FLEXIBILITY IN THE LIGHT REACTIONS OF PHOTOSYNTHESIS

BY

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Abstract

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The conversion of light energy into chemical energy that takes place during photosynthesis involves some of the most oxidizing and reducing, *e.g.* potentially damaging, chemical species known in biology. In addition, photosynthesis must respond to continuously fluctuating biochemical demands, all the while limiting the damaging consequences associated with delitarious side reactions that can occur as a result of various reactive intermediates intrinsic to the system. Such a feat requires a high degree of inherent flexibility. Modulation of q_E sensitivity, the predominant process responsible for achieving variability in the harmless dissipation of excessively captured light energy over short term changes in energetic imbalance, is shown to be attributable to changes in the proton conductivity of the ATP synthase and variable storage of the proton motive force as a proton diffusion potential versus an electric field. Neither of these mechanisms modulates the ATP/NADPH output ratio of the light reactions, for which there is a fluctuating need, a feat that is suggested rather to be attributable to changes in the fractional turnover of cyclic electron flow around photosystem I. These results are discussed in the context of a novel model for regulation of the light reactions.

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Dedication

I dedicate this dissertation to my wife, Jennifer, and my son, Espen. They helped me maintain a proper perspective about *life* by reminding me of things more important than the matter which can be found in the following dissertation. During my time at Washington State University, I was generally greeted upon coming home from a long day at the lab by: a loving wife who had prepared a home cooked meal and the 'pitter pat' of a little boy's foot steps as he sprang to life to meet his 'daddy' at the front door.

PREFACE

Photosynthesis

Photosynthesis processes light energy from the sun into chemical energy that powers our ecosystem (1). The absorption of light is coupled to the storage of energy in redox partners ($NADP^+$/NADPH) and an electrochemical gradient of protons, termed the proton motive force, or *pmf* (2, 3). The output of the light reactions, e.g. ATP and NADPH, is then used to drive various metabolic processes, predominantly of which is the reduction of CO_2 to the level of sugar phosphates in the Calvin-Benson cycle (4).

Recent and Important Discoveries

Although much is known regarding the details of photosynthesis, several relatively recent discoveries have changed how we view various aspects of its mechanistic intricacies. First, for a long time, the *pmf*, predicted to be composed of both pH (ΔpH) and electric field ($\Delta\psi$) components, was thought to be composed solely of ΔpH, e.g. the $\Delta\psi$ component was presumably collapsed by counterion movement (5). However, a transthylakoid $\Delta\psi$ has been shown to exist *in vivo*, a finding that significantly altered our understanding of the complete role of *pmf* in chloroplast bioenergetics (2, 3, 5). Second, information has emerged regarding the structure of the cytochrome b_6f complex (5) and the CF_1-CF_O ATP synthase (6), providing insight into the proton-to-electron ratio (H^+/e^-) associated with electron transfer and the proton/ATP ratio (H^+/ATP) at the ATP synthase, respectively. Based on these findings, a shortfall in ATP, relative to that required to satisfy the ATP/NADPH ratio in the Calvin-Benson cycle, is expected to be produced by linear electron flow (LEF), the predominant pathway for electron transfer

from water to the $NADP^+$/NADPH couple (7, 8). Thus, a regulatory mechanism appears to be necessary involving, for example, alternative proton pumping electron transfer mechanisms (7, 8), a long debated issue in the literature (9-11). Lastly, our understanding of the variability with which the magnitude of the steady state *pmf* can fluctuate was altered by the discovery that the ATP synthase can be variably conductive to protons (12).

Advances in Instrumentation and Techniques

Several of the new discoveries about various aspects of the *pmf* have been made possible due to recently developed spectrophotometers (14, 15) and techniques capable of probing it under steady state conditions (3, 14-17). These techniques are based, in part, on analyses of the electrochromic shift (ECS), a $\Delta\psi$-induced shift in the absorption spectrum of certain thylakoid membrane-associated pigments (18). The ECS responds to transthylakoid charge transfer, whether it be due to electrons or protons. In fact, certain analytical techniques using the ECS can be used to infer charge separation (i.e. electron transfer) in reaction centers (18, 19). Therefore, to specifically associate ECS changes with proton transfer reactions, a technique was developed whereby analyses of the ECS is monitored during brief dark perturbations (i.e. from 300 ms to several seconds depending upon what type of information is being sought) of the steady state, allowing the system to relax in a way that can reveal information about various aspects of the steady state *pmf* (17).

The work contained in this dissertation is based on using these techniques, along with those designed to estimate changes in chlorophyll *a* fluorescence yield (i.e.

techniques capable of estimating electron transfer and efficiency of light capture) (20, 21), to address the mechanisms by which flexibility is achieved in the light reactions. Specifically, the questions addressed are: 1) How is light capture modulated?; and 2) How is the output ratio of ATP/NADPH modulated? Both of these issues are addressed in the context of fluctuations in physiologic demand.

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CHAPTER 1: Integrating the Proton Circuit into Photosynthesis: Progress and Challenges.

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ABSTRACT

The formation of trans-thylakoid proton motive force (*pmf*) is coupled to light-driven electron transfer and both powers the synthesis of ATP and acts as a signal for initiating antenna regulation. This key intermediate has been difficult to study because of its ephemeral and variable qualities. This review covers recent efforts to probe *pmf in vivo* as well as efforts to address one of the key questions in photosynthesis: How does the photosynthetic machinery achieve sufficient flexibility to meet the energetic and regulatory needs of the plant in a varying environment? It is concluded that *pmf* plays a central role in these flexibility mechanisms.

Key-words: CF_1-CF_O ATP synthase proton conductivity; cyclic electron flow around photosystem I; proton motive force.

Abbreviations: CEF1, cyclic electron flow around PS I; cyt, cytochrome; CF_1-CF_O, chloroplast ATP synthase; ΔpH, pH component of *pmf*; Δψ, electric field component of *pmf*; ΔG_{ATP}, the free energy of ATP formation; DIRK, dark interval relaxation kinetics; ECS, electrochromic shift; ECS_t, total magnitude of ECS decay during a light-dark transition; ECS_{ss}, steady state ECS; ECS_{inv}, ECS change from inverted Δψ; Fd, ferredoxin; g_H^+, CF_1-CF_O ATP synthase proton conductivity; LEF, linear electron flow;

LHCs, light harvesting complexes; *n*, number protons required for formation of one ATP; P_{700}, primary electron donor of PS I; P_{700}^{+}, oxidized primary donor of PS I; *pmf*, transthylakoid proton motive force; pmf_{LEF}, *pmf* generated solely by LEF; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; ϕ_I, photochemical yield of PS I; ϕ_{II}, photochemical yield of PS II; q_E, energy-dependent quenching of antenna excitons; τ_{ECS}, time constant for ECS decay in response to a brief dark interruption of steady state ; v_{CEF1}, steady state rate of CEF1; v_H^{+}, steady state rate of proton flux; v_{LEF}, steady state rate of electron flux through LEF

Introduction

The Integrated Reactions of Photosynthesis

The light reactions of photosynthesis catalyze the 'balanced' conversion of light energy into the chemical energy stored in the ATP/(ADP + P_i) and NADPH/$NADP^+$ couples (reviewed in 1). By 'balanced', we mean that energy conversion must meet the absolute and relative demands for ATP and NADPH of the downstream metabolic processes, including not only the reduction of CO_2 to the level of sugar phosphates in the Calvin-Benson cycle, but also nitrogen assimilation, maintenance of ion gradients etc., each with a different relative requirement for ATP and NADPH (reviewed in 2, 3). This requirement is complicated by the tight coupling of ATP and NADPH generation, at least for the predominant light reaction pathway. The light reactions must also be regulated to prevent the buildup of reactive intermediates which can lead to photoinhibition (photodamage), all of which must occur under fluctuating physiologic conditions.

In this review, we argue that understanding how the plant achieves such flexibility requires a cohesive, integrated view of photosynthesis within its environment. Such a view must encompass the interactions among the photosynthetic electron transfer chain, the proton transfer circuit, and downstream metabolic processes. To use more recent jargon, progress in this area will require a 'systems biology' approach, where key intermediates of each of the processes are monitored under natural and manipulated conditions.

Photosynthesis researchers have made remarkable progress towards understanding the machinery of photosynthesis through a large body of elegant experimentation. Exploiting well characterized, isolated systems (thylakoids, membrane

fragments, isolated protein complexes), and sophisticated biochemical and biophysical approaches, a detailed view has emerged of the individual partial reactions of electron transfer. Concurrently, researchers have taken advantage of the light-driven nature of photosynthesis to develop non-invasive techniques to probe many of these partial reactions *in vivo*, even under steady state conditions (reviewed in 4). These converging efforts have led to an unprecedented breadth and depth of understanding of a *system* of vital reactions in a living organism.

Likewise, rapid progress has been made in characterizing the 'dark' reactions of the chloroplast, using biochemical, genomics and metabolomics approaches. From the groundbreaking experiments on CO_2 fixation (e.g. 5, 6, 7), to more recent metabolomics and proteomics approaches (8, 9), a picture is emerging of the chloroplast (and indeed the plant) as a web of interlocking metabolic pathways, transporters, sensors and regulatory systems.

On the other hand, the transthylakoid proton motive force (*pmf*), a central intermediate in photosynthesis that links the light and dark reactions (10), has been very difficult to characterize. The *pmf* is formed by proton translocation coupled to light-driven electron transfer, and performs at least two key roles in photosynthesis (10). First, it provides the essential driving force for the (otherwise) endergonic synthesis of ATP. Second, it is a key signal intermediate in activation of antenna regulation (reviewed in 12, 13, 14) via energy-dependent quenching of antenna excitons (q_E), a mechanism that harmlessly dissipates excessively absorbed light energy as heat (e.g. 15, 16).

The *pmf* is an ephemeral intermediate, and its extent and nature are functions of many complex factors. Not surprisingly, the past lack of specific probes for *pmf* has left

many questions unresolved about the proton circuit, resulting in a proliferation of models for the maintenance and regulation of the light reactions. Recently, new or refined non-invasive *in vivo* spectroscopic tools have been developed for probing the formation, amplitude and composition of the *pmf* (17-23). This review aims to: 1) introduce the concept of the proton circuit in photosynthesis and its importance for sustaining and regulating photosynthesis; 2) introduce the conceptual basis, utility and limitations of new *in vivo* probes of the *pmf*; 3) discuss the impact that measurements made using new *pmf* probes have made on various proposed models for photosynthesis and its regulation; and 4) pose new challenges for proponents of competing models of the proton circuit.

The Proton Circuit of the Light Reactions.

LEF-dependent pmf *formation*. Proton accumulation in the thylakoid lumen of the chloroplast is driven by vectoral electron transfer through the electron transfer chain (reviewed in 1) (See Figure 1). Light energy is absorbed by antennae complexes, specialized assemblages of pigments and proteins that funnel the energy to photosystems (PS) II and I. In linear electron flow (LEF), PS II oxidizes H_2O and delivers the electrons to plastoquinone (PQ) bound to the Q_B site of PS II on the stromal side of the thylakoid membrane. When reduced by two electrons, PQ forms a neutral plastoquinol (PQH_2), with uptake of two protons from the stroma. Following diffusion of PQH_2 from Q_B to the Q_o site of the cytochrome (cyt) b_6f complex, it is sequentially oxidized by the high and low potential chains of the cyt b_6f complex, via the 'Q-cycle' (24, 25). The initial oxidation of PQH_2 at Q_o results in the release of two protons into the lumen, but the transfer of only one electron to the 'high potential chain' (consisting of the Rieske iron

sulfur center and cyt *f*) and on to PS I via plastocyanin. The remaining electron is passed through the low potential chain (consisting of two *b* hemes) to reduce PQ bound at the Q_i site of the cyt b_6f complex on the stromal side of the thylakoid membrane. Two-electron reduction of PQ at Q_i is accompanied by uptake of two protons from the stroma to form PQH_2. Photoexcitation of the PS I reaction center drives transmembrane electron transfer from the primary electron donor, P_{700}, through a series of iron-sulfur centers to ferredoxin (Fd) and eventually $NADP^+$. Oxidized P_{700}^+ is re-reduced by plastocyanin. Overall, 3 protons accumulate in the lumen for each electron that is transferred from H_2O to the reducing side of PS I, one proton for each electron released by the oxidation of H_2O at PS II and two protons for each electron transferred through the high potential chain of cyt b_6f to PS I.

Alternate cycles of the light reactions. In addition to LEF, at least two other electron (and proton) transfer pathways have been proposed and partially characterized. One of these alternative pathways is the Mehler peroxidase reaction, sometimes called the pseudo-cyclic pathway or the water-water cycle, which uses oxygen as an alternate electron acceptor from PS I to decrease the NADPH output of LEF (reviewed in 26). The Mehler peroxidase reaction is technically a cycle because electrons are extracted from H_2O at PS II, which are then used to reduce O_2 to superoxide, which is subsequently reduced to H_2O by superoxide dismutase and ascorbate peroxidase. All of the components of this pathway appear to be characterized and its operation has been demonstrated *in vitro*, but it is quite difficult to measure and significant ambiguity exists as to the extent of its engagement *in vivo* (see review in 27)).

Another alternate pathway is cyclic electron flow around PS I (CEF1), a series of reactions that returns electrons from PS I to the PQ pool, allowing for proton translocation (*pmf* formation) but no net NADPH reduction (28) (Fig. 1). CEF1 uses all of the machinery of LEF with the exception of PS II, which is replaced by a PQ reductase. At least four separate PQ reductases have been proposed to facilitate CEF1 (29): 1) An as of yet unidentified, antimycin A-sensitive Fd-PQ oxidoreductase has been predicted to fulfill this role for some time. *In vitro* activity from this enzyme has been observed as an antimycin A-sensitive reduction of the PQ pool by reduced Fd (28). Recently, Munekage et al. (30) isolated an *Arabidopsis thaliana* mutant, pgr5, which is deficient in Fd-PQ oxidoreductase activity. The PRG5 protein is associated with the thylakoid membrane, but because it is probably not a transmembrane protein, nor does it possess potential ligands for redox cofactors, it is unlikely the Fd-PQ oxidoreductase itself (30). Rather PGR5 might be a regulatory component of the enzyme. 2) A NADPH:PQ oxidoreductase has also been identified as partially homologous to mitochondrial complex I (NADH:UQ oxidoreductase) (31, 32), but a detailed biochemical characterization has been hampered by its very low expression level. Mutation of this enzyme slows the rate of PQ reduction by NADPH in the dark, but has only small effects on steady state photosynthesis (33). 3) A ferredoxin-$NADP^+$ oxidoreductase has also been proposed to participate in CEF1, mainly because of its observed association with the cyt b_6f complex (34). 4) Finally, the cyt b_6f complex itself has been suggested to act as a ferredoxin:PQ oxidoreductase, as supported by the discovery of an unexpected heme group, termed heme c_i or heme x, in the recent x-ray structures (35, 36). The heme is located in a seemingly ideal position to carry out

electron transfer from the stroma to cytochrome b_H and the Q_i site, where PQ is reduced. At present, there is no direct evidence that this heme participates in CEF1, and indeed, earlier studies found no evidence for rapid reduction of cytochrome b_H, which should occur upon reduction of the newly-discovered heme (see review in 29, 37). It is also clear that the majority of non-photochemical PQ reduction is halted by mutation of pgr5 and the NADPH:PQ oxidoreductase (38). Thus, although there appears to be a pathway for electron transfer to PQ via the new heme, for reasons that are unclear, at present there is no evidence that it operates at a substantial rate in higher plant chloroplasts.

Proton efflux and ATP formation. The *pmf* generated by the light reactions drives ATP synthesis through the chloroplast (CF_1-CF_0) ATP synthase (ATP synthase) . According to the generally accepted rotational catalysis/binding exchange mechanism for the ATP synthase (41, 42), proton flux across the membrane occurs by sequential protonation and deprotonation of acidic amino acid residues on transmembrane spanning ring of subunit c (subunit III in chloroplasts) . Each protonation event leads to rotation of the ring by the extent of one subunit III with respect to the α and β subunits. The rotatory motion is transmitted to the F_1 portion of the complex via the γ-subunit of the F_1 portion and the subunit b (either subunit I or II of chloroplast) of the F_O portion (one acts as a rotor, the other as a stator). Rotation of γ within the $\alpha_3\beta_3$ hexamer interconverts a series of three distinct nucleotide binding sites on the hexamer among the following three states: (1) a 'weak' state which binds ADP + P_i, (2) a 'tight' state, which preferentially binds ATP, forcing the equilibrium constant to favor formation of ATP from ADP + P_i and (3) an 'open' state, which only weakly binds nucleotides. Thus, with one full rotation of γ, a loose site will be converted, in series, to a weak site, a tight site, and then

back to a loose site. Each cycle of states corresponds to binding of ADP + P_i, formation of ATP and its subsequent release. Because three catalytic sites exist on the hexamer, each full rotation of γ will yield three molecules of ATP. Since a stoichiometry of 14 has been reported for subunit III in CF_O , this model predicts the number protons required for formation of one ATP by the ATP synthase, *n*, to be 4.67. It should be noted that more recent (and difficult) direct experimental work has not confirmed this value of *n* (43), though an early work obtained precisely this value (44). ATP synthases from other species have different c subunit stoichiometries, and thus are predicted to have different *n* values (45). However, it is very unlikely that the value of *n* can change either rapidly or developmentally (46).

Secondary ion transport. Two other processes should be considered as integral components of the proton circuit. The first is the movement of counterions across the thylakoid membrane in response to light-driven Δψ. The thylakoid membrane has a low electrical capacitance, about 1 $\mu F/cm^2$ (reviewed in 47), so that the movement of even a small number of charges across the membrane will build up a large Δψ (48, 49). On the other hand, the buffering capacity of the lumen is quite large, ~10-30 mM/pH unit, so that a large number of protons must be moved into the lumen to alter its pH (37, 48). With this type of system, *pmf* should be stored overwhelmingly as a Δψ, and indeed this is observed initially after illumination (37 and references within). However, after continuous illumination for seconds or minutes, slow movements of counterions collapse a fraction of the Δψ, allowing ΔpH to build up (10, 37). The steady state ratio of Δψ/ΔpH will depend upon a number of factors, the major variable being the buffering capacity of the lumen, the concentrations of permeable counter ions and the presence of

ion channels, symporters, antiporters etc. (37). Early work suggested that, in contrast to bacterial and mitochondrial energetic membranes, thylakoids stored *pmf* mostly as ΔpH , but more recently this view has been revised, and it appears that about half of the transthylakoid *pmf* is stored as Δψ (10, 21, 22, 37).

It can also be argued that the chloroplast inner envelope should be included in the proton circuit of photosynthesis. ATP-driven proton pumps in the inner envelope generate a substantial *pmf* . This light-modulated process raises the pH of the stroma and generates ion gradients, both of which can contribute to transthylakoid *pmf* or alter the fraction of *pmf* stored as Δψ and ΔpH (see discussion in 37).

The Role of the *pmf* in Regulating Photosynthesis.

In order to prevent excessive excitation of PS II centers under conditions where absorption of light energy exceeds the capacity of downstream metabolism, a series of down regulatory processes, collectively termed non-photochemical quenching of excitons (NPQ) (reviewed in 13), are initiated to harmlessly dissipate the excess excitation energy. In vascular plants, the predominant component of non-photochemical quenching to operate over the minutes-to-hours timescale is q_E which is initiated by buildup of the ΔpH component of *pmf*, i.e. by acidification of the lumen (51) which activates: 1) the conversion of violaxanthin to antheraxanthin and zeaxanthin by the lumen-localized enzyme violaxanthin de-epoxidase (52); and 2) the protonation of lumen-exposed residues of PsbS, a polypeptide associated with the light harvesting complex of PS II (53, 54). In the current model, binding of zeaxanthin to protonated PsbS facilitates energy

transfer from excited chlorophylls to zeaxanthin, which subsequently relaxes to the ground state by heat dissipation (14, 51).

A second antenna regulatory mode, the 'state transition', involves changing the relative distribution of light harvesting complexes (LHCs) between the two photosystems (reviewed in 55). For instance, in the dark, or when the PQ pool is predominantly oxidized, the antenna is in 'state 1', where LHCs associated with PS II (LHC2) deliver absorbed light energy mainly to PS II centers. Under strong light or when electron transfer away from the PQ pool is restricted, the PQ pool becomes largely reduced, and PQH_2 binding to the cyt b_6f complex activates a kinase which phosphorylates LHC2 proteins, leading to a 'state 2' transition, where a fraction of LHC2 migrates to interact with the PS I antenna complex (56, 57). State transitions have two major effects. First, they act to alter the ratio of excitation of PS I and PS II centers, presumably to balance their effective turnover rates. Second, since the oxidized primary donor of PS I, P_{700}^{+}, is an efficient quencher of excitation energy, a state 1 transition will effectively lower the overall efficiency of light capture, as does q_E. State transitions are normally considered to be under redox control, but the redox state of the PQ pool can also be strongly influenced by *pmf*, particularly via its effects on lumen pH, which affects the rate constant for PQH_2 oxidation at the Q_o site of cyt b_6f (10, 47). Thus, state transitions should be controlled, at least partly, by the proton circuit.

In wild type vascular C_3 plants, q_E quenching accounts for the largest fraction of antenna regulation (12), while state transitions have relatively small effects , with maximally only ~20% of LHC2 moving to PS I . In contrast, in some green algae, especially *Chlamydomonas reinhardtii*, state transitions are quite large, with about 80%

of LHC2 moving (57, 58), while q_E is often small or immeasurable, depending upon growth and assay conditions (59).

The Need for Flexibility in the Proton Circuit

An important consequence of a H^+/ATP ratio (i.e. *n*) of 4.67 is that it predicts an ATP/NADPH output ratio for LEF of ~1.3, smaller than that required for steady state turnover of the Calvin-Benson cycle (40, 60). The magnitude of any ATP/NADPH deficit in the cell will depend upon flux to alternate energy sinks with different requirements for ATP/NADPH, especially photorespiration, nitrite reduction, maintenance of ion gradients, transport processes and so on. A recent estimate, which takes into consideration nitrogen assimilation rates, suggested a deficit of about 0.13 ATP/NADPH (as reviewed in 61). Since in LEF production of ATP and NADPH are coupled (i.e. one cannot occur without the other), such a deficit would have severe consequences for C_3 plants if not compensated for by other processes. Thus, the chloroplast requires flexible mechanisms to alter energetic output balance.

One way to balance ATP/NADPH, independently of any changes in the light reactions, is to shuttle reductive power out of the chloroplast through a malate/oxaloacetate exchange, the so-called malate valve, which would then increase the ATP/NADPH ratio in the chloroplast (62, 63). Alternatively, many recent works have proposed that differential engagement of CEF1 or the Mehler peroxidase reaction account for ATP/NADPH balancing (as reviewed in 60).

Plants must also adjust energy dissipation, or q_E, in response to fluctuating environmental conditions to maximize productivity and minimize the potential for

photodamage (64-67). For example, stomatal closure, which occurs in response to drought (67), limits diffusion of CO_2 into leaves, restricting turnover of the Calvin-Benson cycle and thereby consumption of NADPH, effectively lowering LEF. If *pmf* formation were strictly dependent on LEF, then q_E would also be expected to decrease under such conditions, precisely when it was most needed to prevent excessive reduction of the electron transfer chain (29, 65, 67-69). In contrast, q_E has been demonstrated to be robust under conditions that attenuate LEF, *e.g.* its sensitivity with respect to LEF is increased up to 6-fold under low CO_2 or O_2, a phenomenon we term 'q_E modulation' (21, 22).

An integrated view of the proton circuit reveals at least four models that can account for the observed changes in q_E sensitivity (reviewed in 29). Some of these models will also impact ATP/NADPH output balance.

Model 1: Variable antennae response to lumen pH. Changes in the antennae response could be brought about by either changes in the pK_a values on violaxanthin de-epoxidase and/or psbS, by changes in the relative rates of the enzymes controlling zeaxanthin (violaxanthin de-epoxidase and zeaxanthin epoxidase) or total pigment levels. Any of these types of changes could enhance or diminish the q_E response to lumen pH and by extrapolation to ΔpH and *pmf*.

Model 2: Changes in the fractional turnover of alternate electron transfer pathways. As mentioned above, CEF1 has been proposed as a mechanism for adjusting the magnitude of the *pmf* for the purpose of modulating q_E sensitivity when rates of LEF were attenuated (65, 67, 70). However, if solely for the purpose of modulating q_E, such a mechanism is problematic given that, since protons predominantly exit the lumen through

the ATP synthase, it will also necessarily modulate the ATP/NADPH output ratio, possibly causing mismatch problems in downstream metabolism (29). This model predicts discontinuity in the relationship between the measured magnitude of the *pmf* and that generated by LEF alone.

Model 3: Changes in the proton conductivity of the ATP synthase (g_H^+). An often overlooked feature of steady state *pmf* is that changes in its magnitude can be brought about by not only increased flux of protons into the lumen (i.e. Model 2), but also by lowering the conductivity of the ATP synthase to proton efflux, or g_H^+. Such a mechanism that would allow even a small proton flux into the lumen to generate a significant *pmf* without modulating ATP/NADPH output (21, 22, 29) . This model predicts that *pmf* (and thus q_E response) will be a function of proton flux and g_H^+. Operating by itself, the model predicts continuity in the relationship between LEF/g_H^+ and *pmf* with a continuous relationship between q_E and *pmf* (21, 22).

Model 4: Changes in pmf partitioning. Differential partitioning of the *pmf* into ΔpH and Δψ can also modulate q_E (37). Such a model allows for adjustments in q_E without the need for altering the magnitude of total *pmf*. Like Model 1, this model predicts discontinuity in the relationship between total *pmf* and q_E, but it further predicts commensurate changes in the fraction of *pmf* stored as ΔpH.

Progress: Approaches to Probing the Proton Circuit

In principle, any one of the above models could account for q_E modulation, while only Model 2 accounts for ATP/NADPH balancing. Distinguishing between these models requires *in vivo* probes of the formation, storage, and utilization of *pmf* (29). All

available methods for probing the proton circuit are indirect, especially when applied *in vivo*, because the *pmf* itself does not display obvious spectroscopic signals. We are thus limited to probes of the effects of *pmf* on other processes. As a result, the interpretation of *pmf* probes requires at least some assumptions about the overall mechanism. We argue that these limitations are not fatal, but instead require that we test interpretations of results from one technique with those of others. In the following, we will review the available *in vivo* techniques and discuss their respective strengths, weaknesses and assumptions.

Extrapolations from electron transfer measurements. In principle, given the coupling of electron and proton transfer, it should be possible to estimate proton flux from measurements of electron fluxes. Starting with the assumption that proton flux is a linear sum of contributions from LEF (including the Mehler peroxidase reaction) and CEF1, then:

$$v_{H^+} = a \cdot v_{LEF} + b \cdot v_{CEF1} \qquad (1)$$

where a and b are the H^+/e^- ratios coupled to electron flux through LEF (ν_{LEF}) and CEF1 (ν_{CEF1}), respectively. The value of a is most likely constant at 3 (18). The value of b will depend on the PQ reduction pathway used by CEF1 (29). If PQ reduction proceeds through direct transfer from a carrier (*e.g.* Fd-$NADP^+$ oxidoreductase) without additional proton pumping, then b equals 2. However, there is almost certainly sufficient free energy in the NADPH to PQ redox reaction to pump additional protons, as occurs with complex I of mitochondria. If reduction occurs through the chloroplast homologue of

complex I, NADPH-PQ oxidoreductase, *b* could be as high as 4. The situation becomes more complex when one considers that multiple PQ reduction pathways might operate in parallel (reviewed in 29). Nevertheless, it should be possible to obtain at least qualitative estimates of proton flux from CEF1, provided that v_{LEF} and v_{CEF1} can be accurately estimated.

Non-invasive measurements of LEF through PS II, based on saturation pulse-induced changes in chlorophyll *a* fluorescence yield (i.e. from which estimates of the efficiency of PS II electron transfer per quanta absorbed, ϕ_{II}, can be determined), have been available for some time (71). Under a wide range of conditions, these measurements appear to give reasonable estimates for LEF (72, 73), though under more severe conditions, or over long-term acclimatization, inconsistencies have been observed (74).

The greater challenge lies in accurately determining v_{CEF1}. In general, cycles are difficult to probe, because they have no stable or easily measured end product. For CEF1, one approach is to estimate the relative flux of electrons through PS II and PS I (reviewed in 4). Since, in a steady state, LEF will engage both PS II and PS I, whereas CEF1 will only involve PS I, comparative measurements of electron flux through the two photosystems should yield relative estimates of CEF1. There are two commonly used approaches to estimate electron flux through PS I.

One strategy is to measure the fraction of PS I centers in the 'open' state by measuring absorbance changes in the near infrared, which estimates the relative extent of oxidized P_{700}^{+}, under ambient and saturating light, taking care to account for PS I centers closed by reduction of the electron acceptors (75). To a first approximation, the fraction

of open centers should be proportional to the photochemical yield of PS I (ϕ_I), i.e. the fraction of light absorbed in the PS I antenna that results in PS I electron flow. If so, electron flux through PS I should be proportional to the product of light intensity and ϕ_I. One concern is that electron flux through PS I depends not only on the fraction of open centers, but also on the size of the antenna, which as we discussed above, is affected by state transitions. Nevertheless, past research in vascular plants has generally found linear relationships between ϕ_{II} and ϕ_I (see also review in 4, 75, e.g. 76), implying that CEF1 is sluggish or is regulated to be a fairly constant fraction of LEF.

Dark interval relaxation kinetics (DIRK) analysis. Another approach to measuring PS I electron transfer in the steady state is to observe changes in the concentrations of intermediates upon an abrupt (but typically brief) light-dark transition, a method we have termed 'dark interval relaxation kinetics' (DIRK) analysis (19). The basic principle is straightforward. In a steady state, the concentrations of intermediate species are constant because flux into each intermediate is precisely balanced by flux out. With photosynthetic reactions, the flux can be halted by switching off the actinic light. For certain species, the flux into (or out of) an intermediate pool is rapidly halted by light-dark transitions, whereas the flux out of (or into) the pool remains relatively constant for a significant period of time. The *initial* changes in intermediate concentration will, in these cases, reflect steady state flux through the entire system. In principle, several different intermediates could be used as flux probes, but quantitative estimates require accurate and detailed kinetic models for the intermediate reactions (19). Inappropriate kinetic modeling can lead to dramatic errors.

At first glance, P_{700}^{+} would seem to be an especially attractive species for DIRK analysis, because it is easily observed via its near-infrared absorption spectrum (at around 820 nm), and its formation is rapidly halted by a light-dark transition (77). Indeed, estimates of CEF1 made using the initial rate of decay of the 820 nm change have recently been used to argue that large changes in CEF1 contributions to *pmf* controls q_E (up to and above that of LEF) under conditions of restricted LEF, in contradiction with previous results using ϕ_I measurements (78, 79). As we have pointed out previously (29), such large CEF1 contributions should be easily detectable by the ϕ_I method and would also radically alter the ATP/NADPH balance.

Unfortunately, using the 820 nm absorbance change by itself can lead to serious errors in flux measurements because electron transfer to the other high potential chain components must also be considered (18). Several groups have now shown that the degree of redox equilibration among high potential chain components in the steady state is also dependent upon condition (80), rendering flux estimates based solely on P_{700} absorbance changes risky.

One way to circumvent the problem of differential electron partitioning is to sum the initial DIRK rates of each component in the entire high potential chain rather than that into any one component (19). When this approach has been used, the flux through the cyt b_6f complex and PS I was found to be proportional to that through PS II , indicating no changes in CEF1 from low to saturating light intensities (19). However, the technique requires more involved measurement of multiple components and estimates of the *in situ* effective extinction coefficients, which are affected by light scattering-induced path length enhancement and sieve (or flattening) effects, possibly in a species-specific

fashion (81). Moreover, such detailed analyses have not yet been performed under a broad range of conditions where changes in CEF1 contributions to proton flux are expected.

Electric field probes of the proton circuit. Another approach for probing the proton circuit is to follow the effects of proton movements on the transthylakoid $\Delta\psi$. We argue that these techniques, though not without complexities (see below), are much more direct than those based on extrapolations from electron transfer probes. Since protons are charged, their net movement across the thylakoid membrane will affect transthylakoid $\Delta\psi$. Fortunately, such changes are readily measured via the 'electrochromic shift" (ECS), an absorbance signal proportional to changes in $\Delta\psi$ (82). The ECS techniques typically employ a modified DIRK approach, monitoring decay of the ECS during perturbation of the steady state with a light-dark transition. The interpretation of such ECS analysis is based on the concept that, in the steady state, the flux of protons into the lumen (i.e. via LEF, etc.) is precisely balanced by their efflux, rendering a steady baseline ECS signal (18). Briefly shuttering the actinic light off rapidly halts proton flux into the lumen, whereas proton efflux from the lumen continues until *pmf* equilibrates with the free energy of ATP formation, or ΔG_{ATP}. Given that ECS signals based on DIRK analysis are well fit by first order decay kinetics, implying one process predominantly accounts for decay (i.e. collapse of the *pmf* through the ATP synthase), a number of important parameters can be obtained by DIRK analysis of ECS decay (21, 22, 37).

In vivo, steady state *pmf* remains above the activation threshold for the ATP synthase, so that the relationship between *pmf* and efflux of protons through the enzyme is essentially ohmic. As such, *pmf* can be expressed by a force-flux relationship:

$$ECS_t \propto \nu_{H+} \bullet \tau_{ECS} \quad (2)$$

Where ECS_t (i.e. the full amplitude of ECS decay during the dark interval) will reflect the light-induced *pmf*, i.e. that which is generated on top of the dark *pmf*; ν_{H+} represents the flux of protons through the ATP synthase and, assuming steady state conditions, can be interpreted as the sum total flux of protons into the lumen; and τ_{ECS} is the time constant for ECS decay, which is inversely proportional to the conductivity of the ATP synthase to protons, or g_H^+ ($\tau_{ECS} \propto 1/g_H^+$). Assuming the flux of protons into the lumen occurs predominantly via LEF, the above equation yields a very useful proportionality that represents pmf_{LEF}, e.g. the *pmf* generated solely by LEF assuming a constant H^+/e^- and constant fractional turnover of CEF1 (22, 83):

$$ECS_t \propto LEF/g_H^+ = pmf_{LEF} \quad (3)$$

If these assumptions hold true, a continuous (and essentially linear) relationship would be expected between pmf_{LEF} and ECS_t, providing a basis for testing contributions of CEF1 to proton flux in the steady state and *in vivo*.

During longer dark intervals, $\Delta\psi$ decays in two distinct phases that can be readily interpreted using published characteristics of thylakoid membranes (10, 37). Approximately 100 ms after the light-dark transition, the *pmf* in the dark comes into equilibrium with ΔG_{ATP}, i.e. there is no net flux of protons across the membrane, implying that the *pmf* in the dark $\sim \Delta G_{ATP}/n$ (37, 47). Thus, the difference in *pmf* between

light and dark should be reflected in a proportional change in ECS_t, regardless of whether the *pmf* is stored as $\Delta\psi$, ΔpH or a combination of these forms. Because of the proton's charge, the collapse of either form of *pmf* will affect ECS in the same direction. Changes in $\Delta\psi$ will (of course) register directly as a proportional ECS change. Since counterion movements are slower than that of protons (10, 37), ΔpH collapse will induce a change in $\Delta\psi$, positive in the direction of net proton movement, i.e. positive towards the stroma and thus 'inverted' with respect to that generated by the light reactions. The 'inverted' $\Delta\psi$ will grow until it energetically opposes the proton diffusion potential, i.e. until the ΔpH component is balanced by an energetically equivalent inverted field (37).

Over the tens of seconds time scale, the 'inverted' $\Delta\psi$ phase formed by backflow from ΔpH is dissipated by counterion movements. However, changes in ECS related to differences between the steady state and dark $\Delta\psi$ will remain. In this way, the longer-term decay of ECS can be used to estimate the fraction of light-induced *pmf* stored as $\Delta\psi$ and ΔpH (22, 37). The ECS phase which decays over the tens of seconds time scale, termed ECS_{inv}, should be proportional to light-dark differences in ΔpH while the non-decaying phase, termed ECS_{ss} (i.e. because it should be proportional to steady state light-induced $\Delta\psi$), should be proportional to light-dark differences in $\Delta\psi$. The sum of the two accounts for the magnitude of the light-induced *pmf*, ECS_t:

$$ECS_t = ECS_{inv} + ECS_{ss} \propto \Delta pH + \Delta\psi \qquad (4)$$

From such analysis the relative partitioning of *pmf* into ΔpH can be estimated (i.e. ECS_{inv}/ECS_t) (22).

It is important to note that any estimates of *pmf* components measured via the ECS will be offset by the dark *pmf*, which in turn is set by ΔG_{ATP} (10, 37). Fortunately, relatively large changes in ΔG_{ATP} would be needed to significantly alter the dark *pmf*, whereas measured values of ΔG_{ATP} have been found to be relatively constant from light-to-dark and high to low CO_2 (47, 84), so that we expect ECS_t to be a reasonable estimator of $\Delta pmf_{light\text{-}dark}$. Consistent with this view, the response of lumen pH-sensitive reactions (q_E) as a function of ECS measurements were found to be continuous (see below).

Internal consistency in the ECS measurements. As with any technique, the ECS-based probes need to be continuously validated. There are several pitfalls that need to be avoided in ECS measurements. It is important to confirm that the absorbance signal being observed does, in fact, reflect ECS. This is especially important for long-term measurements, where other signals clearly interfere. Key experiments should be repeated using different deconvolution techniques (e.g. 18, 19). It is also important to confirm the spectral shape of the ECS signal especially when using different, or even mutant, species, where the shiftable pigments might differ (82). We recommend a reference spectra measured as a rapid (<1 ms) rise in the ECS upon a single turnover actinic flash, which is relatively uncontaminated by other species (82, 85). Second, (with all other factors being equal) the ECS signal will be proportional to the number of $\Delta\psi$-shiftable pigments in the light path, and thus any changes in this value must be considered. A reasonable approach is to estimate the relative ECS response to a given field, for example by observing its rapid response to a saturating, single-turnover flash (phase *a*) (86), which linearly reflects electron transfer (i.e. charge separation) in PS I and PS II centers (82). With large changes in g_{H^+}, where the rate of proton efflux might become significantly slower than

those of counterion fluxes, truncation of the ECS_{inv} signal is likely (37). Also, because it sets the baseline dark *pmf*, ΔG_{ATP} should remain relatively constant, as is expected to be approximately true (see discussion in 21, 84). Fortunately, the q_E response (i.e. violaxanthin de-epoxidase activation and PsbS protonation) can in many cases be used as a 'standard' pH probe to test for such deviations. At least within species and defined developmental stages, plots of q_E against ECS_{inv} have been constant within the noise level (22), indicating that such factors do not severely impair *pmf* estimates under many conditions.

An Integrated Analysis of the Proton Circuit

The above described ECS techniques were used to test the general models described above. Keeping in mind the limitations of the spectroscopic techniques and the (thus far) limited range of conditions under which they have been employed, we reach the following conclusions.

Model 1: Variable antenna response to lumen pH. Measurements of q_E as a function of estimated lumen pH (ECS_{inv}) made on a single leaf over the short term usually fall on a continuous curve, implying that there is little change in the antenna response to lumen pH (21, 22). Although these data lead us to reject Model 1 as a significant contributor to short-term flexibility responses, clearly acclimation or adaptation can alter these responses (e.g. by changing the levels of xanthophylls or other pigments) (59, 87).

Model 2: Changes in the fractional turnover of alternate electron transfer pathways. Under a wide range of steady state conditions, *e.g.* varying light intensities

and levels of both CO_2 and O_2, relationships between v_H^+ and LEF were constant within 10-15% (21, 22). This implies very little *change* in proton flux contributions from CEF1, in disagreement with large contributions from Model 2 (but see below).

Model 3: Changes in the proton conductivity of the ATP synthase (g_H^+). In *Nicotiana tabacum* (tobacco) plants, lowering CO_2 from 2000 to 0 ppm, while holding O_2 constant at 21%, increased the response of q_E to LEF by about 5-fold (with no evident changes in the contributions from CEF1, see above) (21). Importantly, ECS estimates of g_H^+ were also lowered by ~5-fold over these conditions, completely accounting for the change in q_E sensitivity to LEF. In other words, decreases in g_H^+ allowed a substantial *pmf* to build up even with diminished LEF. We propose that g_H^+ modulation is a predominant mechanism for modulating q_E responses. It is noteworthy that changes in g_H^+ are probably not caused by substantial changes in ΔG_{ATP}, since ATP/ADP remains relatively constant over conditions where we observe large changes in g_H^+. Instead, a regulatory signal is likely involved (see below).

Model 4: Changes in pmf partitioning. Varying both CO_2 and O_2, i.e. from ambient to 50 ppm and 1%, respectively (22), resulted in a ~6-fold increase in q_E sensitivity to LEF, but in this case, changes in g_H^+ could only account for about half of the effect. Again, no changes in CEF1 contributions were observed. However, substantial changes in the fraction of *pmf* stored as $\Delta\psi$ and ΔpH were inferred from ECS_{inv}/ECS_t (i.e. more *pmf* was stored as ΔpH) and these could account for the observed increase in the q_E response that could not be attributed to changes in g_H^+. We have since found evidence for *pmf* partitioning effects under less severe conditions (high light and

low CO_2, but ambient O_2) in other species, in particular *A. thaliana* (K. Takizawa, T. J. Avenson and D. M. Kramer, unpublished).

An Integrated Model for the Proton Circuit.

In this section, we propose a working model that allows for flexible participation of the proton circuit in photosynthesis, as illustrated in Figure 1 (see also 29). This model divides the mechanisms described above into two categories: one that accounts for ATP/NADPH balancing (but has only indirect effects on q_E), and the other that affects the q_E response without affecting ATP/NADPH output. For C_3 vascular plants, we posit that ATP/NADPH output is modulated by a combination of CEF1 and the Mehler peroxidase reaction (Model 2) (in accord with 40, 60). It is important to note that our results comparing v_H^+ with LEF do not reject contributions from CEF1 that are roughly in proportion with LEF, nor do our analyses discount contributions to proton flux from the Mehler peroxidase reaction. Although most *in vitro* assays have shown very slow rates of non-photochemical PQ reduction (reviewed in 29), recently, Joliot and Joliot (23), using modified DIRK assays for v_H^+, found evidence for relatively rapid CEF1 during photosynthetic induction from dark-adapted states. These pre-steady state results suggest a substantial capacity for CEF1, at least under certain conditions. In contrast, our results suggest that the steady state levels are either small or proportional to LEF. We thus propose that CEF1 is highly regulated, in accord with a role in ATP/NADPH balancing (29). How this regulation is achieved remains to be determined, though the redox status of stromal compartments has previously been proposed as a regulating factor (88). In

principle, when ATP/NAPDH output is below that required for metabolism, NADPH will accumulate, leading to a reducing stroma, perhaps activating CEF1.

From the arguments presented above, we propose that g_H^+ (Model 3) and *pmf* partitioning into ΔpH and Δψ (Model 4) constitute the major mechanisms for modulating q_E sensitivity to LEF, which means that LEF remains the major contributor to proton flux in C_3 vascular plants under most physiological conditions. Over the long-term, differential expression of proteins and pigments can alter the q_E response (Model 1). In other types of oxygenic photosynthetic organisms, different mechanisms may predominate to fulfill very different biochemical or regulatory demands. For example, a robust CEF1 pathway has been demonstrated for certain types of C_4 bundle sheath cells, cyanobacteria and green algae where a large ATP/NADPH ratio is required to sustain the dark reactions (reviewed in 29).

We have further speculated that stromal concentration of free P_i and stromal ion balance might act as 'signals' for modulating g_H^+ and ΔpH/Δψ, respectively (21, 29, 37). Conditions that inhibit turnover of the Calvin-Benson cycle will lead to accumulation of phosphorylated intermediates that will deplete the stroma of free P_i, leading to a reduction in the effective rate constant for ATP synthesis and consequently a lowering of g_H^+. A less defined set of conditions might disrupt the normal balance of ions, e.g. increasing free stromal Cl^-, which in turn can pass through across the thylakoid membrane and collapse Δψ (37).

A Possible Test of the Integrated Model.

A very interesting mutant of *A. thaliana* was recently described by Munekage et al. (30, 38) which can be used to test the integrated model (above). This mutant, termed pgr5 (for "proton gradient regulation"), is deficient in both non-photochemical PQ reduction (a required step in CEF1) as well as q_E (see also above). A reasonable inference to draw is that these two deficits are directly related, i.e. that proton translocation by CEF1 contributes substantially to the activation of q_E (30, 38, 89), in contradiction with the integrated model we have proposed above.

Instead, however, we have found that proton flux as a function of LEF is scarcely diminished in pgr5 compared to wild type (22). LEF was diminished by about 50% in pgr5, whereas g_H^+ was increased. A slowed rate of proton pumping (i.e. low rates of LEF) coupled to rapid proton efflux should lead to a decreased *pmf* (as we observe by ECS_t) and a diminished q_E response. It is thus easy to rationalize the pgr5 phenotype within the context of our integrated model. A decrease in CEF1 caused by the loss of PGR5 leads to a small imbalance in the ATP/NADPH output ratio. Over time, this imbalance leads to depletion of $NADP^+$ (buildup of NADPH) and thus a slowing of LEF. The restriction in LEF was, as expected, accompanied by reduction of the electron carriers (38). Interestingly, a similar slowing of LEF is seen when CO_2 is decreased, but in this case g_H^+ is commensurately lowered, possibly as a result of lowered stromal P_i (see above). With prg5, however, P_i should be in excess (due to the ATP/NADPH imbalance), so that the ATP synthase will not be downregulated. The result should be a decrease in LEF with no decrease in g_H^+ (or even an increase), leading to a weak q_E response.

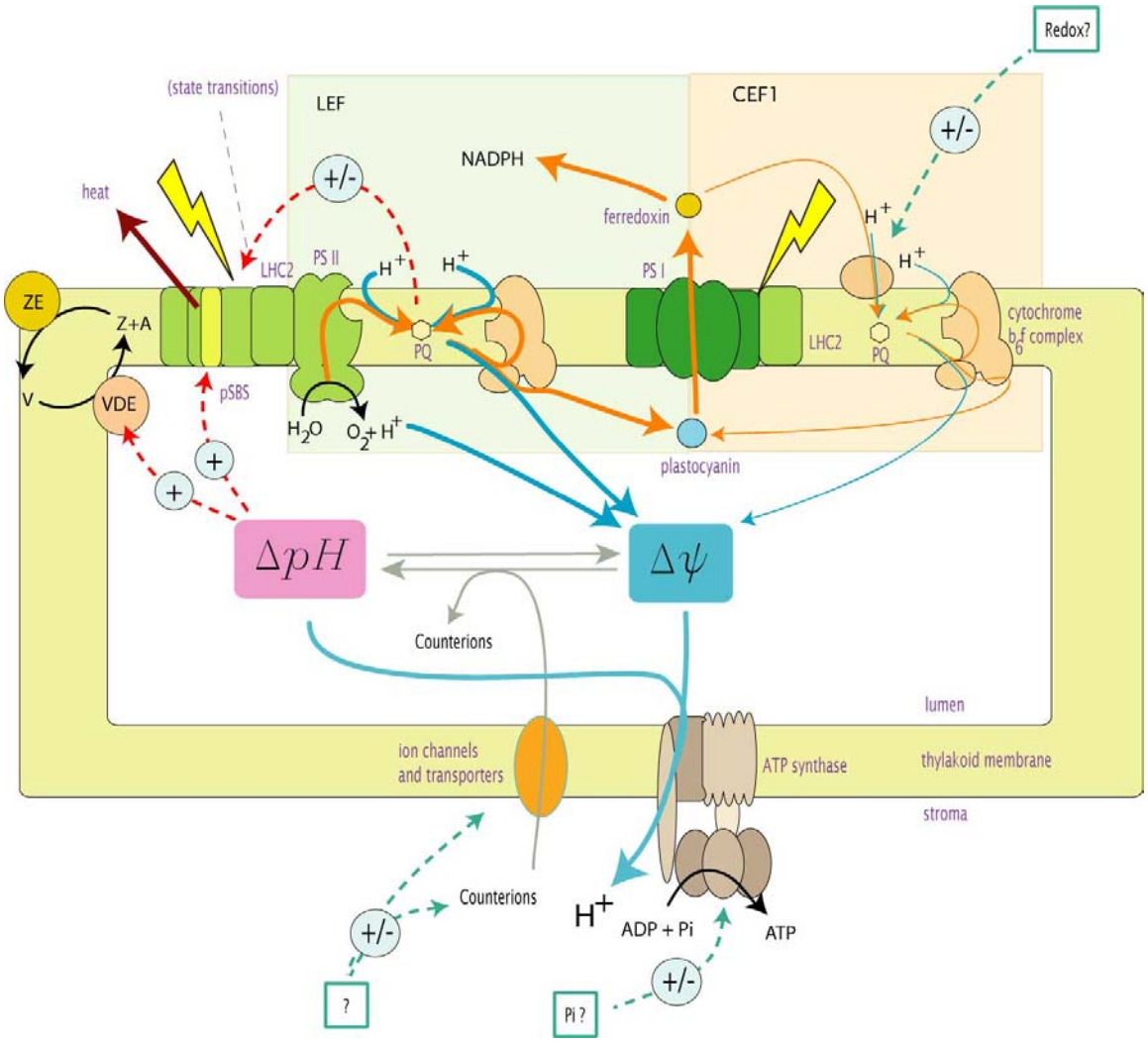
Summary and Challenges

To summarize, it is apparent from recent work that plants have taken advantage of a series (at least four) of mechanisms to achieve the flexibility required to meet the regulatory and energetic needs of the plant in a constantly fluctuating environment. Such mechanisms are applied differentially under specific conditions, e.g. engaging proton translocation when ATP/NADPH balance requires adjustment, whereas proton efflux from the lumen is slowed when increased q_E sensitivity is needed. The current challenge is to address the mechanisms by which each regulatory process is controlled, which at present are only vaguely understood. Also, widely different species will almost certainly utilize different flexibility mechanisms, as exemplified by comparisons of CEF1 among green algae, and C_3 and C_4 vascular plants (see above 68). We know little about these natural variations. Likewise, we need to answer the question: Do variations in these *pmf* regulatory mechanisms alter the fitness of a plant to its environment? For example, do changes in g_{H^+} affect cold acclimation? An obvious way to address many of these issues is to take advantage of new genetics and metabolomics approaches, in combination with the new *pmf* probes described above. At the same time, spectroscopists must continue to test and validate each of the probes described above. Finally, application of these new techniques is currently limited to laboratories with dedicated spectroscopists. Widely disseminating such tools in a simple-to-use form will greatly accelerate their use (as already seen for tremendously successful chlorophyll fluorescence probes) and no doubt lead to a clearer view of the roles of *pmf* in the plant.

Figure Legend

Figure 1. An overview of the photosynthetic proton and electron circuits**.** Transfer of absorbed light energy (lightning bolts) from the light harvesting complexes (LHC2) to photosystem II (PS II) and photosystem (PS I) oxidizes their reaction centers, driving the linear flux of electrons (LEF) from H_2O to NADPH. Depicted are the electron (*orange arrows*) and proton (*blue arrows*) flux circuits of LEF (*blue box*) and cyclic electron flux around PS I (CEF1, *red box*). Electrons originating from the oxidation of H_2O are transferred through PS II reducing plastoquinone (PQ) to a quinol (PQH_2), with uptake of protons from the stroma. Bifurcated oxidation of PQH_2 occurs at the cytochrome (cyt) b_6f complex. Half of the electrons are transferred through the high potential chain (i.e. Reiske Fe_2S_2 protein, cyt *f*), plastocyanin (PC), photoexcited PSI and ferredoxin to reduce $NADP^+$ to NADPH. The other half of the electrons will return to the PQ pool via the low potential chain. With CEF1, electrons from the reducing side of PS I are shunted from the $NADP^+$/NADPH pool to reduce PQ to PQH_2. The cycle is completed with bifurcated oxidation of PQH_2 at cyt b_6f and transfer of electrons to PS I via the high potential chain and PC. Oxidation of H_2O at PS II and PQH_2 at cyt b_6f releases protons into the lumen to establish a proton motive force (*pmf*). Since protons are charged, proton buffering will favor storage of *pmf* as electric field ($\Delta\psi$). However, $\Delta\psi$ will be collapsed by counterion movements that occur in response to it, via channels or transporters (*grey arrows*). Thus, with continued proton influx, the buffering capacity will be exceeded, favoring formation of the pH component of *pmf* (ΔpH). ATP synthesis is coupled to the flux of protons down their electrochemical gradient, via the CF_1-CF_O ATP synthase. Exciton transfer to the reactions centers may be controlled through

exciton dissipation by q_E (*brown arrow*) or changing the fraction of LHC2 associated with PS I and PS II (state transition). State transitions will depend on the redox poise of the PQ/PQH_2 pool, whereby reduction favors LHC2 association with PS I. Induction of q_E, the primary exciton regulatory pathway in higher plants, requires formation of ΔpH since it depends on the pH dependent activity of violaxanthin de-epoxidase (VDE), which reduces violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) and on protonation of PsbS. Several mechanisms have been proposed to augment the response of q_E to LEF: (a) increasing relative proton influx through CEF1, (b) decreasing relative proton efflux from the lumen via modulation of the proton conductivity of the ATP synthase and/or (c) partitioning *pmf* to favor ΔpH. It has been proposed that relative rates of CEF1 may be sensitive to or regulated by the redox balance of the stroma (*blue dashed arrows*), while decreased proton conductivity has been linked tentatively to low stromal concentrations of P_i. No definitive mechanism exists for dynamic control of partitioning, although a likely candidate may involve regulation of chloroplast ionic strength.


Redox?
(state transitions)
LEF
CEF1
+/-
NADPH
heat
ferredoxin
H+
LHC2
PS II
PS I
ZE
Z+A
V
VDE
pSBS
PQ
cytochrome b6f complex
H2O
O2+ H+
plastocyanin
ΔpH
Δψ
Counterions
lumen
ion channels and transporters
ATP synthase
thylakoid membrane
stroma
ADP + Pi
ATP
?
Pi ?

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CHAPTER 2: Modulation of Energy Dependent Quenching of Excitons (q_E) in Antenna of Higher Plants

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ABSTRACT

Energy dependent exciton quenching, or q_E, protects the higher plant photosynthetic apparatus from photodamage. Initiation of q_E involves protonation of violaxanthin de-epoxidase (VDE) and P*sbS*, a component of the photosystem (PS) II antenna complex, as a result of lumen acidification driven by photosynthetic electron transfer. It has become clear that the response of q_E to linear electron flow (LEF), termed "q_E sensitivity", must be modulated in response to fluctuating environmental conditions. Previously, three mechanisms have been proposed to account for q_E modulation: 1) The sensitivity of q_E to the lumen pH is altered; 2) Elevated cyclic electron flow around PS I (CEF1) increases proton translocation into the lumen and; 3) Lowering the conductivity of the thylakoid ATP synthase to protons (g_H^+) allows formation of a larger steady state *pmf*. Kinetic analysis of the electrochromic shift (ECS) of intrinsic thylakoid pigments, a linear indicator of transthylakoid $\Delta\psi$, suggests that when CO_2 alone was lowered from 350 ppm to 50 ppm CO_2, modulation of q_E sensitivity could be explained solely by changes in g_H^+. Lowering both CO_2 (to 50 ppm) and O_2 (to 1%) resulted in an additional increase in q_E sensitivity that could not be explained by changes in g_H^+ or CEF1. Evidence is presented for a fourth mechanism, where changes in q_E sensitivity result from variable partitioning of *pmf* into $\Delta\psi$ and ΔpH. The implications of this mechanism for the storage of *pmf* and the regulation of the light reactions are discussed.

Key words: cyclic electron flow, conductivity of ATP synthase, *pmf* partitioning

Abbreviations: CEF1- cyclic electron flow associated with PSI; DIRK- dark interval relaxation kinetic analysis; ECS- electrochromic shift of carotenoids; ECS_{inv}- inverted ECS signal; ECS_{ss}- steady state ECS signal; ECS_t- amplitude of light-dark ECS signal; g_{H^+}- conductivity of CF_0-CF_1 ATP synthase to proton efflux; H^+/e^-; proton to electron ratio; LC- low CO_2 (50 ppm CO_2, 21% O_2); LEA- low electron acceptor (50 ppm CO_2, 1% O_2); LEF-linear electron flow; NPQ- nonphotochemical quenching of excitation energy; *pmf*- proton motive force; q_E- energy dependent component of NPQ; VDE- violaxanthin de-epoxidase; $\Delta\psi$ & ΔpH- electric field and pH components of *pmf*.

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Introduction

The Dual Roles of the Intermediates of the Light Reactions of Photosynthesis

Plant chloroplasts convert light energy into two forms usable by the biochemical processes of the plant (1, 2). Redox free energy is stored by linear electron flow (LEF) through photosystem (PS) II, the cytochrome b_6f complex, PS I, ferredoxin and finally NADPH. Translocation of protons from the stroma to the lumen is coupled to LEF, resulting in the establishment of transthylakoid proton motive force (*pmf*), which drives the synthesis of ATP from ADP and P_i at the thylakoid CF_O-CF_1 ATP synthase (ATP synthase) (3). It has become clear that certain redox carriers and the *pmf* also play regulatory roles in photosynthesis. The redox status of the electron transfer chain regulates a range of processes via the thioredoxin system (4) and the plastoquinone pool (5). Meanwhile, the ΔpH component of *pmf* regulates the efficiency of light capture via protonation of thylakoid lumen proteins (6). The balancing of these two roles governs the development and efficiency of the photochemical machinery, as well as the avoidance of harmful side reactions.

The Need for Down Regulation of the Photosynthetic Apparatus

Plants are exposed to widely varying environmental conditions, often resulting in light energy capture that exceeds the capacity of the photosynthetic apparatus (7-10), which in turn can lead to photodamage (11, 12). Plants have evolved a series of mechanisms collectively known as non-photochemical exciton quenching, or NPQ (9), to

harmlessly dissipate excessively absorbed light energy as heat and thereby protect plants from photodamage.

'Energy-dependent' exciton quenching (i.e. dependent on the energization of the thylakoid membrane), termed q_E, is arguably the most important and well characterized component of NPQ in higher terrestrial plants (9, 13, 14), though other processes certainly contribute to photoprotection (e.g. state transitions and long-lived quenching phenomena, see ref. (9) for review). The initiation of q_E is dependent upon light-induced lumen acidification (9, 13, 14), which leads to protonation of two key proteins, violaxanthin deepoxidase (VDE) (15) and P*sbS*, a component polypeptide of the PS II-associated light harvesting complex (9, 16, 17). VDE is an integral enzyme of the xanthophyll cycle, and catalyzes the conversion of violaxanthin to antheroxanthin and further to zeaxanthin (18-22). The coincident accumulation of antheraxanthin and zeaxanthin with protonation of P*sbS* activates q_E (16). In the simplest model for q_E activation, photosynthetic proton transfer should increase *pmf*, acidifying the lumen and activating q_E, in effect feedback regulating light capture. If the kinetic constraints of such a model were held constant, a continuous relationship between q_E and LEF would be expected (23).

The Need for Flexibility in Antenna Down Regulation

In contrast, it is generally accepted that antenna down regulation must be flexible to cope with changing biochemical demands (22-25), i.e. that the response of q_E to LEF, which we term 'q_E sensitivity', is regulated. In the absence of such flexibility, the photosynthetic apparatus would be prone to catastrophic failures (23, 26). For example,

conditions which slow turnover of the Calvin-Benson cycle and restrict the availability of PS I electron acceptors should lower the rate of LEF, attenuating lumenal acidification and q_E (23). Subsequently, the increase in 'excitation pressure' (due to loss of quenching) at the reaction centers, compounded by the accumulation of reduced electron carriers, would result in increased photodamage (9). Thus, a flexible or dynamic relationship between q_E and LEF is essential and indeed has been demonstrated to be substantial (24, 26-31). For example, when CO_2 levels were lowered from ambient to near 0 ppm, the sensitivity of q_E to LEF increased by about 5-fold (23). From these observations, four models have been proposed to account for q_E modulation.

Model 1: Variable response of q_E to ΔpH. Changes in the aggregation state of antennae complexes (32) or in pK_a values of key amino acid residues on VDE or P*sbS* could alter the sensitivity of q_E to the ΔpH component of *pmf* (i.e. to lumen pH) (15). Alternatively, a simple change in the maximum activity of q_E-related enzymes (e.g. VDE) could alter q_E sensitivity (22).

Model 2: Modulation of the H^+/e^- ratio. The stoichiometry of protons per electron translocated through the linear pathway could be increased, thus achieving a higher *pmf* (and a more acidic lumen) for a given LEF. This could result from a change in the proton-to-electron stoichiometry (H^+/e^-) of the linear pathway itself, though this seems unlikely given our current understanding of the mechanisms of these processes (reviewed in ref. (15)). Alternatively, increased cyclic electron flow around PS I (CEF1), a process which translocates protons but does not result in net NADPH reduction, could acidify the lumen beyond the capacity of LEF (26). A third possibility is activation of the "Water-Water" cycle (WWC) or Mehler peroxidase reaction (33). In the WWC, electrons are

extracted from water at PSII and subsequently used to reduce O_2 back to water at the reducing side of PSI. Like CEF1, the WWC produces *pmf* without net reduction of $NADP^+$. While, in principle, the WWC can increase q_E, its activity will appear in our assays as LEF (see below) and thus will not affect 'q_E sensitivity' as we have defined it.

Model 3: Modulating conductivity of proton efflux. Because the extent of *pmf* in the steady state is determined by the relative flux of protons into and out of the lumen, changing the kinetic properties of the ATP synthase should alter q_E sensitivity (23). In particular, lowering the enzymatic turnover rate of this enzyme, or effectively its conductivity to proton efflux, should increase *pmf* for a given proton flux (23, 34). This, in turn would increase the sensitivity of q_E to LEF (and also to CEF1 or WWC). This group previously developed a non-invasive technique for estimating relative values of proton conductivity, designated g_{H^+} ((23) see also below). Using this technique, evidence was presented that modification of g_{H^+} by itself could account for essentially all q_E modulation in intact tobacco plants upon alteration of CO_2 levels from 2000 to 0 ppm, while maintaining ambient levels of O_2 (23).

Model 4: Variable partitioning of pmf. Recent work has argued that transthylakoid *pmf* contains significant contributions from the electric field component ($\Delta\psi$) (6, 35). It was further argued that varying the relative partitioning of *pmf* into $\Delta\psi$ and ΔpH would necessarily alter the sensitivity of q_E to total *pmf*. This model, as yet to be tested, states that ΔpH/*pmf* may change with physiological state.

In this work, we explore q_E modulation under low CO_2 and O_2, where several groups over the past few decades (24, 26-31) have observed enhanced sensitivity of q_E to LEF, and attributed this effect to increased activity of CEF1. In contrast, we did not

observe significant increases in CEF1, and concluded that increased q_E sensitivity under these conditions results mainly from changes in both g_H^+ and *pmf* partitioning.

Materials and Methods

Plant Material

Experiments were conducted at room temperature using wild type *Nicotiana tabacum* xanthi (tobacco) plants grown under greenhouse conditions, as described in (23), and dark-adapted over night prior to being used in spectroscopic assays. Young, fully expanded leaves, gently clamped into the measuring chamber of the spectrophotometer described below, were allowed to adjust to the chamber conditions for 5 minutes in the dark prior to being illuminated for ten minutes with actinic light at intensities ranging from 32-820 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). Steady state fluorescence and electrochromic shift (ECS) parameters were measured after this actinic period, after which, the actinic light was turned off for ten minutes in order to measure the fluorescence amplitude indicative of the quickly recovering component of NPQ, i.e. q_E (see below).

Gas Composition

Room air pumped into the measuring chamber was assumed to represent ambient conditions (~372 ppm CO_2/21% O_2). Premixed gases balanced with nitrogen were used to alter the gas composition in the measuring chamber and create a pseudo micro-climate of either 50 ppm CO_2/21% O_2 or 50 ppm CO_2/1% O_2. In all cases the stream of air

entering the measuring chamber was first bubbled through water in order to avoid leaf dehydration.

Spectroscopic Assays

The methods for measuring extents of q_E, rates of LEF, and the relative extents of *pmf* components were as described in (23) except that a newly-developed instrument was used. This instrument, which is preliminarily described in (36), was based on the Non-Focusing Optics Spectrophotometer (NoFOSpec) (37). The current instrument has been modified to allow near-simultaneous measurements of absorbance changes at four different wavelengths. This was accomplished by aiming four separate banks of light emitting diodes (LEDs, HLMP-CM15, Agilent Technologies, Santa Clara, CA), each filtered through a separate 5 nm bandpass interference filter (Omega Optical, Brattleboro, VT), into the entrance of a compound parabolic concentrator. The photodiode detector was protected from direct actinic light by a Schott BG-18 filter. Current from the photodiode was converted to a voltage by an operational amplifier and the resulting signal was AC-filtered to remove background signals, and sampled by a 16-bit analog-to-digital converter on a personal computer data acquisition card (DAS16/16-AO, Measurement Computing, Middleboro, MA). Timing pulses were generated by digital circuitry (PC card D24/CTR 3, Measurement Computing, Middleboro, MA) controlled by software developed in-house. The duration of the probe pulses was set at 10 μs. Actinic illumination was provided by a set of 12 red LEDs (HLMP-EG08-X1000, Agilent Technologies, Santa Clara, CA) and controlled by the timing circuitry. Measuring pulses were typically given at 1-10 ms intervals.

Absorbance changes at only one wavelength, 520 nm, were used to estimate rapid (<1s total trace time) changes in ECS, where its signal predominates on this timescale (37). For longer traces, significant contributions from light scattering have been observed (37). To correct for this, absorbance changes of three wavelengths, 505, 520 and 535 nm were collected. The three wavelength traces were recorded near-simultaneously, with each LED band being pulsed in sequence at 10 ms intervals. Each complete set of three pulses was deconvoluted using the procedure described in (35, 38) to obtain estimates of ECS.

The instrument was also used to measure changes in chlorophyll *a* fluorescence yield using the 520 nm LED bank as a probe beam, as described in (37). Saturation pulses (>30,000 μmole photons m^{-2} s^{-1} PAR) were imposed using light from an electronically shuttered xenon arc lamp, filtered through heat absorbing glass. Actinic light was filtered out using an RG-695 Schott glass filter. Saturation pulse-induced fluorescence yield changes were interpreted as described in (39, 40). The quantum yield of PSII photochemistry (Φ_{II}), a measure of the efficiency of PSII electron transfer per quanta absorbed, and estimates of LEF were calculated as described in (39) and (41), respectively. It should be noted that estimates of LEF made this way contain contributions from the WWC but not from CEF1. The q_E component of NPQ was calculated from the saturation-pulse induced maximum fluorescence yields during steady state illumination (F_m') and ten minutes (F_m") after switching off the actinic light (9, 40).

In vivo Measurements of Proton Flux and *pmf* Characteristics

This work and analyses are made possible by newly introduced techniques that allow us to non-invasively probe the 'proton circuit' of photosynthesis. The theoretical framework for these methods is discussed in (6, 23, 35, 37, 42) and briefly reviewed here. These techniques take advantage of the electrochromic shift (ECS, sometimes called ΔA_{520} or ΔA_{518}) of certain carotenoid species that naturally occur in the thylakoid membranes. The ECS is a linear indicator of changes in transthylakoid $\Delta\psi$ (43, 44) and is particularly useful for our studies because it responds to the transthylakoid movement of protons, as well as other charged species.

We probed the ECS using a previously described technique called Dark Interval Relaxation Kinetic (DIRK) analysis (42), in which steady-state photosynthesis is perturbed by short (typically 0.5 s), dark intervals, allowing the photosynthetic apparatus to relax in ways that reveal information about the system in the steady-state (42). The parameter ECS_t was obtained by taking the total amplitude of the rapid phase of ECS decay from steady state to its quasi-stable level after about 300 ms of darkness (23). As previously discussed, ECS_t should reflect total light-dark *pmf* (i.e. $\Delta\psi + \Delta pH$) (6, 23, 35).

The DIRK technique can also reveal information about the relative conductivity of the ATP synthase to protons, a parameter termed g_{H^+} (23, 35). Since the ATP synthase is the highest conductance proton efflux pathway, decay of the ECS reflects flux through this enzyme (6, 45). ECS decay kinetics during a DIRK experiment are well fit by first-order decay curves, making it possible to approximate the kinetic behavior as a first-order process, i.e. a process that obeys Ohm's law. In this case, we can use a simple force-flux expression to describe the decay (35):

$$pmf \propto \nu_{H+} \cdot 1/g_H^+ = \nu_H^+ \cdot \tau_{ECS} \quad (1)$$

where ν_H^+ represents the flux (current) of protons into and out of the lumen[1] driven by LEF and τ_{ECS} is the decay time of the ECS upon a light-dark transition and is proportional to the 'resistance' (i.e. the inverse of the conductivity, or g_H^+) of the ATP synthase to proton efflux (23).

If H^+/e^- remains constant (42), then the proton flux associated with LEF should be proportional to LEF itself. Taking into account the effective rate constant for proton efflux, or g_H^+, we can then estimate the *pmf* attributable to LEF, or pmf_{LEF} (23) by:

$$pmf_{LEF} \propto LEF/g_H^+ \quad (2)$$

The value of pmf_{LEF} should be proportional to total *pmf* if contributions from CEF1 are also constant. Moreover, a continuous relationship between q_E and pmf_{LEF} would be expected if CEF1, the antenna response to lumen pH, and the relative fraction of *pmf* stored as ΔpH all remain constant, i.e. a deviation in the relationship between pmf_{LEF} and q_E would indicate the participation of other factors, notably activation of models 1, 2 or 4 (23).

DIRK analysis over longer periods of darkness can reveal information regarding the Δψ and ΔpH components of *pmf* (6, 35). Initially, after the onset of illumination, *pmf* is stored predominantly as Δψ, since most protons are buffered and the capacitance of the membrane is relatively low (15). Over time, Δψ relaxes due to relatively slow

[1] At steady state the rate of proton accumulation in the lumen from electron transfer is equal to its rate of efflux.

movements of counterions, allowing the accumulation of free protons and subsequent buildup of ΔpH (6). When the actinic light is rapidly shuttered, proton translocation into the lumen is rapidly halted, but proton efflux continues until *pmf* either completely collapses or comes into equilibrium with the ATP/ADP+P_i couple via the ATP synthase. Because of lumenal proton buffering, Δψ will collapse more rapidly than ΔpH. Even after steady-state Δψ is dissipated, ΔpH will continue to drive proton efflux, establishing an 'inverse' Δψ, positive on the stromal side of the thylakoid membrane. In our measurements, this inverse Δψ phase is measured as an 'inverted' ECS signal, termed ECS_{inv}. Under appropriate conditions (6, 35), the extent of the inverted Δψ should be proportional to the light-driven ΔpH component of *pmf*. We thus used the amplitudes of ECS kinetic components as estimates of light-driven Δψ and ΔpH. ECS kinetics *in vivo* suggest that about 50% of the *pmf* is stored as Δψ (35).

Results and Discussion

Changing the Levels CO_2 and O_2 Alters q_E Sensitivity

Figure 1 shows a plot of q_E against LEF under three atmospheric conditions: 1) ambient (372 ppm CO_2, 21% O_2), 2) low CO_2 (LC, 50 ppm CO_2 and 21% O_2) and 3) low electron acceptor (LEA, 50 ppm CO_2 and 1% O_2). Changing from ambient to LC conditions led to a decrease in the LEF required to achieve $q_E = 0.8$, from about 160 to 90 μmole electrons m^{-2} s^{-1}, representing a ca. 2-fold increase in the sensitivity of q_E to LEF, similar to our previous results under these conditions (23). LEA treatment further increased q_E sensitivity, and a $q_E = 0.8$ was achieved at a LEF of about 30 μmole

electrons m^{-2} s^{-1}, about a 6-fold increase in sensitivity over ambient conditions. The magnitude of this effect was similar to that observed previously, upon lowering CO_2 to essentially 0 ppm, while maintaining O_2 at 21% (23). Our observations are also qualitatively consistent with those of Heber and coworkers (24, 26, 28, 31), who noted that, when both CO_2 and O_2 levels were lowered, total NPQ increased, even though LEF had decreased.

Figure 1 also shows that g_H^+, as estimated from the ECS decay kinetics upon a rapid light-dark transition, decreased by about two-fold, which was sufficient to explain the observed increase in q_E sensitivity from ambient to LC conditions ((23) and below). A further, approximately 2-fold, decrease in g_H^+ accompanied the increase in q_E sensitivity upon transition from LC to LEA conditions. These results are consistent with Model 3, i.e. that changes in g_H^+ alter q_E sensitivity, as previously argued (23).

Heber and coworkers hypothesized that increased q_E sensitivity under LEA conditions was best explained by an increase in proton translocation into the lumen as a result of CEF1 (i.e. Model 2) (24, 26, 28). Figure 2 shows that the relationship between light-induced *pmf*, as estimated from ECS_t, and pmf_{LEF}, i.e. LEF-attributable *pmf* as estimated by Eqn. 2 was, within the noise level, continuous and depended very little on gas composition. The simplest interpretation for these results is that, contrary to Model 2, the relative contributions of CEF1 to proton flux did not change appreciably when CO_2, or when both CO_2 and O_2 were lowered.

Figure 3 shows that the q_E responses as a function of steady state light-induced *pmf* were very similar (essentially continuous) under ambient and LC conditions, as previously observed (23). This strongly suggests that lowering CO_2 alone did not alter

the response of the antenna to *pmf*, consistent with the previous suggestion that changes in g_H^+ could solely account for the majority of q_E modulation upon altering CO_2 levels (23). These results argue against Models 1 and 4 under ambient and LC conditions (23).

In contrast, under LEA conditions q_E was notably more sensitive to light-induced *pmf* (Fig. 3). These data, together with that in Figure 2, implied that q_E sensitivity changes under LEA conditions could not solely be attributed to changes in g_H^+ or CEF1. Instead, the response of q_E to *pmf* appears to have changed. Overall, these results are consistent with either Model 1 or 4 having a role under LEA conditions.

Evidence for Variable Partitioning of *pmf*

We next used an analysis of the ECS decay kinetics developed in our earlier work to estimate the fractions of light-induced *pmf* stored as $\Delta\psi$ and ΔpH ((15, 35) see also Materials and Methods) in order to distinguish between Models 1 and 4 under LEA conditions. The inset to Fig. 4 shows ECS kinetic traces upon rapid light-dark transitions at 520 μmole photons m^{-2} s^{-1} under ambient (Trace A) and LEA (Trace B) conditions. The fraction of *pmf* attributable to ΔpH was ~ 0.3 under ambient and LC (not shown) conditions, reasonably consistent with previous observations (35). On the other hand, the fraction of *pmf* attributable to ΔpH appeared to increase by about 2-fold (~ 0.69) under LEA conditions. This is consistent with Model 4, where the sensitivity of q_E increases under LEA conditions by altering the balance of transthylakoid $\Delta\psi$ and ΔpH.

Figure 4 also shows that the relationship between q_E and our estimate of light-induced ΔpH (ECS_{inv}) remained essentially constant (continuous) under all atmospheric and light conditions. These results strongly suggest that the antenna responses (i.e. at the

level of the pK_a's for VDE and/or P*sbS* protonation) to lumen pH and the relative activity of the enzymes controlling the xanthophyll cycle are constant over ambient, LC and LEA conditions. Taken together, these data argue against Model 1 and instead suggest a role for Model 4, a new mode of modulating q_E sensitivity involving variability in the relative partitioning of *pmf* into $\Delta\psi$ and ΔpH.

Conclusions

No Evidence for Increases in Steady-state CEF1 Under LEA Conditions

A widely cited mechanism of q_E modulation is that CEF1 is more engaged under LEA conditions (24, 26-31) (i.e. Model 2). However, for Model 2 to fully account for the observed 5-6-fold increase in q_E sensitivity (Fig. 1), the turnover rate of the CEF1 pathway would have to increase to several times that of LEF. In contrast, we found little change in the relationship between our estimates of total *pmf*, based on ECS_t, and the *pmf* calculated from Eqn. 2 (Fig. 2). These results imply a constant, fractional turnover of CEF1 (see below) and are therefore inconsistent with a substantial role for changes in CEF1 modulating q_E sensitivity.

Despite the existence of viable models for CEF1 (e.g. 46), evidence for its involvement in q_E modulation is mixed (47-57). In green algae (e.g. *Chlamydomonas*) and cyanobacteria (58, 59), as well as in C_4 plant bundle sheath chloroplasts (52), there is strong evidence for participation of CEF1 in ATP synthesis. The situation in C_3 vascular plants is more confusing. The general consensus based on steady-state comparisons of LEF with PSI activity, cyt b_6f electron transfer (38) or overall proton translocation (38), is

that CEF1 appears to be either negligible or a constant fraction of LEF ((42) but see (60)). Our data generally supports this view. On the other hand, Joliot and Joliot (60) and Makino et al. (61) presented evidence for high CEF1 rates, approaching those of LEF, during the early stages of photosynthetic induction from dark adapted states.

One possibility, which could reconcile these two opposing views, is that CEF1 has a high potential capacity but is tightly regulated in the steady state. In fact, we argue that this situation would be expected since proton efflux from the lumen is tightly coupled to ATP synthesis at the ATP synthase (62). In the steady-state, where consumption of products is matched by their production (63), any increase in proton translocation by CEF1 would require a proportional increase in ATP consumption relative to that of NADPH. This is, of course, the default situation in e.g. C4 bundle sheath cells where ATP, but not reducing power, is needed. In C3 plants, changes in ATP/NADPH out put would necessarily require differential engagement of processes that consume variable ratios of ATP/NADPH, e.g. nitrite reduction, maintenance of ion gradients, etc. Such processes may indeed impose a requirement on flexibility at the level of the light reactions, in which CEF1 may play an important role (64). However, the overall flux through these alternate processes under most conditions is considerably smaller than that through CO_2 fixation in the steady state. During induction, on the other hand, metabolite pools undergo rapid changes, allowing for larger changes in the relative biochemical demands for ATP and NADPH, perhaps imposing substantial changes in CEF1:LEF.

It is worth emphasizing that, while changes in the fractional turnover of CEF1 do not appear to impact q_E sensitivity under our conditions (Figs. 1 and 2), changes in other

modes of q_E modulation will alter the impact of proton translocation, regardless of whether it arises from LEF or from CEF1 (see below). Thus, even a low, constant engagement of CEF1 in the steady state will contribute to the triggering of q_E.

Under a Wide range of Conditions, q_E Sensitivity Changes are Attributable to Modulation of g_H^+

Previously, we have observed substantial changes in g_H^+, which could on their own account for the observed changes in q_E sensitivity (23). We concluded that the CF_O-CF_1 ATP synthase plays a central role in transmitting information about the biochemical status of the stroma to the light reactions. We proposed that decreases in electron acceptor availability cause decreases in g_H^+ which lead to increases in *pmf* at a given LEF, and ultimately to increases in q_E (23). Our current data supports this view, in that, the majority of q_E modulation can be accounted for by changes in g_H^+, especially between ambient and LC conditions (see Figs. 1 and 2).

A New Mechanism of Modulating q_E Sensitivity

In contrast to ambient and LC conditions, q_E appeared more sensitive to light-induced *pmf* under LEA conditions (Fig. 3, closed circles), suggesting that a factor, in addition to changes in g_H^+, influences q_E sensitivity. The data in Fig. 4 suggests that this additional factor is not a change in the response of the antennae to lumen pH or a change in the activities of the enzymes controlling the xanthophyll cycle, but is rather a relative increase in the fraction of *pmf* partitioned into the ΔpH component. Until recently such variable parsing of *pmf* would have seemed inconceivable because the *pmf* was

considered to be composed almost completely of ΔpH, i.e. the Δψ component was considered negligible in thylakoids (6, 15, 35). However, a number of lines of evidence suggest that about half of the *pmf* is stored as Δψ (6, 15, 23, 35). We previously argued that relative changes in the fraction of *pmf* held as ΔpH would alter q_E sensitivity (15, 35). The data in Figs. 3 and 4 are the first *in vivo* evidence for such variable *pmf* partitioning and its expected consequences for regulation of the light reactions. Moreover, our results support the view that the fraction of *pmf* stored as Δψ and ΔpH is important in balancing the dual roles of the *pmf* in allowing sufficient driving force for ATP synthesis while maintaining the pH of the lumen within a range where it can regulate light capture via q_E (15, 35).

The Physiological Basis of q_E Modulation

In this work, we chose to study LEA conditions because they have been previously proposed to support large increases in CEF1. There are good arguments that in terrestrial higher plants, reducing both CO_2 and O_2 to such low levels is unlikely because consumption of O_2 by respiration will produce CO_2 whereas photosynthesis will liberate O_2, while the conductivity of the stomata to the two gases is very similar (65). Aquatic plants, on the other hand, may routinely experience such conditions (66). Lowering CO_2 levels to nearly zero, while maintaining O_2 at 21% induced a change in g_{H^+} (23) comparable to that seen under our LEA condition (Fig. 1), while not inducing the apparent change in *pmf* partitioning seen here (Fig. 3). One explanation to account for this difference is that g_{H^+} cannot be decreased below that seen at 0 ppm CO_2, and other mechanisms must be activated to further increase q_E sensitivity.

Low O_2 may also have secondary effects, in addition to simply reducing PS I electron acceptors, especially at the levels of photorespiration (67) and the WWC (61). Changes in flux through either of these processes would alter the output of ATP/NADPH, and this in turn may effect or trigger changes in *pmf* partitioning. These arguments suggest that this type of imbalance may be seen under other, more physiological, conditions. Indeed, in preliminary work, we have noted changes in ΔpH/*pmf* in intact tobacco and cucumber leaves under wilting conditions (data not shown), hinting at a physiological role.

The mechanism by which thylakoid *pmf* partitioning is accomplished remains unresolved, though *in vitro* experiments have indicated that stromal ionic balance and the lumen proton buffering capacity are likely major effectors (6, 35). This view is consistent with the role of ion homeostasis in maintaining ΔpH and Δψ across eukaryotic and prokaryotic plasma membranes, and we proposed that similar mechanisms work in chloroplasts *in vivo* (reviewed in refs. (6, 35)). By extrapolation, any process which affects ionic balance or lumen proton buffering, either as a consequence of regulation or altered metabolism, could change *pmf* partitioning and thus q_E sensitivity.

The mechanism by which g_{H^+} is influenced by the stromal status is also unclear, but a reasonable working model involves modulation of stromal P_i (a substrate for the ATP synthase) levels (23). It has been proposed some time ago that sequestration of stromal P_i levels into metabolic pools plays a critical role in controlling or regulating both the light and dark reactions of photosynthesis under a variety of conditions (68). If our model proves correct, changes in g_{H^+} (possibly via P_i sequestration) would then constitute an important regulatory link between the light and dark reactions of photosynthesis.

'Balancing' the Two Roles of the *pmf*

The *pmf* is a key intermediate in both energy transduction and feedback regulation of the light reactions. Our results support the view that 'balancing' these two roles plays an important role in maintaining the efficiency and productivity of photosynthesis and avoiding harmful side reactions. Under moderately restrictive conditions, e.g. when lowering CO_2 levels alone, modulation of g_H^+ appears to alter the relationship between light-driven proton flux and the resulting *pmf*. Under more extreme limitations, i.e. when O_2 is also lowered, the relationship between *pmf* and lumen pH appears to be altered. Both mechanisms have the effect of increasing the feedback regulatory effects of limited proton flux.

Acknowledgements: The authors wish to thank Professors Gerald Edwards, John Browse and Krishna Niyogi for important discussions.

Figure Legends

Figure 1. Energy-dependent antenna downregulation (q_E) as a function of linear electron flow (LEF). Measurements of q_E exciton quenching and LEF were performed on intact leaves of tobacco plants over light intensities ranging from 32-820 μmol photons m^{-2} s^{-1}, as described in the text. Gas compositions were 372 ppm CO_2/21% O_2 (open squares), 50 ppm CO_2/21% O_2 (open triangles), 50 ppm CO_2/1% O_2 (closed circles). The sizes of the spheres surrounding the symbols have been set proportional to the conductivity of the ATP synthase to protons (g_H^+) as estimated by the inverse of the decay lifetime of the electrochromic shift signal, as described in the text. The largest diameter symbol was approximately 12.1 s^{-1}, while the smallest was approximately 3.3 s^{-1}.

Figure 2. Total light-induced *pmf* as a function of the *pmf* attributable to LEF. The ECS_t parameter was taken as a measure of light induced *pmf*, whereas the independent measure of *pmf* or pmf_{LEF} (LEF/g_H^+) was derived from analysis of fluorescence and the kinetics of ECS decay upon a rapid light to dark transition (see Materials and Methods). The symbols and conditions are the same as in Figure 1. The error bars represent standard deviations for n = 3-5.

Figure 3. Energy-dependent antenna downregulation (qE) as a function of light-induced *pmf*, as estimated by the ECS_t parameter. Light induced *pmf* (ECS_t) values were derived from analysis of ECS decay kinetics as described in Materials and Methods. The

symbols and conditions were as in Figure 1. The error bars represent standard deviation for n = 3-5.

Figure 4. The relationship between energy-dependent antenna downregulation (q_E) and the ΔpH component of light-induced *pmf*, as estimated by the ECS_{inv} parameter. The symbols and conditions are the same as in Figure 1. The error bars represent SD for n = 3-5. Inset: Kinetic traces of the ECS signal, deconvoluted as described in the text, upon a light-dark transition from steady-state illumination. The extents of the steady state signal (ECS_{ss}) and the inverted region of the signal (ECS_{inv}), which are thought to be proportional to the light-induced Δψ and ΔpH components of *pmf* respectively, are indicated by the vertical arrows. The traces were taken at actinic light intensity of 520 μmol photons m^{-2} s^{-1} at ambient (A) and LEA (B) conditions respectively.

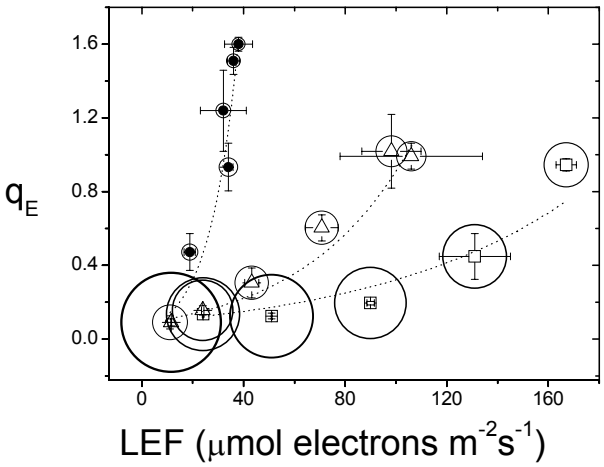


Figure 1

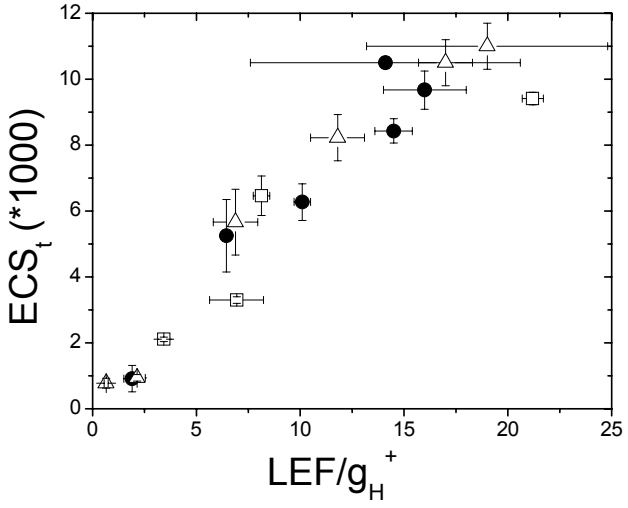


Figure 2

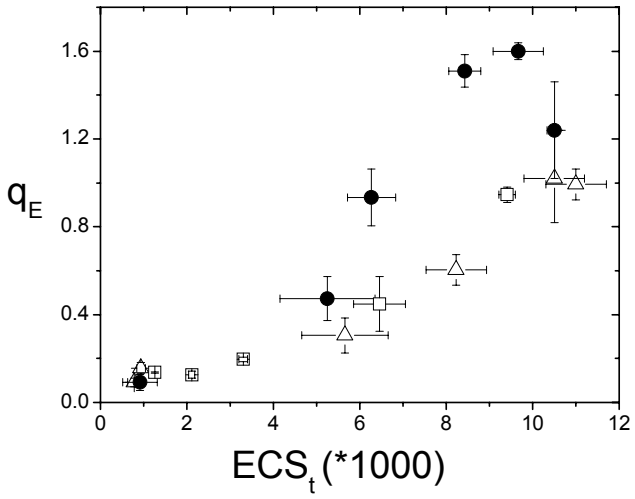
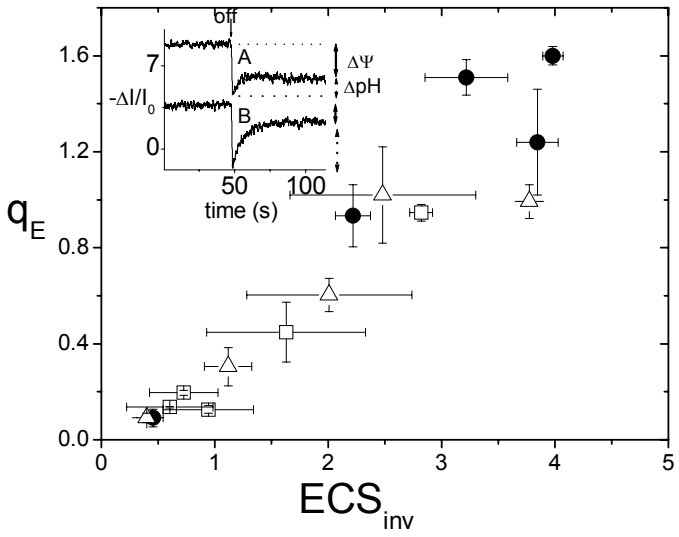


Figure 3


off
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0
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ΔpH
50
100
time (s)
qE
ECSinv
0.0
0.4
0.8
1.2
1.6
0
1
2
3
4
5

Figure 4

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CHAPTER 3: Regulating the Proton Budget of Higher Plant Photosynthesis

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ABSTRACT

In higher plant chloroplasts, transthylakoid proton motive force serves both to drive the synthesis of ATP and to regulate light capture by the photosynthetic antenna to prevent photodamage. *In vivo* probes of the proton circuit in wildtype and a mutant strain of *Arabidopsis thaliana* show that regulation of light capture is modulated primarily by altering the resistance of proton efflux from the thylakoid lumen, whereas modulation of proton influx via cyclic electron flow around photosystem I is suggested to play a role in regulating the ATP/NADPH output ratio of the light reactions.

Key words: cyclic electron flow, conductivity of the ATP synthase, modulation of q_E sensitivity

Abbreviations: CEF1- cyclic electron flow associated with PSI; ECS- electrochromic shift; ECS_t-total amplitude of the ECS change after a 300 ms dark perturbation from steady state; g_H^+- conductivity of CF_1-CF_O ATP synthase to proton efflux as measured by ECS decay; LC- low CO_2 (50 ppm CO_2, 21% O_2); LEF-linear electron flow; PAR, photosynthetically active radiation; PSI and PSII- photosystems I and II; *pmf*- proton motive force; pmf_{LEF}- *pmf* generated by LEF; q_E- 'energy dependent' non-photochemical quenching; $\Delta\psi$ and ΔpH- electric field and pH components of *pmf*

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Introduction

The Light Reactions of Photosynthesis

Photosynthesis converts light energy into chemical energy, ultimately powering the vast majority of our ecosystem (1). Higher plant photosynthesis is initiated via absorption of light by antennae complexes that funnel the energy to photosystem II (PSII) and photosystem I (PSI). The photosystems operate in sequence with the plastoquinone (PQ) pool, the cytochrome b_6f complex and plastocyanin, to oxidize H_2O and reduce $NADP^+$ to NADPH in what is termed linear electron flow (LEF). LEF is coupled to proton translocation, establishing a transthylakoid electrochemical gradient of protons, termed the proton motive force, or *pmf* (2), comprised of electric field ($\Delta\psi$) and pH (ΔpH) gradients (3).

Dual Role of the *pmf*

The *pmf* plays two central roles in higher plant photosynthesis (4). First, *pmf* drives the normally endergonic synthesis of ATP via the CF_1-CF_0 ATP synthase (ATP synthase) (5). Both the ΔpH and $\Delta\psi$ components of *pmf* contribute to ATP synthesis in a thermodynamically, and probably kinetically, equivalent fashion (6). Second, *pmf* is a key signal for initiating photoprotection of the photosynthetic reaction centers via energy dependent quenching of antennae excitons, or q_E, a process that harmlessly dissipates excessively absorbed light energy as heat (7-10). Only the ΔpH component of *pmf*, via acidification of the lumen, is effective in initiating q_E, by activating violaxanthin de-epoxidase (VDE), a lumen-localized enzyme which converts violaxanthin to

antheraxanthin and zeaxanthin, and by protonating lumen-exposed residues of PsbS, a pigment-binding protein of the PS II antenna complex (11).

A Need for Flexibility in the Light Reactions

A major open question concerns how the light reactions achieve the flexibility required to meet regulatory needs and match downstream biochemical demands (12). In LEF to $NADP^+$, the synthesis of ATP and the production of NADPH are coupled, producing a fixed ATP/NADPH output ratio. LEF alone is probably unable to satisfy the variable ATP/NADPH output ratios required to power the sum of the Calvin-Benson cycle (13, 14) and other metabolic processes that are variably engaged under different physiological conditions (12, 15, 16). Failure to match ATP/NADPH output with demand will lead to buildup of products and depletion of substrates for the light reactions, leading to inhibition of the entire process.

The generation of *pmf* is likewise coupled to LEF, so it is clear that the sensitivity of antenna regulation (or q_E) must also be modulated in some way to avoid catastrophic failure of photoprotection (12, 15, 17-19). Longer term acclimation of the q_E response can involve altering the sensitivity of the regulatory machinery to lumen pH by changing the xanthophyll pigment and/or PsbS levels (12, 20). However, dramatic changes in light intensity and/or CO_2 availability can occur over the seconds-to-hours time scale (8), requiring short-term adjustments. Indeed, it has been demonstrated that short-term alteration of CO_2 or O_2 levels can strongly modulate (by up to 6-fold) the sensitivity of q_E with respect to LEF (17, 18).

Two Types of Flexibility Mechanisms

Two general types of models have been proposed to account for the flexibility required to meet these changing demands (12). In 'Type I' mechanisms, proton flux into the lumen is increased via alternate electron transfer pathways, especially cyclic electron flow around PSI (CEF1), a mechanism that returns electrons from PSI to the PQ pool, thereby increasing the magnitude of the *pmf* relative to that generated by LEF alone (12). For C_3 vascular plants, CEF1 has been suggested to supply the relatively small fluxes (10-15% of that supplied by LEF) of protons required to balance ATP/NADPH output for the Calvin-Benson cycle and nitrogen assimilation (13, 14). It is a matter of intense debate (21, 22) as to whether CEF1 can run at sufficiently high rates to alter q_E responses by up to 6-fold, especially given the expected large ATP/NADPH imbalances such large fluxes would likely incur (12, 16).

In Type II mechanisms, lumen acidification with respect to LEF is adjusted without changing the relative flux of protons into the lumen, thus modulating q_E sensitivity without impacting ATP/NADPH output. This is thought to be achieved by varying either the proton conductivity of the ATP synthase (g_{H^+}), i.e. the inverse of the resistance to proton efflux from the lumen, or the relative fraction of *pmf* stored as ΔpH (12, 16-18, 22).

Probing the *pmf* to Gain Insight into the Flexibility Mechanisms

Recently a series of *in vivo* probes of the *pmf* have been introduced (2, 3, 16, 23-26), allowing contributions from Types I and II flexibility mechanisms to be directly assessed. These techniques are based on kinetic analyses of the 'electrochromic shift'

(ECS) (24) of photosynthetic pigments, which yields absorbance changes proportional to changes in transthylakoid $\Delta\psi$ (27). Several useful parameters can be obtained from analysis of ECS decay kinetics during brief dark perturbations of the steady-state, including estimates of the relative flux of protons through the ATP synthase (v_{H+}, which at steady-state equals flux of protons into the lumen), the magnitude of the light-induced *pmf*, the fraction of *pmf* stored as ΔpH and $\Delta\psi$, and g_H^+ (3, 16-18, 23, 24, 26). Combined with standard chlorophyll *a* fluorescence assays, from which estimates of LEF can be obtained (28), one can calculate the *pmf* generated by LEF alone (i.e. $pmf_{LEF} = LEF/g_H^+$), a key parameter for estimating fractional changes in CEF1 turnover (17, 18).

Using these probes of the proton circuit, it was shown that in intact *Nicotiana tabacum* (tobacco) leaves, lowering atmospheric CO_2 from 372 to 0 ppm led to a ~5-fold increase in the dependence of q_E on LEF (17). The effect could be entirely accounted for by a proportional (i.e. 5-fold) decrease in g_H^+, so that even modest rates of LEF generated a substantial *pmf* and a robust q_E response (17, 18). A similar (~6-fold) change in q_E sensitivity was observed when both O_2 and CO_2 were lowered (to 1% and 50 ppm respectively), but in this case, both changes in g_H^+ and increased partitioning of *pmf* into ΔpH were invoked to explain the effect (18). In both cases the ratio of v_H^+/LEF remained essentially constant (within noise levels), indicating that contributions from CEF1 to proton flux were either small or remained a relatively constant fraction of those from LEF, as previously found for tobacco (23). On the whole, these results support a large role for Type II mechanisms in modulating q_E sensitivity upon short term changes in CO_2/O_2 levels, but they do not rule out smaller contributions from Type I mechanisms in balancing ATP/NADPH output (12, 16, 26).

On the other hand, Munekage et al. recently presented partial characterization of a mutant strain of *Arabidopsis thaliana*, termed *pgr*5 for proton gradient regulation, which showed two provocative phenotypes (29, 30). First, non-photochemical reduction of the PQ pool, attributed to the key step in CEF1, was inhibited in *pgr*5. Second, q_E was severely diminished. It is reasonable to hypothesize that loss of PGR5 blocks CEF1 and thereby abolishes a significant flux of protons needed to activate q_E (29, 30). Evidence for such a hypothesis would support a large role for Type I mechanisms in modulating q_E sensitivity (31), while arguing against Type II models (12, 17, 18). On the other hand, mutation of *pgr*5 could indirectly affect q_E by disrupting downstream processes and modulating metabolic pool sizes (29, 30). Here we present the first experimental test for causal links between the loss of PGR5, steady-state proton flux and the q_E response, allowing us to determine the relative roles of Type I and II flexibility responses.

Materials and Methods

Plant Strains and Growth Conditions

Wild type *A. thaliana* (Wt-background strain *gl*1) (29) and *pgr*5 plants were grown in chambers under a 16:8 photoperiod at an average of ~70 μmol photons $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) and at 23°C. Wt (*gl*1) and *pgr*5 seeds were a gift from Dr. T. Shikanai (Nara Institute of Science and Technology, Ikoma, Nara Japan).

Spectroscopic Assays

Fully expanded leaves from ~23-26 day old plants were used in spectroscopic assays. Room air (372 ppm CO_2/21% O_2) or premixed gases from cylinders (i.e. 50 ppm CO_2/21% O_2) were bubbled through water (for humidification) prior to entering the measuring chamber of the spectrophotometer. Leaves were clamped into the measuring chamber of a non-focusing optics spectrophotometer/chlorophyll fluorometer, specifically designed for use on leaves (17, 18, 32). Leaves were first exposed to 26-216 μmol photons $m^{-2}s^{-1}$ PAR from a series of red light emitting diodes (maximum emission wavelength of 637 nm) to reach steady-state conditions (10 minutes). Further pre-illumination had little additional effect. After this actinic period, the steady-state (F_s) and light saturated (F_M') levels of chlorophyll *a* fluorescence yield were obtained (17, 18), from which estimates of the efficiency of PSII photochemistry (Φ_{II}) were calculated (28). Estimates of LEF were obtained using Φ_{II} as in (33). Analyses of the ECS decay kinetics upon perturbation of the steady state with a ~300 ms dark period were performed as described in (17, 18, 24). Absorbance changes at 505, 520, and 535 nm were recorded in series and those attributable to changes in ECS were deconvoluted from background signals according to the following equation (23, 24):

$$\Delta ECS = -\Delta I/I_{o(520)} - ((-\Delta I/I_{o(535)} + -\Delta I/I_{o(505)})/2)) \quad (1)$$

An estimate of steady-state, light-induced *pmf*, termed ECS_t, was taken as the total amplitude of ECS decay from its steady-state level to its minimum quasi-stable level after ~300 ms dark period (16-18). Relative estimates of the conductivity of the thylakoid membrane to protons (g_{H^+}), primarily attributable to the turnover of the ATP

synthase, were obtained by taking the inverse of the time constant for ECS decay (τ_{ECS}) (16-18, 26). Relative estimates of the *pmf* attributable to proton flux from LEF, termed pmf_{LEF}, were calculated using the following equation (16, 18, 26):

$$pmf_{LEF} = LEF/g_H^+ \quad (2)$$

Western Blot Analyses

Crude leaf extracts from Wt and *pgr*5 were prepared as described in (34). Flash-frozen tissue was ground in a mortar and pestle prior to re-suspension in SDS-PAGE sample buffer. 10 μg of protein, as estimated using the BCA Protein Assay Kit (Pierce, Rockford, IL), from each preparation was loaded onto an SDS-Page gel. Protein was transferred to polyvinyl difluoride (PVDF) membranes and probed with antibody directed against the β-subunit of the ATP synthase (a gift from Dr. Alice Barkan, University of Oregon). Immunoreactive bands were detected on radiographic film using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL)**.**

Results and Discussion

Effects of Lowering CO_2 Levels and Loss of PGR5 on LEF and q_E Sensitivity

Fig. 1 (panel A) shows plots of q_E as a function of LEF from 26-216 μmol photons $m^{-2}s^{-1}$ for the wild type (Wt, *gl*1) (29) under ambient air (372 ppm CO_2/21% O_2) and two different treatments that lowered light saturated LEF by about the same extent. Low CO_2 air (LC-50 ppm CO_2/21% O_2) reduced light-saturated LEF in Wt by about

30%, a typical response for *A. thaliana* (33). A similar lowering of light-saturated LEF was obtained using *pgr*5 under ambient air. These conditions were chosen to avoid significant photoinhibition, which appeared in *pgr*5 above 216 μmol photons $m^{-2}s^{-1}$ as well as large changes in the partitioning of the *pmf* into Δψ and ΔpH, a phenomenon that has been previously observed in *N. tabacum* under severe stress (18). Under more extreme conditions (higher light intensities or lower CO_2 levels), results were qualitatively consistent with those presented here (data not shown) as long as partitioning of *pmf* into Δψ and ΔpH was considered (18).

In Wt under ambient air, a flux of ~40 μmol electrons $m^{-2}s^{-1}$ generated a q_E of 0.4, whereas the same level of q_E was achieved at a flux of ~27 μmol electrons $m^{-2}s^{-1}$ under LC air (Fig. 1, panel A). At saturating light q_E was about 35% larger under LC than ambient air, despite having a slower LEF. Thus, similar to previous observations in *N. tabacum* (17, 18), lowering CO_2 in Wt increased the sensitivity of q_E with respect to LEF. In contrast, the ~30% decrease in LEF that occurred in the absence of PGR5 was not accompanied by a corresponding increase in the light saturated q_E response, but was rather 4-6-fold lower in comparison to that in the Wt.

Effects of Lowering CO_2 Levels and Loss of PGR5 on Contributions of CEF1 to the Proton Budget

In Wt, varying the CO_2 levels had no observable effects on the relationship between ν_{H+} and LEF (Fig. 1, panel B), arguing against large CO_2-dependent changes in contributions from Type I modulation (12, 16-18). On the other hand, the slope of ν_{H+} vs. LEF was ~13% smaller ($p < 0.05$) in *pgr*5 than in Wt (Fig. 1, panel B), supporting the

view that PGR5 is important for steady-state proton flux, consistent with a role in CEF1 (29, 30).

This view was supported in separate estimates of proton flux and *pmf*. The data in Fig. 2 shows the relationships between estimates of the *pmf* attributable solely to proton translocation by LEF (pmf_{LEF}) and the total *pmf* (ECS_t), driven by the sum of LEF and other process (i.e. CEF1). Within the noise level, the relationships for Wt under the two CO_2 levels overlapped (analysis of covariance indicated no significant differences in slopes, $p = 0.6$), implying that either LEF accounted for the vast majority of estimated *pmf*, or that contributions from other processes, most notably CEF1, were a constant fraction of LEF. Again, the slope of pmf_{LEF} versus ECS_t was approximately 14% smaller in *pgr*5 in comparison to Wt under ambient conditions, a difference that was statistically significant (analysis of covariance, $p< 0.05$).

It is important to note that the ECS_t estimate of *pmf* is based on the light-dark difference in the amplitude of the ECS signal (17, 18), whereas the pmf_{LEF} estimate of *pmf* is based on ECS decay kinetics (18), i.e. the later is not sensitive to changes in the absolute ECS response. The leaf contents of photosynthetic complexes were equivalent in Wt and *pgr*5 (29) and the amplitudes of the rapid (<1 ms) ECS responses after saturating, single turnover flashes, which reflect charge separation in PSII and PSI centers (35), were indistinguishable, with Wt and *pgr*5 giving 3.5 +/- 0.35 and 3.5 +/- 0.24 ($\Delta I/I_0$ X 1000) respectively, indicating essentially identical responses to $\Delta\psi$. Overall, the constancy of these results supports the validity of comparisons of the ECS-derived parameters between the two strains.

Differences in q_E Senstitivity Between Wt and *pgr*5 can be Largely Attributed to Changes in g_H^+

The above flux estimates suggest differences in contributions to light-induced *pmf* from processes other than LEF, consistent with a difference in CEF1 engagement between Wt and *pgr*5 (29, 30). However, the modest (~13%) decrease in ν_{H+} in the absence of PGR5 was far too small to *directly* account for the corresponding 4-6-fold decrease in the q_E response at light-saturated LEF (Fig. 1, panel A). In this regard, it was striking that the *pgr*5 mutant exhibited lowered LEF without a corresponding increase in q_E sensitivity, in contrast to what was observed in the Wt upon lowering CO_2 (Fig. 1, panel A).

Fig. 3 shows that g_H^+ decreased in the Wt upon lowering CO_2, but substantially increased in *pgr*5, especially at the higher light intensities (Fig. 3). Within the noise level, plots of q_E against pmf_{LEF} for Wt under the two CO_2 levels and *pgr*5 overlapped (Fig. 4), indicating that, as was reported previously (17, 18), changes in g_H^+ could predominantly account for the differences in the q_E response. We thus conclude that in *pgr*5 more facile proton efflux from the lumen through the ATP synthase, accompanied by decreases in LEF and probably CEF1, prevented the buildup of steady-state *pmf* and thus inhibited the q_E response.

In principle, g_H^+ could be modulated by changing the specific activity of ATP synthase or its content in the thylakoids. Hence, a ~ 2-fold increase in the size of the ATP synthase pool could give rise to the observed ~2-fold increase (i.e. at higher light intensities) in g_H^+ in *pgr*5 (Fig. 3). However, ATP synthase content in Wt and *pgr*5 was estimated by western analyses and found to be essentially identical (Fig. 4, inset). In

addition, low light-induced activation of the ATP synthase by thioredoxin and leakage of the thylakoid membrane to protons were indistinguishable between Wt and *pgr*5, essentially as seen for other C_3 plants (35). These data, taken together with the observed similarities in g_H^+ at low light, lead us to conclude that the differences in g_H^+ between Wt and *pgr*5 were caused by alterations in steady-state substrate or affecter concentrations (17).

The decrease in maximal LEF in *pgr*5 is probably due to loss of PSI electron acceptors and a buildup of reduced intermediates (29, 30). A similar decrease in LEF was seen when CO_2 was lowered, but in contrast to the enhanced g_H^+ that occurred in the absence of PGR5, such a decrease in LEF was accompanied by substantial decreases in g_H^+ (Fig. 3), resulting in a net increase in both *pmf* and q_E. These results demonstrate an important role for 'tuning' the activity of the ATP synthase in the signal pathway that regulates light capture (36). Excessive turnover rates (i.e. large g_H^+ values) will result in facile proton efflux, preventing buildup of *pmf* and diminishing the q_E response. On the other hand, inappropriate decreases in ATP synthase turnover rates can result in excessive buildup of *pmf*, over-acidifying the lumen and causing subsequent pH-induced degradation of the photosynthetic apparatus (4, 37).

From the above, we conclude that changes in CEF1 upon loss of PGR5 constitute a flux of protons less than about ~13% of that from LEF, resulting in a commensurate decrease in ATP output. Since consumption of ATP and NADPH by the Calvin-Benson cycle is coupled, even a small ATP/NADPH imbalance could conceivably give rise to not only a buildup of ADP and $[P_i]$, but also a substantial reduction of $NADP^+$, restricting the

availability of PSI electron acceptors and thereby lowering LEF, as was observed in *pgr*5 both here and previously (29).

Conclusions

Possible Causal Relation Between Pgr5$^-$ and g_H^+

We previously proposed (17) that lowering CO_2 will lead to the buildup of phosphorylated metabolites in the stroma, depleting stromal [P_i] below its K_M (~1 mM) at the ATP synthase. This will result in lowering of the effective g_H^+ and subsequent increases in steady-state *pmf* and q_E. A small ATP/NADPH imbalance is expected to result from the absence of the PGR5-mediated CEF1. The deficit is obviously satisfied, but only by substantially slower processes, e.g. alternative cyclic electron transfer processes of export of NADPH (12, 16). We thus expect in *pgr*5 a buildup of stromal [P_i] *above* its K_M at the ATP synthase, maintaining high g_H^+ even when LEF is restricted. Thus, in this model the loss of CEF1 in *pgr*5 indirectly attenuates both steady-state *pmf* and q_E.

These results support a 'division of labor' model for *pmf* modulation, whereby Type I mechanisms act mainly to adjust ATP/NADPH output, whereas Type II mechanisms alter the sensitivity of antenna regulatory pathways, while maintaining *pmf* in an optimal range for energy transduction. Finally, it is clear from these results that a further understanding of the interaction of the photosynthetic apparatus within the plant will require an integrated, yet quantitative, 'systems' approach on the intact plant under true steady-state conditions. Spectroscopic tools, such as we have applied here, will be essential for this progress.

Figure Legends

Figure 1. LEF dependencies of antenna regulation and light-driven proton flux across the thylakoid membrane. Chlorophyll *a* fluorescence yield and ECS analyses were used to obtain estimates of **(A)** Energy-dependent exciton quenching (q_E) and **(B)** steady-state proton flux into the lumen (v_{H+}) respectively, from 26-216 μmol photons $m^{-2}s^{-1}$ on leaves from *A. thaliana* Wt under ambient (372 ppm CO_2/21% O_2) (○) and low CO_2 (LC-50 ppm CO_2/21% O_2) (Δ) air, as well as *pgr*5 under ambient air (■) and plotted as a function of estimated LEF (18). Linear regressions of LEF versus v_{H+} are shown in **(B)**, the regression slopes of which are 2.035 (solid line), 2.038 (dotted line), and 1.774 (dashed line) for Wt ambient air, Wt/LC air, and *pgr*5 ambient air, respectively. Slopes for Wt/atmospheric and *pgr*5/atmospheric were judged by analysis of covariance to be statistically different ($p < 0.05$). Error bars represent SE for n = 3-6.

Figure 2. The relationship between light-induced *pmf* and the *pmf* generated by LEF alone. ECS and chlorophyll *a* fluorescence yield analyses were performed on leaves from *A. thaliana* Wt plants and *pgr*5 in order to estimate light-induced *pmf* (ECS_t) and LEF respectively, from which estimates of the *pmf* generated by LEF alone (pmf_{LEF}) were obtained (i.e. $pmf_{LEF} = LEF/g_H^+$). Linear regressions of pmf_{LEF} versus ECS_t are shown, the slopes of which are 1.972 (solid line), 2.053 (dotted line), and 1.701 (dashed line) for Wt/ambient air, Wt/LC air, and *pgr*5/ambient air, respectively. Slopes for Wt/atmospheric and *pgr*5/atmospheric were ~14% different and judged by analysis of covariance to be statistically different ($p < 0.05$). The small difference (~4%) between

the slopes of Wt/atmospheric versus Wt/LC was not statistically significant ($p = 0.6$). Conditions and symbols are as in Fig. 1. Error bars represent SE for n = 3-6.

Figure 3. The light intensity dependence of the proton conductivity of the ATP synthase (g_H^+). Estimates of g_H^+ in Wt and *pgr*5 from 26-216 μmol photons $m^{-2}s^{-1}$ were obtained by taking the inverse of the time constant for ECS decay during a 300 ms dark perturbation of steady state conditions. Conditions and symbols are as in Fig. 1. Error bars represent SE for n = 3-6.

Figure 4. The relationship between energy dependent exciton quenching and the *pmf* generated solely by LEF. Estimates of energy dependent quenching (q_E) and the *pmf* generated solely by LEF (i.e. pmf_{LEF}) were obtained as in Figs. 1 and 2, respectively. ATP synthase content in Wt (Panel A) and *pgr*5 (panel B) was estimated by western blot analyses using polyclonal serum directed against the β-subunit of the ATP synthase (inset). Conditions and symbols are as in Fig. 1. Error bars represent SE for n = 3-6.

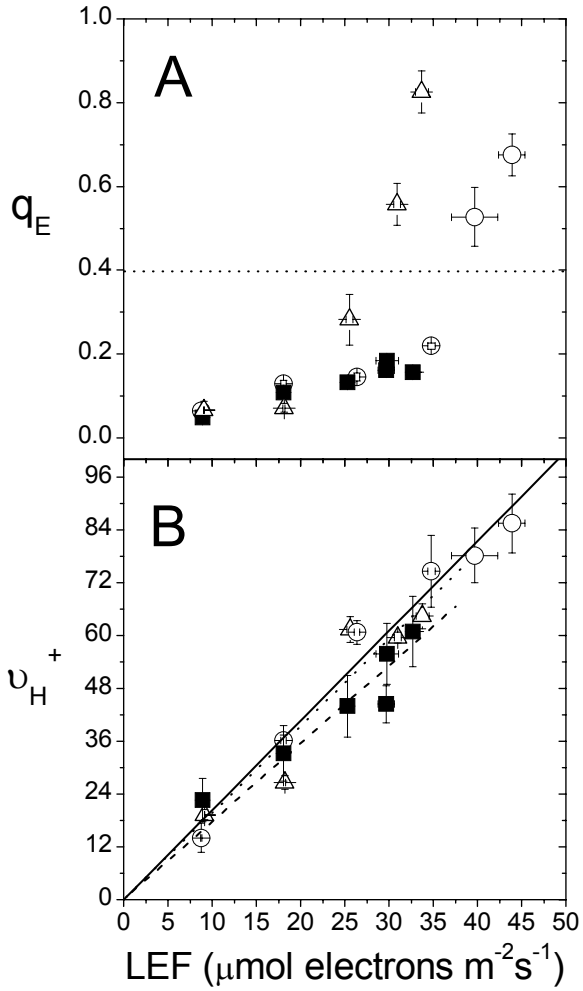
A
B
q_E
υ_H^+
LEF (μmol electrons $m^{-2}s^{-1}$)
1.0
0.8
0.6
0.4
0.2
0.0
96
84
72
60
48
36
24
12
0
0 5 10 15 20 25 30 35 40 45 50

Figure 1

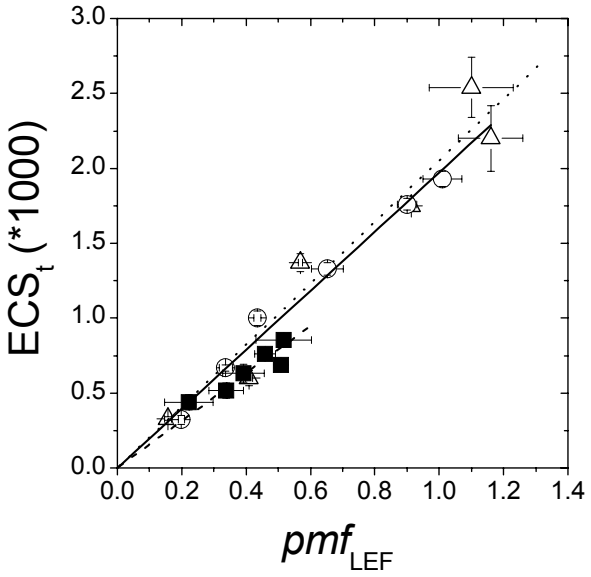

Figure 2

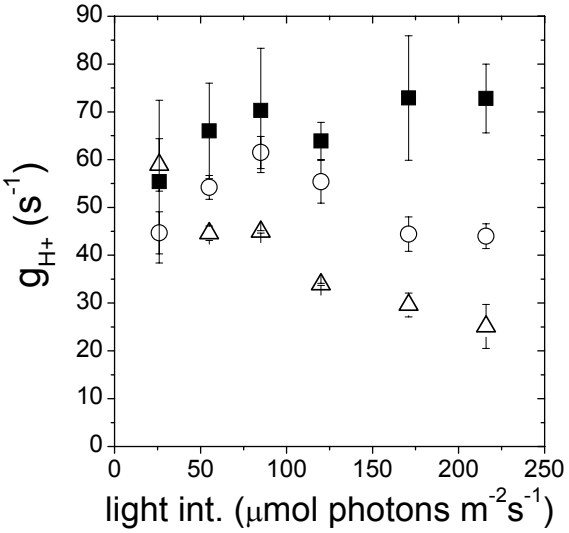
90
80
70
60
50
40
30
20
10
0
g_{H+} (s^{-1})
0
50
100
150
200
250
light int. (μmol photons $m^{-2}s^{-1}$)

Figure 3

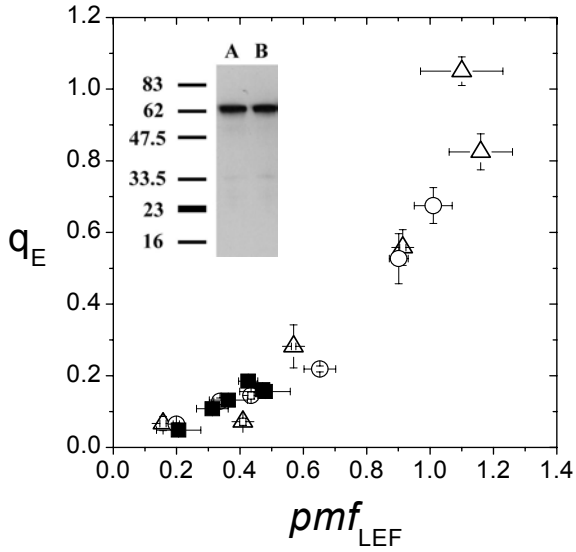

Figure 4

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CHAPTER 4: Unraveling the complexities of photosynthetic regulation through interspecies analyses

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ABSTRACT

The light reactions of photosynthesis must be regulated in order for plants to respond to changes in biochemical demand resulting from natural fluctuations in environmental conditions. Modulation of both q_E sensitivity, the predominant process by which light capture is adjusted, and the ATP/NADPH output ratio of the light reactions comprise such regulation. We show that CO_2-dependent q_E sensitivity modulation is brought about by variability in: 1) the proton conductivity of the ATP synthase; and 2) the storage of proton motive force as a proton diffusion potential. Consistent with previous findings, we observed no evidence for changes in the fractional turnover of cyclic electron flow around photosystem I under these conditions.

Key words: cyclic electron flow around photosystem I, proton motive force partitioning

Abbreviations: CEF1, cyclic electron flow around photosystem I; ΔpH, proton diffusion potential of light-induced *pmf*; Δψ, electrical potential of light-induced *pmf*; ECS, electrochromic shift of thylakoid membrane-associated carotenoid species; ECS_{inv}, inverted ECS signal; ECS_{ss}, steady-state ECS signal; ECS_t, total change in ECS signal during a brief dark perturbation of steady-state; g_H^+, proton conductivity of the CF_O-CF_1 ATP synthase; LEF, linear electron flow from H_2O to $NADP^+$; *pmf*, proton motive force; *pmf* partitioning, the relative storage of *pmf* as Δψ and ΔpH; $pmf_{\Delta pH}$, relative fraction of

light-induced *pmf* stored as a proton diffusion potential; pmf_{LEF}, the *pmf* generated solely by linear electron flow; q_E, energy dependent component of nonphotochemical quenching of excitation energy; q_E sensitivity modulation, variability in the relative response of q_E to linear electron flow; τ_{ECS}, time constant for ECS decay during a brief dark period

Introduction

Photosynthesis converts light energy into the chemical energy that drives our ecosystem (1). In higher plant photosynthesis, light is absorbed by pigment-protein complexes (antennae) (2) that funnel the energy to photosystems (PS) I and II which are capable of rapidly storing the energy via redox chemistry. PSII and PSI are linked in sequence by plastoquinone (PQ), the cytochrome b_6f complex, and plastocycnin, all of which mediate the transfer of electrons from H_2O at PSII to $NADP^+$ at PSI in what is termed linear electron flow (LEF). In addition to generating NADPH, LEF is coupled to the formation of a transthylakoid electrochemical gradient of protons, termed the proton motive force (i.e. *pmf*) (3), consisting of both a proton diffusion potential (ΔpH) and an electrical gradient (Δψ) (3, 4). Although both Δψ and ΔpH components of *pmf* contribute to ATP synthesis (5), the ΔpH component alone plays a role in feedback regulating light capture (6-8) via energy dependent quenching of antenna excitons, or q_E (see below). The ATP and NADPH are subsequently used to drive various metabolic processes, primarily of which is the reduction of CO_2 to the level of sugar phosphates in the Calvin-Benson cycle (9).

A Need for Maintaining Energetic Balance

Plants must delicately balance how much energy they absorb with that of its utilization in downstream metabolism. At the molecular level, the relative size of a chlorophyll molecule (10), even when aggregated into an antenna (i.e. 200-400 chlorophyll molecules), renders incident photon flux density (PFD) the limiting factor in photosynthesis, but *only* at light intensities well below full sunlight. Otherwise, incident

PFD, even under ideal conditions (i.e. permissive temperatures, well watered soil, etc), exceeds a plants capacity to process the energy in downstream metabolism (6). The excess energy can catalyze harmful side reactions at various sites within the photosynthetic apparatus (11, 12), giving rise to the potential for photoinhibition and subsequent diminished plant productivity (13). The situation is exacerbated by constantly fluctuating environmental conditions (i.e. drought, etc.) that can transiently slow downstream metabolism (6), more often than not under circumstances in which light intensities incident upon a particular leaf remain unaffected, enhancing the potential for energetic imbalance. Therefore, photosynthesis is in need of redundant protective mechanisms (11, 12, 14), some of which must be capable of responding to rapidly changing environmental conditions (15).

q_E: A Response to Short Term Energetic Imbalance

It is useful in such discussions to carefully distinguish between *absorption* and *capture* of light energy. Absorption refers to the light-dependent excitation of antennae pigments, e.g. chlorophylls, to their singlet state, whereas capture connotes the subsequent utilization of the absorbed energy to drive downstream electrochemical events, *e.g.* electron/proton transfer. The above-mentioned harmful side reactions result from excessively captured light energy. Over short time-scales (i.e. seconds-minutes) which preclude plants from responding to energetic imbalance by employing, for example, various strategies to avoid light absorption (6), plants are variably efficient at capturing light energy (6, 8). The predominant mechanism for achieving such variable efficiency over short term changes in energetic balance is q_E (15-18), a mechanism that

harmlessly dissipates excess energy once it has been absorbed in the antennae (7, 19). The precise biophysical mechanism of q_E is currently under intense investigation and has recently been suggested to involve de-excitation of bulk antennae pigments through funneling of the energy to chlorophyll-zeaxanthin heterodimers which quench the energy via charge recombination (19, 20). Although q_E is therefore dependent upon the formation of zeaxanthin, the steady-state level of which is controlled primarily by the thylakoid lumen-localized enzyme violoxanthin de-epoxidase (VDE) (21), it has also been shown to be dependent upon protonation of lumen exposed residues of PsbS, a polypeptide associated with the antennae of PSII (22-25).

Modulation of q_E Sensitivity

The pH-dependency of q_E stems from the need to not only protonate lumen exposed residues of psbS (22-25), but also because VDE has a steeply pH dependent rate constant (3, 21). A conceptual paradigm to have emerged in the literature to describe regulation of q_E concerns the observed variability in the relationship that exists between q_E and LEF, the predominant mechanism for acidifying the lumen (16-18, 26, 27). As a first order approximation, a simple model predicts q_E to be a continuous function of LEF, as is in fact observed from low to saturating light intensities under ambient air and permissive temperatures (26, 27). However, various environmental stresses are known to attenuate LEF (i.e. drought) (28), which would also result in, according to this simple model, attenuation of q_E, precisely opposite of what is needed under such circumstances (26-29). In reality, q_E is quite robust under such conditions, implying that its *sensitivity* is

modulated with respect to LEF (i.e. q_E sensivtivity modulation), as has been demonstrated upon short term changes in CO_2 and O_2 availability (26, 27, 29).

A Need for Balancing ATP/NADPH Output

In addition to the need for regulating light capture, plants must also be capable of adjusting the relative output ratio of ATP/NADPH (16, 17). Although reduction of CO_2 to the level of sugar phosphates is the predominant sink for output of the light reactions, a host of other processes consume ATP and NADPH (i.e. nitrogen, lipid metabolism, etc.) at various stoichiometries and may be variably engaged (16, 17). Furthermore, arguments have been made that there is a shortfall of ATP produced by LEF for the purposes of balancing the ATP/NADPH output ratio required to maintain turnover of even the Calvin-Benson cycle alone (16, 17, 30, 31). In short, adjustments in the relative ATP/NADPH output ratio of the light reactions is essential.

Mechanisms for Achieving a Broad Level of Flexibility in the Light Reactions

An integrated view of the proton circuit (18) of photosynthesis reveals at least four general models that can account for broad regulation of the light reactions, some of which can solely account for modulation of q_E sensitivity, while others could impact ATP/NADPH output as well (reviewed in 16, 17, 18).

Model 1: Variable antennae response to lumen pH. Changes in the antennae response to lumen pH could be brought about by either changes in the pK_a values on VDE and/or psbS, by changes in the relative rates of the enzymes controlling zeaxanthin (VDE and zeaxanthin epoxidase) or total pigment levels. Any of these types of changes

could enhance or diminish the q_E response to lumen pH and by extrapolation to ΔpH and *pmf*, effectively modulating q_E sensitivity without affecting ATP/NADPH output.

Model 2: Changes in the fractional turnover of alternate electron transfer pathways. Increased flux of protons into the lumen via cyclic electron flow around PSI (CEF1) has long been thought to be the predominant mechanism for modulating q_E sensitivity (28, 32, 33), a hypothesis that continues to be intensely debated in the literature (34, 35). If solely for the purpose of modulating q_E sensitivity, such a mechanism is problematic given that, since protons predominantly exit the lumen through the ATP synthase, it will also necessarily modulate the ATP/NADPH output ratio, a result for which such a mechanism is ideally suited (16, 17, 30, 31). This model predicts discontinuity in the relationship between the measured magnitude of total *pmf* (i.e. that generated by LEF, CEF1, etc.) and that generated by LEF alone.

Model 3: Changes in the proton conductivity of the ATP synthase (g_{H^+}). A relatively recently discovered feature of steady-state *pmf* is that changes in its magnitude can be brought about by, in contrast to increased flux of protons into the lumen via routes other than LEF (i.e. Model 2), lowering the conductivity of the ATP synthase to proton efflux, or g_{H^+} (26, 27). Such a mechanism would allow for the generation of a significant *pmf* even at modest proton influxes (i.e. low rates of LEF) (16, 26, 27), thereby modulating q_E sensitivity without impacting ATP/NADPH output. Unlike model 2, this model predicts continuity in the relationship between the *pmf* generated by LEF alone and total *pmf*, as well a continuous relationship between q_E and *pmf* (26, 27).

Model 4: Changes in pmf partitioning. The relative partitioning of the light-induced, steady-state *pmf* into Δψ and ΔpH has been suggested to occur in a 1:1 ratio

over a wide range of conditions (26, 27). The subtle importance of variable *pmf* partitioning is that it would allow for adjustments in q_E sensitivity without altering the magnitude of total *pmf* and therefore would not, like Model 3, alter the ATP/NADPH output ratio. Like Model 1, this model predicts discontinuity in the relationship between total *pmf* and q_E, but it further predicts commensurate changes in the fraction of *pmf* stored as ΔpH.

In this work, we test these four models using *Arabidopsis thaliana* as a model system. Although evidence for variable *pmf* partitioning was previously observed in *Nicotiana tabacum* (26), the conditions under which it was observed are unlikely to be experienced by terrestrial plants in nature. In contrast, herein we provide evidence that variable *pmf* partitioning contributes to modulation of q_E sensitivity in *A. thaliana* under conditions of low CO_2, *e.g.* conditions that reflect natural stress.

Materials and Methods

Growth Conditions

Wildtype (Wt) *A. thaliana* plants were housed in a growth chamber using a 16:8 photoperiod under a light intensity of ~70 μmol photons $m^{-2}s^{-1}$ photosynthetically active radiation (PAR). The temperature was maintained at 25°C.

Spectroscopic Assays

Detached leaves from ~3 week old plants were gently clamped into the measuring chamber of a previously described non-focusing optics spectrophotometer (NoFOSpec) (26, 36). Room air (ambient air-372 ppm CO_2/21% O_2) or premixed low CO_2 air (LC: 50

ppm CO_2/21% O_2) were bubbled through water prior to perfusing the measuring chamber of the spectrophotometer. Leaves were first exposed to actinic light intensities ranging from 36-216 μmol photons $m^{-2}s^{-1}$ PAR from a bank of red LED's (maximal emission 633 nm) for ten minutes to reach steady-state. From the steady-state, estimates of the minimum (Fs) and maximum (Fm') yields of chlorophyll *a* fluorescence were obtained using a modulated 520 nm probe beam just prior to and during a saturating pulse of white light, respectively. Estimates of LEF were obtained using Fs and Fm' as in (37, 38). After 10 minutes post-actinic illumination, the light saturated level of chlorophyll *a* fluorescence yield (Fm") was obtained, from which estimates of the energy dependent component (q_E) of nonphotochemical quenching was estimated (i.e. q_E = Fm"-Fm'/Fm') (15).

Probing the Steady-State *pmf*

Estimates of various aspects of the steady-state *pmf* were obtained by kinetic analyses of the electrochromic shift (ECS) of endogenous thylakoid membrane pigments, a linear indicator of transthylakoid $\Delta\psi$ (39). The ECS is a transthylakoid $\Delta\psi$-induced shift in the absorption spectrum of certain carotenoid species that occurs maximally at ~520 nm (i.e. ΔA_{520}). The NoFOSpec is designed with 3 separate banks of green LED's (maximal emission between 500 and 540 nm), located at 19° and above the entrance aperture of a compound parabolic concentrator (CPC) whose exit aperture is positioned right above the leaf surface. Prior to entering the CPC, light from each of the LED banks is passed through separate 5 nm band-pass filters in order to obtain different wavelengths (i.e. 505, 520, 535 nm) of incident light that is then focused onto the leaf via the CPC.

When experiments were performed requiring the measurement of all 3 wavelengths, as in (26), the banks of LEDs were pulsed in sequence by 10 ms, allowing for near simultaneous measurements of absorbance changes associated with all three wavelengths.

ECS changes were assessed by a previously established technique referred to as dark interval relaxation kinetic (DIRK) analysis (40), whereby ECS absorbance changes are measured during perturbations of the steady-state with dark periods of various duration, depending on the type of information being sought (26, 27, 40, 41). Over short dark periods (i.e. >500 ms), the ΔA_{520} signal predominates over background scattering signals, allowing ECS changes to be estimated by monitoring changes in absorbance solely at 520 nm. The resultant signals display several useful characteristics from which information about various aspects of the steady-state *pmf* can be derived (17). For example, ΔA_{520} signals are constant under steady-state illumination, presumably reflecting the fact that the fluxes of protons both into and out of the lumen are precisely balanced in the steady-state. However, during the ensuing brief dark perturbation, the ΔA_{520} signals decay with first order kinetics to a quasi-stable level (i.e. stable after the ~500 ms darkness), presumably reflecting the fact that one process occurs during the short dark perturbation, e.g. equilibration of the light-induced *pmf* with the free energy of ATP synthesis (i.e. ΔG_{ATP}) as protons move down their electrochemical gradient through the ATP synthase. A mathmetical description of light-induced *pmf* based on ECS analyses can be used to derive several useful parameters ( ):

$$pmf\,(ECS_t) = \nu_{H+} \bullet \tau_{ECS} \qquad (1)$$

This equality indicates that the magnitude of the light-induced *pmf* (ECS_t), *e.g.* the total amplitude of ECS decay during the brief light-dark transition, is proportional to the flux of protons into the lumen (v_{H+}), as well as the time constant for proton efflux from the lumen through the ATP synthase (τ_{ECS}), which is inversely proportional to the conductivity of the ATP synthase to protons, or g_{H^+}. Assuming a constant H^+/e^- ratio for LEF and that turnover of other proton pumping processes (i.e. CEF1) are constant fractions of LEF, Eqn. 1 can be rearranged into an equality that expresses the *pmf* generated solely by LEF (pmf_{LEF}) ( ):

$$pmf_{LEF} = LEF/g_{H^+} \quad (2)$$

If the above assumptions hold true, light-induced *pmf* would be expected to be proportional to pmf_{LEF}:

$$ECS_t \propto pmf_{LEF} = LEF/g_{H^+} \quad (3)$$

Therefore, comparisons of pmf_{LEF} and ECS_t, both of which independently estimate the magnitude of the steady-state *pmf*, can provide information regarding changes in the fractional turnover of, for example, CEF1 (26, 27).

Perturbing the steady-state with longer dark periods (i.e. minutes) allows other light scattering processes to significantly contribute to apparent absorbance at 520 nm (26, 40-42). Therefore, absorbance changes at 520 nm were deconvoluted from these background signals according to the following equation:

$$\Delta ECS = -\Delta I/Io_{520} - ((-\Delta I/Io_{535} + -\Delta I/Io_{505})/2) \quad (4)$$

During these longer light-dark transitions, such deconvoluted signals initially decay from the steady-state to a level which reflects ECS_t, but after this initial decay, the signal relaxes over time to a dark stable level that is different in magnitude than the steady-state illuminated ECS level, i.e. the light-dark difference in ECS (ECS_{ss}) is interpreted as being proportional to the $\Delta\psi$ component of light-induced *pmf* (17, 26). Since the ECS signal initially inverts with respect to the ensuing dark stable level (i.e. the ECS level which represents an effective transthylakoid $\Delta\psi$ of 'zero'), the inverted region of the signal (ECS_{inv}) is interpreted as being related to the proton diffusion potential (i.e. the ΔpH component of light-induced *pmf*) coming into equilibrium with reversal of transthylakoid $\Delta\psi$ (i.e. positive on the stromal side of the membrane). Therefore, the relative partitioning of light-induced *pmf* into $\Delta\psi$ and ΔpH can be assessed by such ECS analyses ( ):

$$pmf\ (ECS_t) = \Delta\psi\ (ECS_{ss}) + \Delta pH\ (ECS_{inv}) \quad (5)$$

This information can then be used to estimate the fraction of the *pmf* partitioned into the ΔpH component ($pmf_{\Delta pH}$):

$$pmf_{\Delta pH} = \Delta pH\ (ECS_{inv})/pmf\ (ECS_t) \quad (6)$$

Results and Discussion

Multiple CO_2-Dependent Mechanisms Modulate q_E Sensitivity

Shown in Fig. 1 for wildtype *A. thaliana* is a plot of q_E as a function of LEF, both of which were estimated from 36-216 μmol photons $m^{-2}s^{-1}$ under either ambient (372 ppm CO_2/21% O_2) or low CO_2 (LC: 50 ppm CO_2/21% O_2) air. A flux of ~35 μmol electrons $m^{-2}s^{-1}$ was needed to generate a q_E of 0.5 under ambient air, whereas the same level of q_E was generated by a flux of ~15 μmol electrons $m^{-2}s^{-1}$ under LC air. These data indicate that lowering CO_2 availability increased q_E sensitivity by ~2.5-fold, results that were qualitatively similar to those previously observed in *N. tabacum* upon identical changes in CO_2 availability, results that were shown to be solely attributable to proportional decreases in g_H^+ (26, 27). In contrast, shifting from ambient to LC air in *A. thaliana* resulted in an ~1.5-fold decrease in g_H^+ (Fig.1; spheres surrounding symbols have been set proportional to estimates of g_H^+), suggesting that the magnitude of the observed increase in q_E sensitivity could not be solely attributed to changes in g_H^+. Consistent with this interpretation is the observed discontinuity in the relationship between q_E and the *pmf* generated by LEF alone, e.g. pmf_{LEF} (Fig. 2), results that are predicted *if and only if* changes in g_H^+ are not solely responsible for modulating q_E sensitivity (26, 27).

No Evidence for Changes in the Fractional Turnover of CEF1

Although widely cited in the literature as a mechanism for modulating q_E sensitivity (32, 43, 44), recent work using integrative techniques capable of estimating both the electron and proton transfer reactions suggests that fractional *changes* in CEF1

turnover very likely play no role in modulating q_E sensitivity (26, 27). Consistent with this interpretation is the observation that a continuous, near linear relationship emerged between pmf_{LEF} and light-induced *pmf* (ECS_t), e.g. the total *pmf* generated by LEF and CEF1, upon shifting from ambient to LC air (Fig. 2, inset), essentially the same as was observed in *N. tabaccum* under similar conditions (26). These results imply that CEF1 turnover remained a constant fraction of LEF regardless of lowering CO_2 levels. Therefore, the increase in q_E sensitivity that was observed in *A. thaliana* that could not be attributed to changes in g_{H^+} is not due to fractional changes in CEF1, *e.g.* these observations are inconsistent with Model 2.

Variable *pmf* Partitioning Upon Short Term Perturbations in CO_2

Shown in Fig. 3 is a plot of q_E versus ECS_t, both of which were estimated from 36-216 μmol photons $m^{-2}s^{-1}$ under ambient and LC air. In contrast to the continuous relationship between q_E and ECS_t that was observed in *N. tabaccum* upon similar changes in CO_2 levels (27, 45), a discontinuous relationship emerged between these parameters in *A. thaliana*, e.g. q_E was, in comparison to ambient air, ~2-fold larger at an estimated ECS_t of ~5.0 under LC air. These results are consistent with either of models 1 or 4 upon shifting from ambient to LC air. To distinguish between these models, we estimated the relative fraction of light-induced *pmf* partitioned into ΔpH (i.e. $pmf_{\Delta pH}$) and plotted the relative sizes of the spheres surrounding the symbols in Fig. 3 proportional to such estimates. At an ECS_t of ~5.0, $pmf_{\Delta pH}$ was ~1.5-fold larger under the LC air in comparison to ambient air, changes that are consistent with model 4. In addition, q_E was a continuous function of the estimated ΔpH component of *pmf* (i.e. ECS_{inv}) (Fig. 4),

implying a constant response of q_E to lumen pH over this wide range of conditions (i.e. inconsistent with model 1), essentially as was found in *N. Tabaccum* (26). Taken together, these results are consistent with the enhanced response of q_E to light-induced *pmf* under LC air being due to variable *pmf* partitioning, a phenomenon that was observed previously in *N. tabaccum*, but only under the extreme conditions of low CO_2 and O_2 (i.e. 50 ppm CO_2/1% O_2) (26).

Conclusions

Variable *pmf* Partitioning: a Viable Mechanism for Modulating q_E Sensitivity

Since instrumentation and techniques for estimating both the proton and electron circuits of photosynthesis have become available (4, 36, 40-42), models 1 through 4 have been extensively tested using *N. tabacum* as a model system over a wide range of conditions (26, 27). The preponderance of evidence is consistent with model 3 accounting for the majority of q_E sensitivity modulation (26, 27). However, under the extreme conditions of low CO_2 and O_2 (i.e. 50 ppm CO_2/1% O_2), conditions that are routinely used to assess the role of CEF1 (28, 29, 32), additional evidence consistent with more *pmf* being stored as ΔpH, e.g. model 4, has been obtained (26). The high concentration of O_2 in the atmosphere would seem to preclude terrestrial plants from experiencing such conditions (26), calling into question whether or not variable *pmf* partitioning is a mechanism that occurs in nature. However, we present evidence herein using *A. thaliana* that is consistent with more *pmf* being stored as ΔpH under LC air (Fig. 3), circumstances reflective of what likely occurs in response to natural stress conditions

(i.e. drought, etc.). As such, these results imply that variable *pmf* partitioning is a physiologic mechanism for plants in nature.

Modulation of q_E Sensitivity by Mechanisms Specific for this Purpose

There is currently intense debate in the literature about what mechanisms account for q_E sensitivity modulation (34, 35). Based on our work with *N. tabacum*, in which modulation of q_E sensitivity could be predominantly attributable to changes in g_H^+ (26, 27), except under the extreme conditions of low CO_2 and O_2 (26), we recently proposed a new model for regulation of the light reactions (16). This model consists of two 'Types' of mechanisms, wherein Type I mechanisms (i.e. CEF1, etc) increase the flux of protons into the lumen for the purpose of modulating ATP/NADPH output, whereas Type II mechanisms (i.e. changes in g_H^+ and *pmf* partitioning), which play no role in modulating ATP/NADPH output, are engaged when all that is needed is a change in q_E sensitivity. The interaction between these two Types of mechanisms allows plants to achieve the flexibility necessary to respond to constantly fluctuating biochemical demands.

We recently tested this model by subjecting a mutant strain of *A. thaliana*, termed *pgr*5 for proton gradient regulation, putatively impaired in the main route of CEF1 (46, 47), to our integrated analyses (Avenson et al, submitted). We concluded that the CEF1 pathway mediated by Pgr5 constitutes a flux of protons no more than ~15% that of LEF, changes that were insufficient on their own to account for the observed ~5-6 fold lowering of q_E in the *pgr*5 mutant (46, 47). However, if a modest turnover CEF1 is needed to balance the ATP/NADPH output ratio required for maintaining even normal turnover of the Calvin-Benson cycle, then its absence would be expected to result in

metabolic congestion (16, 17), evidence for which we and others have indeed observed (45-47). Our results with *pgr*5 are therefore consistent with just such a modest turnover of CEF1, consistent with the proposed mechanism of CEF1 in the above mentioned model as a means of modulating ATP/NADPH output.

Similarly, our results using wildtype *A. thaliana* further bolster this new model for regulation of the light reactions. We show that an ~2.5-fold increase in q_E sensitivity (Fig. 1) occurs in *A. thaliana* upon shifting from ambient to LC air, a change that could *not* be solely attributed to commensurate decreases in g_H^+ (Fig. 1, sizes of spheres). Rather than this discrepancy being explained by enhanced turnover of CEF1, which was ruled out by the observation that proton flux associated with LEF could completely account for estimates of light-induced *pmf* over the entire range of conditions tested (Fig. 2, inset), the LC conditions resulted in more of the *pmf* being partitioned into the ΔpH component (Fig. 3). These changes, coupled with the observation that the antenna responded constantly to lumen pH (Fig. 4), could account for the increase in q_E sensitivity that was *not* attributable to changes in g_H^+. Therefore, q_E sensitivity modulation in *A. thaliana* upon short term fluctuations in CO_2 can be attributed to a combination of Type 2 mechanisms, as described in the above mentioned model (16).

Learning Lessons from Interspecies Differences

Analyses of interspecies differences has been proposed as a way for answering questions that are intractable by studying one particular species (48). For example, an active area of research is aimed at understanding more precisely the functional role of the PsbS protein in q_E (22-25). Although much of this research is being done with *A.*

thaliana (22-24), the species in which the link between q_E and the PsbS protein was initially characterized (24), a PsbS homolog was recently discovered in *Chlamydomonas reinhardtii* (48). It has been suggested that having two 'fronts' upon which to study the function of PsbS should lead to progress in understanding not only the functional significance of PsbS, but the q_E mechanism itself, an essential mechanism for maintaining plant viability in a constantly fluctuating environment (49).

Similarly, the search for what controls g_H^+ and *pmf* partitioning, difficult problems in and of themselves, is well under way. The intractability of such endeavors is marked by the fact that each of these processes is putatively controlled by mechanisms that are intricately linked to a host of other metabolic processes. For example, modulation of stromal [P_i], an intermediate of many different processes, is the current model for what controls changes in g_H^+ (27). Under low CO_2, when the Calvin-Benson cycle is attenuated, diminished consumption of ATP is thought to shift the intermediates of the ATP synthesis reaction away from the reactants (i.e. lowered amounts of P_i). Since [ADP] has been suggested to remain constant under such conditions (27), a decrease in [P_i] below its Km at the ATP synthase is thought to slow turnover of the ATP synthase, effectively lowering apparent g_H^+ (27).

Variability in *pmf* partitioning was initially proposed to result from changes in the ionic strength of the chloroplast (4). In thylakoids a steady-state transthylakoid $\Delta\psi$ was observed, using ECS analyses, to be progressively collapsed by increasing the ionic strength of the buffer in which the thylakoids were suspended (4). Since discovering similar changes in *pmf* partitioning *in vivo* (26), we have begun to search for mutants defective in thylakoid membrane ion transporters, channels, etc. However, questions

about what controls partitioning are complicated by the fact that the ionic strength of the chloroplast can also be affected by chloroplast inner/outer envelope transporters/channels, which provide a link between the chloroplast and the cytosol, further complicating elucidation of what controls *pmf* partitioning.

Therefore, the observed differences in modulation of q_E sensivity between *N. tabacuum* and *A. thaliana* provide a means for addressing some of these questions. In *N. tabaccum*, modulation of q_E sensitivity upon shifting from ambient to LC air can be completely accounted for by commensurate changes in g_H^+ (26, 27). Although under more extreme conditions of low CO_2 and O_2 variable partitioning of *pmf* contributes to q_E sensitivity modulation in *N. tabacum* (26), these conditions are unlikely to be experienced by terrestrial plants in nature (26). In contrast, under conditions resembling what plants likely experience in nature under various conditions (i.e. drought, etc.), modulation of q_E sensivitity in *A. thaliana* upon shifting from ambient to LC air is explained only on the basis of *simultaneous* changes in both g_H^+ and *pmf* partitioning. Why? Are there differences between the two species in what controls the ionic strength of the chloroplast? Are there ion transporters in *A. thaliana* that are not present in *N. tabacum*? A systematic study of differences in growth conditions between the two species would also be needed to rule out differences in expression of putative transporters/channels under different growth conditions, etc. One thing is clear though: answering such questions in the context of regulating the light reactions will only be achieved through integrated analyses of both proton and electron transfer (16-18).

Moving Forward Through Integrated Analyses

Regulation of the light reactions has been the subject of intense research for decades (see references in 16, 17, 18). At the center of this research, even up to the present (26, 27, 34, 35, 43, 44), has been much debate concerning the role of CEF1 in modulating q_E sensitivity. Through advances in instrumentation and techniques capable of estimating both the proton and electron transfer reactions of photosynthesis, a range of models previously un-testable are no longer so (26, 27). Therefore, rather than focusing on one particular model, the scientific community can now objectively test alternative hypotheses, an approach previously suggested to result in rapid scientific progress (50). This notion would seem to be superfluous given the sentiment that we already know everything there is to know about photosynthesis, with the mechanism of q_E being one of the 'last mysteries of photosynthesis' (25). On the contrary, uncovering what controls variability in g_{H^+} and *pmf* partitioning, the predominant mechanisms for modulating q_E sensitivity, will likely require questioning long held assumptions and broadening our understanding what controls photosynthesis in nature.

Figure Legends

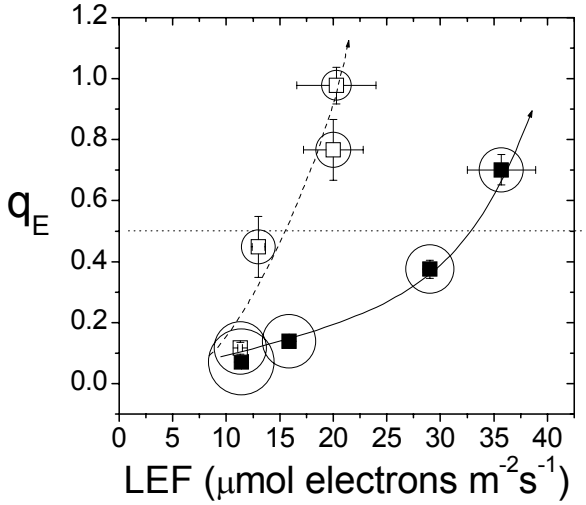
Figure 1. Modulation of q_E sensitivity is accompanied by diminished g_H^+. LEF and q_E were estimated from changes in chlorophyll *a* fluorescence yield (as in 26) in leaves from wildtype *A. thaliana* from 36-216 μmol photons $m^{-2}s^{-1}$ under ambient (372 ppm CO_2/21% O_2-closed symbols) and low CO_2 (50 ppm CO_2/21% O_2- open symbols) air. Relative estimates of g_H^+ were obtained from DIRK analyses (40) of the ECS using ~300 ms dark perturbations and have been plotted proportional to the relative sizes of the spheres surrounding the symbols. Maximum g_H^+ (i.e at low light intensities) was 68.7 s^{-1} and 53.6 s^{-1} under ambient and low CO_2 air, respectively. The horizontal line marks a q_E of 0.5. Error bars are SE for LEF and q_E for n = 5-6.

Figure 2. The dependence of q_E on the *pmf* generated solely by LEF. Estimates of q_E, LEF and g_H^+ were obtained as described in Fig. 1 from 36-216 μmol photons m^{-2} s^{-1}. The pmf_{LEF} parameter was derived by dividing LEF by g_H^+ (26, 27). Inset: Estimates of the light-induced *pmf* (i.e. ECS_t), taken as the total amplitude of ECS decay upon a ~300 ms dark perturbation of steady-state conditions, are plotted as a function of pmf_{LEF}. Symbols and conditions are as in Fig. 1. Error bars are SE for ECS_t, pmf_{LEF} and q_E for n = 5-6.

Figure 3. The dependence of q_E on total, light-induced *pmf*. q_E and ECS_t were estimated as in Fig. 1 and 2, respectively, from 36-216 μmol photons $m^{-2}s^{-1}$. The spheres surrounding the symbols have been set proportional to estimates of the fraction of light-induced *pmf* partitioned into ΔpH (i.e. $pmf_{\Delta pH}$), derived by dividing estimates of the light-induced ΔpH component of *pmf* (i.e. ECS_{inv}) by the total magnitude of light-induced *pmf*

(i.e. ECS_t). Symbols and conditions are as in Fig. 1. Error bars are SE for ECS_t and q_E for n = 5-6.

Figure 4. The dependence of q_E on the light-induced ΔpH component of *pmf*. q_E and the ΔpH component of light-induced *pmf* (i.e. ECS_{inv}) were estimated as described in Figs. 1 and 3, respectively, from 36-216 μmol photons $m^{-2}s^{-1}$. Symbols and conditions are as in Fig. 1. Error bars are SE for ECS_{inv} and q_E for n = 5-6.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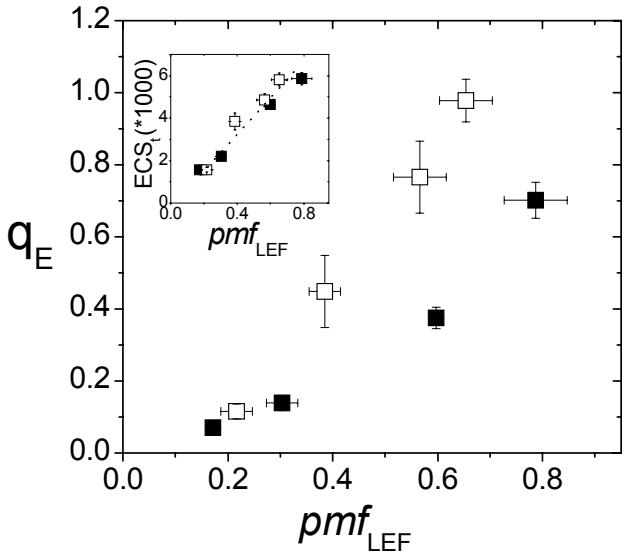
LEF (μmol electrons $m^{-2}s^{-1}$)

Figure 1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pmf_{LEF}

Figure 2

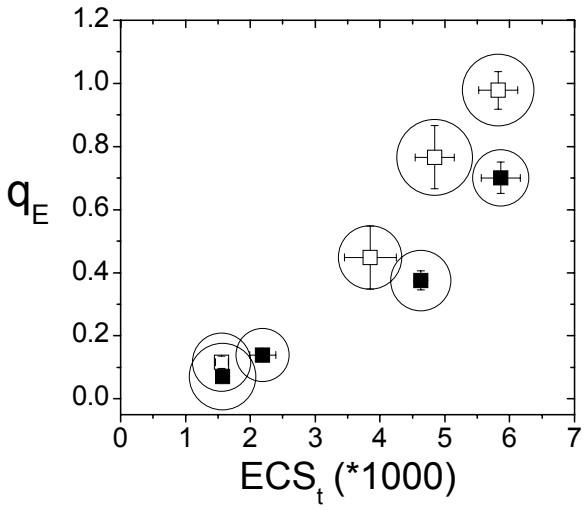

Figure 3

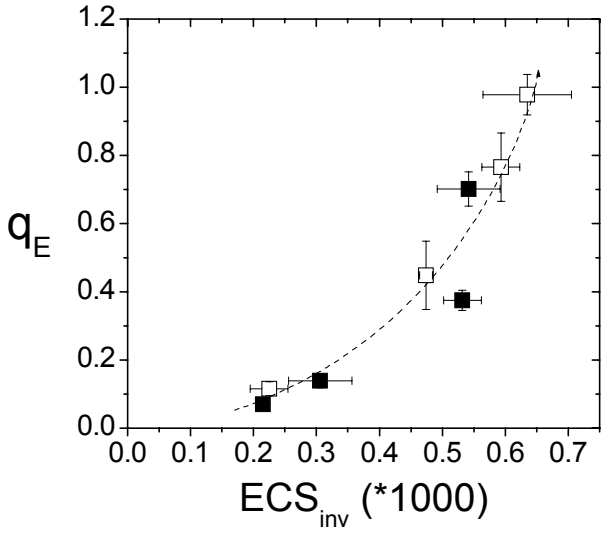

Figure 4

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CHAPTER 5: Integrating the role of the unique thylakoid membrane lipid matrix into the light reactions of photosynthesis

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ABSTRACT

The light reactions of photosynthesis occur within a unique lipid environment, the thylakoid membrane, comprised of lipids with fatty acid side chains that are ~75-80% poly unsaturated. We combined two mutant alleles, Fad2-5 and Fad6, which control the extent of lipid polyunsaturation, in a single genetic background. The resulting double mutant, *Fad*2-5/*Fad*6, had significantly attenuated levels of polyunsaturated fatty acids in its predominant thylakoid membrane lipids, monogalactosyldiacylglycerol and digalactodiacylglycerol, but was capable of photoautotrophic growth on soil, facilitating an *in vivo* analyses of the role of polyunsaturated fatty acids in photosynthesis. Using flash-induced analyses of the electrochromic shift we provide evidence that the *Fad*2-5/*Fad*6 thylakoid membranes are slightly leaky to protons. In contrast to increased sensitization of energy dependent quenching, a mechanism for harmlessly dissipating excessively absorbed energy, to electron transfer, as demonstrated in the wild type upon lowering CO_2, a desensitization of energy dependent quenching occurred in *Fad*2-5/*Fad*6, results which were accompanied by enhanced proton conductivity of the ATP synthase. These combined results are consistent with metabolic congestion occurring in *Fad*2-5/*Fad*6, resulting very likely from slightly leaky thylakoid membranes to proton efflux, implying that the high degree of polyunsaturation of the thylakoid membrane facilitates the very tight coupling between the output of the light reactions (ATP/NADPH) with that of their downstream consumption.

Key-words: CF_1-CF_O ATP synthase proton conductivity; cyclic electron flow around photosystem I; polyunsaturated fatty acids

Abbreviations: CEF1, cyclic electron flow around PS I; CF_1-CF_O, chloroplast ATP synthase; ΔpH, pH component of *pmf*; Δψ, electric field component of *pmf*; ΔG_{ATP}, the free energy of ATP formation; DIRK, dark interval relaxation kinetics; ECS, electrochromic shift; ECS_t, total magnitude of ECS decay during a light-dark transition; ECS_{ss}, steady state ECS; ECS_{inv}, ECS change from inverted Δψ; g_{H^+}, CF_1-CF_O ATP synthase proton conductivity; LEF, linear electron flow; *pmf*, transthylakoid proton motive force; pmf_{LEF}, *pmf* generated solely by LEF; PQ, plastoquinone; PS, photosystem; ϕ_{II}, photochemical yield of PS II; q_E, energy-dependent quenching of antenna excitons; τ_{ECS}, time constant for ECS decay in response to a brief dark interruption of steady state ; v_{H^+}, steady state rate of proton flux;

Introduction

The light reactions of photosynthesis

Photosynthesis converts light energy into chemical energy that powers our ecosystem and it produces the oxygen we breathe as a 'by-product' (1). Light energy is absorbed by pigment-protein complexes (antennae) (2-5) that resonate the energy to reaction centers, photosystems (PS) II and I (6), which very quickly store the energy via redox chemistry (1, 7). In conjunction with the cytochrome b_6f complex, the plastoquinone (PQ) pool, and plastocyanin, PS II and I operate in sequence to mediate the light-driven transfer of electrons from H_2O at PSII to $NADP^+$ at PSI in what is termed linear electron flow (LEF) (8, 9). LEF establishes a transthylakoid electrochemical gradient of protons, or proton motive force (*pmf*) (10, 11), comprised of both proton (ΔpH) and electrical ($\Delta\psi$) gradients (10, 12, 13). Total *pmf* (i.e. $\Delta pH + \Delta\psi$) (14) drives the synthesis of ATP as protons move down their electrochemical gradient through the CF_1-CF_O ATP synthase (ATP synthase) (15-17), whereas the ΔpH component alone also plays a pivotal role in regulating light capture (see below) (10, 18, 19). The ATP and NADPH are subsequently used to drive various downstream metabolic processes, predominantly of which is the reduction of CO_2 from the atmosphere to the level of sugar phosphates in the Calvin-Benson cycle (20).

Flexibility in the light reactions

Two general levels of flexibility are requisite in the light reactions of photosynthesis (11, 21-23). The are strong arguments based on the mechanisms of LEF

and the rotary catalytic mechanism of ATP synthesis (8, 9, 15) that the production of ATP by LEF alone is insufficient to balance the relative ATP/NADPH output ratio required to sustain turnover of even the Calvin-Benson cycle alone (8, 9, 11, 21-23). Given that consumption of ATP and NADPH is coupled in the Calvin-Benson cycle, as well as other biochemical processes (23), such an imbalance could result in metabolic congestion, effectively depleting the light reactions of substrates, predicting catastrophic failure of the entire system. Therefore, flexibility in the light reactions exists at the level of modulating ATP/NADPH output.

Flexibility in the light reactions also exists at the level of regulating light capture due to the potential for plants to absorb, even under ideal conditions, more energy than can actually be processed in downstream metabolism (5, 18, 19, 24-27). The excess energy can drive harmful side reactions that, in some cases, involve toxic species of oxygen which can give rise to a cascade of damage throughout the chloroplast (24, 25, 28). Plants are equipped with a robust antioxidant system to protect themselves from such damage (24, 25, 28), but they also possess a preventative mechanism referred to as energy dependent quenching (q_E) which, over short periods of time (29), harmlessly dissipates excessively absorbed energy (18, 19, 30-33).

The mechanism of q_E is dependent on the conversion of violaxanthin to zeaxanthin (26, 31, 34), which is predominantly controlled in the steady-state by the thylakoid lumen-localized enzyme violaxanthin de-epoxidase (VDE) (10, 12), and protonation of lumen exposed residues of psbS, a polypeptide associated with the light harvesting complex of PSII (18, 19, 24, 30, 31, 35). Because both of these processes are controlled by the pH of the thylakoid lumem (10, 12, 18, 25), a simple model predicts q_E

to be a continuous function of LEF (i.e. the predominant pathway for acidifying the lumen) (36, 37), yet many natural conditions (i.e. drought) attenuate LEF, predicting commensurate decreases in q_E precisely when it is most needed (38, 39). However, flexibility in the relative sensitivity of q_E to LEF has been well established (39-41), a phenomenon that has been termed *modulation of q_E sensitivity* (21-23, 36, 37).

Mechanisms for achieving flexibility in the light reactions

Four models have been proposed to account for this broad range of flexibility in the light reactions, the mechanisms of which have been extensively tested over a wide range of conditions (21-23, 36, 37). The results from such analyses established that complex, yet predictable, relationships exist between various components of the system (i.e. between LEF and q_E, *pmf* and q_E, etc) (21-23, 36, 37). Based on this information, a multitude of questions can be addressed by exploring what factors account for such predictability in the system. All four models predict discontinuity in the relationship between q_E and LEF (i.e. modulation of q_E sensitivity), but they can be further distinguished from each other based on other predicted relationships that are unique to each model. The four models are:

Model 1: Variable response of antennae (i.e. the q_E response) to lumen pH. Short term responses (37) that could account for this model involve changes in the pKa's of amino acid residues on psbS and/or VDE, or by changes in the relative rates of the enzymes of the xanthophyll cycle. Such changes would modulate q_E sensitivity without changing the ATP/NADPH output ratio. This model predicts discontinuity in the

relationship between q_E and *pmf*, as well as that between q_E and the ΔpH component of *pmf*.

Model 2: Variable turnover of alternative electron transfer pathways. In addition to LEF, there are several alternative routes of electron transfer that can contribute to lumen acidification, including the putative involvement of a terminal plastid oxidase in what is termed chlororespiration (42, 43), all of which can potentially modulating either ATP/NADPH output and/or q_E sensitivity (8, 9, 28, 39-41, 44, 45). One such mechanism that is under intense debate in the literature (46, 47) is referred to as cyclic electron flow around PSI, or CEF1, a mechanism that returns electrons from the stromal (i.e. reducing) side of PSI to the PQ pool (45, 48), thereby enhancing the flux of protons into the lumen over that of LEF (23, 39, 48). Variable engagement of such a mechanism predicts discontinuity in the *pmf* generated solely by LEF and total *pmf* (i.e. that which is generated by contributions from LEF, CEF1, etc.)

Model 3: Variable conductivity of the ATP synthase to proton efflux. An important finding to have emerged based on integrated analyses of the light reactions is that the thylakoid membrane is variably resistant to proton efflux (49), which in the steady-state has been shown to be controlled by the conductivity of the ATP synthase to proton efflux (g_H^+) (36, 37). This model predicts that an increase in the magnitude of the steady-state *pmf* could be achieved at a constant flux of LEF simply by decreasing g_H^+ (36, 37, 50). As such, this mechanism would modulate q_E sensitivity, but would do so without altering the relative ATP/NADPH output ratio. Model 3 predicts continuity in the relationship between the *pmf* generated by LEF and total *pmf*, but it further predicts that

discontinuity in the relationship between LEF and q_E will be accompanied by commensurate decreases in g_H^+.

Model 4: Variable pmf partitioning. The transthylakoid *pmf* was thought for a long time to be composed solely of ΔpH (12), but this view has changed and it is now generally accepted that *in vivo*, under steady-state conditions, *pmf* is composed of the both ΔpH and Δψ (13), the partitioning of which varies with physiologic status (36, 51). This model predicts discontinuity in the relationship between *pmf* and q_E, but it also predicts, unlike model 1, continuity in the relationship between q_E and the ΔpH component of *pmf*.

The light reactions occur in a unique lipid matrix

The thylakoid membrane is the matrix within which the light reactions occur and it is composed of unique lipids that are derived from what is referred to as the prokaryotic (i.e. chloroplast) and eukaryotic (i.e. ER-endoplasmic reticulum) pathways (52). The major lipids comprising the thylakoid membrane are monogalactosyldiacylglycerol (MGD) and digalactosyldiaclyglycerol (DGD), making up approximately 75% of the total thylakoid lipid (53). These lipids are further distinct from other cellular lipids in that their fatty acid side chains are highly unsaturated, consisting of ~75-80% polyunsaturated fatty acids (PUFA's) (54), the functional significance of which remains equivocal (52, 55). Although mutant analyses offers a powerful way to elucidate this functionality *in vivo* (52, 55), the bipartite pathway for production of these unique lipids has required combining multiple mutant alleles in a single background in order to observe functional defects (52, 55). One such mutant, referred to as *Fad*2-2/*Fad*6, which is

impaired in 18:1 (ER) and 16:1 (chloroplast) desaturase activity, has only 6% PUFA's in its membranes (52, 55). While *Fad*2-2/*Fad*6 is unable to grow photoautotrophically, it can grow vegetatively on agar plates supplemented with sucrose (52, 55). Although severely chlorotic, growth of *Fad*2-2/*Fad*6 under these conditions was characterized as being 'robust', implying that photosynthesis was the primary process that is dependent upon membranes with highly unsaturated lipids (55).

An *in vivo* characterization of the role of PUFA's in photosynthesis under physiologic conditions using *Fad*2-2/*Fad*6 is hampered by its inability to grow photoautotrophically on soil (55). Therefore, in this study we combined the Fad2-5 allele (56) with the Fad6 allele (57), resulting in a double mutant (*Fad*2-5/*Fad*6) with significantly attenuated levels of PUFA's in its predominant thylakoid membrane lipids (i.e. MGD & DGD), but that could grow photoautotrophically on soil. Using this mutant, we performed integrated analyses of the light reactions (21) to probe for irregularities in the predictability of the system (21-23, 36, 37) in order to assess the function of the unique lipid environment of the thylakoid membrane in photosynthesis. Our results are consistent with the high degree of unsaturation of the thylakoid membrane lipid matrix being integral to maintaining its impermeability to intrinsic proton 'leak', which facilitates tight coupling between the output of the light reactions and downstream metabolism, in the absence of which *pmf* generation and light capture are improperly regulated.

Materials and Methods

Construction of *Fad*2-5/*Fad*6

The *Arabidopsis thaliana* lines used in this study originally descended from Columbia wildtype (Wt). The *Fad*6 mutant was isolated previously from M_2 populations after mutagenesis with ethyl methane sulfonate (EMS) (57), whereas the *Fad*2-5 mutant was isolated from a population of plants with T-DNA insertions in their genomic DNA (56). The *Fad*2-5/*Fad*6 double mutant was generated by crossing *Fad*6 with *Fad*2-5.

Growth conditions

Wildtype (Wt) *A. thaliana* Columbia ecotype and *Fad*2-5/*Fad*6 plants were housed in a growth chamber using a 16:8 photoperiod under a light intensity of ~70 μmol photons $m^{-2}s^{-1}$ photosynthetically active radiation (PAR). The flats within which the plants were grown were kept covered using transparent lids to optimize growth of *Fad*2-5/*Fad*6. The temperature within the growth chamber was maintained at 25°C.

Lipid and fatty acid analyses

Wt and *Fad*2-5/*Fad*6 lipids were extracted and analyzed from leaf tissue as described previously (58).

Spectroscopic Assays

Detached leaves from ~4 week old plants were gently clamped into the measuring chamber of a previously described non-focusing optics spectrophotometer (NoFOSpec) (36, 59). Room air (ambient air-372 ppm CO_2/21% O_2) or premixed low CO_2 air (LC: 50 ppm CO_2/21% O_2) were bubbled through water prior to perfusing the measuring chamber

of the spectrophotometer. Leaves were first exposed to actinic light intensities ranging from 32-216 μmol photons $m^{-2}s^{-1}$ PAR from a bank of red LED's (maximal emission 633 nm) for ten minutes to reach steady-state. From the steady-state, estimates of the minimum (Fs) and maximum (Fm') yields of chlorophyll *a* fluorescence were obtained using a modulated 520 nm probe beam just prior to and during a saturating pulse of white light, respectively. Estimates of LEF were obtained using Fs and Fm' as in (60, 61). After 10 minutes post-actinic illumination, the light saturated level of chlorophyll *a* fluorescence yield (Fm") was obtained, from which estimates of the rapidly recovering, energy dependent component (q_E) of nonphotochemical quenching was estimated (i.e. q_E = Fm"-Fm'/Fm') (19, 36, 37).

Probing the steady-state *pmf*

Estimates of various aspects of the steady-state *pmf* were obtained by kinetic analyses of the electrochromic shift (ECS) of endogenous thylakoid membrane pigments, a linear indicator of transthylakoid Δψ (62). The ECS is a transthylakoid Δψ-induced shift in the absorption spectrum of certain endogenous carotenoid species that occurs maximally at ~520 nm (i.e. ΔA_{520}) (62). The NoFOSpec is designed with 3 separate banks of green LED's (maximal emission between 500 and 540 nm), located at 19° and above the entrance aperture of a compound parabolic concentrator (CPC) whose exit aperture is positioned right above the leaf surface. Prior to entering the CPC, light from each of the LED banks is passed through separate band-pass filters in order to obtain different wavelengths (i.e. 505, 520, 535 nm) of incident light that is then focused onto the leaf via the CPC. When experiments were performed requiring measurement of

absorbance changes at all 3 wavelengths, as in (36, 63), the banks of LEDs were pulsed out of sequence by 10 ms, allowing for near simultaneous measurements of absorbance changes associated with all three wavelengths.

ECS changes were assessed by a previously established technique referred to as dark interval relaxation kinetic (DIRK) analysis (64), whereby ECS absorbance changes are measured during perturbations of the steady-state with dark periods of various duration, depending on the type of information being sought (36, 37, 64, 65). Over short dark periods (i.e. >500 ms), the ΔA_{520} signal predominates over background scattering signals, allowing ECS changes to be estimated by monitoring changes in absorbance solely at 520 nm. The resultant signals display several useful characteristics (22). For example, such ΔA_{520} signals are constant under steady-state illumination, presumably reflecting the fact that the fluxes of protons both into and out of the lumen are precisely balanced in the steady-state. However, during the ensuing brief dark perturbation, the ΔA_{520} signals decay with first order kinetics to a quasi-stable level (i.e. stable after the ~500 ms darkness), presumably reflecting the fact that one process occurs during the short dark perturbation, e.g. equilibration of the light-induced *pmf* with the free energy of ATP synthesis (i.e. ΔG_{ATP}) as protons move down their electrochemical gradient through the ATP synthase. A mathematical description of light-induced *pmf* based on ECS analyses can be used to derive several useful parameters (21-23, 36, 37):

$$pmf\,(ECS_t) = \nu_{H+} \bullet \tau_{ECS} \qquad (1)$$

This equality indicates that the magnitude of the light-induced *pmf* (ECS_t), *e.g.* the total amplitude of ECS decay during the brief light-dark transition, is proportional to the flux of protons into the lumen (v_{H+}), as well as the time constant for proton efflux from the lumen through the ATP synthase (τ_{ECS}), which is inversely proportional to the conductivity of the ATP synthase to protons, or g_{H^+}. Assuming a constant H^+/e^- ratio for LEF and that turnover of other proton pumping processes (i.e. CEF1, etc) are constant fractions of LEF, Eqn. 1 can be rearranged into an equality that expresses the *pmf* generated solely by LEF (pmf_{LEF}) (36, 37):

$$pmf_{LEF} = LEF/g_{H^+} \quad (2)$$

If these assumptions hold true, light-induced *pmf* (i.e. that which is generated by contributions from LEF, CEF1, etc) would be expected to be proportional to pmf_{LEF}:

$$ECS_t \propto pmf_{LEF} = LEF/g_{H^+} \quad (3)$$

Therefore, comparisons of pmf_{LEF} and ECS_t, both of which independently estimate the magnitude of the steady-state *pmf*, can provide information regarding changes in the fractional turnover of CEF1 (36, 37). Variability in the relative ECS responses between the Wt and mutants, attributable to differences in chlorophyll content (see above), were accounted for by normalizing the signals relative to the initial, rapid rise in the ECS in response to a saturating xenon flash, essentially as described in (21).

Perturbing the steady-state with longer dark periods (i.e. minutes) allows other light scattering processes to significantly contribute to apparent absorbance at 520 nm (36, 64-66). Therefore, absorbance changes at 520 nm were deconvoluted from these background signals as in (21, 36, 64). During these longer light-dark transitions, such deconvoluted signals initially decay from the steady-state to a level which reflects ECS_t (22), but after this initial decay, the signal relaxes over time to a dark stable level that is different in magnitude than the steady-state illuminated ECS level, i.e. the light-dark difference in ECS (ECS_{ss}) is interpreted as being proportional to the $\Delta\psi$ component of light-induced *pmf* (22, 36). Since the ECS signal initially inverts with respect to the ensuing dark stable level (i.e. the ECS level which represents an effective transthylakoid $\Delta\psi$ of 'zero'), the inverted region of the signal (ECS_{inv}) is interpreted as being related to the proton diffusion potential (i.e. the ΔpH component of light-induced *pmf*) coming into equilibrium with reversal of transthylakoid $\Delta\psi$ (i.e. positive on the stromal side of the membrane). Therefore, the relative partitioning of light-induced *pmf* into $\Delta\psi$ and ΔpH (i.e. ECS_{inv}/ECS_t represents the fraction of light-induced *pmf* partitioned into ΔpH) can be assessed by such ECS analyses (22).

Probing the pre-steady-state *pmf*

Flash-induced analyses of the ECS (67) were performed to assess the pre-steady-state kinetics of *pmf* dissipation using a kinetic spectrophotometer constructed in-house similar in design to the NoFOSpec (59), a notable exception of which is that a xenon arc lamp was connected to the spectrophotometer via a fiber optic bundle that terminated directly above the entrance aperture of a CPC, allowing for delivery of short (10 μsec),

saturating flashes of actinic light to the leaf surface. A bank of green LED's positioned above the CPC provided the 520 nm probe beam whose timing was controlled by computer software constructed in-house. Current at the photodiode detector was integrated and amplified by an operational amplifier and sampled by a 16-bit resolution analog-digital converter.

In order to estimate the intrinsic yield of proton 'leak' (Φ_{PL}) across the thylakoid membrane, the kinetics of ECS decay following a short (10 usec) saturating xenon flash were monitored on leaves that had either been dark-adapted for ~2 hours or that had been exposed to 30 seconds of actinic light at an intensity of 300 μmol photons $m^{-2}s^{-1}$. An assumption of these analyses is that there are two primary routes through which flash-induced *pmf* formation can be dissipated, one of which is through activated ATP synthase enzymes, whereas the other is passive proton leak across the thylakoid membrane. Since dark-adaptation inactivates the ATP synthase (67), flash-induced ECS decay kinetics under such conditions were interpreted as being indicative of passive dissipation of *pmf* across the thylakoid membrane, whereas the decay kinetics from leaves that had been light adapted for 30 seconds were interpreted as representing *pmf* dissipation through activated enzymes. Proton 'slip' through inactivated enzymes (i.e. dark-adapted leaves) was ruled out based on the observation that infiltration of leaves with dicyclohexylcarbodiimide (DCCD-SIGMA Aldrich), an inhibitor of the CF_O ring of the ATP synthase (15), had no effect on the kinetics of ECS decay (data not shown). Therefore, rate constants for passive proton leak across the thylakoid membrane and that through activated ATP synthase enzymes were calculated from the time constants of

single exponential fits of the flash-induced ECS decay kinetics, which were then used to estimate Φ_{PL}:

$$\Phi_{PL} = k_{PL}/(k_{PL} + k_E) \quad (4)$$

k_{PL} and k_E are rate constants for dissipation of flash-induced *pmf* via passive proton leak across the thylakoid membrane (i.e. dark-adapted leaves) and through activated enzymes (i.e. leaves exposed to actinic light), respectively.

Results and Discussion

Attenuation of [PUFA] in lipids specific for the thylakloid membrane in *Fad*2-5/*Fad*6

Shown in Table 1 are the fatty acid contents (on a percentage basis) of the predominant thylakoid membrane lipids, *e.g*. MGD and DGD (53), in Wt and *Fad*2-5/*Fad*6. Consistent with the 18:1 and 16:1 desaturase activities of FAD2 and FAD6, respectively (52), 18:1 and 16:1 in MGD were, in comparison to Wt, 9-fold and 19-fold larger in *Fad*2-5/*Fad*6, whereas 18:3 and 16:3 levels were ~3.7-fold and 33-fold lower. A similar qualitative pattern is shown for DGD. These results indicate that combining the Fad6 and Fad2-5 mutant alleles in one background significantly attenuated the PUFA content of the predominant thylakoid membrane lipids. Unlike the *Fad*2-2/*Fad*6 double mutant (55), *Fad*2-5/*Fad*6 was capable of photoautotrophic growth on soil, although it

was severely chlorotic, e.g. chlorophyll concentration in *Fad*2-5/*Fad*6 was only ~40% that in the Wt (data not shown).

Desensitization of q_E to LEF in *Fad*2-5/*Fad*6 was accompanied by modest decreases in g_H^+

Shown in Fig. 1 are estimates of q_E as a function of LEF from 32-216 μmol photons $m^{-2}s^{-1}$ for Wt under ambient (372 ppm CO_2/21% O_2) and low CO_2 (LC: 50 ppm CO_2/21% O_2) air, as well as that of *Fad*2-5/*Fad*6 under ambient air. A q_E of ~0.5 was generated in the Wt by a flux of ~30 μmol electrons $m^{-2}s^{-1}$ under ambient air, whereas the same level of q_E was generated by a flux of ~15 μmol electrons $m^{-2}s^{-1}$ under the LC air, indicating a ~2-fold increase in q_E sensitivity. These results are qualitatively similar to previous observations in both tobacco (36, 37) and *A. thaliana* (51). Accompanying this CO_2-dependent increase in q_E sensitivity was a ~1.5 fold decrease in g_H^+ (Fig. 2), implying that such changes, while important, were insufficient on their own to account for the observed 2-fold increase in q_E sensitivity. These combined results imply that q_E sensitivity modulation in *A. thaliana* is achieved by mechanisms other than changes in g_H^+ (see below), as was suggested previously (51).

Although light saturated LEF was attenuated in *Fad*2-5/*Fad*6 to the same extent as that in the Wt under LC conditions (Fig. 1), its light saturated q_E response was ~4-fold lower than that of the Wt under LC air, indicating that, in contrast to the CO_2-dependent increase in q_E sensitivity in Wt, the q_E response was actually *desensitized* to LEF in *Fad*2-5/*Fad*6. Moreover, in contrast to the CO_2-dependent ~1.5-fold decrease in g_H^+ in Wt, estimates of g_H^+ in *Fad*2-5/*Fad*6 were indistinguishable from that in the Wt under

ambient conditions (Fig. 2), except at higher light intensities where g_H^+ was observed to modestly decrease in *Fad*2-5/*Fad*6, although such changes were not to the extent of the CO_2-dependent decrease in g_H^+ in the Wt.

Defective compensatory changes in LEF and g_H^+ in *Fad*2-5/*Fad*6 solely account for attenuation of q_E

A unique parameter referred to as pmf_{LEF}, or the *pmf* generated by LEF alone, can be derived from integrating the proton and electron circuits of photosynthesis (21-23, 36, 37). Comparisons of pmf_{LEF} with other photosynthetic parameters can provide valuable information about how the light reactions are adjusted (21-23, 36, 37). Using *N. tabacum* as a model system, Kanazawa and Kramer provided evidence that a continuous relationship exists between q_E and pmf_{LEF} over a wide range of CO_2 levels (37). These results were interpreted as being consistent with the observed CO_2-dependent modulation of q_E sensitivity being solely attributable to the fact that decreases in LEF were accompanied by commensurate decreases in g_H^+, which effectively allowed the low rates of LEF to build-up significant *pmf* for the purposes of down-regulating light capture (37). These results imply that variability in the magnitude of the steady state *pmf* over this wide range of conditions was controlled by compensatory changes in the rate of proton flux into the lumen (i.e. driven by LEF) and the resistance to proton efflux from the lumen (i.e. controlled by g_H^+).

In contrast to the situation in *N. tabacum*, discontinuity between q_E and pmf_{LEF} was observed in Wt *A. thaliana* upon lowering CO_2 from ambient to LC levels (Fig. 3). These results are predicted *if and only if* changes in g_H^+ do not solely account for

modulation of q_E sensitivity (36, 37), consistent with the discrepancy in the 2-fold increase in q_E sensitivity being accompanied by only a ~1.5-fold decrease in g_H^+ (above). These results are similar to what was observed previously in *A. thaliana* upon identical changes in CO_2 levels, results which were shown to be solely attributable to enhanced partitioning of the light-induced *pmf* (i.e. ECS_t) into ΔpH (51). Consistent with this interpretation, a continuous relationship between not only ECS_t and pmf_{LEF} was observed upon lowering CO_2 in the Wt (Fig. 3 inset), but a continuous relationship was also observed between q_E and estimates of the ΔpH component of *pmf* (i.e. ECS_{inv}) (Fig. 4). Taken together, these results suggest that alternative electron transfer pathways (i.e. CEF1) and the relative sensitivity of the q_E response to lumen pH, respectively, both remained constant under these conditions, implying that the observed enhanced partitioning of the light-induced *pmf* into ΔpH upon lowering CO_2 (Fig. 5) solely accounts for the discontinuity in the relationship between q_E and pmf_{LEF}. Overall, these combined results are consistent with CO_2-dependent q_E sensitivity modulation in *A. thaliana* occurring via simultaneous changes in both g_H^+ and *pmf* partitioning, as has been suggested (51).

A continuous relationship between q_E and pmf_{LEF} was observed for the Wt and *Fad*2-5/*Fad*6 under ambient air (Fig. 3). In addition, not only was a continuous relationship observed between ECS_t and pmf_{LEF} (Fig. 3 inset) and q_E and ECS_{inv} (Fig. 4), but the relative partitioning of light-induced *pmf* into ΔpH in *Fad*2-5/*Fad*6 was also indistinguishable from that in the Wt (Fig. 5). These results imply that relative changes in neither the topology of electron transfer (i.e. CEF1), the antenna response to lumen pH, nor variable *pmf* partitioning, respectively, account for attenuation of q_E in *Fad*2-5/*Fad*6,

consistent with q_E being lower in the mutant simply because the magnitude of the steady-state *pmf* is lower (Fig. 3). Furthermore, these combined results (i.e. Figs. 1-3) are consistent with the diminished magnitude of light-induced *pmf* (i.e. pmf_{LEF}) in *Fad*2-5/*Fad*6 being solely attributable to the fact that decreases in light saturated LEF were accompanied by, in contrast to the CO_2-dependent 1.5-fold decrease in g_H^+ in the Wt (Fig. 2), only modest, at best, decreases in g_H^+ (Fig. 2). In effect, the modest decreases in g_H^+ were incapable of compensating for the observed decreases in LEF, resulting in attenuation of the steady-state *pmf* and commensurate decreases in q_E.

Enhanced proton permeability of *Fad*2-5/*Fad*6 thylakoid membranes

Shown in Table 2 are relative estimates of the rate constants for dissipation of flash-induced *pmf* through the thylakoid membrane (k_{PL}), through activated ATP synthase enzymes (k_E), as well as relative estimates of the yield of passive proton leak across the thylakoid membrane (Φ_{PL}) for both the Wt and *Fad*2-5/*Fad*6. Consistent with passive proton leak being very slow, Φ_{PL} was ~0.014 for the Wt, whereas that in *Fad*2-5/*Fad*6 was ~0.04. Estimates of Φ_{PL} in *Fad*2-5/*Fad*6 were ~3-fold larger than that in the Wt, representing an effective ~3-fold increase in the leakiness of the mutant thylakoid membranes to passive proton efflux.

Conclusions

Lack of compensatory change in LEF:g_H^+ in *Fad*2-5/*Fad*6 is not *directly* due to leakiness of the thylakoid membranes to proton efflux

The majority of the CO_2-dependent increase in q_E sensitivity upon shifting Wt from ambient to LC air could be attributed to a decrease in g_H^+, an effect which partially (see below) compensated for the lowering of light saturated LEF (Figs. 1 & 2). In contrast, although light saturated LEF was attenuated in *Fad*2-5/*Fad*6 to the same extent as that of the Wt at low CO_2, g_H^+ decreased only modestly, at best (Fig. 2). It is tempting to ascribe this lack of compensatory change in the LEF:g_H^+ relationship to the fact that there was evidence for the thylakoid membranes of *Fad*2-5/*Fad*6 being slightly leaky to proton efflux (Table 2), which could, assuming that such a leak was robust, maintain high effective g_H^+ even when LEF was low. However, we believe that the yield of the leak, while being 3-fold higher than that in the Wt, is still far too small (Φ_{PL} = 0.04) to *directly* account for the effect on the steady-state estimates of g_H^+ in *Fad*2-5/*Fad*6.

Modest leakiness of *Fad*2-5/*Fad*6 thylakoid membranes to proton efflux results in a system failure

An alternative explanation for the defect in the compensatory change in the LEF:g_H^+ relationship in *Fad*2-5/*Fad*6 involves a failure at the *system* level. For example, the compensatory change in g_H^+ in the Wt in response to a lowering of LEF has been suggested to involve a decrease in stromal levels of P_i below its K_M at the ATP synthase, decreasing the turnover rate of the enzyme and effectively g_H^+, allowing low rates of LEF to generate significant *pmf* so that regulation of light capture (i.e. via q_E) occurs properly (36, 37). This interpretation assumes a biochemical demand-induced system response, whereby the light reactions are controlled by downstream events that modulate stromal levels of P_i (36, 37).

We recently reported on a mutant (63), referred to as *pgr*5 for proton gradient regulation, putatively impaired in the main route of CEF1 (68), the results from which bolster this system view of the light reactions. Based on our analyses, the gene product of PGR5 mediates a flux of protons no more than ~15% that of LEF (63). However, in the absence of such a modest flux, a shift in the intermediates of the ATP synthesis reaction could be expected to occur toward that of reactants, *e.g*. accumulation P_i, possibly above its K_M at the ATP synthase, maintaining effective g_H^+ at high levels. Because ATP and NADPH consumption are coupled in the Calvin-Benson cycle, such a shift could also deplete LEF of electron acceptors (i.e. $NADP^+$). Consistent with this interpretation, LEF was attenuated in the *pgr*5 mutant and g_H^+ was indistinguishable from that of the Wt, except that at higher light intensities g_H^+ was actually enhanced in *pgr*5 (63). These combined changes were shown to result in attenuation of the magnitude of the steady-state *pmf*, resulting in commensurate lowering of the q_E response (63). These results imply that that the system is very tightly regulated in the steady state and when even slight perturbations occur (i.e. a slight decrease in CEF1), metabolic congestion can ensue which prevents the system from responding properly.

Similarly, the combined results for *Fad*2-5/*Fad*6 confirm very tight regulation of the system and that the high levels of PUFA's in the lipid matrix of energy transduction (i.e. the thylakoid membrane) are important for such regulation. For example, our results suggest that the thylakoid membranes in *Fad*2-5/*Fad*6 are slightly leaky to proton efflux (Table 2), an effect that, like the *pgr*5 phenotype, could be expected to result in accumulation of ADP and P_i due to a slight uncoupling of *pmf* dissipation with that of ATP synthesis. Once again, because ATP and NADPH consumption are coupled in the

Calvin-Benson cycle, such a shift could restrict the availability of electron acceptors, thereby accounting for the decrease in light saturated LEF in *Fad*2-5/*Fad*6 (Fig. 1). Moreover, accumulation of P_i above its K_M at the ATP synthase could prevent g_H^+ from decreasing to levels that would compensate for the attenuated rates of LEF (Fig. 2). The combined effect of such a defective compensatory change would be a lowering of the q_E response simply because steady-state *pmf* was lower (Fig. 3). This interpretation implies a failure of the system resulting from slight imbalances in ATP:NADPH output with that of downstream metabolism, an effect for which the highly unsaturated character of the lipids comprising the thylakoid membrane help to prevent by maintaining the impermeability of the membrane to proton efflux.

A failure in the system could also occur at the level of either ion homeostasis or lumenal proton buffering capacity. The attenuated rates of LEF upon shifting Wt from ambient to LC air were capable of generating an enhanced q_E response not only because g_H^+ was lower (Fig. 1 & 2), but there was also evidence for more of the *pmf* being stored as ΔpH under the LC conditions (Fig. 5). Mathematical modeling indicates that variable storage of *pmf* as ΔpH:Δψ is most dependent on ion homeostasis within the chloroplast and/or proton buffering capacity of the thylakoid lumen (10, 12, 13). Attenuated rates of LEF in *Fad*2-5/*Fad*6 (Fig. 1) were not, in contrast to the situation in the Wt upon shifting to LC air, accompanied by enhanced partitioning of the *pmf* into ΔpH (Fig. 5), implying that perturbations likely occurred in the system at the level of either ion homeostasis or proton buffering capacity.

Understanding the complexities of steady state photosynthesis requires a systems approach

For several decades the predominant mechanism thought responsible for modulating q_E sensitivity was CEF1 (23), and intense debate persists in the literature as to its precise role, even up until very recently (46, 47). Based on work in our lab, we recently presented a series of reviews in which we proposed, rather, that CEF1 is predominantly responsible for modulating output of ATP and NADPH (21-23), a hypothesis that was supported by our work with the *pgr*5 mutant (63). In this model, modulation of q_E sensitivity is relegated to changes in g_H^+ and *pmf* partitioning, neither of which alter ATP:NADPH output (21-23). The basis for proposing such a model was an extensive body of research aimed at integrating the proton *and* electron circuits of the light reactions into our understanding of *in vivo*, steady-state photosynthesis (13, 36, 37, 59, 64-66). In essence, these conclusions required analyses of the entire system.

Similarly, understanding the functional significance of the high levels of poly-unsaturation in the lipids which comprise the thylakoid membrane in photosynthesis requires such an integrated approach. Although photosynthesis was previously demonstrated to be the predominant process absolutely dependent upon high levels of poly-unsaturated fatty acids, the evidence was equivocal with respect to demonstrating precisely why they were essential (55). It was even speculated that CO_2 metabolism may be altered, indirectly impacting the light reactions (55). In contrast, our results using *Fad*2-5/*Fad*6 indicate that the highly un-saturated nature of the thylakoid membrane is essential for properly balancing ATP:NADPH output of the light reactions with that of their downstream consumption, an interpretation that is 180° opposite of what was

originally thought (55). This interpretation is based on the observation that the *Fad*2-5/*Fad*6 thylakoid membranes were slightly more leaky to passive proton efflux (Table 2). The results of the flash-induced analyses of ECS decay kinetics from which this interpretation was derived could be interpreted by more complex models involving counterion movements (13). However, the simplest interpretation based on integrated analyses of other aspects of the system is that the highly unsaturated nature of the thylakoid membrane facilitates the very tight coupling of *pmf* dissipation through the ATP synthase, allowing for appropriate balancing of ATP:NADPH output with that of their downstream consumption. Our results are consistent with the notion that, in the absence of such balancing, metabolic congestion ensues which results in failure of the system at the level of electron transfer (Fig. 1) and regulation of the proton conductivity of the ATP synthase (Fig. 2), both of which account for attenuation of the magnitude of the steady-state *pmf* (Fig. 3). Failure also occurs at the level of *pmf* partitioning (Fig. 5) which, when coupled with attenuated levels of *pmf*, results in a diminished q_E response (Fig. 3).

Figure/Table Legends

Table 1. Fatty acid composition of thylakoid membrane lipids. The predominant thylakoid membrane lipids monogalactosyldiacylglycerol (MGD) and digalactosyldiacylglycerol (DGD) (53) were isolated from leaves of wild type and *Fad*2-5/*Fad*6, followed by analyses of their fatty acid composition as described in (58). ND = not detectable.

Table 2. Kinetic parameters derived from flash-induced analyses of the electrochromic shift (ECS). The kinetics of ECS decay following a 10 μsec xenon flash were analyzed in leaves from wild type and *Fad*2-5/*Fad*6 that had been either dark-adapted for ~2 hours or exposed to 30 seconds of actinic light at a flux of ~300 μmol photons $m^{-2}s^{-1}$. Time constants were obtained from single exponential fits of the decay kinetics, by which rate constants for passive proton leak (k_{PL}) and proton flux through activated ATP synthase enzymes (k_E) were derived from dark-adapted and light-adapted samples, respectively. The yield of passive proton leak through thylakoid membranes (Φ_{PL}) was estimated as described in Materials and Methods using these rate constants.

Figure 1. The relationship between energy dependent quenching and electron transfer. Energy dependent quenching (q_E) and linear electron flow (LEF) were estimated from 32-216 μmol photons $m^{-2}s^{-1}$ via analyses of chlorophyll *a* fluorescence yield (36) in wild type under ambient (372 ppm CO_2/21% O_2) and low CO_2 (LC-50 ppm CO_2/21% O_2) air, as well as *Fad*2-5/*Fad*6 under ambient air. Closed squares and circles represent wild

type under ambient and LC conditions, respectively, whereas open squares represent *Fad*2-5/*Fad*6 under ambient air. Horizontal and vertical error bars represent SE for LEF and q_E, respectively (n = 4-7).

Figure 2. Relative estimates of the proton conductivity of the ATP synthase as a function of light intensity. Estimates of the proton conductivity of the ATP synthase (g_H^+) were derived by taking the inverse of the time constants from the single-exponential fits of the ECS decay kinetics during a 300 ms dark perturbation of the steady-state (36, 37). Conditions and symbols are the same as in Fig. 1. Error bars are SE for n = 4-7.

Figure 3. The relationships between the *pmf* generated by LEF alone and both energy dependent quenching and total *pmf*. Estimates of q_E, LEF, and g_H^+ were obtained as described in Figure legends 1 & 2, respectively. Estimates of the *pmf* generated solely by LEF (pmf_{LEF}) were obtained by dividing LEF by g_H^+ (36, 37). Total, light-induced *pmf* (ECS_t) was estimated as the total amplitude of ECS decay during a 300 ms perturbation of steady-state illumination (36, 37). Conditions and symbols are the same as in Fig. 1. Error bars are SE for n = 4-7.

Figure 4. The relationship between energy dependent quenching and the ΔpH component of light-induced *pmf*. q_E was estimated as described in the legend from figure 1. The ΔpH component of light-induced *pmf* (ECS_{inv}) was estimated from the deconvoluted ECS decay kinetics after a ~2 minute dark perturbation of steady-state as

described in (22, 36). Conditions and symbols are the same as in Fig. 1. Horizontal and vertical error bars are SE for ECS_{inv} and q_E, respectively (n = 4-7).

Figure 5. The fraction of light-induced *pmf* partitioned into ΔpH as a function of light intensity. The fraction of light induced *pmf* partitioned into ΔpH (ECS_{inv}/ECS_t) was obtained from 32-216 μmol photons $m^{-2}s^{-1}$ via kinetic analyses of the ECS during a ~2 minute dark perturbation of steady-state illumination as described in (22, 36). Light-induced *pmf*, ECS_t, was taken as the total amplitude of ECS decay after ~30 ms, whereas the ΔpH component of *pmf* (ECS_{inv}) was taken as the portion of the ECS signal (i.e. after ~30 ms) that inverts with respect to the ensuing dark stable level (i.e. after ~ 2minutes in the dark) (22, 36). Error bars are SE for n = 4-7.

	MGD		DGD	
Fatty acid	Wild type	*Fad*2-5/*Fad*6	Wild type	*Fad*2-5/*Fad*6
% 16:1	1.8	16.2	1.2	6.2
% 18:1	2.8	53.8	1.5	52.3
% 16:3	33.8	ND	4.2	ND
% 18:3	49.2	13.2	77.8	18.5

Table 1

	k_{PL} (s^{-1})	k_E (s^{-1})	Φ_{PL}
Wild type	0.00115	0.081	0.014
*Fad*2-5/*Fad*6	0.00303	0.074	0.040

Table 2

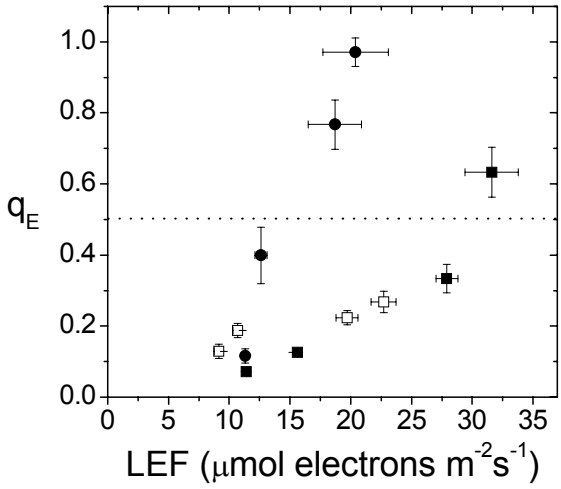

Figure 1

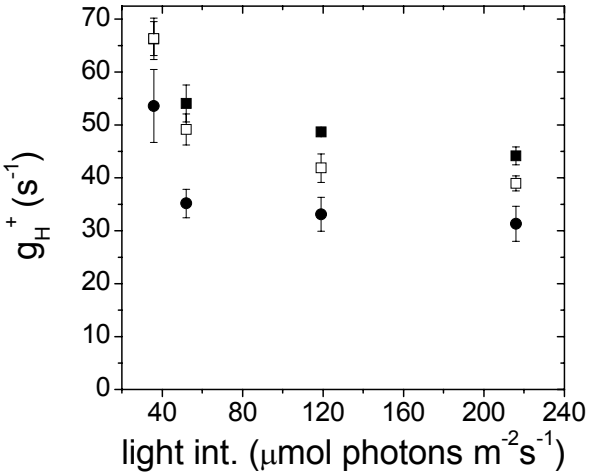
70
60
50
40
30
20
10
0
40
80
120
160
200
240
g_H^+ (s^{-1})
light int. (μmol photons $m^{-2}s^{-1}$)

Figure 2

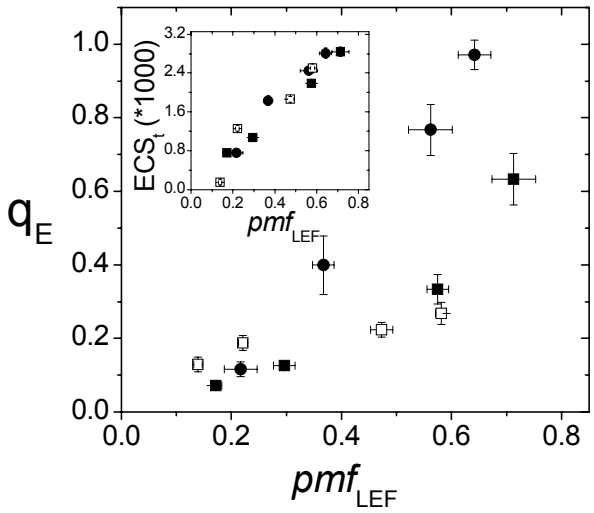

Figure 3

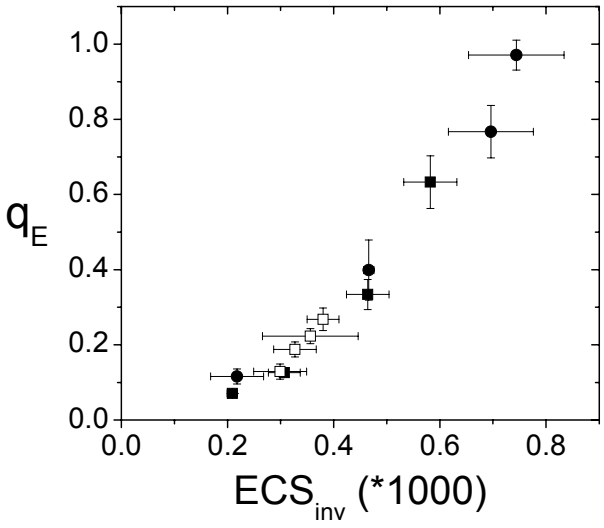

Figure 4

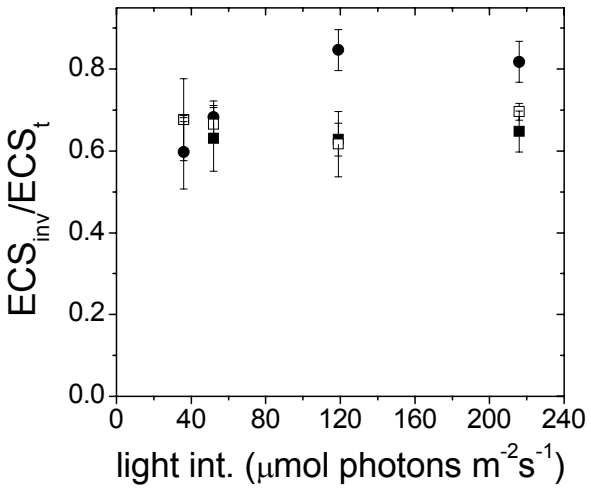
ECS$_{inv}$/ECS$_t$
0.0
0.2
0.4
0.6
0.8
0
40
80
120
160
200
240
light int. (μmol photons m^{-2}s^{-1})

Figure 5

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Conclusions

We approach scientific truth asymptotically and our goal as a scientific community should be to progressively move along this continuum by rigorously applying the scientific method, whereby alternative hypotheses are proposed for a given observation and subsequently tested in a systematic fashion. This approach has been previously suggested to lead to rapid scientific progress (1). A pernicious period of time occurs during the discovery of new instruments and techniques for measuring previously un-testable hypotheses because long held views may be in need of reconsideration, even if doing so requires rethinking decades of previous research. The danger involves the temptation to hold on to long held beliefs, even if new data is to the contrary. We are protected from such temptation by succumbing to the notion that we are approaching, not arriving at, truth.

Research aimed at understanding how the light reactions of photosynthesis are regulated is right in the middle of just such a period in history. Cyclic electron flow around photosystem I (CEF1) has been acknowledged in the literature for decades (as reviewed in 2), and a predominant role ascribed to it has been in regulating light capture in the antennae (3-6). This hypothesis was based on the observation that antennae regulation was actually enhanced under conditions that attenuated linear electron flow (LEF) (i.e. low CO_2, etc.), an observation that could logically be attributed to an increase in the fractional turnover of CEF1 under such conditions. However, with advances in instruments and techniques designed to estimate various aspects of the steady-state *pmf* (7-13), Kanazawa and Kramer (12) showed, in a seminole study, that the turnover rate of the CF_O-CF_1 ATP synthase (ATP synthase), *e.g.* its effective conductivity to protons, or

g_H^+, decreased commensurately with that of LEF upon lowering CO_2 from 2000 to 0 ppm. In essence, this discovery demanded a paradigm shift in how we think of antennae regulation, from viewing it as occurring by an *increase* in the flux of protons into the lumen by, for example, CEF1, to viewing it as being predominantly due to a *diminished* rate constant for proton efflux from the lumen, a response that allows low rates of proton flux into the lumen to generate a substantial *pmf*.

The work presented in this dissertation is consistent with these initial findings regarding the role of CO_2-dependent changes in g_H^+, but it also extends these new findings by demonstrating that in addition to changes in g_H^+, antennae regulation is also modulated by variability in the relative partitioning of *pmf* into a proton diffusion potential (i.e. ΔpH). Regulation of light capture by changes in these two mechanisms preserves the ATP/NADPH output ratio of the light reactions, the modulation of which is herein suggested to be due to variability in the fractional turnover of CEF1. The interplay between these two types of mechanisms allows plants to survive in a dynamic environment (14-16).

In closing, integration of the role of the thylakoid membrane in the light reactions of photosynthesis represents a move forward, utilizing the observed predictability that has been established in the relationships between various components of the system to understand what components account such predictability.

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