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, monoxygenases, 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; Chilappagari 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-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...
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-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, 2008). 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 COP1-T2 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; Casruita 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 c₆ (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* (Kropp 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 c₆ (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-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 caa₃-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 *sll0639*). 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...
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 slr0788 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 CuSO4), 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 copP (COPR strain), copS and copP (COPS strain), or carrying a mutation in the catalytic His (COPS<sub>H227A</sub>) 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<sup>−1</sup> 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<sub>H227A</sub> 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<sub>H227A</sub> strains (Fig. 1D), suggesting that CopRS controls its own induction in response to copper.

**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<sub>H227A</sub>, PCOP, and GCOP strains to copper was examined. Ten-fold serial dilutions of a suspension of 1 μg chlorophyll mL<sup>−1</sup> 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<sub>H227A</sub> strains. Total RNA was isolated from wild-type, COPR, COPS, and COPS<sub>H227A</sub> 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.]
CopRS Controls the Expression of an HME-RND Efflux System Involved in Copper Resistance

Downstream of pcpMRS, 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 pcpMRS. 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 dramatically 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 ± 8 versus 339 ± 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.

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 pcpMRS transcripts start 27 nucleotides upstream of the predicted copM or pcpM 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
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 (CopS38-183). We tested whether CopS38-183 was able to bind metals using metal chromatography. The protein was retained by beads charged with 0.5 mM of Cu2+ but not by Zn2+, Ni2+, and Co2+ charged beads (Fig. 4, A and B). To further analyze CopS38-183 interaction with copper we used ligand competition with apo-4-(2-pyridylazo)-resorcinol (PAR). CopS38-183 was able to extract one equivalent of Cu2+ from PAR, suggesting that one atom of copper binds to one molecule of CopS38-183 (Fig. 4C). Titration of Cu2+-loaded PAR with increasing amounts of CopS38-183 revealed a concentration-dependent decrease in PAR-Cu2+ concentration (Fig. 4D; Supplemental Fig. S4), and allowed us to calculate an apparent dissociation constant (K_dapp) for CopS38-183 of 2.3 \times 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 plastoquinone 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 \mu M 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 \mu M 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.

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 \mu M 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 \mu M 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-CopR\DeltaN. E, Band-shift assay of the copBAC promoter region with increasing quantities GST-CopR\DeltaN.
The Response of CopS to Plastocyanin Protein Levels

Photosynthetic electron transport between cytochrome b_{6}f 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_{6}f, 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,
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 PI-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 ± 1.8 versus 70 ± 14 μg mg⁻¹ dry weight), while the SAS strain accumulated the same amount as the wild type (71.4 ± 9.1 μg mg⁻¹ dry weight). Even more, the SAS strain failed to do the switch from petJ...
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, COPSH227A) 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...
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 c₆ (petF), 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 Cu²⁺ 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-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 to 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
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 CuSO₄), and BG11C-N (lacking NaNO₃) medium (Rippka et al., 1979) at 30°C under continuous illumination (50 μmol m⁻² s⁻¹) and bubbled with a stream of 1% (v/v) CO₂ 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 CuSO₄, NiSO₄, ZnSO₄, CdCl₂, and CoCl₂ when indicated. Experiments were performed using cultures from the midlogarithmic phase (3–5 × 10⁶ cells mL⁻¹) and incubated for 2 h at 4°C with gentle agitation. Then beads were washed baked glass beads (0.25–0.42 mm) and incubated for 2 h at 4°C with gentle agitation. 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 CopRAN**

The complete DNA-binding domain from copR was cloned from *Synechocystis* DNA after PCR amplification with oligonucleotides COPR3 and NIY2 and cloned into *BamHl*·*SalI* pGE006. GST-CopRAN 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-β-D-thiogalactopyranoside for 2.5 h, harvested by centrifugation, and resuspended in 5 mL of phosphate-buffered saline buffer (150 mM NaCl, 16 mM NaHPO₄, 4 mM NaH₂PO₄, 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 (Amerham) 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 8.0) containing 10 μM of reduced glutathione.

**Gene Retardation Assays**

Probes were PCR synthesized using oligonucleotides NIY4 and NIY5, for copMRS promoter, and COPA4 and COPA5 for copBAC promoter, which in-roduce an NcoI restriction site in both cases. The resulting DNA was digested with *NcoI* and end labeled with [α-32P]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-CopRAN. 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 (CopS38–153)**

A 462-bp band coding for the CopS periplasmic domain was PCR amplified from genomic DNA with oligonucleotides CopSp38–2–CopSp152, digested with *BamHl* and *SalI* and cloned into pET51 digested with the same enzymes. CopSp38–153 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-β-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 μM 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 at 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 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.
CopS38-183 was eluted with 10 mL of 1× Step-Tag elution buffer (IBA GmbH). CopS38-183 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 CopS38-183 with Cu²⁺, Ni²⁺, Zn²⁺, and Co²⁺ 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 CuSO₄, NiSO₄, ZnSO₄, or CoCl₂ in water and then equilibrated in 25 mM Tris HCl (pH 8), 500 mM NaCl (buffer A). About 10 µg of purified CopS38-183 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 B. 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 CopS38-183 Cu²⁺ binding was obtained via colorimetric titration similar to that described previously with the divergent metal ligand PAR (Totty 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 Cu²⁺-PAR (500 nm) was plotted against [Cu²⁺]. Titration was repeated in the same way but with the addition of 10 µM apo-CopS38-183. The apparent dissociation constant (Kₘ) of CopS38-183 for Cu²⁺ was estimated using competition experiments as described previously (Turski et al., 2012). The quantitative release of the 1:1 Cu²⁺-PAR complex upon titration of apoCopS38-183 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 Cu²⁺-PAR complexes (formation constant [J]) is 3.2 × 10⁵ M⁻¹, and the Cu²⁺ binding affinity was calibrated using a spectroscopically silent ligand EDTA, with a known affinity for Cu²⁺ of 1.6 × 10¹⁴ M⁻¹ (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 immunooblotted (Sambrook et al., 1989) with antibodies against: HA (1:1,000; Sigma catalog number 19398), 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; Mériva et al., 1990), the ECL Plus immunoblotting (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 Ni²⁺, Co²⁺, and Zn²⁺.

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

Supplemental Figure S4. Spectral changes of the Cu²⁺-PAR complex on the CopS38-183 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 are 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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LITERATURE CITED

Andrés-Colás N, Sancenón V, Rodriguez-Navarro S, Mayo S, Thiele DJ, Ecker JR, Fuig S, Peñarrubia L (2006) The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. Plant J 45: 225–236

Bagai I, Rensing C, Blackburn NJ, McEvoy MM (2008) Direct metal transfer between periplasmic proteins identifies a bacterial copper chaperone. Biochemistry 47: 11408–11414

Bernal M, Casero D, Singh V, Wilson GT, Grande A, Yang H, Dodani SC, Pellegrini M, Hueijer P, Cowelolly EL, et al (2012) Transcriptome sequencing identifies SPL7-regulated copper acquisition genes FRO1/ FRO5 and the copper dependence of iron homeostasis in Arabidopsis. Plant Cell 23: 738–761

Blanco AG, Canals A, Bernués J, Solá M, Coll M (2011) The structure of a transcription activation subcomplex reveals how α(70) is recruited to PhoB promoters. EMBO J 30: 3776–3786

Castañeda M, Casero against Apowicz SJ, Krapaj T, Vieler A, Hsieh SJ, Yan W, Kokos S, Luo JA, Benning C, et al (2011) Systems biology approach in Chlamydomonas reveals connections between copper nutrition and multiple metabolic steps. Plant Cell 23: 1273–1292

Chillappapari S, Seubert A, Trip H, Kuipers OP, Marahiel MA, Mietheke M (2010) Copper stress affects iron homeostasis by destabilizing iron-sulfur cluster formation in Bacillus subtilis. J Bacteriol 192: 2512–2524

Chong LX, Ash MR, Maher MJ, Hinds MG, Xiao Z, Wedge AG (2009) Unprecedented binding cooperativity between Cu(I) and Cu(II) in the copper resistance protein CopK from Cupriavidus metallidurans CH34: implications from structural studies by NMR spectroscopy and x-ray crystallography. J Am Chem Soc 131: 3549–3564

del Pozo T, Cambiasso V, González M (2010) Gene expression profiling analysis of copper homeostasis in Arabidopsis thaliana. Biochem Biophys Res Commun 393: 248–252

Durán RV, Hervás M, De La Rosa MA, Navarro JA (2004) The efficient functioning of photosynthesis and respiration in Synechocystis sp. PCC 6803 strictly requires the presence of either cytochrome c6 or plastocyanin. J Biol Chem 279: 7229–7233

Ferino F, Chauvat F (1989) A promoter-probe vector-host system for the cyanobacterium, Synechocystis sp. PCC6803. Gene 84: 257–266

Finazzi G, Sommer F, Hippler M (2005) Release of oxidized plastocyanin from photosystem I limits electron transfer between photosystem I and cytochrome b6f complex in vivo. Proc Natl Acad Sci USA 102: 7031–7036

Franke S, Grass G, Rensing C, Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli. J Bacteriol 185: 3804–3812

Garcia-Dominguez M, Florencio FJ (1997) Nitrogen availability and electron transport control the expression of gshB gene (encoding PIIP protein) in the cyanobacterium Synechocystis sp. PCC 6803. Plant Mol Biol 38: 723–734

García-Dominguez M, Lopez-Maury L, Florencio FJ, Reyes JC (2004) The ef

García-Dominguez M, Florencio FJ, Reyes JC (2000) A gene cluster involved in metal homeostasis in the cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol 182: 1507–1514

Grass G, Rensing C (2003) Genes involved in copper homeostasis in Escherichia coli. J Bacteriol 185: 2145–2147

Hihara Y, Sonoike K, Kanehisa M, Ikekuchi M (2003) DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol 185: 1719–1725

Plant Physiol. Vol. 159, 2012 1817
Imlay JA (2003) Pathways of oxidative damage. Annu Rev Microbiol 57: 395–418

Kahlon S, Beer K, Okhawa H, Hihara Y, Murik O, Suzuki I, Ogawa T, Kaplan A (2006) A putative sensor kinase, Hik31, is involved in the response of Synechocystis sp. strain PCC 6803 to the presence of glucose. Microbiology 152: 647–655

Kaneko T, Nakamura Y, Sasamoto S, Watanabe A, Kohara M, Matsumoto M, Shimpo S, Yamada M, Tabata S (2003) Structural analysis of four large plasmids harboring in a unicellular cyanobacterium, Synechocystis sp. PCC 6803. DNA Res 10: 221–228

Kenney LJ (2002) Structure/function relationships in OmpR and other winged-helix transcription factors. Curr Opin Microbiol 5: 135–141

Kropat J, Tottey S, Birkenbilir RP, Depèvre N, Huijser P, Merchant S (2005) A regulator of nutritional copper signaling in Chlamydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. Proc Natl Acad Sci USA 102: 18730–18735

Krupi J, Chitnis PR, Lagoutte B, Rögnér M, Boekema EJ (1997) Structural organization of the major subunits in cyanobacterial photosystem 1: localization of subunits PsA-D, E, F, and G. J Biol Chem 272: 17061–17069

Loftin IR, Franke S, Roberts SA, Sancenón V, Andrés-Colás N, García-Molina A, Peñafrancia L (2007a) Copper and iron homeostasis in Arabidopsis: responses to metal deficiencies, interactions and biotechnological applications. Plant Cell Environ 30: 271–290

Puig S, Mira H, Dorcey E, Sancenón V, Andrés-Colás N, Garcia-Molina A, Burkhead JL, Gogolin KA, Abdel-Ghany SE, Thieie DJ, et al (2007b) Higher plants possess two different types of ATX1-like copper chaperones. Biochem Biophys Res Commun 354: 385–390

Puig S, Peñafrancia L (2009) Placing metal micronutrients in context: transport and distribution in plants. Curr Opin Plant Biol 12: 299–306

Rensing C, Fan B, Sharma R, Mitra B, Rosen BP (2000) CopA: an Escherichia coli (translocating) P-type ATPase. Proc Natl Acad Sci USA 97: 652–656

Rensing C, Grass G (2003) Escherichia coli mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev 27: 197–213

Ripka R, Deruelle J, Waterbury JB, Herman M, Stanier RY (1979) Genomic assignment, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111: 1–61

Robinson NJ, Winge DR (2010) Copper metallochaperones. Annu Rev Biochem 79: 537–562

Rutherford JC, Cavel JS, Robinson NJ (1999) Cobalt-dependent transcriptional switching by a dual-effector Mer-like protein regulates a cobalt-exporting variant Cpx-like ATPase. J Biol Chem 274: 25827–25832

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sanz M, Abicht HK, Mermod M, Mancini S (2010) Response of gram-positive bacteria to copper stress. J Biol Inorg Chem 15: 3–14

Su CC, Long F, Zimmermann MT, Rajashankar KR, Jernigan RL, Yu EW (2011) Crystal structure of the CusBA heavy-metal efflux complex of Synechococcus sp. Nature 470: 559–562

Summerfield TC, Nagarajan S, Sherman LA (2011) Gene expression under low-oxygen conditions in the cyanobacterium Synechocystis sp. PCC 6803 demonstrates Hik31-dependent and -independent responses. Microbiology 157: 301–312

Thelwell C, Robinson NJ, Turner-Cavel JS (1998) An SmtB-like repressor from Synechocystis PCC 6803 regulates a zinc exporter. Proc Natl Acad Sci USA 95: 10728–10733

Tottey S, Patterson CJ, Banci L, Bertini I, Felli IC, Pavelkova A, Dainty SJ, Pernil R, Waldron KJ, Foster AW, et al (2012) Cyanobacterial metallochaperone inhibits deleterious side reactions of copper. Proc Natl Acad Sci USA 109: 95–100

Tottey S, Rich PR, Rondet SA, Robinson NJ (2001) Two Menkes-type at-pases supply copper for photosynthesis in Synechocystis PCC 6803. J Biol Chem 276: 19999–20004

Tottey S, Rondet SA, Borretly GP, Robinson PJ, Rich PR, Robinson NJ (2002) A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. J Biol Chem 277: 5490–5497

Tottey S, Waldron KJ, Firbank SJ, Reale B, Bessant C, Sat0 K, Cheek TR, Gray J, Banfield MJ, Dennison C, et al (2008) Protein-folding location can regulate manganese-binding versus copper- or zinc-binding. Nature 455: 1138–1142

Trebst A (2007) Inhibitors in the functional dissection of the photosynthetic electron transport system. Photosynth Res 92: 217–224

Turski ML, Brady DC, Kim HJ, Kim BE, Nose Y, Counter CM, Winge DR, Thieie DJ (2012) A novel role for copper in Ras/mitogen-activated protein kinase signaling. Mol Cell Biol 32: 1284–1295

Waldron KJ, Tottey S, Yanagisawa S, Dennison C, Robinson NJ (2007) A periplasmic iron-binding protein contributes toward inward copper supply. J Biol Chem 282: 3837–3846

Yamashita H, Hayashi M, Fukazawa M, Kobayashi Y, Shikina T (2009) SQUAMOSA promoter binding Protein-Like is a central regulator for copper homeostasis in Arabidopsis. Plant Cell 21: 347–361

Zhang L, McSpadden PB, Pakrasi HB, Whitmarsh J (1992) Copper-mediated regulation of cytochrome c553 and plastocyanin in the cyanobacterium Synechocystis sp. PCC 6803. J Biol Chem 267: 19054–19059

Zhang Z, Pendse ND, Phillips KN, Cotner JB, Khodursky A (2008) Gene expression patterns of sulfur starvation in Synechocystis sp. PCC 6803. BMC Genomics 9: 344