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Review

Dark complexes of the Calvin-Benson cycle in a physiological perspective

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ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK) are two enzymes of the Calvin Benson cycle that stand out for some peculiar properties they have in common: (i) they both use the products of light reactions for catalysis (NADPH for GAPDH, ATP for PRK), (ii) they are both light-regulated through thioredoxins and (iii) they are both involved in the formation of regulatory supramolecular complexes in the dark or low photosynthetic conditions, with or without the regulatory protein CP12. In the complexes, enzymes are transiently inactivated but ready to recover full activity after complex dissociation. Fully active GAPDH and PRK are in large excess for the functioning of the Calvin-Benson cycle, but they can limit the cycle upon complex formation. Complex dissociation contributes to photosynthetic induction. CP12 also controls PRK concentration in model photosynthetic organisms like *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*. The review combines *in vivo* and *in vitro* data into an integrated physiological view of the role of GAPDH and PRK dark complexes in the regulation of photosynthesis.

1. Introduction

The carboxylating activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) depends on two substrates with different origin: CO_2 that diffuses from the atmosphere into the leaves through stomata, and ribulose-1,5-bisphosphate (RuBP) that is synthesized by Calvin-Benson (CB) cycle enzymes starting from 3-phosphoglycerate (PGA), the product of Rubisco itself. In C_3 plants in particular, the oxygenating activity of Rubisco competes with its carboxylating activity and generates besides PGA, a different acid (2-phosphoglycolate, 2PG). The 2PG is converted to PGA with consumption of energy (ATP) and reducing power (NADPH) in the photorespiratory pathway. Whichever its origin, the conversion of PGA into RuBP requires further ATP and NADPH molecules provided by thylakoid reactions in the light. The net output of the CB cycle plus photorespiration are sugar-phosphates that can leave the cycle for further use once the substrate of Rubisco has been properly reconstituted (Fig. 1).

The conversion of PGA into RuBP includes a *reduction phase*, catalyzed by phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and a *regeneration phase*, in which 8 enzymes catalyze 10 reactions, starting from glyceraldehyde-3-phosphate (GAP) and ending with RuBP. In land plants, Rubisco and four additional enzymes of the PGA to RuBP conversion (*reduction* plus *regeneration*) are activated by light and transiently inactivated in the dark, namely GAPDH, fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase) and phosphoribulokinase (PRK) [1]. The regulation of GAPDH and PRK is peculiar because it is both autonomous and mediated by the chloroplast protein 12 (CP12), a non-enzymatic protein that coordinately regulates GAPDH and PRK by assembling a complex in which both enzymes are temporarily inactivated [1–3].

The dependence on light of the CB cycle is therefore twofold. First, because light reactions generate the NADPH and ATP required by the cycle; second, because light provides the conditions for the activation of the regulated enzymes of the cycle that in this way can express their full activity [1,4]. Both types of light dependence of the CB cycle are mediated by the photosynthetic electron transport. Chloroplast ATP-synthase, which is responsible for the chemiosmotic production of ATP for the CB cycle and photorespiration, needs also to be activated by light [5] (Fig. 1). Moreover, photosynthetic metabolism is also controlled by the circadian clock, besides being directly regulated by light [6,7]. However, underlying mechanisms are not yet fully understood and will not be covered in this review.

Light activation of ATP synthase and CB cycle enzymes is in general a

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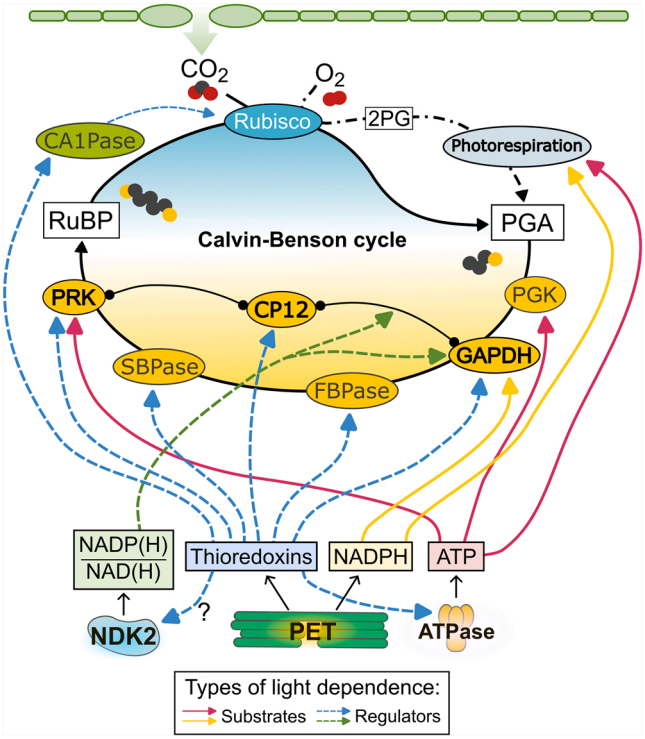

Fig. 1. The twofold light-dependence of the CB cycle. The reactions converting PGA to RuBP are regulated and influenced by several endogenous and exogenous stimuli. While stomata conductance controls the concentration of CO_2 in leaves, light exerts a twofold control *via* substrates production (full arrows: ATP and NADPH) and production of reduced thioredoxins and activators (NADP(H):NAD(H) ratio)(dotted arrows). Thioredoxins activate A_2B_2-GAPDH, CP12, PRK, FBPase, SBPase, CA1Pase, γ-subunit of ATPase and possibly NDK2 [78] by reducing inhibitory disulfide bonds [30].

slow process, slower than the light response of the photosynthetic electron transport. During a dark to light transition this contributes to the lag observed in reaching the steady state photosynthesis (photosynthetic induction) [8]. Steady state photosynthesis shows a typical response to variations of light intensity at constant (*e.g.* ambient) CO_2 concentration. At low light intensities photosynthesis tend to be limited by the capacity of the system to generate RuBP for Rubisco, while at high light intensities photosynthesis becomes limited by CO_2, the second substrate of Rubisco [9].

This review is focused on the role played by GAPDH and PRK in the generation of RuBP from PGA, and in the potential role played by the coordinated regulation of GAPDH and PRK in the general regulation of photosynthesis. These two enzymes exemplify the concept of the dual light-dependency of the CB cycle: they both need light for activity (NADPH for GAPDH, ATP for PRK) and both need light for activation through reduced thioredoxins (TRXs) and high NADP(H)/NAD(H) ratios [1] (Fig. 1). An effort was made to combine *in vitro* and *in vivo* evidence in an integrated physiological view.

2. GAPDH, PRK and the limitation of photosynthesis by RuBP

GAPDH and PRK catalyze the second and the last step of the PGA to RuBP conversion. These two enzymes are finely regulated, either independently or in a coordinated way by means of CP12 [3]. Thanks to their position in the cycle, and to their regulation, GAPDH and PRK have thus the potential to control photosynthesis by determining the rate of RuBP regeneration in the CB cycle (Fig. 1).

The problem of whether single CB cycle enzymes could control, and to which extent, the flux through the pathway was addressed by studying mutant plants obtained by antisense technology or RNA interference [10–12]. These approaches allowed obtaining a set of transgenic plants that contained variable levels of single enzymes under study. The flux through the pathway, *e.g.* the CO_2 assimilation rate, could then be correlated to the maximal activity of the enzyme, which was measured in raw extracts obtained from the different transgenic plants [10]. Except for ribulose-5-phosphate 3-epimerase and ribose-5-phosphate isomerase, all CB cycle enzymes have been investigated with this approach in several species, especially tobacco [10] and rice [11,12]. Among the enzymes of the PGA to RuBP conversion, transketolase (TK), fructose-1,6-bisphosphate aldolase (FBA/SBA), and SBPase were found to exert the strongest control on the pathway, meaning that relatively small reductions of these enzymes caused significant reductions of CO_2 assimilation [13–15]. These enzymes are considered promising targets to enhance photosynthetic efficiency following their overexpression. Both FBA/SBA and SBPase were confirmed by several studies to have positive effects on photosynthesis and growth in those species in which they have been overexpressed [16].

The other enzymes of the PGA to RuBP conversion pathway that have been analyzed in the same way, including GAPDH and PRK, show under similar conditions, lower control than TK, FBA/SBA and SBPase on CO_2 assimilation [10]. Nevertheless, these studies are informative. In tobacco plants with chloroplast GAPDH activity ranging from 10 % to 100 % of wild type levels, CO_2 assimilation was little affected unless GAPDH activity was reduced to less than 35 % of wild type levels (Fig. 2A) [17,18]. Similar results were obtained both in ambient and high CO_2 concentrations [18]. Interestingly, GAPDH exerted a strong control on RuBP regeneration (Fig. 2C), but as long as RuBP was sufficient to saturate Rubisco, CO_2 assimilation was not affected [17,18]. In other words, GAPDH of wild type tobacco plants appeared to be largely in excess compared to the needs of the CB cycle under tested conditions. Only dramatic reductions of GAPDH activity could cause RuBP to drop at levels that reduced Rubisco activity [17]. As a result, photosynthesis in tobacco plants with 7 % GAPDH activity was strongly inhibited over a wide range of light intensities (Fig. 2D) [17,18].

Analogous experiments conducted with PRK gave similar results (Fig. 2B,D,F) [19–21]. Only plants with residual levels of PRK below 10 % of wild type levels were affected in CO_2 assimilation (Fig. 2B), apparently because RuBP dropped below the threshold of Rubisco saturation (Fig. 2D). By testing both GAPDH and PRK antisense plants at varying light intensities it was apparent that neither GAPDH nor PRK could limit photosynthesis at very low light (Fig. 2E,F). Under these conditions, photosynthesis is limited by RuBP and the regeneration of RuBP is limited by the rate of the electron transport [9]. Even low levels of GAPDH and PRK are enough to cope with the slow production of ATP and NADPH by thylakoid reactions. Conversely, CO_2 assimilation was dramatically reduced in both GAPDH and PRK-antisense plants exposed to high light intensities (Fig. 2E,F). This reduction resulted from the fact that neither GAPDH nor PRK could match the rate of electron transport. A new type of RuBP limitation, in this case dependent on CB cycle enzymes and not on electron transport, thus came into play [17–19,21].

Both GAPDH and PRK thus appear to be in excess for the CB cycle flux. Careful determination of CB cycle intermediates and enzymes in illuminated *Chlamydomonas* cells show that neither GAPDH nor PRK are saturated by their respective substrates 1,3-bisphosphoglycerate (BPGA) and ribulose-5-phosphate (Ru5P) [22,23]. GAPDH is indeed 10-fold more concentrated than BPGA, and PRK, although less concentrated than Ru5P, has a K_m for Ru5P exceeding the typical Ru5P concentrations in the light (Tables 1 and 2) [23]. Based on these considerations, any increase of GAPDH and/or PRK activities above wild type levels is not predicted to stimulate the CO_2 assimilation rate.

Experimentally, this prediction was confirmed in rice [24]. In this specie, the combined over-expression of A and B subunits of GAPDH (see Box 1) caused a 4-fold increase of enzyme activity with no effects on CO_2 assimilation under normal CO_2 conditions. A very limited increase (*ca.* 10 %) was only observed when photosynthesis was tested at very high

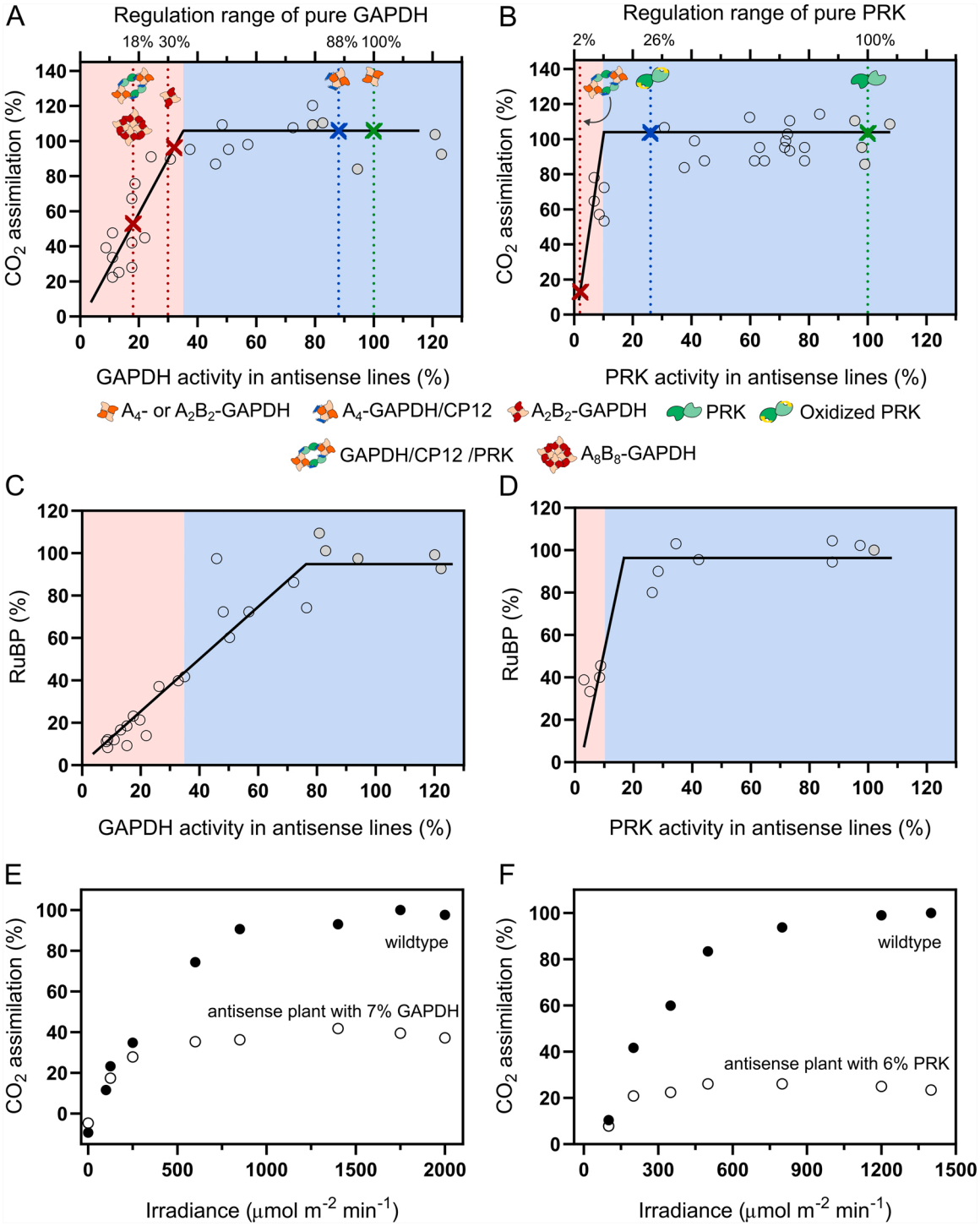

Fig. 2. Control of CB cycle by GAPDH and PRK: relevance of inhibitory complexes. Panel A: CO_2 assimilation as a function of maximal GAPDH activity in antisense plants (lower x-axis). Experimental data and manual fitting are taken from [17]. Empty circles represent antisense lines expressing different levels of GAPDH normalized on wild type plants (grey circles). Red background defines the range in which GAPDH activity controls the CB cycle. Blue background shows activity range not affecting the CB cycle. The upper x-axis represents the activity of GAPDH in the different association states [29,43] showing that 100 % maximal GAPDH activity in plants (lower x-axis) may correspond to different actual activities depending on regulation (upper x-axis). Different regulatory states are represented as cartoons. Panel B: as in (A) but referred to PRK and based on data taken from [19]. Panel C and panel D as panel A and panel B, respectively, except for RuBP concentration substituting CO_2 assimilation in the y-axis [17,19]. Panel E and panel F show CO_2 assimilation in two antisense plants with minimal GAPDH (E) and PRK (F) activity [15,19]. All data were redrawn and expressed as percentage of wild type plants (grey or black circles).

CO_2 (800–1200 ppm; [24]). Similar experiments have not yet been performed on PRK. Quite surprisingly, however, transgenic overexpression of CP12 in the tropical leguminous plant *Stylosanthes guianensis,* was found correlated with increased CO_2 assimilation, increased GAPDH and PRK activities, and chilling stress resistance [25]. To our best knowledge, this is the first and only report in which an increase in GAPDH and/or PRK activities correlates with an increase in productivity (Box 2 and 3).

Table 1
Biochemical parameters of GAPDH.

Species	K_M (NADPH) [μM]	K_M (NADH) [μM]	K_M (BPGA) [μM]	V_{max} [μmol min^{-1} mg^{-1}]	E'_m [mV]
Arabidopsis thaliana (A_4)	29 [104]	140 [104]	15 [104]	-	-
Spinacia oleracea (A_2B_2)	19 [105]-50 [38]	-	20 [38]	13 [105]	-293 [38]
Sinapis alba (A_2B_2)	23 [106]	300 [106]	-	-	-
Licopersicum esculentum (A_2B_2)	-	-	-	-	-290 [107]
Spinacia oleracea[a] (B_4)	30 [72]	-	15 [72]	-	-287 [72]
Chlamydomonas reinhardtii	23[b] [88]	128[b] [88]	-	-	-
Synechococcus PCC 7942	62 [108]	420 [108]	-	150 [108]	-
Thermosynechococcus elongates	40 [83]	37 [83]	-	-	-
Median value	**29**	**138**	**15**	**144**	**-290**

[a] Recombinant form.
[b] Mean value from [88].

Table 2
Biochemical parameters of PRK.

Species	$K_{M\ (Ru5P)}$ [μM]	$K_{M\ (ATP)}$ [μM]	V_{max} [μmol min^{-1} mg^{-1}]	E'_m [mV]
Spinacia oleracea	220 [109]	280 [109]	410 [110]	-290 [73]
Pisum sativum	170 [111]	69 [111]	-	-
Arabidopsis thaliana	55 [37]	56 [37]	212 [37]	-270 [29]
Triticum aestivum	65[a] [112]	70 [112]	588 [112]	-
Licopersicum esculentum	-	-	-	-255 [107]
Chlamydomonas reinhardtii	56 [113]–87.5 [58]	33.8 [113]–62 [58]	465 [113]	-273 [56]
Heterosigma carterae[b]	226 [114]	208 [114]	218 [114]	-
Odontella sinensis	118 [115]	84 [115]	300 [115]	-257 [115]
Synechococcus PCC7942	270 [116]	90 [116]	72.6 [117]–231 [118]	-
Thermosynechococcus elongatus	-	40 [83]	-	-
Median value	**118**	**70**	**300**	**-270**

[a] Mean value from [112].
[b] Partially pure enzyme [114].

As far as chilling sensitivity is concerned, the limited activation of regulated enzymes of the PGA to RuBP regeneration pathway was proposed to be the cause of chilling sensitivity in warm-climate plants [26]. Diminished levels of CP12 correlate with lower levels of PRK in both *Arabidopsis* [27] and *Chlamydomonas* [28] even at normal temperatures (see Section 5), and it is possible that a protective role exerted by CP12 on PRK may help understanding the results with the warm-climate adapted plant *Stylosanthes guianensis* [25]. More generally, these experiments suggest that the role of CP12 (and PRK) in plants overexpressing these proteins might deserve further studies, with a focus on productivity under normal and chilling stress conditions.

GAPDH and PRK may control photosynthesis only when their maximal activity is reduced below 35% and 10% of wild type levels, respectively. For non-regulated enzymes, dramatic reductions of activity can result from varying transcription, while post-translational regulation can give rise to the same effects in regulated enzymes. *In vitro*, GAPDH and PRK loose about 80 % and 98 % of their maximal activity, respectively, when they are associated in a ternary complex with CP12 [29]. This dramatic drop of activity suggests that both GAPDH and PRK have the potential to post-translationally control the CB cycle. Not surprisingly, PRK-antisense plants were found to spontaneously compensate for the reduction of maximal PRK activity by strongly increasing its activation state [20].

3. GAPDH and PRK regulation, photosynthetic induction and light/dark regulation of the CB cycle

Light activated enzymes of the CB cycle exist in multiple states, each with a different catalytic activity, and light can indirectly affect the balance between the different forms. A common mechanism of light activation is mediated by TRXs [1]. In the light, specific TRXs like the subtype TRX f [30,31] transfer electrons derived from the linear electron transport to the disulfide bonds of target enzymes. For CB cycle target enzymes, reduction generally equals to activation. In the dark, other TRXs (*e.g.* thioredoxin-like 2 and atypical Cys His-rich thioredoxin, ACHT) do the opposite by transferring electron pairs derived from the dithiols of target enzymes to 2-Cys peroxiredoxin and hence hydrogen peroxide [32–34]. Rubisco has its own peculiar mechanisms of activation based on the carbamylation of a specific lysine in the active site. As such, Rubisco is insensitive to thioredoxins, but two proteins regulating Rubisco activation, namely Rubisco activase and carboxyarabinitol 1P phosphatase (CA1Pase) are, in some specie at least, regulated by thioredoxins through dithiol/disulfide interchange reactions [1,30]. However, the light activation of the CB cycle cannot be explained in terms of thioredoxins alone, as other physiological factors concur to the regulation of the whole process. These factors include the light shifts of stromal pH, magnesium concentration, and the change in concentration of crucial metabolites, including nucleotides (see Section 3.5) [4].

Upon a dark to light transition, the photosynthetic electron transport is immediately activated and responds to light much quicker than the CB

Box 1
GAPDH

GAPDH is represented by 4 different isoforms in oxygenic photosynthetic organisms. The ancestor of all GAPDH isoforms is the NAD-specific isoform C, found in eubacteria and in eukaryotes' cytoplasm, and it is involved in the glycolytic pathway [98]. The isoform A instead is bispecific for NADP(H) and NAD(H) and takes part in the CB cycle. A-GAPDH is expressed in cyanobacteria, algae and land plants [3]. The third isoform, B-GAPDH, is involved in the CB cycle too and evolved from A-GAPDH and CP12. In this way, B-GAPDH acquired 2 regulatory cysteines conferring autonomous redox regulation [3]. The fourth isoform derived from C-GAPDH but is localized in plastids, and is termed Cp-GAPDH [98]. GAPDH always forms homotetramers (C_4-, A_4-, Cp_4-GAPDH) except for B subunits that are combined with A subunits (A_2B_2 and A_8B_8).

In the CB cycle, GAPDH catalyzes the reduction of BPGA to glyceraldehyde-3-phosphate, consuming NADPH and releasing inorganic phosphate. A_4- and A_2B_2-GAPDH show comparable affinity for BPGA (Table 1) and bispecificity for pyridine nucleotides with preference for NADPH (Table 1).

Box 2
PRK

PRK is the last enzyme of the CB cycle, ultimately responsible for the regeneration of the substrate of Rubisco, RuBP. PRK is found in pathways that use Rubisco to fix CO_2, *i.e.* the CB cycle common to cyanobacteria, algae and land plants, but also autotrophic proteobacteria; and novel pathways like the reductive hexulose-phosphate pathway found in Archaea [99,100]. Evolution in different cellular environments gave rise to three PRK types: the plant type, found in the Plantae kingdom, in cyanobacteria and in photosynthetic protists; the bacterial type, widespread in proteobacteria and α-cyanobacteria, and the archaeal type [81,101,102]. The GAPDH-CP12-PRK complex is found in organisms coding for plant type PRKs.

PRK catalyzes the phosphorylation of Ru5P on carbon-1 producing RuBP. The binding of substrates is ordered, with Mg^{2+}-ATP binding first [57]. Kinetic parameters have been measured in many organisms (Table 2). Optimal pH values for catalysis are around 8 [56].

Box 3
CP12

All organisms performing oxygenic photosynthesis harbor CP12 genes, from cyanobacteria to land plants [98]. A single gene is found in algae and cyanobacteria, while in angiosperms CP12 genes form a small family. Canonical CP12 contains two N-terminal and two C-terminal disulfide-forming cysteines and a consensus sequence AWD_VEEL, which is important for PRK binding [57,83].

CP12 is classified as a conditionally disordered protein [103] that adopts an ordered conformation upon binding with GAPDH and PRK [57,60,83,98]. The two disulfides have different standard redox potential at pH 7.0 (E_m'). Once the C-terminal disulfide is formed (E_m' − 299 mV), CP12 can interact with GAPDH forming a binary complex. When the environment becomes more oxidizing, the N-terminal disulfide (E_m' − 273 mV) promotes the binding between CP12/GAPDH and PRK [61]. While this happens in land plants, non-canonical CP12 deprived of N-terminal cysteines (*e.g.* in Cyanobacteria), can still form ternary complexes demonstrating that the N-terminal disulfide may be dispensable [80].

cycle, which usually requires several minutes to reach its steady state activity [30]. This typical behavior of photosynthetic systems is called photosynthetic induction. During photosynthetic induction, the photosynthetic electron transport is limited by the slowness of the CB cycle, and non-photochemical quenching mechanisms must be transiently activated to relax the system. The CB cycle intermediates are slowly built up, thioredoxins get reduced and light-activated enzymes slowly recover their maximal activity potential by shifting form dark-adapted inhibited forms to fully active conformations. All together, these modifications constitute the biochemical limitation of photosynthetic induction which is usually overcome in several minutes after the onset of light. The second type of limitation depends on stomata that constitute the major resistance to CO_2 diffusion. Stomata opening is also regulated by light, though with mechanisms that are largely different from those activating the CB cycle in mesophyll cells [35]. Stomata limitation is a slower component of photosynthetic induction and usually requires longer times than biochemical limitation to be fully overcome [8].

Light activation of GAPDH and PRK is a component of the biochemical limitation of photosynthetic induction. Though the principles of light activation are well established [1], many issues are still unsolved. Our current knowledge is based on a combination of *in vivo*, *ex vivo* and *in vitro* studies [3,31].

3.1. Photosynthetic induction: light activation of GAPDH and PRK extractable activities

One of the simplest but not trivial measures that have been repeatedly carried out in several species are assays of enzyme activities in extracts from leaves or isolated chloroplasts of different species. These measurements were conducted under conditions that were believed to keep the original activation state of the enzymes. Crucial in this respect is the composition of the extraction buffer, which should contain a minimum amount of thiolic reducing agents to avoid oxidation of catalytic protein cysteines [36,37] without reducing regulatory disulfides [38,39]. In these conditions, the activities of both GAPDH and PRK assayed in extracts from illuminated leaves or chloroplasts are typically several-fold higher than in dark-adapted samples. The kinetics of light activation were usually completed in less than ten minutes, a reasonable timeframe for contributing to the biochemical limitation of photosynthetic induction [40–47]. It is worth mentioning that besides extraction, also the assays should be performed under non-activating conditions, and for GAPDH this may be difficult to achieve because BPGA and NADPH are both substrates and activating compounds [48,49]. However, since activation is slower than catalysis, the problem is usually overcome by recording initial enzyme activities before activation occurs.

3.2. GAPDH and PRK regulation and complex formation in vitro

In vitro studies have revealed essential molecular details of GAPDH and PRK regulation (Tables 1 and 2) [1–3]. The major chloroplast GAPDH isoform of land plants has A_2B_2 subunit composition and is regulated by the C-terminal extension (CTE) of B subunits which contains two redox-active cysteines [38,50,51]. Chloroplast GAPDH can use both NADP(H) and NAD(H) as coenzymes, but mechanisms of regulation are specific for the NADP(H)-activity [48]. When the cysteines of the CTE are engaged in a disulfide bridge, the CTE adopts a position that hinders the access of the substrate BPGA to the active site. If the coenzyme binding site is occupied by NADP(H), whether reduced or oxidized, inhibition by oxidized CTE is partial [51]. In contrast, if NAD(H) replaces NADP(H), the CTE can fit deeply into the active site and inhibition is stronger [52]. In these conditions (the CTE is oxidized and NAD(H) is bound to the protein), A_2B_2 tetramers self-assemble into tetramers of tetramers (A_8B_8) [46] as each CTE slips into the active sites of an adjacent tetramer. A_8B_8-GAPDH is therefore stabilized by the CTEs acting as cross-linkers [52].

Reactivation of inhibited A_8B_8-GAPDH requires displacement of the oxidized CTE from the active site and consequent recovery of the tetrameric conformation (A_2B_2-GAPDH). This can be accomplished by ligands like NADP(H), BPGA and ATP that compete at physiological

concentrations with the binding of the CTE [38,44,47–49,53]. Thioredoxin *f* does also contribute to A_8B_8-GAPDH dissociation by specifically reducing the regulatory disulfide of the CTE. In the absence of activating ligands however, the effect of reduced TRX *f* is minimal or very slow [39,44,47,49] indicating that oxidized CTEs in A_8B_8-GAPDH are not readily accessible to thioredoxins [52]. In any case, biochemical reactivation of A_8B_8-GAPDH is a relatively slow process that usually takes minutes to complete, despite the synergistic effect of activating ligands and TRX *f* [44].

The autonomous regulation of PRK appears simpler than that of GAPDH. Two regulatory cysteines, Cys16 and Cys55, reside in the N-terminal portion of the protein. Being part of the ATP- (Cys16) and Ru5P-binding sites (Cys55), both cysteines facilitate catalysis [54,55]. Formation of a disulfide bridge between Cys16 and Cys55 constitutes the essential regulatory mechanism that inhibits catalysis. Reduction and hence reactivation is accomplished by both *f*- and *m*-type thioredoxins [39]. A second disulfide, also controlled by thioredoxins, can be formed in the C-terminal part of the protein [56–58] without affecting activity, in *Arabidopsis* at least [59]. In *Anabaena* PRK, this second disulfide was instead found to regulate the activity of the enzyme [59].

On top of these mechanisms of autonomous regulation, GAPDH and PRK are also regulated through the formation of a supramolecular complex with a third protein, CP12 [3]. The interaction between CP12 and GAPDH resembles that of AB-GAPDH with its own CTE [52], but does only occur when GAPDH binds NAD(H), and is prevented by NADP(H) [29,60]. Like the CTE, also CP12 must first bear a disulfide in its C-terminus before binding to GAPDH [61]. The GAPDH-$CP12_2$ binary complex has only a slight reduction in the NADPH-dependent activity but shows high affinity for PRK to form the $GAPDH_2$-$CP12_4$-PRK_2 complex [29,61]. In land plants at least, recruitment of PRK requires a second disulfide to be formed in the N-terminal part of CP12 [61]. In the ternary complex, the four CP12s fully obstruct all active sites of PRK (which are four since PRKs are dimers and half of the active sites of GAPDH, which are four per tetramer and eight in the complex [57]. As a result, PRK activity is very low and GAPDH is also substantially inhibited [29]. Both activities can be recovered upon complex dissociation by reduced thioredoxins or compounds that displace CP12 from its binding site like BPGA, NADP(H) or ATP [29,39]. It should be noted that PRK can be part of the complex in both reduced and oxidized state [47]. CP12 provides a way to regulate the GAPDH isoform A_4 which is not autonomously regulated [2]. The regulated isoform A_2B_2-GAPDH can also be complexed with CP12 and PRK as long as its active sites are not already occupied by the CTEs [62].

With respect to light/dark regulation, some synthetic conclusions can be drawn from *in vitro* studies. GAPDH and PRK are inhibited by oxidized thioredoxins or other oxidizing compounds (*e.g.* H_2O_2, [37]) that favor the formation of disulfides in A_2B_2-GAPDH, CP12 and PRK. GAPDH and PRK are also indirectly inhibited by NAD(H) that is functional to the formation of the inhibited complexes A_8B_8-GAPDH and $GAPDH_2$-$CP12_4$-PRK_2 [3]. Reactivation of GAPDH and PRK can be mediated by reduced thioredoxins (which reduce disulfides in AB-GAPDH, CP12 and PRK) and/or by NADPH, BPGA or ATP (which displace the CTE/CP12 from GAPDH/PRK active sites); in both cases the reactivation involves the dissociation of the complexes. Whether these are also the mechanisms that underlie the light/dark regulation *in vivo* is probably true but not yet fully demonstrated.

3.3. Photosynthetic induction and in vivo detection of GAPDH/PRK complexes

Since the inhibited forms of GAPDH and PRK can be associated into large complexes, detecting these complexes *in vivo* may be informative of GAPDH and PRK activation states. Unfortunately, these complexes are not covalently bound and must be extracted under native conditions. Detecting these complexes has thus similar limitations as recording the activities of enzymes with variable activation states (see Section 3.1). In particular, complexes are stable when GAPDH binds NAD(H), and this condition might be difficult to maintain during extraction, with the risk of complex dissociation before detection.

With proper extraction methods, AB-GAPDH and GAPDH/PRK complexes could be identified in different plant species, either by size-exclusion chromatography followed by enzyme activity assays [43,63] or Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE) followed by immunoblotting [47,64]. In all investigated species, GAPDH extracted from dark-adapted leaves was found in different conformations, including high molecular weight complexes (A_8B_8, A_4B_4,) and tetrameric forms (A_2B_2, A_4) [43,47,63,64]. PRK was often found associated with GAPDH in CP12-complexes, but in few species like *Arabidopsis* and tobacco, the $GAPDH_2$-$CP12_4$-PRK_2 complex could not be detected under native conditions in the dark [64]. The doubt remains as to whether in species like Arabidopsis, which contains three CP12 expressed genes in its genome [2,65,66], the $GAPDH_2$-$CP12_4$-PRK_2 complex does not form at all, or it is just hard to detect. The $GAPDH_2$-$CP12_4$-PRK_2 complex made *in vitro* from *Arabidopsis* recombinant proteins was crystallized and its structure solved at 3.5 Å resolution [57]. Therefore, it is very likely that such complex exists also *in vivo* although it has so far eluded detection. Anyway, in all species in which high molecular weight complexes with GAPDH and/or PRK were identified in the dark, these complexes tend to dissociate in the light [47,64]. In terms of kinetics, $GAPDH_2$-$CP12_4$-PRK_2 complex dissociation in pea leaves upon dark to light transition was accomplished in a timeframe of minutes, followed by a somehow slower recovery of GAPDH and PRK activity [47]. Overall, these results seem to suggest that GAPDH/PRK regulation through complex formation/dissociation, an aspect that we know mostly from *in vitro* studies, is part of the photosynthetic induction response of leaves subject to dark/light transitions.

3.4. Photosynthetic induction and in vivo detection of GAPDH/PRK redox states

While supramolecular complexes may be labile and difficult to detect in raw extracts, disulfide bridges are stable covalent bonds that stand denaturing conditions, which are necessary to take a snapshot of the metabolic state of the tissue. Moreover, with alkylating agents such as N-ethylmaleimide (NEM) reduced cysteines are blocked and the formation of spurious disulfides during the extraction is prevented. Native disulfides in extracted proteins can then be reduced by dithiothreitol (DTT), alkylated with high molecular weight compounds like poly(ethylene glycol) methyl ether maleimide (Mal-PEG) and finally detected by immunoblot or mass spectrometry analyses [30,67]. This approach was used to determine the redox state of B-GAPDH subunits and PRK in *Arabidopsis* and other species in various conditions of illumination, and further confirmed that both B-GAPDH and PRK are more reduced in the light than in the dark [30,68]. Again, kinetics of light-reduction and dark-oxidation were compatible with the kinetics of enzymes activation/inhibition observed with activity assays [32,33] (see Section 3.1). Experiments of this type have also shown the indirect role of NADPH-dependent thioredoxin reductase C (NTRC) in favoring the reduction of GAPDH and PRK *in vivo* [69] and the role of atypical thioredoxins [70] and 2-cys peroxiredoxins [32,33] in allowing the rapid formation of disulfide bridges upon light to dark transitions [67].

In spite of the great potential of the technique, it must be recalled that the redox state of both B-GAPDH and PRK may not exactly correspond to the real activity of GAPDH and PRK *in vivo*. A complication arises from the presence of the thioredoxin-insensitive GAPDH isoform (A_4-GAPDH) that is possibly regulated by CP12 but does not form its own disulfide [38,71]. Moreover, the formation of the complexes (with or without CP12) depends on pyridine nucleotides, in addition to disulfides, and there is no doubt that complexes affect enzyme activities. Oxidized B subunits of AB-GAPDH can have different levels of residual activity depending on whether the enzyme binds NADP(H) and is tetrameric (A_2B_2), or binds NAD(H) and is hexadecameric (A_8B_8) or in

other oligomeric conformations [38,52,72]. Associating the PRK redox state with its activity may also be problematic if we consider that reduced PRK can be part of the $GAPDH_2$-$CP12_4$-PRK_2 complex [47,57] and thus be inhibited by protein-protein interactions rather than disulfide formation. These limitations have to be taken into account when the *in vivo* redox states of GAPDH and PRK under varying illumination conditions are interpreted in a physiological framework.

A recent proteomic application of this approach on tobacco leaves is a good example of how the *in vivo* determination of thiol/disulfide redox states can be combined with data obtained from redox titrations of purified proteins, to derive an integrated picture of photosynthetic induction [30]. In this work, twenty chloroplast proteins were identified by shotgun redox proteomics for having a disulfide bridge in the dark that got reduced during ten minutes of illumination at low intensity. Interestingly, many of the major players of CB cycle light activation were identified: thioredoxin f, the four thioredoxin-regulated enzymes of the CB cycle (including B-GAPDH and PRK), CA1Pase involved in Rubisco regulation and the γ-subunit of ATPase (Fig. 1). The reduction state of these proteins was analyzed at different time points during a 10 min transition from dark to low light, *i.e.* during photosynthetic induction. Thioredoxin *f* and PRK were among the fastest proteins to get reduced in the light: both reached a maximum reduction level in less than one minute and largely maintained their reduced state for the following minutes. B-GAPDH reduction was much slower [28]. It is worth mentioning that *Arabidopsis* PRK, embedded in the GAPDH/CP12/PRK complex, and B_8-GAPDH, followed similar kinetics of activation by thioredoxin f *in vitro* compared to their respective counterparts *in vivo* [39]. Both types of evidence converge in pointing to thioredoxin f as a physiological activator of B-GAPDH and PRK, associated into "dark" complexes [30,39].

Based on the reduction states of the different proteins measured *in vivo*, and on the standard redox potentials (E_m') determined *in vitro*, *in vivo* redox potentials (E′) can be calculated. Similar values of *in vivo* redox potentials indicate equilibrium between two interacting proteins. This was the case for TRX *f* (≈ 40 % reduced after 30 s; E′ of − 285 mV based on E_m' of − 290 mV [73]) and PRK (≈ 80 % reduced after 30 s; E′ of − 288 mV based on E_m' of − 270 mV) (Table 2). Based on these numbers, TRX *f* and PRK are predicted to rapidly equilibrate after the onset of light, in a similar way as they do it *in vitro*. Predictions may be slightly different depending on the values of E_m' used for calculations [30]. For B-GAPDH the prediction is different because its redox state increased continuously during the photosynthetic induction and hence it was not in equilibrium with thioredoxin f. A plausible explanation is that GAPDH ligands like BPGA and NADPH, that do also change in concentration during the photosynthetic induction, may affect the kinetics of reduction and/or the standard redox potential of the regulatory disulfide, making any prediction uncertain [30]. Light reduction of GAPDH *in vivo* is therefore still less understood than PRK.

3.5. The role of metabolites in the light activation of GAPDH and PRK

Though GAPDH and PRK are regulated by thioredoxins, GAPDH is also very sensitive to metabolites like pyridine nucleotides, ATP and BPGA and since these metabolites have a large influence on the stability of the CP12-assembled ternary complex, PRK is indirectly regulated by GAPDH ligands too [29]. *In vitro*, the binding of NAD^+ or NADH by GAPDH is a necessary but not sufficient condition for the formation of A_8B_8 and CP12-complexes. The presence of a disulfide in the CTE of B-GAPDH subunits or in the C-terminus of CP12 is also necessary, but again not sufficient [29,38,48,50,52,57,61]. NADP(H) dissociates both types of complexes by substituting NAD(H), hence removing one of the two necessary conditions for complex stability. Reduced TRX *f* does the same, with high efficiency with the CP12 ternary complex but low efficiency with A_8B_8-GAPDH [39]. In conclusion, association of the different types of GAPDH/PRK complexes require both NAD(H) binding and formation of disulfides, while dissociation of the same complexes requires either NADP(H) or reduction of dilsulfides.

The chloroplast NADP(H)/NAD(H) ratio rather than pyridine nucleotides redox states is the relevant parameter for GAPDH and PRK regulation through complex dynamics. Chloroplast NADP(H)/NAD(H) ratios were found to increase several-fold in the light in respect to darkened samples [74–76], an effect that is likely due to the light activation of chloroplast NAD kinase 2 which catalyzes the ATP-dependent phosphorylation of NAD^+ into $NADP^+$ [77–79] (Fig. 1). Similar light/-dark oscillations of NADP(H)/NAD(H) ratios measured in the cyanobacterium *Synechococcus* PCC7942 and reproduced *in vitro* were shown to induce association/dissociation of the CP12 ternary complex starting from isolated GAPDH, PRK and CP12 proteins from the same organism [80].

4. The GAPDH/PRK regulatory system in other organisms than land plants

Cyanobacteria contain a great diversity of CP12 genes [81]. The marine cyanobacterium *Synechococcus* PCC7942 contains a CP12 gene with no N-terminal regulatory cysteines, besides a canonical CP12 with both N- and C-terminal cysteine pairs [82]. The former CP12 isoform was shown to assemble a ternary complex with GAPDH and PRK with similar features to those of land plants [57,83]. Interestingly, inactivation of this gene in *Synechococcus* PCC7942 led to growth inhibition in light/dark cycles but not in continuous low light, in agreement with CP12 playing a role in light/dark regulation [80]. The role of the canonical four-Cys CP12 of *Synechococcus* PCC7942 has not yet been investigated.

On the other hand, in the distantly related cyanobacterium *Synechocystis* PCC6803, deletion of the single canonical CP12 gene caused no growth defects in either continuous or intermittent light (12 h/12 h light/dark cycles) [82,84]. In wild type *Synechocystis* PCC6803 cells, both GAPDH and PRK (eYFP-tagged) formed fluorescent aggregates in the dark [84]. Aggregation was impaired in Δ*cp12* mutants and was reverted in the light. Both dark-induced aggregation and light-induced disaggregation occurred in a timeframe of seconds to minutes. Dark-induced aggregation was faster for GAPDH than PRK, in agreement with the sequence of formation of binary (GAPDH/CP12) and ternary CP12 complexes (GAPDH/CP12/PRK) *in vitro* [3]. However, since only few fluorescent spots were observed per cell, the nature of these dark- and CP12-dependent aggregates of GAPDH and PRK is still uncertain. Obviously, they cannot be assimilated to the classical CP12 complexes of GAPDH and PRK which may possibly occur in millions of copies per cell [22]. Further studies will hopefully reveal the nature of these dark- and CP12-dependent aggregates, for the first time described in *Synechocystis* PCC6803 [84].

A clear phenotypic trait shown by Δ*cp12* mutants of *Synechocystis* PCC6803 consists in the inability to grow mixotrophically in the presence of glucose and low light, suggesting a role of CP12 in fine tuning the co-existence of photosynthetic carbon reduction and carbohydrate oxidation in the same cell [82,84]. Interestingly, a similar role of CP12 in regulating the partitioning between the CB cycle and the oxidative pentose phosphate pathway was experimentally disproven in tobacco plants with antisense suppression of CP12 [85]. These contrasting results clearly suggest that CP12, though ubiquitous in oxygenic photosynthetic organisms, may play different physiological roles in different biological contexts.

Although the role of CP12 in the light/dark regulation of the CB cycle in *Synechocystis* PCC6803 is questioned by the recent observations just reported [82,84], the light-regulation of PRK was recently supported by metabolic analyses performed in the same specie. Notably, the symmetrical increase of Ru5P, and decrease of RuBP during a light to dark transition clearly showed that the CB cycle was blocked at the level of PRK, already after 1 min of darkening [86].

CP12 complexes certainly exist in green algae and have been thoroughly characterized in *Chlamydomonas reinhardtii* [87,88].

Fundamental features of the complex *in vitro* appear analogous to those of cyanobacteria or land plants [56,89]. *Chlamydomonas* contain a single CP12 gene and is one of the few organisms for which knock out mutants have been characterized [28]. Cells with no CP12 grew normally in either continuous or alternating light conditions, questioning that CP12 may have any relevant role in light/dark regulation of the CB cycle in this model green algae [28]. However, in conditions in which the stability of the complexes in the extracts could be preserved, algal cells with no CP12 were found to contain more GAPDH activity and less PRK activity than wild type cells [28]. The increase in GAPDH activity can be explained by the absence of CP12 ternary complex that would inhibit GAPDH activity. The decrease of PRK activity was tentatively explained in terms of decrease of PRK protein in CP12 knock out cells [28], an interesting effect that could bring together *Chlamydomonas* and *Arabidopsis* CP12 ([27] see Section 5).

A CP12 complex with GAPDH and PRK was also detected in the unicellular red algae *Galdieria sulphuraria* having a CP12 with no N-terminal cysteines [90] but no GAPDH/CP12/PRK complexes have been found yet in diatoms, although they do contain CP12 [91,92]. An alternative complex involving GAPDH, CP12 and ferredoxin $NADP^+$ reductase was detected in *Asterionella formosa* and found to allow GAPDH to be regulated by NADPH [91]. Different from plants, thioredoxins appear to play a minor role in the light regulation of the CB cycle in diatoms [91,93].

5. Beyond light-regulation: the role of CP12 in PRK stability and/or abundance

The unique CP12 of *Chlamydomonas* was recently knocked out by CRISPR-CAS9 [28] and cells with no CP12 grew normally in variable light regimes, questioning whether CP12 could play any relevant role in light/dark regulation of the CB cycle in this algae, though it clearly regulates GAPDH activity [28]. However, the lack of CP12 caused a wide rearrangement of the proteome, including a reduction of PRK concentration to 15% of its native level but no effects on GAPDH [28]. The normal growth of the *Chlamydomonas* strain with only 15% residual PRK indeed suggests that also in this algae, like in tobacco [19], PRK may be largely in excess for the functioning of the CB cycle (Fig. 2). *In vitro,* CP12 preserves GAPDH from heat-induced aggregation [94] and also PRK from its slow inactivation at 4 °C [28]. In both cases, the effect of CP12 is independent from its redox state [28,94]. CP12 binds PRK by means of an α-helix [57] that though more stable in oxidized CP12, shortly exists also in reduced CP12 that is much more dynamic [95].

The effect of CP12 on PRK concentration in Chlamydomonas is reminiscent of a similar effect first observed in Arabidopsis [27], which contains three CP12 isoforms with similar biochemical properties [65]. The triple *cp12-1/-2/-3* mutant was found to contain lower amounts of PRK proteins but similar amounts of PRK transcripts [27]. Whether the dramatic decrease of PRK was due to lower stability of the protein or slower translation of the messenger is still an open question. In any case, the CP12 mutations also caused a strong inhibition of CO_2 assimilation, probably because PRK dropped below the threshold for optimal CB cycle functioning (Fig. 2) [27].

In vitro, CP12 complexes reconstituted with recombinant GAPDH and PRK from either Arabidopsis or Chlamydomonas, are somehow protected from oxidation by H_2O_2 or GSNO [37]. The same CP12 mutant strain of *Synechococcus elongatus* PCC7942 that grows normally under continuous low light, cannot stand few hours in high light (100 μmol m^{-2} s^{-1}), because of ROS accumulation and rapid chlorophyll degradation [96]. It is not known whether CP12 provides protection to PRK also in cyanobacteria, but based on the similar structure of binary and ternary CP12 complexes in cyanobacteria and plants [57,60,83,97] the hypothesis that these complexes have a wide protecting role in photosynthetic organisms seems worth investigating.

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Conflict of interest

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