The CopRS Two-Component System Is Responsible for Resistance to Copper in the Cyanobacterium *Synechocystis* sp. PCC 6803[1][C][W][OA]

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Photosynthetic organisms need copper for cytochrome oxidase and for plastocyanin in the fundamental processes of respiration and photosynthesis. However, excess of free copper is detrimental inside the cells and therefore organisms have developed homeostatic mechanisms to tightly regulate its acquisition, sequestration, and efflux. Herein we show that the CopRS two-component system (also known as Hik31-Rre34) is essential for copper resistance in *Synechocystis* sp. PCC 6803. It regulates expression of a putative heavy-metal efflux-resistance nodulation and division type copper efflux system (encoded by *copBAC*) as well as its own expression (in the *copMRS* operon) in response to the presence of copper in the media. Mutants in this two-component system or the efflux system render cells more sensitive to the presence of copper in the media and accumulate more intracellular copper than the wild type. Furthermore, CopS periplasmic domain is able to bind copper, suggesting that CopS could be able to detect copper directly. Both operons (*copMRS* and *copBAC*) are also induced by the photosynthetic inhibitor 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone but this induction requires the presence of copper in the media. The reduced response of two mutant strains to copper, one lacking plastocyanin and a second one impaired in copper transport to the thylakoid, due to the absence of the P_I-type ATPases PacS and CtaA, suggests that CopS can detect intracellular copper. In addition, a tagged version of CopS with a triple HA epitope localizes to both the plasma and the thylakoid membranes, suggesting that CopS could be involved in copper detection in both the periplasm and the thylakoid lumen.

Copper is an element required for essential biological processes such as respiration, through the cytochrome oxidase, or in photosynthesis through the electron transfer protein plastocyanin in plants, some algae, and cyanobacteria. It is also used as a metal cofactor of different enzymes including oxidases, monooxygenases, dioxygenases, and superoxide dismutases. The ability of copper to alternate between its cuprous Cu(I) and cupric Cu(II) oxidation states makes it an excellent biological cofactor. However, when unbound within a cell redox cycling means copper is toxic, largely due to its ability to catalyze Fenton-like reaction, causing the production of highly reactive hydroxyl radicals that damage biomolecules such as DNA, proteins, and lipids (Imlay, 2003). An alternative copper toxicity mechanism has been also demonstrated in some bacteria in which copper interferes with the formation of catalytic iron-sulfur clusters, damaging essential enzymatic activities and also leading to the generation of reactive oxygen species (Macomber and Imlay, 2009; Chillappagari et al., 2010; Tottey et al., 2012). As a result, microorganisms have developed diverse mechanisms for the control of copper homeostasis.

Copper homeostasis is a complex process involving acquisition, sequestration, and efflux of the metal ion. In bacteria, active efflux is one of the key mechanisms for copper resistance and three nonrelated families of export system have been implicated in copper resistance and homeostasis: P_I-type ATPases, such as *Escherichia coli* CopA (Rensing et al., 2000; Grass and Rensing, 2001; Rensing and Grass, 2003), heavy-metal efflux-resistance nodulation and division (HME-RND) efflux systems, such as CusBAC (Grass and Rensing, 2001), and membrane proteins such as CopB and CopD from *Pseudomonas syringae* (Mills et al., 1993; Osman and Cavet, 2008). Periplasmic copper metabolism also has an important role in copper homeostasis, since most copper-containing proteins are periplasmic or plasma membrane proteins. In fact, copper homeostasis

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systems usually contain periplasmic copper-binding proteins, and in some cases, copper oxidases, which oxidize Cu(I) to the less toxic Cu(II) (Osman and Cavet, 2008; Kim et al., 2010). In addition, some bacteria contain intracellular copper chaperones, which deliver intracellular copper to target proteins (Robinson and Winge, 2010). These copper resistance systems are, in general, regulated by metalloregulatory proteins that are able to bind the metal. Two unrelated families of copper-responsive repressors have been described: CopY, a winged helix DNA-binding protein, and CsoR, which belongs to a new family of transcriptional repressors (Solioz et al., 2010). In addition, two other regulatory systems that work as activators have been also described: CueR, a MerR family copper-dependent activator (Outten et al., 2000), and CopRS, a two-component copper-responsive system (Osman and Cavet, 2008). CueR, CopY, and CsoR detect cytoplasmic copper levels, while CopRS is thought to detect periplasmic copper.

Photosynthetic organisms have high intracellular copper requirements, mainly for the photosynthetic electron transfer protein plastocyanin, and they have adapted to accommodate variable copper concentrations in the environment. In plants, copper import requires the action of several transporters at different locations in the plant. The import of copper in the roots is mediated by the CTR and ZIP families of transporters while the P_I-type ATPases PAA1 and PAA2 are involved in copper transport into the chloroplast (Pilon et al., 2006, 2009; Puig and Peñarrubia, 2009). Copper transport systems from roots to shoots are much less characterized (Puig and Peñarrubia, 2009). As in other organisms, copper chaperones assist the trafficking and loading of copper to proteins in the cytosol (ATX1, CCH1, CCS1), the mitochondria (COX17), or the chloroplast (CCS1; Puig et al., 2007b). Most of these genes are regulated at the transcriptional level after copper excess. Thus, transporters such as COPT1-2 and COPT4, ZIP2 and 4, and PAA1, PAA2, and HMA1 are down-regulated, while copper chaperones are induced (del Pozo et al., 2010). Under copper-deficiency conditions, photosynthetic organisms express alternative isoenzymes that use different metal cofactors to copper and also induce copper import proteins (Yamasaki et al., 2009; Castruita et al., 2011; Bernal et al., 2012) to save copper for plastocyanin that is strictly required for photosynthesis in plants (Puig et al., 2007a). Some algae and cyanobacteria can also express an alternative electron transfer protein: a heme-containing cytochrome c_6 (Merchant and Bogorad, 1986; Zhang et al., 1992; Merchant et al., 2006). This response is regulated by homologous transcriptional factors in eukaryotic photosynthetic organisms: CRR1 in *Chlamydomonas reinhardtii* (Kropat et al., 2005) and SPL7 in Arabidopsis (*Arabidopsis thaliana*; Yamasaki et al., 2009; Bernal et al., 2012). In contrast, very little is known about copper gene regulation in cyanobacteria despite the early discovery of the switch in gene expression between plastocyanin (encoded by *petE*) and cytochrome c_6 (encoded by *petJ*) depending on copper availability (Zhang et al., 1992). In cyanobacteria, copper metabolism has been analyzed mainly in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). Copper import is mediated by two P_I-type ATPases, CtaA and PacS, a small soluble copper metallochaperone, Atx1 (SynAtx1; Tottey et al., 2002), and a periplasmic iron-containing protein, FutA2 (Waldron et al., 2007). These proteins are required for normal photosynthetic electron transfer via plastocyanin and for the activity of a second thylakoid-located copper protein, a caa_3-type cytochrome oxidase (Tottey et al., 2001, 2002, 2012; Waldron et al., 2007), although the exact role of the periplasmic protein FutA2 is not completely clear (Waldron et al., 2007). Copper is imported inside the cell by CtaA, which delivers it to SynAtx1, which is then thought to transfer it to PacS, which in turn transports it to the thylakoid lumen. Recently, glutathione has been shown to cooperate with SynAtx1 to buffer cytoplasmic copper levels, preventing deleterious side reactions (Tottey et al., 2012).

Here we present evidence that the Hik31/Rre34 two-component system (designated CopRS here) is involved in copper resistance in *Synechocystis* by directly regulating an HME-RND export system, CopBAC (encoded by open reading frames [ORFs] *slr6042*, *slr6043*, *and slr6044*), and a protein of unknown function, CopM (encoded by ORFs *sll0788* and *slr6039*). Although responsive to copper, CopRS is neither involved in the regulation of copper import system nor in the switch between *petE* and *petJ*. Furthermore, using a combination of different genetic and molecular biology approaches, we show that CopS is able to bind copper and partially localizes to thylakoid membranes. *copMRS* is also induced by conditions that alter the electron transport rate around PSI, which indicates that these genes are under redox control. Under these conditions, plastocyanin protein levels decrease, and this mirrors *copMRS* induction. This induction strictly requires the presence of copper in the media and CopRS. Furthermore, induction of *copMRS* after a low copper addition is diminished in mutants with reduced levels of plastocyanin, suggesting that part of the signal detected by CopS needs copper to be incorporated into plastocyanin.

RESULTS

CopRS Is Involved in Copper Resistance

A gene cluster involved in metal resistance in *Synechocystis* was previously characterized (Thelwell et al., 1998; Rutherford et al., 1999; García-Domínguez et al., 2000). The two-component system *hik31-rre34* (*sll0789* and *sll0790*) is located next to the metal resistance cluster, downstream of *ziaA* (Fig. 1A), and code for the closest homolog to the NrsRS two-component system in *Synechocystis* (46% identity; 64% similarity). Upstream of these two genes there is an additional ORF (*sll0788*) that contains two DUF305 domains of unknown function and likely forms an operon with

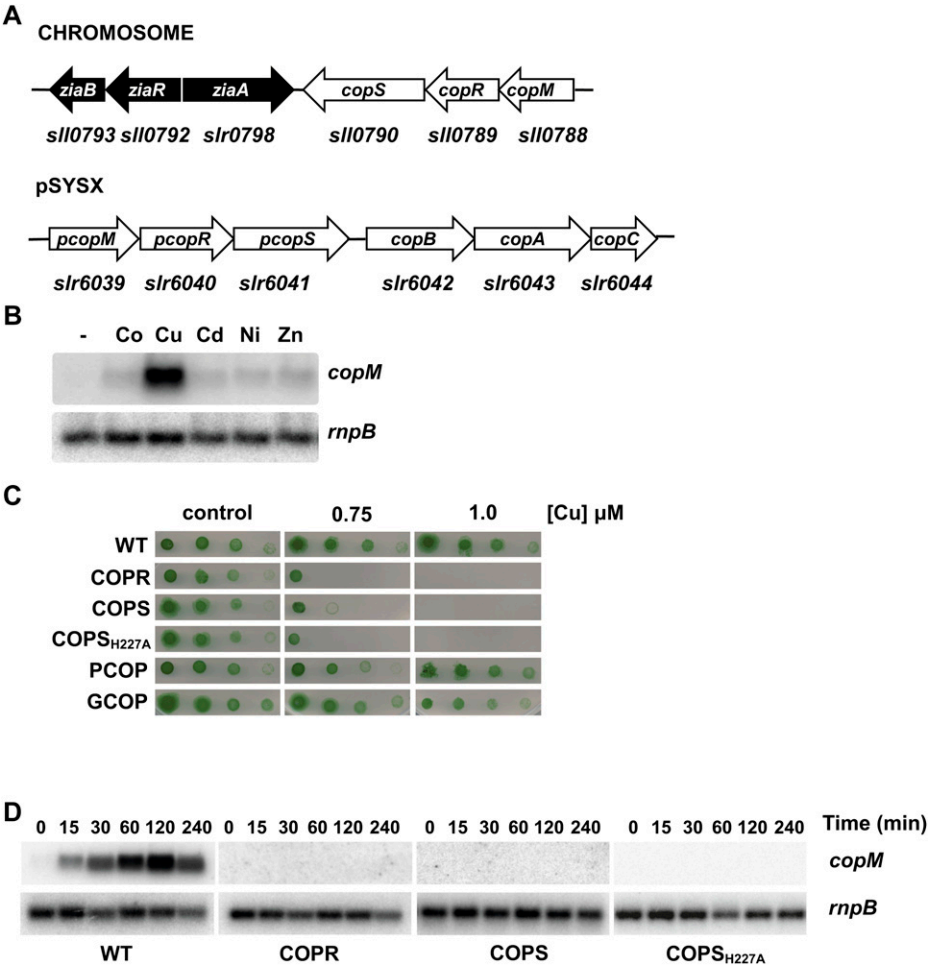


Figure 1. CopRS is involved in copper resistance. A, Schematic representation of *copMRS* and *pcopMRS-copBAC* genomic regions. B, Northern-blot analysis of the expression of *copM*. Total RNA was isolated from wild-type cells grown in BG11C-Cu medium and exposed for 90 min to 3 μM of the indicated metal ions. Control cells were not exposed to added metals (−).The filter was hybridized with a *copM* probe and subsequently stripped and rehybridized with an *rnpB* probe as a control. C, Phenotypic characterization of mutants in *copRS*. Tolerance of wild-type, COPR, COPS, $COPS_{H227A}$, PCOP, and GCOP strains to copper was examined. Tenfold serial dilutions of a suspension of 1 μg chlorophyll mL^{-1} cells were spotted onto BG11C-Cu supplemented with the indicated copper concentrations. Plates were photographed after 5 d of growth. D, Loss of *copM* induction in COPR, COPS, and $COPS_{H227A}$ strains. Total RNA was isolated from wild-type, COPR, COPS, and $COPS_{H227A}$ strains grown in BG11C-Cu medium after addition of 3 μM of copper. Samples were taken at the indicated times. The filter was hybridized with a *copM* probe and subsequently stripped and rehybridized with an *rnpB* probe as a control. [See online article for color version of this figure.]

them. These three genes are repeated in one of the *Synechocystis* endogenous plasmids (Kaneko et al., 2003), pSYSX (*slr6039*, *slr6040*, and *slr6041* with a 93% identity at the nucleotide level, including 71 pb before the starting GTG for *sll0788* and *slr6039*, and 95% at the amino acid sequence level). We have named these genes *copMRS* and *pcopMRS*, respectively. Their location and homology led us to study its putative role in metal resistance. As a first step we have analyzed their expression in response to different metals in the media. We analyzed expression of both *copMRS* and *pcopMRS* since their high sequence homology did not allow us to distinguish between them (therefore we will refer to both copies simply as *copMRS* when analyzing gene expression). As shown in Figure 1B, *copM* expression was induced in the presence of an excess of copper (3 μM $CuSO_4$), but induction by other metals was negligible (Fig. 1B). Furthermore, northern and reverse-transcription-PCR analysis confirmed that *copM* was cotranscribed with *copR* and *copS* and therefore the three genes form an operon (Supplemental Fig. S1; Summerfield et al., 2011). To further study their role in metal homeostasis we analyzed growth of mutant strains lacking one or both copies of these genes (Supplemental Table S1) in the presence of different metals in the media. Mutants lacking a functional copy of *copMRS* (GCOP strain) or *pcopMRS* (PCOP strain; Fig. 1C) are indistinguishable from the wild type. In contrast, double mutants lacking functional copies of both *copR* and *pcopR* (COPR strain), *copS* and *pcopS* (COPS strain), or carrying a mutation in the catalytic His ($COPS_{H227A}$) showed reduced growth at 0.75 μM of copper and failed to grow at 1 μM of the metal (Fig. 1C), showing that this two-component system is essential for copper resistance, but not to other metals (Supplemental Fig. S2). Moreover, COPR cells accumulated about twice the amount of copper than wild-type cells (576 ± 43 versus 339 ± 14 μg copper mg^{-1} dry weight) after a 5-h exposure to 3 μM of copper, suggesting that CopRS controls a copper resistance system.

Two-component systems are often autoregulated in a positive feedback loop, and to test whether CopRS regulated its own expression we analyzed *copM* expression in the COPR, COPS, and $COPS_{H227A}$ strains. *copM* mRNA levels increased (75-fold induction) at least during the first 2 h after addition of 3 μM of copper in wild-type cells, but this induction was completely lost in the COPR, COPS, and $COPS_{H227A}$ strains (Fig. 1D), suggesting that CopRS controls its own induction in response to copper.

CopRS Controls the Expression of an HME-RND Efflux System Involved in Copper Resistance

Downstream of *pcopMRS*, in the plasmid pSYSX, there are three ORFs (*slr6042*, *slr6043*, and *slr6044*) that code for a putative HME-RND transport system (Fig. 1A). These three ORFs code for proteins with homology to a membrane fusion protein, an RND protein, and an outer membrane protein, respectively. We have designated these three genes as *copB*, *copA*, and *copC*. To test if *copBAC* was involved in metal resistance, we analyzed its expression in response to the presence of different metals in the media. *copB* was induced in response to the presence of copper, and, to a lesser extent, zinc, while induction by other metals was negligible (Fig. 2A). Northern and reverse transcription-PCR analysis showed that *copA* and *copC* were also induced by copper, composing a single transcriptional unit with *copB* (Supplemental Fig. S3). Since they were induced by copper, we wanted to test if they were regulated by the CopRS system. *copBAC* expression increased (14-fold induction) after an addition of 3 μM of copper during at least the first 4 h, although with delayed kinetics when compared with *copMRS*. This induction was lost in the COPR strain (Fig. 2B), showing that CopRS is involved in *copBAC* induction in response to copper. To further clarify their role in metal homeostasis, we constructed mutants in all three genes (Supplemental Table S1) and tested their sensitivity to different metals. These strains were sensitive to the presence of copper, but its tolerance to other metals was not drastically different from the wild type (Supplemental Fig. S2). COPB and COPA strains presented growth defects in the presence of 3.5 μM or higher copper concentrations (Fig. 2C). However, the COPC strain showed lower sensitivity to copper, because it was able to grow on 3.5 μM of copper and only at 5 μM of copper was its growth fully inhibited (Fig. 2C). We also analyzed the copper content of COPB cells (which lack expression of *copBAC*) in liquid media and these cells also accumulated 20% more intracellular copper than wild-type cells (400 $\pm$ 8 versus 339 $\pm$ 14 μg copper mg^{-1} dry weight) when challenged with 3 μM of copper for 5 h, although to a lesser extent than COPR cells, which is in agreement with the lower sensitivity of COPB cells to copper in our plate assay.

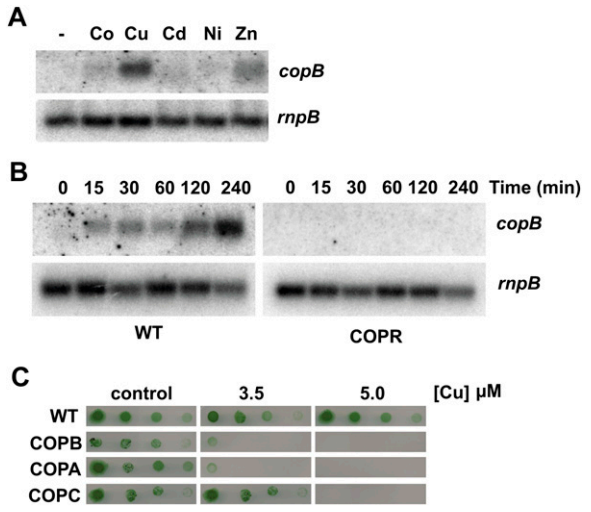


Figure 2. A new RND system involved in copper resistance. A, Northern-blot analysis of the expression of *copB*. Total RNA was isolated from wild-type cells grown in BG11C-Cu medium and exposed for 90 min to 3 μM of the indicated metal ions. Control cells were not exposed to added metals (−).The filter was hybridized with a *copB* probe and subsequently stripped and rehybridized with an *rnpB* probe as a control. B, Loss of *copB* induction in the COPR strain. Total RNA was isolated from wild-type and COPR strains grown in BG11C-Cu medium after addition of 3 μM of copper. Samples were taken at the indicated times. The filter was hybridized with a *copB* probe subsequently stripped and rehybridized with an *rnpB* probe as a control. C, Phenotypic characterization of *copBAC* mutants. Tolerance of wild-type, COPB, COPA, and COPC strains to copper was examined. Ten-fold serial dilutions of a 1 μg chlorophyll mL^{-1} cells suspension were spotted onto BG11C-Cu supplemented with the indicated copper concentrations. Plates were photographed after 5 d of growth. [See online article for color version of this figure.]

CopR Binds to copMRS and copBAC Promoters

The transcription start points were determined by primer extension to establish the location of *copMRS* and *copBAC* promoters. Both *copMRS* and *pcopMRS* transcripts start 27 nucleotides upstream of the predicted *copM* or *pcopM* starting codon (Fig. 3A), since these sequences are identical and we could not distinguish between them. *copBAC* transcripts start 19 nucleotides upstream of the putative *copB* starting codon (Fig. 3B). No consensus −10 and −35 boxes could be identified in these promoters but two repeats, in the form of TTTCAT separated by 5 bp, are present in both promoters, replacing −35 boxes (Fig. 3C). CopR belongs to the OmpR family of response regulators that binds to direct repeats around the −35 boxes in promoters to activate transcription (Kenney, 2002; Blanco et al., 2011). To test whether CopR binds to these promoters, we purified a truncated version lacking the amino terminal receiver domain fused to glutathione *S*-transferase (GST; CopRΔN; as we were unable to obtain a soluble full-length protein preparation) and used it in electrophoretic mobility shift assays. CopRΔN was able to bind to probes containing *copM* and *copB* promoters (Fig. 3, D and E) and therefore the repeated sequences found in *copMRS* and *copBAC* promoters are likely to be CopR binding sites to regulate their transcription.

CopS Periplasmic Domain Binds Metals

CopS is composed of two protein domains: a carboxy-terminal domain containing the His kinase catalytic site and amino-terminal sensor domain. This sensor domain contains two putative transmembrane segments (residues 15–37 and 185–207) and a putative periplasmic region. To test if the periplasmic region

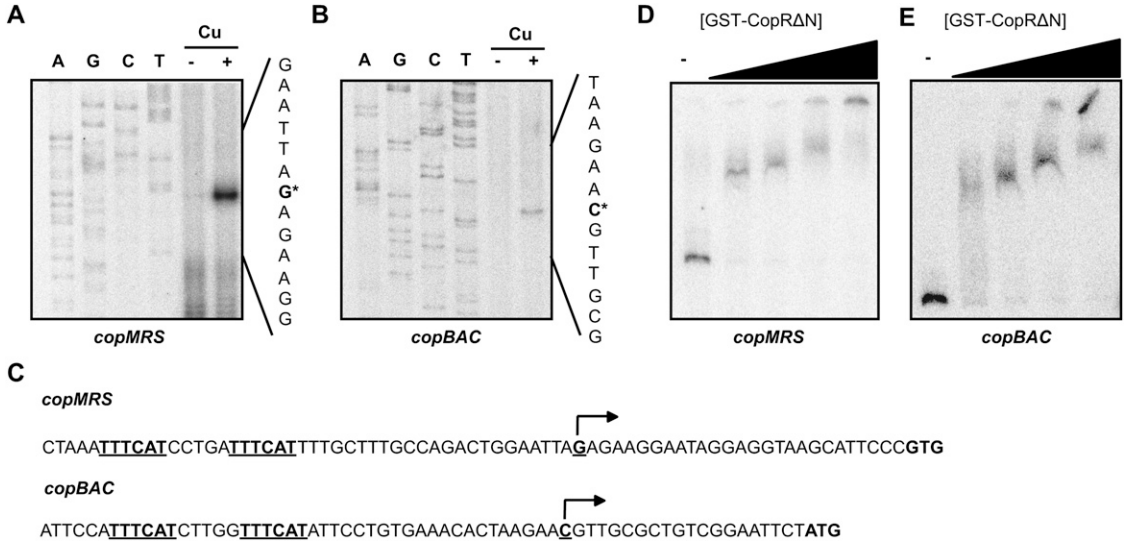
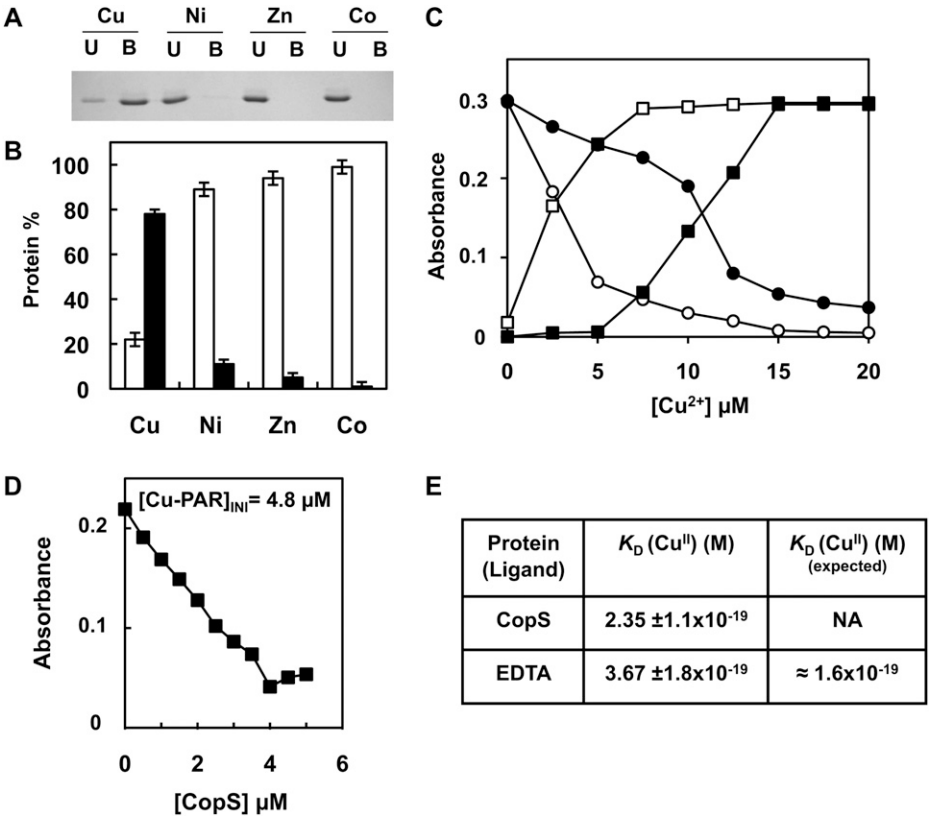


Figure 3. CopR regulates directly *copMRS* and *copBAC* promoters. A, Primer extension of *copMRS* and *pcopMRS* transcripts from wild-type cells grown in BG11C-Cu medium and exposed to copper 3 μM for 1 h. Sequencing ladders generated with the same oligonucleotide used for primer extension is also shown. B, Primer extension of *copBAC* transcript from wild-type cells grown in BG11C-Cu medium and exposed to copper 3 μM for 1 h. Sequencing ladders generated with the same oligonucleotide used for primer extension is also shown. C, Sequences of the *copMRS* and *copBAC* promoters. Transcriptional start sites are marked with an arrow and direct repeated sequences are underlined. D, Band-shift assay of the *copMRS* promoter region with increasing quantities GST-CopRΔN. E, Band-shift assay of the *copBAC* promoter region with increasing quantities GST-CopRΔN.

was able to bind metals, we have expressed and purified the region between the transmembrane segments (expanding from residues 38–183) fused to a strep-tag to facilitate its purification ($CopS_{38\text{-}183}$). We tested whether $CopS_{38\text{-}183}$ was able to bind metals using metal chromatography. The protein was retained by beads charged with 0.5 mM of Cu^{2+} but not by Zn^{2+}, Ni^{2+}, and Co^{2+} charged beads (Fig. 4, A and B). To further analyze $CopS_{38\text{-}183}$ interaction with copper we used ligand competition with apo-4-(2-pyridylazo)-resorcinol (PAR). $CopS_{38\text{-}183}$ was able to extract one equivalent of Cu^{2+} from PAR, suggesting that one atom of copper binds to one molecule of $CopS_{38\text{-}183}$ (Fig. 4C). Titration of Cu^{2+}-loaded PAR with increasing amounts of $CopS_{38\text{-}183}$ revealed a concentration-dependent decrease in PAR-Cu^{2+} concentration (Fig. 4D; Supplemental Fig. S4), and allowed us to calculate an apparent dissociation constant (K_{Dapp}) for $CopS_{38\text{-}183}$ of $2.3 \cdot 10^{-19}$ after calibration of the assay with EDTA (Fig. 4, D and E). These data demonstrated that CopS periplasmic region is able to bind copper with high affinity in vitro.

Redox Induction of copMRS Depends on the Presence of Copper

Previous microarray studies have shown that *copMRS* operon is highly induced by 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB; which blocks electron transfer from the plastoquinone pool to the cytochrome b_6f), but not by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; which blocks electron transfer from PSII to the platoquinone pool; Hihara et al., 2003), suggesting that these genes were controlled by the redox state of the plastoquinone pool. Having established that *copMRS* had a role in copper homeostasis, we wanted to investigate if there was any interaction between copper metabolism and DBMIB induction of *copMRS*. First, we confirmed that the addition of 10 μM DBMIB to a *Synechocystis* culture induced expression of *copMRS* (Fig. 5A; Supplemental Fig. S5A) and *copBAC* (Supplemental Fig. S5A), but these genes were not induced by the addition of 10 μM DCMU (Supplemental Fig. S6). Second, when the DBMIB treatment was performed in a medium without copper (BG11C-Cu) plus bathocuproinedisulfonic acid (BCSA), a copper chelator, to avoid any residual copper in the media (Durán et al., 2004), neither *copMRS* nor *copBAC* operons were induced, as determined by *copM* and *copB* expression (Fig. 5A; Supplemental Fig. S5A). DBMIB treatment in the COPR strain was also ineffective at inducing the expression of both *copM* and *copB* (Supplemental Fig. S7). However, *sll0528*, another gene induced by DBMIB in the microarray analysis (Hihara et al., 2003), was still fully induced in both cases (Fig. 5A; Supplemental Figs. S5 and S6). These results suggested that induction after DBMIB treatment of both *copMRS* and *copBAC* was related to copper metabolism, rather than a direct effect of the redox state of the plastoquinone pool, and that it was dependent on CopRS.



Protein (Ligand)	K_D (Cu^{II}) (M)	K_D (Cu^{II}) (M) (expected)
CopS	2.35 ±1.1x10^{-19}	NA
EDTA	3.67 ±1.8x10^{-19}	≈ 1.6x10^{-19}

Figure 4. CopS periplasmic domain binds copper. A, Analysis of $CopS_{(38-183)}$ protein interaction with metals. His-Bind resin columns were loaded with 0.5 mM $CuSO_4$, $NiSO_4$, $ZnSO_4$, and $CoCl_2$. About 10 μg of purified $CopS_{(38-183)}$ protein was applied to the columns. Unbound (U lanes) and bound (B lanes) fractions were analyzed by 15% SDS-PAGE and Coomassie Blue staining. B, Quantification of CopS in bound and unbound fractions. Coomassie-stained gel was scanned and the intensity of the bands was quantified using ImageJ program; the graph represents the average of two experiments. Unbound fraction (white), bound fraction (black). C, Titration of PAR, which absorbs at 410 nm (circles), to its copper form absorbing at 500 nm (squares), in the absence (white symbols) and presence (black symbols) of 10 μM CopS. D, Determination of the Cu^{2+} dissociation constant, K_D, of CopS by titration into a solution of 10 μM PAR. The graph shows the decrease at 500 nm relative to CopS additions for a $[Cu\text{-}PAR]_{TOTAL}$ of 0.9 μM. E, Apparent K_D CopS and EDTA at pH 7.5 derived from competition titration using Cu^{2+}-PAR. Dissociation constant, K_D, was estimated as described in "Materials and Methods" from four independent experiments like the one shown in D. NA, Not available.

The Response of CopS to Plastocyanin Protein Levels

Photosynthetic electron transport between cytochrome b_6f complex and PSI is mediated by plastocyanin or cytochrome c_6 depending on the availability of copper in *Synechocystis* (Zhang et al., 1992; Waldron et al., 2007). DBMIB blocks the electron transfer between the plastoquinone pool and cytochrome b_6f, and therefore impairs the plastocyanin and cytochrome c_6 reduction, causing their accumulation in the oxidized form (Trebst, 2007). Plastocyanin is the main copper-containing protein in *Synechocystis* cells and it is confined to the thylakoid lumen (Waldron et al., 2007). To test if DBMIB treatment induces plastocyanin degradation, we analyzed plastocyanin protein levels by western blot. As shown in Figure 5C the amount of plastocyanin rapidly declined after DBMIB treatment. To test whether reduction of plastocyanin levels were responsible for *copM* induction, lincomycin, a protein synthesis inhibitor, was added to *Synechocystis* cells growing in copper-containing medium. Induction of *copM* and *copB* and the decrease in plastocyanin levels occurred in parallel after lincomycin treatment (Fig. 5, B and D; Supplemental Fig. S5), but with delayed time course respective of the DBMIB treatment (Fig. 5). In agreement to this, plastocyanin half-life was 3 times longer in lincomycin-treated cells ($t_{1/2}$=182 min) compared with DBMIB-treated cells ($t_{1/2}$=59 min; Fig. 5F). Similar to the DBMIB treatment, no induction of *copM* and *copB* expression was observed when lincomycin was added to cells growing in medium without copper + BCSA (Supplemental Fig. S5B). Furthermore, we analyzed whether plastocyanin was required for *copM* and *copB* induction. For that, a *Synechocystis* mutant lacking plastocyanin was constructed (PETE) and *copM* induction was followed after the addition of 200 nM of copper, since higher copper concentrations were toxic to the PETE strain. As shown in Figure 6,

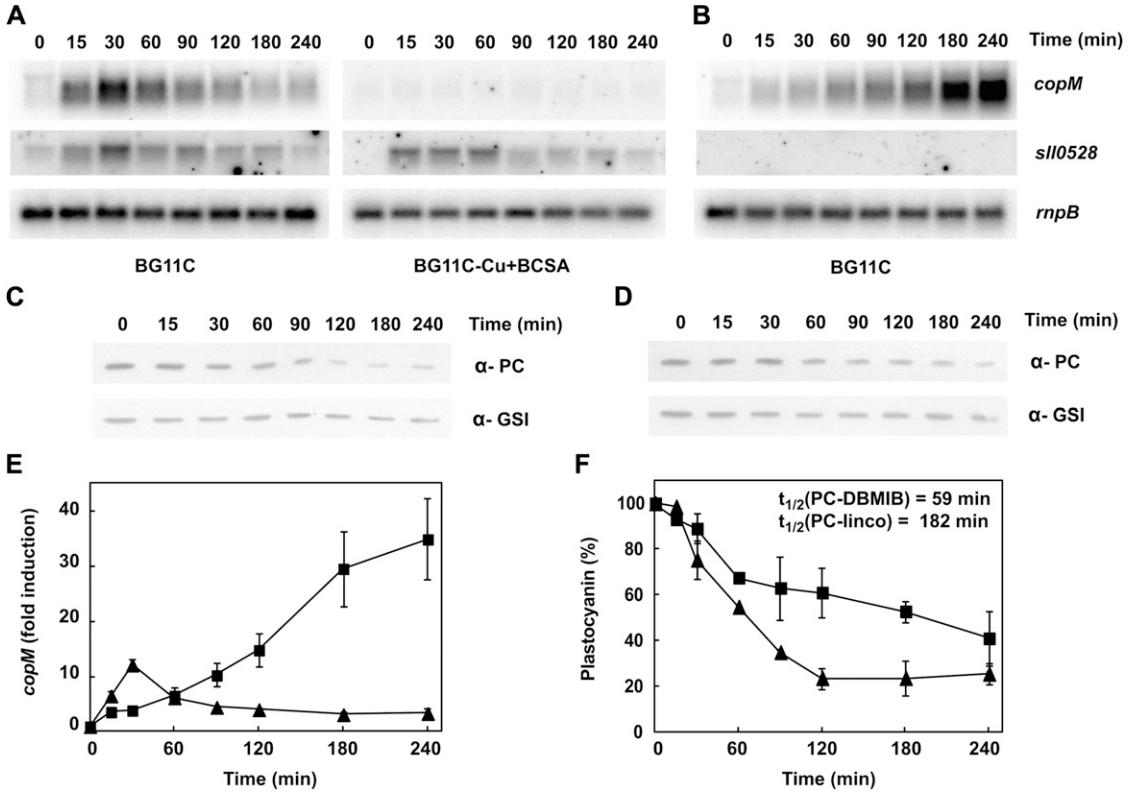


Figure 5. CopS responds to plastocyanin protein levels. A, Northern-blot analysis of the expression of *copM* and *sll0528* after DBMIB addition. Total RNA was isolated from wild-type cells grown in BG11C or BG11C-Cu + BCSA medium after addition of DBMIB 10 μM. Samples were taken at the indicated times. The filters were hybridized with *copM* and *sll0528* probes and subsequently stripped and rehybridized with an *rnpB* probe as a control. B, Northern-blot analysis of the expression of *copM* and *sll0528* after lincomycin addition. Total RNA was isolated from wild-type cells grown in BG11C medium after addition of lincomycin 250 μg mL^{-1}. Samples were taken at the indicated times. The filters were hybridized with *copM* and *sll0528* probes and subsequently stripped and rehybridized with an *rnpB* probe as a control. C, Western-blot analysis of plastocyanin levels after DBMIB addition. Wild-type cells were grown in BG11C medium and exposed for 4 h to DBMIB 10 μM. Cells were harvested at the indicated times, and 5 μg of total protein from soluble extracts was separated by 15% SDS-PAGE and subjected to western blot to detect plastocyanin or glutamine synthetase type I (GSI). D, Western-blot analysis of plastocyanin levels after lincomycin addition. Wild-type cells were grown in BG11C medium and exposed for 4 h to lincomycin 250 μg mL^{-1}. Cells were harvested at the indicated times, and 5 μg of total protein from soluble extracts was separated by 15% SDS-PAGE and subjected to western blot to detect plastocyanin or GSI. E, Quantification of relative mRNA levels of *copM*, in response to DBMIB and lincomycin addition in wild-type strain. Radioactive signals of three independent experiments for each strain were quantified and averaged. RNA levels were normalized with the *rnpB* signal. Plots of relative mRNA levels versus time were drawn; error bars represent SE. DBMIB treatment (triangles), lincomycin treatment (squares). F, Quantification of plastocyanin levels, in response to DBMIB and lincomycin addition in wild-type strain. Western-blot signal of three independent experiments were quantified using Image J program. Plastocyanin levels were normalized with the GSI signal. Error bars represent SE. DBMIB treatment (triangles), lincomycin treatment (squares). Half-life, $t_{1/2}$, of plastocyanin was estimated as described in "Materials and Methods" from three independent experiments.

copM expression was lower in the PETE strain (about 60% of the wild-type induction), although it followed the same kinetics of the wild-type strain (Fig. 6B), suggesting that part of the signal sensed by CopS depends on the presence of plastocyanin in the thylakoid lumen. Copper is delivered to plastocyanin by the sequential action of two P_I-type ATPases, CtaA and PacS, and mutant strains lacking these genes have reduced levels of plastocyanin (Tottey et al., 2001, 2012). We constructed a double mutant lacking both ATPases (SAS strain) to test whether copper import was needed for CopS activation. After the addition of 200 nM of copper to the SAS strain, *copM* induction was also lower (about 50% of the wild-type induction), similar to the PETE strain behavior and with the same kinetics as the wild-type strain (Fig. 6B). Although the behavior of both strains was similar, they accumulated different amounts of intracellular copper after this treatment: The PETE strain accumulated only 60% of the wild-type copper (42.7 $\pm$ 1.8 versus 70 $\pm$ 14 μg mg^{-1} dry weight), while the SAS strain accumulated the same amount as the wild type (71.4 $\pm$ 9.1 μg mg^{-1} dry weight). Even more, the SAS strain failed to do the switch from *petJ*

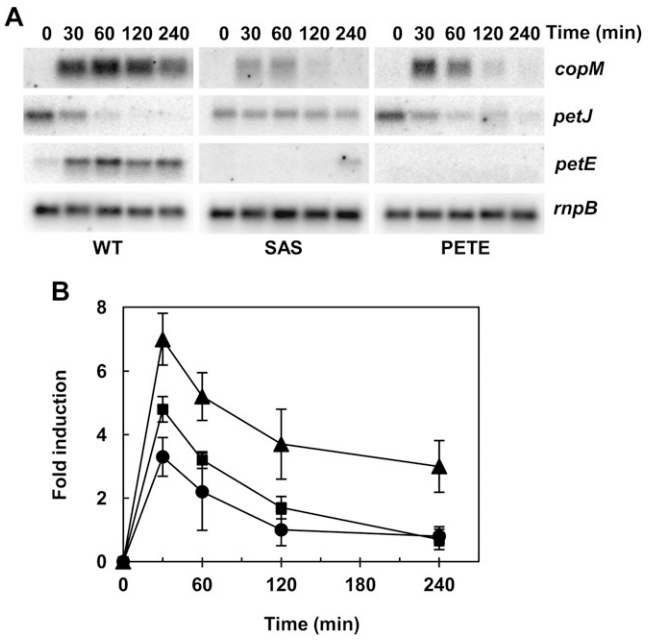


Figure 6. CopS responds to intracellular copper. A, Northern-blot analysis of the expression of *copM*, *petE*, and *petJ* in response to copper addition in wild-type, SAS, and PETE strains. Total RNA was isolated from wild-type, SAS, and PETE cells grown in BG11C-Cu medium after addition of copper 200 nM. Samples were taken at the indicated times. The filters were hybridized with *copM*, *petE*, and *petJ* probes and subsequently stripped and rehybridized with an *rnpB* probe as a control. B, Quantification of relative mRNA levels of *copM* in response to copper addition in wild-type, SAS, and PETE strains. Radioactive signals of three independent experiments for each strain were quantified and averaged. RNA levels were normalized with the *rnpB* signal in all strains. Plots of relative mRNA levels versus time were drawn; error bars represent SE. Wild-type strain (triangles), SAS strain (circles), PETE strain (squares).

to *petE* expression after this low copper addition, unlike the wild-type and PETE strains (Fig. 6). Single mutants in these two ATPases have been shown to accumulate similar copper contents but reduced copper-loaded plastocyanin (Tottey et al., 2001, 2012), and our double mutant (SAS strain) did not express *petE*, reinforcing that copper loading into plastocyanin is needed for activation of CopS.

CopS Is Localized to Both Plasma and Thylakoid Membranes

All of the aforementioned results demonstrated that CopS could detect signals both at the periplasmic space and at the thylakoid lumen (where plastocyanin is located). In that way, CopS would need to be inserted into both the plasma and thylakoid membranes. With the aim of determining the subcellular localization of CopS, we constructed a strain (COPSHA) that expresses CopS fused to a triple HA epitope (CopS-3HA) under control of the *nrsBACD* promoter that is induced by nickel (García-Domínguez et al., 2000; López-Maury et al., 2002). After the addition of 2 μM of nickel for 4 h to the COPSHA strain, thylakoid and plasma membrane fractions were prepared by Suc density gradient centrifugation and aqueous polymer two-phase partitioning (Norling et al., 1998). As shown in Figure 7, a single protein band of the corresponding molecular mass of CopS-3HA (56 kD) was detected in both thylakoids (about 25% of total fraction) and plasma membranes, while marker proteins PsaC (a PSI protein; Kruip et al., 1997) and NtrA (a plasma membrane attached protein; Norling et al., 1998) were exclusively detected in thylakoid fraction and plasma membrane fraction, respectively. This result shows that CopS is localized to both thylakoid and plasma membranes and therefore could perceive signals in both compartments.

DISCUSSION

This work shows the existence of a copper resistance system in *Synechocystis* comprised of a two-component system (CopRS), an HME-RND transport system (CopBAC), and a protein of an unknown function, CopM. CopRS is essential for the expression of both *copBAC* and *copMRS* operons. The system responds specifically to the presence of copper but not to other metals (Figs. 1 and 2). Mutant strains affecting the regulatory system (COPR, COPS, $COPS_{H227A}$) are more sensitive to the presence of copper in the media than strains lacking components of the CopBAC transport system (Figs. 1C and 2C), suggesting that CopRS might control more genes involved in copper homeostasis. These strains lack expression of both *copBAC* and *copM* (Figs. 1D and 2B), and therefore the more likely candidate to be involved in copper resistance is

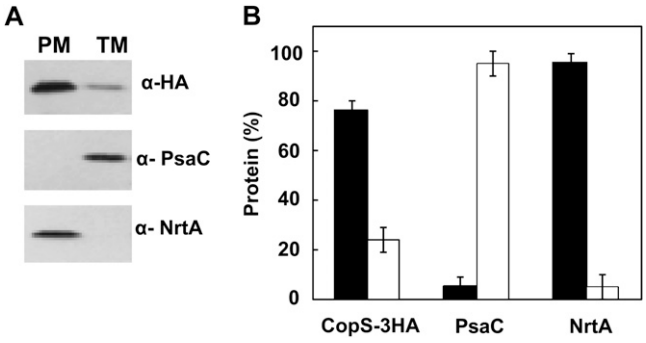


Figure 7. CopS is localized to plasma and thylakoid membranes. A, Membrane localization of CopS. Membrane fractions from COPSHA strain induced for 4 h with 2 μM of nickel were prepared by Suc density gradient and aqueous polymer two-phase partitioning. Five micrograms of total protein were loaded and separated by SDS-PAGE. CopS-3HA, NrtA, and PsaC proteins were detected by western blot. PM, Plasma membrane; TM, thylakoid membrane. B, Quantification of CopS in different membrane fractions. Western-blot signal of three independent experiments were quantified using Image J program and averaged; error bars represent SE. Plasma membrane (black); thylakoid membrane (white).

CopM. CopM contains an uncharacterized Duf305 domain that is present in conserved proteins in several other cyanobacteria (Nagarajan et al., 2012) and bacteria, but the function of proteins containing the Duf305 domain has not been reported. CopM contained an elevated number of Met and His residues and a signal peptide that will target it to the periplasmic and/or thylakoid compartment. In other copper resistance systems, periplasmic proteins with an elevated number of these residues work as copper chaperones, acting either as a buffer and/or transferring periplasmic copper to RND transport systems (Loftin et al., 2005; Bagai et al., 2008; Chong et al., 2009; Mealman et al., 2011) that efflux it outside the cell. Attempts to delete *copM* without affecting *copRS* expression have been unsuccessful, and for that reason we could not determine the contribution of CopM to copper resistance. The fact that COPR strains accumulate more copper than wild-type or COPB cells suggests that either CopM contributes to copper extrusion or that CopRS controls other genes involved in copper transport. Other obvious candidates to be controlled by CopRS are genes that code for proteins required for copper import (*ctaA*, *pacS*, *atx1*), cytochrome c_6 (*petJ*), and plastocyanin (*petE*), all of which are regulated by the presence of copper in the media. We tested whether the expression of these genes was under CopRS control but they behaved similarly in wild-type and COPR strains (Supplemental Fig. S8). On the other hand, mutant strains in *copB* or *copA* tolerated up to 3.5 μM of copper, while mutant strains in *copC* resisted up to 5 μM. *copC* codes for an outer membrane protein, which in other HME-RND systems connects the RND protein to the outer membrane and allows extrusion of metals outside of the cells. In this regard, recent structural and functional studies show that the *E. coli* CusBA complex could be able to transport copper from the cytosol to the periplasm in the absence of CusC, the homolog of *Synechocystis* CopC (Franke et al., 2003; Su et al., 2011), where it could be buffered by CopM.

The CopRS two-component system (previously known as Hik31-Rre34) was reported as affecting *Synechocystis* cell growth under mixotrophic and heterotrophic conditions (Kahlon et al., 2006; Nagarajan et al., 2012), and also in the regulation of the response to low-oxygen conditions (Summerfield et al., 2011). Even more, their mutants lack the expression of *icfG*, a gene essential for Glc metabolism (Kahlon et al., 2006). In our hands, the COPR strain is able to grow in the presence of Glc and expresses the *icfG* gene to levels similar to the wild type, both in the presence and absence of Glc (Supplemental Fig. S9). It has been previously shown that differences in strain genetic background affect Glc sensitivity in *Synechocystis* (Kahlon et al., 2006), and this could explain these discrepancies. Nagarajan et al. also showed that their single and double mutants of the *copMRS* genes presented different metal sensitivities to nickel, cobalt, zinc, and cadmium (Nagarajan et al., 2012), but our mutants in both *copRS* or *copBAC* were as resistant as the wild type to all metals except copper (Figs. 1 and 2; Supplemental Fig. S2).

In contrast to most bacteria, cyanobacteria have high intracellular copper requirements in the form of the electron transfer protein plastocyanin (Waldron et al., 2007). This protein is localized to the thylakoid lumen and is essential for electron transfer reaction during photosynthesis in copper-containing media (Durán et al., 2004). *copMRS* has been described to be highly induced by different conditions, all of which alter the photosynthetic electron transport, such as treatment with DBMIB (Fig. 5; Hihara et al., 2003), nitrogen starvation (Supplemental Fig. S10; Osanai et al., 2006), and sulfur starvation (Zhang et al., 2008). We have shown here that induction in DBMIB-treated cells (Fig. 5) and nitrogen-starved cells (Supplemental Fig. S10) is dependent on the presence of copper in the media, thus establishing that this induction is related to copper metabolism and not to other factors. All of these conditions have in common a general decrease in photosynthetic electron flux (or a complete blockage, in the case of DBMIB) that will probably lead to accumulation of oxidized plastocyanin. We have shown that under these conditions plastocyanin protein levels are reduced in vivo (Fig. 5; Supplemental Fig. S10). This reduction in plastocyanin protein levels leads to activation of CopS (Fig. 5). Further support for this comes from the induction of *copM* and *copB* after treatment with the translation inhibitor lincomycin, which also causes a reduction in plastocyanin protein levels (Fig. 5D). In both cases, induction of these genes correlates with plastocyanin degradation, although the response is maintained after lincomycin treatment since it completely blocks translation and therefore cells are not able to respond to this treatment. Furthermore, these results are reinforced with our genetic data about *copM* and *copB* induction using the PETE and SAS strains (Fig. 6). Both of these mutants lack copper plastocyanin in the thylakoid lumen (Fig. 6; Waldron et al., 2007; Tottey et al., 2012) and show a reduced induction of the *copMRS* operon, even if they accumulate less (PETE mutant), or the same amount of (SAS mutant), copper than the wild-type strain. These data strongly suggest that copper needs to be incorporated into plastocyanin to be detected by CopS. Plastocyanin degradation will probably release copper into the thylakoid lumen and this copper could be detected by CopS. In addition, we have shown that the CopS periplasmic domain is able to bind one atom of Cu^{2+} with comparable affinity to the recently described MAP kinase (Turski et al., 2012), supporting that CopS detects copper directly (Fig. 4). All of these data, together with the localization of CopS to both plasma and thylakoid membranes (Fig. 7), showed that this protein responds to copper (probably by direct binding to it) in both the periplasm and the thylakoid lumen (Fig. 8). Since plastocyanin levels have been estimated to be in the high micromolar range inside the thylakoid (Durán et al., 2004; Finazzi et al., 2005),

even a small decrease in plastocyanin levels could generate large amounts of free copper ions in the thylakoid lumen. This copper could be enough to activate CopS, even if it is present at low levels in the thylakoid membrane (Fig. 7). Why is CopS detecting thylakoid copper levels? The thylakoid lumen contains numerous proteins that are highly sensitive to oxidative damage (Nishiyama et al., 2001), and therefore copper will be highly toxic in this compartment. CopS activation will induce *copMRS* and *copBAC*. CopBAC efflux system is unlikely to be able to detoxify copper from the thylakoid lumen, but it will at least export the surplus of copper that could be accumulated in the periplasm and the cytosol, creating a positive concentration gradient for copper efflux from the thylakoid. In addition, CopM could have an unidentified role in detoxifying thylakoid copper, preventing damage in this compartment. Finally, we cannot rule out that CopRS controls other unknown genes involved in copper homeostasis.

Whether the responses described here are conserved in photosynthetic eukaryotes is unknown, but copper trafficking in the chloroplast is also mediated by P_I-type ATPases, homologous to CtaA and PacS, and copper chaperones (Puig et al., 2007a). Therefore, it seems reasonable to expect that the drastic reduction in the photosynthetic electron flux that leads to accumulation of oxidized plastocyanin could lead to its degradation, releasing free copper in the thylakoid lumen. It is also anticipated that this excess of free copper could be detected and a response similar the one observed here could be launched to detoxify this copper. The proteins studied here are only conserved in some cyanobacteria (Nagarajan et al., 2012; J. Giner-Lamia, L. López-Maury, and F.J. Florencio, unpublished data), and therefore the response in photosynthetic eukaryotes is likely mediated by a different set of regulatory proteins and effectors, in the same way the *petE* to *petJ* switch is conserved between cyanobacteria and *Chlamydomonas* but the regulatory mechanisms are not (Merchant and Bogorad, 1986; Zhang et al., 1992; Merchant et al., 2006).

CONCLUSION

In summary, we have shown that the CopRS two-component system is essential for copper resistance in *Synechocystis* by regulating expression of *copMRS* and

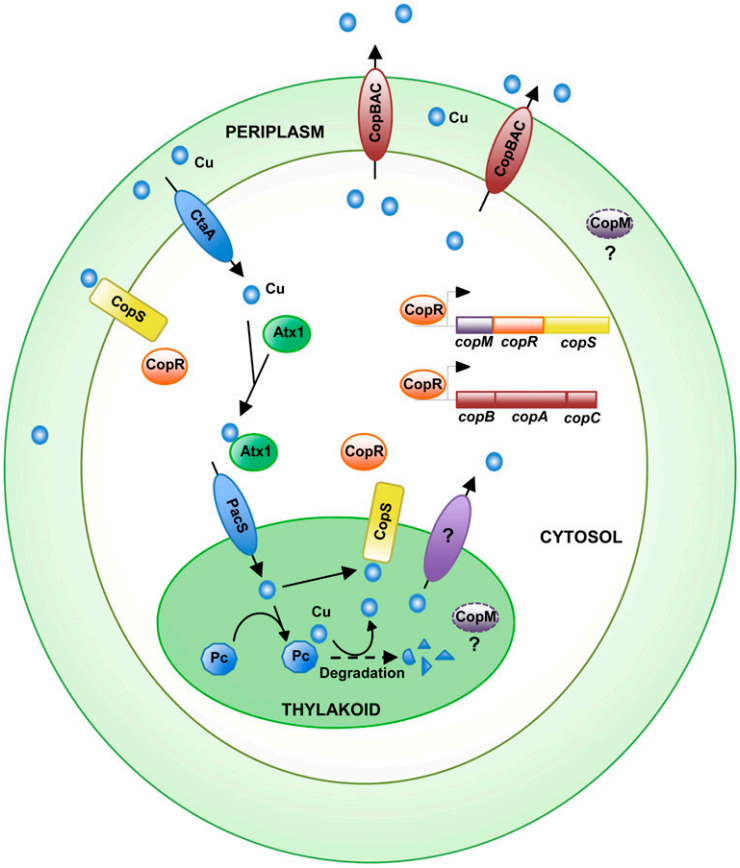


Figure 8. Simplified model showing the copper transport proteins and its relation to CopRS and CopBAC resistance systems. [See online article for color version of this figure.]

copBAC operons in response to copper. CopS is probably detecting copper directly, as its putative periplasmic sensor domain is able to bind copper in vitro. We also present evidence that redox induction of *copMRS* is strictly dependent on the presence of copper and that this induction is probably related to plastocyanin degradation. Furthermore, we show that CopS localized to both plasma and thylakoid membranes and therefore could respond to copper both in the periplasm and in the thylakoid lumen. Whether CopRS controls additional mechanisms involved in thylakoid copper detoxification remains to be elucidated. To our knowledge, CopS is the first His kinase detecting events directly inside the thylakoid lumen in cyanobacteria, despite the extensive regulation mediated by changes that occur in this compartment in photosynthetic organisms.

MATERIALS AND METHODS

Strains and Culture Conditions

Synechocystis cells were grown photoautotrophically on BG11C, BG11C-Cu (lacking $CuSO_4$), and BG11C-N (lacking $NaNO_3$) medium (Rippka et al., 1979) at 30°C under continuous illumination (50 μE m^{-2} s^{-1}) and bubbled with a stream of 1% (v/v) CO_2 in air. For Supplemental Figures S5 to S7 and S10, BG11C-Cu or BG11C-Cu-N was supplemented with 300 μM BCSA as a chelating agent to eliminate any traces of copper (Durán et al., 2004). For plate cultures, medium was supplemented with 1% (w/v) agar. Kanamycin, chloramphenicol, and spectinomycin were added to a final concentration of 50 μg mL^{-1}, 20 μg mL^{-1}, and 5 μg mL^{-1}, respectively. BG11C-Cu medium was supplemented with different concentrations of $CuSO_4$, $NiSO_4$, $ZnSO_4$, $CdCl_2$, and $CoCl_2$ when indicated. Experiments were performed using cultures from the midlogarithmic phase (3–5 μg chlorophyll mL^{-1}). Glc, DBMIB, DCMU, and lincomycin were added to a final concentration of 5 mM, 10 μM, 10 μM, and 250 μg mL^{-1}, respectively. *Synechocystis* strains and their relevant genotypes are described in Supplemental Table S1. *Escherichia coli* DH5α or BL21 cells were grown in Luria broth medium and supplemented with 100 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin, 20 μg mL^{-1} chloramphenicol, and 100 μg mL^{-1} spectinomycin when required.

Construction of *Synechocystis* Strains

Synechocystis cells were transformed as described in Ferino and Chauvat (1989). Plasmid construction is detailed in Supplemental Materials and Methods S1. All the oligonucleotides used in this work are described in Supplemental Table S2.

RNA Isolation and Northern-Blot Analysis

Total RNA was isolated from 30-mL samples of *Synechocystis* cultures in the midexponential growth phase (3–5 μg chlorophyll mL^{-1}). Extractions were performed by vortexing cells in the presence of phenol chloroform and acid-washed baked glass beads (0.25–0.3 mm diameter) as previously described (García-Domínguez and Florencio, 1997). Five micrograms of total RNA was loaded per lane and electrophoresed in 1.2% agarose denaturing formaldehyde gels (Sambrook et al., 1989) and transferred to nylon membranes (Hybond N-Plus; Amersham). Prehybridization, hybridization, and washes were in accordance with Amersham instruction manuals. All probes were synthesized by PCR and oligonucleotide pairs used are described in Supplemental Table S3. Hybridization signals were quantified with a Cyclone Phosphor system (Packard).

Determination of Cellular Copper Content

The cellular copper contents were determined from 800 mL of exponentially growing cells that were treated with 200 nM of copper for 1 h (wild type, SAS, and PETE strains) or 3 μM of copper for 5 h (wild type, COPR, and COPB strains). Cells were centrifuged at 5,000*g*, washed twice with BG11C-Cu, and dried overnight in an oven at 85°C. One hundred micrograms of dried cells was microwave digested, dissolved in suprapure HNO_3, and analyzed by induced coupled plasma (ICP) in an ICP-OES Varian ICP 720-ES (Tottey et al., 2001; Andrés-Colás et al., 2006). Data shown represent the average ± SE.

Primer Extension Analysis of copMRS and copBAC Transcripts

Oligonucleotides NIY3 and COPA3, end labeled with T4 polynucleotide kinase and [γ-^{32}P]-dATP (3,000 Ci $mmol^{-1}$) following standard procedures (Sambrook et al., 1989), were used for primer extension analysis of *copMRS* or *copBAC* promoters, respectively. For annealing, a 10-μL mixture containing 0.15 M KCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA, 20 μg of total RNA, and about 2 pmol of oligonucleotides (10^6 cpm) was prepared. The annealing mixture was heated for 2 min at 90°C in a water bath and cooled slowly to 50°C. For extension, a 10-μL mixture was prepared with half of the annealing mixture: 10 mM dithiothreitol, 0.5 mM each dNTP, 2 mg mL^{-1} of actinomycin D, 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 100 units of Superscript II RNase H-Reverse Transcriptase (Invitrogen). The mixture was incubated for 45 min at 45°C, and the reaction was stopped by adding 4 μL of formamide-loading buffer. Half of the reaction was electrophoresed on a 6% polyacrylamide sequencing gel together with a sequencing reaction of the *copMRS* or *copBAC* promoter regions using the same oligonucleotides.

Cloning and Purification of CopRΔN

The complete DNA-binding domain from *copR* was cloned from *Synechocystis* DNA after PCR amplification with oligonucleotides COPR3 and NIY2 and cloned into *Bam*HI-*Sal*I pGEX6P. GST-CopRΔN fusion protein was expressed in *E. coli* DH5α. Two-hundred milliliters of culture was grown in Luria broth medium to an optical density at 600 nm of 0.6, induced with 0.5 mM isopropyl-b-D-thiogalactopyranoside for 2.5 h, harvested by centrifugation, and resuspended in 5 mL of phosphate-buffered saline buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 4 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol) supplemented with 0.1% Triton X-100. Cells were broken by sonication on ice, and insoluble debris were pelleted by centrifugation. Extracts were mixed with 1 mL of glutathione agarose beads (Amersham) and incubated for 2 h at 4°C with gentle agitation. Then beads were transferred to a column and washed extensively with phosphate-buffered saline buffer until no more protein was eluted from the column. GST fusion proteins were eluted with 3 mL of 50 mM Tris HCl (pH 8) containing 10 mM of reduced glutathione.

Gel Retardation Assays

Probes were PCR synthesized using oligonucleotides NIY4 and NIY5, for *copMRS* promoter, and COPA4 and COPA5 for *copBAC* promoter, which introduce an *Nco*I restriction site in both cases. The resulting DNA was digested with *Nco*I and end labeled with [α-^{32}P]-dCTP (3,000 Ci $mmol^{-1}$) using Klenow fragment. The binding reaction was carried out in a final volume of 25 μL containing 4 ng of labeled DNA and 4 μg salmon sperm DNA in 20 mM Tris HCl (pH 8.0), 150 mM KCl, 10 mM spermidine, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and different amounts (from 0.2–1 μg) of partially purified GST-CopRΔN. The mixtures were incubated for 25 min at 4°C and loaded on a nondenaturing 6% polyacrylamide gel. Electrophoresis was carried out at 4°C and 200 V in 0.25× Tris-borate/EDTA. Gels were transferred to a Whatman 3 MM paper, dried, and autoradiographed.

Cloning, Purification, and Metal-Binding Assays of CopS Periplasmic Domain ($CopS_{38\text{-}183}$)

A 462-pb band coding for the CopS periplasmic domain was PCR amplified from genomic DNA with oligonucleotides CopSperiF2-CopSperiR2, digested with *BamHI* and *Sac*I, and cloned into pET51 digested with the same enzymes. $CopS_{38\text{-}183}$ was expressed in *E. coli* BL21. A total of 1.5 L of culture was grown in Luria broth medium to an optical density at 600 nm of 0.6, induced with 0.2 mM isopropyl-b-D-thiogalactopyranoside, and incubated for 6 h at 25°C; cells were harvested by centrifugation and frozen at −20°C. Frozen pellets were resuspended in 40 mL of 100 mM Tris HCl (pH 8), 150 mM NaCl, 1 mM BCSA, 1 mM EDTA, and 2 mM Tris(2-carboxyethyl)-phosphine (buffer S) and broken by sonication. The suspension was centrifuged 30 min at 30,000*g* at 4°C and the supernatant was loaded into a 5-mL streptavidin beads (IBA GmbH) column equilibrated in buffer S. Beads were washed with 50 mL of buffer S and

$CopS_{38-183}$ was eluted with 10 mL of 1× Strep-Tag elution buffer (IBA GmbH). $CopS_{38-183}$ was further purified by gel filtration in a Hi-Load Superdex 75 (GE-Healthcare) column equilibrated with 20 mM Tris HCl (pH 8), 150 mM NaCl. The purified protein was concentrated using a 3K Vivaspin concentrator.

Interaction of $CopS_{38-183}$ with Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} was investigated by immobilized metal ion affinity chromatography. A 100-μL aliquot of His-bind resin (Novagen) was loaded with 0.5 mL of 0.5 mM of $CuSO_4$, $NiSO_4$, $ZnSO_4$, or $CoCl_2$ in water and then equilibrated in 25 mM Tris HCl (pH 8), 500 mM NaCl (buffer A). About 10 μg of purified $CopS_{38-183}$ were applied to the columns. Unbound proteins were removed by washing with 2 mL of buffer A. Bound proteins were eluted with 100 μL of 0.4 M imidazole in buffer A. Fifteen microliters of the imidazole eluted and flowthrough fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Quantities of bound and unbound proteins were determined by densitometry.

Analysis of $CopS_{38-183}$ Cu^{2+} binding was obtained via colorimetric titration similar to that described previously with the divalent metal ligand PAR (Tottey et al., 2008). PAR (10 μM) in 20 mM Tris HCl (pH 7.5), 50 mM NaCl (buffer B) was titrated against copper (0–20 μM) measuring absorbance in the 350 to 600 nm range. Absorbance of PAR (410 nm) and Cu^{2+}-PAR (500 nm) were plotted against [Cu^{2+}]. Titration was repeated in the same way but with the addition of 10 μM apo-$CopS_{38-183}$. The apparent dissociation constant (K_D) of $CopS_{38-183}$ for Cu^{2+} was estimated using competition experiments as described previously (Turski et al., 2012). The quantitative release of the 1:1 Cu^{2+}/PAR complex upon titration of apo$CopS_{38-183}$ was monitored spectrophotometrically at 500 nm in buffer B. The samples were equilibrated for 5 min at room temperature before the measure. The affinity of Cu^{2+}-PAR complex (formation constant [β]) is 3.2×10^{17}, and the Cu^{2+} binding affinity was calibrated using a spectroscopically silent ligand EDTA, with a known affinity for Cu^{2+} of 1.6×10^{-19} (Turski et al., 2012).

Membrane Fractionation and Western Blotting

Thylakoid and plasma membranes were prepared from *Synechocystis* as described previously (Norling et al., 1998). For western-blot analysis, proteins were fractionated on SDS-PAGE and immunoblotted (Sambrook et al., 1989) with antibodies against: HA (1:1,000; Sigma catalog number H9658), NrtA (1:10,000; Omata et al., 1989), PsaC (1:3,000; Mata-Cabana et al., 2007), plastocyanin (1:12,000; Durán et al., 2004), or *Synechococcus* sp. PCC 6301 Gln synthetase I (1:20,000; Mérida et al., 1990). The ECL Plus immunoblotting system (Amersham) was used to detect the different antigens with anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:10,000). Films were scanned and quantified using Image J software.

The genes named in this article can be found in the Cyanobase database (http://genome.kazusa.or.jp/cyanobase/).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *copMRS* are expressed as a single transcriptional unit.

Supplemental Figure S2. Mutants in *cop* genes are not differentially affected with respect to the wild-type strain by Ni^{2+}, Co^{2+}, and Zn^{2+}.

Supplemental Figure S3. *copBAC* are expressed as a single transcriptional unit.

Supplemental Figure S4. Spectral changes of the Cu^{2+}-PAR complex on the $CopS_{(38-183)}$ titration.

Supplemental Figure S5. Redox induction of *copMRS* and *copBAC* expression depends on the presence of copper in the medium.

Supplemental Figure S6. *copMRS* and *copBAC* expression is not induced after DCMU treatment.

Supplemental Figure S7. *copM* and *copB* induction depends on CopR after DBMIB treatment.

Supplemental Figure S8. CopRS do not control copper-related genes.

Supplemental Figure S9. Growth of COPR is not affected by Glc.

Supplemental Figure S10. Nitrogen starvation leads to *copM*, *copB* induction, and plastocyanin degradation.

Supplemental Table S1. *Synechocystis* strains used in this work.

Supplemental Table S2. Oligonucleotides used in this work.

Supplemental Table S3. Oligonucleotides pairs used to synthesize probes used for northern-blot analysis.

Supplemental Materials and Methods S1. Insertional mutagenesis and reverse transcription-PCR.

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