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A. Project Narrative: PROGRESS ON SPECIFIC AIMS

We have made substantial progress on all of our scientific goals, as detailed below. Several open questions remain, and we will address in the final year of the proposed work and beyond. The work thus far has resulted in several publications citing funding from this grant (*1-12*) with several more near publication (*13-15*), as well as the introduction of new spectroscopic tools and methods to explore the photosynthetic energy budget *in vivo*.

A.1. Specific Aim 1: Why do *hcef* mutants have increased CEF1?

*Rationale: Determining the loci of hcef mutants will tell us which processes can affect CEF1 capacity or activation***.**

Progress: Using multi-stage selection of chemically-mutagenized Arabidopsis plants, we isolated a new class of mutant, *hcef* for high CEF1, which shows constitutively elevated CEF1 (*11*). This class of mutants has the potential to revolutionize the field of CEF1 for three important reasons. First, CEF1 is substantial in these mutants, allowing us for the first time to measure with confidence, its function, properties and control. Second, the dramatic increase in CEF1 in these mutants provides important clues about the regulation of CEF1. Third, the increase in activity can also tell us which of the many pathways are involved in CEF1.

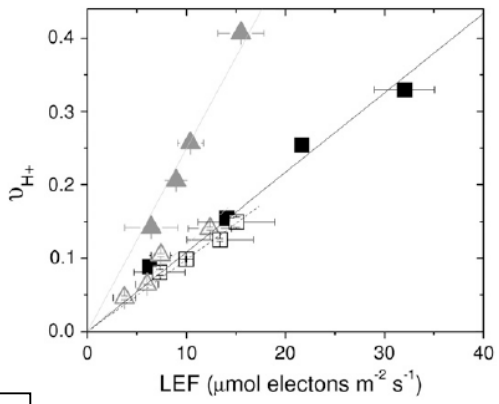


Figure 1 Evidence for Elevated CEF1 in *hcef1:* The Relationship between Light-Driven Proton Translocation across the Thylakoid (v_H^+) and LEF.

We have identified the genetic loci of three of the four high CEF1 mutants. Interestingly, the fourth mutant, *hcef4*, resulted in sterile phenotype when crossed into Ler. We must therefore take a different approach to cloning this gene. Nevertheless, the three *hcef* mutants we have cloned have allowed us to approach all of our proposed goals. Two of these mutations occur at different points in the Benson-Calvin cycle. The *hcef1* mutation was found at fructose-1,3-bisphosphatase (*11*), and that for *hcef2* was found within glyceraldehyde-3-phosphate dehydrogenase (GADPH) (*12*). The mutation for *hcef3* was found in a gene annotated as 2-phosphoglycerate kinase (2PGK). This enzyme is not involved in the formation of 1,3-bisphosphoglycerate in the Benson-Calvin cycle, but is proposed to catalyze the phosphorylation

of phosphoglycerate to 2,3-phosphoglycerate (2,3-BPG). Importantly, 2,3- BPG is thought to play a regulatory (rather than strictly metabolic) role in some species.

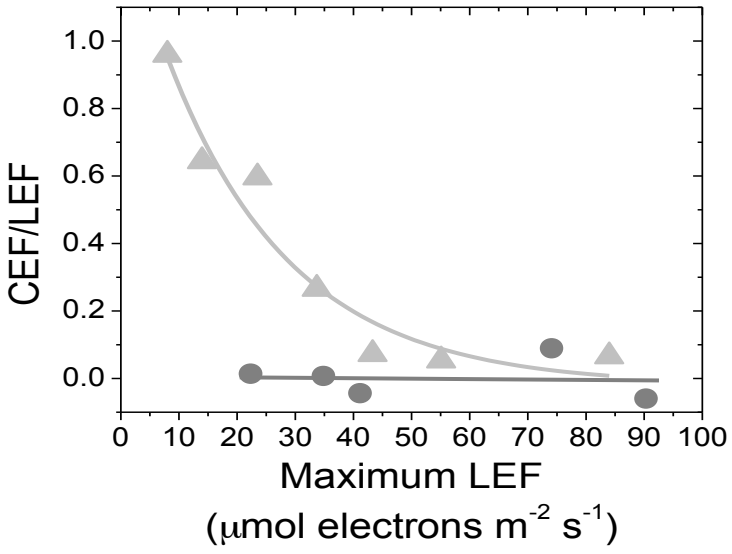


Figure 2. The fraction of CEF/LEF in the mutants, ssuR and gapR compared to maximum rate of LEF. The mutants ssuR (●) and gapR (▲) are compared over a variable range of inhibition which is inversely proportional to their maximum level of LEF.

The cloning of *hcef1* and *hcef2* led us to use antisense suppression and reverse genetics on other Benson-Calvin cycle and related targets. As we reported in (*13*) and illustrated in Fig. 2, suppression in tobacco of GAPDH (in the GAPR mutant lines) led to a decrease in maximal linear electron flow (LEF, X-axis), accompanied by a dramatic increase in CEF1 as a fraction of LEF (Y-axis). Suppression of Rubisco small subunit (in the ssuR antisense lines) led to similar decreases in maximal LEF, but no increase in CEF. This contrast in effects indicate that simply slowing photosynthesis is not sufficient to increase CEF. Rather, specific signals or metabolites are responsible for regulating CEF1.

In light of these results, we have begun to assess the effects of many of the steps in assimilation. Figure 3 briefly illustrates our findings so far. The red circles indicate the steps where antisense suppression (or in the cases of FNR1 and FNR2, complete knockout) led to a suppression of photosynthesis with an accompanying increase in CEF1 (GAPDH, aldolase, fructose-1,6-bisphosphatase, transketolase, ferredoxin NADP+ oxidoreductase (FNR1 or FNR2)). The yellow circles indicate steps where photosynthesis could be suppressed to similar extents but with no increase in CEF1 (Rubisco small subunit, cytochrome b_6f complex, ATP gamma subunit). These results strongly support our conclusion that CEF1 is upregulated by specific metabolites or signals, and not by simply suppressing overall photosynthesis.

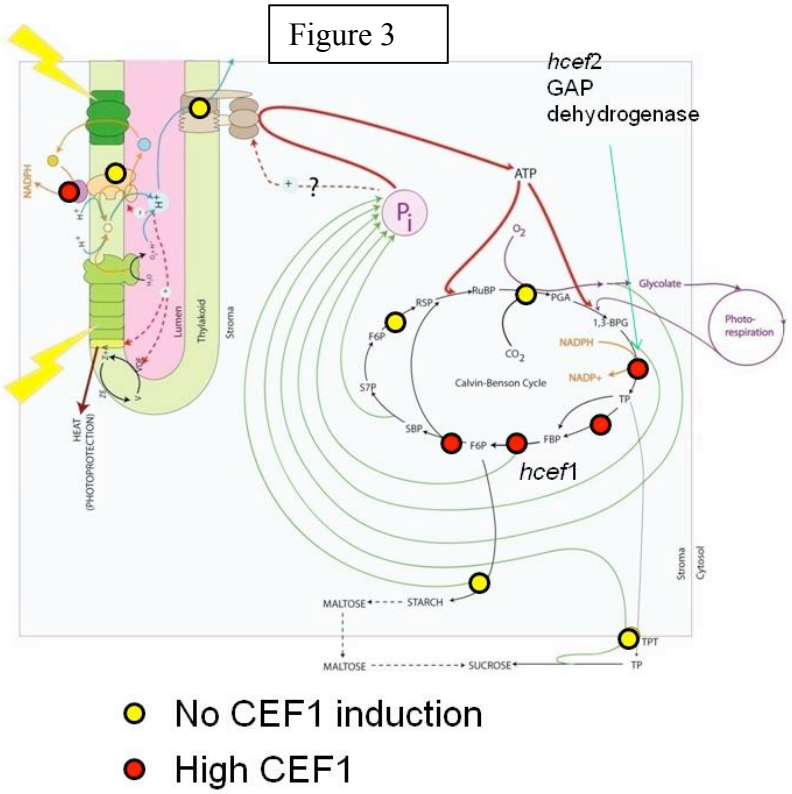
A.2. Is increased CEF1 caused by elevated expression or altered regulation of CEF1 components?

Rationale: The increases in rates seen in high CEF1 mutants could be caused either by an increase in the expression levels of PQR enzymes, or up-regulation of Wt enzymes levels. We will distinguish between these by assaying for expression levels and in vitro activities.

Progress: In the high CEF1 mutant, *hcef1*, we demonstrated a dramatic increase in accumulation of NDH proteins, but a slight *decrease* in PGR5, FNR and cytochrome b_6f proteins (*11*). These results are consistent with a major role for the NDH complex, but not the other possible CEF1 pathways (*11*).

A.3. Which metabolic pools can be regulators of CEF1?

Figure 3



Rationale: Our preliminary work showed that restricting flux through some parts of the Calvin cycle, but not others, triggered elevated CEF1, leading us to hypothesize that specific metabolic pools govern CEF1, either directly (by interacting with CEF1 components) or indirectly (via signal cascades). We will narrow down which components could regulate CEF1 by testing for correlations between CEF1 activity and metabolic pool sizes in a series of mutant plants with elevated or 'normal' CEF1.

Progress. We have performed comparative metabolite profiling, using techniques developed in this grant (*3*) on several mutants with impaired photosynthesis, both with elevated and unchanged CEF1 (*13*). These results allowed us to exclude most of the Calvin-Benson cycle intermediates, ATP and NADPH as regulators of CEF1.

Eliminating these possibilities leaves us with feredoxin, thioredoxin or reactive oxygen species as the most likely candidates for CEF1 regulation.

We found a strong correlation between H_2O_2 production (measured as DAB staining) and elevated CEF1 in leaves of wild type and *hcef1/2* mutants (Figure 4). These results could indicate either that H_2O_2 is the trigger for or a consequence of CEF1.

We thus used *in vivo* spectroscopy to assay the initiation of CEF1 in the presence of elevated H_2O_2 to test whether this reactive oxygen species could be the regulator of CEF1. We found that infiltration of H_2O_2 into leaves led to strong up-regulation of CEF1, suggesting that H_2O_2 may trigger CEF1. Because infiltration with H_2O_2 is not very specific, and can thus potentially affect a large number of processes, we sought a more targeted approach. We took advantage of a series of mutants produced in the Maurino laboratory that express peroxisomal glycolate oxidase (GO) in the chloroplast (GO5, GO16, and GO20) and consequently accumulate varying amounts of H_2O_2 (*16*). As illustrated in Fig. 5, we again saw an excellent correlation between H2O2 production (DAB staining, top panels) and increases CEF1 (as indicated by the increase in proton translocation as a function of LEF (see red arrows). The effect was inhibited by addition of methyl viologen, which inhibits CEF1 by shunting PSI

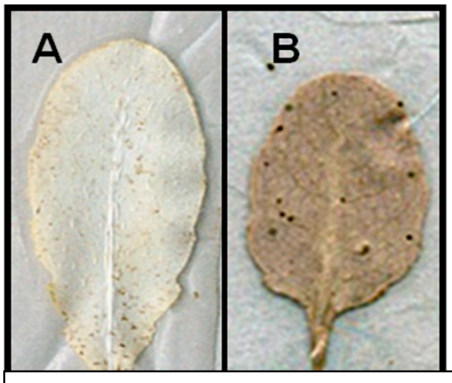


Figure 4. **Hydrogen Peroxide production in Columbia wild-type and *hcef1*.** DAB staining of illuminated leaves of **(A)** Columbia wild-type, **(B)** the *hcef1* mutant.

electrons to O_2 (+MV, green arrows). These results strongly suggest that H_2O_2 is a regulator of CEF1. This work serves the basis of a publication in preparation (*14*) as well as a new line of research, which we will outline in our next proposal.

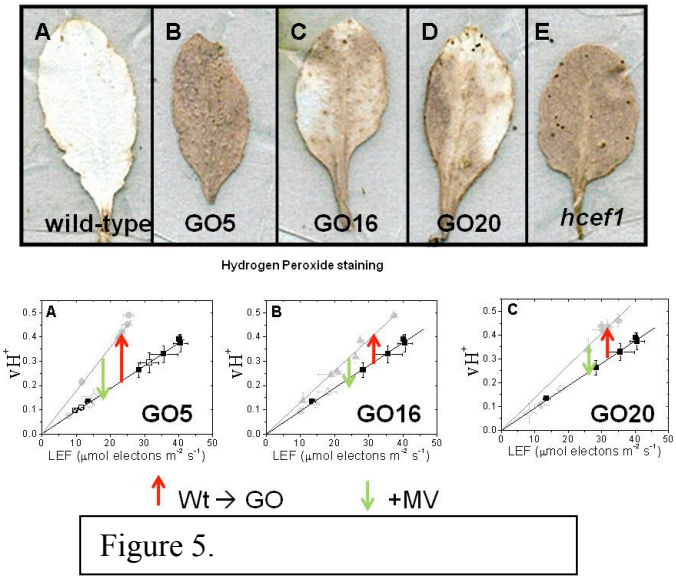


Figure 5.

A.4. Do metabolites influence CEF1 directly or indirectly?

Rationale: Metabolite pools that change with activation of CEF1 could act directly on CEF1 components, or indirectly via an unidentified signal cascade.

Expected results: Observing acceleration of in vitro CEF1 rates by addition of a metabolite would support a direct role in regulation in vivo. Failure to observe these effects would suggest that, if the metabolite is regulatory, it affects rates indirectly, via other components.

Progress. Consistent with our *in vivo* metabolic work, described in A.3. we observed no effects of ADP/ATP, $NADP^+$/NADHP, commercially-available Calvin-Benson cycle intermediates and dithiothreitol (mimicking thioredoxin modulation) on CEF1 in wild type tobacco thylakoids (unpublished results). It is thus likely that none of these *directly* controls CEF1. In contrast, our progress in A.3 strongly suggests that H_2O_2 plays a direct role in activating CEF1.

A.5. Which CEF1 pathways are activated in high CEF1 mutants?

Rationale: The strong acceleration of CEF1 seen in hcef and antisense-suppressed plants should allow us to investigate mechanistic aspects of the process. In this section, we aim to determine which CEF1 pathway(s) are activated in each mutant, by crossing high CEF1 mutants with those lacking specific activities, by assessing the inhibitor-sensitivity of CEF1, and by observing changes in spectroscopic signatures for each process.

Expected results: Lack of effects on CEF1 in hcef*1/2/3:pgr5 and* hcef*1/2/3:crr1 will indicate that the FQR or NDH pathways, respectively, are probably not involved in elevated CEF1. Likewise, observing no effect of antimycin A on elevated CEF1 in high CEF1 mutants, FQR can be likely ruled out as the activated pathway, leaving NDH and the direct cytochrome b_6f pathways. Inhibition of CEF1 by antimycin A would support participation of FQR, but does not prove it since this inhibitor has several potential sites of action in the cell, and secondary effects (e.g. on metabolic pools) cannot be ruled out. If FNR/cytochrome b_6f complex are involved in elevated CEF1, we expect to see changes in the rates of heme c_i and b_6 reduction upon addition of reduced Fd/NADPH.*

Progress: We have crossed several high CEF1 mutants with specific mutants lacking proposed CEF1 pathways (*11, 12*). The results (Fig. 6) imply that NDH rather than PGR5 or FNR is the likely plastoquinone reductase for CEF1 in higher plant chloroplasts. The *hcef1pgr5* double mutant showed the same high CEF1 phenotype of *hcef1*, i.e. loss of PGR5 had no effect on high CEF1 in *hcef1*. We observed similar effects upon crossing *pgr5* with *hcef2* (*14*), *hcef3* (manuscript in preparation), *fnr1* (null for FNR1, manuscript in preparation) and *fnr2* (null for FNR2, manuscript in preparation). In all cases, we found no effect on CEF1 of knocking out PGR5. However, knocking out PGR5 did observe a large effect on the regulation of the ATP synthase, consistent with our previously report (*17*), that *pgr5* affects the *pmf* and thus q_E by preventing the down-regulation of the ATP synthase, rather than through changes

to CEF1. These results were further supported by our observation that elevated CEF1 in tobacco plants with anti-sense suppressed GAPDH showed no sensitivity to antimycin A, an inhibitor of the FQR pathway (*13*). From these results we conclude that PGR5 is not involved in elevated CEF1.

In contrast to the results on *pgr5*, the crossing *crr2-2*, which is deficient in NDH expression, with *hcef1* (*11*) or *hcef2* (*12*) generated double mutants with severe inhibition of photosynthesis and a complete loss of the high CEF1 phenotype.

Figure 6

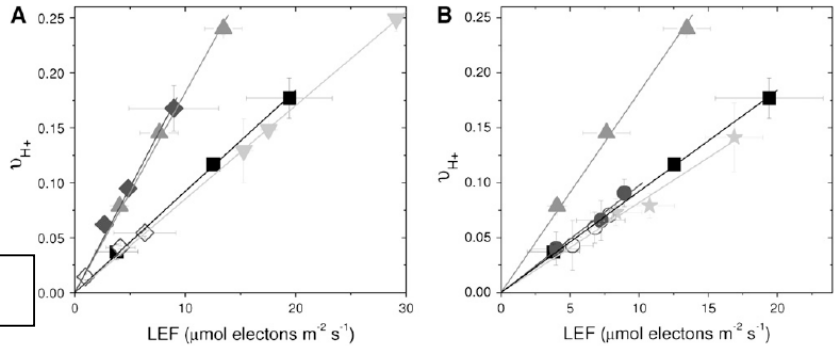


Figure 6. Effects of Double Mutations on Elevated CEF1.

The relationships between light-driven proton translocation across the thylakoid (v_H^+) and LEF were assessed in leaves of Col (squares), *hcef1* (upwards triangles), *pgr5* (downwards triangles), *crr2-2* (star), the *hcef1 pgr5* double mutant (diamonds), and the *hcef1 crr2-2* double mutant (circles). All data indicated by the closed symbols were obtained from leaves infiltrated with distilled water. The double mutants were also treated with MV, as described in Figure 3, as indicated by the open symbols. The data for Col and *hcef1* are reproduced in both **(A)** and **(B)** for comparison with the other mutants. For all linear fits, $P < 0.05$. Error bars represent SD, with $n = 3$, with individual plants.
(A) The *hcef1 pgr5* double mutant compared with Col, *pgr5*, and *hcef1*.
(B) The *crr2-2* mutant and the *hcef1 crr2-2* double mutant show no increases in CEF1.

A.6. Is PQR a proton pump?

Rationale: The ability of CEF1 to balance ATP/NADPH depends directly on how many protons can be pumped per electron cycled. Our high CEF1 mutants display sufficient, sustained rates of CEF1 to make the first estimates of the proton-to-electron stoichiometry (H^+/e^-). Our recent results strongly imply the NDH in this process. One might thus expect a proton pumping CEF1 since NDH is homologous with Complex I or bacteria and mitochondria, which pumps $2H^+/e^-$ (in addition to the scalar protons taken up onto quinone).

Expected results. LEF has a proton to electron stoichiometry (H+/e-) of 3 (18).
If the plastoquinone reductase pumps protons, CEF1 should have an overall H+/e- ratio of 4 (higher than LEF), whereas if it does not, H+/e- should be 2 (lower than LEF). We have developed assays that estimate both electron and proton transfer through LEF and CEF1, allowing us to distinguish these possibilities (19).

Progress: High CEF1 in *hcef2* increased proton transfer to a larger extent than electron transfer through photosystem I, indicating that the plastoquinone reductase very likely is a proton pump, like Complex I. Importantly, none of the other proposed plastoquinone reductase systems are expected to be proton pumps. These results strongly support NDH as the plastoquinone reductase. They also give the first indication of an important bioenergetic factor: the energy budget for CEF1. We are currently writing up these results for publication.

A.7. Is elevated CEF1 activated by state transitions?

Rationale: In Chlamydomonas (20), CEF1 appears to be triggered by phosphorylation that regulates state transitions of the photosynthetic antenna, but the case with higher plants is unresolved.

We will use reverse genetics and biochemical/biophysical approaches to this issue. We will test whether loss of state transitions affects the high CEF1 phenotype of *hcef*1/2/3 by constructing the double mutants, *hcef*1/2/3:*stn*7. We will also directly test for changes in state transitions *hcef*1/2/3 and in

tobacco antisense mutants showing high or low CEF1. State transitions will be assessed using 77K fluorescence emission spectra, avoiding fluorescence reabsorption artifacts via the 'diluted leaf powder' technique of Weiss (*21*).

Expected results. If CEF1 in higher C_3 plants is regulated by the state transitions, knocking out the specific kinase should eliminate elevated CEF1 in our mutants. If this is the only major process involved in CEF1 regulation, we should see tight correlations between CEF1 rates and state transitions.

Progress: Crossing hcef2 with stn7, which is deficient in the kinase responsible for state transitions, did not diminish CEF, indicating that elevated CEF1 does not require a state 2 transition. We also crossed *hcef2* with tap38 , a kinase involved in dephosphorylating LHCII and reversing state transitions. We did not see an increase in CEF1 in the double mutant. Interestingly, we observed small increases in CEF1 in both the *stn7* and *tap38* single mutants, suggesting that secondary effects, possible via reactive oxygen production, could trigger CEF1, possibly consistent with our results in H_2O_2 production. This work has been presented at the International Congress on Photosynthesis Research and a manuscript is in preparation.

B. Revised Plan of Work for Year 3

B.1. Specific Aim 1: Why do *hcef* mutants have increased CEF1?

All but one *hcef* mutant has been cloned. We were unable to cross the remaining mutant, *hcef4*, into Ler. We will attempt to map the hcef4 mutant by a combination of rough mapping with crossing against a range of available ecotypes and deep sequencing using the MSU genomics core facility.

B.2. Is increased CEF1 caused by elevated expression or altered regulation of CEF1 components?

We observed a large increase in NDH expression in *hcef1*. We are extending these studies to all the other *hcef* mutants as well as the forward genetics mutants that we found showed high CEF1. In addition, we have initiated a preliminary chloroplast proteomics study of *hcef1*, *hcef2* and *hcef3* in collaboration with Dr. Mary Lipton at Pacific Northwest National Laboratory.

B.3. Which metabolic pools can be regulators of CEF1?

We feel our data rules out direct participation of measureable Calvin-Benson cycle metabolites in regulating CEF1. We are thus concentrating our efforts on H_2O_2, which we have strong evidence may be involved in regulating both expression and activation of NDH complex. Currently, we ware writing a manuscript on these findings. To support this work, we are repeating key experiments exploring how generation of H_2O_2 affects the expression of CEF1-related proteins as well as the induction of CEF1 in a several mutant lines, including *pgr5*, *gl1* (the background line for *pgr5*), and *crr2-2*.

B.4. Do metabolites influence CEF1 directly or indirectly?

Because of our findings about H_2O_2, this aim is now included as a sub-aim in Aim 3.

B.5. Which CEF1 pathways are activated in high CEF1 mutants?

We have already published two papers related to this aim. To determine if NDH is the predominant route for CEF1, or if other can participate under different conditions, we are crossing all of the high CEF1 mutants we have found against pgr5, crr2-2 and *nda1*. Results thus far show that in each case NDH is the major pathway for CEF1. When results are complete we will publish in a series of papers.

B.6. Is PQR a proton pump?

Our results strongly suggest that the PQR is a proton pump. We will work to further test this by comparing results from higher plants, in which PQR is likely NDH, and a proton pump) with those from Chlamydomonas, which lacks NDH but contains a type-2 NADPH-PQ oxidoreductase which cannot pump protons.

B.7. Is elevated CEF1 activated by state transitions?

Our results so far indicate that CEF1 is not regulated by state transitions. We are currently preparing a manuscript on these results.

C. Anticipation of Unexpended Funds.

We will continue our work immediately and expect to complete our goals in the remaining one-year period. However, because of the necessary delays incurred by the PI's move to MSU, we might request a short (2-6 month) no-cost extension.

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