Inverse transcriptional activities during complementary chromatic adaptation are controlled by the response regulator RcaC binding to red and green light-responsive promoters

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Summary

Complementary chromatic adaptation (CCA) provides cyanobacteria with the ability to shift between red and blue-green phenotypes that are optimized for absorption of different wavelengths of light. Controlled by the ratio of green to red light, this process results from differential expression of two groups of operons, many of which encode proteins involved in photosynthetic light harvesting antennae biogenesis. In the freshwater species Fremyella diplosiphon, the inverse regulation of these two classes is complex and occurs through different mechanisms. It also involves a two-component pathway that includes a phytochrome-class photoreceptor and the response regulator RcaC. Here we uncover the mechanism through which this system controls CCA by demonstrating that RcaC binds to the L Box within promoters of both classes of light-regulated operons. We provide functional evidence that complementary regulation of these operons occurs by RcaC’s simultaneous activation and repression of transcription in red light. We identify rcaC and L Boxes in the genome of a marine cyanobacterium capable of CCA, suggesting widespread use of this control system. These results provide important insights into the long-standing enigma of CCA regulation and complete the first description of an entire two-component system controlled by a phytochrome-class photoreceptor.

Introduction

One of the most striking of all physiological responses, the process of complementary chromatic adaptation (CCA) was first described over 125 years ago (Engelmann, 1883; Gaidukov, 1903). During CCA, which occurs in many species of cyanobacteria, cells undergo a dramatic colour change, switching between brick red and green colouration in response to shifts in the ratio of red light (RL; wavelength absorption maximum ($\lambda_{\text{max}}$) = 650–660 nm) to green light (GL; $\lambda_{\text{max}}$ = 540–550 nm) in their environment. This phenotype change is the result of cells adjusting the composition of their photosynthetic light harvesting antennae or phycobilisomes (Gantt, 1981; Glazer, 1984; Sidler, 1994), becoming the colour that is complementary to ambient light. Thus, CCA apparently provides a selective advantage by maximizing photon capture and photosynthetic activity in varying light colours (Campbell et al., 1993). Similar chromatic acclimation processes are widespread in the environment, occurring in freshwater, terrestrial and marine cyanobacterial species (Tandeau de Marsac, 1977; Palenik, 2001; Everroad et al., 2006), but virtually nothing is known about the mechanisms that control such processes in most species.

Complementary chromatic adaptation is the best characterized response of phycobilisomes, which consist of linker proteins and chromophorylated phycobiliproteins, to changing light conditions (Cohen-Bazire and Bryant, 1982; Tandeau de Marsac and Houmard, 1993; Grossman et al., 2003). The molecular events that underlie phycobilisome biogenesis during CCA have been closely studied in the filamentous freshwater cyanobacterium Fremyella diplosiphon/Tolyposiphon sp. PCC 7601 (Tandeau de Marsac and Houmard, 1993; Grossman, 2003; Kehoe and Gutu, 2006). The antennae in this species consist of an internal region called the core and structures called rods that extend outward from the core. Only the composition of the rods changes during CCA. They may contain three types of phycobiliproteins: two types of phycocyanin (PC, $\lambda_{\text{max}}$ = 620 nm) and one type of phycoerythrin (PE, $\lambda_{\text{max}}$ = 566 nm). Constitutive PC, or
PC1, and its associated linker proteins, encoded by the cpc1 operon, make up the core–proximal region of the rod and are not CCA-controlled (Conley et al., 1986; Mazel et al., 1988). Only the core–distal rod segments change during CCA, with inducible PC (PC2) and its linkers accumulating in RL but not GL, while PE and its linkers are abundant in GL and absent in RL. The expression of the operons encoding PC2 and its linkers (cpc2) and PE (cpeBA) were found to parallel the accumulation patterns of the proteins they encoded and to be transcriptionally regulated (Oelmüller et al., 1988a; Casey and Grossman, 1994; Alvey et al., 2007).

However, the mechanism(s) underlying the diametric transcriptional regulation of cpc2 and cpeBA still remains unclear. Three regulatory proteins specific for CCA have been isolated: RcaE, RcaF and RcaC (Chiang et al., 1992; Kehoe and Grossman, 1996; Kehoe and Grossman, 1997). Each has some effect on both cpc2 and cpeBA expression and operates in a two-component phosphorelay, with RcaE functioning as a phytochrome-class photoreceptor and sensor kinase, and RcaF and RcaC as response regulators (Seib and Kehoe, 2002; Alvey et al., 2003; Terauchi et al., 2004; Li and Kehoe, 2005). RcaC also contains an OmpR family DNA binding motif, but was considered unlikely to be a transcription factor controlling both RL and GL CCA responses for two reasons. First, studies failed to uncover any common DNA sequence element within the cpc2 and cpeBA promoters or within the promoters of cpeBA and cpeCDEF-STR (hereafter cpeC), which includes the genes encoding the PE linkers and is also upregulated in GL (Federspiel and Grossman, 1990; Mazel et al., 1990; Sobczyk et al., 1993). Second, there are significantly different kinetics of cpc2 and cpeBA mRNA abundance changes during CCA (Oelmüller et al., 1988a). Together, these findings suggested that multiple transcription factors were required for the control of gene expression during CCA.

More recently, the upregulation of cpeBA in GL was found to depend on cpeC activation (Cobleby et al., 2002; Seib and Kehoe, 2002), suggesting that key elements for CCA regulation might directly control the expression of cpeC rather than cpeBA (Cobleby et al., 2002). This hypothesis was further strengthened by the identification of a CCA regulatory element that activates RL-responsive genes called the L Box (5′TTGCACAN₄TTGCACA3′) 23–24 bp upstream of transcription start within the promoters of cpc2 and pcyA (encoding phycocyanobilin oxidoreductase) (Alvey et al., 2007). A slightly degenerate L Box was also uncovered at −71 (relative to the presumptive transcription start site) of the cpeC promoter in the reverse orientation, reopening the possibility that a single regulatory element and transcription factor might play a major role in the diometric light regulation of transcription during CCA.

Here we report that RcaC binds to the L Boxes present within the cpc2, pcyA and cpeC promoters. We also provide functional data that demonstrate that the L Box is a CCA regulatory element for GL-induced genes and that it acts by repressing cpeC expression during RL growth. Our findings demonstrate that RcaC and L Boxes are the terminal regulatory components responsible for co-ordinating RL and GL transcriptional responses during CCA. We also identify these regulatory components in a marine cyanobacterium that is capable of CCA but not closely related to F. diplosiphon, suggesting widespread use of this control system. These results provide the first clear mechanism of action for a phosphorelay system controlled by a prokaryotic phytochrome family member and significantly advance our understanding of the molecular basis of light regulation of phycobilisome biogenesis and CCA regulation.

Results

RcaC binds to red light-activated promoters

Protein extracts from F. diplosiphon cells grown in RL bind to the −86 to +15 region of the cpc2 promoter, while protein from GL-grown cells do not (Casey and Grossman, 1994). We determined that this RL-specific binding was absent in a rcaC null mutant, proving that RcaC was required for this activity (Fig. 1A). This region of the cpc2 promoter contains the L Box, a RL activation element (Alvey et al., 2007), and this binding activity was specific for DNA containing an L Box (Fig. 1B). Because it was not possible to purify large amounts of soluble, full-length RcaC, we purified a form of RcaC containing the amino-terminal receiver module and DNA binding motif that we have named RcaCΔ (Fig. 2A) and performed DNase I footprinting studies using this protein and the cpeBA promoter. Both the top (template) and bottom (coding) strands were strongly protected from cleavage over the area encompassing the L Box (Fig. 2B and C). Protection of both strands was detectable at a minimum concentration of 1−3 μM RcaCΔ. The bottom strand protection was from −47 to −22, while the top strand was protected from −43 to −16. The OmpR/PhoB-class binding domain of RcaC was responsible for this protection, as a truncated form of RcaC containing the DNA binding domain (RcaCΔD) alone protected the bottom strand over the same region of the promoter (Fig. S1). The RcaCΔ–DNA interaction also created a hypersensitive site downstream of the L Box on the bottom strand at −10 (Fig. 2B).

We tested whether RcaCΔ also bound the L Box present upstream of pcyA, which is fivefold activated by RL (Alvey et al., 2007). DNase I footprinting analysis revealed that this protein protected a region centred over the pcyA L Box extending from −44 to −18 on the bottom strand and
from -44 to -22 on the top strand (Fig. 3). RcaC\textsuperscript{5} binding was specifically to the L Box region of this DNA fragment, as no protection was observed in footprinting studies using the same pcyA promoter DNA region with a mutated L Box sequence (Fig. 4). RcaC\textsuperscript{5} interaction with the pcyA L Box region appears to be weaker than with the cpc2 L Box as protection from cleavage was not observed below a protein concentration of 6–12 \(\mu\text{M}\). RcaC binding to the pcyA L Box also resulted in the creation of at least one hypersensitive cleavage site upstream of the L Box on the top strand (Fig. 3).

**An L Box controls the light colour regulation of cpeC**

The L Box was shown to be a positively acting element that confers CCA regulation to the RL-activated cpc2 and pcyA promoters (Alvey *et al*., 2007). The associated discovery of a slightly degenerate L Box at -71 within the cpeC promoter, in the opposite orientation from those in the RL-activated promoters, raised the possibility that this element might also be involved in the transcriptional regulation of the cpeC operon. We tested this by fusing the region from -421 to +38 to gusA and assaying *F. diplosiphon* cells transformed with a plasmid containing this construct, p400cpeCGUS, for light regulation of GUS activity. These transformants had twofold greater GUS activity in GL than when grown in RL (Fig. 5). This difference was statistically significant (\(P = 0.0035\)). The role of the L Box in the light regulation of this promoter was tested by substituting random nucleotide sequences for the L Box direct repeats and testing *F. diplosiphon* cells transformed with this modified form of p400cpeCGUS for GUS activity after growth in RL and GL. Mutation of

![Fig. 1](image1.png)

**Fig. 1.** A. cpc2 promoter binding by partially purified protein extracts from wild-type (WT) cells grown in RL or GL and rcaC null mutant cells grown in RL. B. Specificity of binding is demonstrated by competition using unlabelled specific (probe) or non-specific (pcyA) DNA. The region of pcyA used did not contain an L Box. Molar ratios of labelled to unlabelled DNA are provided. Free probe is indicated by the closed arrow, protein–DNA complex by the open arrow.

![Fig. 2](image2.png)

**Fig. 2.** A. Schematic diagram of RcaC and RcaC\textsuperscript{5}. Receiver domains (RD), DNA binding domain (DBD) and histidine-containing phosphotransfer (HPT) domain are shown. N, amino terminus; C, carboxy terminus. DNase I footprinting assays of the cpc2 promoter (B) bottom and (C) top strands. G + A indicates a guanine and adenine chemical cleavage reaction. Concentration of RcaC\textsuperscript{5} protein (\(\mu\text{M}\)) used in the assay is at top. Thick lines denote protected regions and numbering is relative to transcription start site. Arrowhead indicates hypersensitive site location. Each assay was repeated independently two or three times.
the L Box repeats resulted in the loss of light regulation \((P = 0.778)\), which occurred as a result of the loss of RL downregulation rather than GL activation (Fig. 5). This demonstrates that the \(cpeC\) L Box is a CCA regulatory element, but that unlike the \(cpc2\) and \(pcyA\) L Boxes, it represses transcription in RL.

**RcaC binds the L Box within the cpeC promoter**

Our recent discovery of a L Box upstream of the \(cpeC\) operon (Alvey *et al.*, 2007) suggested that RcaC also could be directly binding to this region. This hypothesis is supported by previous findings that the Rca system downregulates the expression of this operon in RL (Seib and Kehoe, 2002; Alvey *et al.*, 2003). The presumptive \(cpeC\) transcription start site, determined using a ribonuclease protection assay, was 187 bp upstream of the CpeC translation start (Fig. S2). This placed the direct repeats of the \(cpeC\) L Box, which are in the opposite orientation relative to those found within the \(cpc2\) and \(pcyA\) promoters, at \(-71\) to \(-88\) relative to transcription start. DNase I footprinting was conducted to determine whether RcaC\(^8\) bound to the \(cpeC\) L Box. There was nuclease protection from \(-68\) to \(-88\) on the bottom strand and from \(-71\) to \(-93\) on the top strand, which is centred over the L Box region (Fig. 6). RcaC\(^8\) binding affinity for this region appeared to be weak relative to the \(cpc2\) and \(pcyA\) L Box regions, as a greater concentration (9–12 \(\mu M\)) of RcaC\(^8\) was required for significant protection from cleavage. RcaC\(^8\) binding also created a hypersensitive cleavage site on the bottom strand at \(-60\). A summary of the regions 5′ of \(cpc2\), \(pcyA\) and \(cpeC\) that were protected from DNase I cleavage by RcaC\(^8\) is provided in Fig. 7.

**RcaC and L Boxes are also present in a marine species capable of CCA**

Analysis of the genome of a marine cyanobacterial species capable of CCA, *Synechococcus* sp. PCC 7335 (https://research.venterinstitute.org/moore/SingleOrganism.do?speciesTag=S7335&pageAttr=pageMain), suggests that the regulation of CCA by the Rca system and L Boxes is very likely to be common in freshwater and marine envi-
Ronments. In this species, the genes encoding RcaE, RcaF and RcaC are clustered in the genome and contiguous with pcyA and cpc2 (Fig. 8A). In addition, an L Box is present upstream of both pcyA and cpc2 in the same orientation as those adjacent to the orthologous genes in *F. diplosiphon*, indicating that these genes are likely activated by RcaC in RL through a similar mechanism. The L Box sequences are not as highly conserved at the first position of each repeat in *Synechococcus* sp. PCC 7335 as in *F. diplosiphon* (Alvey et al., 2007). Interestingly, although no L Box appears to be present upstream of cpeC in this species (data not shown), one is present, in the reverse orientation from those upstream of pcyA and cpc2, upstream of the pebAB operon (encoding phycoerythrobilin synthesis enzymes) (Fig. 8). This suggests that in *Synechococcus* sp. PCC 7335, RcaC may downregulate pebAB expression in RL through a mechanism similar to that used to downregulate the expression of the cpeC operon in RL in *F. diplosiphon*.

**Discussion**

The results presented here reveal the role of RcaC in the regulation of CCA. By binding to L Boxes in CCA-regulated promoters, RcaC activates the expression of RL-upregulated genes and, in conjunction with the Cgi system (Kehoe and Gutu, 2006), represses the expression of the key GL-upregulated operon cpeC, leading to decreased expression of additional GL-induced operons (Cobley et al., 2002; Seib and Kehoe, 2002; Alvey et al., 2003). A model summarizing our current understanding of CCA regulation is provided in Fig. 9.

RcaC appears to bind with greater affinity to the cpc2 L Box than to the pcyA L Box (Figs 2 and 3), and with lesser affinity to the cpeC L Box (Fig. 6). For cpc2 and pcyA, these differences in apparent binding affinity are due to variation in the sequences flanking or separating the L Box repeats, as the direct repeat sequences in these two L Boxes are identical. There is also variation in the sequences surrounding the cpeC L Box repeats, as well...
as a single nucleotide change in one of the cpeC L Box repeats that may contribute to the lower apparent binding affinity of RcaC for this region. It is possible that these apparent differences in binding affinity play a role in the extent of RL transcriptional activation or repression. An affinity hypothesis invoked to explain OmpR action has been brought into question by biochemical studies (Head et al., 1998). However, there may be fundamentally different mechanisms of action for OmpR and RcaC. There are multiple, usually clustered OmpR binding sites upstream of ompF and ompC (Ostrow et al., 1986; Maeda and Mizuno, 1988; Mizuno et al., 1988; Tsung et al., 1989; Maeda and Mizuno, 1990; Huang et al., 1994; Huang and Igo, 1996), while no RcaC binding site clusters appear to exist. OmpR family members, in general, appear to have many different modes of action (Robinson et al., 2003).

The RcaC used in these studies was apparently not phosphorylated, as phosphatase pre-treatment did not affect DNA binding activity (data not shown). However, it is highly likely that this binding is physiologically relevant, as the concentration of RcaC required for DNase I protection in our assays was very similar to those previously reported for unphosphorylated OmpR (Aiba et al., 1989; Huang and Igo, 1996). We were unable to phosphorylate either full-length RcaC or RcaC\textsuperscript{in vitro} with either RcaE and RcaF or a variety of small molecule phosphodonors, and there were no differences between RcaC\textsuperscript{3} binding footprints conducted in the presence or absence of beryllium fluoride (Yan et al., 1999) (data not shown). Also, based on the fact that aspartate (D) to glutamate (E) changes partially mimic the phosphorylated, and D to asparagine (N) changes fully mimic the unphosphorylated forms of response regulators (Klose et al., 1993; Moore et al., 1993), site-directed mutagenesis studies indicate that D51 is the probable site of regulatory phosphorylation activity and that this residue is phosphorylated in RL and dephosphorylated in GL (Li and Kehoe, 2005). Therefore, we conducted DNase I footprinting studies using the cpc2 L Box region and forms of RcaC with either a D51E or D51N mutation.
provided the cpc2 promoter with equivalent protection from DNase I digestion (data not shown). The reason for this is currently unclear.

The 459 bp cpeC promoter region transcriptionally fused to gusA conferred only a twofold increase in GUS activity in GL (Fig. 5), although cpeC mRNA is 10 times more abundant in GL in wild-type cells. All upstream regulatory elements were likely included as this fragment extends upstream of cpeC to the adjacent open reading frame (GenBank Accession No. EF531616). Also, GUS activities were probably accurate reflections of mRNA levels for this reporter gene, as we have never observed such discrepancies for the cpc2, cpeBA or pcyA promoters (Casey and Grossman, 1994; Alvey et al., 2007) (L.O. Seib and D.M. Kehoe, unpubl. res.). Although CCA regulation of the cpc2 and cpeBA operons was shown to be transcriptional (Oelmüller et al., 1988a; Casey and Grossman, 1994), the level of regulation through which CCA

Fig. 9. Model of the mechanism controlling CCA-responsive gene expression in F. diplosiphon. RcaC (black circle) phosphorylation and binding to L Boxes (striped boxes) in RL leads to the simultaneous activation of pcyA and cpc2 for the production of PC-containing phycobilisomes (PBS), and repression of cpeC. In GL, decreased phosphorylation of RcaC and cellular RcaC levels (Li and Kehoe, 2005) result in reduced binding to L Boxes and the loss of pcyA and cpc2 transcription. The reduction in binding of RcaC to the cpec L Box results in the derepression of cpeC, with subsequent activation of cpeBA and pebAB via the action of CpeR and production of PE-containing phycobilisomes. The Cgi system acts cooperatively with the Rca system to regulate GL-activated gene expression. Its mechanism of action, including whether it activates in GL or represses in RL, or both, is currently unknown. However, it may operate post-transcriptionally. Bent arrows, transcription activity; arrowheads denote L Box orientations (see Fig. 8 legend).
controls cpeC expression has never been tested. We predict that control of the 10-fold increase in cpeC RNA in GL is both transcriptional and post-transcriptional. As the GL induction of cpeC is co-regulated by the Rca and Cgi systems (Seib and Kehoe, 2002; Alvey et al., 2003; Kehoe and Gutu, 2006), and the Rca system alone controls the twofold CCA transcriptional regulation of cpeC (Fig. 5), we envisage that the Cgi system regulates cpeC expression post-transcriptionally.

We did not identify any L Boxes in the regions upstream of two other GL-activated operons regulated by the Rca system, cpeBA and pebAB (Mazel et al., 1986; Alvey et al., 2003), consistent with the hypothesis (Cobley et al., 2002) that GL activation of cpeC (and CpeR production) is required for the expression of these operons (Cobley et al., 2002; Seib and Kehoe, 2002; Alvey et al., 2003). Thus, the cpeC operon appears to be the key control site for Rca system regulation of GL-activated genes during CCA.

In RL, RcaC both activates and represses gene expression (Fig. 5) (Alvey et al., 2007), similar to OmpR, which diametrically regulates the porin-encoding genes ompC and ompF (Feng et al., 2003). For RcaC, two factors are important to which role it plays. The first is the intrinsic activities of the cpc2 and cpeC promoters in the absence of RcaC: cpc2 is not expressed in the absence of RcaC (Li and Kehoe, 2005) and thus has the capacity to be activated, while the cpeC promoter is active in the absence of RcaC binding (Fig. 5) and thus can be repressed by this protein. The second factor is the location and perhaps the orientation of the L Box relative to transcription start. For pcyA and cpc2, the L Box is likely to position RcaC so that it positively interacts with RNA polymerase, as do other OmpR/PhoB family members (Matsuyama and Mizushima, 1987; Makino et al., 1993). The opposite orientation of the L Box of cpeC and its greater distance from the transcription start site may position RcaC so that it interferes with a positively acting factor(s) that bind upstream of the L Box, thereby decreasing transcriptional activity. Alternatively, RcaC binding may cause DNA bending, as suggested by the hypersensitive sites resulting from RcaC binding (Figs 2, 3 and 6) and as appears to be the case for OmpR and PhoB (Slauch and Silhavy, 1991; Blanco et al., 2002). Such topology changes could lead to transcriptional activation or repression.

The DNA binding results presented here provide the final link in the first description of a two-component system controlled by a phytochrome-class photoreceptor in a prokaryote and further expand the known roles of OmpR/PhoB-class transcription factors in environmental responses. As many cyanobacterial genomes contain large numbers of OmpR/PhoB-class transcription factors [50% of identified transcription factors in Synechococcus elongatus PCC 7942, 32% of those identified in Nostoc punctiforme ATCC29133 and 36% of those identified in Anabaena variabilis ATCC29413 (http://genome.jgi-psf.org/mic_home.html)], such transcription factors are likely to be important for light regulation of gene expression. Indeed, two OmpR-class proteins, RpaA and RpaB, have been shown to be involved in light energy transfer during photosynthesis and present in a wide range of cyanobacteria (Ashby and Mullineaux, 1999; Ashby et al., 2002). This work also provides critical insights into the central mechanisms controlling CCA in F. diplosiphon and makes an unexpected contribution to the long-standing debate of whether the process of CCA is controlled by a single or dual photosensory systems (Bogorad, 1975; Vogelmüller and Scheibe, 1978; Oelmüller et al., 1988b; 1989) by demonstrating that although two signal transduction systems are involved, one of these, the Rca system, controls the expression of both RL- and GL-upregulated operons through specifically located L Boxes. In addition, the differences in the placement of L Boxes within the genomes of F. diplosiphon and Synechococcus sp. PCC 7335 (Figs 8 and 9), which inhabit freshwater lake and marine intertidal regions respectively, indicates that while the Rca system and L Boxes are clearly pervasive and central elements of CCA regulation, variation in L Box location and primary sequence are likely to be important mechanisms for generating subtle variation in the CCA response in different environmental niches.

Experimental procedures

Cyanobacterial growth conditions

Fremyella diplosiphon UTEX 481/Tolyphothrix sp. PCC 7601 cultures were grown as previously described (Seib and Kehoe, 2002). Wild type was Fd33 (also called SF33) (Cobley et al., 1993), an F. diplosiphon mutant that has normal photoresponses but forms discrete colonies on plates.

DNA electrophoretic mobility shift assays

Total soluble protein from either wild-type cells grown in RL or GL or the rcaC null mutant (CR2) grown in RL was extracted as previously described (Li and Kehoe, 2005), then fractionated using ammonium sulphate precipitation. Each fraction was dialysed against 1000 times volume of Binding Buffer (6 mM HEPES pH 8.0, 60 mM KCl, 1 mM EDTA, 10% w/v glycerol) overnight at 4°C with one buffer change. Protein concentration was determined using Bio-Rad Protein Assay reagent (Hercules, CA) and concentrated by centrifugation in a Microcon column (Millipore, Billerica, MA). The 35–65% fraction was used in DNA electrophoretic mobility shift assays as described (Casey and Grossman, 1994; Manna et al., 2000) with slight modification. Primer set cpc2gs5 and cpc2gs3 was used to polymerase chain reaction (PCR)-amplify the region of the cpc2 promoter containing the L Box. Oligonucleotide sequences are listed in Table 1. PCR-
amplified DNA fragments were digested with Xmal and end-labelled using the Klenow fragment of DNA polymerase, [\( ^{32}P \)]-dCTP and [\( ^{32}P \)]-dGTP. Five nanograms of radiolabelled DNA (from \( ^{32}P \) to \( ^{+} \)) were incubated with or without 2 \( \mu \)g of the 35–65% fraction of either wild-type or CR2 proteins in the presence of 2 \( \mu \)M EDTA. Primer set pcyA101gsU and pcyA101gsL (Novagen) to make pETRcaCS. The N-terminus of RcaCS contains an extra alanine and six histidine residues after the translation start, from pPLrcaC (Li and Kehoe, 2005) using primer set Ab1up and Ab1dn. Amplification products were cut with XmaI and end-labelled DNA (from cpc2-400 to cpc2-3P) were used to PCR-amplify the mutated form of the cpc2 promoter region (Alvey et al., 2007). Primer set CDEpcboxmut1 and CDEpcboxmut2 was used to PCR-amplify the promoter region of pcyA using ppcyA-LGUS (Alvey et al., 2007). which contains an extra alanine and six histidine residues between the methionine and leucine at its N-terminal end. The nucleotide sequences of all plasmids generated using PCR were checked by sequencing.

**Production and purification of truncated forms of RcaC**

A truncated form of RcaC (RcaC\(^{\beta} \)) was used for DNA binding studies. RcaC\(^{\beta} \) contains the N-terminal 252 amino acids of RcaC, encompassing the receiver domain and the OmpR/PhoB DNA binding motif (Chiang et al., 1992; Kehoe and Grossman, 1997). RcaC\(^{\beta} \) was synthesized by PCR-amplification of nucleotides 1–756 of rcaC, based on translation start, from pPLrcaC (Li and Kehoe, 2005) using primer set Ab1up and Ab1dn. Amplification products were cut with Ncol and Xhol and ligated into similarly cut pET28a\((+)\) (Novagen) to make pETRcaC\(^{\beta} \). Primer set cpc2gs5 and cpc2gs3 was used to PCR-amplify the cpc2 promoter sequence encompassing –86 to +15 relative to transcription start (Alvey et al., 2007). Primer set pcyAFP1 and pcyApboxxs2 was used to PCR-amplify the promoter region of pcyA (\( –130 \) to +20) relative to red light-specific transcription start (Alvey et al., 2007). Primer set CDEpcboxgs1 and CDEpcboxgs2 was used to PCR-amplify the cpeCDE promoter (\( –129 \) to –30) relative to transcription start. For footprinting of the mutated L Box, the same pcyA primers were used to PCR-amplify the mutated form of the pcyA promoter using ppcyA-LGUS (Alvey et al., 2007) as a template.

**Table 1. List of oligonucleotides used in this study.**

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<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'→3')</th>
<th>Notes</th>
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<tr>
<td>Ab1up</td>
<td>5'-GCCCATGGGCACACCATCACATCCATCACATTGAAAAATCTTGCTTGAGATGAT-3'</td>
<td>(Ncol site underlined, six-histidine tag in bold)</td>
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<td>Ab1dn</td>
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<td>(XhoI site underlined, stop codon in bold)</td>
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<td>5'-GGGCCTAGAAGCCAGGAGTGAAGTGGG-3'</td>
<td>(XbaI site underlined)</td>
</tr>
<tr>
<td>rpacpec2seq</td>
<td>5'-GATTGTGTTTCTGCTCTCTTGG-3'</td>
<td></td>
</tr>
<tr>
<td>cpeC-400</td>
<td>5'-GCGCCGATGCAATCTGGCCACATCGGTGGTGTACATCAAGATTTTTGACGGG-3'</td>
<td>(PstI site underlined)</td>
</tr>
<tr>
<td>cpeC-3P</td>
<td>5'-GCTCTGAGATGATGCTCAAGGTTTACGCG-3'</td>
<td>(Sphi site underlined)</td>
</tr>
<tr>
<td>CDEpcboxx1</td>
<td>5'-AATGAGAAATTGATGCTCAAAATGAGAATTTCTGTAAT-3'</td>
<td>(mutated L Box sequence in bold)</td>
</tr>
<tr>
<td>CDEpcboxx2</td>
<td>5'-ATTGAGAAATTGATGCTCAAAATGAGAATTTCTGTAAT-3'</td>
<td>(mutated L Box sequence in bold)</td>
</tr>
</tbody>
</table>
DNase I protection experiments

The 6.25 μM primers (upstream for bottom strand analyses and downstream for top strand analyses) were labelled with 60 μCi of [γ-32P]-ATP (10 mCi ml⁻¹, Amer sham Biosciences) using 10 units of New England Biolabs T4 polynucleotide kinase. PCR-amplifications were conducted with 0.625 μM labelled and 0.6 μM unlabelled primers and Roche High Fidelity Taq polymerase. Amplification products were passed through a Qiagen PCR clean-up column and checked on a 5% non-denaturing polyacrylamide gel.

DNase I footprinting analyses were performed as described (Galas and Schmitz, 1978) with several modifications. Binding reactions were in 20 μl containing 25 mM Tris pH 8.0, 50 mM KCl, 10 mM MgCl₂, 1% DTT, 62 nM labelled RNA, 2 μg sheared salmon sperm DNA and RcaC5 or RcaC60 at various concentrations (see figures). DNA binding reactions were allowed to proceed at RT for 35 min, then 0.5 units of DNase I (Promega) was added. The digestion was stopped after 3 min by addition of 40 μl of stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS and 100 μg ml⁻¹ yeast RNA).

DNA was phenol/chloroform-extracted, ethanol-precipitated, resuspended in 10 μl loading dye containing 0.2 M urea and electrophoresed in a 6% 7 M urea-Tris-borate-EDTA polyacrylamide gel. G + A ladders were prepared using a previously described method (Maxam and Gilbert, 1977).

Ribonuclease protection assays

Primer set Rpacpec2R and RpacpecL was used to PCR-amplify a region encompassing –228 to +289 relative to the cpeC transcription start site. This product was digested with BamH1 and XbaI and ligated into similarly digested pGEM-7Zf(+) (Promega) to make pGEMCDE. The PCR product sequence was checked by sequencing. Radiolabelled RNA probe was made using the Riboprobe Combination System-SP6/T7 Kit from Promega. Probes were passed through a Princeton Separations Centri-Sep column, then diluted 100-fold in distilled water before use.

An Ambion RPAIII kit was used for cpeC transcription start site determinations. Total RNA was isolated from wild-type F. diplosiphon cells grown under RL or GL as described (Seib and Kehoe, 2002). And 50 μg of RNA was mixed with 2 ng of riboprobe and ethanol-precipitated. RPA was carried out over the manufacturer’s instructions and the products electrophoresed through a 5% 8 M urea-Tris-borate-EDTA polyacrylamide gel. A sequence ladder was prepared using the USB Sequenase version 2.0 DNA Sequencing Kit and primer rpacpec2seq as directed by the manufacturer.

Promoter-reporter gene construction and GUS assays

The cpeC promoter region was PCR-amplified using primer set cpeC-400 and cpeC-3P. The product was digested with Sphl and PstI and inserted into the same sites in the plasmid pL (Casey and Grossman, 1994) to make pUC-cpeCprom. This plasmid was digested with Sphl and BamH1, and the fragment containing the cpeC promoter was gel-purified and inserted into p2.7GI (Casey and Grossman, 1994) that had been similarly cut to make p400cpeCGUS.

Primer set CDEpcboxmut1 and CDEpcboxmut2 was used to alter the L Box in the cpeC promoter. Primer set cpeC-400 and CDEpcboxmut1 was used to PCR-amplify the upstream region of the cpeC promoter with L Box mutations, and primer set cpeC-3P and CDEpcboxmut2 to PCR-amplify the downstream region of the cpeC promoter with complementary mutations. These two products were combined and used as templates in a PCR reaction that used primer set cpeC-400 and cpeC-3P to amplify the –421 to +38 region of cpeC promoter containing a mutated L Box. This product was cut with Sphl and PstI and used to replace the same region within p400cpeCGUS, making p400cpeCLBKO. The sequences of all plasmids generated using PCR were confirmed by DNA sequencing.

GUS assays were carried out essentially as described (Casey and Grossman, 1994). F. diplosiphon transformants harbouring either p400cpeGUS or p400cpeCLBKO were grown (see Cyanobacterial growth conditions section) in 50 ml of liquid BG-11 with kanamycin (10 μg ml⁻¹) to an A₅₇₀ –0.8, then 0.25 ml of each culture was centrifuged at RT for 1 min at 12 000 g. Pellets were resuspended in 1 ml of GUS assay buffer (50 mM NaPO₄ pH 7.0, 1 mM EDTA) with 6.25 μg ml⁻¹ chloramphenicol and centrifuged as before. Pellets were resuspended in 1 ml of GUS assay buffer, then two drops of 0.1% SDS and four drops of chloroform were added and samples vortexed for 10 s. Assays were conducted in 96-well microtitre plates. Three replicates were conducted for each transformant, and each plasmid was tested in at least three independently transformed lines. In each assay, 20 μl of cell lysate was added to 180 μl of GUS assay buffer containing 1.25 mM α-p-Nitrophenylglycerine and incubated at 37°C. Absorption at 405 nm was recorded every 2 min with a Molecular Devices SpectraMax 190. Sample protein concentrations were measured using a Pierce BCA protein assay kit.

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References


**Supplementary material**

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2008.06151.x

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