

The higher plant plastid NAD(P)H dehydrogenase-like complex (NDH) is a high efficiency proton pump that increases ATP production by cyclic electron flow

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## ABSTRACT

Cyclic electron flow around photosystem I (CEF) is critical for balancing the photosynthetic energy budget of the chloroplast, by generating ATP without net production of NADPH. We demonstrate that the chloroplast NADPH dehydrogenase complex (NDH), a homolog to respiratory Complex I, pumps approximately two protons from the chloroplast stroma to the lumen per electron transferred from ferredoxin to plastoquinone, effectively increasing the efficiency of ATP production via CEF by two-fold compared to CEF pathways involving non-proton-pumping plastoquinone reductases. By virtue of this proton-pumping stoichiometry, we hypothesize that NDH not only efficiently contributes to ATP production, but operates near thermodynamic reversibility, with potentially important consequences for remediating mismatches in the thylakoid energy budget.

electron transfer reactions are coupled to proton transfer, generating an electrochemical gradient of protons—the proton motive force ( $\Delta p$ )—that drives the synthesis of ATP. Because of the strong coupling of electron and proton transfer reactions (2, 3), LEF should produce a fixed (or rigid) ratio of ATP/NADPH [reviewed in (4)]. In contrast, downstream metabolic reactions impose variable demands for ATP and NADPH, requiring dynamic adjustments of the photosynthetic energy budget to avoid “metabolic congestion” that can lead to buildup of products or depletion of substrates that can result in photodamage (2, 3). Indeed, LEF alone should not be able to power the Calvin-Benson cycle. With one proton being deposited in the lumen during water oxidation and two being translocated by cytochrome *b<sub>f</sub>* complex catalyzes through the Q-cycle, LEF should result in the translocation of 3 protons for each electron transferred to NADPH (5 - 7); with a  $H^+/ATP$  ratio of 4.67 at the ATP synthase (8), LEF should produce 2.6 ATP for every 2 NADPH, a deficit of 0.4 ATP/2 NADPH compared to the ratio required to sustain the Calvin-Benson cycle.

The canonical ‘Z-scheme’ model of photosynthetic linear electron flow (LEF, Figure S1A) uses a series of two photochemical reaction centers to extract electrons from water at photosystem II (PSII), and transfer them to NADPH through photosystem I (PSI) (1). The

There is substantial evidence that cyclic electron flow around photosystem I (CEF) plays an important role in balancing the ATP/NADPH energy budget (3, 4, 9,10). The generally accepted model for CEF involves the transfer of highly

reducing electrons from photoexcited PSI centers to plastoquinone (PQ) through a PQ reductase, resulting in the formation of plastoquinol (PQH<sub>2</sub>) with the uptake of protons from the chloroplast stroma. The PQH<sub>2</sub> is then oxidized by the cytochrome *bf* complex and returned to the oxidizing site of PSI via plastocyanin or cytochrome *c*<sub>6</sub>.

There are several proposed CEF pathways in higher plant chloroplasts that differ at the level of the plastoquinone reductase [reviewed in (11), see also Figure S1B, S1C]. One of these involves the thylakoid ferredoxin:plastoquinone oxidoreductase complex, for historical reasons called the NADPH dehydrogenase complex (NDH, see Discussion), which is homologous to respiratory Complex I (12, 13). Another pathway, termed the ferredoxin:quinone reductase (FQR) is sensitive to antimycin A (AA), and proposed to involve a complex of the PGR5/PGRL1 proteins that is able to transfer electrons from ferredoxin (Fd) to PQ (11, 14 - 17).

Both of these CEF routes are conserved across the flowering plants, even though they may appear redundant, i.e. both function in CEF by transferring electrons from ferredoxin to PQ (15, 17 - 18). However, the PQ reductases in these two pathways are structurally distinct, possibly giving clues about their relative functions. Of particular interest is the homology of NDH to the bacterial or respiratory type I NADH:quinone reductases (Complex I) (12, 19, 20) that are known to couple reduction of quinones to the pumping of up to two protons for each electron transferred to quinone, efficiently generating  $\Delta p$  to drive ATP synthesis (21, 22). In contrast, the FQR is likely to catalyze a simpler, more direct mode of PQ reduction (14, 17) that is very unlikely to be coupled to proton translocation, much like the type II (non-proton-pumping) NAD(P)H:quinone oxidoreductases such as NDA2 and NDH2, as found in the mitochondrial respiratory chains of plants, certain fungi and protozoa. Non-protonomotive NDA/NDH2-type enzymes are also widespread in bacteria and the plastids of certain green algae (23, 24).

If the NDH is functionally similar to Complex I, it could have the capability to use the

energy liberated during PQ reduction to translocate protons across the thylakoid against the electrochemical gradient and thus increase the H<sup>+</sup>/e<sup>-</sup> stoichiometry of CEF. Though proton-pumping activity for NDH has been proposed in previous work (25, 26) it has not been experimentally demonstrated. Such activity would have a large impact on the energy balance of the chloroplast, by allowing for more efficient balancing of the ATP/NADPH budget with relatively low turnover rates. On the other hand, as discussed below, a high coupling ratio for proton-pumping to electron transfer could impose significant thermodynamic and kinetic limitations, or even allow plastoquinol to reduce NADP<sup>+</sup>. In this work, we used a series of complementary approaches to assess the possibility that NDH acts as a proton pump both *in vitro* and *in vivo*.

## RESULTS

### *Sequence conservation in NDH of residues essential for proton-pumping*

Figure 1 shows a structural cartoon of *Arabidopsis thaliana* NDH indicating the likely organization and conservation of charged residues within the membrane domain, which are considered to be essential for proton translocation in respiratory Complex I. Complete sequence alignments for the membrane subunits depicted in Figure 1 for the respective NDH subunits from *A. thaliana*, *Spinacia oleracea* (spinach), and *Nicotiana tabacum* (tobacco) and Complex I from *Escherichia coli*, *Thermus thermophilus*, *Yarrowia lipolytica* (yeast), and *Bos taurus* are presented in Figure S2(A-E).

Four discrete proton channels, exhibiting highly characteristic sequence conservation, have been identified in the atomic structures of the membrane domains of *T. thermophilus* and *Y. lipolytica* Complex I (21, 27, 28). Three of these channels are contained within the 'antiporter-like' subunits equivalent to NdhB, -D, and -F, and the fourth from an association of subunits equivalent to NdhA, -C, -E and -G. This latter group also forms part of the quinone binding site within the enzyme. The lysine and glutamate residues located within the center of the lipid bilayer are key

mechanistic features of these proton channels, and are conserved amongst Complex I and NDH (displayed in Figure 1). Mutagenesis of these residues has been shown to impair the proton-pumping capacity of *E. coli* Complex I [reviewed in (22)]. The conservation of these intramembrane lysine and glutamate residues between Complex I and NDH provides circumstantial evidence for the protonmotive of the latter enzyme, which was then investigated experimentally as described below.

*Proton-pumping activity of chloroplast NDH probed in vitro.*

*In vitro* ATP generation in the dark via protonmotive NDH activity. The low abundance of NDH in plant thylakoids generally precludes the use of proteoliposome reconstitution experiments to directly assess protonmotive activity, as has been used for respiratory Complex I (19, 29). As such, we developed an *in vitro* assay (illustrated in Figure 2A) for use with coupled thylakoid preparations designed to report ATP generation only in the presence of a proton-pumping, NDH-type plastoquinone reductase. ATP synthesis was monitored using firefly luciferase luminescence in spinach thylakoids. Spinach was chosen as it provides a reliable (and abundant) source for the isolation of coupled thylakoids. To avoid interference from light-driven ATP production through photophosphorylation, experiments were conducted in strict darkness in the presence of dichloromethyl urea (DCMU), an inhibitor of PSII, and/or tridecyl stigmatellin (an inhibitor of the cytochrome *b<sub>f</sub>* complex, see below). The NDH reaction was initiated by addition of exogenous (oxidized) Fd, NADPH and the PQ analogue decyl-plastoquinone (dPQ). Oxidised Fd (the electron donor to NDH) was reduced by NADPH through endogenous FNR activity. The suspension medium was well buffered so that the uptake of protons onto dPQ should not have affected the pH of the extra-luminal space and thus  $\Delta p$  should only be produced by the transfer of protons in the thylakoid lumen. As shown in Figure 2B, addition of Fd and NADPH alone did not induce luminescence, but further addition of dPQ resulted in a sustained, reproducible increase in luminescence, reflecting the production of about 10 nmol ATP mg chlorophyll<sup>-1</sup> min<sup>-1</sup>. This rate

was approximately 1% of the steady-state (LEF) ATP production rate reported for spinach thylakoid suspensions illuminated at 250  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>, using ferricyanide as the Hill oxidant (30). The (comparatively) low rate of ATP production supported by NDH activity in the *in vitro* assay described in this section should be understood in terms of i) the energetically unfavourable reduction of ferredoxin by NADPH, and thus low electron donor concentration and ii) the low abundance of NDH- and the predominance of the (non-protonmotive) FQR CEF pathway in spinach (see discussion below).

For the *in vitro* proton pumping assay described here the order of substrate addition was not critical, but that NADPH, Fd and dPQ were all required, implying that the oxidation of NADPH through Fd to dPQ was coupled to the synthesis of ATP (Figure S3). This result is consistent with the previously reported requirements of Fd for NDH activity (18).

The observed ATP synthesis was dependent on the generation of  $\Delta p$ , as demonstrated by its abolition upon addition of uncouplers (valinomycin with nigericin, see arrow marked “unc” in Figure 2B). A number of additional controls were performed to verify the identities of the processes participating to the ATP generation observed in this luciferase-based assay. FQR activity was not expected to contribute to the  $\Delta p$  generation observed here, and as expected ATP generation was insensitive to the addition of 10  $\mu$ M antimycin A (considered an inhibitor of FQR activity (17)) (Fig. S4). The ATP synthesis was insensitive to tridecyl stigmatellin (Figure S5), a potent inhibitor of the *b<sub>f</sub>* complex, indicating that the observed ATP synthesis also did not involve electron or proton translocation by the *b<sub>f</sub>* complex. Likewise, the activity was insensitive to oligomycin (Figure S5), a specific inhibitor of mitochondrial ATP synthase, indicating that the reaction was not catalyzed by contaminant mitochondrial respiration.

*Linkage of NDH activity to generation of  $\Delta p$  probed by post illumination fluorescence kinetics.* As a second, independent approach to determining the proton coupling of NDH, we assessed the effects of  $\Delta p$  on the reduction of the

PQ pool using the “post-illumination fluorescence” signal, a transient rise in chlorophyll fluorescence observed in the dark after a sustained period of actinic illumination in leaves and chloroplast preparations. This fluorescence signal is generally considered to be related to the activity of NDH, and results from the transfer of electrons from stromal donors to the PQ pool, which equilibrate with the  $Q_A$  quinone molecule in photosystem II, resulting in elevated chlorophyll *a* fluorescence yield (Figure 3A, also; (12)).

Figure 3B compares the kinetics of fluorescence yield changes in thylakoid preparations from *S. oleracea*, *Amaranthus hybridus*, *A. thaliana* (wildtype Col-0 and the NDH-knockout *ndhm* (31)) using 100  $\mu$ M NADPH as the electron donor in the presence of exogenous (spinach) Fd.

The experiments were initiated by measurements of the fluorescence level in the dark,  $F_0$ , followed by a brief pulse saturating actinic light to estimate for the maximal fluorescence yield,  $F_M$ , allowing for normalization of results from preparation to preparation. This treatment was followed by illumination with approximately 250  $\mu$ mol photons  $m^{-2} s^{-1}$  red light for 60 s to establish steady state electron transfer. After illumination, the actinic light was switched off and chlorophyll fluorescence yield were recorded. Upon switching off the actinic light, the fluorescence yield values decayed initially to a level well above the  $F_0$  value, likely indicating  $Q_A$  was not completely reoxidized in the dark. To fully oxidize PQ, we periodically illuminated with far-red light (50  $\mu$ mol photons  $m^{-2} s^{-1}$ ), which preferentially excites PSI, resulted in quenching of the fluorescence signal. Following the far-red light, fluorescence yield increased again, reflecting re-reduction of the PQ pool and  $Q_A$  by NADPH/Fd. These re-reduction responses, which we term Post-illumination Fluorescence Rise (PIFR) were maintained over multiple cycles of far-red illumination and dark recovery, indicating that the pool of reductant was not depleted during the experiments.

The largest PIFR response was observed in *A. hybridus*, a NAD-ME  $C_4$  species with an elevated level of NDH in mesophyll chloroplasts

compared to bundle-sheath (32). The amplitude of the PIFR in *S. oleracea* and *A. thaliana* (Col-0) was approximately 65% and 50% of that observed in *A. hybridus* respectively. The PIFR in Arabidopsis was diminished in the *ndhm* line, which lacks a functional NDH complex (31), confirming that the PIFR was related to NDH activity. The residual (20%) PIFR in *ndhm* presumably reflects the activity of the (non-protonmotive) FQR pathway or the recently demonstrated direct reduction of PSII electron acceptors, (33). To test these assignments, we repeated experiments in the presence of 1 mM hydroxylamine (HA) and 10  $\mu$ M DCMU, abolishing variable fluorescence from PSII. The PIFR was inhibited under these conditions (Figure S6), confirming that the PIFR observed in the absence of DCMU and HA was related to PQ pool reduction (mediated by NDH) and subsequent redox equilibration with  $Q_A$  (34). An anaerobic control for plastid terminal (plastoquinol) oxidase (PTOX) activity on the kinetics of the PIFR was performed in spinach and *A. hybridus* thylakoid preparations (Figure S7). When normalizing the fluorescence data to  $F_0'$ , the extent of the *A. hybridus* PIFR was increased by 15-20% during the 20s transient under anaerobic conditions compared to aerobic. The initial rate of the PIFR was also observed to increase approximately 1.5-fold under anaerobic conditions in this species. The PIFR extents were unaffected by anaerobiosis in *S. oleracea* thylakoid preparations. These data presumably reflect higher PTOX activity in *A. hybridus* thylakoid preparations compared to *S. oleracea*.

It is generally considered that the FQR-dependent pathway of CEF is sensitive to AA, whereas the NDH-dependent pathway is insensitive. Addition of 10  $\mu$ M AA reduced the amplitude of the PIFR by approximately 60% in *S. oleracea* and *A. thaliana* (Col-0) thylakoids, and almost completely in *A. thaliana* (*ndhm*) thylakoid preparations (Figure 4A-D). In contrast, the amplitude of the rise in *A. hybridus* thylakoid preparations was largely unaffected by AA, consistent with an NDH-dominated CEF pathway in this species (32). These data provide a useful example for a point that is often overlooked in the literature; that is that both NDH- and FQR activity contribute to the PIFR (which is to be expected,



since both activities result in quinone reduction)(35), and the contribution of these activities varies between species. Accordingly, the PIFR is reported to be diminished (but not abolished) in the *A. thaliana pgr5* mutant (36), which is considered to be affected in CEF.

Collapsing the transthylakoid  $\Delta p$  through the use of valinomycin and nigericin in the presence of 10 mM  $K^+$ , which should uncouple the thylakoid membranes, resulted in a rapid, two-fold increase in the amplitude and rate of the PIFR observed in *S. oleracea* and *A. hybridus* chloroplast preparations (Figure 4A-D). Dissipation of the  $\Delta p$  allows the protonmotive NDH complex to operate unimpeded by thermodynamic 'back pressure' created by the by the light-induced proton gradient across the thylakoid membrane. The PIFR was less sensitive to proton uncoupling in *A. thaliana* (Col-0) and *ndhm* mutant (Figure 4C, D), likely reflecting the larger contribution from the AA-sensitive, and non proton-pumping FQR pathways, which should not be hindered by  $\Delta p$  backpressure.

*Proton-pumping activity of chloroplast NDH probed in vivo.*

As discussed in (6, 37), the initial slope of the Dark Interval Relaxation Kinetics (DIRK) of the electrochromic shift (ECS) signal during brief dark intervals can be analyzed to obtain  $v_{H^+}$ , an estimate of the relative fluxes of protons generated by the light reactions. Previous work (38) showed the rates of CEF can be estimated by comparing  $v_{H^+}$ , which is driven by both LEF and CEF, with electron transfer through PSII, which only contributes to LEF. Here, we call this method the "proton-LEF" method.

Here, we introduce a method, modified from the proton-LEF approach, to estimate the ratio of proton translocation to electron transfer through LEF and CEF. In the new approach, we compared  $v_{H^+}$  with light-driven proton fluxes to electron transfer through PSI ( $v_{P700}$ ), measured using the method modified by that of Fan et al. (65). Each turnover of LEF or CEF should result in one electron transferred to  $P_{700}$ , so that:

$$v_{P700} \propto CEF + LEF \quad (1)$$

By contrast,  $v_{H^+}$  should depend on both the rates and proton translocation stoichiometries of CEF and LEF, so that but also on may contribute unequally to  $v_{H^+}$  should be a function of CEF and LEF, but the contributions from

$$v_{H^+} \propto n_{CEF}CEF + n_{LEF}LEF \quad (2)$$

where  $n_{CEF}$  and  $n_{LEF}$  represent the  $H^+/e^-$  ratios for CEF and LEF. The slope of the relationship between to  $v_{H^+}$  and  $v_{P700}$  will be:

$$m = \frac{n_{CEF}CEF + n_{LEF}LEF}{CEF + LEF} \quad (3)$$

where  $m = \frac{v_{H^+}}{v_{P700}}$ . Our previous work showed that, in wild type *A. thaliana*, under non-stressed conditions, CEF is less than 10% of LEF (37). By contrast, in the High Cyclic Electron Flow 1 (*hcef1*) mutant, CEF is elevated, while LEF is decreased so that CEF contributes about 90% of electron and proton fluxes (38). Thus, to the first approximation, Col-0 and *hcef1* have essentially all LEF and CEF, respectively, and one can easily derive a rough estimate the relative proton to electron stoichiometries for CEF and LEF

$$\frac{n_{CEF}}{n_{LEF}} \approx \frac{m_{hcef1}}{m_{Col-0}}$$

where  $n_{CEF}$  and  $n_{LEF}$  are the proton to electron stoichiometries for CEF and LEF, and  $m_{Col-0}$  and  $m_{hcef1}$  are the ratios of  $v_{H^+}$  to  $v_{P700}$  (for Col-0 and *hcef1* respectively). (We also derived a more complex equation that considers the residual levels of LEF and CEF in Col-0 and *hcef1*, but the results were within 20% of those using the simplified equation above). The value of  $n_{LEF}$  is likely to be 3 (6), i.e. for each electron through LEF, one proton is released from water oxidation and two from the Q-cycle. In the absence of a proton pumping PQ reductase,  $n_{CEF}=2$ , i.e. 2 protons transported by the Q-cycle, and thus, we would expect  $m_{hcef1} < m_{Col-0}$ . If CEF occurs through an NDH that pumps on proton per electron, we would expect  $m_{hcef1} \approx m_{Col-0}$ . Finally, if NDH pumps 2 protons per  $e^-$  (i.e. 4 protons per NAD(P)H) we would expect

$m_{hcef1} > m_{Col-0}$ , as we clearly observe. If one assumes that the contributions to CEF were constant throughout the experiments, then the ratio of slopes for the relationship between  $v_{H^+}$  and  $v_{P700}$  should approximate the ratio of  $n$  ( $H^+/e^-$ ) values. Assuming  $n=3$  for LEF, fitting the data in Figure 5 yields a wide range of possible values for  $H^+/e^-$  ratio of slopes, from about 4.5 to 8.8. Given this broad uncertainty, it is probably not possible to obtain an accurate estimate of  $n$  from this data. However, because the points for *hcef1* (predominantly CEF), all fall above the curve for the wild type, which (predominantly LEF), we can make the quantitative conclusion that  $H^+/e^-$  for CEF is larger than that for LEF in this mutant.

## DISCUSSION

The function of plastid NDH complex has been the subject of considerable controversy (39). NDH is known to catalyze the reduction of PQ by reduced Fd, and thus has often been suggested to play a role in CEF (18, 35, 38, 40). However, other CEF pathways, particularly that involving the FQR, have been proposed to constitute the major route for CEF (14 - 16, 41), though it is clear that CEF can proceed rapidly even in the absence of FQR, through the NDH complex (35, 38, 40, 42).

The fact that NDH is homologous to proton-pumping Complex I (NADH:UQ oxidoreductase) of mitochondria and related prokaryotic systems, suggests proton-pumping capacity may also be conserved in plastids, with substantial consequences for the energetics of CEF. In support of this possibility, key residues required for proton-pumping (22, 28, 43) are also conserved in the plastid complex (Figures 1 and S2).

In this work we used several complementary approaches to provide the first direct evidence for the predicted proton-pumping capacity of NDH. In one approach, we developed an *in vitro* assay that reported dark ATP synthesis in coupled, thylakoid preparations under conditions strictly dependent upon NDH activity (Figures 2, S3 - S5). Secondly, the extent of the PIFR, a chlorophyll fluorescence phenomenon

arising from the NDH-mediated reduction of the PQ pool (Figure 3), increased on addition of uncouplers to thylakoid preparations from *S. oleracea*, *A. hybridus*, and *A. thaliana* (Col-0) (Figure 4). This latter observation is consistent with a protonmotive NDH complex with a pumping ( $H^+/e^-$ ) stoichiometry of two or more, so that its enzymatic activity is controlled by the thermodynamic back-pressure from transmembrane  $\Delta p$ . (As discussed below, proton-pumping capacities of less than 2  $H^+/e^-$  should not be thermodynamically limited by physiologically accessible  $\Delta p$  extents.) Finally, *in vivo* data obtained on the high CEF *hcef1* mutant indicated a  $H^+/e^-$  stoichiometry for CEF of about four (Figure 5), requiring an NDH that pumps about two protons per electron transferred to PQ, consistent with the published ratios for respiratory Complex I of about 2  $H^+/e^-$  (22, 43, 44).

A proton-pumping NDH should have several important consequences. First, it effectively doubles the amount of ATP produced for a given rate of CEF, making it a more efficient mechanism to balance the ATP/NADPH budget, i.e. compared to non-proton-pumping FQR, fewer turnovers would be required to sustain the required ATP production. The actual contribution of NDH-related CEF to chloroplast bioenergetics has been in dispute. Estimates suggest a chloroplast NDH content of about 0.05 per PSI in *Nicotiana tabacum* (12). In order to meet the ATP demands imposed by the CBB cycle, NDH-mediated CEF would need to translocate protons at a rate of ~13% the LEF rate (37). With a fixed ratio of 3  $H^+/e^-$  for LEF, a 1  $H^+/e^-$  NDH would need to turnover 13% the rate of LEF, while a 2  $H^+/e^-$  NDH would need to turnover at 9% of the rate of LEF. Assuming NDH has an activity similar to that of respiratory Complex I, which has maximal sustained turnover numbers of about 200  $e^- s^{-1}$  (45, 46) we estimate that wild type levels of NDH could support a minimum rate of CEF of about 4  $e^- s^{-1}$  per PSI complex, or about 4% of maximal LEF. This rate, in the absence of environmental stresses, is reasonable, as NDH likely works in parallel with the FQR complex and other processes like the malate shunt or the water-water cycle [see review in (4)] to balance the chloroplast energy budget. Under stressed conditions leading to ROS formation (47, 48), in chloroplasts of certain  $C_4$

plants (32), and in the high CEF mutant *hcefl* (38, 40), where high rates of CEF are observed, the content of NDH subunits increase substantially, likely allowing the bulk transfer of electrons through NDH-mediated CEF to be much higher. For example, approximately 15-fold higher levels of NDH were observed in the high CEF mutant *hcefl* (38).

Furthermore, a proton-pumping NDH is expected to be *required* for non-photosynthetic energy transduction in higher plant plastids, as in the proposed chlororespiratory pathway (49 - 51) to involve NDH and the plastid terminal oxidase. Our finding that NDH is a proton pump adds critical support for this possibility because without coupling PQ reduction to proton translocation the proposed pathway would not be able to conserve energy in  $\Delta p$  and ATP synthesis [see also (26)].

While a proton-pumping NDH provides a higher ATP output for CEF, the coupling of the forward reaction to the generation of  $\Delta p$  should also thermodynamically constrain the extent of the overall reaction. At equilibrium, the relationship between protons translocated into the lumen against  $\Delta p$  per electron traversing a redox span of  $\Delta E_h$  mV is given by equation (E1):

$$\Delta E_h \geq n\Delta p \quad \text{Equation (E1)}$$

where  $n$  represents the  $H^+/e^-$  ratio. With Fd as electron donor to NDH,  $\Delta E_h$  equals 520 mV assuming 90% reduction of both the PQ and Fd pools (conditions which may be met during photosynthetic induction) at a stromal pH of 7.5. Assuming a  $\Delta p$  of 180 mV across the thylakoid membrane in the light, NDH would be capable of acting as a  $2H^+/e^-$  pump. This assertion also holds for a predominantly oxidized (90%) Fd pool. However, if the redox state of Fd comes into equilibrium with that of NADPH, as would be expected under many conditions (e.g. when LEF is limited by turnover of the CBB cycle, diminishing kinetic controlling factors on NDH activity), then the NDH energetics become significantly more constrained. As illustrated in Figure S8, certain physiologically relevant combinations of redox poise and  $\Delta p$  would limit the forward (plastoquinone reductase) NDH reaction, and may even allow for reverse reactions, as discussed

further below. One may expect these conditions to be produced during rapid light transients where large  $\Delta p$  can be generated (1, 9).

The predicted thermodynamic linkage between PQ reduction and proton translocation is clearly demonstrated in our results showing that PIFR associated with CEF is constrained by  $\Delta p$  (Figure 4). It is noteworthy that we would not expect to observe significant thermodynamic backpressure if  $H^+/e^-$  was lower than 2 (44). For example, if  $H^+/e^-$  for NDH was 1, the equilibrium constant for reduction of PQ by NADPH with  $\Delta p = 180\text{mV}$  should be  $5 \times 10^6$ , allowing for essentially full reduction of PQ and  $Q_A$ . This analysis thus strongly supports the involvement of a NDH with a coupling stoichiometry of  $2H^+/e^-$ . In addition, the quinone reductase reaction catalyzed by a  $1H^+/e^-$  NDH (or non-protonmotive enzyme such as NDA/NDH2 (23)) would be insensitive to the presence of uncouplers under physiological conditions and would not manifest the transient post-illumination fluorescence rise response observed in Figure 4 upon valinomycin and nigericin addition. Similarly, CEF catalyzed by the PGR5/FQR-pathway as observed *via* the PIFR would be expected to be uncoupler insensitive.

This thermodynamic backpressure effect on NDH also likely explains the unusual kinetics of the transient PIFR seen *in vivo*, which appears to indicate a rate of PQ reduction that is much slower than could sustain expected rates of CEF [see Discussion in (52)]. More likely, the slow kinetics represents a combination of NDH activity and the relaxation of  $\Delta p$  in the dark, which allows the reaction to proceed over a slow time course dictated by dissipation of  $\Delta p$  in the dark. As such, the previous conclusion (52) that the PIFR represents a very slow rate of PQ pool reduction should be reassessed as these kinetics probably reflect, not a slow NDH, but the shifting of equilibrium between stromal reductants and the PQ pool as  $\Delta p$  is dissipated in the dark. Plastid terminal oxidase (PTOX) activity, acting as a slow drain of electrons from the PQ pool under aerobic conditions (49,50), may also be expected to modify the kinetics of the fluorescence transients observed in Fig. 3, although the effect of anaerobiosis on the PIFR transients was observed

to be relatively minor in *S. oleracea* and *A. hybridus* (Fig S7).

The thermodynamic sensitivity of the reaction catalyzed by NDH would provide a simple “self-control” mechanism for regulation of the chloroplast energy budget under conditions of low ATP demand (i.e. high  $\Delta G_{\text{ATP}}$ ) and favors the partitioning of electrons into downstream metabolism. For instance, a deficit of ATP would favor a low  $\Delta p$  and a more reduced NADPH pool, allowing the NDH reaction to proceed. An excess of ATP might result in high  $\Delta p$ , constraining the quinone reductase activity of NDH. The fact that the extent of the NDH reaction is limited by  $\Delta p$  implies that the system reaches quasi-equilibrium; the enzyme can be considered to be approaching the 'static head' condition at normal operating  $\Delta p$  ( $\approx 180$  mV). As such, increasing  $\Delta p$  should favor the operation of NDH in the “reverse” direction (assuming no other limiting kinetic factors), consuming  $\Delta p$ , and functioning as a plastoquinol:NADP<sup>+</sup> oxidoreductase under conditions of a moderately high  $\Delta p$  ( $> 180$  mV) when the PQ pool is predominantly (90%) reduced (Figure S8A-C). This condition would fall within the expected ranges of redox and  $\Delta p$  conditions expected in vivo, and might be expected to occur under fluctuating environmental conditions. Although in the current study we do not directly demonstrate that the reverse reaction occurs under physiological conditions, we note that functional reversal of NDH-like complexes has been observed in respiratory homologs of this enzyme (53- 55).

A  $1\text{H}^+/\text{e}^-$  coupled (or non-protonmotive) NDH would be energetically incapable of significant reverse reaction under physiological conditions. The reverse reaction catalyzed by the  $2\text{H}^+/\text{e}^-$  thylakoid NDH becomes increasingly energetically favorable as the pool of oxidized acceptor (NADP<sup>+</sup>)- and magnitude of  $\Delta p$  increases. With Fd as the electron acceptor, the reversibility of NDH is expected to be strongly thermodynamically limited below a  $\Delta p$  of  $\approx 200$  mV (Figure S8C). However, rapid oxidation of Fd molecules (by FNR and other downstream processes) reduced by PSI and NDH activity would facilitate this reverse reaction. Thus, under conditions of high  $\Delta p$ , we propose that

chloroplasts may conduct electron flow from water, via PSII, to NADPH without the direct participation of PSI, effectively a 'pseudo-linear' pathway for photosynthetic electron transfer that could operate under special conditions of high  $\Delta p$  and reduced PQH<sub>2</sub>. NDH may be considered to be acting as a 'metabolic buffer' enzyme in this hypothesis, smoothing oscillations in ATP/NADPH supply and demand. For example, activation of the pseudo-linear pathway may have large consequences for regulation of metabolic processes that are modulated by redox state of NADPH through the thioredoxin system, possibly explaining why NDH knockouts exhibit strong phenotypes during the induction of photosynthesis, when high  $\Delta p$  and imbalances of redox state are expected, or when subjected to environmental stress, such as decreased CO<sub>2</sub>, fluctuating light intensity or chilling (56, 57). A similar 'pseudo-linear' electron transfer pathway for photoreduction of NADP<sup>+</sup> in the absence of photosystem I activity has been postulated to exist in the alga *Chlamydomonas reinhardtii*, although this organism was later found to lack a protonmotive plastid NDH complex (50, 58).

The trade-offs between efficiency and thermodynamic constraints may also explain why there are multiple routes of differentially-regulated CEF pathways in chloroplasts. Additionally, as noted by Allen, the redox poise of the electron donor- and acceptor pool will also affect CEF activity, with FQR and NDH potentially in competition for substrate (59). Under some conditions, highly efficient, but slower, synthesis of ATP via the NDH complex would be favored. However, NDH activation may be slow, taking approximately 20 minutes (40), and a more rapidly-activated pathway may be needed for rapid responses. In addition, there may also be conditions where higher  $\Delta p$  than that afforded by the NDH pathway may be beneficial, perhaps to acidify the lumen and down-regulate photosynthesis (9). We thus propose that the NDH and FQR modes of CEF are activated step-wise when the downstream metabolism decreases ATP/NADPH. First, a reducing environment is generated as NADPH accumulates, and CEF activated through the non-proton-pumping, FQR-dependent (antimycin A-sensitive) pathway. Under conditions in which this route of CEF is not



able to augment the ATP deficit, and restore redox homeostasis to the chloroplast, ROS is generated and activates the proton-pumping NDH complex. This would increase the yield of ATP formation per  $e^-$  transfer, increasing the efficiency of ATP production via CEF. This route of CEF is not as rapidly activated as the FQR, and long term ROS generation leads to not only activation of already assembled complexes, but an increase in total NDH content (40, 47, 48).

## EXPERIMENTAL PROCEDURES

### *Reagents, plant materials and growth conditions*

Reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise noted.

*Arabidopsis thaliana* and *Amaranthus hybridus* (the latter supplied by Swallowtail Garden Seeds, Santa Rosa, CA) plants were grown in a 16:8 light:dark photoperiod (white fluorescent illumination at  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at  $21^\circ\text{C}$ . Experiments using Col-0 and *A. hybridus* and were performed at 3-4 weeks of age, while slow-growing *hcefl* was used at the same developmental stage as Col-0 (around 6-7 weeks of age). Chloroplasts were prepared from *A. hybridus*, Col-0 and *ndhm* (Salk\_087707) plants 3 weeks after germination. Spinach was obtained from a local market, with chloroplasts prepared on the morning of purchase.

Chloroplasts were prepared from *S. oleracea*, *A. thaliana* and *A. hybridus* leaves as described in (60), and chlorophyll determined as in (61). Chloroplast intactness with regards to proton coupling was routinely monitored using the  $\Delta p$ -dependent acridine dye fluorescence quenching assay described (62)

### *Spectroscopic measurements*

All spectroscopic measurements were made using an integrated diode emitter array (IDEA) spectrophotometer/fluorimeter (63). Plants were poised by illuminating with 700 nm actinic light to favor oxidation of PSI and enable measurement of electron flux through PS I by analysis of dark interval relaxation kinetics.

Transthylakoid proton flux ( $v_H^+$ ,  $\Delta A_{520 \text{ nm}} \text{ m}^{-2} \text{s}^{-1}$ ) was calculated using the electrochromic shift (ECS) of the carotenoids at 520 nm as described in [(40, 64), and references within, Figure S9]. To correct for variability in leaf pigmentation between Col-0 and *hcefl* total extent of ECS in *hcefl* was normalized to the fraction of Col-0 per area leaf chlorophyll content as described previously for *hcefl* (38, 42).

Redox state of PSI was monitored using absorbance changes at 820 nm in a protocol modified from (65). The initial rate of  $P700^+$  re-reduction kinetics were used as the relative rate of electron transfer through PSI ( $v_{P700}$ ,  $\Delta A_{820 \text{ nm}} \text{ m}^{-2} \text{s}^{-1}$ ) (Figure S9).

Postillumination chlorophyll *a* fluorescence transients were measured as described in (12,66) with modifications. Thylakoids were preilluminated with  $250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (620 nm), for 1 minute. After the light to dark transition, the measuring beam ( $505 \text{ nm}$ ,  $0.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was pulsed at a 10 Hz interval. Chlorophyll fluorescence changes during the dark interval were monitored for 145 seconds, with 5 second pulses of far-red illumination ( $720 \text{ nm}$ ,  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) every 20 seconds.

### *In vitro ATP synthesis assays*

Proton-pumping *in vitro* was measured as ATP production in the dark using the Promega ('Enliten') luciferase/luciferin reagent kit with a laboratory-constructed photomultiplier-based phosphoroscope. Osmotically ruptured *S. oleracea* chloroplasts (prepared in the presence of 2 mM reduced DTT) were present at a chlorophyll concentration of  $50 \mu\text{g/ml}$ . DCMU was present at  $10 \mu\text{M}$ . Fd and NADPH were present at  $5 \mu\text{M}$  and  $100 \mu\text{M}$  respectively. The assay buffer (pH 7.5) consisted of 10 mM HEPES, 2 mM potassium phosphate, 10 mM KCl, 5 mM  $\text{MgCl}_2$ , 2 mM DTT and was supplemented with 2 mM ADP and  $100 \mu\text{M}$  diadenosinepentaphosphate (an adenylate kinase inhibitor). Premixed 'Enliten' recombinant luciferase/luciferin reagent (used as supplied by Promega) was added to a final concentration of 8% (v/v). Proton-pumping was initiated by the addition of  $50 \mu\text{M}$  decylplastoquinone (dPQ), and

the proton gradient was collapsed by the addition of 10  $\mu$ M nigericin and 10  $\mu$ M valinomycin. The plastid ATP synthase was activated by red actinic illumination (50  $\mu$ mol photons  $\text{m}^{-2} \text{m}^{-1}$ , 625 nm) of

the chloroplast suspension for two minutes immediately prior to the addition of DCMU, ADP, 'Enliten' reagent and the start of data collection.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

## AUTHOR CONTRIBUTIONS

D.D.S and N.F. designed experiments, collected-, analysed- and interpreted data. D.M.K. designed experiments, constructed instrumentation, analysed- and interpreted data. All authors contributed to the drafting of the manuscript, and all authors reviewed the results and approved the final version of the manuscript.

## FOOTNOTES

The abbreviations used are:  $\Delta p$ , protonmotive force;  $v_H^+$ , transthylakoid proton flux;  $v_{P700}$ , rate of PSI turnover; AA, antimycin A; CEF, cyclic electron flow; DCMU, dichloromethyl urea; (d)PQ, (decyl)plastoquinone; DTT, dithiothreitol; ECS, electrochromic shift; Fd, ferredoxin; FQR, ferredoxin:(plasto)quinone reductase; HA, hydroxylamine; LEF, linear electron flow; NDA/NDH2, non-protonmotive NAD(P)H dehydrogenases; NDH, (protonmotive) NAD(P)H dehydrogenase complex; PIFR, post-illumination fluorescence rise; PSI, Photosystem I; PSII, Photosystem II; PTOX, plastid terminal (plastoquinol) oxidase

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## FIGURE LEGENDS

**Figure 1.** Structural cartoon of NDH showing conserved residues essential for proton-pumping within the membrane domain (grey) and likely organization of the electron donor domain (yellow). Subunits equivalent to the NADH-binding domain of respiratory Complex I (the 'N-module' in the terminology of Brandt (67)) have not been identified in NDH. The (plasto)quinone-binding 'Q-module' is extant in NDH, and formed from NdhH, -I, -J and -K. Structural motifs for the Complex I iron-sulfur clusters N6a, N6b (NdhI) and N2 (NdhK) are conserved in NDH, although their presence has yet to be confirmed spectroscopically. As such, the 'h' prefix for these putative clusters indicates their homology to Complex I. The subunits NDF4 and CRR31/NdhS are unique to NDH. NDF4 is likely to contain an iron-sulfur (FeS) cluster, and CRR31/NdhS has been proposed to be a constituent of the Fd binding site (although it may not be a redox-active participant) (68, 69). Other stromal subunits that have been identified as NDH components but are unlikely to directly participate in the electron transfer pathway are not shown here (70, 71). Highlighted charged residues within NdhB, -D, -E and -F in the membrane domain are conserved between NDH and Complex I, and are considered essential for proton translocation. Residues are numbered according to the *A. thaliana* sequence data. Helices are numbered according to their Complex I homologues. Predicted discontinuities in helices 7 and 12 of NdhB, -D and -F as observed in the atomic structure of *T. thermophilus* Complex I are shown. Note that the NdhB gene is duplicated in Arabidopsis (the sequences of B-01 and B-02 are identical). Complete predicted sequence alignments for the membrane domain subunits shown here are presented in Figure S2.

**Figure 2. Evidence for involvement of a proton pump in CEF.** (A) Cartoon of experimental rationale showing the coupling of proton-pumping NDH activity with ATP synthesis and subsequent detection by luciferase-mediated luminescence. A non-protonmotive NDH would be incapable of generating the

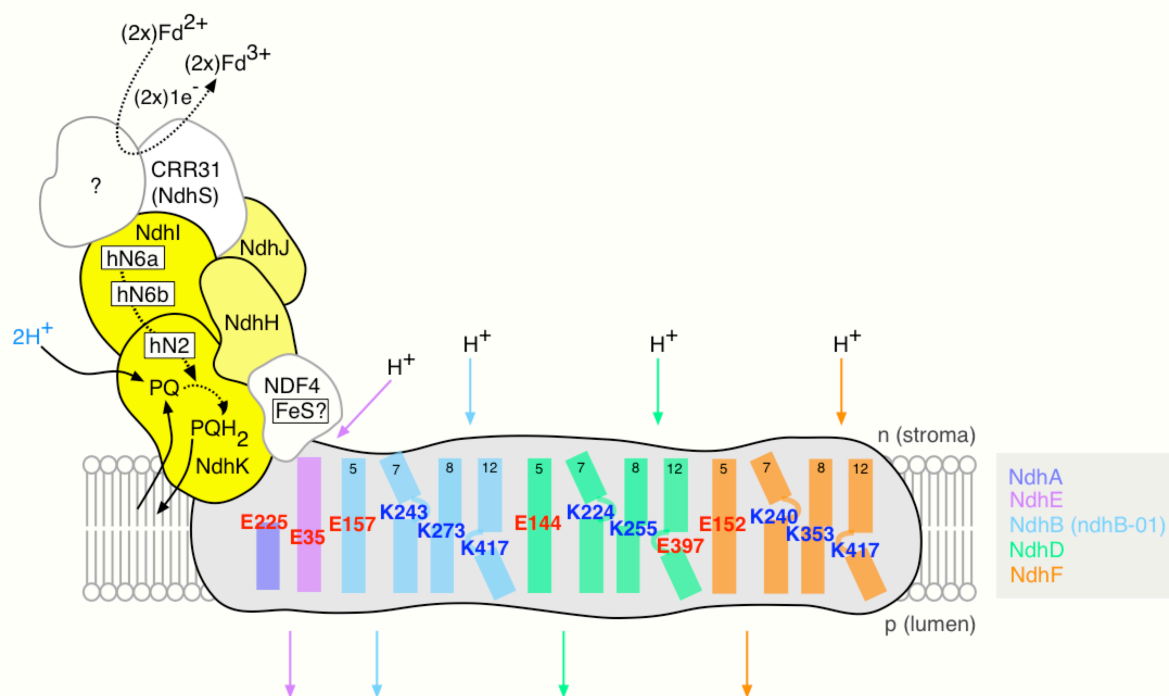
proton gradient required for ATP synthesis. (B) ATP synthesis, monitored by luciferase luminescence, in DCMU-treated *S. oleracea* thylakoids (50 µg Chl/ml) in the dark after addition of 5 µM ferredoxin (Fd), 100 µM NADPH and 50 µM decylplastoquinone (dPQ). The assay buffer consisted of 10 mM HEPES (pH 7.5), 10 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM ADP, 2 mM potassium phosphate, 2 mM DTT, 100 µM diadenosinepentaphosphate and 10 µM DCMU. Premixed 'Enliten' recombinant luciferase/luciferin reagent (used as supplied by Promega) was added to a final concentration of 8% (v/v). Addition of valinomycin and nigericin (10 µM each) is indicated by 'unc'. Representative data (fitted in Kaleidagraph (Synergy Software, PA) by the locally weighted least squared error method after application of a 5-point smooth) are shown; discontinuities in the data are due to the removal of mixing artifacts on substrate addition. The rate of ATP synthesis on dPQ addition was approximately 10 nmol ATP/mg Chl/min.

**Figure 3.** (A) Mechanism of association of NDH-mediated PQ pool reduction and the post illumination fluorescence rise. PSII, Q<sub>A</sub> and Q<sub>B</sub> represent photosystem II and the primary and second quinone electron acceptors in PSII respectively. As a protonmotive enzyme, NDH activity is subject to thermodynamic back pressure from the transthylakoid protonmotive force ( $\Delta p$ ). (B) The post illumination fluorescence rise in *Spinacia oleracea* (*S.o.*), *Amaranthus hybridus* (*A.h.*), *Arabidopsis thaliana*- Columbia (*A.t.* Col-0) and *A.t. ndhM* thylakoid preparations. Assay conditions consisted of thylakoids suspended at 50 µg Chl/ml in 10 mM HEPES (pH 7.5), 10 mM KCl, 5 mM MgCl<sub>2</sub> supplemented with 5 µM Fd and 100 µM NADPH. The periods of actinic- (620 nm, 250 µmol photons m<sup>-2</sup> s<sup>-1</sup>) and far-red (720 nm, 50 µmol photons m<sup>-2</sup> s<sup>-1</sup>) illumination are indicated by bright- and dark red bars under the fluorescence data. Periods of darkness are indicated by black bars. 'SP' refers to a saturating actinic flash (5000 µmol photons m<sup>-2</sup> s<sup>-1</sup>). F<sub>o</sub>, F<sub>m</sub>, F<sub>s</sub> and F<sub>o</sub>' indicate the fluorescence levels in the dark, during the saturating flash, during the steady state under actinic illumination and under far-red illumination respectively. The position of F<sub>o</sub> is indicated by the grey bar. Representative unsmoothed data (normalized to F<sub>o</sub>') from three biological replicates are presented.

**Figure 4.** The effect of uncoupling (unc, 10 µM nigericin + 10 µM valinomycin) and 10 µM antimycin A (AA) on the post illumination fluorescence rise in (A) *Spinacia oleracea* (*S.o.*), (B) *Amaranthus hybridus* (*A.h.*), (C) *Arabidopsis thaliana*- Columbia (*A.t.* Col-0) and (D) *A.t. ndhM* thylakoid preparations. Effectors were added during the course of the experiments as indicated by the asterisked arrow above the fluorescence data. Other conditions as in Figure 3.

**Figure 5. Evidence for involvement of a proton pump in CEF *in vivo*.** Relative rates of light-induced thylakoid proton flux ( $v_H^+$ ) were estimated by the initial slope of the decay of the electrochromic shift. The rates of turnover PSI ( $v_{P700}$ ) were estimated by the rate of decay of the P<sub>700</sub><sup>+</sup> absorbance changes upon rapid transition from light to dark, under conditions where P<sub>700</sub><sup>+</sup> accumulates (700 nm illumination). Data are from intact leaves of Col-0 (black) and *hcef1* (grey) under increasing light intensities. Lines represent best free fit of the data (solid lines, slopes of 1.8 and 2.75 for Col-0 and *hcef1*) or forced through the origin (dashed lines, slopes of 1.6 +/- 0.3 and 4.7 +/- 0.8 for Col-0 and *hcef1*).

### Figure 1





**Figure 2**

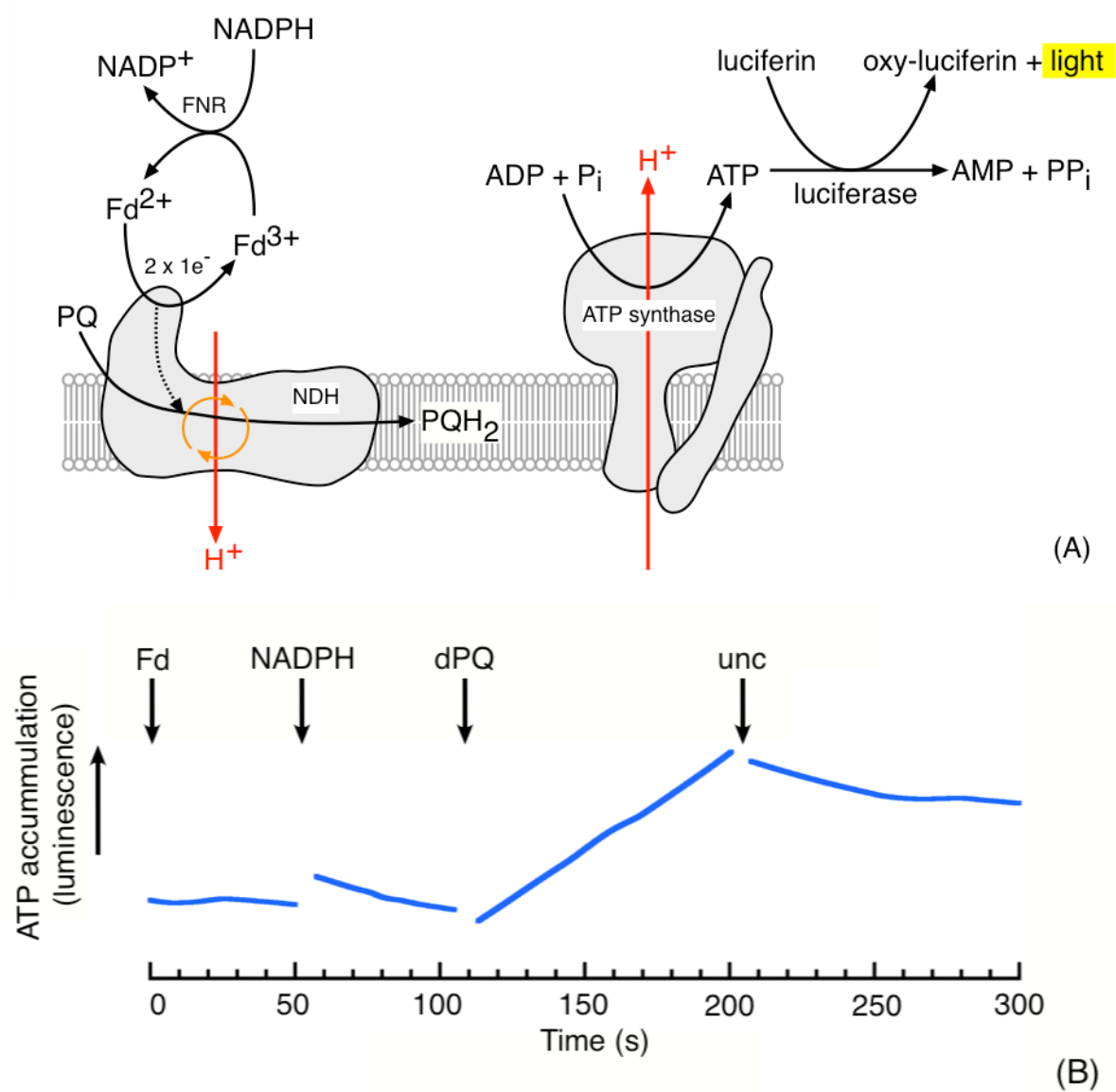
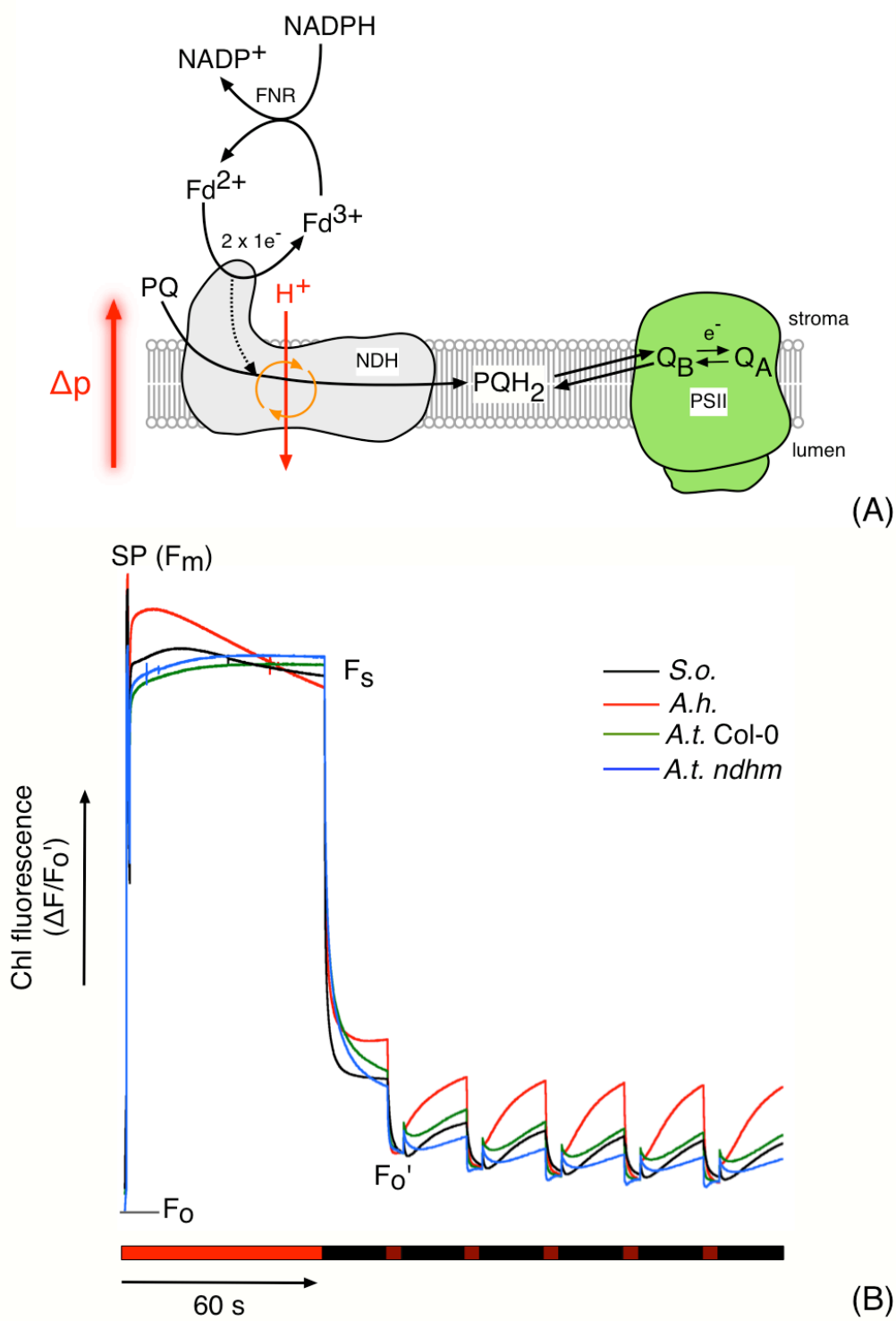
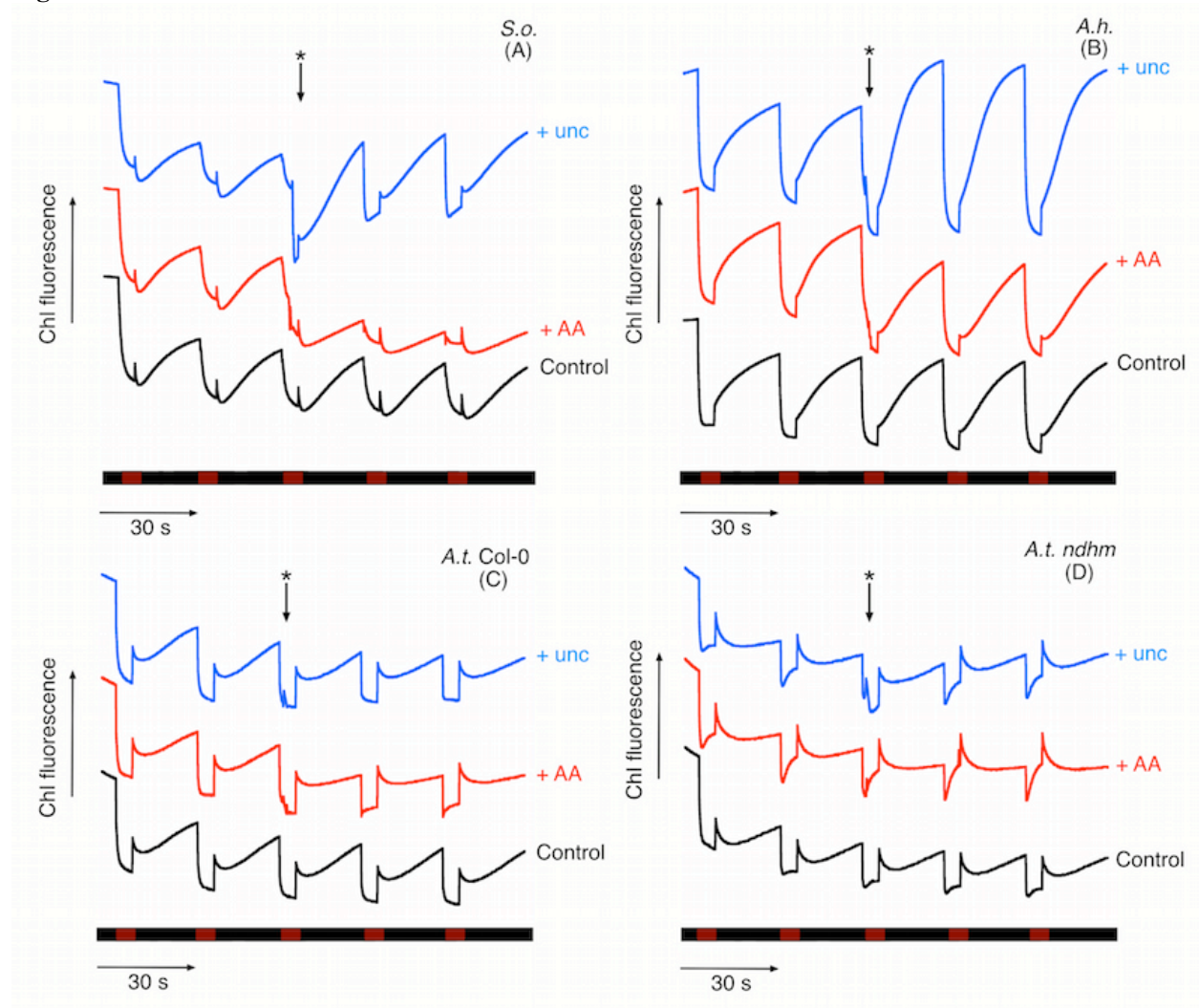


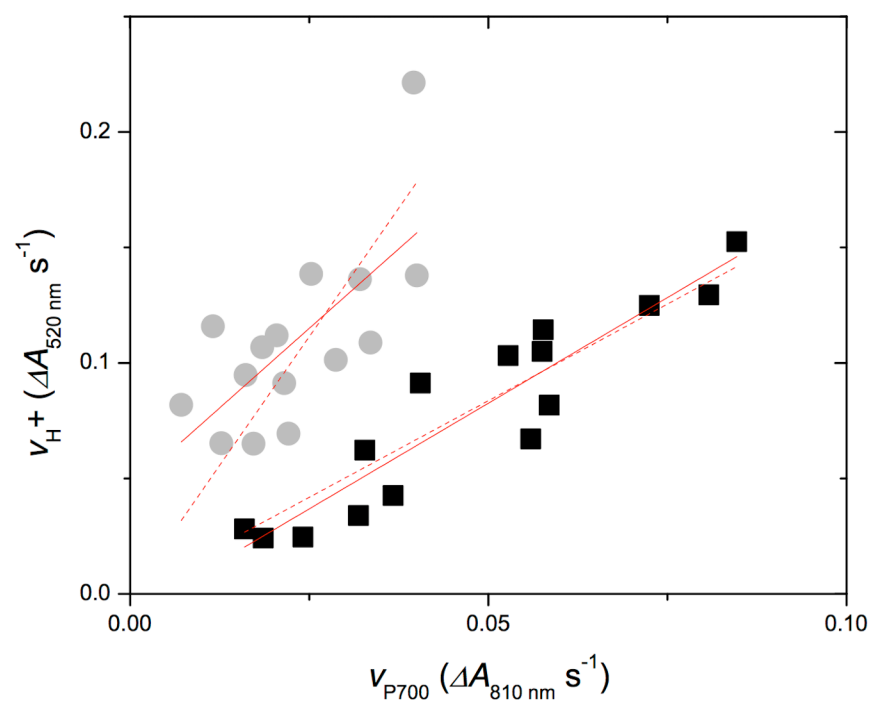
Figure 3



**Figure 4**



**Figure 5**





**The higher plant plastid NAD(P)H dehydrogenase-like complex (NDH) is a high efficiency proton pump that increases ATP production by cyclic electron flow**

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