Genetically Programmed Changes in Photosynthetic Cofactor Metabolism in Copper-deficient Chlamydomonas*

Received for publication, January 26, 2016, and in revised form, July 18, 2016 Published, JBC Papers in Press, July 20, 2016, DOI 10.1074/jbc.M116.717413

Daniela Strenkert‡§1, Clariss Ann Limso5, Abdelhak Fatihi1, Stefan Schmollinger‡§2, Gilles J. Basset4, and Sabeeha S. Merchant‡§3

From the 1Institute for Genomics and Proteomics, University of California, Los Angeles, California 90095, the 5Department of Chemistry and Biochemistry, UCLA, Los Angeles, California 90095, the 2Institut Jean-Pierre Bourgin, UMR1318 INRA-AgroParisTech, 78026 Versailles Cedex, France, and the 3Horticultural Sciences Department, University of Florida, Gainesville, Florida 32611

Copper is an essential cofactor for most forms of life because of its function as a catalyst of oxygen chemistry and redox reactions (1). In photosynthetic organs of land plants or in unicellular phototrophs like algae and cyanobacteria, plastocyanin is a major copper-requiring protein (2). Many algae and cyanobacteria reduce their dependence on copper (the copper quota) by replacing plastocyanin with a heme protein called cytochrome (Cyt) (2) c6 (reviewed in Ref. 3). In Chlamydomonas, this switch is controlled by copper response elements associated with the CYC6 gene encoding Cyt c6 and a transcription factor, copper response regulator 1 (CRR1) (4–6). CRR1 has a QUAMOSA promoter binding protein (SBP) domain that is conserved in the green lineage (6). Its homologue in Arabidopsis, SPL7, controls nutritional copper homeostasis as well (7, 8). Transcriptome experiments revealed multiple targets of this transcription factor in both organisms but besides the genes encoding assimilatory copper transporters (COPT/CTR), which are dramatically up-regulated in both organisms under copper deficiency (9, 10), the target genes are distinct (8, 11). This may be because modification of the photosynthetic apparatus is a key response in Chlamydomonas but not in Arabidopsis.

We noted previously that redox proteins, especially O2-dependent metabolic enzymes, are enriched among the CRR1 targets in Chlamydomonas (11). Among these are CRD1 and CPX1, encoding enzymes regulating two rate-limiting steps in tetrpyrrole biosynthesis: crd1 mutants are chlorotic in copper-deficient cells because of reduced chlorophyll (Chl) content accompanied by reduced abundance of PSI and LHCI (12, 13). The abundance of CAO1 mRNA encoding another O2-dependent enzyme in Chl biosynthesis is also increased in copper deficiency but this may occur independently of CRR1 (11). The motivation for regulation of tetrpyrrole biosynthesis is not clear, but one hypothesis is that copper deficiency necessitates modification of photosystem biogenesis pathways. FAB2, another CRR1 target, encodes an acyl-ACP desaturase, whose activity is required for the synthesis of unsaturated fatty acids in the chloroplast. Indeed, the galactolipids of the thylakoid membrane from copper-deficient cells are more unsaturated relative to those from copper-replete cells (11). Again, the underlying significance is not known, but because of the importance of unsaturated fatty acids for thylakoid membrane function, we hypothesized that replacement of plastocyanin with Cyt c6 may require structural modifications of the membrane.

In this work, we probe the function of two other CRR1 target genes encoding enzymes in pathways affecting pigment and...
l lipid metabolism in the chloroplast, CGL78 (also called LCAA and Ycf54 in Arabidopsis/tobacco and cyanobacteria), a conserved protein that has been proposed to function with CRD1 in tetrapyrrole biosynthesis at the Mg Protoporphyrin IX monomethyl cyclase step (14–16), and HPPD1, encoding one of two isoforms of 4-hydroxyphenylpyruvate dioxygenase (HPPD), which catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate (17). Although HPPD is critical for tyrosine catabolism in animals, in plants, this enzyme catalyzes the rate-limiting step of an anabolic branch that forms plastoquinone-9 (PQ-9) and tocopherols. PQ-9 is the mobile carrier of 2 reducing eq between PSII and the Cyt b₆f complex, whereas tocopherols have antioxidant activity by scavenging singlet oxygen produced by excess excitation energy input into PSII and protecting membrane lipids from peroxidation (18–21). There are four tocopherol types, α-, β-, γ-, and δ-tocopherols, based on the number and position of methyl groups on the chromanol ring. The increase in abundance of α-tocopherols in response to high light is well documented for both algae and plants (22–24), and there is also a role for tocopherols in signaling pathways, although the mechanistic aspects are not yet fully elucidated (25).

For CGL78, we used an artificial micro-RNA-mediated reverse genetics approach to dissect its function, whereas for HPPD, we monitored pathway end products under conditions affecting its expression to validate its contribution to plastoquinone and tocopherol biosynthesis. We discovered: 1) that LHCSR3 accumulation is increased in copper-deficient cells and 2) that LHCSR3 accumulation was especially sensitive to loss of CGL78 as it accumulates before PSاك, a connector between PSI and LHCl. This phenotype is similar to that of the crd1 mutant, which is blocked at the same step in Chl biosynthesis. For HPPD, we noted that, an increase of HPPD1 is, surprisingly, not accompanied by an increase in all end products of the tocopherol/plastoquinone biosynthesis pathway. Rather, copper-deficient cells accumulate specifically γ-tocopherols. Moreover, when HPPD1 expression is blocked, as in the crd1 mutant, γ-tocopherol accumulation is abolished as well, supportive of a causal connection between HPPD abundance and γ-tocopherol accumulation. We speculate that operation of the light reactions is less effective in copper-deficient cells, requiring subtle modifications of mechanisms for handling excess excitation energy and stress.

Results

**Reduced CGL78 Expression Impacts PSI/II-LHC Super-complex Formation**—To assess the function of CGL78 in Chlamydomonas, we used an inducible amiRNA system to generate strains with conditionally reduced abundance of CGL78 mRNA. Because the amiRNA is driven by the NIT1 promoter, it is ammonium repressible (26). Therefore, strains cgl78-ami11 and cgl78-ami13 carrying the knock-down constructs accumulate CGL78 mRNA at normal abundance in medium with ammonium but have reduced abundance in medium lacking ammonium (8 and 5% as compared with control strains, CGL78, respectively (Fig. 1A). Accordingly, the cgl78-ami strains are chlorotic on ammonium-free medium (Fig. 1B) with significantly reduced Chl content relative to CGL78 (36%), although the ratio of Chl a to b is unaffected (Fig. 1C). There is no effect of cgl78-ami knock-down on growth (Fig. 1D), making it unlikely that loss of CGL78 affects any other pathway. The chlorotic phenotype indicates a direct role of CGL78 in chlorophyll-binding protein accumulation, but the photosystem II capacity was unaffected in cgl78-ami lines (Fig. 1E). To distinguish which chlorophyll-binding proteins might be reduced in cgl78-ami strains, we analyzed thylakoid membrane proteins after separation by BN-PAGE, which resolves various photosystem-containing complexes (27). We noticed a striking loss of PSI/II-LHC super-complexes (marked with a red arrow) with a corresponding increase in PSII core monomers (Fig. 2, compare unfilled with filled arrows).

Because CGL78 is a target of CRR1 and both mRNA and protein abundances are increased 7- and 4-fold, respectively, in copper-deficient medium (Refs. 11 and 28 and Fig. 3A), we tested whether the cgl78-ami phenotype was affected by copper nutrition status. The copper status was verified by expression of the previously characterized sentinel gene CYC6 and accumulation of the corresponding protein (29) (Fig. 3, A and C). Nev-
ertheless, copper neither exacerbated nor ameliorated the phenotype (Fig. 3B). Separation of chlorophyll-binding proteins by BN-PAGE revealed that copper deficiency did not affect the PSII/I-LHC super-complex formation in wild-type lines (Fig. 3D, red arrow), but copper-deficient cgl78-amiRNA lines showed reduced amounts of PSII/I-LHC super-complexes (Fig. 3D, marked by an asterisk). The pattern of PSII/I-LHC super-complexes of thylakoids isolated from cgl78-ami lines in copper-depleted versus copper-replete grown cells look similar except that PS core subunits are more enriched in the former (Fig. 3D, circle). On the other hand, accumulation of PSII was the same in cgl78-ami strains and control lines based on the abundance of D1 (Fig. 4).

In previous work, we observed that PSI-LHCI complexes are destabilized in iron-deficient cells, coincident with a loss of PSI-LHCI complexes prompted us to monitor PSAK, which is the subunit connecting PSI and LHCI. The loss of PSAK, which is the subunit connecting PSI and LHCI. The loss of CGL78 seems not to be interconnected with that of CTH1/CRD1 and we can attribute the cgl78 molecular phenotype in copper deficiency directly to loss of CGL78 rather than indirectly to loss of its partner protein(s) in the complex.

In previous work, we showed that the presence of PSAK was related to functional association of the LHCI antenna with PSI (30). For instance, in iron-deficient cells, which are impaired in chlorophyll biosynthesis, PSAK is reduced, resulting in less energy transfer from LHCI to PSI. Therefore, we collected fluorescence emission spectra to assess PSI-LHCI interactions in wild-type versus knock-down lines as a function of copper nutrition. The copper-replete control cells (CGL78) showed characteristic peaks at 685 and 711 nm, resulting from fluorescence of LHCII attached to PSII and of LHCI to PSI, respectively (Ref. 31 and Fig. 7A). Copper-depleted control cells showed the same changes of the emission spectra that we had observed in copper-depleted wild-type cells in previous work (13), namely an increase in intensity of the peak at 710 nm relative to the peak at 685 nm, indicating modifications of the PSI-LHCI interaction in copper-deficient cells (Fig. 7B). In cgl78-ami lines, the PSI-LHCI peak at 710 nm was blue-shifted (32) and reduced in intensity under both, copper-depleted and copper-replete conditions (Fig. 7, A and B). The latter is consistent with the observation that PSAK is reduced in cgl78-ami lines. When wild-type strains are grown in high light, we note reduced intensity and a blue-shift of the peak corresponding to PSI-LHCI (Fig. 7C), whereas cgl78-ami lines subject to high light show a blue-shift of the PSII-LHCCI peak to 682 nm and loss of the PSI-LHCCI peak (Fig. 7C). The latter situation is reminiscent of iron-starved cells (Ref. 30 and Fig. 7D), and is consistent with a complete loss in both cases of PSAK (Ref. 30 and Fig. 6C).

**Copper-deficient Cells Accumulate a Stress-related Antenna Protein, LHCSR3**—Besides the change in PSI-LHCI interactions noted above, a survey of chlorophyll-protein accumulation revealed a previously unrecognized difference between copper-depleted and copper-replete cells. Specifically, LHCSR3 is increased in copper-deficient wild-type (CGL78) cells severalfold, but not in copper-deficient cgl78-ami strains (Fig. 4). Quantification by a dilution series indicates that LHCSR3 abundance in cgl78-ami strains is about 12.5% of the level in the control strains (Fig. 4, A and B).

When we surveyed the copper-deficiency transcriptome relative to the iron-deficiency transcriptome, where up-regulation of LHCSR3 has already been described (33), we noted a small but consistent increase in LHCSR3.1 and LHCSR3.2 mRNA abundance in copper-deficient relative to copper-replete cells (Table 1). The change is smaller than that documented for iron-starved cells (33) and substantially less than that in cells exposed to high light, but it is statistically significant. When we compared the abundance of LHCSR3 in copper-deficient cells to the abundance in iron-limited cells, again the increase was
correspondingly lower in the copper-deficient situation (Fig. 5). A simple explanation might be that copper-deficient cells are also secondarily iron-deficient, but measurements of the iron content in copper-deficient cells indicate that the iron content in copper-replete cells (10.6 ± 1.4 × 10^7 atoms/cell) does not differ from the iron content in copper-depleted cells (10.4 ± 1.1 × 10^7 atoms/cell). Therefore, we conclude a direct consequence of copper nutrition on the performance of the PSI-LHCII complex.

Because LHCSR3 seemed to be the most affected chlorophyll-binding protein in copper-deficient cgl78-ami lines, and LHCSR3 has a known role in photo-protection (34–38) we wondered if cgl78-ami strains are more sensitive to high light relative to the control. Indeed, if we grew cgl78-ami lines in high light, we observed that the cells became bleached (Fig. 6A). The chlorophyll content of cgl78-ami strains that were grown for 20 h in high light is reduced to 5.5%, as compared with CGL78 wild-type lines (Fig. 6B), concomitant with a strong reduction of LHCSR3 abundance and nearly complete absence of PSAK (Figs. 6C and 7, A and D). Reduced abundance of LHCSR3 likely occurs at the level of mRNA accumulation because the increased LHCSR3 expression in copper deficiency is blocked in the cgl78-ami lines. In fact, the high light responsive increase in LHCSR3 mRNA is also blocked in the cgl78-ami lines (Fig. 6C). This prompted us to test LHCSR3 expression in the crd1 mutant, and we noted that this molecular phenotype is recapitulated in crd1. The latter suggests that the cyclase in Chl biosynthesis might signal to the LHCSR3 gene. Another possibility is that a functional electron transport chain in PSI is required for increased LHCSR3 expression (39).

Tocopherol Metabolism Is Altered in Copper-deficient Chlamydomonas—Besides Chl biosynthetic enzymes, previous transcriptome analyses had identified other changes in cofactor metabolizing enzymes in copper-deficient cells. One of these is HPPD1, a likely target of CRR1, and one of two genes encoding HPPD, which catalyzes the first step in a branched pathway leading to the terpenoid cofactors, plastoquinone and tocopherols (Fig. 8). Sequence analysis indicates that both genes are more highly related to homologues in plants versus animals. The two isoforms are paralogues; they are most closely related to algal homologues but even more closely related to each other (90% identity at the nucleotide level and 93.5% similarity at the protein level) consistent with a recent duplication event. Previous transcriptome and proteome profiling indicated that HPPD1 mRNA and the corresponding protein are increased in copper-deficient cells and in cells that experienced hypoxia (11, 28) (Fig. 9A), which was validated in this work by real time PCR with paralog-specific primers and immunoblotting (Fig. 9, B and C). Antibodies raised against the carrot protein recognized an approximate 46-kDa protein whose abundance is increased in copper-deficient relative to copper-replete Chlamydomonas cells (Fig. 9C). The impact of CRR1-dependent regulation is an ~2-fold increase in mRNA templates encoding HPPD in copper-deficient growth conditions (Fig. 10B). Given the function
of these molecules in electron transfer and photoprotection, we measured the abundance of the end products of the pathway to assess the impact of increased expression.

Surprisingly, despite the increase in mRNA and protein at a rate-limiting step, the total plastoquinone or tocopherol content was not changed in copper-deficient versus copper-replete conditions.
Metabolic Changes in Copper-deficient Chlamydomonas

Chlamydomonas (Fig. 9, D and E). Nevertheless, we noted a consistently higher proportion of \( \gamma \)-tocopherol (42% increase on average) in copper-deficient cells of strain 4532 (wild-type for CRR1) (Fig. 9E). To evaluate the contribution of HPPD1 to \( \gamma \)-tocopherol content, we took advantage of the \( crr1 \) mutant (because HPPD1 is a CRR1 target) and two independent complemented lines, \( CRR1 \) (in which the wild-type gene rescued \( crr1 \)) and \( crr1-\Delta \text{Cys} \) (in which a gene encoding a C terminally truncated version of CRR1 complements the \( crr1 \) mutation). The copper status of the cultures was validated on the basis of sentinel protein accumulation (Fig. 10A). Both complemented lines, but not the \( crr1 \) mutant, show normal \( CRR1 \) - and copper-responsive expression of HPPD1 and the sentinel CYC6 gene (Fig. 10, A–C). The increase in \( \gamma \)-tocopherol mirrors CRR1 function, namely observed in both complemented lines but not in the \( crr1 \) mutant (Fig. 10D). Therefore, we conclude that the change in the \( \gamma \)-tocopherol to \( \alpha \)-tocopherol ratio is dependent on copper nutrition and \( CRR1 \) and is an intrinsic response to poor copper nutrition.

When we surveyed plastochlorin species in \( crr1 \) versus \( CRR1 \), we found that the total PQ-9 content was unaffected (Fig. 11A), consistent with a lack of effect of copper nutrition on plastochlorin content, but we did observe a new metabolite, plastochromanol-8 (PC-8), previously not observed in algae (Fig. 11B). PC-8 is a stress metabolite generated from plastochlorin-9 by a cyclization reaction catalyzed by VTE1. We hypothesize that PC-8 accumulation in copper-deficient \( crr1 \) is part of a general stress acclimation response in this situation because it is independent of an increase in HPPD expression. Its abundance relative to PQ-9 is small (0.35% of total) and is found only in copper-deficient \( crr1 \) cells, a situation in which HPPD1 is not up-regulated and \( \gamma \)-tocopherol increase is not observed. One possibility is that PC-8 is produced for a photo/ROS-protective function in copper-deficient \( crr1 \) strains, a hypothesis that is consistent with the up-regulation of \( LHCSR3 \) and reduced PSAK in this situation (Fig. 11C).

HPPD1 expression can be increased also in hypoxia in a CRR1-dependent pathway (40) (Fig. 12, A and B). Nevertheless, in this situation the enzymatic activity should actually be compromised because of low \( O_2 \), a substrate of the reaction. Indeed, wild-type hypoxic cells have less \( \gamma \)-tocopherol than do normoxic cells (Fig. 12C, 66% reduced), which confirms that an oxygen-dependent step is required for the change in tocopherol composition beyond simply increased mRNA accumulation. When we tested \( crr1 \) mutants versus complemented strains, again the hypoxic increase in HPPD1 mRNA abundance is dependent on \( CRR1 \) (Fig. 13A), but in the absence of oxygen, \( \gamma \)-tocopherol content is decreased rather than increased relative to normoxic cells (Fig. 13B). When we looked at the plastochlorin profile in the hypoxic cells, we noted that the total PQ-9 content is unchanged in \( crr1 \) mutants or complemented strains (Fig. 13C), but the ratio of oxidized to reduced is dramatically reduced in hypoxic cells of all genotypes, which validates the physiology of the treatment.

Discussion

Acclimation to Copper Deficiency

Cyt \( c_6 \) and Acyl-ACP Desaturase—Plastocyanin is the most abundant copper protein in photosynthetic cells and accordingly photosynthesis is dependent on copper in most organisms, except where there is a genetically programmed pathway to accommodate copper deficiency (41). The copper deficiency response occurs in many cyanobacteria and algae (42). The
most well recognized change in the pathway of the light reactions in copper-deficient cells is the replacement of plastocyanin with a copper-independent substitute, a soluble c-type cytochrome or Cyt \(_{c_6}\) (43–45). In this and previous work, we show that there are, in addition, subtler changes of the photosynthetic apparatus. These changes may be required to accommodate the use of structurally distinct mobile electron donors to PSI or to accommodate Cyt \(_{c_6}\) as a less effective catalyst.

Although it is not yet evident in the laboratory setting, the absence of Cyt \(_{c_6}\) in land plants, which makes photosynthesis strictly copper dependent, suggests that plastocyanin offers a selective advantage.

Whole transcriptome analyses of copper-replete versus copper-deficient and \(CRR1\) versus \(crr1\) strains revealed dozens of genes with candidate copper-response elements that are likely targets of nutritional copper signaling (11). Many of these encode key enzymes in plastid lipid or lipid cofactor metabolism, such as coprogen oxidase (CPX1), CRD1 and CGL78 in tetrapyrrole biosynthesis, acyl-ACP desaturase (FAB2), and HPPD (discussed below). There are two possible rationales for increasing the expression of metabolic enzymes in copper deficiency: 1) increased expression is a compensatory mechanism that allows maintenance of end products because the pathway is compromised in copper-deficient cells or 2) increased expression allows increased accumulation of end products, more of which are required in copper-deficient cells. The two models are distinguished by end product analyses in wild-type versus \(crr1\) mutants, and previous analyses showed that the latter is the situation for FAB2 and CRD1. Indeed, there are more unsaturated fatty acids in copper-deficient \(Chlamydomonas\) cells, whereas in the \(crr1\) mutant the level of desaturation is unchanged (11). Lipid-profiling indicated that the enrichment was restricted to the galactolipids in the thylakoid membrane, one of the first indications that acclimation to copper deficiency requires modification of the photosynthetic membranes.

\(\gamma\)-Tocopherol and Plastochromanol-8—In this work, we document other subtle but measurable modifications to the photosynthetic apparatus. Increased expression of \(HPPD1\) is (like for FAB2) causally connected with increased \(\gamma\)-tocopherol content. The proportion of \(\gamma\)-tocopherol is small (17% of the total tocopherol pool) and the increase therefore is also small, but it is reproducible and statistically significant, comparable with the magnitude of the change in the galactolipid desaturation. If Cyt \(_{c_6}\) is a less effective catalyst relative to plastocyanin, the change in proportion of \(\gamma\)-tocopherol may serve to fine tune photosynthetic physiology by adjusting antioxidant or stress...
We conclude that like the situation for FAB2 and CRD1, increased expression of HPPD is required for increased accumulation of an end product (in this case \( \gamma \)-tocopherol).

Plastochromanol-8 has not previously been found in algae, but was revealed here in the \( \text{crr1} \) mutant and only in copper deficiency where \( \text{crr1} \) grows poorly. Because it is implicated in stress protection (46), its production may be a downstream consequence of stress in \( \text{crr1} \) mutants. It would be interesting to test whether other stress situations also allow PC-8 accumulation and to distinguish the relevant regulatory mechanisms. In Arabidopsis, PC-8 levels are regulated by the activity of a type II NADPH dehydrogenase C1 (ortholog is NDA5 in Chlamydomonas) (47, 48) and a plastid ABC1-like kinase (49).

We had expected increased HPPD1 expression to yield increased total tocopherol, but this is not the case. It is possible that the biosynthetic pathways for \( \alpha \)-versus \( \gamma \)-tocopherol in Chlamydomonas are branched at the level of suborganellar organization of enzyme complexes rather than at the level of the VTE3 enzyme. Increased expression of HPPD1 is not by itself sufficient to change the proportion of \( \gamma \)-tocopherol. In hypoxic cells, expression of HPPD1 is also increased but in the absence of the substrate O\(_2\), \( \gamma \)-tocopherol is not increased (Fig. 12C).

**Chlorophyll-binding Proteins**

We describe two other examples of adjustment of stress protection mechanisms in Chlamydomonas. First, copper-deficient cells have increased LHCSR3 content (Fig. 4). Although the increase is not as dramatic as in iron-deficient Chlamydomonas cells (50), it is notable, and more importantly, it is blocked in \( \text{cgl78} \)-ami lines and in \( \text{crd1} \) mutants (Fig. 6C), suggestive of a signaling function of the cyclase with respect to expression of particular chlorophyll proteins. The operation of the tetrapyrrole pathway has been linked to nuclear gene expression in many works; this work documents a new regulatory connection (51).

LHCSR3 functions in photoprotection (35) involving photosystem I in Physcomitrella patens (52); its adjustment in copper-deficient cells may serve as pre-emptive fine tuning to accommodate Cyt \( c_6 \) as a less effective donor to PSI relative to plastocyanin. The up-regulation of the tetrapyrrole pathway, documented for coprogen oxidase and the aerobic oxidative cyclase (CPX1, CRD1, CGL78) at the level of RNA and protein (28, 29) may allow adjustment of the abundance of specific chlorophyll-binding proteins to optimize the operation of the light reactions in a copper-deficient cell.

Second is de-stabilization of the LHCI-PSI interaction. Survey of photosystem I in the copper-replete situation showed a specific loss of PSAK, leading to de-stabilization of PSI-LHCI super-complexes. The \( \text{cgl78} \)-ami strains are also deficient in PSAK (Fig. 4), perhaps contributing to the phenotype noted by blue native gel separation of Chl-protein complexes and the observed blue-shift of the PSI-LHCI emission at 77 K. The loss...
Metabolic Changes in Copper-deficient Chlamydomonas

A

HPPD1
HPPD2

+Cu
- Cu

+O₂
- O₂

(light)
(dark)

B

C

aHPPD
Ponceau S

Cu + -

relative transcript abundance

CyC6
HPPD1
HPPD2

10⁶
10⁵
10⁴
10³
10²
10¹

CC4532

D

E

-tocopherol
α-tocopherol

Cu

CC4532

FIGURE 9. HPPD accumulation correlates with HPPD1 abundance and increased γ-tocopherol in copper-deficient Chlamydomonas. A–D, change in HPPD encoding transcripts in response to growth in copper-deficient medium or dark anoxic conditions. A, data from RNA-seq experiments in Chlamydomonas strain CC4532. The sizes of the circles are proportional to the relative abundance (RPKM) of the total HPPD transcript pool in each condition. B, abundance of CyC6, HPPD1, and HPPD2 transcripts was also estimated in independent samples by qRT-PCR. CC4532 was grown in TAP medium in the condition indicated: -Cu (II) (black squares) or + Cu(II) (white squares). Cells were collected after reaching a density of 5–8 x 10⁶ cells/ml and analyzed for RNA abundance by real-time PCR. Each symbol represents an independent experiment analyzed in technical duplicates. C, proteins were extracted from copper-depleted or copper-replete conditions and 10 µg of soluble protein was separated by 10% PAGE, followed by immuno-detection with an antibody against Arabidopsis HPPD. D, plastoquinone, and E, α- and γ-tocopherol contents were measured in extracts of Chlamydomonas cells. Data are the averages of three experimental replicates ± S.D.

consistent with its location in the complex (53), and hence serve a role in photo-protection.

We conclude that replacement of plastocyanin with Cyt c₆ in response to copper deficiency requires considerable adjustments of the photosynthetic apparatus and photoprotective mechanisms. Although CGL78 is 5-fold up-regulated in copper-deficient cells, the cgl78-ami mutants are chlorotic independent of copper nutrition (Fig. 3). This contrasts with the copper nutrition-conditional crd1 phenotype, but this might be because CTH1, the reciprocally expressed paralog of CRD1, covers the loss of function in copper-replete situations.

Experimental Procedures

Strains and Culture Conditions—A miRNA targeting Chlamydomonas CGL78 was designed according to Refs. 26 and 54 using the WMD3 tool at wmd3.weigelworld.org. Resulting oligonucleotides CGL78amiFor, ctgtaTAGATGGTACAGGTAAGGTGACATCAGTAT-GAAGTGATCCACACCATCTAg and CGL78amiRev, ctggAGATGGTACAGGTAAGGTGACATCAGTAT-ggcgtcgccgaagagtckgacccgtaTAT-GAAGTGATCCACACCATCTAa (uppercase letters representing miRNA*/miRNA sequences) were annealed by boiling and slowly cooling down in a thermocycler and ligated into SpeI-digested pMS539, yielding pDS1. pDS1 was linearized by digestion with HindIII and transformed into Chlamydomonas strain CC4532 by transformation with meganucleases (55).

CGL78-amiRNA strains and Chlamydomonas reinhardtii strains CC4532, crr1–2 (CC5068), CRR1 (CC5071), and crrΔcys (CC5073) (56) were grown in Tris acetate/phosphate (TAP) with constant agitation in an Innova incubator (180 rpm, New Brunswick Scientific, Edison, NJ) at 24 °C in continuous light (90 µmol m⁻² s⁻¹), provided by cool white fluorescent bulbs (4100 K) and warm white fluorescent bulbs (3000 K) in the ratio of 2:1, unless stated otherwise. High light (600 µmol m⁻² s⁻¹) was provided by a white fluorescent bulb (3800 K). TAP medium with or without copper was used with revised trace elements (Special K) instead of Hutner’s trace elements according to Ref. 56. Cell density (number of cells per milliliter of culture) was determined with a hemocytometer.

Chlorophyll Measurements—Chlorophyll was extracted from whole cells using an 80/20 (v/v) acetone/methanol mixture. Chlorophyll content was estimated according to Ref. 58 from the absorbances at 646.6 and 663.6 nm measured on a PerkinElmer LAMBDA 25 UV/visual spectrometer. The absorption at 750 nm was used as a reference.

RNA Extraction and cDNA Synthesis—2–4 x 10⁷ cells were collected by centrifugation for 5 min at 2450 x g, 4 °C. RNA was extracted using the TRIZol reagent as described previously (59), treated with Turbo DNase (Ambion), concentrated, and cleaned with the Zymo Research RNA Clean & Concentrator™. 5
Kit according to the manufacturer’s instructions. Reverse transcription was primed with oligo(dT)18 using 2.5 μg of total RNA and SuperScript III (Invitrogen) according to the manufacturer’s instructions.

Quantitative Real-time PCR—cDNA was diluted 10-fold before use. qRT-PCR contained 5 μl of cDNA, 6 pmol of each forward and reverse primer, 2 μl of Taq polymerase, 0.5 μl of 10 mM deoxynucleotide triphosphate (New England Biolabs), 2 μl of 1/1000 10 Ex Taq buffer (Mg2+) (TaKaRa), 2 μl of 1/1000 10 SYBR mix (0.1% (w/v) SYBR Green 1 Nucleic Acid Gel Stain from Cambrex, 1% (w/v) Tween 20, 1 mg ml−1 of BSA, and 50% (v/v) DMSO) in a 20-μl volume. The following program was used: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 65 °C for 60 s. Fluorescence was measured after each cycle at 65 °C. A melting curve analysis was performed afterward from 65 to 95 °C with fluorescence reads every 0.5 °C. Relative abundances were calculated using LinReg. The abundance (No) of RACK1 (CBLP, Cre13.g599400) served as a reference transcript.

Primers used in this study are as follows: CYC6For, CAGGTCTTCAACGGCAACTGT; CYC6Rev, ATCGCCCCCTTGCCAT, HPPD1For, GGTCGCGTCGATTGGGTTAC; HPPD1Rev, TGAGAACTCGTGGAAGCCACA; HPPD2For, ACCTCCTTCGGCCTGCAAC; HPPD2Rev, CACGTCCTCCGCAACAACT; CGL78For, CCTGGACCGCGTGCTGAAGA; CGL78Rev, TACCGGGCGTAAGGGGCAGT; LHCSR3f, CACAACACCTTGATGCGAGATG; LHCSR3r, CCGTGTCTTGTCAGTCCTG; CRD1For, CGTAGGTAGCTGGACTGCGTT; CRD1Rev, GTCATTTATGCGCAGCCCTTG; CTH1(2)F, AGCTGTGCGTCGCGGGCTTGT; CTH1(2)R, ATCCGCGTGTTCCGAAGAAAC; CTH1(3)F, ACGCAGCAGCACAGCTCACT; and CTH1(3)R, TCCCCAAGTCTAGCCCGATG. Thylakoid Membrane Preparation and Blue Native Gel Electrophoresis—Thylakoid membranes were prepared as described in Ref. 27 with the following modification: cells were collected by centrifugation at 3100 × g at 4 °C and re-sus-
Metabolic Changes in Copper-deficient Chlamydomonas

A

\[ \text{relative transcript abundance} \]

\begin{tabular}{ccc}
        & CYC6 & HPPD1 & HPPD2 \\
\hline
\hline
O_2 & high & low & high \\
\hline
\end{tabular}

\begin{flushright}
\textit{FIGURE 12}. HPPD1 is also regulated by dark hypoxia but \( \gamma \)-tocopherol content is decreased in dark hypoxic Chlamydomonas. A, abundance of CYC6, HPPD1, and HPPD2 transcripts. Independent cultures corresponding to Chlamydomonas wild-type strain CC4532 were grown in dark hypoxia. Cells were collected after reaching a density of \( 2 \times 10^8 \) cells/ml and analyzed for RNA abundance. Each symbol represents an independent experiment analyzed in technical triplicates. B, transcript abundance of genes encoding enzymes of the tocopherol biosynthesis pathway were determined by RNA sequencing in dark grown and dark anoxic Chlamydomonas strain CC4532 (38). The sizes of the circles are proportional to the relative abundance (RPKM) of the total transcript pool of genes encoding enzymes regulating the plastochinone/tocopherol pathway as shown in Fig. 8. C, the tocopherol content in Chlamydomonas CC4532 that was either grown in the dark, normoxic (high) or in the dark, hypoxic (low) as indicated.
\end{flushright}

B

\begin{flushright}
\textit{FIGURE 13}. The decrease of \( \gamma \)-tocopherol in dark hypoxia is independent of CRR1. A, abundance of HPPD1 transcripts as determined by quantitative real time PCR. Independent cultures corresponding to CRR1 (black circles), crr1 (triangles apex up), and crr1-\( \Delta \text{Cys} \) (triangles apex down) were grown in TAP medium in the conditions indicated: dark hypoxia (white symbols) relative to dark (black symbols). Cells were collected after reaching a density of \( 2 \times 10^8 \) cells/ml and analyzed for RNA abundance. Each symbol represents an independent experiment analyzed in technical triplicates. Tocopherol (B) and plastochinone (C) content in Chlamydomonas crr1 and corresponding strains that have been complemented with full-length CRR1 (crr1(CRR1)) or with a truncated version of CRR1 lacking a cysteine-rich domain (crr1-\( \Delta \text{Cys} \)). Respective strains were either grown in the dark, normoxic (high) or hypoxic (low) as indicated. Data are the averages of three biological replicates \( \pm \) S.D.
\end{flushright}

membranes twice for 15 min each time. The secondary antibody, used at 1:10,000, was goat anti-rabbit conjugated to alkaline phosphatase. Antibodies directed against CF1 (1:15,000), Plastocyanin (1:5,000), OEEl (1:5,000), PsAF (1:4000, F. A. Wollman), Lhca3 (1:8000, M. Hippler), LHCSR3 (1:1000, Agrisera), CP29/Lchb4 (1:4000, J. D. Rochaix), D1 (1:1,000, A. Wollman), Lhcb2 (1:5,000, Agrisera), PSAK (1:2,000, M. Hippler), CP29/Lcb4 (1:4000, F. A. Wollman), Lhca3 (1:8000, M. Hippler), LHCSR3 (1:1000, M. Hippler), CRD1 (1:1,000), and Cyt \( _c \) (1:2,000) and HPPD (affinity purified against Arabidopsis HPPD, kindly provided by M. Matringe) (1:1,000) were used.

Quantitative Metal, Phosphorus and Sulfur Content Analysis—1 \times 10^8 Chlamydomonas cells of a culture at a density of \( 3 \times 10^8 \) cells/ml were collected by centrifugation at 2450 \( \times g \) for 3 min in a 50-ml Falcon tube. The cells were washed three times in 1 mM Na_2-EDTA, pH 8 (to remove cell surface-associated metals), and once in Milli-Q water. The cell pellet, after removing the water, was overlaid with 286 \( \mu l \) of 70% nitric acid and digested at room temperature for 24 h at 65 °C for about 2 h before being diluted to a final nitric acid concen-
tation of 2% (v/v) with Milli-Q water. Aliquots of fresh or spent culture medium were treated with nitric acid to a final concentration of 2% (v/v). Metal, sulfur, and phosphorous contents were determined by inductively coupled plasma mass spectrometry on an Agilent 8800 Triple Quadrupole ICP-MS instrument, in comparison to an environmental calibration standard (Agilent 5183–4688), a sulfur (Inorganic Ventures MSY-100PPM), and phosphorus (Inorganic Ventures CGP1) standard, using 89Y (the 89 isotope of the chemical element Yttrium) as an internal standard (Inorganic Ventures MSY-100PPM). The lev-

tiation between these replicates are depicted in figures.

the variation in between cultures, average, and standard devia-

ticate samples (independent cultures) were used to determine

each individual biological sample, the average variation

final version of the manuscript.

per deficiency and high light experiments. S. S. performed and ana-

performed hypoxia experiments. D. S. and C. A. L. performed cop-

truita for critical reading of the manuscript.

Acknowledgments—We thank Prof. Michel Matringe for the Arabi-

dopsis thaliana HPPD immunopurified antibody and Dr. Madeli Cas-

References


mydomonas is mediated by the same element. J. Biol. Chem. 275, 6080–6089


mydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. Proc. Natl. Acad. Sci. U.S.A. 102, 18730–18735


hardtii. EMBO J. 19, 2139–2151


ing complex accumulation. Plant Cell 14, 673–688


plast open-reading frame ycf54 is required for activity of the magnesium protoporphyrin monomethylster oxidative cyclase in Synechocystis PCC 6803. J. Biol. Chem. 287, 27823–27833

16. Bolívar, D., Braumann, I., Berendt, K., Gough, S. P., and Hansson, M. (2014) The Ycf54 protein is part of the membrane component of Mg-

protoporphyrin IX monomethyl ester cyclase from barley (Hordeum vul-

gare L.). FEBS J. 281, 2377–2386


ols protect Synechocystis sp. strain PCC 6803 from lipid peroxidation. Plant Physiol 138, 1422–1435


Genetically Programmed Changes in Photosynthetic Cofactor Metabolism in Copper-deficient *Chlamydomonas*
Daniela Strenkert, Clariss Ann Limso, Abdelhak Fatihi, Stefan Schmollinger, Gilles J.
Basset and Sabeeha S. Merchant

doi: 10.1074/jbc.M116.717413 originally published online July 20, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.717413

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 32 of which can be accessed free at [http://www.jbc.org/content/291/36/19118.full.html#ref-list-1](http://www.jbc.org/content/291/36/19118.full.html#ref-list-1)