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Author for correspondence:
Sujith Puthiyaveetil
e-mail: s.puthiyaveetil@wsu.edu

†Present address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164, USA.

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Evolutionary rewiring: a modified prokaryotic gene-regulatory pathway in chloroplasts

Sujith Puthiyaveetil[1,†], Iskander M. Ibrahim[1] and John F. Allen[1,2]

[1]School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK
[2]Research Department of Genetics, Evolution and Environment, University College London, Gower Street, London WC1E 6BT, UK

Photosynthetic electron transport regulates chloroplast gene transcription through the action of a bacterial-type sensor kinase known as chloroplast sensor kinase (CSK). CSK represses photosystem I (PS I) gene transcription in PS I light and thus initiates photosystem stoichiometry adjustment. In cyanobacteria and in non-green algae, CSK homologues co-exist with their response regulator partners in canonical bacterial two-component systems. In green algae and plants, however, no response regulator partner of CSK is found. Yeast two-hybrid analysis has revealed interaction of CSK with sigma factor 1 (SIG1) of chloroplast RNA polymerase. Here we present further evidence for the interaction between CSK and SIG1. We also show that CSK interacts with quinone. *Arabidopsis* SIG1 becomes phosphorylated in PS I light, which then specifically represses transcription of PS I genes. In view of the identical signalling properties of CSK and SIG1 and of their interactions, we suggest that CSK is a SIG1 kinase. We propose that the selective repression of PS I genes arises from the operation of a gene-regulatory phosphoswitch in SIG1. The CSK-SIG1 system represents a novel, rewired chloroplast-signalling pathway created by evolutionary tinkering. This regulatory system supports a proposal for the selection pressure behind the evolutionary stasis of chloroplast genes.

1. Gene regulation in chloroplasts

Photosynthesis is the conversion of light energy into chemical energy by plants, algae and certain bacteria. In plants and algae, photosynthesis takes place in cytoplasmic organelles known as chloroplasts. Chloroplasts originated from free-living cyanobacteria, which established an endosymbiotic relationship with a eukaryotic host cell around 1.2 billion years ago [1,2]. As evidence of their bacterial origin, chloroplasts contain functional genomes, which are nevertheless greatly reduced in coding capacity when compared with the genomes of free-living cyanobacteria [3–5]. Although chloroplast genomes are miniscule by eukaryotic standards, they encode some of the core proteins of the photosynthetic machinery [6,7]. The photosystems are the functional units of photosynthesis where the initial light-driven electron transfer reactions take place [8,9], and their major protein subunits are always chloroplast-encoded [6,7]. Chloroplast genes retain prokaryotic genetic organization, and are transcribed from bacterial-type gene promoters by a eubacterial multisubunit RNA polymerase known as the plastid-encoded polymerase (PEP) [10–12]. As its name indicates, all subunits of the PEP, except its sigma factor subunit, are products of chloroplast genes. The sigma factor subunit of the PEP recognizes the bacterial-type gene promoters of chloroplast genes. In chloroplasts of the model plant *Arabidopsis thaliana*, as many as six sigma factors are found [13–15]. Some chloroplasts also contain a second, phage-type single-subunit RNA polymerase known as the nuclear encoded polymerase (NEP), which transcribes DNA from distinct promoter elements found in some chloroplast genes [10,16].

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Even though chloroplasts contain an elaborate transcriptional machinery capable of regulatory control, the principal mode of gene regulation in chloroplasts has long been considered to be post-transcriptional [17,18]. Transcriptional regulation in plant chloroplasts was thought to occur only during their early development, when there is a global increase in transcription of chloroplast genes. This view, however, has to be abandoned in the light of a series of experiments showing robust transcriptional regulation of genes in mature plant chloroplasts [19–21]. The first such experiment was a demonstration that chloroplast genes respond to increasing light intensity by increasing their rate of transcription [19,22]. Increased global transcription of chloroplast genes in high light enables chloroplasts to keep up with an increased demand for components of the photosynthetic machinery as the rate of photosynthesis increases. The increase in light intensity is sensed by a eukaryotic serine/threonine protein kinase known as the plastid transcription kinase (PTK) through the redox state of the stromal electron carrier glutathione [19]. PTK regulates chloroplast gene transcription by phosphorylation of the sigma factor and other subunits of the PEP [19,23,24]. In addition to its role in global regulation of chloroplast transcription, PTK has recently been shown to regulate distinct subsets of chloroplast genes depending on which sigma factor it phosphorylates [25].

The blue light-induced transcription of the *psbD-psbC* operon is another instance of chloroplast transcriptional regulation [20]. In response to intense blue light, an increase occurs in transcription of the *psbD-psbC* operon that encodes the D2 and CP43 proteins of photosystem II. This increase is probably mediated by cytoplasmic photoreceptors, which perceive the blue light and induce transcription of the nuclear gene that encodes the sigma factor 5 (SIG5) [26–29]. SIG5 then transcribes the *psbD-psbC* operon from a blue-light-responsive promoter element [30]. The higher rate of transcription of this operon in strong blue light compensates for the high turnover of the D2 and CP43 proteins as they suffer photodamage.

In an acclimatory response to changes in light quality, chloroplasts regulate the transcription of genes that encode proteins of the core photochemical reaction centres of the photosystems [21,31–34]. In this acclimatory response, termed photosystem stoichiometry adjustment, the relative abundance of the two photosystems—photosystem II (PS II) and photosystem I (PS I)—becomes adjusted. Chloroplasts perceive changes in the quality of light through changes in the redox state of the electron carrier plastoquinone (PQ) [21]. In oxygenic photosynthesis, PS II and PS I are connected in series for linear electron transport from water to $NADP^+$ [35]. Each photosystem has a distinct action spectrum, and yet the two photosystems must convert light energy at an equal rate in order for efficient linear electron transport to occur. In photic environments with gradients of light quality, any imbalance in the excitation of an individual photosystem is sensed by changes in the redox state of the PQ pool [21]. The PQ pool then regulates the transcription of the photosystem genes in such a way as to adjust the stoichiometry of the two photosystems, which eventually corrects this excitation imbalance [21,31]. Photosystem stoichiometry adjustment increases the efficiency of photosynthesis in limiting light [36]. The physiological importance of this acclimatory response and the role that the transcriptional regulation of photosystem genes plays in it are relatively well understood, and we are beginning to unravel the precise molecular mechanism by which the redox state of PQ is conveyed to the chloroplast transcription machinery [37–39].

2. A two-component gene-regulatory system in chloroplasts

Chloroplast sensor kinase (CSK) is a two-component sensor kinase discovered during the search for the regulatory components underlying photosystem stoichiometry adjustment [39]. Two-component systems comprise a class of bacterial signal transduction proteins, each system consisting of a sensor histidine kinase and a response regulator [40]. Upon sensing an internal or external stimulus, the sensor kinase undergoes an ATP-dependent autophosphorylation at a conserved histidine residue. The phosphate group from the sensor kinase is then transferred to a conserved aspartate residue in the response regulator. The phosphorylation event in the response regulator modulates the activity of its effector domain, which in most cases is a DNA-binding transcription factor module [40]. Responses mediated by two-component systems therefore usually involve regulation of genes at the transcriptional level. These regulatory systems can be considered to be on/off switches of transcription.

As part of a hypothesis that seeks to explain the evolutionary retention of genes in chloroplasts and mitochondria, two-component systems have been predicted to exist in these organelles [41,42]. The hypothesis of co-location for redox regulation (CoRR) predicts that chloroplasts have inherited bacterial-type gene-regulatory systems from their cyanobacterial ancestors to keep the chloroplast gene expression under the direct control of photosynthetic electron transport [41,43]. The selection pressure that retains genes in organelles, according to CoRR, is the requirement for regulatory coupling of redox chemistry with organellar gene expression, in order for the stoichiometry of electron transfer complexes to be adjusted to the rate of electron transfer reactions [6].

Prior to the discovery of CSK, two-component systems were unknown in the chloroplasts of green algae and plants. Reports of chloroplast two-component systems were limited to a few instances in non-green algae, where these components are chloroplast-encoded and their functional roles remain uncertain [44,45]. In contrast, inactivation of the nuclear *CSK* gene in *Arabidopsis* results in plants that cannot suppress PS I gene (*psaA*) transcription in light that preferentially excites PS I (light 1) [39]. During photosystem stoichiometry adjustment, oxidized PQ represses *psaA* gene transcription and the reduced PQ pool releases that repression [21]. Thus light 1 causes transient oxidation of the PQ pool while light specific for PS II, light 2, drives the quinone pool to a more reduced state. This light-quality-controlled transcription of PS I gene expression through the redox state of the PQ pool is therefore an essential component of photosystem stoichiometry adjustment. Since *CSK* knockout plants cannot repress *psaA* in light 1, CSK has been suggested to act as the repressor of PS I gene transcription in light 1 [37–39]. An overexpression line of CSK shows a remarkable shade avoidance phenotype, characteristic of plants grown in light 1 [46]. It can be assumed that overexpression of CSK overrides the PQ control of chloroplast transcription, causing constitutive repression of PS I genes and resulting in a shade avoidance phenotype. This phenotype of the

CSK overexpression line underscores the function of CSK as a light 1 acclimator. A *CSK* knockout line of the model moss *Physcomitrella patens*, like the *Arabidopsis CSK* null mutants, has been found to be unable to regulate *psaA* transcription in response to changes in light quality (A.C. Cuming, S. Puthiyaveetil and J.F. Allen; unpublished results). CSK homologues are found in all major lineages of photosynthetic eukaryotes, and a homologue of CSK is seen also in cyanobacteria [38,39]. Histidine kinase 2 (hik2) is the closest cyanobacterial homologue of CSK [39].

3. Rewiring chloroplast gene regulation by evolutionary tinkering

The working of natural selection has been likened to *bricolage*, or tinkering [47]. The origin and evolution of novel cell signalling pathways are consistent with this view of evolution as a tinkerer. Protein–protein interaction studies in cyanobacteria reveal that the response regulator 1 (rre1) is the functional partner of hik2, the cyanobacterial homologue of CSK [48]. Genome-wide analysis of photosynthetic eukaryotes shows that rre1 has survived in the chloroplasts of non-green algae as the product of a chloroplast gene called ycf29 [45]. However, no ycf29 homologue has been found in the genomes of green algae or plants [38]. The apparent loss of ycf29 seems to correlate with the loss of the conserved histidine residue in CSK, and with a predicted change in its kinase activity [38]. This finding has prompted the search for non-response regulator proteins that might act as functional partners of CSK. Thus a yeast two-hybrid analysis has identified interaction of CSK with the sigma factor 1 (SIG1) subunit of the chloroplast RNA polymerase; with PTK; and with itself [38]. Using an *in vitro* pull-down assay we provide further evidence for the interactions identified in the earlier yeast two-hybrid analysis [38].

Figure 1*a* shows the overexpressed and purified glutathione S-transferase (GST) protein, CSK, and PTK in lanes 1, 2 and 3 respectively. CSK and PTK are overexpressed and purified as GST fusion proteins, and are used as baits in our pull-down assay. The GST protein itself represents a control bait to show that any interaction we identify arises from specific interaction with the bait protein, and not from an interaction with the affinity purification tag GST to which the baits are fused. As the prey protein SIG1 is expressed as an insoluble protein in bacteria, we synthesized SIG1 *in vitro* using a coupled transcription–translation system (for materials and methods, see the electronic supplementary material). The translated SIG1 protein has been labelled with the ^{35}S-methionine (figure 1*b*, lane 1). The bait proteins are bound to a glutathione affinity column and can be purified by affinity chromatography. When the SIG1 prey protein is incubated in the glutathione column along with the bound bait proteins, it co-purifies with the CSK and PTK bait proteins (figure 1*b*, lanes 3 and 4), but not with the GST control bait protein (lane 2), supporting the specific CSK/SIG1, and PTK/SIG1 interactions identified in the earlier yeast two-hybrid study [38]. PTK has been shown to phosphorylate the SIG1 protein [23,24]; our PTK-SIG1 pull-down result provides independent evidence for the required interaction.

Figure 2 shows the results of a second pull-down assay that independently verifies the CSK/CSK and CSK/PTK

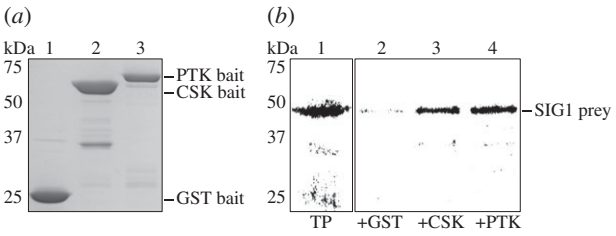

Figure 1. CSK specifically interacts with the SIG1 component of PEP. An *in vitro* pull-down assay shows specific interaction between CSK and SIG1, and PTK and SIG1. (*a*) Coomassie-stained SDS–PAGE gel showing overexpressed and purified bait proteins. The positions of molecular weight markers are indicated on the left and the position of the bait proteins are labelled on the right. Lanes are labelled numerically at the top of the gel. The second most prominent band in lane 2, at around 37 kDa and seen along with the CSK-GST bait, is the truncated kinase domain of CSK without the GST tag. (*b*) An autoradiograph of the SDS–PAGE gel that separated the products of the pull-down assay. The position of the mature *in vitro* translated and radiolabelled SIG1 prey protein is indicated on the right. Numbers at the top of the autoradiograph indicate individual lanes of the gel. Lane 1 shows the translation product (TP) from the *in vitro* synthesis reaction of SIG1. Lanes 2, 3 and 4 show the SIG1 eluate that resulted from each pull-down assay. The bait used in the assay is shown at the bottom of each lane, with the '+' sign. Note that the assay with the control bait GST recovers very little SIG1 prey protein.

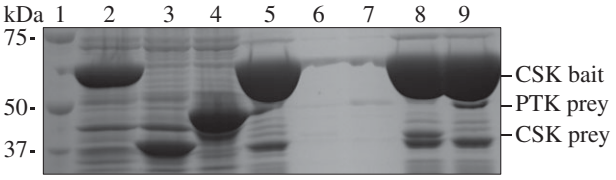

Figure 2. Interaction of CSK with itself and with PTK. Coomassie-stained SDS–PAGE gel with the products of the second pull-down assay that shows specific interactions of CSK with itself and with PTK. The positions of protein molecular weight markers are indicated on left, and the position of each individual bait or prey protein is marked on the right. Lanes are labelled numerically at the top of the gel. Lane 1 contains protein molecular weight markers. Lanes 2, 3 and 4 shows bacterial cell lysates overexpressing CSK-GST bait, CSK-His prey and PTK-His prey proteins, respectively. In lane 5, the bait CSK-GST has been purified from a glutathione affinity column. Lanes 6 and 7 show eluates from the purification of the prey proteins CSK-His and PTK-His, respectively, in the absence of the CSK-GST bait protein. Lanes 8 and 9 contain eluates from the purification of the prey protein CSK-His and PTK-His, respectively, in the presence of the CSK-GST bait protein. Note that, in the absence of the bait protein CSK-GST, very little or no prey proteins are recovered from the glutathione column.

interactions identified in the yeast two-hybrid assay [38]. Figure 2, lane 2 shows bacterial lysate containing the overexpressed, GST-tagged CSK bait protein. Lanes 3 and 4 show lysates with overexpressed, His-tagged CSK and PTK prey proteins, respectively (for materials and methods, see the electronic supplementary material). The CSK-GST bait protein binds to the glutathione agarose column and can be eluted by the addition of excess glutathione (lane 5). In contrast, the His-tagged CSK and PTK prey proteins do not bind to the glutathione column and therefore their elutions are devoid of overexpressed proteins (lanes 6 and 7). However, in the presence of the CSK-GST bait protein in the column, the prey proteins CSK-His and PTK-His bind to the column and can be eluted along with the CSK bait protein (lanes 8

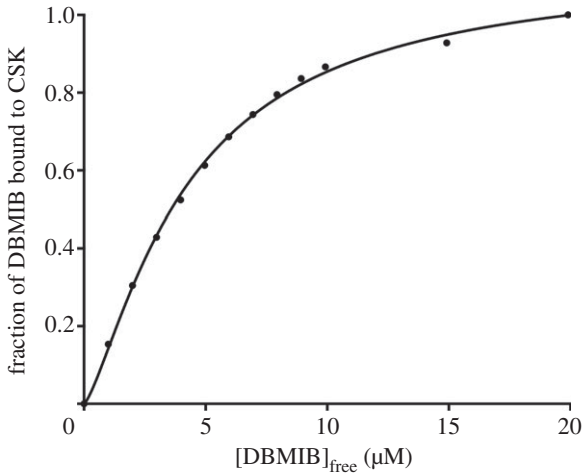

Figure 3. CSK binds a quinone analogue. The DBMIB-binding kinetics of CSK are presented as a plot diagram. The fraction of DBMIB bound to CSK, shown on the *y*-axis, is calculated from the quenching of tryptophan fluorescence in CSK, caused by DBMIB binding. The *x*-axis is the concentration of the unbound, free DBMIB.

and 9, respectively), thus supporting the specific CSK/CSK, and CSK/PTK interactions noted in the yeast two-hybrid assay [38].

4. CSK links PQ with chloroplast transcription: the basis of photosystem stoichiometry adjustment in green algae and plants

The interaction of CSK with the SIG1 subunit of the chloroplast RNA polymerase ([38]; figure 1) suggests a means by which the signal sensed by CSK can be transduced to the transcriptional machinery to regulate chloroplast genes. The exact nature of the regulatory signal sensed by CSK, however, remains to de determined. The demonstration that CSK regulates chloroplast transcription in response to changes in light quality that affect the redox state of the PQ pool suggests that the regulatory signal is likely to be the redox state of the PQ pool itself [39]. Here, we demonstrate direct binding of a quinone analogue 2,5-dibromo-3-methyl-5-isopropyl-*p*-benzoquinone (DBMIB) by CSK (figure 3). Overexpressed and purified CSK protein was incubated with DBMIB at increasing concentrations. The intrinsic fluorescence from tryptophan residues in CSK was monitored by fluorescence spectroscopy (for materials and methods, see the electronic supplementary material). Since fluorescence from tryptophan residues is sensitive to structural perturbations resulting from protein–ligand interaction, it can be used as a proxy to analyse whether a protein binds a given ligand. Increasing concentrations of DBMIB were observed to quench CSK tryptophan fluorescence, indicating binding of DBMIB by CSK. The dissociation constant (K_d) for the binding was calculated to be 3.66 μM, which is within a physiological range of quinone concentration and comparable with K_d values of known quinone-binding proteins such as the cyanobacterial circadian clock regulator KiaA, the multifunctional bacterial regulator PutA and the electron transfer flavoprotein [49–51].

Functional studies of *CSK* knockout mutants [39], and the interaction data presented here (figures 1–3) and elsewhere [38], suggest the signalling scheme shown in figure 4 for the transcriptional component of photosystem stoichiometry adjustment. The signalling pathway, in its simplest form, consists of the following components and regulatory events (figure 4). Light 1 causes transient oxidation of the PQ pool and the oxidized PQ activates CSK (figure 4*a*). The activated CSK undergoes autophosphorylation, possibly on serine/threonine residues, and transphosphorylates the SIG1 subunit of the chloroplast RNA polymerase. The phosphorylated SIG1 specifically represses PS I gene transcription, while PS II transcription is unaffected by phosphorylation of SIG1 [53]. Repression of PS I transcription decreases the stoichiometry of PS I to PS II, giving an acclimation to light 1 (figure 4*a*). When plants are moved to the opposite light condition, light 2, the PQ pool becomes momentarily more reduced (figure 4*b*). CSK is then inactive as a protein kinase. A phospho-SIG1 phosphatase is predicted to dephosphorylate phospho-SIG1 and thereby remove the transcriptional repression of PS I genes in light 2 (figure 4*b*). This in turn increases PS I gene transcription and, with it, the ratio of PS I to PS II. A higher PS I/PS II ratio is advantageous in light 2. The predicted SIG1 phosphatase, which is the antagonist of CSK, is likely to be constitutively active in both light conditions [37,38]. However, the kinase activity of CSK can be assumed to be faster than the dephosphorylation reaction of the phosphatase in light 1. The net result is that SIG1 is maintained predominantly in its phosphorylated form. In contrast, in the absence of the kinase activity of CSK, the dephosphorylation reaction dominates in light 2, and the net result is dephosphorylated SIG1.

Although biochemical evidence is, as yet, lacking for the SIG1 phosphorylation by CSK, and although some of the premises of this scheme remain to be tested, the scenario discussed above may be sufficient to explain the transcriptional regulation of PS I genes during photosystem stoichiometry adjustment. We do not yet know the functional implication for the interaction of CSK with PTK ([38]; figure 2). PTK phosphorylates SIG1 and other subunits of PEP to down-regulate global transcription of chloroplast genes in low light [19,22,54]. It may be that CSK interacts with PTK since both kinases act on the same substrate—SIG1. We have earlier raised the possibility that the phosphorylation of PTK by CSK in light 1 inactivates PTK and thereby removes non-specific suppression of chloroplast transcription [37,38]. This effect would produce specific repression of PS I gene transcription by CSK as part of photosystem stoichiometry adjustment. This hypothesis is based on the observation that phosphorylation inactivates PTK as a SIG1/PEP kinase [19,54]. However, the observed phosphorylation of PTK was a result of the action *in vitro* of protein kinase A [19,54], and, to our knowledge, no evidence exists for phosphorylation of PTK *in vitro* or *in vivo* by CSK or any other chloroplast kinase. Phosphorylation of PTK by CSK is a possibility that remains to be pursued, while it is unlikely to require any substantial change to our regulatory scheme for photosystem stoichiometry adjustment (figure 4).

5. How does SIG1 phosphorylation confer photosystem promoter specificity?

SIG1, the most abundant sigma factor in *Arabidopsis* chloroplasts, becomes phosphorylated in PQ oxidizing conditions

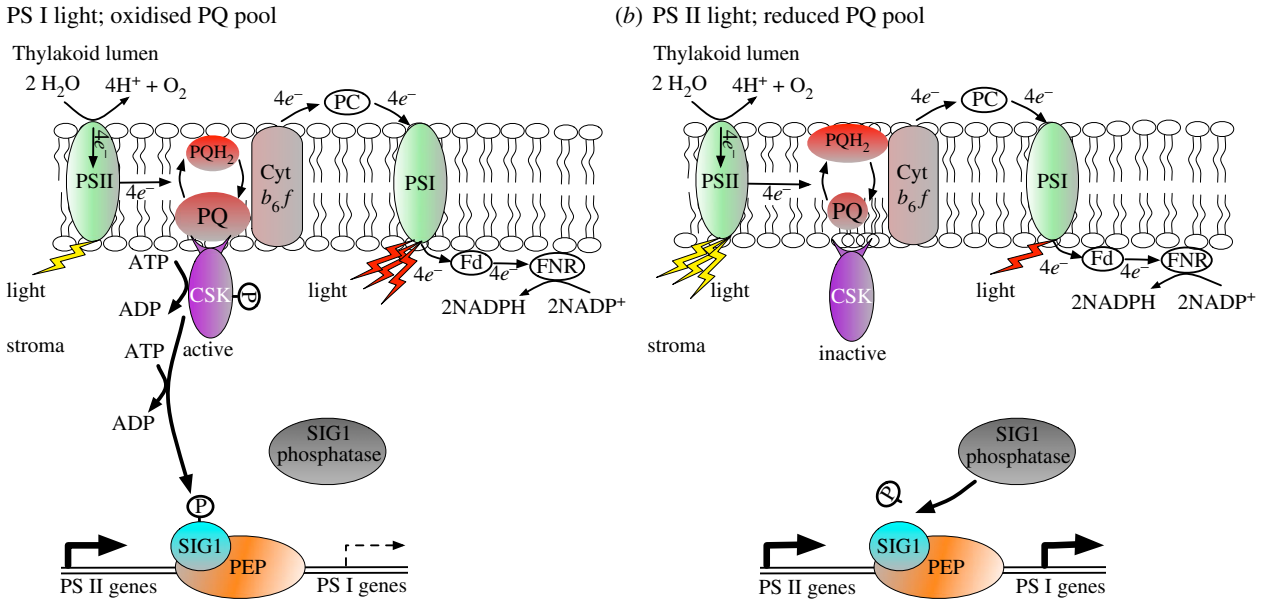

Figure 4. A regulatory scheme for plant and algal photosystem stoichiometry adjustment. Photosynthetic electron transport is depicted diagrammatically, with electrons flowing from photosystem II (PS II) to photosystem I (PS I) via cytochrome b_6f complex (cyt b_6f). PC is plastocyanin, Fd, ferredoxin, and FNR, ferredoxin $NADP^+$ reductase. 'PEP' stands for plastid-encoded RNA polymerase, and SIG1 phosphatase is the predicted protein phosphatase that dephosphorylatates phospho-sigma factor 1 (SIG1). The plastoquinone (PQ) pool regulates the transcription of photosystem genes via chloroplast sensor kinase (CSK). Adapted from [52]. (*a*) Light 1, which preferentially excites PS I, causes transient oxidation of the PQ pool. The oxidized PQ pool activates CSK, which then phosphorylates itself and the SIG1 subunit of PEP. This results in specific repression of PS I genes and, as a result, the stoichiometry of PS I to PS II decreases. (*b*) Light 2, selective for PS II, shifts the PQ pool momentarily to a more reduced state. CSK becomes inactive as a SIG1 kinase in this condition. In the absence of this kinase activity, the SIG1 phosphatase steadily dephosphorylates phospho-SIG1, thus removing the repression of PS I gene transcription. As a result the stoichiometry of PS I to PS II increases.

as part of photosystem stoichiometry adjustment [53]. Though phosphorylated SIG1 recognizes and binds to both PS gene promoters, it specifically represses transcription of PS I genes while leaving the transcription of PS II genes unaffected [53]. What is the molecular mechanism that underlies this specificity? Presuming that the site where SIG1 becomes phosphorylated may hold clues to answer this question, we examined the phosphorylation site of SIG1. SIG1 is phosphorylated on threonine 170, which is located in the N-terminal region of SIG1 [53].

Sigma factors recognize and bind to promoter regions of genes, and thereby enable the RNA polymerase to start transcription at the correct place in the genome [55]. Sigma factors also help the polymerase to melt the double-stranded DNA into the single-stranded template. Sigma factors contain four distinct functional domains known as region 1, 2, 3 and 4. Regions 2 and 4 recognize and bind to the −10 and −35 elements of the bacterial promoters, respectively [55]. Region 1 is found at the N termini of sigma factors, and contains a distinct subregion known as region 1.1, which lies at the extreme N terminus [56]. Region 1.1 is a 100 amino acid-long, poorly conserved, acidic region. Because of its enrichment with acidic residues, it has a net negative charge. Region 1.1 occupies the catalytic cavity of the RNA polymerase, which is lined with basic residues and complements the region 1.1 with a net positive charge [56]. The RNA polymerase exists in two functional states—the closed and open conformations. In the closed conformation, the DNA is bound by the sigma factor, but has not yet entered into the catalytic cavity, which is now occupied by the region 1.1. In the open complex conformation, the DNA, which is negatively charged, enters the catalytic cavity and the negatively charged region 1.1 is ejected [56]. The DNA is then melted and forms the characteristic transcription bubble. The open complex is the transcriptionally competent state of the RNA polymerase.

Region 1.1 is found only in primary—class I—sigma factors. It has been noted that when region 1.1 is present, the efficiency of open complex formation correlates with how well the −10 and −35 elements of the bacterial promoters match the consensus sequences [57]. This means that more open complex formation and transcriptional initiation occur at promoters that match the consensus sequences well, while promoters that have diverged and become weaker have fewer of these transcriptional events. It is assumed that, by some means, the strength of binding of region 2 and 4 to −10 and −35 elements is conveyed to region 1.1. This information determines whether region 1.1 should occupy the catalytic cavity or not [56,57]. If the binding is stronger owing to truly corresponding promoter elements, region 1.1 leaves the catalytic cavity so that DNA can now enter the cavity for transcription. Conversely, if the binding is weaker due to weaker elements, region 1.1 does not leave the cavity, thus preventing the DNA from entering. Region 1.1 has therefore been regarded as the 'gatekeeper' of the catalytic site, as it discourages transcription from weaker promoters and DNA sequences that are similar to promoter elements but are not actual promoters [57].

For this bacterial model to apply to chloroplasts, two requirements must be met. The first is that SIG1 has region 1.1 and the second is that PS II and PS I gene promoters differ in their promoter strengths. SIG1 is a class I sigma factor; however, it has been claimed that it lacks region 1.1 [13], even though it contains an acidic patch in its N-terminal region that corresponds to a region 1.1 in other sigma factors (figure 5). This conclusion was based on the fact that SIG1

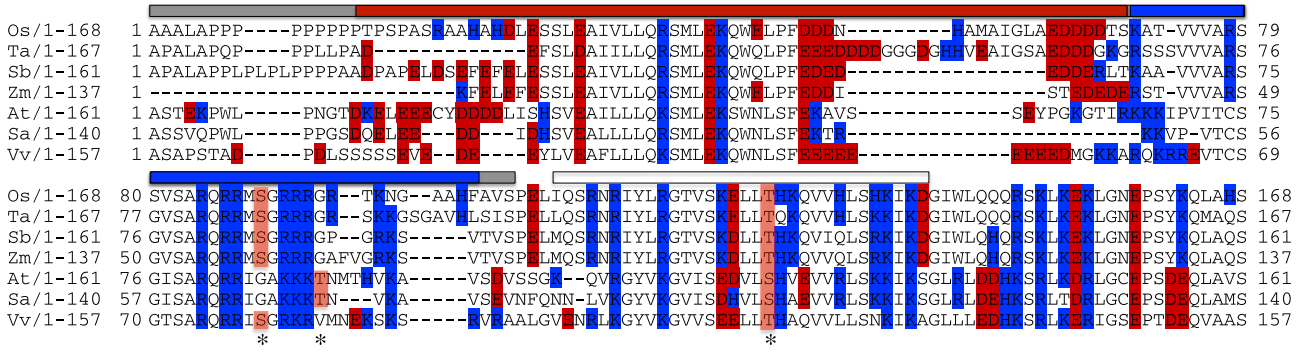

Figure 5. The acidic, basic and phosphorylation site components of the N-terminal region of SIG1. A multiple sequence alignment of SIG1 homologues shows the acidic and basic patches and the phosphorylation site in the putative region 1.1 and 1.2 of SIG1. The acidic residues (D and E) are shaded red and the basic residues (H, K, and R) are in blue. The predicted and observed CSK and PTK phosphorylation sites on SIG1 N-terminal region are shaded light red and marked with a star sign (*) underneath. Note that the CSK phosphorylation site is in region 1.1 and the PTK site in region 1.2. Region 1.1 is indicated by a grey-filled rectangle above the alignment. The red and blue areas on the grey rectangle correspond to acidic and basic patches, respectively. Region 1.2 is marked with an unfilled rectangle. The SIG1 homologues were retrieved by BLAST searches, and the alignment was constructed by ClustalX program. Jalview was used to edit the alignment. Os, *Oryza sativa*; Ta, *Triticum aestivum*; Sb, *Sorghum bicolor*; Zm, *Zea mays*; At, *Arabidopsis thaliana*; Sa, *Sinapis alba*; Va, *Vitis vinifera*.

also contains an extensive patch of basic residues at the C-terminal end of its putative region 1.1 (figure 5). A substantial patch of basic residues makes this amino acid segment an atypical region 1.1, hence it has been instead designated as an un-conserved region (UCR) of SIG1 [13]. This UCR is a 100 amino acid-long region—which is the right size for a region 1.1—with an acidic and basic patch lying side by side (figure 5).

6. A gene-regulatory phosphoswitch in SIG1: a structural model

We propose that the UCR behaves as a region 1.1 in light 1. The basis for this suggestion is as follows. Examination of the phosphorylation site of SIG1, Thr170, reveals that it is located within the basic patch of UCR (figure 5). Phosphorylation is two negative charges at pH values above 7.5. Chloroplast stroma where the phosphorylation of SIG1 takes place, usually has a pH of 8.0 during photosynthesis. We predict that phosphorylation of one or more threonine or serine residues within the basic patch imparts upon UCR the function of a region 1.1. In light 2, the basic patch and the acidic patch form a complex through electrostatic interactions that prevent the acidic patch from entering into the catalytic cavity and engage with the basic residues that line the catalytic cavity (figure 6*a*). In light 1, when the PQ pool becomes oxidized, CSK phosphorylates SIG1 on one or more amino acids within the basic patch. This phosphorylation destabilizes the attraction between basic and acidic patches, thus liberating the acidic patch to engage with the catalytic cavity. When inside the catalytic cavity, the acidic patch acts as the gatekeeper of the catalytic cavity and discriminates between strong and weak promoters (figure 6*a*). When plants are moved from light 1 to light 2, thus reducing the PQ pool in the process, CSK is inactive as a SIG1 kinase. The phospho-SIG1 phosphatase dephosphorylates the basic patch, which then allows it, once again, to form a complex with the acidic patch. In this position, the acidic patch can no longer engage the catalytic cavity and discriminate strong and weak promoters (figure 6*a*).

A smaller patch of basic residues is also seen in the region 1.1 of the primary sigma factor SIGA of cyanobacteria; and its function remains uncertain [59]. For example, in *Nostoc punctiforme* ATCC 29133, the acidic and basic residues are found in a ratio of 2.3 (23 acidic residues and 10 basic residues). Therefore, its region 1.1 still has a net negative charge. However, the UCR of *Arabidopsis* SIG1 has acidic and basic amino acids in a ratio of 0.8 (15 acidic residues and 19 basic residues). Here, the number of acidic residues has gone down when compared with *Nostoc* and the number of basic residues has nearly doubled. SIGAs from other cyanobacterial species show similarly high ratios of acidic-to-basic residues in their regions 1.1 [59], while green algal and plant chloroplast SIG1 show ratios that are closer to unity. It thus appears that cyanobacterial SIGA homologues have net negative charges and that unphosphorylated chloroplast SIG1s have no net charge, as the number of basic and acidic residues has evolved to become equal. We suggest that the selection pressure to evolve the gene-regulatory phosphoswitch drove this change in the composition of amino acids in the putative region 1.1 of SIG1.

The second requirement of our hypothesis is that the photosystem gene promoters differ in their promoter strength. This requirement is satisfied by analysis of the PS II (*psbA*) and PS I (*psaA*) gene promoters (figure 6*b*). The consensus −10 and −35 elements have the following sequence: TATAAT and TTGACA, respectively (figure 6*b*). The nucleotide bases in the −10 box, TATAAT, occur with the following likelihoods: 82|89|52|59|49|89, which means, for example, that the probability of finding a 'T' in position 1 of the TATAAT box is 82% [58]. The *Arabidopsis psbA* −10 box is TATACT and the −35 box, TTGACA (figure 6*b*) [60]. The −10 box of *psbA* has its 5th base as 'C', instead of an 'A' as in the consensus sequence. However, the chance of finding 'A' in that position is only 49%, which is the lowest likelihood among all the bases in the −10 box. The −35 box of *psbA* is fully conserved. This means that the *psbA* promoter is a strong promoter. In contrast, the analysis of the *Arabidopsis psaA* promoter reveals it to be weaker (figure 6*b*). The −10 box of *psaA* is CATAAT and the −35 box, TCCGTT [60]. The −10 box has a cytosine, instead of the consensus thymine, as its first base. The probability of finding the thymine in that position is one of the highest among all other bases. The −35 box of the *psaA* gene, in contrast to that of the *psbA* gene, shows a high degree of divergence, with five out of six bases replaced (figure 6*b*). The same pattern of photosystem promoter divergence is seen in other plant and algal species [60].

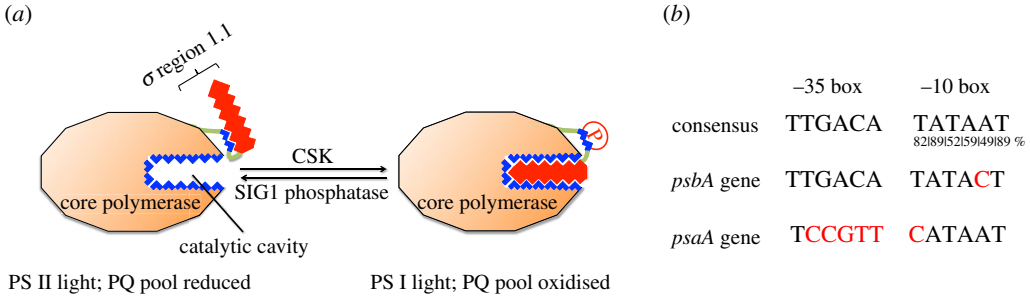

Figure 6. The operation of a gene-regulatory SIG1 phosphoswitch on photosystem gene promoters ensures their differential transcription. (*a*) The operation of the proposed SIG1 phosphoswitch is shown schematically. The core region of the chloroplast RNA polymerase (PEP) is represented with an oval shape, and region 1.1 with a double-edged saw. The red and blue serrations on the region 1.1 and on the inside of the catalytic cavity denote negative and positive amino acid residues, respectively. The region 1.1 is predicted to adopt two conformations depending on the phosphorylation state of its basic patch. CSK and the SIG1 phosphatase are depicted as catalyzing the transition between these conformational states. In PS II light, the acidic patch forms a complex with the basic patch through electrostatic interactions. In this conformation, the acidic patch is kept away from the catalytic cavity by the basic patch. The phosphorylation of the basic patch by CSK in PS I light prohibits its interaction with the acidic patch, in turn freeing up the acidic patch to engage with the basic residues lining the catalytic cavity. When inside the catalytic cavity, the acidic patch acts as the gatekeeper and inhibits transcription from weak PS I gene promoters. The predicted SIG1 phosphatase dephosphorylates the basic patch of region 1.1 and thereby allows it, once again, to form a complex with the acidic patch, a region 1.1 conformation characteristic of PS II light. (*b*) The promoter component of the gene-regulatory phosphoswitch. The −10 and −35 boxes of the consensus *psbA* (PS II) and *psaA* (PS I) gene promoters are shown. The nucleotides that have diverged from the consensus sequence are given in red. The numbers below the −10 consensus sequence represent the probability of occurrences of particular nucleotides in those positions [58].

7. Specific versus general transcriptional response and further SIG phosphoswitches

The two requirements of the bacterial model of gene regulation (figure 6) have been met in chloroplasts, as (i) SIG1 is likely to have a region 1.1-like function in light 1 and (ii) the photosystem gene promoters differ in their promoter strengths. It is now easier to envisage how, as part of photosystem stoichiometry adjustment, in light 1, the region 1.1 will inhibit transcription from the weaker PS I gene promoter, while leaving transcription from the stronger PS II gene promoters unaffected. Conversely, dephosphorylation of region 1.1 in light 2 will derepress the transcription of PS I genes, as region 1.1 no longer retains its gate-keeping role for the catalytic cavity. The functional implication behind the divergence of chloroplast gene promoters also becomes clear in the light of our regulatory model [60]. Our regulatory model (figure 6) also explains why the effect of SIG1 phosphorylation by PTK could be different from that by CSK, as the predicted PTK phosphorylation site on SIG1, S201, lies in a different region, the region 1.2, of SIG1 (figure 5) [25]. The function of region 1.2 is unclear; however, its phosphorylation is likely to affect all chloroplast genes, as the effect of SIG1 phosphorylation by PTK suggests [19,22,23]. We predict that gene regulation via sigma factor phosphoswitches may emerge as a common theme in chloroplasts, as all six chloroplast sigma factors contain predicted phosphorylation sites in their putative region 1.1, 1.2 and 4 [13,61]. These phosphoswitches, acting on diverged chloroplast gene promoters, may generate extensive transcriptional regulation of chloroplast genes, hitherto unanticipated.

8. Evidence for interaction of CSK with SIG1 region 1.1

A key component of the gene-regulatory SIG1 phosphoswitch, as depicted in figure 6*a*, is the phosphorylation of SIG1 by CSK. So far, there is no direct evidence for this. However, evidence exists for the interaction of CSK with SIG1 ([38]; figure 1). In figure 7 we present further evidence for the specific interaction of CSK with the putative region 1.1 of SIG1, using *in vitro* pull-down assay. The bait protein CSK is expressed and purified as a GST fusion protein (lane 1, figure 7) (for materials and methods, see the electronic supplementary material). GST protein on its own is taken as a control bait (lane 2). The prey protein, Region 1.1 of SIG1, is expressed and purified as a maltose binding protein (MBP) fusion protein (lane 3). Region 1.1, in contrast to the mature SIG1, is soluble when expressed in bacteria.

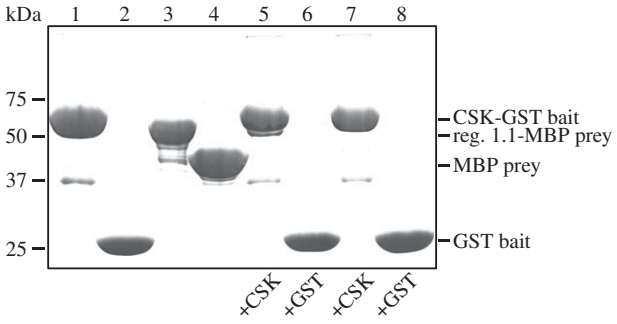

Figure 7. Specific interaction of CSK with the region 1.1 of SIG1. Coomassie-stained SDS–PAGE gel with the products of the third pull-down assay that shows specific interaction between CSK and region 1.1 of SIG1. The positions of molecular weight markers are indicated on the left, and positions of the individual bait and prey proteins are shown on the right. Lanes are labelled numerically at the top of the gel. In lanes 1 and 2, the overexpressed and purified CSK-GST and GST control baits are seen. As in figure 1, the CSK-GST eluate in lane 1 contains a second band at around 37 kDa, indicative of the CSK protein devoid of the GST tag. Lanes 3 and 4 contain the overexpressed and purified region 1.1-MBP and MBP control preys, respectively. Lanes 5–8 show products of the pull-down assay. The baits used in each assay is shown at the bottom of the lane with a '+' sign. In lane 5, the region 1.1-MBP prey protein co-purifies with the CSK-GST bait, but not with the GST control bait, as shown in lane 6. The control prey MBP does not co-purify with either the CSK-G ST bait (lane 7) or the GST bait (lane 8).

The MBP on its own is used as a control prey (lane 4). The region 1.1-MBP prey protein co-purifies with the target bait CSK-GST (lane 5), but not with the control bait GST (lane 6), thus illustrating specific recognition and interaction of region 1.1 with CSK. To rule out the possibility that this interaction (lane 5) might be the result of CSK interacting non-specifically with the MBP tag of the region 1.1-MBP fusion protein, rather than specifically with the region 1.1 itself, we incubated the control prey MBP with both the target and control baits, CSK-GST and GST, respectively. Neither CSK-GST (lane 7) nor GST alone (lane 8) recovers the control prey MBP in its eluate, confirming that the interaction found between CSK and region 1.1 (lane 5) is specific.

9. Conclusions and future perspectives: the rewiring of reaction centre gene regulation

CSK is a cyanobacterial two-component sensor kinase retained by chloroplasts to couple photosynthetic electron transport to the expression of reaction centre genes during photosystem stoichiometry adjustment [37–39,45]. In non-green algae and cyanobacteria, CSK homologues are likely to operate with their response regulator partners in canonical two-component pathways [38,45], and to adjust photosystem stoichiometry through as-yet-unknown regulatory mechanisms. In plants and green algae, CSK is likely to function with a non-response regulator partner, SIG1 [37,38]. It is not clear why the response regulator partner of CSK has been lost in the green lineage. Response regulators usually function in proximity with the RNA polymerase to regulate transcription. Once CSK's response regulator partner has been lost, it is not difficult to envisage how CSK might start phosphorylating a component of the RNA polymerase. The CSK-SIG1 signalling scheme (figure 4) for photosystem stoichiometry adjustment in plants and green algae makes many predictions, which can easily be tested. If this signalling scheme stands up to scrutiny, it will be a prime example of a signalling pathway rewired by evolutionary tinkering. Even though the CSK-SIG1 signalling scheme offers a clear pathway from PQ redox state to reaction centre gene transcription in chloroplasts of green algae and plants, many facets of this regulatory system remain to be uncovered, including but not limited to the precise nature of the regulatory signal sensed by CSK, the signal-sensing mechanism of CSK, the nature of the kinase activity in CSK, the number of target genes under this regulatory system and the existence and operation of the predicted gene-regulatory phosphoswitch in SIG1 (figure 6*a*).

The question of how CSK senses quinone signals from the photosynthetic membrane becomes more pertinent when we consider the fact that CSK is a soluble protein of the chloroplast stroma [37]. Another pressing question concerns the nature of the kinase activity in modified CSKs, which have lost the conserved histidine residue. The basis of the earlier reported ^{32}P labelling in CSK remains ambiguous [39]. Diatom CSKs retain the conserved histidine residue and all characteristic motifs of ATP-binding. An attempt to autophosphorylate unmodified diatom CSKs, *in vitro*, was unsuccessful (S. Puthiyaveetil and I. M. Ibrahim, unpublished results). It thus seems that regardless of the catalytic modification, the bacterially overexpressed and purified CSKs retain no detectable autophosphorylation activity *in vitro*. It may be that the bacterially overexpressed proteins are not folded properly for kinase activity or that we were unable to provide the correct regulatory signal for activation of CSK. New experimental approaches to monitor autophosphorylation both *in vitro* and *in vivo* and new autophosphorylation conditions may be necessary to solve this conundrum. Along these lines, a kinase profiling approach using a fluorescence-based reporter assay in *Arabidopsis* reports serine/threonine autophosphorylation in CSK [62]. However, before interpreting this observation at face value, it is important for it to be verified independently using other assays for kinase activity.

Our current model, developed here from previous proposals [37,38], predicts the presence of a phosphatase activity on SIG1 (figure 4). It has been suggested that the PS II phosphatase, PBCP, is an ideal candidate for SIG1 phosphatase [63]. PBCP is a soluble phosphatase that dephosphorylates the PS II core proteins as part of the PS II repair cycle [64]. If SIG1 phosphatase is indeed the PBCP, it is likely to be present wherever CSK exists as a modified histidine kinase, since only modified CSKs require an antagonist in the form of the SIG1 phosphatase (figure 4). If it turns out that only PS I genes are regulated as part of photosystem stoichiometry adjustment in plants and algae [32,34,53,65,66], as originally proposed for cyanobacteria [67], then regulation of PS I genes may operate by means of a mechanism that was directly inherited by chloroplasts from their cyanobacterial antecedants. The involvement of post-transcriptional regulation of PS I genes by the state transition kinase, Stn7, cannot yet be ruled out in plant and green algal photosystem stoichiometry adjustment. It may also be that the aberrant photosystem gene transcription seen in *Stn7* knockout mutants [68] is the pleiotropic effect of an overreduced PQ pool. Photosystem stoichiometry adjustment in cyanobacteria also involves regulation of genes encoding the light-harvesting antenna of PS II [69]. In this regard, the regulation of the nuclear *Lhc* (*cab)* genes, which encode the light-harvesting complex II (LHC II) antenna of PSII, could be an important part of photosystem stoichiometry adjustment in algae and plants [70]. Plastid-to-nuclear signalling pathways are, however, just beginning to be unravelled [71].

How photosystem stoichiometry is adjusted in algae that do not have CSK is unclear. The completed genomes of the haptophyte alga *Emiliania huxleyi* [72] and green alga *Chlamydomonas reinhardtii* [73] reveal no readily identifiable CSK homologues. Though *Emiliania huxleyi* contains a chloroplast-encoded sensor histidine kinase, ycf26, its role in photosystem stoichiometry adjustment remains to be determined [38,45]. *Chlamydomonas*, likewise, contains chlamyopsin proteins with histidine kinase domains. The functional role of these proteins in light acclimation is far from clear [74]. *Chlamydomonas* may also use post-transcriptional regulation of PS I genes to control photosystem stoichiometry [75]. Notwithstanding these peculiarities and exceptions, the CSK signalling system is a remarkable functional relic from the cyanobacterial ancestry of chloroplasts, and its role in the continued coupling of photosynthetic electron transport and reaction centre gene transcription in cyanobacteria and in chloroplasts is a clear example of the tenacity of the selection pressure that retains genes in organelles [41,43].

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