## Review Article

# Efficient photosynthesis in dynamic light environments: a chloroplast's perspective

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In nature, light availability for photosynthesis can undergo massive changes on a very short timescale. Photosynthesis in such dynamic light environments requires that plants can respond swiftly. Expanding our knowledge of the rapid responses that underlie dynamic photosynthesis is an important endeavor: it provides insights into nature's design of a highly dynamic energy conversion system and hereby can open up new strategies for improving photosynthesis in the field. The present review focuses on three processes that have previously been identified as promising engineering targets for enhancing crop yield by accelerating dynamic photosynthesis, all three of them involving or being linked to processes in the chloroplast, i.e. relaxation of non-photochemical quenching, Calvin–Benson–Bassham cycle enzyme activation/deactivation and dynamics of stomatal conductance. We dissect these three processes on the functional and molecular level to reveal gaps in our understanding and critically discuss current strategies to improve photosynthesis in the field.

## Introduction

The biological process photosynthesis provides for most of the atmospheric oxygen and metabolic energy on our planet. It is fueled by the plenty of energy reaching Earth every day in the form of sunlight. In most environments, including farmland, light energy supply for photosynthesis can vary tremendously. Changes in solar position and dynamic movement of plants with the wind can lead to large fluctuations in incident light on very short time scales [1]. A comprehensive understanding of how plant photosynthesis reacts to such strong light fluctuations is warranted on the molecular level. Firstly, this is critical to assess the potential of engineering photosynthetic responses for improving photosynthesis in the field. Enhancing field photosynthesis holds great promise for increasing crop yield and thereby helping address global challenges related to overpopulation and climate change [2,3]. Secondly, it allows basic insights into nature's design for highly dynamic and efficient energy conversion and thereby carries potential for inspiring artificial photosynthesis and, more broadly, energy systems engineering.

While our understanding of the rapid responses of photosynthesis is still limited, the basic processes of plant photosynthesis are well understood (see recent reviews in [1,4–6]). In summary, light energy drives charge separations at the two photosystems in the thylakoid membrane and electrons are transported from water to $NADP^+$ along the electron transport chain, including plastoquinone (PQ), the cytochrome $b_6f$ complex and plastocyanin [5]. Concurrently, protons are pumped into the thylakoid lumen, generating the proton motive force ($pmf$) used by the ATP synthase for ATP production [7]. The chloroplast $pmf$ consists of two components: the proton concentration gradient ($\Delta pH$) and the membrane potential ($\Delta\Psi$) [4]. The net proton concentration in the lumen has an additional regulatory effect: a high proton concentration (i.e. low pH) down-regulates light harvesting by activating a non-photochemical quenching (NPQ) mechanism that dissipates absorbed light energy as heat [8–10]. Additionally, high lumen [$H^+$] inhibits plastoquinol oxidation at the $Cytb_6f$ complex [11–13]. ATP and NADPH, the products of the thylakoid-localized electron/proton transport reactions, drive cellular

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metabolism including the carbon fixation reactions of the Calvin–Benson–Bassham (CBB) cycle. NADPH serves an additional regulatory function by donating electrons to the reductive activation of photosynthetic components including the ATP synthase and many CBB enzymes [14,15].

For improving dynamic photosynthesis, three main strategies have been identified [1,16]: (i) the acceleration of NPQ relaxation upon transition to low light. A model of canopy photosynthesis predicted that slow NPQ relaxation kinetics constrain crop photosynthesis by decreasing the quantum efficiency of photosynthesis in low-light periods [17]. (ii) The acceleration of CBB enzyme activation/deactivation. (iii) The acceleration of stomatal dynamics. While the acceleration of NPQ relaxation is expected to improve dynamic photosynthesis of both C3 and C4 plants, alterations to CBB enzyme activation and stomatal conductance mainly apply for improving C3 photosynthesis. This review lays out our current understanding of the molecular processes that underlie NPQ and particularly its relaxation, the activation of CBB enzymes and recent advances in the two light-dependent responses of stomatal opening (red and blue light responses). We point out gaps in our understanding and draw connections to other physiological processes that may be affected by current enhancement approaches.

# The acceleration of NPQ relaxation to enhance light use efficiency

Photosynthesis in full sunlight is safeguarded by thermal dissipation of absorbed light in the PSII antenna, the NPQ process which prevents transfer of excess light energy to the photosystems and thus avoids detrimental side reactions (reviewed in [18,19]). In dynamic light environments, however, the efficiency of photosynthesis will depend (at least theoretically) on the speed by which NPQ can be reduced upon shading events. Once cells find themselves in light-limited conditions, any residual NPQ will reduce their photosynthetic efficiency by dissipating some of the available light energy as heat. The idea that slow NPQ relaxation kinetics could represent a limiting factor for plant photosynthesis and crop yield was first inferred from a computational model of light distribution in a crop canopy by Zhu et al. [17]. As a result, the acceleration of NPQ relaxation kinetics has been pushed forward as a key strategy for enhancing photosynthesis in the field [16,20,21].

## Mechanisms that contribute to plant NPQ

The main contributor to plant NPQ is the energy-dependent quenching (qE, 70–80% of total NPQ [8–10,22–36]. qE is the fastest responding NPQ component and relaxes within 5 min in the dark. More slowly relaxing NPQ components include the zeaxanthin-dependent quenching qZ [23] and the photoinhibitory component qI, the latter relating to the damage of the photosystem II (PSII)-D1 protein by reactive oxygen species and the resulting inactivation of PSII [24–26]. Such photodamaged PSII is a potent quencher of absorbed light energy [27], but in terms of maintaining efficient photosynthesis in dynamic light environments, the targeted destruction of the PSII-D1 protein must be a 'last resort' measure, only to be employed when other dissipative mechanisms have failed (reviewed by Ruban [28]). The relaxation of qI, i.e. the repair of photodamaged D1, is energy costly and relatively slow. While the acceleration of PSII repair may theoretically be one option to speed up the recovery of photosynthetic efficiency after prolonged stress, practically this is extremely difficult to approach as many different processes and organizational layers (i.e. protein degradation, expression and complex reassembly) are involved (reviewed by Murchie and Niyogi [29]). While there are additional NPQ components with slower relaxation kinetics than qE [30–32], and their manipulation may well have potential for accelerating NPQ relaxation kinetics, these are outside the scope of this review. We will focus this part of the review on the major NPQ contributor in plant leaves, qE, and its core components PSII subunit S (PsbS), xanthophylls and lumenal pH. We will discuss existing strategies to accelerate NPQ relaxation and point out gaps in our molecular understanding, including (i) PsbS and the molecular mechanism by which light energy gets converted into heat, (ii) the regulation and distribution of the zeaxanthin epoxidase (ZEP) in the chloroplast and (iii) the contribution of thylakoid ion transport to lumenal pH dynamics (Figure 1).

## The role of PsbS and LHCII–PSII supercomplexes in qE

The absorption of light energy by chlorophylls (Chl) results in Chl excited states. When light energy gets absorbed in excess of the photosynthetic electron and proton transfer reactions and ultimately $CO_2$ fixation (and other energy-requiring metabolic processes), Chl excited states are harmlessly dissipated as heat by the qE
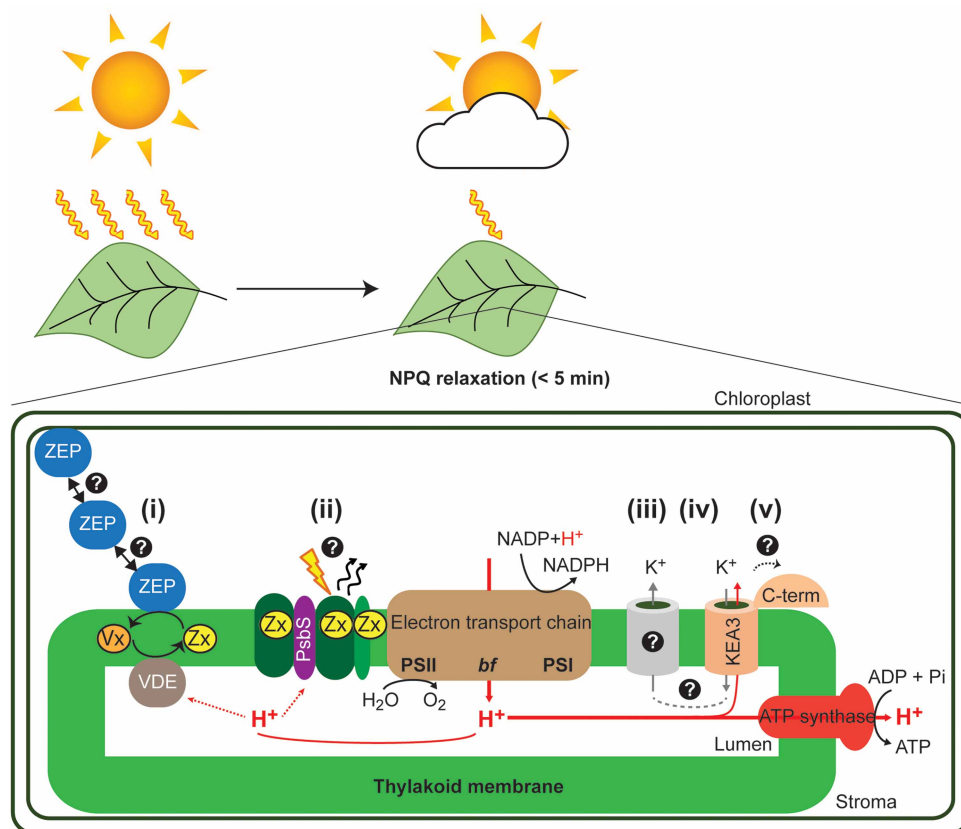
**Figure 1. Summary of chloroplast processes involved in NPQ relaxation.**

The figure summarizes known components involved in rapid NPQ relaxation and points to five main questions: (i) How is the zeaxanthin epoxidase (ZEP) distributed among the three chloroplast compartments?, (ii) How is light energy converted into thermal energy?, (iii) Which is the molecular identity of the $K^+$ channel in the thylakoid membrane?, (iv) Does KEA3 act in concert with a $K^+$ channel? and (v) How is KEA3 activity regulated? VDE, violaxanthin de-epoxidase; Vx, violaxanthin; Zx, zeaxanthin; PSII, photosystem II; *bf*, cytochrome $b_6f$ complex; PSI, photosystem I; KEA3, $K^+$ efflux antiporter 3.

mechanism. This mechanism requires a conformational change within the PSII–light harvesting supercomplexes (PSII–LHCII) [33]. These supercomplexes are composed of a dimeric oxygen-evolving PSII core complex, four trimers of the major light harvesting antenna proteins (LHCII) Lhcb1, Lhcb2 and Lhcb3, and two of each minor antenna proteins CP24, CP26 and CP29 [34]. The PsbS, a four-helix transmembrane protein from the LHC family, is essential for qE induction [35–37]. The precise localization of PsbS within the supercomplex and its mechanism during qE activation have not been completely elucidated; however, several studies provide insight, which can be summarized as follows. In the dark, inactive PsbS dimers weakly bind to the PSII–LHCII supercomplexes [38,39]. Upon illumination, lumen acidification leads to the protonation of at least two glutamate residues of PsbS at the luminal side [40,41], which may cause PsbS monomerization [39,42,43]. PsbS is activated upon proton binding and induces the reorganization of the PSII–LHCII supercomplex by interacting with Lhcb1 and Lhcb3 [39,44,45]. These interactions are predicted to be responsible for the initial quenching phase that is observed already after short illuminations of one minute or less [46,47]. Upon prolonged light exposure, violaxanthin (Vx) is converted to zeaxanthin (Zx) by the violaxanthin de-epoxidase (VDE), a process also catalyzed by low lumenal pH [10,48]. Zx accumulation correlates with an increased interaction of PsbS with minor antenna proteins, but mainly CP29 [39,45,49]. This interaction likely allows for further rearrangements within the PSII–LHCII supercomplex thereby completing the switch to a thermal dissipating state [46,47]. Altogether, PsbS can be defined as a sensor for lumenal pH, as well as a catalyzer that promotes the conformational reorganization of PSII–LHCII complexes required for qE activation. Conformational changes within the PSII–LHCII complexes occur in response to pH-induced structural changes within the

antenna proteins, which may be facilitated by protonated PsbS [50,51]. However, how this mechanism works in detail remains to be elucidated.

PsbS overexpression results in increased qE capacity and thereby reduces photoinhibition [52]. In line with this finding, qE was shown to be important for plant fitness in dynamic light environments [53], i.e. Arabidopsis plants lacking PsbS or VDE set fewer seeds when grown in the field or under controlled light fluctuations [53]. Therefore, in order to generate plants with improved fitness characteristics in the field, PsbS was overexpressed in multiple plant species with interesting and revealing results. In tobacco (*Nicotiana tabacum*), PsbS overexpression reduces stomatal conductance, resulting in increased water use efficiency [54]. This is likely due to an increase in PSII acceptor availability (i.e. more oxidized PQ pool), which serves as a signal triggering stomata closure [54], a process also referred to as stomata red light response (see the paragraph on *Red light response*). How exactly the redox state of the PQ pool determines stomatal aperture remains to be determined. However, field-grown tobacco plants that overexpressed PsbS, despite having higher water use efficiency, did not show any increase in biomass [54].

In another study, PsbS was overexpressed in *Oryza sativa* (rice). These transgenic rice plants displayed increased biomass and grain yield in the field during one growth season [55]. There may be multiple explanations for the discrepancies between the two reports including (i) the effect of PsbS overexpression on plant yield may depend on environmental conditions such as light and water availability and these may have greatly differed between the two reports and (ii) the two species tobacco and rice may respond differently to PsbS overexpression. While increases to NPQ capacity, i.e. by the overexpression of PsbS, may not constitute a robust approach to enhancing plant yield, the acceleration of NPQ induction and particularly NPQ relaxation may pose a promising alternative. Because PsbS (and its rapid function in catalyzing qE) is dependent on lumenal pH, this may be achieved by changing the kinetics of lumenal pH or pH sensitivity and reactivity of the qE components. For such approaches, it will be crucial to understand how PsbS is activated upon protonation and how the resulting conformational change elicits the activation of the qE process at the site of the PSII–LHCII supercomplex.

## The role of the xanthophyll cycle in qE and qZ

The xanthophylls Vx, antheraxanthin and Zx are oxygenated β-carotenes and mainly bound at specific sites of PSII major and minor light harvesting antenna proteins [56–58]. Like other carotenoids, xanthophylls are C40 hydrocarbon compounds. Xanthophylls are synthesized through the condensation of isoprenoids and thus have common precursors with the chlorophyll and gibberellic acid biosynthesis pathways [59]. Aside from their important role in plant photoprotection, they also serve as precursors for the plant hormone abscisic acid (ABA) [60]. Thus, xanthophyll cycle enzymes are tightly integrated into metabolic networks contributing to plant hormone biosynthesis. This is important, because any results that derive from the modulation of plant NPQ kinetics by altering the activity of xanthophyll cycle enzymes require careful interpretation as this may additionally affect plant hormone levels. Synthesis of Zx occurs from β-carotene by the carotenoid β-hydroxylase, while Ax and Vx are generated from Zx via a two-step epoxidation reaction catalyzed by the ZEP [61]. In the thylakoid-localized xanthophyll cycle, the formation of Zx from Vx occurs in response to high light or other stresses and as detailed in the previous section is required for a maximum qE response [10,36]. If Zx is produced for a prolonged time, a Zx-dependent sustained quenching component (qZ) is activated, which may be explained by the replacement of minor antenna-bound Vx with Zx [23], a process that is much slower than Vx to Zx exchange at the site of LHCII [62]. The specific role of Zx in qE is still under debate [63,64]. While Zx was first described to be part of the quenching mechanism [65,66], recent evidence suggests that Zx instead plays an indirect role as an allosteric modulator of qE by promoting antenna aggregation [45,64]. Thus, the direct molecular mechanism by which excitation energy is converted to thermal energy remains to be entirely resolved [63]. A recent report suggests that the quenching of excess light energy involves changes in carotenoid structure as the result of lumen pH-induced conformational reorganization of antenna proteins [67].

Upon transition from high to low light, reconversion of Zx to Vx is important for full NPQ relaxation [23]. This reaction is catalyzed by ZEP, which is localized at the stromal side of the thylakoid membrane [68]. In contrast with VDE, relatively little is known about the regulation of ZEP activity. However, it has been shown that high-light exposure down-regulates the xanthophyll epoxidation reaction, which suggests that ZEP is inactivated under such conditions [69]. Interestingly, a recent report identified ZEP as a 'fast turn-over' protein, which, similar to the D1 protein of PSII, has a much higher degradation rate than most other plant proteins [29,70]. In line with this report, ZEP activity may be regulated via protein stability. Because of the key role that

ZEP plays in both qE and qZ, its manipulation appears as an attractive entry point for accelerating NPQ relaxation. In a previous study, ZEP was overexpressed together with VDE and PsbS and data showed that field-grown plants generated more biomass [71]. However, as outlined above, changes to xanthophyll enzyme levels and activities may also affect plant hormone levels. Fortunately, there may be the possibility to isolate the activity of ZEP involved in NPQ relaxation from its general carotenogenic and ABA-related function. This is because for ZEP to be active in the xanthophyll cycle, it has to be localized at the thylakoid membrane [60,72], whereas its function in carotenoid and ABA biosynthesis is mainly restricted to the chloroplast envelope. Proteomics analyses of chloroplast subfractions found nearly all enzymes upstream and downstream of ZEP at the inner chloroplast envelope [73], spurring the idea that this is the prominent site for ABA and carotenoid syntheses [72]. In fractionated chloroplasts, the ZEP is partitioned about half-and-half between stroma and thylakoid membrane, with envelope levels only detectable when plants are exposed to high light [60]. The thylakoid ZEP has been shown to weakly bind to the stromal side of the membrane via hydrophobic interactions [60,74]. Thus, one possibility for locally increasing ZEP activity in the xanthophyll cycle would be through selectively manipulating its thylakoid-binding properties to achieve binding of a larger pool of ZEP to the thylakoid membrane. This would preferably occur in conditions where ZEP activity is required to rapidly down-regulate qE and qZ, such as in low-light periods. Such manipulation of ZEP activity may appear promising for accelerating NPQ relaxation, however, in order to engineer this property, we currently lack sufficient understanding of (i) how the ZEP binds to the thylakoid membrane (i.e. via interacting proteins or lipids) and (ii) which mechanisms contribute to the formation of different ZEP pools within the chloroplast. Future research is needed to characterize any posttranslational modifications and/or protein interactions, which may determine the localization of the ZEP. ZEP has been found phosphorylated [75] and can be reduced *in vitro* by the NADPH thioredoxin reductase C (NTRC, [76], see *Redox regulation of photosynthesis*). However, currently, there is no information about whether these posttranslational modifications affect ZEP binding and/or enzymatic properties.

## Overexpression of three key components of qE increases plant biomass in the field

The combined overexpression of three key genetic elements involved in qE, i.e. PsbS and the two xanthophyll cycle enzymes ZEP and VDE, increased the biomass production of field-grown tobacco plants by 15–20% [71]. These so-called VPZ transgenic plants, when analyzed under laboratory conditions, displayed accelerated NPQ relaxation kinetics and increased leaf carbon dioxide uptake after transitions from high to low light. This finding appeared as a breakthrough for work on manipulating NPQ relaxation, because it was the first to show that the acceleration of NPQ relaxation correlated with increased biomass when plants were grown under field conditions. However, several further information are needed to support the notion of Kromdijk et al. [71] that NPQ relaxation is actually causal for their observed increase in biomass: (i) Phenotypes of single or double transgenic lines (not shown in the publication). These will help evaluating (a) what is the contribution of single or double overexpression on NPQ relaxation and growth and (b) whether the speed of NPQ decay directly correlates with the biomass increase. (ii) Hormone levels of VPZ plants. It is known that the overexpression of ZEP increases whole plant levels of ABA [77]. While at high doses, ABA has inhibitory effects on plant growth, it can stimulate shoot growth if supplied at lower doses (reviewed by Humplik et al. [78]). Adding complexity, the overexpression of another carotenogenic enzyme, the lycopene β-cyclase, which acts further upstream of the xanthophyll cycle and draws from the same precursors as the gibberellin biosynthesis pathway, increases levels of the growth-promoting hormone gibberellin, proposedly via a regulatory feedback loop [79]. Thus, it remains to be investigated, whether VPZ plants show altered hormone levels and if so, whether changes in the hormonal status of these plants contribute to the observed increase in biomass. Changes on the levels of plant hormones could either be direct, through 'push or pull' effects of the overactive xanthophyll cycle enzymes (i.e. for ABA and gibberellin), or alternatively, as has been proposed recently, indirect by alterations at the levels of ROS signal production in the VPZ plants [24]. Taken together, further experiments are needed to dissect the cross-talk between photoprotection and plant hormone homeostasis via the xanthophyll cycle.

## Accelerating NPQ relaxation by speeding up luminal pH kinetics

As discussed above, qE is proportional to the proton concentration of the lumen, because both, the PsbS protein and Zx synthesis via VDE, are activated upon protonation of key residues [40,80]. Thus, a further

maybe even superior strategy for the acceleration of NPQ relaxation (when compared with the modification of qE functional components) may be realized by speeding up lumenal pH dynamics after sudden decreases in light intensity. Support for this notion comes from our studies of mutants lacking the putative $H^+/K^+$ anti-porter in the thylakoid membrane, KEA3 [81,82]. Our data show that, after a transition from high to low light, KEA3 accelerates NPQ relaxation by decreasing the fraction of *pmf* stored as ΔpH, thereby increasing $CO_2$ fixation [81]. KEA3 activity appears to be tightly regulated via a mechanism involving its C-terminus: KEA3 is inactive in high light, but becomes activated quickly (within seconds) upon transition to lower light intensities [82]. Taken together, these data clearly demonstrate that by means of KEA3 and its regulation, plants possess an intrinsic mechanism to accelerate NPQ relaxation by speeding up lumenal pH dynamics. In fluctuating light, this optimizing mechanism benefits plant growth, as seen by decreased growth of mutants lacking KEA3 when compared with wild-type (WT) specifically under this condition [82]. This prompted us to investigate whether we could speed up NPQ relaxation even further by overexpressing KEA3. This approach was carried out transiently in tobacco and stably in Arabidopsis and, during high to low-light transitions, yielded even faster NPQ relaxation kinetics when compared with control or WT, respectively [82]. The transient decrease in NPQ in leaf cells overexpressing KEA3 was accompanied by increased PSII quantum yield [82]. However, despite lowering NPQ and increasing PSII quantum efficiency during high to low-light transitions, the overexpression of KEA3 did not yield increased growth of Arabidopsis plants in fluctuating light [82]. The reasons for this may be numerous and in the following, we will outline some of the possible explanations.

## Photosynthetic or other metabolic constraints downstream of PSII independent of KEA3

An increased PSII quantum efficiency will only translate into growth, if no other reactions downstream of PSII are limiting. Hypothetically, such limitations could be species-dependent and in Arabidopsis could be constraining photosynthetic efficiency downstream of PSII or growth.

## Negative effects of high KEA3 activity on ATP production

While Arabidopsis plants with increased KEA3 levels have higher PSII quantum efficiency during high to low-light transitions, they may fail to produce the ATP levels required for increased $CO_2$ fixation. This could have multiple reasons. For example, upon a high to low-light transition, rapid proton efflux from the lumen may be causing an inversion of the membrane potential counteracting further proton efflux via the ATP synthase and thus ATP production. This scenario is supported by data from Stitt et al. [83], which show that right after a high to low-light shift, ATP levels transiently drop. Then, it is also conceivable that the overexpression of KEA3 itself negatively affects the production of ATP. This scenario is unlikely if KEA3 works reversibly by exporting protons from the lumen during high to low-light transitions and subsequently uses the generated $K^+$ gradient to import protons again. If working this way, enhanced KEA3 activity should not disturb the relative production of ATP and NADPH. The situation is different if KEA3 works in concert with a $K^+$ channel, which dissipates the KEA3-generated $K^+$ gradient. Both activities together would result in net *pmf* loss. Even if temporally separated (KEA3 on during transitions from high to low light, channel on during low to high light), an increase in KEA3 activity should negatively affect *pmf* and thus ATP production, particularly in highly fluctuating light conditions. At least four experimental reports support the presence of a $K^+$ transporting channel in the thylakoid membrane: (i) light-driven proton influx into the thylakoid lumen was shown to be accompanied by an increase in $K^+$ in the surrounding buffer and (ii) $K^+$ channel activity was independently measured by three groups in isolated thylakoids [84–86]. Additionally, a recent computational simulation of thylakoid $K^+$ channel activity underlines the role of $K^+$ flux for dissipating thylakoid membrane potential (ΔΨ) in the light [87]. Such activity is required for part of the *pmf* to be stored as ΔpH, and this in turn is necessary for the induction of qE [87]. Another function of thylakoid ion channel activity relates to minimizing charge recombinations within PSII, which are favored by high ΔΨ [25]. While an earlier report found the Arabidopsis Two-Pore $K^+$ channel 3 (TPK3) in the thylakoid membrane [88], we could recently show that TPK3 is instead targeted to the tonoplast (as was shown before [89,90]) and does not affect photosynthesis [91]. Thus, the molecular identity of the thylakoid $K^+$ channel remains to be determined.

## Activation of energy-consuming processes by KEA3 overexpression

Additionally, KEA3 may have a function outside of photosynthesis that could counteract the enhancing effects that higher KEA3 levels have on photosynthetic efficiency. In line with such an explanation, KEA3 was shown

to positively affect $Ca^{2+}$ transient generation in response to hyperosmotic stress [92]. Consequently, enhanced KEA3 levels may lower the threshold for $Ca^{2+}$ transient generation, thereby prematurely activating energy-consuming stress responses. According to this scenario, the surplus in energy generated by the enhancement of photosynthesis by overexpressing KEA3 would be consumed by costly stress responses.

Do these hypotheses for explaining the lack of growth increments in Arabidopsis KEA3 overexpressors in fluctuating light exclude KEA3 as a target for the improvement of photosynthesis? According to *hypothesis I* (i.e. photosynthetic or metabolic constraints downstream of PSII independent of KEA3), enhancing KEA3 activity may still be a viable option for improving plant growth in species that are not subjected to such constraints. If we consider faster NPQ relaxation in the VPZ tobacco plants [71] as responsible for their increased biomass, and *hypothesis I* applies in the context of species-dependency, KEA3 overexpression should result in increased biomass accumulation in tobacco (and potentially other species). Hypotheses II and III (i.e. *negative effects of high KEA3 activity on ATP production* or *activation of energy-consuming processes by KEA3 overexpression*, respectively) argue against KEA3 overexpression alone as a sustainable strategy to increase biomass production. However, these hypotheses would still allow for strategies targeted at accelerating KEA3 activation, while avoiding higher overall activity. We have collected evidence that KEA3 is specifically activated upon transitions to shade by a mechanism involving its C-terminus [82]. Plants that overexpress a KEA3 version lacking the C-terminus show reduced NPQ in high light, while plants that overexpress the KEA3 full-length version show NPQ levels similar to WT. Upon transition to low light, NPQ relaxation kinetics correlate with levels of KEA3. The KEA3 C-terminus contains the conserved $K^+$ transport nucleotide binding (KTN) domain, which is similar to the regulation of $K^+$ conductance (RCK) domain, with both constituting ubiquitous regulatory components of $K^+$ transport systems [93]. These domains have been shown to gate $K^+$ transport in response to nucleotide binding. In nearly all reported cases, the regulating nucleotide ligand contained an adenosine phosphate moiety (i.e. c-di-AMP [94], ATP [95] or NADH [96]). High-energy adenosine phosphate derivatives in the form of ATP and NADPH are the products of light-dependent photosynthetic reactions. Together with their low-energy counterparts ADP and $NADP^+$, these molecules may serve as reporters of the chloroplast energy status and trigger responses to counteract low energy supply. One of these responses may be the activation of KEA3 activity in order to increase light harvesting and thus energy input into the system. This idea is supported by the analysis of these nucleotides during high to low-light transitions, which demonstrated that (i) the ATP/ADP ratio drops transiently directly after the shift and (ii) $NADP^+$ levels rise permanently in low light [83]. Thus, KEA3 activity after a high to low light transition coincides with decreased ATP/ADP and NADPH/$NADP^+$ ratios. Understanding the mechanism by which KEA3 is regulated may allow for strategies targeted at accelerating activation and deactivation of KEA3. Such versions of KEA3 would optimally accelerate lumenal pH dynamics without increasing net ion flux.

# Redox regulation of photosynthesis

The intermediates produced by photosynthetic proton and electron transport, ATP and NADPH, are used in the CBB to regenerate the five-carbon-compound $CO_2$ acceptor molecule, ribulose-1,5 bisphosphate (RuBP). RuBP is carboxylated by the ribulose-1,5 bisphosphate carboxylase oxygenase (Rubisco), with oxygenation of RuBP as a side product. Given the strong dependence of photosynthetic electron and proton transport on light, it is not surprising that the activity of several enzymes in the CBB is light intensity regulated. Posttranslational redox regulation by the thioredoxin (Trx) system is highly conserved throughout life, but it is nowhere as complex and diversified as in chloroplasts [97].

Chloroplast redox regulation has long been considered important for plant photosynthesis in dynamic light conditions, because of its role in the light-dependent activation of CBB enzymes [98,99]. Low CBB enzyme activity in low light and slow reactivation upon shifts from low to high light can lead to a transiently reduced turnover of metabolites in the CBB, thereby reducing carbon gain in fluctuating light [100]. CBB enzymes that are subject to redox control and whose activity may be limiting photosynthesis under fluctuating light are sedoheptulose-1,7 bisphosphatase (SBPase), fructose-1,6 bisphosphatase (FBPase), phosphoribulokinase (PRK) and glyceraldehyde-3-phosphate dehydrogenase (GADPH), as well as Rubisco activase (Rca, see below). Recently, new exciting roles for Trx systems have emerged that are important for photosynthesis in fluctuating light and unrelated to CBB enzyme regulation. As there are several excellent recent reviews on the topic [97,101,102], here we only review some developments that in our view are important for photosynthesis in fluctuating light.

## Two thioredoxin systems with differing redox potentials confer flexibility for chloroplast redox regulation

In the ferredoxin (Fd)–Trx system, electrons are transferred from the primary PSI acceptor, Fd, to Trx by engaging the ferredoxin–thioredoxin reductase (FTR). Trxs then pass the electrons onto their target enzymes. As it requires the supply of electrons by photosynthesis, this system is only active in the light. Arabidopsis chloroplasts contain 10 isoforms of Trx: two f, four m, two y and each one x and z-type Trx. These isoforms have a large range of target proteins, some specific and some redundant, and are all reduced by the same FTR [103], albeit with very different efficiencies that correlate with the midpoint redox potentials of the different Trx [15].

A second system engaging the NADPH-dependent thioredoxin reductase (NTRC) combines all functions of the Trx system in one protein: it contains an N-terminal NADPH thioredoxin reductase domain which can oxidize NADPH and a C-terminal Trx domain with which it can reduce its targets. NTRC is active in low light (below ∼150 $\mu$mol m$^{-2}$ s$^{-1}$ [76,104]), when the Fd–FTR–Trx system is still inactive. Thus, it can already activate enzymes, such as the ATP synthase, the NDH complex and several CBB enzymes in shade conditions [102]. Indeed, plants overexpressing NTRC showed much faster induction of electron transport, and higher net $CO_2$ fixation in low light intensity when compared with WT [105]. These results suggest that crops with increased amounts of NTRC could grow faster under naturally fluctuating light intensities, but this remains to be shown experimentally. Whether NTRC overexpression can increase photosynthesis in fluctuating light may depend on the specific light regime [102].

The Fd–FTR–Trx system gets activated at higher light intensities. It is important for full activation of photosynthesis during the high-light phases of fluctuating light [106]. Double mutants lacking the Trx m1 and m2 isoforms, when grown in fluctuating light, have lower PSII quantum efficiency in high-light periods when compared with WT. However, in low-light periods, they show increased PSII quantum efficiency [106]. Together, this suggests that while lack of Trx m decreases photosynthetic efficiency in high light periods, it also elicits a compensatory response that results in increased light usage in low-light periods.

Together, the two Trx systems are involved in the regulation of CBB enzyme activity, ATP synthase activity, starch synthesis, the malate valve and cyclic electron flow, all of which can exert some control over photosynthesis in fluctuating light [97,101,102]. The manipulation of Trxs may thus play an important role for strategies to improve photosynthesis in dynamic light environments. However, many questions remain unanswered, particularly regarding the target selectivity of the Fd–FTR–Trx system, including (i) which are the targets of the different Trx isoforms? Although many specific Trx-target protein couples have already been identified, clearly, there are more to be found. For example, just recently, the thylakoid STN7 was revealed as a specific target of Trxm [107]. (ii) How specific is the Trx-target interaction and to what extent does redundancy exist and (iii) along these lines, can target selectivity be modified to allow for a selective over- or underactivation of specific enzymes?

## Deactivating redox-regulated enzymes upon shading: the role of thiol oxidation

Research on redox regulation has predominantly focused on the activation of chloroplast enzymes by reduction in the light, while the reverse process, their deactivation in the dark or low light by oxidation of redox-sensitive thiols has been unclear. Recently, three landmark publications [108–110] have helped in elucidating this process. Firstly, Ojeda et al. [108] demonstrated that double mutants lacking both A and B isoforms of 2-cys peroxiredoxin (2CP) showed reduced oxidation of several redox-regulated enzymes upon darkening. Concomitant with slower enzyme deactivation in the dark, these plants displayed faster induction of photosynthesis under fluctuating light, compared with WT [108,109]. *In vitro*, incubating reduced target enzymes with different Trx isoforms, 2CP and $H_2O_2$ revealed that reducing equivalents could be successfully transferred from the enzymes to Trx, then to 2CP and finally to $H_2O_2$[108,109], suggesting this to be the *in vivo* oxidation cascade. Additionally, work by Yoshida et al. [110] identified a previously unknown Trx-like protein termed TrxL2 (for Thioredoxin-like2), which has a higher midpoint redox potential (approx. −250 mV) than NTRC (−275 mV) and different Trx isoforms (between −280 and −340 mV), suggesting TrxL2 to be a very efficient oxidant. Also, TrxL2 interacted with many redox-regulated enzymes, but the only enzyme it could reduce, and do so with high efficiency, was 2CP [110]. The overall model emerging from these results was that upon darkening, enzymes are predominantly oxidized by TrxL2, which then reduces 2CP, which finally passes the
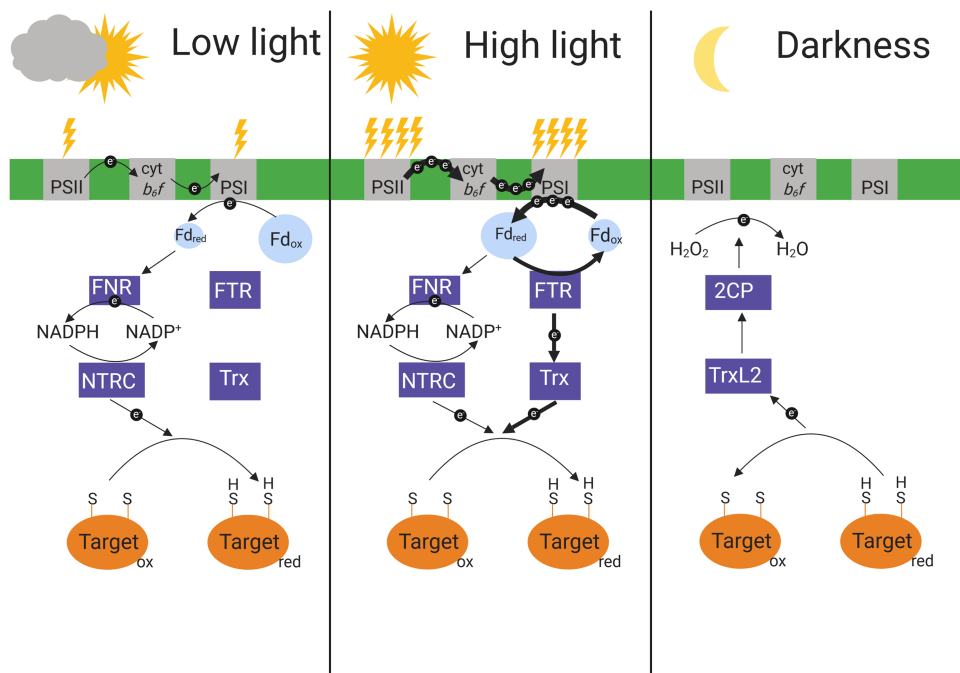
**Figure 2. Summary of chloroplast redox regulation in response to light availability.**
Under low irradiance, electrons flow to NADPH, the NTRC is activated and reduces target enzymes. Under high irradiance, reduced ferredoxin ($Fd_{red}$) is oxidized by the FTR and Trx reduces target enzymes. In the dark, reduced target enzymes are reoxidized by a cascade involving thioredoxin L2 (TrxL2) and 2 Cys-peroxiredoxins (2CP) and a final transfer of electrons to $H_2O_2$. PSII, photosystem II; cyt$b_6f$, cytochrome $b_6f$ complex; PSI, photosystem I; $Fd_{ox}$, oxidized ferredoxin.

reducing power on to $H_2O_2$ to form $H_2O$ ([110], Figure 2). This redox cascade was additionally shown to oxidatively activate the glucose-6-phosphate dehydrogenase, an important enzyme of chloroplast energy metabolism in the dark [111]. These results are clearly a breakthrough for understanding oxidative enzyme deactivation and activation upon decreases in light intensity. Also, the *2cp* mutant represents an interesting system for studying the 'fast phase' of photosynthetic induction [99]. In WT leaves exposed to low-light intensities, some of the enzymes controlling the rate of RuBP regeneration (FBPase, SBPase, PRK, GADPH) deactivate within 1–2 min and then initially limit the rate of photosynthetic induction after re-illumination [98]. In *2cp*, the deactivation of these enzymes in the shade is much slower, conferring faster induction of electron transport after re-illumination [108]. Additionally, it was shown that *2cp* plants, which grow smaller than WT under various light conditions, show similar growth as WT under highly fluctuating light, suggesting that the lack of 2CP benefits photosynthesis in dynamic light environments [109]. However, how exactly the lack of 2CP positively affects photosynthesis in fluctuating light and whether this is dependent on changes in the RuBP regeneration activation state remains to be determined.

# Rubisco activation

The enzyme central to photosynthetic $CO_2$ fixation, Rubisco, is a large protein complex with eight active sites. Each active site requires carbamylation (binding of $Mg^{2+}$ and $CO_2$) at a lysine residue to be catalytically competent. In the absence of carbamylation, RuBP or other sugar phosphates can bind tightly to the uncarbamylated active sites and inhibit their activity [112]. In low light, Rubisco activity decreases slowly due to inhibitor binding at active sites, and reaches a species-dependent residual activation state of 30–50% [113]. The binding of inhibitors in low light may be a protection mechanism to prevent Rubisco degradation [112]. Reactivation of inhibited Rubisco active sites is facilitated by the ATPase Rubisco activase (Rca), which removes inhibitory sugar phosphates [114]. The action of Rca is important to uphold Rubisco activity in the light, but also for reactivation after phases of low light or darkness. Upon shifts from high to low-light intensities, Rca activity decreases, probably due to the formation of higher-order Rca oligomers, which inactivate the enzyme and may

act as its storage form [115]. When light intensity increases, Rca is reduced by the Fd–FTR–Trx system and gradually reactivates Rubisco, a process that takes several minutes and therefore limits photosynthetic $CO_2$ fixation under fluctuating light [113].

In many plants, two Rca isoforms exist: one longer α-isoform (46–47 kDa) and one shorter β-isoform (41–43 kDa). Only the α-isoform is redox-regulated and in species containing both isoforms (most species tested so far), the α-isoform regulates the activation state of the β-isoform. Intriguingly, there is large interspecific variation for Rca isoforms: in Arabidopsis, spinach and rice, both isoforms are produced from an identical gene by alternative splicing whereas in maize, cotton and barley, two separate genes exist for the two isoforms [116,117]. Furthermore, in tobacco, only the β-isoform exists, but it is encoded by three separate genes; for a more thorough discussion on Rca isoform distribution, see [118]. Why there is such a large diversity for Rca isoform composition, as well as for the genes that encode these isoforms, is unclear. Also, it remains to be tested whether the diversity in Rca isoform distribution can be used to increase crop photosynthesis in fluctuating light.

Two strategies for manipulating Rca to improve dynamic photosynthesis have been proposed: eliminating Rca redox regulation and changing the Rca/Rubisco ratio. As for the first strategy, in *rwt43*, transgenic Arabidopsis plants lacking the α-isoform, the β-isoform was not redox-regulated and Rca activation state therefore remained high in low light [119]. Photosynthetic induction after low to high-light transitions was faster in *rwt43* compared with WT [113,120]. Interestingly, light use efficiency was higher in *rwt34* plants growing under fluctuating light than in *rwt43* growing under constant light, while the opposite was demonstrated for WT. However, a functional α-isoform appears to be critical for optimal growth (at least in Arabidopsis), as *rwt34* plants were smaller than WT plants under either constant or fluctuating light [118].

The second strategy is based on the idea that there is a tradeoff for protein allocation between Rubisco and Rca: Rubisco makes up 25–40% and ∼14% of total soluble protein in $C_3$ and $C_4$ plants, respectively [118,121,122], while Rca accounts for only 1–2% of total soluble protein [123,124]. Sacrificing small amounts of Rubisco could thus dramatically increase Rca concentrations and may speed up the rate of photosynthetic induction, but this might reduce photosynthesis under constantly high light [125]. A combination of novel *in silico* studies, using realistic wind-induced leaf movement [126], detailed modeling of photosynthesis dynamics as well as evolutionary algorithms that can predict optimal nitrogen distribution across generations may improve our understanding of optimal Rca/Rubisco ratios. Additionally, to what extent acclimation to different environments affects the Rca/Rubisco ratio is not known.

Genetic variation may be another modulator of the Rca/Rubisco ratio: a recent study in wheat found that Rubisco activation kinetics (measured as the time needed to reach 95% of steady-state carboxylation capacity, $V_{cmax}$) varied roughly twofold in the 10 cultivars tested (5.2–9.5 min; [127]). Whether this variation in $V_{cmax}$ induction times is caused by variation in Rca or Rubisco concentrations remains to be shown, as this was not determined. Also, model calculations suggested that accelerating $V_{cmax}$ induction could increase diurnal $CO_2$ fixation by at most 3.4% [127]. While this does not seem like a large increment per day, when integrated over a whole growing season, this may cause a large increase in biomass. Further investigations into the natural variation in Rubisco activation dynamics therefore seem promising.

## Stomatal conductance and light

Stomata are microscopically small pores on the leaf surface that dynamically balance the influx of $CO_2$ into and the efflux of water vapor out of the leaf. A stoma is formed by two guard cells, which can increase or decrease their turgidity and thereby change the size of the stomatal pore. Stomata continuously balance $CO_2$ supply for photosynthesis against water loss, which for most species and conditions necessitates that they adjust their aperture depending on a large number of environmental factors (e.g. light intensity, light spectrum, humidity, temperature, soil salinity) and intrinsic cues (e.g. photosynthetic electron transport, leaf and plant water status). Stomata typically close in the shade and open upon high-light exposure. Stomatal opening in the light is achieved through import of $K^+$ (using malate$^{2-}$ as counterion) into the guard cells, with subsequent water import leading to guard cell swelling and an increase in stomatal pore size. Given that (i) guard cells contain chloroplasts whose interaction with light plays a major role in regulating stomatal pore width and (ii) stomatal opening has a strong effect on $CO_2$ fixation under fluctuating light [128], here we review the responses of stomata to light. Two separate responses to light are known, one based on light intensity ('quantitative' or 'red light' response) and one solely responding to the presence of blue light (blue light response; reviewed in [129,130]).

## Red light response

When exposed to light not containing blue light, stomatal conductance to water vapor ($g_s$) increases near-linearly up to intensities exceeding full sunlight, a response that has been termed 'red light' or 'quantitative'. The driver for the red light response has been debated heavily, as it was often argued that it must be related to the rate of net $CO_2$ fixation, but several papers showed this assumption to be inconsistent with experimental results (reviewed in [130]). Currently, the tissue-specific origin of the 'red light signal' is still unclear. However, recently Busch [131], after a critical review of published data, suggested that the stomatal red light response correlated strongly with the reduction state of the plastoquinone pool and thus the $Q_A$ redox state. $Q_A$ is the primary acceptor of electrons from PSII and its availability is expressed by the chlorophyll fluorescence parameter qL [132].

Support for the claim by Busch [131] recently came in the form of experimental and modeling data. When using transgenic tobacco with a range of PsbS concentrations (which strongly affected the $Q_A$ redox state), PsbS antisense plants showed an increase in 1-qL and $g_s$, whereas PsbS overexpressors showed the opposite ([54], see also the section *The role of PsbS and LHCII–PSII supercomplexes in qE*). The same study demonstrated a strong linear correlation between qL and $g_s$ across genotypes and light intensities, while no such correlation between net $CO_2$ fixation and $g_s$ exists [54]. The same authors recently demonstrated that including qL as a factor significantly improved model-based estimations of $g_s$ [133]. While these results are potentially exciting for phenotyping and modeling purposes, from our viewpoint, two major questions remain to be addressed: (i) given that qL changes quickly after a change in light intensity and this does not instantaneously translate to $g_s$ [134], how long do changes in qL have to persist in order to elicit changes in $g_s$? (ii) —and this may help understand the timeframe of signal transduction from qL to $g_s$—which molecular processes are involved in the signaling cascade?

## Blue light response

Provided there is sufficient red background light, stomata strongly increase their pore size in response to blue light. Unlike the red light response, the blue light response saturates at low blue light intensities and is independent of photosynthesis [135]. Blue light is sensed by the phototropins 1 and 2, which start a signaling cascade that ultimately leads to guard cell swelling and stomatal opening [129]. At the beginning of the photoperiod, it is blue light that specifically triggers the breakdown of starch granules in guard cell chloroplasts of Arabidopsis (within 30 min) [136]. This appears to be important for increasing stomatal opening by providing (i) malate to serve as a counterion for $K^+$ uptake, and (ii) sugars to increase guard cell osmotic potential [137].

Recently, the expression of an additional, light-induced $K^+$ channel in Arabidopsis guard cells accelerated stomatal opening under blue light, as well as stomatal closure upon shading, conferring a growth advantage under low light that fluctuates slowly [138]. This result is potentially very exciting, as increasing the kinetics of stomatal opening and closure—instead of $g_s$ as such—has been predicted to lead to strongly improved light- and water use efficiencies in the field [139]. However, given that the plants in Papanatsiou et al. [138] were grown under relatively low-light intensity, and that increases in plant growth were observed under conditions where illumination was switched slowly between two relatively low intensities (10 and 150 $\mu$mol m$^{-2}$ s$^{-1}$, every 60 min), it remains to be investigated whether results can be translated to crops grown in the field. Indeed, stomata of climate-chamber grown WT Arabidopsis plants in this study [138] showed relatively slow opening and closure (half-times of 25–40 min), which is reportedly much slower than that of many crop species grown under natural light conditions (~2–10 min [140]). Further experiments including the introduction of the light-induced $K^+$ channel into crop species and growing such transgenic plants under controlled conditions of strongly fluctuating light are needed to predict the effect that the synthetic $K^+$ channels may have on stomatal kinetics and photosynthesis under field conditions. Alternatively, such transgenic plants could be analyzed directly under field conditions.

Since the blue light response of stomatal opening saturates at light intensities <1% of full sunlight, it has been suggested to be important for predawn stomatal opening, and increasing stomatal aperture in shaded conditions, which in both cases would contribute to the rapid uptake of $CO_2$ once leaves are exposed to full sunlight [141,142]. However, at least to our knowledge, these hypotheses have not been verified and the ecophysiological role of the stomatal blue light response has not been demonstrated conclusively.

While the modulation of red and blue light responses to increase stomatal opening in low light may be a strategy to accelerate full photosynthesis induction upon transition to high light, such a strategy may be

disadvantageous under water-limited conditions. Also, there are several possible explanations for why nature has not already devised faster stomatal responses [139]: (i) in line with our arguments: in a quickly fluctuating light environment, the rapid closure of stomata upon shading would greatly limit $CO_2$ fixation during subsequent high-light periods, i.e. leaves closing their stomata slowly upon shading should be able to use subsequent periods of high light more efficiently [98]. (ii) Leaf temperatures can change strongly in fluctuating light, and evaporative cooling due to larger $g_s$ may be in place to buffer temperature fluctuations that may otherwise negatively affect photosynthesis [143]. (iii) Active stomatal movement requires a change in guard cell osmotic potential, which is energy costly [144] and in a fluctuating environment, instantaneously responding stomata may be consuming high amounts of energy, which may be too costly for plant metabolism [145].

## Summary and future directions

The aim of this review was to (i) summarize key molecular mechanisms contributing to photosynthesis in dynamic light environments, (ii) reveal gaps in our understanding of dynamic photosynthesis and (iii) discuss existing strategies targeted at improving photosynthesis in the field. For the latter point, two major conclusions can be drawn: firstly, previous attempts to increase dynamic photosynthesis by overexpressing or removing a singular gene have not succeeded in enhancing plant biomass production (as shown for overexpressing PsbS [54], KEA3 [82], NTRC (as discussed in [102]) and removing the Rca α-isoform [113,120]). Secondly and very importantly, processes involved in dynamic photosynthesis tightly interact with other physiological processes. This requires for the future that results, obtained in the context of improving photosynthesis and plant yield, are carefully discussed and integrated into whole plant physiology. Accordingly, rather than changing levels of proteins involved in dynamic photosynthesis, we propose adjusting proteins to activate/deactivate more rapidly in response to light intensity changes, as a promising strategy to improve field photosynthesis. In line with topics discussed in this review, examples for potential target processes include the ZEP activity in the xanthophyll cycle, LHCII–PSII supercomplex reorganization, KEA3-dependent lumenal pH dynamics (Figure 1), the CBB cycle and stomatal aperture. However, currently, we are still lacking important information necessary for the modulation of the kinetics of these processes.

So, how can we fill these gaps in knowledge? One great opportunity lies in exploiting genetic variation. Nature very likely has already devised mechanisms, by which the dynamic responses can be modulated. Already within the Arabidopsis species, photosynthesis acclimation to high light differs greatly between accessions [146]. Much more differences are expected to emerge by cross-species comparisons. Thus, a detailed characterization of photosynthetic responses across diverse species under multiple dynamic light environments is warranted. Such approaches, combined with the mapping of genes that are responsible for phenotypic variation, appear highly promising for increasing our understanding of dynamic photosynthesis.

Furthermore, there may be additional regulatory signals generated in response to light intensity changes (apart from lumenal pH and redox) that have previously been overlooked. For example, KEA3-dependent lumenal pH dynamics may be regulated via ATP/ADP or $NADPH/NADP^+$ binding. It appears plausible that levels of these nucleotides serve as a signal for the energy status of the chloroplast and elicit compensatory responses if needed by directly binding to target proteins. However, whether KEA3 or any other components of photosynthesis are regulated directly via interaction with ATP, ADP, NADPH and/ or $NADP^+$ remains to be determined.

### Abbreviations

2CP, 2-cys peroxiredoxin; ABA, abscisic acid; CBB, Calvin–Benson–Bassham cycle; Chl, chlorophyll; FBPase, fructose-1,6 bisphosphatase; Fd, ferredoxin; FTR, ferredoxin–thioredoxin reductase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; gs, stomatal conductance to water vapor; KEA3, $K^+$ efflux antiporter; LHCII, light harvesting complex II; NPQ, non-photochemical quenching; NTRC, NADPH-dependent thioredoxin reductase; *pmf*, proton motive force; PQ, plastoquinone; PRK, phosphoribulokinase; PsbS, PSII subunit S; PSII, photosystem II; qE, energy-dependent quenching; qI, photoinhibition; qZ, zeaxanthin-dependent quenching; Rca, Rubisco activase; Rubisco, ribulose-1,5 bisphosphate carboxylase oxygenase; RuBP, ribulose-1,5 bisphosphate; SBPase, sedoheptulose-1,7 bisphosphatase; TPK3, two-pore $K^+$ channel 3; Trx, thioredoxin; Vx, violaxanthin; WT, wild type; ZEP, zeaxanthin epoxidase; Zx, zeaxanthin.

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## Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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