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

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Genetic and genomic studies indicate that copper deficiency triggers changes in the expression of genes encoding key enzymes in various chloroplast-localized lipid/pigment biosynthetic pathways. Among these are CGL78 involved in chlorophyll biosynthesis and HPPD1, encoding 4-hydroxyphenylpyruvate dioxygenase catalyzing the committed step of plastoquinone and tocopherol biosyntheses. Copper deficiency in wild-type cells does not change the chlorophyll content, but a survey of chlorophyll protein accumulation in this situation revealed increased accumulation of LHCSR3, which is blocked at the level of mRNA accumulation when either CGL78 expression is reduced or in the crd1 mutant, which has a copper-nutrition conditional defect at the same step in chlorophyll biosynthesis. Again, like copper-deficient crd1 strains, cgl78 knock-down lines also have reduced chlorophyll content concomitant with loss of PSI-LHCI super-complexes and reduced abundance of a chlorophyll binding subunit of PSI, PSAK, which connects LHCI to PSI. For HPPD1, increased mRNA results in increased abundance of the corresponding protein in copper-deficient cells concomitant with CRR1-dependent increased accumulation of γ-tocopherols, but not plastoquinone-9 nor total tocopherols. In crr1 mutants, where increased HPPD1 expression is blocked, plastochromanol-8, derived from plastoquinone-9 and purported to also have an antioxidant function, is found instead. Although not previously found in algae, this metabolite may occur only in stress conditions.

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) 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 SQUAMOSA 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 tetrapyrrole 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 tetrapyrrole 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
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 equivalents 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 was the accumulation of PSAK, a connector between PSI and LHCl. This phenotype is similar to that of the crr1 mutant, which is blocked at the same step in Chl biosynthesis. For HPPD, we noted 3) 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 crr1 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 indicated 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-
whether functions in concert with CRD1 and CTH1, we tested reciprocally regulated, also by CRR1 (13). Because CGL78 cyclase. Its expression is up-regulated by poor copper nutrition via CRR1 (12). The other isoform, named CTH1, is 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 pattern of PSI/II-LHC super-complexes is reduced when CGL78 expression is down-regulated. Thylakoid membranes extracted from control (CGL78) or cgl78-ami lines reveal reduced chlorophyll content in PSI/II-LHC super complexes (red arrow). Super-complex composition of selected proteins was verified by second dimension, denaturing SDS-PAGE. Shown are immuno-detections with anti-LHCB2 (LHCII marker) and anti-PSAF (PSI marker) and anti-D1 (PSII marker). All experiments shown were performed at least twice either with cgl78-ami11 or cgl78-ami13.

Nevertheless, 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 PSI/II-LHC super-complex formation in wild-type lines (Fig. 3D, red arrow), but copper-deficient cgl78-amiRNA lines showed reduced amounts of PSI/II-LHC super-complexes (Fig. 3D, marked by an asterisk). The pattern of PSI/II-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 PSAK, which is the subunit connecting PSI and LHCI. The loss of PSI-LHCI complexes prompted us to monitor PSAK accumulation in cgl78-ami lines, where we found it to be decreased to 50% (Figs. 4 and 6C). This molecular phenotype is reminiscent of the crd1 mutant (30). CRD1 is one of two isoforms of a subunit of Mg Protoporphyrin IX monomethyl cyclase. Its expression is up-regulated by poor copper nutrition via CRR1 (12). The other isoform, named CTH1, is reciprocally regulated, also by CRR1 (13). Because CGL78 functions in concert with CRD1 and CTH1, we tested whether CRD1 and CTH1 expression might be affected in the cgl78-ami lines (Fig. 4C). We did not see any significant change in CRD1/CTH1 abundances in cgl78-ami lines (Fig. 4, A and B) nor in the pattern of mRNA accumulation as assessed by qRT-PCR (Fig. 4C). Likewise, CGL78 mRNA abundance is also not changed in crd1 as compared with CRD1 cells (Fig. 4D). Therefore, the expression 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 PSI-LHCl peak to 682 nm and loss of the PSI-LHCI 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 lines (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 ± 1.1 × 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 (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 HPPD, 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
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Chlamydomonas (Fig. 9, D and E). Nevertheless, we noted a consistently higher proportion of γ-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 γ-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-ΔCys (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 γ-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 γ-tocopherol to α-tocopherol ratio is dependent on copper nutrition and CRR1 and is an intrinsic response to poor copper nutrition.

When we surveyed plastoquinone 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 plastoquinone 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 plastoquinol-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 γ-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 O₂, a substrate of the reaction. Indeed, wild-type hypoxic cells have less γ-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, γ-tocopherol content is decreased rather than increased relative to normoxic cells (Fig. 13B). When we looked at the plastoquinone 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 c₆ 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.

**FIGURE 7.** Weaker antenna-reaction center association in copper-deficient cells. 77 K fluorescence emission spectra of samples prepared from _CGL78_ in black, _cgl78-ami11_ in bright blue, and _cgl78-ami13_ in dark blue. Cells are grown in TAP medium with copper (+ Cu) (A) or without copper (−Cu) supplementation (B) at a PFD of 100 μmol m⁻² s⁻¹. Cells grown in TAP medium with copper were exposed for 24 h to high light (600 μmol m⁻² s⁻¹) (C). D, _CGL78_ lines were grown in TAP medium containing 20 (black) or 0.25 μM (orange) iron. The excitation wavelength was 435 nm. Fluorescence emission was normalized to the value at 685 nm. The peaks are labeled with the wavelength of the maximum if applicable.

**γ-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 γ-tocopherol content. The proportion of γ-tocopherol is small (17% of the total tocopherol pool) and the increase therefore is 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 γ-tocopherol may serve to fine tune photosynthetic physiology by adjusting antioxidant or stress...
capacity. 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 α-tocopherol).

Plastochromanol-8 has not previously been found in algae, but was revealed here in the crr1 mutant and only in copper deficiency where crr1 grows poorly. Because it is implicated in stress protection (46), its production may be a downstream consequence of stress in 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 α- versus γ-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 γ-tocopherol. In hypoxic cells, expression of HPPD1 is also increased but in the absence of the substrate O_2, γ-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 cgl78-ami lines and in 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).

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 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

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**FIGURE 8. Overview of the plastoquinone and tocopherol biosynthesis pathway in Chlamydomonas.** HGA, homogentisate; HST, homogentisate solanesyl transferase; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; HPPD, 4-hydroxyphenylpyruvate dioxygenase; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinol; VTE1, tocopherol cyclase; VTE2, homogentisate phytoltransferase (HPT); VTE3, 2-methyl-6-phytylplastoquinol methyltransferase (MPBQMT); VTE4, γ-tocopherol methyltransferase (γ-TMT).
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A

|        | HPPD1 | HPPD2 |
|--------|-------|-------|
| +Cu    |       |       |
| -Cu    |       |       |
| +O2    |       |       |
| -O2    |       |       |

(light) (dark)

B

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 (black squares) or +Cu (white squares). Cells were collected after reaching a density of 5–8 \( \times 10^6 \) 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.

C

\( \text{Cu} + - \)

Ponceau S

aHPPD

C

D

E

\( \text{a-HPPD} \)

\( \text{HPPD1} \)

\( \text{HPPD2} \)

\( \alpha\)-tocopherol

\( \gamma\)-tocopherol

\( \text{Cu} + - \)

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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 10 Ex Taq buffer (Mg2+ plus) (TaKaRa), 2 μl of 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, CAGGTCTTACGCAACTGT; CYC6Rev, ATCGCCCCCTTGCCAT; HPPD1For, GGTCGCGTCGATTGGGTTAC; HPPD1Rev, TGAGAACTCGTGGAAGCCACA; HPPD2For, ACCTCCTTCGGCCTGCAAC; HPPD2Rev, CACGTCCTCCGCAACA-CT; CGL78For, CCTGGACCGCGTGCTGAAGA; CGL78Rev, TACCGGGCGTAAGGGGCAGT; LHCSR3f, CACAACACCTTGATGCGAGATG; LHCSR3r, CCGTGTCTTGTCAGTCCCTG; CRD1For, CGTAGGTAGGCTGACTGCGTTG; CRD1Rev, GTCACTTATGCGACGGCCTTG; CTH1(2)F, AGCTGTGCGTCGCGGGCTTGT; CTH1(2)R, ATCCGCGTGTTCCGAAGAAAC; CTH1(3)F, ACGCAGCAGCACAGCTCACT; and CTH1(3)R, TCCCAGAAGTCTAGCCCGATG.

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-
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FIGURE 12. HPPD1 is also regulated by dark hypoxia but γ-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 2–6 × 10^6 cells/ml and analyzed for RNA abundance. Each symbol represents an independent experiment analyzed in technical triplicates. B, transcript abundance of genes encoding enzymes regulating the plastoquinone/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.

FIGURE 13. The decrease of γ-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-ΔCys (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 2–6 × 10^6 cells/ml and analyzed for RNA abundance. Each symbol represents an independent experiment analyzed in technical triplicates. Tocopherol (B) and plastoquinone (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-ΔCys). Respective strains were either grown in the dark, normoxic (high) or hypoxic (low) as indicated. Data are the averages of three biological replicates ± S.D.
turation 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 CGS1), and phosphorus (Inorganic Ventures CGP1) standard, using $^{89}Y$ (the 89 isotope of the chemical element Yttrium) as an internal standard (Inorganic Ventures MSY-100PPM). The levels of all analytes were determined in MS/MS mode, where $^{63}$Cu were measured directly using helium in the collision reaction cell, whereas $^{56}$Fe was directly determined using H2 as a cell gas. The average of 4 technical replicate measurements was used for each individual biological sample, the average variation between the technical replicate measurements was 1.1% for all analytes and never exceeded 5% for an individual sample. Triplicate samples (independent cultures) were used to determine the variation in between cultures, average, and standard deviation between these replicates are depicted in figures.

**Fluorescence Measurements**—Chlorophyll fluorescence emission spectra at 77 K were recorded using a TECANXP-HP8000 using intact cells diluted to a concentration of 5 μg of chlorophyll/ml in 30% glycerol, 5 mM Hepes, pH 7.5, and 10 mM EDTA and snap frozen in liquid nitrogen in a customized 96-well plate. The samples were excited at 435 nm (bandwidth 5 nm), and emission was monitored between 640 and 760 nm (bandpass 1 nm). The gain was manually adjusted to 150, the Z-position was 22,000 μm. The signals obtained were processed with the Adjacent-Average function (OriginLab) and normalized to the value obtained at a wavelength of 685 nm.

**Plastoquinone-9 and Tocochromanol Analyses**—Chlamydomonas cells were grown to a density of 4–8 × 10^6 cells·ml⁻¹ and 50–100 ml were collected by centrifugation (5 min at 2450 × g, 4°C). For normalization, 1 ml of each culture was used for chlorophyll determination according to Ref. 58. For plastoquinone-9 and tocochromanol analyses, frozen cell pellets were resuspended in 500 μl of 95% (v/v) ethanol, spiked with 50 μl of 121 μM ubiquinone-10 (6.05 nmol) as an internal standard, and homogenized in a 5-ml Pyrex tissue grinder. The grinder was then rinsed with 500 μl of 95% (v/v) ethanol, and the wash was combined to the initial extract. Insoluble material was removed by centrifugation (5 min at 18,000 × g) and the supernatants were immediately analyzed by HPLC as previously described (60).

**Author Contributions**—D. S. and S. S. M. designed the study. D. S. designed and constructed vectors for conditional knock-down and performed hypoxia experiments. D. S. and C. A. L. performed copper deficiency and high light experiments. S. S. performed and analyzed ICP-MS measurements. A. F. and G. J. B. performed the tocopherol and plastoquinone experiments. D. S. and S. S. M. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank Prof. Michel Matringe for the Arabidopsis thaliana HPPD immunopurified antibody and Dr. Madeli Casttruita for critical reading of the manuscript.

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