hypothesis

Metabolic regulation of photosynthetic membrane structure tunes electron transfer function.

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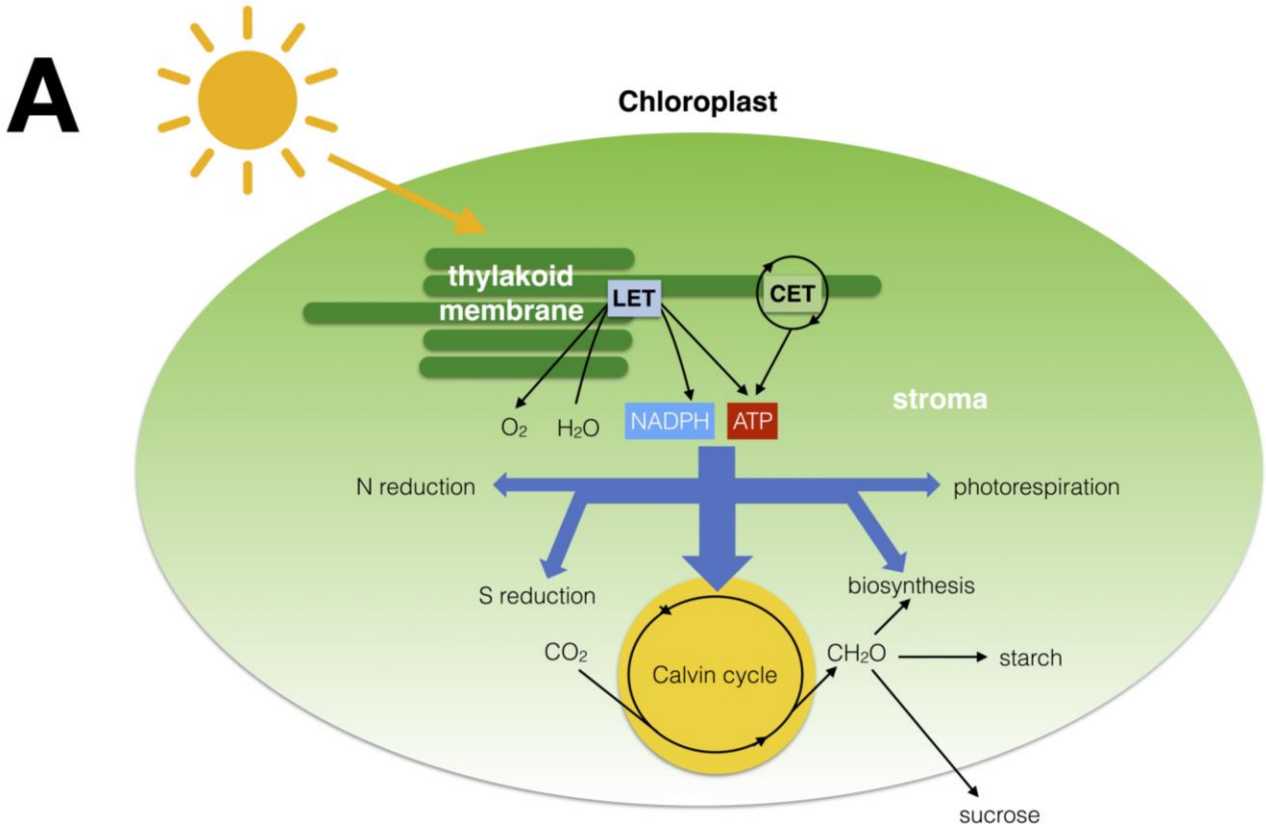
The photosynthetic chloroplast thylakoid membrane of higher plants is a complex 3-dimensional structure that is morphologically dynamic on a timescale of just a few minutes. The membrane dynamics are driven by the phosphorylation of light harvesting complex II (LHCII) by the STN7 kinase, which controls the size of the stacked grana region relative to the unstacked stromal lamellae region. Here I hypothesise that the functional significance of these membrane dynamics is in controlling the partition of electrons between photosynthetic linear and cyclic electron transfer (LET and CET), which determines the ratio of NADPH/ ATP produced. The STN7 kinase responds to the metabolic state of the chloroplast by sensing the stromal redox state. High NADPH/ ATP ratio leads to reduction of thioredoxin f (TRX*f*), which reduces a CxxxC motif in the stromal domain of STN7 leading to its inactivation, whereas low NADPH/ATP ratio leads to oxidation of TRX*f* and STN7 activation. Phosphorylation of LHCII leads to smaller grana, which favour LET by speeding up diffusion of electron carriers plastoquinone (PQ) and plastocyanin (PC) between the domains. In contrast, dephosphorylation of LHCII leads to larger grana that slow the diffusion of PQ and PC, leaving the PQ pool in the stroma more oxidised, thus enhancing the efficiency of CET. The feedback regulation of electron transfer by the downstream metabolism is crucial to plant fitness, since perturbations in the NADPH/ATP ratio can rapidly lead to the inhibition of photosynthesis and photo-oxidative stress.

Introduction

The plant chloroplast thylakoid membrane houses the photosynthetic electron transfer reactions (PET) that provide the NADPH and ATP required for CO_2 fixation by the Calvin-Benson-Bassham (CBB) cycle. Generally, PET and the CBB cycle are conceptualised and studied separately, even though the two processes are intimately coupled. Interactions take place not only at the level of shared turnover of the $NADP^+$/NADPH and ADP/ATP pools in the chloroplast, but also in the feedforward and feedback regulation the two processes impart on one another [1-3]. Feedforward regulation of the CBB by PET involves the alteration of the stromal environment in terms of redox potential, pH and ionic status, which modulates the activity of numerous enzymes involved in CO_2 assimilation [3]. In turn, feedback regulation of PET by the CBB cycle influences the redox state of the electron carriers and the size of the proton motive force (Δp) of the thylakoid membrane [1,2]. These factors control the balance between light energy utilisation in the photochemical reactions and the dissipation of this energy as heat by non-photochemical quenching (NPQ) through regulation of the LHCII antenna system and the ATP synthase [4-8]. In principle, the CBB cycle in C3 plants consumes ATP to NADPH in the strict ratio of 1.5:1, yet the linear electron transfer pathway (LET) from H_2O to $NADP^+$ during photosynthesis only produces 1.28 ATP per NADPH if the H^+/ATP ratio is 4.67, as suggested by early biochemical studies and later the structure of the c-ring of the spinach ATP synthase [9,10]. In addition to production of glyceraldehyde 3-phosphate by the CBB cycle, a range of other metabolic processes also make varying demands on the chloroplast NADPH and ATP pools, including photorespiration, nitrogen and sulphur reduction, and protein, lipid and carbohydrate biosynthesis [1,11,12] (Fig. 1A). The activity of these pathways varies depending on environmental conditions and developmental state of the leaf, thus affecting the ratio of ATP to NADPH required by the cell [1,11,12]. Since the ATP and NADPH pools turnover so rapidly any mismatch in the ATP/NADPH ratio can rapidly inhibit photosynthesis [12-14]. Therefore, several regulatory mechanisms exist in the plant cell for the dissipation of the metabolite in excess or the augmentation of the metabolite in deficit. These mechanisms

include the water-water cycle (WWC or Mehler reaction) [15], the malate valve [16], chlororespiration via plastoquinol oxidase (PTOX) [17] and cyclic electron transfer (CET) [18,19]. In plants CET appears to be the dominant pathway for ATP augmentation and has been shown to be crucial to plant fitness, particularly in fluctuating light conditions [20-22]. Whereas LET involves photosystem II (PSII), plastoquinone (PQ), cytochrome *b*$_6$*f* (cyt*b*$_6$*f*), plastocyanin (PC), photosystem I (PSI), ferredoxin (Fd) and ferredoxin-NADP$^+$ reductase (FNR) complexes acting in series and generates NADPH and a proton gradient that is utilised by ATP synthase to make ATP, CET transfers electrons from Fd back to PQ, forming a cycle around PSI. This allows proton transfer and thus synthesis of ~~, allowing~~ ATP ~~to be produced~~ without net NADPH formation (Fig. 1B) [18,19]. Therefore, by controlling the balance between LET and CET plants can adjust the ATP/NADPH ratio and contribute to the fulfilment of metabolic demand [1,4,11]. A greatly lowered capacity for electron transfer and photoprotection is observed when CET is knocked out by genetic mutation, suggesting that the balance between LET and CET is central to the proper regulation of photosynthesis *in vivo* [20-22].

In plants two major routes of CET have been discovered to date, the antimycin A-(AA) sensitive Proton Gradient Regulation complex (PGRL1/PGR5)–dependent pathway and AA-insensitive NADPH-like dehydrogenase (NDH)-dependent pathway [18,19] (Fig. 1B). There is evidence that PGRL1/PGR5 acts as an Fd-PQ oxidoreductase that can transiently interact with both PSI and cyt*b*$_6$*f* [23,24]. Alternatively, it has been suggested that PGR5 may be a regulator of the cyt*b*$_6$*f* complex [21] and that CET may involve transfer of electrons from a Fd-FNR complex to the Q_n site of cyt*b*$_6$*f* [25,26], a pathway which may involve the unusual haem c [27,28] (Fig. 1B). The NDH (NADPH-like dehydrogenase complex) is an Fd-PQ oxidoreductase that forms a supercomplex with PSI and shows similarity to Complex I in mitochondria [29-31]. The NDH complex has been shown to act as a proton pump, thus increasing the H^+/e^- ratio of this CET pathway compared to the PGR5/PGRL1 and Fd/FNR/cyt*b*$_6$*f* routes, which only translocate protons at cyt*b*$_6$*f* [32].



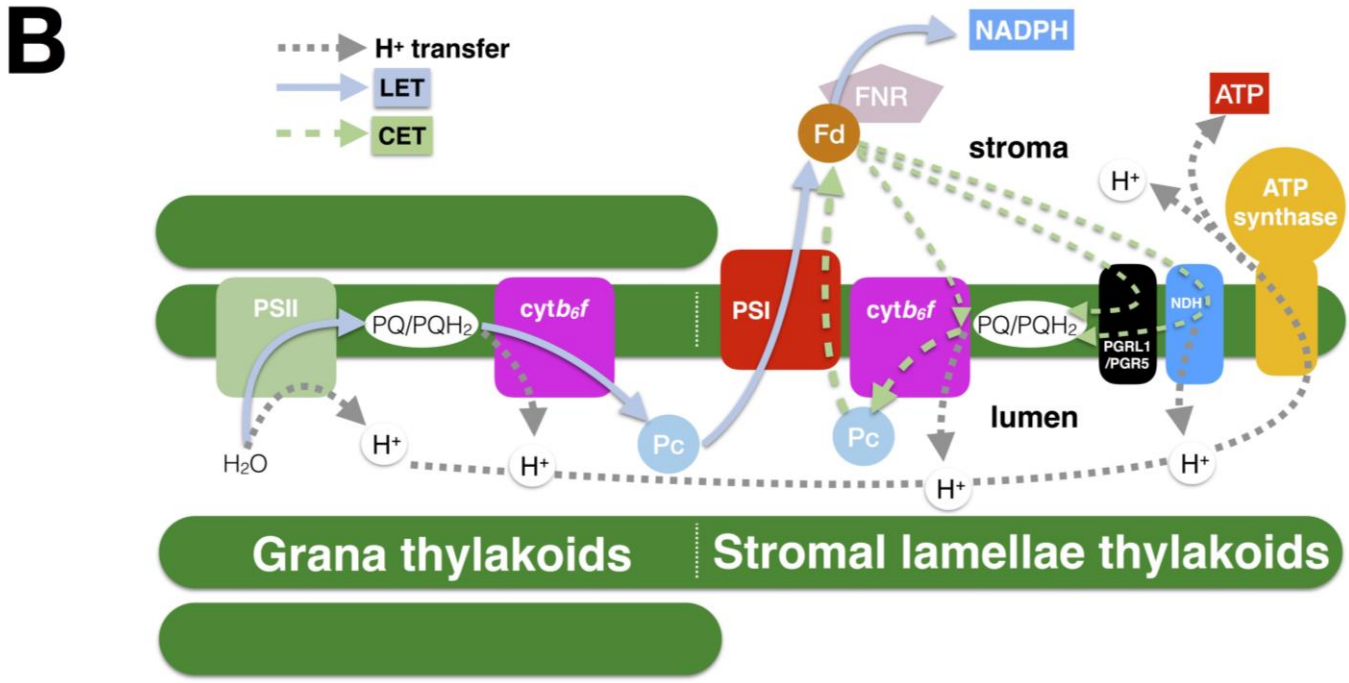


Figure 1. Role of linear and cyclic electron transfer (LET and CET) in photosynthesis. (A) Sunlight initiates electron transfer in the chloroplast thylakoid membrane that leads to the synthesis of NADPH and ATP by LET, with ATP production further augmented by CET. The thylakoid membrane must continually adjust the LET / CET balance to provide the correct ratio of NADPH/ ATP to meet changeable metabolic demand that depends on the relative activity of the Calvin cycle, N and S fixation, photorespiration and biosynthesis in the chloroplast stroma, which vary according to the environmental and developmental state of the plant. **(B)** Lateral heterogeneity in organisation of photosynthetic complexes in the thylakoid membrane. PSII is located in the grana stacks, PSI, ATP synthase and PGRL1/PGR5 and NDH are located in the stromal lamellae and cyt*b*$_6$*f* is present in both domains. LET involves electron transfer from water to $NADP^+$ via both PSII and PSI, with water oxidation at PSII and PQH_2 oxidation at cyt*b*$_6$*f* leading to proton accumulation in the lumen, which is utilised by ATP synthase to drive ATP synthesis. CET involves recycling electrons from Fd to the stromal PQ pool and therefore produces ATP without net NADPH synthesis. Three possible CET pathways based on the NDH, PGRL1/PGR5 and FNR/cytb$_6$f are shown.

Current models for regulation of the LET/CET balance.

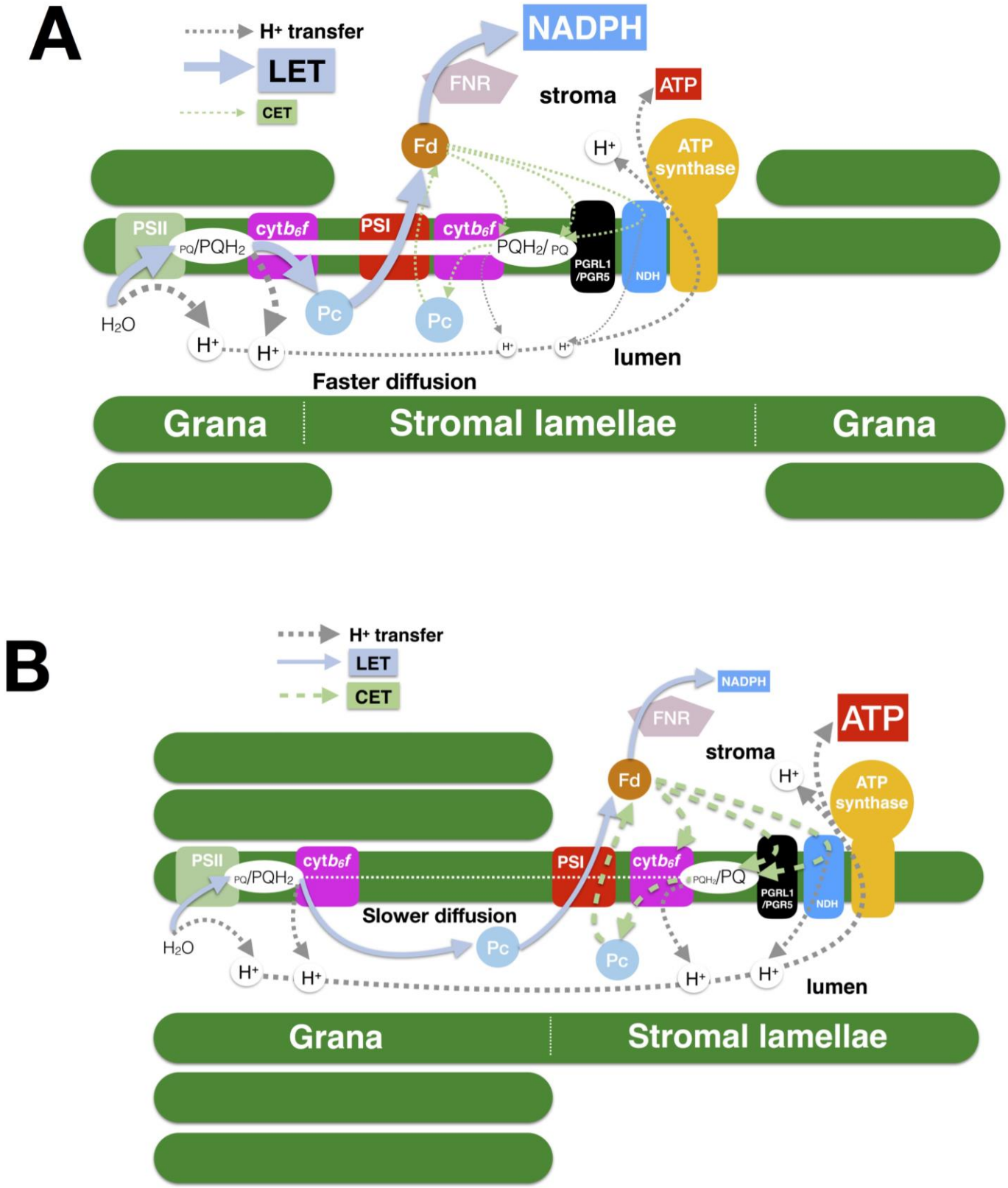
Since the CBB cycle uses 1.5 ATP/NADPH and LET only produces a ratio of 1.28, a constant portion of CET can be expected even in the steady state, estimated at ~15% of the total LET flux [4]. Indeed, different isoforms of Fd and FNR genes, which encode proteins with activities devoted to LET or CET [33] could provide the basis for a constant portion of CET regulated by protein copy number. However, as the developmental state of the leaf and its environment can change with time so the relative demand for ATP/NADPH and so the ratio of LET/CET can be expected to dynamically vary [1,4,18,19]. Several metabolic signals, indicative of an imbalance in the NADPH/ ATP ratio, have been suggested to regulate the amount of CET including the PQ redox state [1, 34], ADP/ATP ratio [35], stromal redox state [36-38], reactive oxygen species [39,40], phosphorylation of NDH [41] and PGRL1 [42] and calcium signalling [43].

The suggested regulation of the LET/CET balance via the PQ redox state is based on the state transition [1, 34]. State transitions in plants involve the dynamic reallocation of LHCII between PSI and PSII, provoked by preferential excitation of PSI (far-red light) or PSII (red/ blue light). PQ reduction in red/blue light activates STN7 kinase [44,45] mediated phosphorylation of LHCII, favouring its association with PSI (State II). PQ oxidation in far-red light inactivates STN7 allowing the constitutively active TAP38 [46,47] to dephosphorylate LHCII, favouring its association with PSII (State I). Reduction of PQ was suggested to

parallel PSI acceptor side limitation due to a high NADPH/ ATP ratio [1,34]. The transition to State II would then lead to an increased excitation of PSI relative to PSII, thus increasing CET and making good the ATP deficit. In contrast, a low NADPH/ATP ratio would lead to PQ oxidation via removal of the acceptor side limitation of PSI and a transition to State I and thus enhanced LET to augment NADPH production [1,34]. In line with this, anoxic conditions that lead to induction of State II in the green alga *Chlamydomonas,* also cause increased CET flux and accumulation of a supercomplex containing PGRL1, PSI and cytb_6f [37, 48]. However, the CET supercomplex formation and enhanced CET flux still occurred in the absence of state transitions in *Chlamydomonas* [37], similarly in *Arabidopsis* CET activity was unaffected by the absence of the STN7 kinase [49].

An alternative means through which metabolic signals may regulate the LET/CET balance is via control of the partition of electrons between the pathways. CET is sensitive to the 'redox poise' of the electron transfer chain, thus it is inhibited by over-reduction or over-oxidation [50]. Therefore, when high NADPH levels inhibit LET, PQ will tend to be over-reduced thus potentially curtailing the ability of CET to operate. For this reason, LET and CET may require some degree of structural compartmentalisation. One possibility is through the formation of supercomplexes dedicated to CET [29-31,37,48] with a sequestered PQ pool. Yet to date no counterpart to the PGRL1/PSI/cytb_6f supercomplex in *Chlamydomonas* [37,48] has been found in plants, although associations between PGRL1 and PSI, PGRL1 and *cytb_6f*, NDH and PSI [29-31], cytb_6f and FNR [51], NDH and FNR [52], PSI and FNR [53], and more recently between PSI and *cytb_6f* [31] have been described, although none of these has yet been shown to be more abundant in conditions favouring CET. Another possibility is the maintenance of a separate PQ pools within membrane domains [54, 55]. The thylakoid membrane in plants is divided into two domains; the stacked grana and the interconnecting stromal lamellae with the various electron transfer components heterogeneously distributed among them [56]. PSII is found mainly within the grana stacks while PSI and ATP synthase is found mainly within the stromal lamellae, and cytb_6f is found within both domains [56, 57]. There is evidence for compartmentation of PQ in dark adapted thylakoids, with ~70% of the PQ pool available for rapid reduction by PSII (t½=25 ms) and the remaining 30% only slowly reduced (t½=6 s) [54]. These fast and slow PQ pools were suggested to arise from the grana and stromal lamellae respectively, with diffusion of PQ confined by the extreme protein crowding within the grana [54, 58]. Joliot suggested that the presence of a distinct PQ pool associated with cytb_6f in the stromal lamellae, isolated by diffusion limitation from PSII in the grana, could create the conditions necessary for CET to co-exist with LET [54]. In this view, CET and LET would share a common pool of PC and PSI, with injection of electrons into the CET pathways determined by competition at the level of Fd [18]. Importantly the restricted diffusion of PQ is largely abolished upon either unstacking of thylakoids [58] or light adaptation [54], suggesting that changes in membrane organisation can significantly influence the rate of diffusion and potentially therefore the balance between CET and LET.

Figure 2. Variable grana stacking alters the partition of the thylakoid PQ pool and so LET/CET balance. (A) Smaller grana, create a larger area of contact between the grana and stromal lamellae membranes. Under these conditions the diffusion of PC and PQ between grana and stromal lamellae is relatively fast, engaging cytb_6f in both domains efficiently in LET which predominates over CET. **(B)** Larger grana reduce the area of contact between the grana and stromal lamellae membranes. Under these conditions the diffusion of PC and PQ between grana and stromal lamellae is relatively slow, leaving the stromal lamellae PQ pool in a more oxidised state that promotes more efficient CET.



A new model for the regulation of the LET/CET balance based on dynamic thylakoid stacking.

Here I propose that dynamic changes in thylakoid stacking regulate the LET/ CET balance by altering the degree of partition of the PQ pool. Rapid changes in thylakoid stacking have been observed in spinach upon transition from darkness to low light and from low light to high light [59-61]. Darkness and high light led to larger grana stacks, whereas low light led to smaller grana stacks. These changes parallel the trends in LHCII phosphorylation, which peak at low light and are decreased in high light and darkness [62, 63]. Phosphorylation regulates the surface charge on LHCII and so the cation dependency of stacking [64,65].

Smaller grana have been shown to facilitate faster diffusion of PQH_2 and PC from the grana to stromal lamellae thylakoids and more rapid reduction of PSI, i.e. reduced partition of electron carriers between domains [61] (Fig. 2A). Since the rate-limiting step of LET is the diffusion of PQH_2 from PSII to cytb_6f and its oxidation therein, any change in the diffusion time has the potential to affect the overall rate considerably [66]. Indeed, more efficient LET has also been observed in ΔTAP38 *Arabidopsis* plants [46], which possess smaller grana, whereas LET is compromised in the ΔCURT mutant with larger grana [67]. In contrast to enhanced LET, smaller grana and reduced stacking negatively impact on the ability to induce CET [61]. The rate of PSI reduction following far-red illumination was slower when the grana size was smaller indicating reduced CET and transient NPQ generation upon moderate light challenge, which is a proxy for ΔpH generation by CET [22], was also compromised [61]. Thus, larger grana may benefit CET through increased partition of electron carriers between the domains, leaving the stromal PQ pool in a relatively more oxidised state, that is more competitive for electrons from Fd (Fig. 2B). Larger grana are also favoured in high light and darkness conditions [59], where there is evidence CET is upregulated [20,35,68].

How might the STN7 kinase be regulated to allow these dynamics? STN7 is activated when PQH_2 binds to the Q_O site of the cytb_6f complex [69], wherein it is suggested to reduce a lumenal facing disulphide linkage between two cysteines in the kinase [70]. Therefore, increasing reduction of the PQ pool with light intensity should upregulate STN7. However, a second negative feedback loop exists that sees STN7 inactivated by thioredoxin *f* (TRX*f*) upon a build-up of reducing power on the PSI acceptor side with increasing light intensity [71, 72]. TRX*f* is suggested to reduce a buried disulphide linkage between cysteines near the ATP-binding motif on the stromal side of STN7 [70, 72]. STN7 is therefore maximally active at around 100-200 μmols photons m^{-2} s^{-1} in white light and inactivated in darkness (by PQ oxidation) and in high light (by TRX*f*) [44, 62, 71, 72]. Interestingly, changes in white light intensity cause no alterations in relative PSI and PSII antenna sizes in contrast to the preferential excitation of the photosystems described above [8, 63]. Thus, under natural white light conditions the hyper-phosphorylation of LHCII, and thus the state transition, is prevented by the TRX*f* regulation of STN7. Thus, white light driven LHCII phosphorylation is sufficient for changes in grana size, but insufficient to provoke the state transition in plants. This situation contrasts with that in *Chlamydomonas* where the STT7 LHCII kinase (counterpart of STN7) is not inhibited by TRX*f* and therefore state transitions and stromal over-reduction (and therefore increased CET) co-exist [37, 48].

I suggest that the prevailing NADPH/ ATP ratio in the chloroplast controls the activity of STN7 and thus the size of the grana. In this view, PQ reduction in the light activates STN7 and its activity is then largely regulated by the stromal redox state and therefore the extent of TRX*f* reduction. Thus, when the NADPH/ ATP ratio is low, TRX*f* is oxidised, allowing ATP binding and phosphorylation of LHCII (Fig. 3A). In contrast, when ATP is in deficit (high NADPH/ ATP ratio), STN7 is inactivated by reduced TRX*f* leading to larger grana stacks and increased CET. The amount thylakoid membrane stacking and thus LET/ CET balance is therefore regulated by the metabolic state of the chloroplast. Evidence for metabolic regulation of STN7 already exists in the literature. For instance, treatment of thylakoids with reduced dithiothreitol (DTT) resulted in decreased LHCII phosphorylation and increased CET activation [38, 71], while oxidised DTT stimulated LHCII phosphorylation [62, 71]. In turn, addition of ADP or an ATP sink such as ribulose-5-phosphate, which would elevate the NADPH/ATP ratio, also provokes dephosphorylation of LHCII [73, 74], while addition of ATP or increased adenylate pool energy charge stimulates LHCII phosphorylation through consumption of NADPH and therefore oxidation of TRX*f* [73]. It is also possible that the PGR5/PGRL1 complex may play a crucial role in sensing the stromal redox state [26] and directly or indirectly regulating STN7 [63]. Such a role for PGR5 would be consistent with two other observations; the lack of LHCII dephosphorylation upon shift from low to high light in the ΔPGR5 mutant of *Arabidopsis* and the stimulation of LHCII phosphorylation in the presence of antimycin A [63, 75]. If grana size remains small in the ΔPGR5 muant in high light this may partly explain the inefficient CET in this mutant.

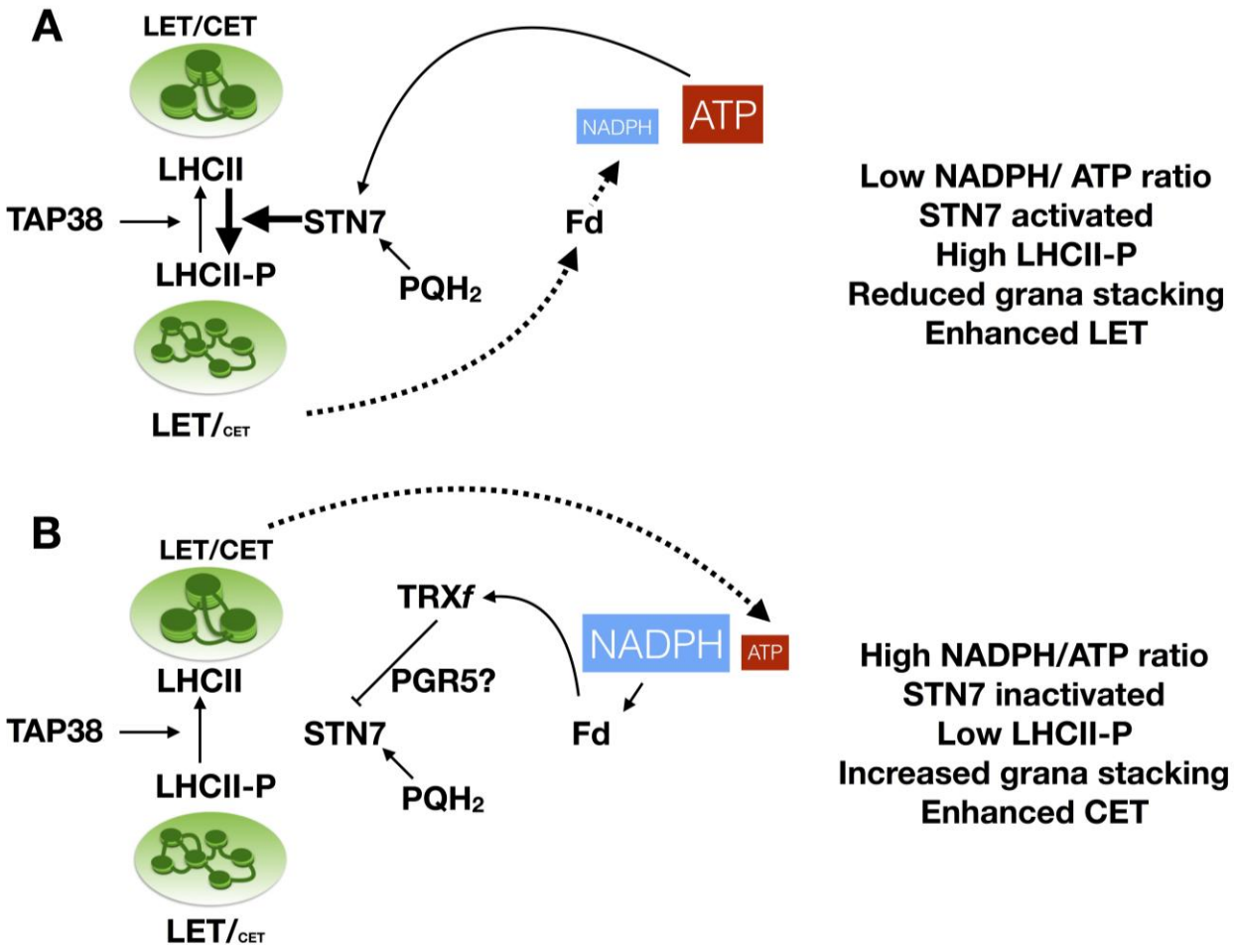


Figure 3. Metabolic regulation of the Stn7 kinase. In the light PQ reduction leads to STN7 activation. Two conditions can then ensue **(A)** When the NADPH/ ATP ratio is low, the STN7 kinase is active and LHCII phosphorylation is increased leading to smaller grana that favour LET augmenting NADPH production (dashed arrow) **(B)** When the NADPH/ ATP ratio is high, reduced TRX *f* inactivates the STN7 kinase and the constitutively active TAP38 phosphatase dephosphorylates LHCII leading to larger grana that favour CET, augmenting ATP production (dashed arrow). The PGR5/PGRL1 complex appears to have some as yet undefined regulatory role in sensing the stromal redox poise and its absence [63] or inhibition by antimycin A [76] prevents the inactivation of STN7 by TRX *f*.

Concluding remarks

I have suggested a novel hypothesis that links grana dynamics with LHCII phosphorylation and the regulation of the LET/ CET balance. The key player in this regulatory model is the effect of TRX*f* on STN7 activity, which provides information on the NADPH/ATP ratio in the stroma. There are several important implications of this hypothesis both in terms of future experiments and biological function. The hypothesis predicts a central role for grana stacking in controlling the degree of partition of the PQ pool and thus control of the relative efficiencies of CET versus LET. Therefore, CET should be less competitive for electrons in the ΔTAP38 and oeCURT mutants of *Arabidopsis,* which have smaller grana. It is equally important to establish whether the absence of STN7 and /or TAP38 prevent dynamic changes in stacking and understand whether changing PSII (and possibly CURT) phosphorylation play any role. In principle, by manipulating the metabolic state of the chloroplast, it should be possible to manipulate the extent of grana stacking, while keeping the light intensity constant. Moreover, with modern metabolomic approaches it should now be possible to accurately quantify the dynamic transients in metabolite pools that elicit changes in LHCII phosphorylation upon fluctuations in light intensity.

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