2. Plots of data from figure 2.1 to show the relationships among different parameters. Panel A shows the relationship between activities of the two photosystems per four electrons in response to changes in light intensity. Panel B compares PS II activity with the proton flux (v_{H+}, determined from the initial rate of ECS_t decay during the light-dark transient). Included are data for qE versus APFD (panel C) and a plot showing an increase in qE with increasing *pmf* (ECS_t, panel D).

There was a ~3-fold increase in the magnitude of the pH gradient (ECS_t) with increasing PFD from 100 to 900 μmol $m^{-2}s^{-1}$.

In all three species there was a linear increase in J_{PS1} versus J_{O2} in response to varying light with the two malic enzyme type C_4 species having similar slopes, while *P. texanum* had a lower slope which indicates a lower J_{PS1}/J_{O2} ratio (Fig 2.2A). With increasing J_{O2} there was a linear increase in the velocity of proton flux through the ATP synthase (v_{H+}) with the higher flux in the malic enzyme type C_4 plants and a lower flux in the PEP-CK species (Fig. 2.2B). Also, with increasing light intensity there was a near linear increase in NPQ up to full sunlight, which was essentially the same for all C_4 types (Fig. 2.2C). Also, with increase in ECS_t there was a transition from a slow to a more rapid response in qE in all three subtypes (Fig. 2.2D).

CO_2 response

The response of the three C_4 subtype species to varying levels of CO_2 were made at two light intensities, ~400 and 800 PPFD. In *S. bicolor*, the NADP-ME type C_4 species, rates of J_{O2} and net rates of CO_2 assimilation (A) became saturated at ~325 μbar CO_2 at 440 PFD and 450 μbar CO_2 at 770 PFD (Fig. 2.3A, D). Gross rates of O_2 evolution measured by chlorophyll fluorescence analysis (J_{O2}) were higher than net rates of CO_2 assimilation (A); J_{O2} and A show the same pattern of change with increasing levels of CO_2. With increasing levels of CO_2, g_H^+ increased reaching a maximum level at ~500 μbar CO_2, whereas ECS_t showed a reversed pattern (Fig. 2.3B, E).

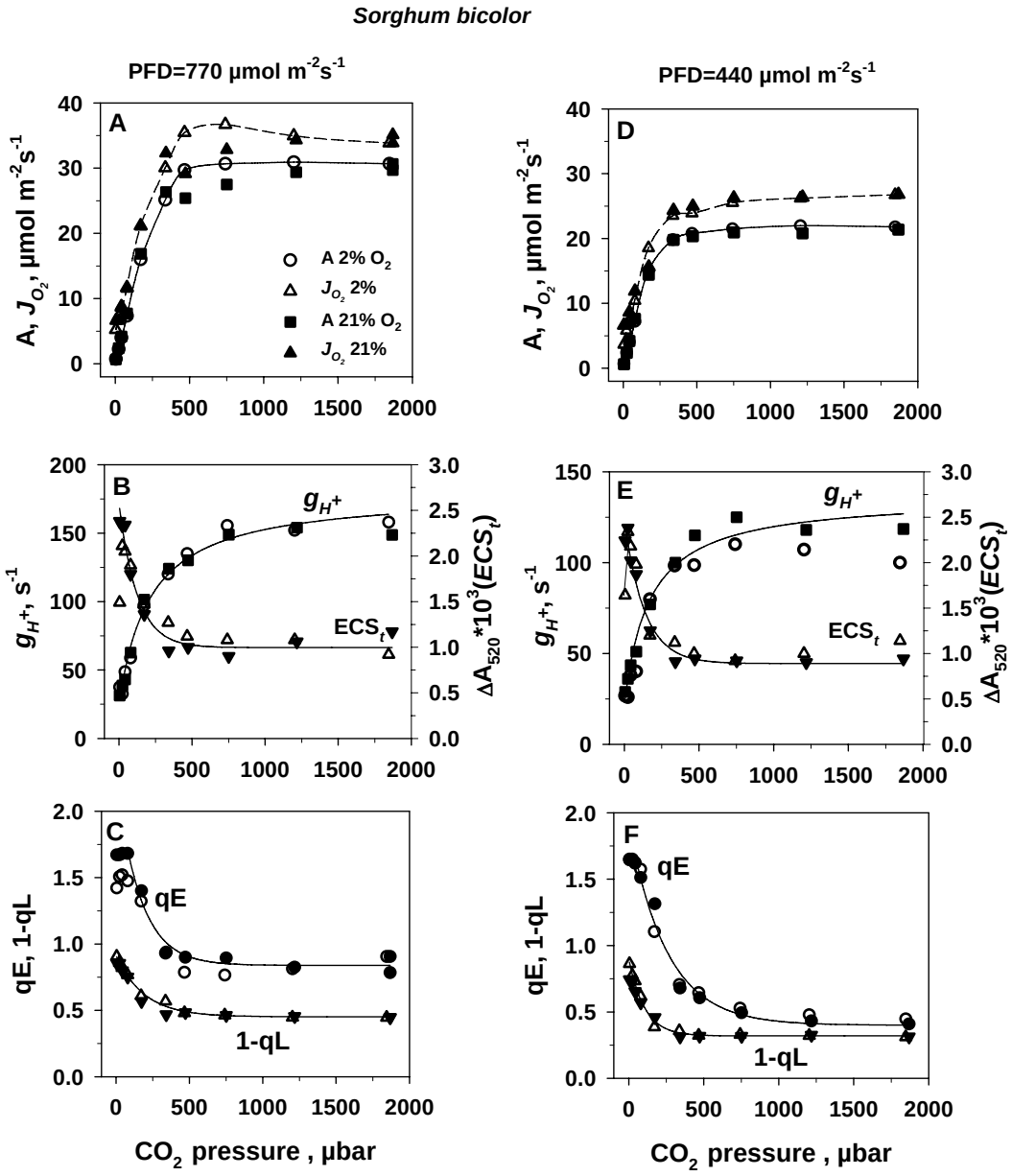

Figure 2.3 continued.

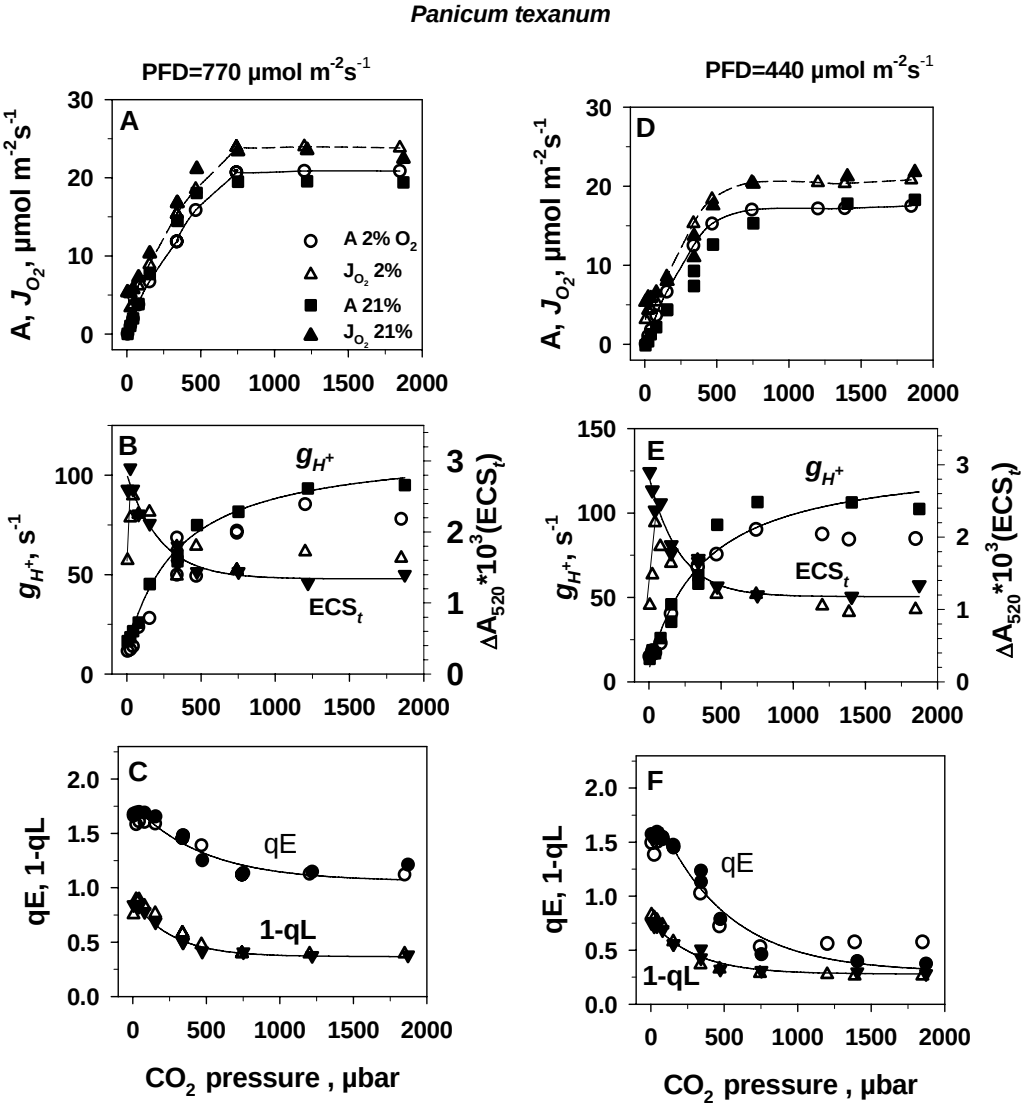

Figure 2.3 continued.

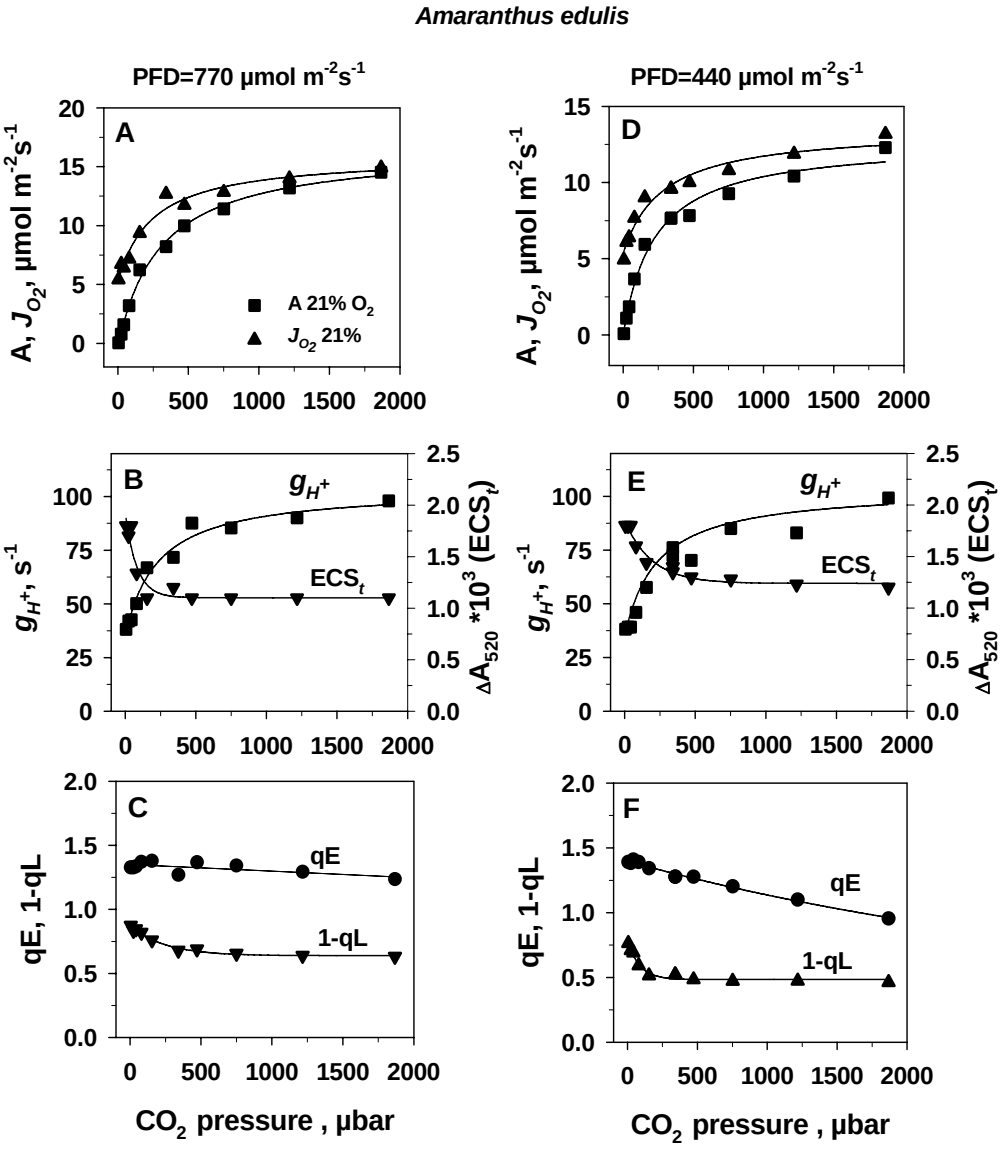

Figure 2.3. The response of multiple photosynthetic parameters to varying CO_2 for the three C_4 subtype species. The data were collected at two light intensities 770 and 440 µmol $m^{-2}s^{-1}$ (absorbed light) and two O_2 concentrations 21% (black symbols) versus 2% (white symbols). Leaf temperature was 24.4°C for all experiments.

The parameters qE and 1-qL which relate to exiton quenching (Fig. 2.3 C, F), increased in parallel with a decrease in CO_2 levels and CO_2 assimilation, with qE reaching a maximum at very low CO_2 values. The plants with lower CO_2 assimilation capacity, *P. texanum* and *A. edulis*, had higher qE values at CO_2 saturation. For the parameters studied, there was little or no difference between 21% versus 2% O_2. With increasing levels of CO_2 there was a linear increase in J_{O2} versus g_H^+ with values coinciding at the two light levels (Fig. 4 left panels). The relationship between PS II electron flux represented by J_{O2} (from fluorescence yield measurements) and proton flux v_H^+ (where $v_H^+ = g_H^+ * ECS_t$) was essentially linear considering the experimental errors (Fig. 4 right panels). There may be some increase in proton flux relative to linear electron flux at low CO_2 concentrations which might be explained by over-estimating the value of ECS_t at low CO_2 concentrations, or some increase in over-cycling of the C_4 pump could occur at low CO_2.

1. Oxygen response

Oxygen had a very similar effect on the electron transport rates at low levels of CO_2 in all three species. Most of extra electron transport with respect to CO_2 assimilation at low CO_2 can be explained by re-assimilation of respiratory CO_2 and Mehler reaction. Photorespiration seems to be effectively suppressed even at very low CO_2 concentrations in C_4 plants.

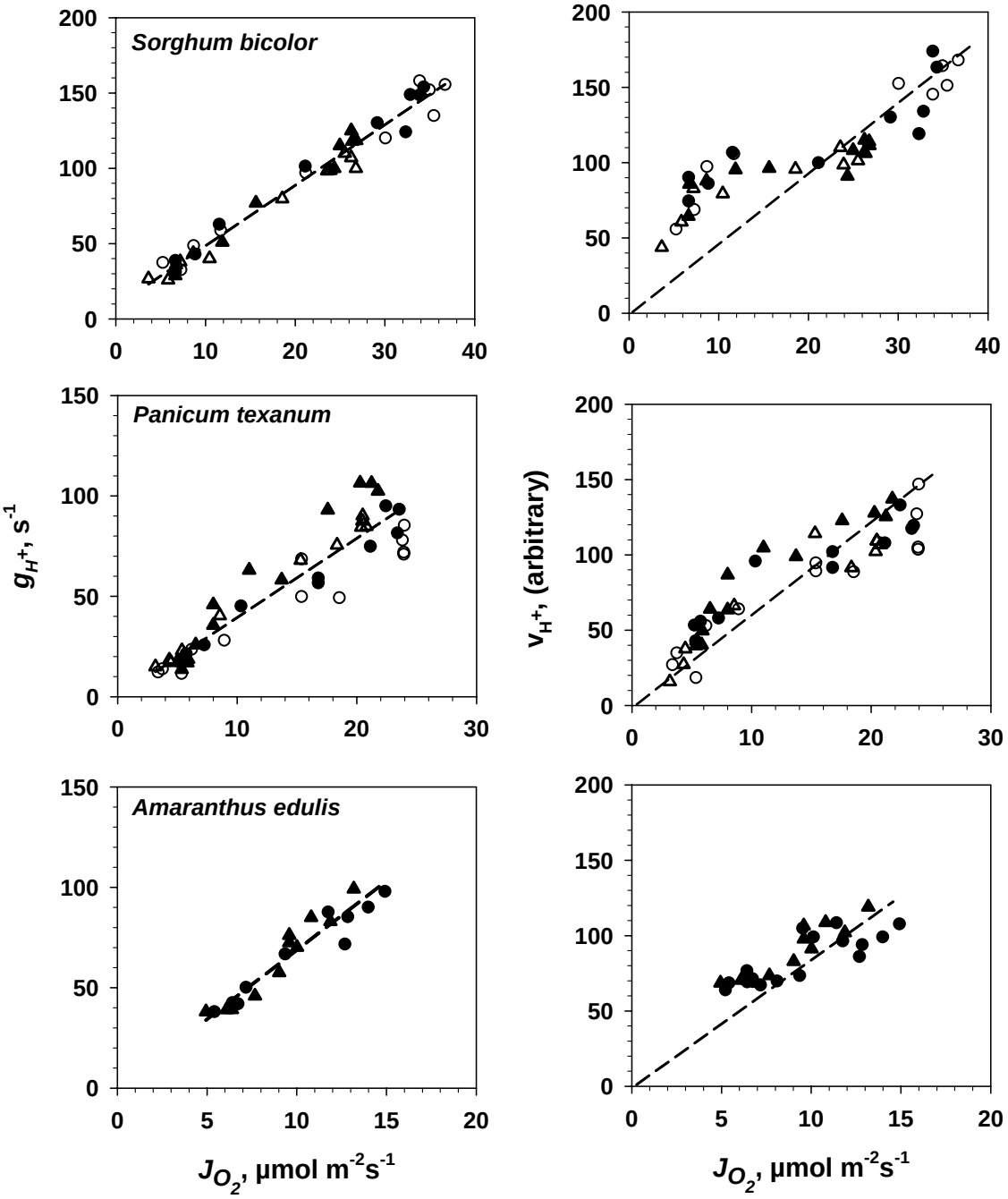

Figure 2.4. Plots of gross rates of O_2 evolution (J_{O2}) against proton conductivity and proton steady state flux (data from experiment Fig. 2.3) for the three C_4 subtype species. The panels combine the data from two O_2 concentrations (2% O_2 – white symbols, 21%

O_2 black symbols) and two light intensities (circles 770 µmol $m^{-2}s^{-1}$, triangles 440 µmol $m^{-2}s^{-1}$).

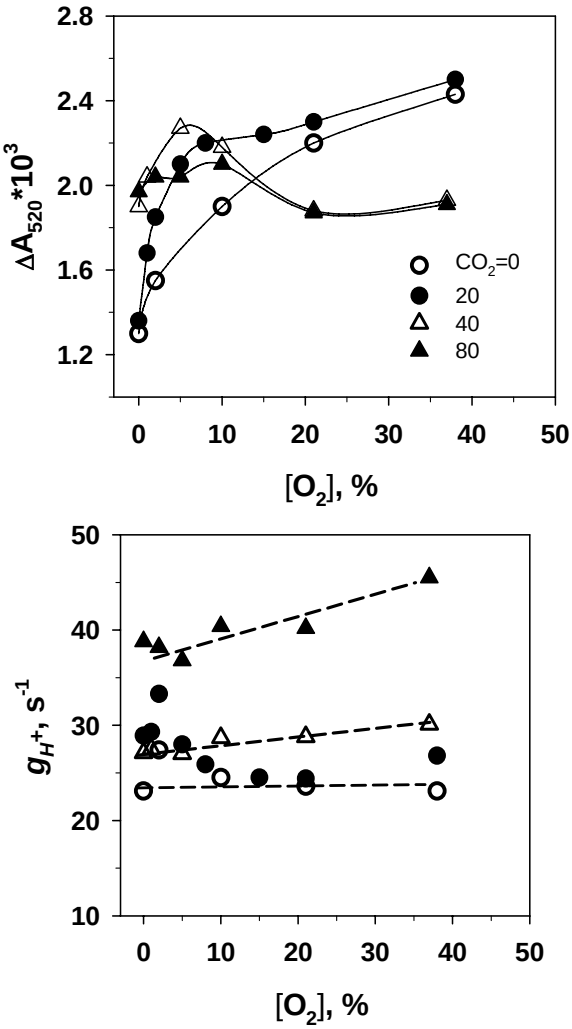

Figure 2.5. The response of leaf ECS_t (ΔA_{520}) and thylakoid proton conductivity (g_{H^+}) to increasing O_2 in CO_2 free gas and at 20, 40 and 80 µbar CO_2 in the C_4 species *Sorghum bicolor*. Leaf temperature was 24.5 ^oC, PFD was 800 µmol $m^{-2}s^{-1}$.

It can be seen from Fig. 2.3 (panel E for *Sorghum bicolor* and *Panicum texanum*) that the ECS_t signal drops significantly when there is very low CO_2 combined with low oxygen concentration. This occurs in all three C_4 subtypes (not shown for *A. edulis*). This decrease in ESC_t was not accompanied by a decrease in qE. Previously, a similar phenomenon was reported for C_3 plants (Avenson *et al*., 2004), and a decrease in the ratio of the thylakoid membrane electrical potential relative to the proton concentration gradient was found (ECS_t measures the electrical component of the total *pmf* only).

With *Sorghum bicolor*, and analysis was made of the effect of increasing O_2 from 0 to 40% in CO_2 free nitrogen and at three low CO_2 levels on ECS_t and g_H^+. There was measurable proton conductivity (about 15% compared to maximum at high CO_2) in CO_2 and oxygen free gas. Addition of either CO_2 or oxygen eliminated the initial decrease of ECS_t. It can be seen (Fig. 2.5) that the initial drop in ECS_t can be eliminated by adding either 40 μbar CO_2, or 21% O_2, in a N_2 background. For *S. bicolor*, either 40 μbar CO_2 or 21% O_2 in N_2 produced about equal rates of J_{O2} (5 μmol O_2 m^{-2} s^{-1}). We conclude that this level of electron flow is required to prevent the above described phenomenon. Thus, this electron flow can be coupled to either RuBP carboxylase or RuBP oxygenase activity. The Mehler reaction potentially could be involved in some capacity; but it is not necessary for ECS_t recovery as adding CO_2 has the same effect as O_2.

DISCUSSION

The mesophyll chloroplasts and bundle sheath chloroplasts in C_4 plants differ by their pigment composition. Also, the special arrangement differs; the mesophyll chloroplasts are more scattered while bundle sheath chloroplasts are concentrated close to

the vascular tissue bundles. The heterogeneity of the system raises the question if the optical measurements adequately reflect the rates of the electron transport processes. It has been shown that PS II electron flow calculations using the C_4 leaf fluorescence yield data and Genty *et al*. (1989) formulas adequately predict CO_2 assimilation rates (Edwards and Baker, 1993, Oberhuber *et al*., 1993). This adds confidence that PS I rates can be predicted as well using P700 absorption.

It is generally agreed that the extra ATP needed in C_4 plants for CO_2 concentrating mechanism is provided by cyclic photophosphorylation related to PS I activity (Kanai and Edwards, 1999). The details and the efficiency of cyclic electron transport around PS I are not fully established. The measurement of the maximum quantum yield of C_4 photosynthesis helps to differentiate between different possible mechanisms. It has been suggested that NDH pathway as opposed to Fd/PQ pathway is involved. Takabayashi *et al.* (2005) found that NDH was highly expressed in mesophyll cells in the NAD-ME species and in bundle sheath cells in NADP-ME species that coincides with the locations where the extra ATP is needed. Considering the electron transport model where Q-cycle is fully engaged NDH cyclic pathway would carry $2H^+$ per e^- (Heber *et al*., 1995). With linear electron flow $3H^+/e^-$ we need to run PS I twice as fast to provide 5 ATP per one O_2 evolved (4 e^-) assuming 4 H^+ per ATP. If the ATP production requires 4.66 H^+ per ATP (Kramer *et al.* 2004) the cyclic process must run 2.8 times faster than linear electron flow. There are also extra costs of ATP related to glycosidic bond formation in starch and sucrose synthesis and C_4 cycle "over-cycling" due to the leakage of bundle sheath cell walls. Furbank *et al*. (1990) estimate the ATP expense for polysaccharide synthesis to be 0.17 moles ATP per mol of CO_2 fixed and the C_4 cycle needs to run about 25% faster

than the net rate of photosynthesis to compensate CO_2 losses through leakage. The measured quantum efficiencies for O_2 evolution 0.072-0.075 (~13.5 quanta per O_2 evolved) and PS I rate slightly more than 2 times linear electron flow (Fig. 2.1) would require H^+/e^- ratio higher than 2 for cyclic process or ATP synthase H^+/ATP 4 instead of 4.66 to satisfy above mechanism.

The linear relationship between electron fluxes of PS II and PS I and PS II and proton flux through ATP synthase (Fig. 2.2) are expected as there is no possibility for CO_2 limitation at 3% CO_2 and both the Calvin cycle and C_4 pump mechanism are only limited by light availability. The range of light intensities that was used for ECS measurements was limited to 100- 800 μmol $m^{-2}s^{-1}$ due to the limitations of equipment used. The relationship between ECS_t and NPQ is similar to C_3 response but Fig. 2.2 covers only raising part of the typical S-shaped curve recorded for C_3 species (Takizawa *et al*., 2007) due to limited range of illumination used. The fit of the data points from different species on almost the same line should be considered coincidental as ECS signal is not calibrated and it depends on the content of carotenoids in thylakoid membranes and on optical properties of the leaves.

CO_2 response of all three species show slightly higher PS II activity in compared to net CO_2 fixation (Fig. 2.3). It can be explained by dark respiration and some relatively small Mehler reaction. The data agree with relatively small role of Mehler type reaction in ATP production in C_4 species (Laisk and Edwards, 1998). ATP synthase proton conductivity followed closely linear electron transport rate at both light intensities used (Fig. 2.3) and there was a linear correlation between linear electron transport rate (represented by J_{O2}) and g_H^+. This relationship between J_{O2} and g_H^+ closely resembles

the same in C_3 species (see Chapter 1 and 2). The explanation we provided for C_3 species was that ATP synthase under these conditions is limited from chloroplast stroma side and mostly by the availability of the substrate P_i while ADP is essentially saturating and ΔG for ATP synthesis does not change substantially in the range of conditions used. The decrease of ATP synthase conductance at low CO_2 helps to maintain high proton pool size inside thylakoid lumen necessary for excess energy dissipation via NPQ mechanism. Previously it has been argued that the proton pumping via PS I cyclic pathway is necessary for NPQ development at CO_2 limitation (Heber and Walker, 1992). Being part of the proton pumping mechanism the cyclic photophosphorylation has importance in pH gradient generation. It can be argued that as a regulator ATP synthase conductance variation has more importance. It is supported by the relatively unchanged NADPH/NADP ratio on CO_2 response curve (need to check the reference).

The apparent decrease in ECS_t at very low CO_2 and O_2 can be overcome if the electron flow is increased either with adding CO_2 or O_2 (Fig. 2.5). About 5 μmol $m^{-2}s^{-1}$ O_2 evolution rate was sufficient to eliminate the effect. The ECS_t decrease could be explained by some proton leakage that can be overcome if more pumping activity is increased. At very high PQ reduction (1-qL in Fig. 2.3) about 90% Q-cycle may be not fully operational as well (Heber and Walker, 1992). The proton flux with 21% O_2 added at 0 CO_2 background creates O_2 oxygenation activity about equal to 40 μbar CO_2 at 0 O_2 background. Both eliminate the observed ECS_t decrease. Therefore Meler type proton pumping is not necessary in this case.

The ECS_t drop at very low CO_2 and O_2 occurs also in C_3 plants. It has been shown (Avenson *et al*., 2005) that there is no drop in actual proton pool size but *pmf* distribution

between electrical and H^+ concentration component changes toward concentration component that creates ECS_t decrease as ECS records only electrical component. This is also in agreement with the observation that there is no corresponding change in NPQ that would decrease with proton concentration drop. The mechanism of electrical component decrease is not clear.

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CHAPTER 3

The use of photosynthetic mutants to elucidate the mechanism of regulation of photosynthesis by ATP synthase under variable climatic conditions

ABSTRACT

The chloroplast thylakoid membrane CF_0-CF_1 ATP synthase participates in co-regulation of the electron transport reactions and dark reactions in plant photosynthesis. It has been shown that the proton conductivity of the enzyme varies depending on environmental conditions. Proton conductivity of the ATP synthase modulates non-photochemical exciton quenching by directly affecting the steady state proton pool size (pH) inside the thylakoid lumen. The mechanism of dynamic change of ATP synthase conductance has not yet been conclusively established.

This work included measurements of rates of CO_2 exchange, photosystem II activity and gross rates of O_2 evolution (by chlorophyll fluorescence analysis) and non-photochemical dissipation of energy as heat (qE). Thylakoid membrane proton conductivity was measured using the carotenoid electrochromic shift signal. Analysis of the post-illumination CO_2 uptake signal allowed us to determine the status of the Calvin cycle RuBP pool sizes. We used two photosynthetic mutants which were compared to wild type plants: the Arabidopsis *pgr5* protein deficient mutant which affects the capacity for cyclic electron transport, and the tobacco low Rubisco antisense mutant. The above mentioned photosynthetic parameters were measured under different light, CO_2 and O_2

conditions. The Arabidopsis *pgr5* mutant, compared to wild type, had similar CO_2 uptake rates and RuBP pool sizes; but, in the mutant the steady state proton concentration in the thylakoids was reduced along with lower NPQ levels. The lower pool of protons in the thylakoids in the mutant was fully compensated by an increase in ATP synthase proton conductivity; so, that the photosynthetic rates did not differ much between mutant and wild type *Arabidopsis*. The lower maximum quantum efficiency for CO_2 assimilation for the *pgr5* mutant (0.063) compared to wild type (0.072) was explained by photo-inhibition in the mutant. The data are discussed relative to possible models for regulation of ATP synthase.

INTRODUCTION

While *pmf* is the driving force for ATP synthesis, its ΔpH component serves at the same time as an essential part of the non-photochemical energy dissipating mechanism. Intrathylakoid proton concentration holds the central role in this regulatory process by activating conversion of violaxanthin to zeaxanthin in the xanthophyll cycle, and by modifying protonation of certain PS II LHC proteins involved in the process (Demmig-Adams and Adams, 1996, Horton *et al*., 1996). Under stressful conditions where CO_2 assimilation is restricted by low intracellular levels of CO_2, by cold temperatures, or other factors under excess light, increased acidification of the thylakoid lumen (resulting in a high pH gradient, *pmf*) is needed in order to induce high non-photochemical dissipation of the light energy. To achieve this, the proton conductance (g_H^+) of the ATP-synthase must be reduced. Kanazawa and Kramer (2002) described the process of modulating g_H^+ under several CO_2 and light levels and proposed a model that includes modulation of

ATP-synthase activity on the stromal side by change in metabolite pools with inorganic phosphate (P_i) being the most likely limiting factor. As P_i is a regulator for several other processes (starch synthesis, triose phosphate export from the chloroplasts, Paul and Foyer, 2001), this finding has important consequences for general understanding of the regulation of photosynthesis as a whole. Besides regulation by P_i there are other possibilities for regulation of ATP synthase activity. It may also be controlled by free energy change (ΔG) of ATP synthesis in the chloroplast stroma or by direct allosteric regulation of ATP synthase activity (Bunney *et al*., 2001).

The alternative explanations offered for NPQ regulation under low CO_2 and stressful situations and excess light is increasing activities of cyclic or pseudocyclic electron transport, which could potentially increase *pmf* and acidification of the lumen (Heber and Walker, 1992; Ott *et al*., 1999; Miyake *et al*., 2005).

There is potential to increase understanding of the mechanisms of excess energy dissipation during photosynthesis by testing how mutations in photochemistry and carbon assimilation affect ATP synthase and energy dissipation. We used two mutants in this study, one affecting photochemistry, *Arabidopsis pgr5* mutant with altered cyclic electron transport (Munekage *et al*., 2002); and, the other affecting capacity for carbon fixation (low Rubisco mutant, Hudson *et al*., 1992). Due to an imbalance between light and dark reactions capacity, there is a possibility of activating PS I cyclic or Mehler type electron transport pathways as a means of inducing energy dissipation. The *Pgr5* mutant interferes with the ferredoxin-plastoquinone oxidoreductase catalyzed cyclic pathway. A feature of this mutant is up-regulated ATP synthase proton conductivity which results in low *pmf* and reduced non-photochemical quenching mediated by violaxanthin/zeaxanthin cycle

(Munekage *et al*., 2002; Munekage *et al*., 2004). The association of an elevated ATP synthase proton conductivity with decreased NPQ clearly provides support for function of ATP synthase in regulation of NPQ. The mechanism of how regulation of ATP synthase occurs is still uncertain.

METHODS

Tobacco low Rubisco activity (~15% of wt) mutant (Hudson *et al*., 1992) and Arabidopsis *pgr5* mutant with modified cyclic electron transport (low ΔpH and increased NPQ, Munekage *et al*., 2002) were used. *Plants* of *Nicotiana tabacum* were grown in Econair (Winnipeg, Canada) growth chambers in fertilized potting soil in 8 L pots (one plant per pot), with a 14/10 h day/night cycle at 28/22 °C, 50% relative humidity, 380 μbar CO_2, and an incident photosynthetic quantum flux density (PFD) of 800 μmol m^{-2} s^{-1}. *Arabidopsis* plants were grown with a 14/10 h day/night cycle at 24/18 °C, 50% relative humidity, 380 μbar CO_2, and PFD of 350 μmol m^{-2} s^{-1} in 1 L pots (one plant per pot).

The following leaf photosynthetic parameters were measured: A, rate of CO_2 fixation; AC, assimilatory charge-RuBP pool (post-illumination uptake of CO_2); qE, non-photochemical quenching; 1-qL, reductive state of the plastoquinone pool; g_H^+, ATP synthase conductance; ECS_t, electrochromic shift a *pmf*.

Leaf photosynthetic parameters were recorded with the FastEst gas system (described in detail in Laisk and Oja, 1998). The system is equipped with a Li-Cor 6251 (Lincoln, Nebraska) CO_2 analyzer, Applied Electrochemistry Inc. (Sunnyvale, California) S-3A O_2 ceramic heated zirconium oxide analyzer and Walz PAM 101

(Effeltrich, Germany) flurometer that can be used to measure fluorescence yield associated with PSII or A_{820} measurements associated with PSI. The true rates of O_2 evolution from PSII (J_{O_2}) were calculated as:

$$J_{O_2} = APFD \cdot Y_{II} \cdot (F_m' - F_s)/F_m')/4$$

where $(F_m' - F_s)/F_m'$ is the yield of PSII, F_s is fluorescence yield of steady state photosynthesis, F_m' is maximal fluorescence yield by exposure to a 1 s pulse of 15,000 μmol $m^{-2}s^{-1}$ light and *APFD* is the absorbed photosynthetic quantum flux density at steady state (Genty *et al.*, 1989). Y_{II} is the relative optical cross-section of PSII (the fraction of light absorbed by PSII) as determined in (Laisk *et al.*, 2002) from light response curves for leaf O_2 evolution under a low O_2 background. Absorbed photosynthetic quantum flux density (*APFD*) was calculated with absorption coefficient determined by using an integrating sphere (Labsphere, North Sutton, NH). qE and qL were calculated as in Kramer *et al.* (2004). qE for all experiments represents the energy dependent component of NPQ where for NPQ calculations F_m was measured after 15 min of darkness.

The rate of PS I per four electrons transported was calculated as

$J_{PSI} = APFD \cdot Y_I \cdot (P/P_m)/4$.

P/P_m is quantum yield of PS I according to the saturating pulse method (Klughammer and Schreiber, 1994). P_m represents leaf 830 nm maximum absorbance change (from dark level to the level obtained under Far red light combined with a saturating flash of white light) and P represents maximum absorbance change during illumination with a saturating flash for 50 ms at 10,000 μmol quanta m^{-2} s^{-1}. The absorbance change during the saturating flash (P) represents PS I centers that are efficient in electron transport. The

centers have reduced donor side and oxidized acceptor side (Klughammer and Schreiber, 1994).

Y_I was calculated as 1-(Y_{II}-0.05) assuming 5% of light is being absorbed by non-photosynthesizing components of the leaf.

The assimilatory charge (AC) during photosynthesis in the light was determined at a given time as described in (Laisk *et al*., 2002) by measuring the magnitude of the post-illumination uptake of CO_2.

Steady state light-driven *pmf* and g_H^+ were estimated by following the absorbance changes attributable to *ECS*, at 520 nm, with rapid light to dark transitions. The diffused-optics spectrophotometer, which was constructed in-house (Kramer and Sacksteder, 1998; Sacksteder *et al.*, 2001) has a 1 cm^2 window that was clamped on the leaf. A small air space (3 mm thickness) was left on the lower side of the leaf and gas with known humidity, O_2 and CO_2 content (mixed with the FastEst system) was constantly passed over the lower leaf surface at a rate of 10 cm^3 s^{-1}. The window was illuminated by actinic diffuse red LED light. The modulated measurement light at 520 nm was provided by another set of LEDs. The experiments for measurements of *ECS* were performed at room temperature 24 ^oC.

RESULTS AND DISCUSSION

Regulation of ATP synthase conductance in pgr5 mutant of Arabidopsis

The *Pgr5* mutant of *Arabidopsis* was initially considered to have impaired cyclic electron transport (CET) due to the PGR5 protein being direct involved in the mechanism (Munekage *et al*., 2002; Munekage *et al*., 2004). This view was challenged by Nadha *et al.* (2007) who showed PGR5 protein participates not directly in CET; but, rather in a

regulatory capacity. Low CET was suggested to be the result of high reduction state of electron transport carriers. They suggested that the low *pmf* in *pgr5* mutant was a result of reduced CET.

Figure 3.1. Arabidopsis *pgr5* mutant and wild type (left) used in the experiments.

It has been shown that the thylakoid ATP synthase conductance is high in *pgr5* mutant (Avenson *et al.*, 2005), for unknown reasons. We were interested in this phenomenon, since high thylakoid membrane proton conductivity could be the sole reason for reduced *pmf* and qE.

Under the growth conditions used, the *pgr5* mutants grew only slightly slower than wild type plants. The chlorophyll content of *pgr5* was significantly reduced: 135 μmol m^{-2} versus 248 μmol m^{-2} in the wild type. The aim of this set of experiments was to analyze possible reasons for the high proton conductivity in the mutants. Either partial uncoupling of ATP synthase or proton leakage across thylakoid membranes should cause a low quantum efficiency under limiting light. Hypothetical models for *pmf* regulation

involving proton "slippage" (partial uncoupling) have been offered (Evron *et al*., 2000). For testing this in the *pgr5* mutant, light response curves were measured (Fig. 3.2).

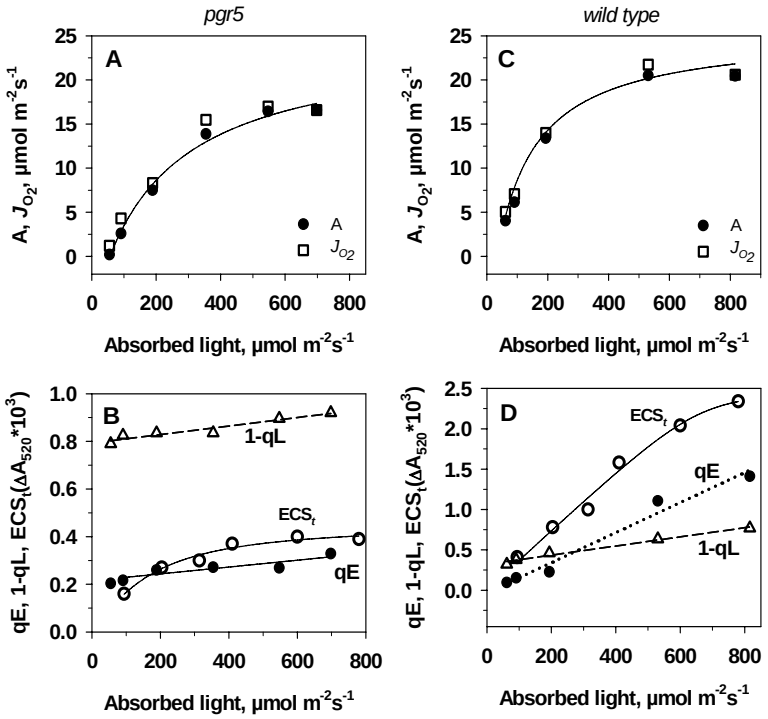

Figure 3.2. Light response of *Arabidopsis pgr5* mutant (A, B) compared to wild type (C, D) photosynthetic parameters at 1000 µbar CO_2, 2% O_2, and 24 ^oC. Panels A and B describe light response of CO_2 assimilation rate (A, circles) and oxygen evolution related to PS II activity (J_{O2}, squares) calculated from simultaneous recording of the fluorescence yield. Panels C and D describe corresponding changes in ECS_t ~*pmf* (opened circles), qE (closed circles) and 1-qL (triangles).

Panels A and C (Fig. 3.2) describe light response of some parameters of the *pgr5* mutant versus wild type. The maximum quantum efficiency for CO_2 assimilation (initial slope of the curve) for *pgr5* mutant was 0.063 CO_2/quanta in compared to 0.072

CO_2/quanta in the wild type. This 12% decrease in quantum efficiency in the mutant can be accounted for by photo-inhibition, rather than by a decrease in efficiency of use of *pmf*.

The additional panels B and D in Figure 3.2 show with increasing light, the mutant in comparison to wild type does not build up a substantial *pmf* (measured as ECS_t) and as a result it does not develop qE as light intensity increases. In addition the mutant has a highly reduced plastoquinone pool (based on 1 – qL), even at low light intensities.

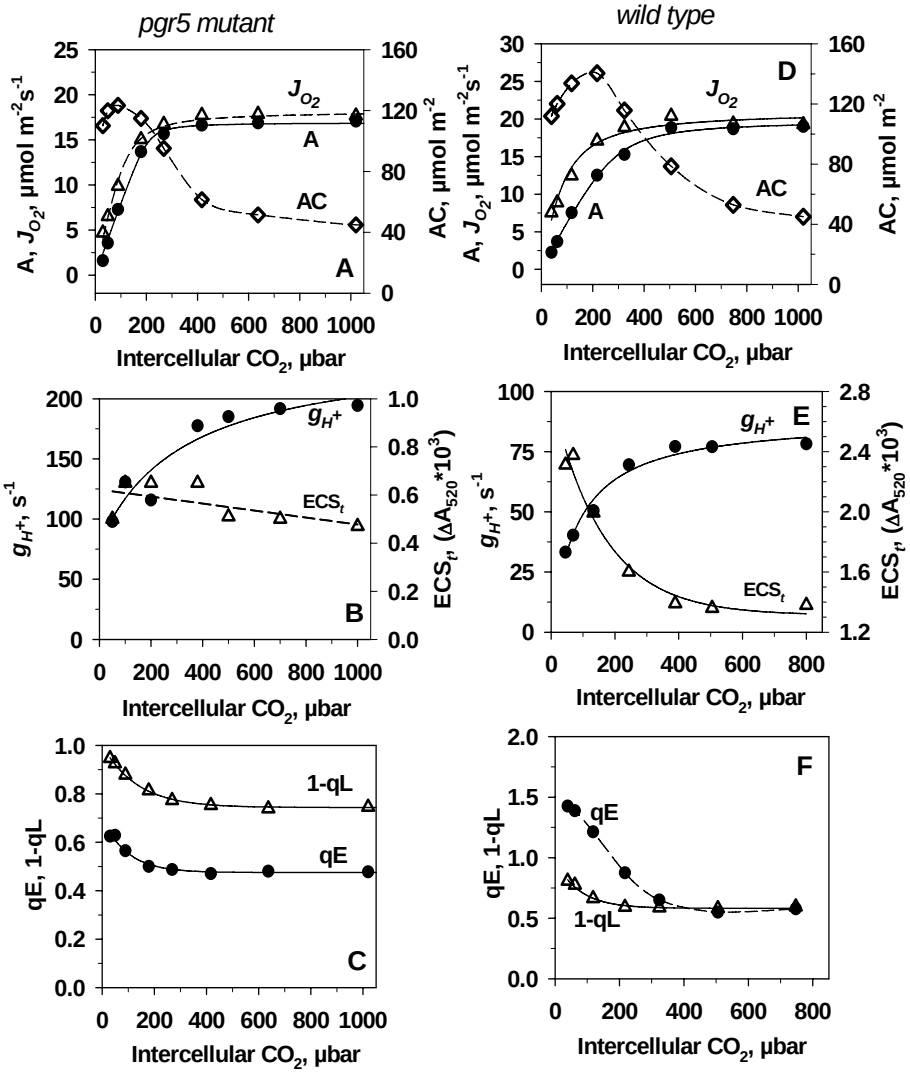

Figure 3.3. CO_2 response of *Arabidopsis pgr5* mutant (A, B, C) compared to wild type (D, E, F). Photosynthetic parameters were measured at 360 μmol $m^{-2}s^{-1}$ APFD, 2% O_2, and 24 ^oC. Panels A and B describe light response of CO_2 assimilation rate (A, circles) and oxygen evolution related to PS II activity (J_{O2}, squares) calculated from simultaneous recording of the fluorescence yield. Panels C and D describe corresponding changes in ECS_t ~*pmf* (opened circles), qE (closed circles) and 1-qL (triangles).

One of the hypotheses offered (Avenson *et al*., 2005) to explain the high proton conductivity in *pgr5* has been that it may have an unusually high level of inorganic phosphate (P_i) in the chloroplast stroma which allows high ATP synthase turnover rates. High P_i levels may also cause high levels of organic-P in the Calvin cycle, as it could inhibit utilization of triose-P for starch synthesis (P_i is a negative effector for AGP pyrophosphorylase), and efflux of triose-P from chloroplasts through the phosphate translocator. We used a gas-exchange parameter "assimilatory charge" (determined from the post-illumination CO_2 uptake) as a measure of RuBP pool size (Viil *et al*., 1986; Laisk *et al*., 2002). Figure 3.3, panels A and D, show that the rates of CO_2 uptake, oxygen evolution and assimilatory charge over the range of CO_2 concentrations used are similar between *pgr5* and wild type. The small differences may be explained by variability in plant material. Panels B and E (Fig. 3.3) describe corresponding changes in ECS_t ~*pmf* and proton conductivity g_H^+ as a response to change in levels of CO_2. Proton conductivity in the mutant under a given condition was about 2.5 times higher than in the wild type. It is noteworthy that there was still regulation of proton conductivity in the mutant as it increased with increasing CO_2 assimilation. There was only a small change in *pmf* in the

mutant in response to CO_2 change, which was reduced about 3-fold compared to wild type.

With decreasing CO_2 to low levels, the non-photochemical quenching in the mutant increased only very slightly compared to wild type which developed a high qE. The PQ reduction state (1-qL) was high in the mutant, and it increased even further at low CO_2 reaching 95%, while in wild type 1-qL increased only moderately at low CO_2 (Fig. 3.3, panels C and F).

A possible explanation for the high ATP synthase conductance in the mutant is a higher pool of P_i which is a substrate for the ATP synthase reaction. High levels of P_i could occur if there is a reduction in the levels of organic-P, e.g. RuBP. The assimilatory charge was similar in mutant and wild type, suggesting the higher ATP synthase conductance in the mutant may not be due to high levels of P_i. However, detailed chloroplast metabolite profiling is needed to test the high P_i hypothesis as an explanation for high ATP synthase activity in the mutant. Another possible explanation of the high thylakoid proton conductance in the mutant is that it has higher levels of ATP synthase than the wild type; however, this has been tested and shown not to be the case (Avenson *et al*., 2005). There is also a possibility that ATP synthase operates in a higher activity form in the mutant compared to wild type. Different activation forms of ATP synthase have been demonstrated in experiments with isolated thylakoids (Schwartz and Strotmann, 1998).

Regulation of ATP synthase conductance in low Rubisco activity mutant of Nicotiana tabacum

A tobacco mutant having low Rubisco activity (Rubisco small subunit antisense, Hudson *et al*., 1992) with about six times reduced Rubisco content were used. A direct Rubisco assay from freeze-clamped leaves under growth conditions gave Rubisco activity 14.8±1.7μmol/m^2 s (SD, n=3) and a Rubisco activation state of 75%±3.2% compared to wild type tobacco grown in parallel under the same conditions with activity of 85.0±4.8 μmol/m^2 s (SD, n=3) and activation state of 86%±2.7% . The mutant plants grew slowly at ambient CO_2 conditions at 28^oC; however, plants appeared healthy except that chlorophyll content was reduced (158 μmol/m^2) in comparison to wild type (287 μmol/m^2). The specific aim of the studies with this mutant was to evaluate possible mechanisms of regulation of ATP synthase regulation when there are restrictions on the side of utilization of assimilatory power. One might propose there would be high ratios of ATP/ADP and NADPH/NADP in the mutant as the capacity of Calvin cycle is reduced relative to electron transport/ATP synthesis capacity. A high reduction state of NADP and the electron transport chain is favorable for occurrence of the Mehler type (pseudo-cyclic) and cyclic electron transport.

Electron transport rates were estimated independently, using fluorescence yield for analysis of PSII and steady state A_{830} measurements for analysis of PSI, and compared to CO_2 assimilation rates. In addition, measurements were made of qE and 1-qL which reflect the state of energy dissipation and state of reduction of the electron transport chain on the acceptor side of PSII, and electrochromic shift parameters (ECSt, and g_H^+) which reflect *pmf* and ATP synthase proton conductivity. Figure 3.4 shows responses to changes in levels of CO_2 in the Rubisco anti-sense tobacco mutant. Linear electron transport (shown as J_{O2}) is higher than v_c+v_o especially at 21% O_2 (Fig. 3.4 A,

D). Part of this difference can be explained by dark respiration; but, it is also possible that some Mehler type electron transport occurs. Electrochromic shift related parameters (ECS_t and g_H^+) and exciton quenching parameters (qE, 1-qL) were measured at two light intensities in order to provide information on interaction between light and CO_2. The lower light intensity (240 μmol $m^{-2}s^{-1}$) was partially limiting at the higher CO_2 concentrations and it is reflected in an increase in g_H^+ while ECS_t decreases which is accompanied by a decrease in qE. Panels B and E show clearly that g_H^+ is changing in parallel with the CO_2 assimilation rate (panels A, D) indicating a tight regulation of ATP synthase activity

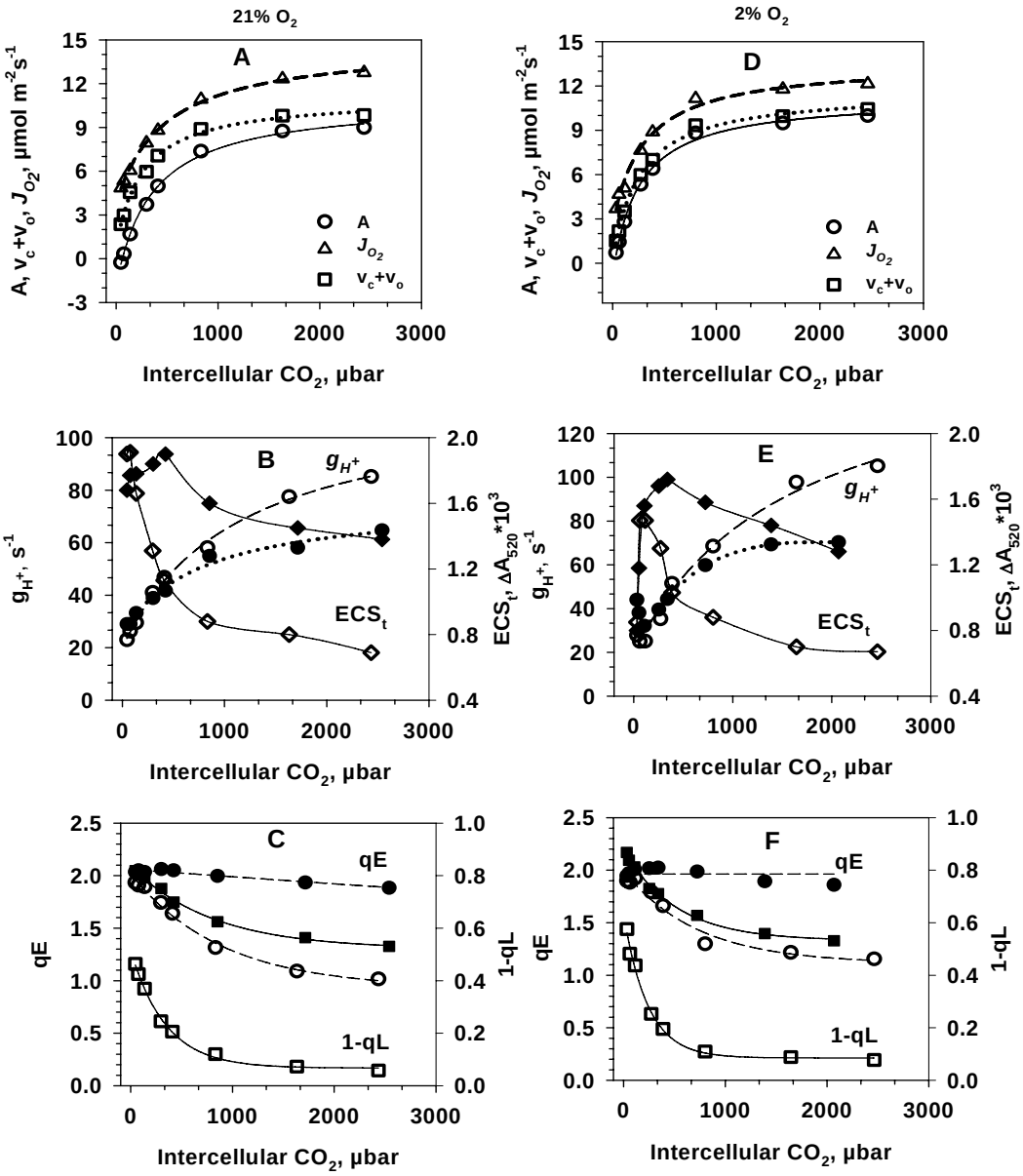

Figure 3.4. Response of photosynthetic parameter to changes in CO_2 in tobacco Rubisco antisense mutant at 240 (opened symbols) and 840 (closed symbols) µmol quanta $m^{-2}s^{-1}$

light, 21% O_2, (A, B, C) and 2% O_2 (D, E, F) at 24 ^oC. Panels A and D represent leaf CO_2 assimilation rate (A, circles), O_2 evolution rate (J_{O2}, triangles) and calculated sum of RuBP carboxylation and oxygenation rates (v_c+v_o) at *APFD* 240 μmol $m^{-2}s^{-1}$ and two O_2 concentrations (2 and 21%). Panels B and E represent thylakoid membrane proton conductivity (g_H^+, circles) and *pmf* measured as ECS_t (fast light-dark ΔA_{520nm}), closed symbols (*APFD*=840 μmol $m^{-2}s^{-1}$); opened symbols (*APFD*=240 μmol $m^{-2}s^{-1}$). Panels C presents corresponding changes in PS II energy dependent exciton non-photochemical quenching (qE) and panel F photochemical quenching constant 1-qL which describes relative reduction state of the plastoquinone pool in thylakoid membrane.

In order to evaluate the possibility that cyclic electron flow may be involved in energy dissipation, the data from Fig. 3.4 were plotted as linear electron flux (represented as J_{O2}) versus proton flux ($v_H^+ = ECS_t * g_H^+$) (Fig. 3.5). The proton flux units are arbitrary. Under 21% O_2 and high light there is an increase in proton flux relative to PSII activity (Fig. 3.5B), which suggests some activation of proton pumping via cyclic electron flow. The same parameters measured at light limited conditions (panel C and D) reveal a relatively good linear fit that suggests deviation from 2% and 21% under high light may in fact reflect some cyclic electron transport activity. The replicate experiment gave the same result.

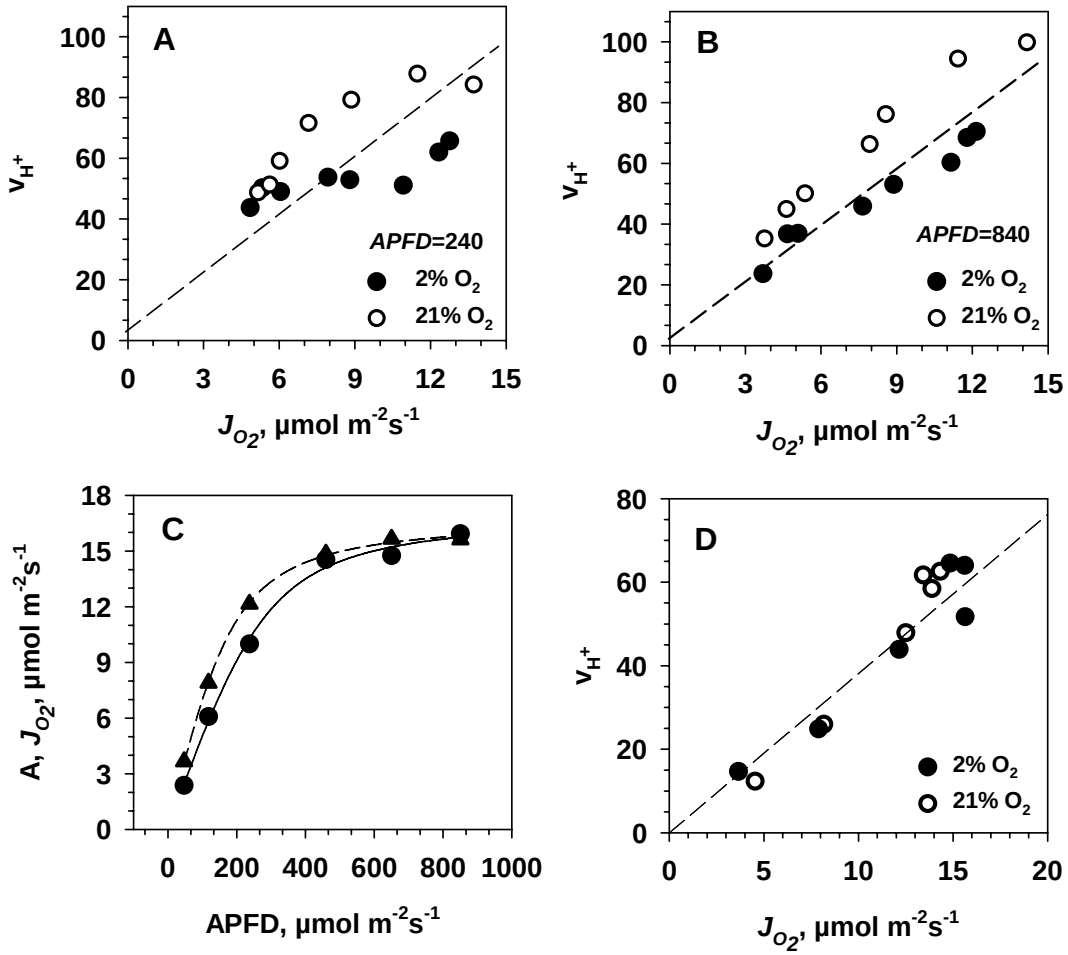

Figure 3.5. Relationship between linear electron transport rates (expressed as gross rates of oxygen evolution rate J_{O2}) relative to proton efflux rate v_H^+ (arbitrary values) at two light intensities 240 (panel A) and 840 µmol $m^{-2}s^{-1}$ (panel B) and two oxygen concentrations 2 and 21% (data from figure 3, $v_H^+ = ECS_t * g_H^+$). Panel C represents light response curve of leaf CO_2 assimilation rates at 2800 µbar CO_2 and 2% O_2 (circles) with corresponding O_2 evolution rates (from fluorescence yield measurements). Panel D describes relationship between electron flux (J_{O2} = 4 e^-) and proton flux v_H^+ (arbitrary units).

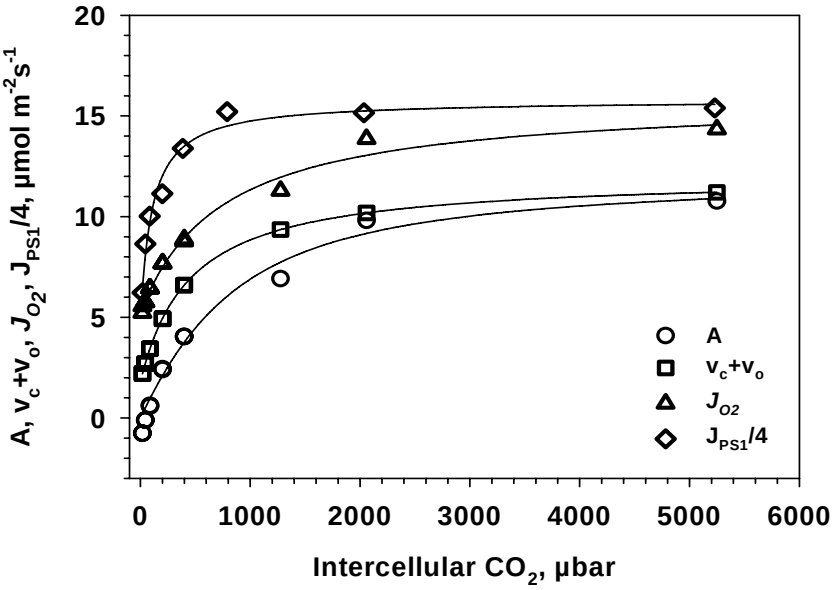

Figure 3.6. CO_2 response of tobacco Rubisco anti-sense mutant photosynthetic parameters at 380 µmol $m^{-2}s^{-1}$ APFD, 21% O_2 and 23°C. The figure compares CO_2 assimilation rate (A, circles), calculated sum of RuBP carboxylation and oxygenation rates $v_c + v_o$ (squares), O_2 evolution rate, J_{O2} (triangles) and the rate of PS I turnover per 4 electrons (J_{PS1}, diamonds).

In order to further test possible increases in cyclic relative to linear electron transport activity in the low Rubisco activity mutant, PS I electron transport rates were calculated by measuring steady state changes in the A_{830} signal as described in the Methods section. Elevated PS I electron transport activity relative to linear electron

transport appears to occur (Fig. 3.6), especially on the shoulder region of the A/C_i curve. The method for measuring PS I flux has known difficulties to reliably reflect the true absolute quantum efficiencies of PSI (reviewed in Baker *et al*., 2007); so, the reliability of the results may be questioned. However, from the results in Fig. 3.6, together with the experiment showing extra proton flux relative to PSII activity at 21% O_2 compared to 2% (Fig. 3.5), we consider this as evidence for occurrence of PS I cyclic photophosphorylation in the Rubisco antisense mutant under 21% O_2.

The PS I acceptor side reduction was small (3 – 5% of total centers) over most part of the CO_2 response curve; but, it increased sharply at low CO_2 and O_2 concentrations when light was saturating and J_{O2} fell below 5 μmol $m^{-2}s^{-1}$ (data not shown). This situation was accompanied by a decrease in ECS_t signal (Fig. 3.4 E). It has been shown that the decrease in ECS_t under these conditions is not a reflection of decreased *pmf*; rather there is increased partitioning from the electrical to the ΔpH component of *pmf* (ECS_t reflects electrical component only). Since under these conditions the electron transport chain becomes highly reduced (indicated by high 1-qL in Fig. 3.4 F), it can be considered as evidence of an additional mechanism for achieving qE without an increase in the total *pmf* under stressful conditions (Cruz *et al*, 2004).

In the Rubisco antisense mutant, ATP synthase conductance (measured as g_{H^+}) follows essentially the same pattern as in wild type tobacco and C_4 plants (Ch. 1, 2), that is decreasing as CO_2 assimilation becomes reduced. The relationship between Calvin cycle activity (v_c+v_o) and g_{H^+} was close to a linear relationship (not shown). It shows tight regulation of ATP synthase activity with Rubisco activity which is the major use of assimilatory power. Rubisco activity appears to be a major factor for regulating steady

state ΔpH and qE, while the contribution from CET and the Mehler reaction appears small. A low level of Mehler reaction activity in this Rubisco antisense mutant has also been demonstrated by Ruuska *et al*. (2000).

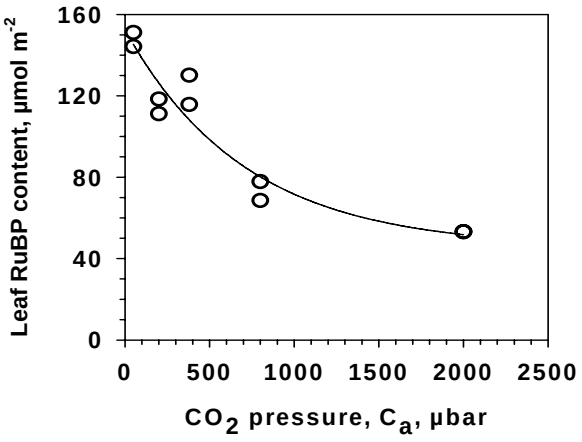

Figure 3.7. RuBP content in leaves of the Rubisco anti-sense mutant of tobacco as a function of the CO_2 partial pressure on the leaf surface (C_a). RuBP was measured from freeze clamped leaves using ^{14}C incorporation method with purified Rubisco (according to Wirtz *et al.*, 1980). O_2 concentration was 2%, leaf temperature was 24 ^oC.

ATP synthase can be regulated by P_i level in the chloroplast stroma. When capacity of Rubisco is limiting, there is potential for a high ΔG for the ATP synthesis reaction.

The RuBP content in the mutant at light saturation (Fig. 3.7) was measured to test for P_i involvement in ATP synthase regulation. The RuBP concentration decreases with increasing CO_2 concentration. The decrease in RuBP with increasing CO_2 may result in

an increase in the P_i pool, which could explain increased ATP synthase proton conductance in Fig. 3.4.

In this chapter, a mutant of *Arabidopsis* which affects CEF, and a low Rubisco mutant of tobacco, provides further evidence that regulation of ATP synthase conductance has a key role in photoprotection and dissipation of excess energy.

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APPENDIX

Other research contributions during Ph.D. studies

1. **Kramer DM, G Johnson, O Kiirats and GE Edwards** (2004) New fluorescence parameters for the determination of Q_A redox status and excitation energy fluxes. Photosynthesis Research **79**:209-218.

I contributed the experimental work in the paper and to discussions involving development of a new parameter for analyzing the reductive state of photosystem II by chlorophyll fluorescence.

2. **Voznesenskaya EV, SDX Chuong, O Kiirats, VR Franceschi, GE Edwards** (2005) Evidence that C_4 species in genus *Stipagrostis*, family Poaceae, is NADP-malic enzyme subtype with nonclassical type of Kranz anatomy (Stipagrostoid). Plant Science **168**: 731-739.

I contributed to discovery of a new photosynthetic form of C_4 in family Poaceae through enzymatic analysis of photosynthetic enzymes.

3. **Boyd, CN, VR Franceschi, SDX Chuong, H Akhani, O Kiirats, M Smith and GE Edwards** (2007) Flowers of *Bienertia cycloptera* and *Suaeda aralocaspica* (Chenopodiaceae) complete the life cycle performing single-cell C_4 photosynthesis.

Special Issue in Memory of Vincent R. Franceschi. Functional Plant Biology **34**: 268-281.

I contributed to demonstration of single-cell C_4 photosynthesis in flowers of two chenopod species through assay of $^{14}CO_2$ fixation.

4. **Sickler, CM, GE Edwards, O Kiirats, Z Gao and W Loescher** (2007) Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Special Issue in Memory of Vincent R. Franceschi. Functional Plant Biology **34**: 382-391.

I contributed to the understanding of mechanism of tolerance to salinity in Arabidopsis plants transformed to produce mannitol by analysis of rates of photosynthesis and stomatal conductance.

5. **Maricle, BR, RW Lee, CE Hellquist, O Kiirats, GE Edwards** (2007) Effects of salinity on chlorophyll fluorescence and CO_2 fixation in C_4 estuarine grasses. Photosynthetica **45**: 433-440.

I contributed to a study of salinity tolerance in C_4 estuarine grasses by collaborating on analyses of photochemistry by chlorophyll fluorescence, and CO_2 exchange.

6. **Edwards, GE, E Voznesenskaya, M Smith, N Koteyeva, Y Park, J-H Park, O Kiirats, TW Okita and SDX Chuong** (2007) Breaking the Kranz paradigm in terrestrial C_4 plants: Does it hold promise for C_4 rice? In: *Charting New Pathways to C_4 Rice*.

(Sheehy JE, Mitchell PL and Hardy B, editors). International Rice Research Institute, Makati City, Philippines, pp.249-273.

I contributed to studies on the mechanism of single-cell C_4 photosynthesis by analyzing the diffusive resistance which is required to minimize CO_2 leakage in the C_4 cycle.

7. **Voznesenskaya, EV, H Akhani, NK Koteyeva, SDX Chuong, EH Roalson, O Kiirats, VR Franceschi and GE Edwards (2008)** Structural, biochemical and physiological characterization of photosynthesis in C_3 and C_4 species of the genus *Tecticornia* (family Chenopodiaceae) and a phylogenetic placement of the photosynthetic types. Journal of Experimental Botany Special Issue on CAM and C_4 photosynthesis **59**: 1715-1734.

I contributed to understanding the mechanism of C_4 photosynthesis in subfamily Salicornioideae by collaborations and gas exchange analyses.