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

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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 \( \gamma \)-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) (3). In *Chlamydomonas*, this switch is controlled by copper response elements associated with the *CYC6* gene encoding Cyt (4) 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 \( O_2 \)-dependent metabolic enzymes, are enriched among the CRR1 targets in *Chlamydomonas* (11). Among these are *CRD1* and *CPRX1*, encoding enzymes regulating two rate-limiting steps in tetrapyrrole biosynthesis: *crr1* 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 \( O_2 \)-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 \( c_6 \) 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 electron donor and a component of the LHC. Thus, defects in HPPD activity result in a loss of PQ-9, leading to the accumulation of LHCSR3. This phenotype is similar to that of the crd1 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 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). According, 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-
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 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 PSIII 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 in the abundance of D1 (Fig. 4). 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).

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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 × 10⁷ atoms/cell) does not differ from the iron content in copper-depleted cells (10.4 ± 1.1 × 10⁷ atoms/cell). Therefore, we conclude a direct consequence of copper nutrition on the performance of the PSI-LHCI complex.

Because LHCSR3 seemed to be the most affected chlorophyll-binding protein in copper-deficient cgl78-am1 lines, and LHCSR3 has a known role in photo-protection (34–38) we wondered if cgl78-am1 strains are more sensitive to high light relative to the control. Indeed, if we grew cgl78-am1 lines in high light, we observed that the cells became bleached (Fig. 6A). The chlorophyll content of cgl78-am1 strains that were grown for 20 h in high light is reduced to 5.5%, as compared with the wild-type lines (Fig. 6B). The latter suggests that the cyclase in Chl biosynthetic enzymes, previous transcriptome analyses had identified other changes in cofactor-encoding 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. Previ-ous 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 synthesis 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
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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 crl1 mutant (because HPPD1 is a CRR1 target) and two independent complemented lines, CRR1 (in which the wild-type gene rescued crl1) and crl1-ΔCys (in which a gene encoding a C terminally truncated version of CRR1 complements the crl1 mutation). The copper status of the cultures was validated on the basis of sentinel protein accumulation (Fig. 10A). Both complemented lines, but not the crl1 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 crl1 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 crl1 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 crl1 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 crl1 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 crl1 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 crl1 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 crl1 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_c6 (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_c6 as a less effective catalyst.

Although it is not yet evident in the laboratory setting, the absence of Cyt_c6 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_c6 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.
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 \( \alpha \)-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 \( \alpha \)-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 \( cgl78 \)-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).

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 \( cgl78 \)-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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A

+Cu

HPPD1

HPPD2

−Cu

+Cu

−O

O

B

C

Ponceau S

Cu

−

−

−

E

γ-tocopherol

α-tocopherol

Cu

−

−

CC4532

CC4532

FIGURE 9. HPPD accumulation correlates with HPPD1 abundance and increased γ-tocopherol in copper-deficient Chlamydomonas. A–D, changes in HPPD1-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 HPPD1 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 × 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.

of PSAK is even more dramatic in cgl78-ami lines subject to a high light treatment (Fig. 6). We had suggested previously that PSAK may modulate the connection between LHCI and PSI, 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, ccgtagATGTGTCACCCGTACATTcgcgtatcgcaccatgaggtgtggttgagccgtcmetaTATGACGTGATCCACATCTAa and CGL78amiRev, ctagcTGATGTCACCTCAATcgcgtatcgcaccacccca-cgtggtggtggtgatcaccacccca-ctagtGACGTGATCCACATCTAa (uppercase letters representing miRNA*/miRNA sequences) were annealed by boiling and slowly cooling down in a thermocycler and ligated into SpeI-digested pMSS39, yielding PDS1. PDS1 was linearized by digestion with HindIII and transformed into Chlamydomonas strain CC4351 by protoplast transformation (55). CGL78-amiRNA strains and Chlamydomonas reinhardtii strains CC4352, crl1–2 (CC5068), CR1 (CC5071), and crl−Δcys (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 (57). CGL78-amiRNA strains were either inoculated from a plate into standard TAP(NH₄) to repress the artificial micro-RNA or a modified TAP medium, where nitrate was substituted instead of ammonium as the sole nitrogen source, TAP(NO₃). Dark hypoxic cells were grown in TAP medium for 24 h in 1% air, 2% CO₂, and 97% N₂ by bubbling before collection as described in 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 × 10^7 cells were collected by centrifugation for 5 min at 2450 × 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+ plus) (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, CAGGTCTTACCGCAACTGT; CYC6Rev, ATCGCCCCCTTGCCAT; HPPD1For, GGTCGCGTCGATTGGGTTAC; HPPD1Rev, TGAGAACTCGTGGAAGCCACA; HPPD2For, ACCTCCTTCGGCCTGCAAC; HPPD2Rev, CACGTCCTCCGCAACAACT; CGL78For, CCTGGACCGCGTGCTGAAGA; CGL78Rev, TACCGGGCGTAAGGGGCAGT; LHCSR3f, CACAACACCTTGATGCGAGATG; LHCSR3r, CCGTGTCTTGTCAGTCCCTG; CRD1For, CGTAGGTAGGCGACTGCGATT; CRD1Rev, GTATTATGCGCAGCCTTGG; CTH1(2)F, ACGTGTGCGTCGGGAGCTTGT; CTH1(2)R, ATCCGGCGTGGTTCCGAAGAAAC; CTH1(3)F, ACGCAGCAGCACAGCTCACT; and CTH1(3)R, TCCCAAGTCTAGCGCCGATG.

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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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 \( 2 - 6 \times 10^{6} \) cells/ml and analyzed for dna 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 dna 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 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.

membranes in 20 mm na-tricine, ph 8, 10 mm mgcl2, 0.4 m sorbitol; 0.2% bsa before sonication (two cycles of 5 s, 50% power). thylakoid membranes were mildly solubilized for 15 min on ice in the dark with 1.5% \( \beta \)-dodecyl-d-maltoside (\( \beta \)-dm). the unsolubilized membranes were removed by centrifugation at 12,100 \( \times \) g for 5 min, and the solubilized thylakoid membranes equivalent to 20 \( \mu \)g of chlorophyll were separated by bn-page.

protein analyses—sd-page was performed using 20 \( \mu \)g of total protein for each lane and transferred in a semi-dry blotter to nitrocellulose membranes (amersham biosciences protran 0.1 nc). the membrane was blocked 30 min with 3% dried milk inpbs (137 mm nacl, 2.7 mm kcl, 10 mm na2hpo4, 1.8 mm kh2po4) containing 0.1% (w/v) tween 20 and incubated in primary antiserum; this solution was used as the diluent for both primary and secondary antibodies, for 1 h, respectively. pbs containing 0.1% (w/v) tween 20 was used for washing 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), pasef (1:4000, j. d. rochaix), d1 (1:1,000, agrisera), lhcb2 (1:5,000, agrisera), psek (1:2,000, m. hippler), cp29/lhcb4 (1:4000, f. a. wollman), lhca3 (1:8000, m. hippler), lhcsr3 (1:1000, m. hippler), crr1 (1:5000), crd1 (1:1000), and cyt c (1:2000) and hppd (affinity purified against arabadopsis hppd, kindly provided by m. matringe) (1:1000) were used.

quantitative metal, phosphorus and sulfur content analysis—\( 1 \times 10^{8} \) chlamydomonas cells of a culture at a density of \( 3 - 5 \times 10^{6} \) 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 na2-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 concentra-
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References

singlet oxygen produced by the triplet states of chlorophyll in the PSI reaction centre. J. Exp. Bot. 57, 1677–1684


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