Cyclic electron flow (CEF) has been proposed to balance the chloroplast energy budget, but the pathway, mechanism, and physiological role remain unclear. We isolated a new class of mutant in *Arabidopsis thaliana*, *hcef* for high CEF1, which shows constitutively elevated CEF1. The first of these, *hcef1*, was mapped to chloroplast fructose-1,6-bisphosphatase. Crossing *hcef1* with *pgr5*, which is deficient in the antimycin A–sensitive pathway for plastoquinone reduction, resulted in a double mutant that maintained the high CEF1 phenotype, implying that the PGR5-dependent pathway is not involved. By contrast, crossing *hcef1* with *crr2-2*, deficient in thylakoid NADPH dehydrogenase (NDH) complex, results in a double mutant that is highly light sensitive and lacks elevated CEF1, suggesting that NDH plays a direct role in catalyzing or regulating CEF1. Additionally, the NdhI component of the NDH complex was highly expressed in the *hcef1 crr2-2* double mutant, whereas other photosynthetic complexes, as well as PGR5, decreased. We propose that (1) NDH is specifically upregulated in *hcef1*, allowing for increased CEF1; (2) the *hcef1* mutation imposes an elevated ATP demand that may trigger CEF1; and (3) alternative mechanisms for augmenting ATP cannot compensate for the loss of CEF1 through NDH.

**INTRODUCTION**

The majority of photosynthetic energy in green plants is stored by the chloroplast in a process termed linear electron flow (LEF). LEF involves light-stimulated electron transfer in two separate reaction centers: photosystem II (PSII) and photosystem I (PSI). Photoexcitation of PSII leads to the extraction of electrons from water, producing molecular oxygen, and the reduction of plastoquinone (PQ). Meanwhile, photoexcitation of PSI oxidizes plastocyanin and reduces ferredoxin. The redox reactions at the two photocenters are linked in series by the cytochrome b6f complex, which transfers electrons from PQH2 to plastocyanin. Ferredoxin reduces NADP+ to NADPH via ferredoxin:NADP+ oxidoreductase (Ort and Yocum, 1996). The electron transfer reactions of LEF are coupled to the translocation of protons from the stroma into the lumen, leading to the establishment of an electrochemical gradient of protons or proton motive force (*pmf*) (Mitchell, 1972; Cruz et al., 2001).

The *pmf* generated by the light reactions drives the synthesis of ATP via the chloroplast CF0 CF1-ATP synthase (ATP synthase) (Jagendorf and Uribe, 1966). The *pmf* also acts as a major regulator of photosynthesis, slowing electron transfer at the cytochrome b6f complex (Hope et al., 1994; Takizawa et al., 2007) and triggering photoprotective *qE* quenching of excitation energy (Crofts and Yerkes, 1994). The *qE* response is activated by acidification of the lumen via the conversion of violaxanthin to zeaxanthin by violaxanthin deepoxidase (Gilmore, 1997) and protonation of the PsbS protein (Li et al., 2000).

The production of ATP and NADPH is tightly coupled in LEF, resulting in a fixed ATP/NADPH output ratio. This rigidity can lead to metabolic congestion and inhibition of photosynthesis if the relative consumption rates of ATP and NADPH do not match their production rates (Edwards and Walker, 1983; Noctor and Foyer, 1998). Recent work on the mechanism of the ATP synthase suggests that 4.67 protons are required for the production of one molecule of ATP (Seelert et al., 2000; but see Berry and Rumph, 1996; Turina et al., 2003), resulting in an ATP/NADPH ratio of 1.29 for LEF. By contrast, the Calvin-Benson cycle requires a 1.5 ratio of ATP/NADPH, leading to a substantial shortfall in ATP/NADPH production. Even after considering the energy requirements of photosynthesis and nitrate assimilation, the ATP/NADPH demand is estimated to be ~1.43 for *C3* plants (Edwards and Walker, 1983). This shortfall may be exacerbated under environmental stress, when additional ATP is needed to drive protein repair and transport. Without mechanisms to produce additional ATP/NADPH, the chloroplast would be unable to balance its energy budget (Kramer et al., 2004).

Three main mechanisms are proposed to account for balancing the ATP/NADPH output ratio: (1) the water-water cycle, in which electrons from LEF reduce O2 to H2O in the chloroplast (Asada, 2000); (2) the malate shunt, in which electrons from LEF are shuttled to oxidative phosphorylation in the mitochondrion (Scheibe, 2004); and (3) cyclic electron flux around PSI (CEF1) (Allen, 2003). In this work, we focus on CEF1, a process in which electrons from the reducing side of PSI are shuttled back into the PQ pool via a PQ reductase, forming PQH2. The cycle is...
completed by oxidation of POH₂ via the cytochrome b⁶f complex and plastocyanin, which transfers electrons back to PSI. Proton translocation associated with CEF1 drives ATP synthesis without net reduction of NADPH, increasing the ATP/NADPH output ratio and initiating photoprotection by acidification of the lumen (Heber and Walker, 1992).

Some groups have reported substantial increases in CEF1 under environmental stress, such as drought (Jia et al., 2008; Kohzuma et al., 2008) or high light (Baker and Ort, 1992), or during the induction of photosynthesis from prolonged dark acclimation (Joët et al., 2002; Joliot and Joliot, 2002). Others have found only small contributions of CEF1 to the photosynthetic energy budget, especially under steady state conditions (Genty et al., 1989; Habrinson et al., 1989; Avenson et al., 2005a). The confusion may partly be due to the difficulty in measuring cyclic processes, such as CEF1 (Baker et al., 2007), or to real differences in CEF1 activity between species or conditions (Kramer et al., 2004). In C₄ plants (Kubicki et al., 1996) and green algae (Finazzi et al., 2002), CEF1 is required to generate the ATP necessary to drive the CO₂-concentrating mechanisms. How could this be done? One mechanism by which CEF1 is regulated is also unknown, but several plausible models have been proposed, including via stromal ADP or ATP levels (Joliot and Joliot, 2006), the redox state of NADPH/NADP⁺ (Munekage et al., 2004), or the availability of PSI electron acceptors (Breyton et al., 2006). It is clear, though, that regulation of CEF1 is essential to fulfill its proposed role in balancing the ATP/NADPH output ratio; too much activity will result in depletion of ADP, while too little will result in over-reduction of the electron transfer chain (Kramer et al., 2004). One may thus expect random mutagenesis to produce strains both with lower than wild-type CEF1 activity (e.g., pgr5; Munekage et al., 2002) as well as those with high activity, as we report here.

RESULTS

Genetic Selection of hcef1 Mutants

The first aim of this work was to select mutants that displayed high levels of CEF1 under permissive nonstress conditions. Approximately 20,000 ethyl methanesulfonate (EMS)-mutagenized Arabidopsis thaliana plants (ecotype Columbia [Col]) were screened for high q₂ phenotypes using chlorophyll fluorescence imaging (as described in Methods; see Supplemental Figure 1 online), yielding ~300 stable lines. A secondary screen, using electrochromic shift (ECS) decay kinetics, was used to select mutants that had high levels of light-driven pmf and relatively small changes in thylakoid proton conductivity (qₑ), which would indicate increased proton translocation. The secondary screen yielded 12 stable lines.

Finally, we conducted a tertiary screen, comparing LEF and light-induced proton translocation reflected in the rate of ECS decay (Takizawa et al., 2008), as described below. The tertiary screen yielded only five lines with increased CEF1 rates, which we have designated hcef1 (for high cyclic electron flow) mutants. Here, we describe the characterization of hcef1, the first of the hcef1 mutants to be extensively studied.

Growth of hcef1

The hcef1 mutant grew photoautotrophically in soil, but with a diminished growth rate, reaching a rosette diameter at maturity ~25% of Col (see Supplemental Figure 2 online). Bolting was delayed in hcef1 (35 to 40 d) compared with Col (24 to 28 d).

Responses of Photosynthetic Electron Transport and Photoprotection

The maximal photochemical efficiency of PSII in extensively dark-adapted leaves was unaffected by the hcef1 mutation, with both Col and hcef1 retaining values near 0.8. However, light-saturated LEF was approximately fourfold lower in hcef1 (Figure 1A) compared with Col, indicating a limitation in photosynthesis downstream of PSII. The light half-saturation point for LEF in hcef1 (~50 μmol photons m⁻² s⁻¹) was approximately one-third that of Col (~150 μmol photons m⁻² s⁻¹).

The photoprotective qₑ response developed to a larger extent and at lower light intensities in hcef1 than in Col. In hcef1, a qₑ value of 0.5 was attained at ~70 μmol photons m⁻² s⁻¹, whereas in Col, this point was not reached until more than 300 μmol photons m⁻² s⁻¹ (Figure 1B). The qₑ half-saturation was reached at an approximately threefold lower light intensity in hcef1 (~50 μmol photons m⁻² s⁻¹) than for Col (~175 μmol photons m⁻² s⁻¹). At saturating light, qₑ reached a level of ~0.7 in Col, consistent with previous values for plants grown under these (relatively low light) conditions (Takizawa et al., 2008), but was substantially higher (~1.2) in hcef1. The difference in extent of qₑ was particularly pronounced at ~150 μmol photons m⁻² s⁻¹ where the extent in hcef1 was approximately fivefold higher than in Col. In hcef1, the extent of qₑ decreased between 300 and 500 μmol photons m⁻² s⁻¹, most likely reflecting the onset of increased photodamage, the slowly reversible form of excitation quenching associated with photodamage (Müller et al., 2001). This was supported by the fact that hcef1 showed a twofold increase in q₁ when light intensity was increased from 110 to 480 μmol photons m⁻² s⁻¹, whereas the wild type showed no significant change in q₁ under these conditions (see Supplemental Figure 3 online).
Comparison of the Photosynthetic Electron and Proton Circuits in hcef1 and Col

We measured chlorophyll fluorescence yields and the kinetics of the thylakoid ECS signal as probed by the electron and proton transfer reactions in leaves. The ECS techniques (reviewed in Sacksteder and Kramer, 2000; Cruz et al., 2005) monitor changes in the thylakoid electric field and can be used to estimate light-driven fluxes of protons through the photosynthetic apparatus. In a typical experiment, the kinetics of the ECS signal are measured during a brief dark interval punctuating steady state actinic illumination, an experiment called DIRK for dark-interval relaxation kinetics. The total amplitude of the DIRK signal, over a few hundred milliseconds, gives a parameter termed ECSt, that is used to estimate the light-driven pmf across the thylakoid (Sacksteder and Kramer, 2000; Cruz et al., 2005). The initial rate of change in the ECS signal can be used to estimate the light-driven proton flux, \( v_{H^+} \). The ECS decay lifetime yields an estimate of the conductivity of the thylakoid to proton efflux, \( g_{H^+} \), attributed predominantly to the activity of the chloroplast ATP synthase (reviewed in Kramer and Crofts, 1996; Cruz et al., 2001).

Figure 1C shows that light-induced pmf, as estimated by the ECS parameter, was higher and more sensitive to increasing light in hcef1 than in Col (Figure 1C). The half-saturation point for hcef1 was reached at \( \sim 75 \) \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) compared with over \( 150 \) \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) for Col. Near the growth light intensity (50 to 150 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)), light-driven pmf was threefold to fourfold larger in hcef1 than in Col.

Figure 1D shows that, for both Col and hcef1, the conductivity of the thylakoid to protons, \( g_{H^+} \), reflecting mainly the activity of the chloroplast ATP synthase, decreased with increasing light intensity. However, \( g_{H^+} \) in hcef1 remained consistently lower, \( \sim 60 \) to 70\% of that in Col (Figure 1D).

Analysis of Photosynthetic Parameters of hcef1 and Col

To reveal relationships among measured photosynthetic parameters, we replotted data from Figures 1A, 1B, and 1C to produce Figure 2. Figure 2A shows that hcef1 displayed an \( \sim 10 \)-fold higher sensitivity of \( \alpha_E \) to LEF than Col. However, as shown in Figure 2B, the extent of \( \alpha_E \) (taken from Figure 1B) to pmf (ECS; Figure 1C) fall upon the same curve for hcef1 and Col (i.e., the relationship between \( \alpha_E \) and ECS remained continuous). Figure 2C shows that hcef1 produces approximately fivefold higher light-driven pmf (ECS, values from Figure 1C) for a given LEF (from Figure 1A).

Because the activity of the ATP synthase is ohmic with respect to pmf (i.e., the rate is proportional to the force and the
conductivity), under steady state conditions, the pmf produced by a given proton flux should be proportional to 1/gH+ (Kanazawa and Kramer, 2002; Cruz et al., 2005). Given a constant stoichiometry of proton translocation for LEF (Sacksteder et al., 2000), the relative pmf expected from LEF alone can be estimated given by the term pmf<sub>LEF</sub> = LEF/gH+ (Avenson et al., 2005a), and the relationship of pmf<sub>LEF</sub> (the pmf expected from LEF alone) against ECS<sub>i</sub> (a measure of total pmf) should be linear with a slope proportional to the proton-to-electron stoichiometry for LEF (Figure 2D). Upwards deviations from this curve will indicate proton transfer above that supported by LEF alone. Figure 2D shows that hcef1 produced approximately twofold higher pmf than can be accounted for by LEF alone (i.e., the slope of pmf against pmf<sub>LEF</sub> was twofold higher in hcef1 than Col), consistent with the activation of CEF1 in hcef1 (see Discussion).

The hcef1 Mutant Shows Higher Light-Driven Proton Fluxes from CEF1

Figure 3 shows the relationship between relative light-driven proton flux (v<sub>H+</sub>) and LEF. This plot is devised to test for contributions from CEF1, since v<sub>H+</sub> should reflect proton flux generated by turnover of both CEF1 and LEF, while the chlorophyll fluorescence-derived LEF parameter only measures electron transfer from PSII. The hcef1 mutant showed an approximately twofold higher v<sub>H+</sub> as a function of LEF (slopes of the linear regression of LEF versus v<sub>H+</sub> in units of ΔA/μmol e·m<sup>-2</sup>·s<sup>-2</sup> equal to 0.025) over Col (slope equal to 0.011) (analysis of covariance [ANCOVA], P < 0.05), suggesting that hcef1 has higher light-driven proton fluxes than can be attributed to LEF alone. Upon infiltration of methyl viologen (MV) to inhibit CEF1, the slopes for hcef1 (slope of 0.012) and Col (slope of 0.0095), without MV, became statistically indistinguishable (ANCOVA, P > 0.1). The observed MV sensitivity implicates CEF1 as the origin of the increased proton fluxes observed in hcef1 (see Discussion).

Comparison of PSII Photochemical Efficiency, Φ<sub>I</sub>, with PSI Redox State Confirms Increased CEF1 in hcef1

Figure 4 plots estimated PSI quantum efficiency (Φ<sub>I</sub>), as determined by Klughammer and Schreiber (1993) against Φ<sub>I</sub>, the photochemical efficiency of PSII measured by saturation-pulse chlorophyll fluorescence yields. It should be noted that this assay probes the fraction of PSI centers in photochemically active states, and not Φ<sub>I</sub> per se, but should provide good estimates of Φ<sub>I</sub> as long as PSI antenna size and efficiency

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**Figure 2.** Analysis of Photosynthetic Regulation in Col and hcef1.

Data from Col (squares) and hcef1 (triangles) were replotted to show the following relationships.

(A) qE versus LEF.

(B) qE versus light-driven pmf (ECS<sub>i</sub>).

(C) Light-driven pmf (ECS<sub>i</sub>) and LEF.

(D) Light-driven pmf and the pmf expected from LEF along (pmf<sub>LEF</sub>).

All data represent averages of n = 4 individual plants ± SD.
hcef1 showed little change in relative ADP/ATP concentrations or (FBPase). Despite this large change in stromal metabolic profiles, phosphate (FBP), suggesting a lesion at fructose-1,6-bisphosphatase but a dramatic, threefold increase in fructose 1,6-bisphosphate sugars (HEXP). Similar metabolic effects were seen in a transgenic potato line that had an anti-sense knockdown for the chloroplast form of fructose 1,6-bisphosphatase (Kossmann et al., 1994).

**Map-Based Cloning and Complementation of hcef1**

Using map-based cloning, the hcef1 mutation was localized to At3g54050, the chloroplast-targeted FBPase, consistent with our observation of large accumulation of FBP in this mutant (Figure 5) and similar effects on photosynthetic performance (nonphotochemical quenching and qE) seen in tobacco (Nicotiana tabacum) and potato (Solanum tuberosum) antisense-FBPase mutants (Bilger et al., 1995; Fisahn et al., 1995). Upon sequencing the gene encoding FBPase, we found a G-to-A transition, resulting in substitution of Lys for Arg at amino acid 361 (see Supplemental Figure 4 online). This residue is in the highly conserved substrate binding pocket of FBPase (Villeret et al., 1995).

The correct identification of the hcef1 mutation was confirmed by complementation with a construct of the gene At3g54050 and screening using kanamycin resistance (Clough and Bent, 1998). Analysis of three independent hcef1-complemented lines showed a return to wild-type growth and photosynthetic rates as well as a reversal of the additional proton flux, higher qE responses, and extent of light-induced pmf seen in hcef1 (see Supplemental Figure 5 online). An independent allele of this mutation was provided by Arabidopsis seed stock from The Arabidopsis Information Resource (TAIR), CS836161, in which the FBPase gene (At3g54050) is interrupted with a T-DNA insertion in the promoter region, directly before the start of the gene between all promoter elements and the gene. The T-DNA line, which should have little to no FBPase activity, showed a phenotype similar to hcef1 (i.e., slowed photosynthesis and

**Chloroplast Metabolites**

Figure 5 shows relative contents of major stromal metabolites from Col and hcef1 in rapidly frozen leaves exposed to continuous actinic light (500 μmol photons m⁻² s⁻¹) obtained as described by Cruz et al. (2008). It should be noted that these profiles were not calibrated for changes in metabolite sensitivity (Cruz et al., 2008) and were only intended to give estimates of relative changes, not in absolute concentrations. Compared with Col, hcef1 showed ~80% decrease and ribulose 1,5-bisphosphate but a dramatic, threefold increase in fructose 1,6-bisphosphate (FBP), suggesting a lesion at fructose-1,6-bisphosphatase (FBPase). Despite this large change in stromal metabolic profiles, hcef1 showed little change in relative ADP/ATP concentrations or six-carbon single phosphate sugars (HEXP). Similar metabolic effects were seen in a transgenic potato line that had an anti-

![Figure 3](image1.png)

**Figure 3.** Evidence for Elevated CEF1 in hcef1: The Relationship between Light-Driven Proton Translocation across the Thylakoid (vH⁺) and LEF.

Col (squares) and hcef1 (triangles) leaves were infused with either water (closed symbols) or MV (open symbols). To avoid photodamage, particularly in the MV-treated leaves; data were taken at light intensities below the light saturation point for LEF in Col (i.e., between 0 and ~120 μmol photons m⁻² s⁻¹). Only hcef1 infused with water was significantly different. Error bars represent SD, with n = 3 individual plants.

![Figure 4](image2.png)

**Figure 4.** Comparison of PSII Photochemical Efficiency, Φh with PSI Redox State: The Relationship between Estimated Photochemical Efficiencies of PSI (Φh) and PSII (Φ).
increased CEF1 and qE) (see Supplemental Figure 6 online). It is noteworthy that Serrato et al. (2009) recently described cpFBPaseII, a redox-independent isoform of FBPase, capable of dephosphorylating FBP, thus explaining the photosynthetic competence of the FBPase T-DNA insertion line and possibly hcef1.

Characterization of Double Mutants hcef1 pgr5 and hcef1 crr2-2

To test whether hcef1 used the PGR5-dependent pathway for CEF1, the hcef1 mutant was crossed with the PGR5 knockout line pgr5 to obtain the hcef1 pgr5 double mutant. Double homozygous lines were screened using mutation-specific PCR and confirmed by sequencing. Three independent crosses were found to be phenotypically and genotypically identical; thus, results from only one line are shown. The hcef1 pgr5 line displays ~40% reduction in light-saturated LEF compared with hcef1. Notably, however, hcef1 pgr5 retained the MV-sensitive increases in proton flux VH+ as a function of LEF seen in hcef1 (Figure 6A), with a slope indistinguishable from that of hcef1 (ANCOVA, P > 0.1). Infiltration with MV decreased the slope for the hcef1 pgr5 double mutant to nearly match that of wild-type Col (ANCOVA, P > 0.1).

We also constructed the hcef1 crr2-2 double mutant (with lesions in both FBPase and NDH) to test if hcef1 used the NDH dependent pathway to run CEF1. Three independent crosses (double homozygous hcef1 crr2-2, confirmed by genotype-specific PCR and sequencing) were selected and were found to be phenotypically indistinguishable. The hcef1 crr2-2 double mutant showed severe growth impairment and light sensitivity, requiring very low growth light (<50 μmol photon m⁻² s⁻¹) to avoid photobleaching. When grown at 40 μmol photon m⁻² s⁻¹, maximal LEF in hcef1 was is ~17%, while that in hcef1 crr2-2 is ~9% of Col. Strikingly, hcef1 crr2-2 showed a complete loss of elevated proton flux VH+ relative to LEF (showing a slope similar to Col [ANCOVA, P > 0.1]), associated with increased CEF1, which is characteristic of hcef1 (Figure 6B). Infiltration of MV did not significantly alter the relationship between VH+ (Figure 6B) (ANCOVA, P > 0.1), indicating that CEF1 was undetectable in hcef1 crr2-2 under our conditions.

NDH but Not Other Photosynthetic Components Is Upregulated in hcef1

We determined relative changes in representative protein levels for key photosynthetic complexes using protein gel blot analysis,
applying equal amounts of total protein for hcef1 and Col. Under our relative low light growth conditions (85 to 90 μmol photons m⁻² s⁻¹), we observed no significant change between Col and hcef1 in the PSI component OEC33 and the cytochrome b₆f complex subunit PetD (Figure 7A). We measured small decreases in the β- and ε-subunits of the ATP synthase (Figure 7A), consistent with the decreases in ATP synthase activity observed in vivo by ECS decay (Figure 1D). In Figure 7A, we also observed a small (~50%) decrease in PGR5, associated with the antimycin A-sensitive CEF1 pathway (Munekage et al., 2002).

Under our growth conditions (low-light conditions required to maintain hcef1, we were unable to detect NDH expression in Col even when loading 75 μg protein per lane, whereas we still saw a clear band with hcef1 diluted to 5 μg protein (Figure 7B). This means that hcef1 has at least a 15-fold increase in the accumulation of the NDH-I component of the NDH complex compared with Col.

**DISCUSSION**

**Mutants with High CEF1: Implications for the Role of CEF1**

CEF1 is proposed to balance the chloroplast ATP/NADPH output and thus must be finely regulated, probably via metabolic signals (Kramer et al., 2004). Therefore, we expected to find mutants not only with low CEF1, as have already been reported (Munekage et al., 2002), but also those with higher than normal, or even excessive, CEF1. Our results show that high CEF1 mutants can be isolated via a straightforward process. Presented here is the characterization of the first of these mutants, hcef1.

**Proton Translocation, in Addition to That Attributable to LEF, Increases qₑ Sensitivity in hcef1**

Figure 2A shows the hcef1 mutant displayed a higher sensitivity of qₑ to LEF than Col. This would be expected for a mutant with excess CEF1, since additional proton translocation by CEF1 should acidify the lumen. However, an elevated qₑ sensitivity can also result from an increase in sensitivity of the qₑ response (activation of violaxanthin deepoxidase or protonation of PsbS) to lumen pH, an increase in the fraction of pmf stored as ΔpH, or a decrease in the conductivity of the thylakoid membrane to proton efflux (gₑ⁺) (Kanazawa and Kramer, 2002; Avenson et al., 2004, 2005b). However, Figure 2B shows that the responses of qₑ to estimated light-induced pmf were nearly identical in hcef1 and Col, implying that neither changes in partitioning of pmf toward ΔpH nor qₑ responses to lumen pH played large roles in changing the overall qₑ response in hcef1.

Strikingly, hcef1 produced much higher pmf for a given LEF than Col (Figure 2C), indicating either a decrease in proton conductivity through the ATP synthase or an increase in proton pumping via CEF1 (Kanazawa and Kramer, 2002). The conductivity for proton efflux, gₑ⁺, was indeed decreased in hcef1 by ~40% with respect to that seen in Col (Figure 1D), but this difference could not by itself explain the observed threefold to fivelfold increase in pmf and qₑ in the mutant (Figures 1B and 1C). After eliminating other plausible explanations for increased qₑ and pmf in hcef1, we used three complementary approaches to test directly for increased CEF1. First, we compared estimates of light-driven pmf with that expected by LEF alone (with no CEF1) (reviewed in Baker et al., 2007), determined by pmf_EL. The term pmf_EL is equal to LEF divided by conductivity of protons through the ATP synthase (gₑ⁺); it is an estimate of the pmf generated by LEF-coupled proton influx taking into account the control of proton efflux by gₑ⁺. Additional proton pumping through CEF1 should cause an engagement of EGS, resulting in an increase of pmf above that expected from LEF alone and, thus, a steeper slope in the relationship between ECS and pmf_EL (Avenson et al., 2005a). We observed an approximately twofold larger pmf than could be explained by LEF (Figure 2D), suggesting a substantial increase in CEF1.

As an alternative measure of CEF1, we analyzed DIRK of the ECS signal, using the initial rate of decay of ECS as an indicator of the total light-driven flux of protons (vₑ⁺) (Takizawa et al., 2008). We observed an approximately twofold higher vₑ⁺ as a function of LEF in hcef1 compared with Col (Figure 3), indicating a higher proton flux in hcef1 than can be explained by LEF alone. Importantly, infiltration of MV, which blocks CEF1 by shunting PSI electrons away from the PQ pool to O₂, completely abolished the excess proton translocation in hcef1 but had no detectable effects on Col (Figure 3). Comparison of the slope of vₑ⁺ against LEF in the control leaves (with both CEF1 and LEF) and those infiltrated with MV (with LEF only) was used to estimate the extent
of proton translocation contributed by CEF1 (see dashed lines in Figure 3). From this analysis, we conclude that CEF1 was minimally engaged in CoI, contributing less than 10% of proton flux, consistent with previous results (Avenson et al., 2005a). In hcef1, by contrast, the absolute rate of CEF1 was greatly enhanced and contributed about the same extent to proton translocation as LEF.

As a third test for increased CEF1 in hcef1, we measured the PSI redox state using dark-interval and saturation pulse-induced absorbance changes in the near infrared, which are often used to obtain estimates of CEF1 (Klughammer and Schreiber, 1993). Provided that the effective size and efficiency of the PSI-associated antenna remain constant, the fraction of PSI centers in photochemically open states (i.e., with reduced P700 and oxidized FeS centers) will give an estimate of PSI photosynthetic efficiency (ΦPSI). Under steady state photosynthetic conditions with LEF only, ΦPSI and ΦII should be equal since electron flux through the two photosystems is balanced. Engagement of CEF1 requires PSI to turn over faster than PSII, increasing ΦPSI over ΦII. Thus, CEF1 should register as an increase in ΦPSI versus ΦII. This is observed in Figure 4 as the increase in slope of estimated ΦPSI against ΦII. Overall, three complementary approaches qualitatively confirmed a substantial increase in CEF1 in hcef1.

Interestingly, the three different spectroscopic approaches to measuring CEF1 suggested different energetic contributions from CEF1. Those based on the proton circuit (νH+ versus LEF, Figure 3; pmfH+/t versus ECS, Figure 2D) suggested that in hcef1, proton flux from CEF1 is about equal to that from LEF. The ΦII versus ΦIIH+ assay (Figure 4) suggested that about electron transfer through CEF1 was ~50% that through LEF. At this point, we cannot tell whether these differences are due to inaccuracies in any of the methods or to an elevated proton pumping capacity for CEF1.

**hcef1 Is a Metabolic Mutant in FBP**

Map-based cloning and subsequent sequencing, together with complementation studies and known alleles (see Supplemental Figures 5 and 6 online), demonstrated that the hcef1 mutation resides in the gene for chloroplast FBPase. This assignment also explains the observation that hcef1 accumulated large levels of FBP, while being depleted of many other stromal intermediates (Figure 5).

The reduction of FBPase activity limits the overall photosynthesis, presumably at the Calvin-Benson cycle. However, previous work on Arabidopsis showed that decreasing assimilation by itself (e.g., by lowering CO2 levels) does not substantially activate CEF1 (Avenson et al., 2005a). Rather, we propose that hindering the Calvin-Benson cycle at FBPase resulted in a higher demand for ATP or the accumulation of specific metabolites that activate CEF1.

The hcef1 and crn2-2 mutants show phototrophic growth under the normal light conditions used here (90 μmol photons m−2 s−1), whereas the hcef1 crn2-2 double mutant is severely compromised and cannot survive at this intensity. These results suggest that the simplest model is one where (1) hcef1 has a requirement for extra ATP, but CEF1 is able to meet that need; (2) the crn2-2 mutant is deficient in NDH, but since the normal demand for ATP is nearly met by LEF, other processes may compensate for the loss of CEF1; and (3) the hcef1 crn2-2 double mutant has an increased requirement for ATP but cannot meet this demand via CEF1, probably because it is deficient in NDH activity.

One plausible model suggested by the metabolite profiling data (Figure 5) involves the feedback-induced disruption of regulation of glyceraldehyde-3-phosphate dehydrogenase, resulting in the depletion of phosphoglycerate (PGA) and the accumulation of 1,3-bisphosphoglycerate. Since 1,3-bisphosphoglycerate is unstable, it will be hydrolyzed back to PGA. In fact, mutants with decreased glyceraldehyde-3-phosphate dehydrogenase function showed a large decrease in PGA (Ruuska et al., 2000; Cruz et al., 2008). This futile cycle would consume ATP without NADPH, requiring an additional ATP source to maintain photosynthesis.

We observed no major changes in the whole leaf ATP/ADP ratios in hcef1 (Figure 5), suggesting that the supplementation of ATP (e.g., by increased CEF1) was able to compensate for any extra ATP demand or that changes in ATP levels in the stroma were compensated for by those in the cytosol. These results suggest that the ATP/ADP ratio itself might not be the regulatory trigger that activated CEF1, in contrast with earlier suggestions (Joliot and Joliot, 2006). It is also possible that an ATP deficit induces changes in the redox state of the PSI acceptor pool, leading to redox-induced upregulation of CEF1, as previously suggested (Breyton et al., 2006). Additionally, a lack of oxidized electron acceptors at the reducing side of PSI could lead to an increase in the formation of reactive oxygen species, such as H2O2, which has been shown to modulate NDH expression and activity (Lascano et al., 2003). More detailed analysis of the metabolic intermediates in hcef1 may allow us to directly assess these questions.

**Elevated CEF1 in hcef1 Involves NDH, but Not the PGR5, Pathway**

Past work has identified several CEF1 pathways, leading from the reducing side of PSI back into the PQ pool, namely, the FQR pathway mediated by PGR5 and the NDH-mediated pathway. Upon crossing hcef1 with the FQR impaired mutant pgp5, we found no decrease in the elevated CEF1 (Figure 6A). These results suggest that additional CEF1 induced by hcef1 does not involve PGR5. By contrast, crossing with a knockout of chloroplast NDH, crn2-2, resulted in double mutants that lost the increased CEF1 observed in hcef1 (Figure 6B), implying that NDH is an important component of increased CEF1. This is strongly supported by protein gel blot analyses, which show that hcef1 has, on a total protein level, decreased PGR5 (Figure 7A), but strongly upregulated (>15×) Ndhh, a component of NDH (Figure 7B).

We conclude that expression of NDH is specifically upregulated in hcef1, allowing for higher CEF1 capacity under specific conditions. This conclusion is consistent with the reported strong correlation between the expression levels of NDH, but not PGR5, and the extent of CEF1 in C4 plants (Takabayashi et al., 2005; Darie et al., 2006). Our results may also explain the apparent discrepancy between the apparent function of NDH, as a NAD(P)
H-plastoquinone oxidoreductase, and its very low expression levels under permissive conditions (Sazanov et al., 1996; Quiles, 2005) where the need for CEF1 is minimal (Kramer et al., 2004) but strongly upregulated under stress where ATP demand may be high (Sazanov et al., 1998; Nixon, 2000; Quiles, 2006), requiring the activation of CEF1 or chlororespiration. Our results do not rule out the participation of PGR5 in CEF1 under other conditions, but because we observe high CEF1 and qE responses in hcef1 pgr5, this protein does not appear to be essential either for CEF1 or for induction of photoprotection (Munekage et al., 2002).

CEF1 Involving NDH Appears to Be Critical for Maintaining the Energy Budget of C3 Photosynthesis in hcef1

Our results (Figure 3) showed that CEF1 was minimally engaged in Col under permissive conditions, in line with previous observations (Harbinson and Foyer, 1991; Kramer et al., 2004; Avenson et al., 2005a). These results are consistent with the ability of cr2-2 in Arabidopsis and the Ndhl mutant of tobacco to grow well photosynthetically under permissive conditions but poorly under stress (Endo et al., 1998; Shikanai et al., 1998; Nixon, 2000). By contrast, introducing the cr2-2 mutation into hcef1 (hcef1 cr2-2, Figure 6B) resulted in severely hindered photosynthesis, suggesting that hcef1 imposes a requirement for additional ATP that is fulfilled via CEF1 that involves NDH. Similar increases in CEF1, presumably refracting increased chloroplast ATP demand, have been reported upon imposing environmental stresses, such as drought (Jia et al., 2008; Kohzuma et al., 2008). At least in the case of hcef1, alternative ATP/NADPH balancing mechanisms (e.g., water-potatocycle [Asada, 2000] and the malate shunt [Scheibe, 2004]) were apparently unable to compensate for the loss of CEF1 upon mutation of NDH. Our results thus support the proposal (Allen, 2003; Kramer et al., 2004; Joliot and Joliot, 2006; Baker et al., 2007) that CEF1 is critical for maintaining the energy budget of C3 photosynthesis under varying ATP demands.

METHODS

Plant Material and Growth Conditions

Wild-type Arabidopsis thaliana (Col ecotype) and derived mutants were grown on soil under a 16 h:8 h light:dark photoperiod at 85 to 90 μmol photons m⁻² s⁻¹ at 23°C. Double mutants pgr5 hcef1 and cr2-2 hcef1, along with hcef1 and the wild type for comparison, were grown under 16:8 photoperiod at 35 to 40 μmol photons m⁻² s⁻¹ at 23°C. The pgr5 and cr2-2 (for chlororespiratory reduction2-2) mutants were provided by T. Shikanai (Kyoto University).

EMS Mutagenesis of Arabidopsis and Screening of Mutants

Wild-type Col was mutagenized by EMS as previously described (Kim et al., 2006), and those with high CEF1 were selected through a three-stage screening process. In the first stage of screening, EMS mutants with high qE responses were selected via chlorophyll fluorescence imaging screening using an approach similar to that employed earlier (Lokstein et al., 1993; Niyogi et al., 1998). The fluorescence image was constructed in-house using a Sony monochrome camera (Minato) filtered with a 750-nm interference filter (750FS40-25; Andover). Both saturating (~5000 μmol photons m⁻² s⁻¹) and background actinic light (~100 μmol photons m⁻² s⁻¹) were provided by four banks of nine red (626 nm) light emitting diodes (Red Luxeon STAR/O, LXHL-ND94; Philips Lumiled Lighting Company). To achieve a higher dynamic range, the electronic shutter speed of the video camera was varied electronically, using a longer shutter opening during weak or steady state illumination and a shorter shutter speed during saturation pulses.

Trays of intact, mutagenized plants, ~12 d after planting, were placed into the darkened chamber of the video imager and given weak and saturating pulses (as above). Fluorescence images were recorded during these light treatments to estimate minimal (F₀) and maximal fluorescence yields (Lokstein et al., 1993). The plants were then illuminated with actinic light (~100 μmol photons m⁻² s⁻¹) for 20 min, with saturating pulses every 4 min, during which fluorescence images were recorded to estimate steady state photosynthetic conditions (Krall and Edwards, 1992) and pulse-induced (Fm') fluorescence yields. After 20 min, the actinic light was switched off, and fluorescence images were recorded during a series of 10 saturating pulses at one minute intervals to probe the relaxation of NPQ, estimating the Fm'' parameter (Genty et al., 1989). The Fm’ and Fm” images were used to calculate false-color representations of the qE responses as described previously (Niyogi et al., 1998). In the second stage of screening, high qE plants were tested for the buildup of elevated light-driven pmf using our ECS probes, as described by Sacksteder and Kramer (2000) and Cruz et al. (2005) and in detail in the following section. In the third screening stage, mutants were selected in which the proton flux, estimated by the ν_pmf parameter, exceeded that expected by LEF alone (Takizawa et al., 2008), as described in the following section.

In Vivo Spectroscopic Assays

Fully expanded leaves between 24 and 28 d old at 23°C were placed in the leaf chamber of an in-house constructed nonfocusing optics spectrophotometer/fluorometer (NoFOSpec) (Sacksteder and Kramer, 2000; Kanazawa and Kramer, 2002), modified to allow continuously flowing humidified air. Saturation-pulse chlorophyll fluorescence yield changes were used to calculate qE and the photochemical yield of PSII (Genty et al., 1989; Kanazawa and Kramer, 2002; Avenson et al., 2005a). Chlorophyll fluorescence was excited with 30-μs pulses of green (525 nm) light from an LED (Hewlett-Packard HLMP-CMR) and detected with a photodiode protected from actinic light by an infrared filter (RG730; Schott). The pulsed fluorescence signal was filtered electronically and measured by an analog-to-digital converter (Kramer and Crofts, 1998). An Ulbricht integrating sphere was used (Knapp and Carter, 1998) to estimate the relative absorbivities of Col and mutant hcef1 at 0.5 and 0.4, respectively, allowing us to calculate rates of LEF from Fv/Fm (Ziyu et al., 2004).

LEF was measured after ~15 min of actinic illumination to establish steady state photosynthetic conditions. The extent of qE was estimated as described previously (Genty et al., 1989; Kramer and Crofts, 1996; Kanazawa and Kramer, 2002), taking Fm’ just before switching off the actinic light and Fm” after 10 min of dark relaxation. Fv/Fm and qE measurements were taken from plants that were dark adapted for 12 h.

Steady state, light-induced pmf was estimated from DIRK changes in absorbance associated with the ECS at around 520 nm, as described previously (Sacksteder and Kramer, 2000; Cruz et al., 2005). The maximal extent of the ECS signal over an ~300-ms dark interval, termed ECSs, is proportional to the light-induced pmf. The conductivity of the thylakoid membrane to protons (gH⁺), attributable to the activity of the ATP synthase, was estimated from the inverse of the constant of ECS decay (τ_ECS) (Kramer and Crofts, 1996; Kanazawa and Kramer, 2002; Cruz et al., 2005). The amplitude of pmf contributed by LEF, termed pmf_LEF, was estimated by dividing LEF by gH⁺ (Avenson et al., 2005a). The
The relative value of steady state proton flux across the thylakoid membrane ($\text{p}_4$) was estimated from the initial slope of the ECS decay (Takizawa et al., 2008). To account for variations in leaf thickness and pigmentation, ECS measurements were normalized either to the extent of the rapid rise in ECS induced by a saturating, single-turnover flash (Kramer and Crofts, 1989) or to leaf chlorophyll content (Porra et al., 1989), resulting in very similar corrections, with $hcef1$ showing ECS responses relative to Col of 54 and 56%, respectively (see Supplemental Table 1 online).

The redox state of PSI was measured using the technique of Klughammer and Schreiber (1993) using a probe beam provided by an 810-nm LED (ELD-810-525, Rothenier). Light reaching the detector was filtered using an infrared–transmitting filter (RT-830; Edmund Optics). Data were collected and calculated as described by Peterson (1991) and Klughammer and Schreiber (1993). Key experiments were repeated using the two-wavelength deconvolutions described by Oja et al. (2004) [$\Delta \lambda$ (820-940 nm)] with similar results, indicating that absorbance changes from plastocyanin or other components did not substantially affect the measurements.

Introduction of MV into Leaves

Where indicated, plants were infused by placing them between two Kimwipes (Kimberly-Clark) saturated either with distilled water or a solution of 100 mM MV, and incubated under dim light (~5 $\mu$mol photon m$^{-2}$ s$^{-1}$) for 1 h. After infiltration, leaves were gently blotted to remove excess liquid prior to experimentation.

Map-Based Cloning

The $hcef1$ mutant was mapped using molecular markers based on single nucleotide polymorphisms (Drenkard et al., 2000) and cleaved amplified polymorphic sequences (Baumbusch et al., 2001; Jander et al., 2002). F2 plants were derived from breeding homozygous $hcef1$ (Col background) and wild-type (Landsberg erecta background). The $hcef1$ mutation was found to be recessive. Genomic DNA was isolated from homozygous F2 plants ($hcef1$ $hcef1$) that expressed a high qE response using chlorophyll fluorescence imaging (Niyogi et al., 1998). $hcef1$ was PCR amplified from wild-type and $hcef1$ genomic DNA using AmpliTaq Gold PCR Master Mix (Applied Biosystems). The PCR products were sequenced using Big Dye Reagent and were run on an ABI Prism 377 (reagent and Master Mix (Applied Biosystems)).

Metabolic Profiling

Metabolic profiling of the Calvin–Benson Cycle intermediates was accomplished as previously described (Cruz et al., 2008), except that 1 g of fresh weight Arabidopsis plants was used in place of tobacco leaf disks. Plants were dark adapted for 24 h and place for 20 min under 500 $\mu$mol photon m$^{-2}$ s$^{-1}$ to avoid chilling the mortar. Extraction and liquid chromatography–mass spectrometry assays were completed as described by Cruz et al. (2008), yield relative changes of metabolites (but not absolute concentrations, since no spike-recovery assays were performed).

Measurement of ATP and ADP Content

ATP and ADP levels in leaf tissue extracts were determined using the luciferase assay as described previously (Lundin and Thor, 1975) with the following modifications. Extracts were incubated for 30 min at room temperature in buffer containing 50 mM Tris–HCl, pH 8.0, 5 mM MgCl$_2$, 4 mM KCl, and 50 $\mu$M phosphoenol pyruvate in the presence or absence of 4 units of pyruvate kinase (PK) (Sigma–Aldrich) in a total volume of 450 $\mu$L. Fifty microliters of Enliten Luciferase reagent (Promega) was added, and luminescence was measured immediately using an LKB 1250 Luminometer, linked to a computer via a USB–1608FS (Measurement Computing). The amplitude of the luminescent signal in the PK(–) samples was proportional to ATP content. To obtain relative ADP content, the amplitudes from samples without PK were subtracted from the corresponding samples with PK.

Complementation of hcef1

The $hcef1$ mutation was complemented with the wild-type Columbia coding sequence of the gene At3g54050, the chloroplast-targeted FBPass. The coding sequence was created using the Thermoscript RT–PCR system (Invitrogen), primers 5’-CAGAAACCAGCAGAGAACCC-3’ and 5’-TTATGTATCTAGATC AAGCCAAGTACTTC-3’, with RNA isolated from wild-type Col (RNA was isolated using the RNasey plant mini kit; Qiagen). The PCR product was verified by sequencing and subcloned into pAVA 121 to add a tobacco etch virus translational enhancer, a double 3SS promoter regions, and the nopalin synthase terminator (Tnos) to the construct. The resulting construct was then subcloned into the pCAMBIA 2300 plasmid (www.cambia.org) and transferred to Agrobacterium tumefaciens GV3101 (pMP90) strain. A. tumefaciens cells were suspended at OD$_600$ = 0.85 and then used to transform homogenous $hcef1$ plants using the floral dip method as described by Clough and Bent (1998). The transformed plants were then selected for using 50 $\mu$g/mL kanamycin with 0.43% Murashige and Skoog salts and 1.0% sucrose (Clough and Bent, 1998).

hcef1 Crosses

Croses of $pg5$ $hcef1$ and $crr2-2$ $hcef1$ were screened by genetic analysis for the presence of each mutation in the double cross. The following primers were used: $pg5$ forward 5’-CTTTTGGAAACTGATTGAATGC-3’ and reverse 5’-AACCGGCAACGAGAAGAC-3’, specific 5’-GACCCTAAGCAGGAAAACC-3’; $hcef1$ forward 5’-GCTGCCTGTCTACTGTGTTGTTGTT-3’ and reverse 5’-AGGACCAATATCTGAACTT-3’, specific 5’-GCAAGAGCAGAAAATGGAAGGACTTGAAG-3’; $crr2-2$ forward 5’-CCTTTGGGAACGAA-3’ and reverse 5’-AGGACACAACATTCCGCGTT-3’, specific 5’-CCGTCCTTTTATCTAAGATGAT-3’. The presence of both mutations in the double mutants was confirmed by PCR and sequencing. Both double mutant varieties were grown at ~40 $\mu$mol photon m$^{-2}$ s$^{-1}$.

Protein Extraction and Protein Gel Blot Analyses

For immunoblot analyses, leaf total protein was extracted by grinding fresh Arabidopsis leaves under liquid nitrogen and extracted with 100 mM Tricine–KOH, pH 7.5, 2 mM MgCl$_2$, 10 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 10 mM $\beta$-mercaptoethanol. The suspension was microcentrifuged for 5 min at 13,000 rpm, and the resulting pellet containing the leaf insoluble proteins was dissolved in sample buffer (50 mm Tris–HCl, pH 6.8, 2% SDS, 10 mm $\beta$-mercaptoethanol, 10% glycerol, and 0.04% bromophenol blue). Proteins were separated by 12% SDS–PAGE and then blotted onto polyvinylidene difluoride membranes (Invitrogen). The amount of protein loaded was revealed by Ponceau S staining (Sigma–Aldrich).

The membranes were then probed with the following antibodies: NdhI (from Peter Nixon and Mako Boehm, Imperial College London), PetD (from Alice Barkan, University of Oregon), OEC33 and Cft-1 (from Akiko Yokota, Nara Institute of Science and Technology), and Cft-1 (from T. Shikanai, Kyoto University). Using a conjugated anti-rabbit secondary antibody, immunoblots were cast onto x-ray films (Kodak) by an ECL–chemiluminescence kit (Amersham Biosciences).
The amount of total protein introduced into each lane, determined by the Lowry assay (Lowry et al., 1951), was optimized to give the best signals for each target protein. For detection of CF1-β and CF1-ε, we introduced 5 μg of protein, whereas with NdhI, OEC33 and the Rieske iron-sulfur protein (PetD) we loaded 10 μg of protein.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: chloroplast-targeted FBPase, AY039934 (At3g54050); PGR5, BX821933 (At2g05620); and CRR2-2, AK228825 (At3g46790). The T-DNA insertion line in this gene was obtained from TAIR as stock number CS836161. The pgr5 and crr2-2 mutants were provided by T. Shikanai (Kyoto University).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Selection of hcef1.

Supplemental Figure 2. Growth of hcef1.

Supplemental Figure 3. Measurement of qE in hcef1.

Supplemental Figure 4. hcef1 Mutation in Chloroplast FBPase.

Supplemental Figure 5. Complementation of hcef1.

Supplemental Figure 6. Comparison of Proton Pumping as a Function of LEF for hcef1, Col, and Known FBPase Knockout (CS836161).

Supplemental Table 1. Phenotypic Comparison of Photosynthetic Traits of Col, hcef1, hcef1 pgr5, and hcef1 crr2-2.

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