Defects in the Cytochrome b₆/f Complex Prevent Light-Induced Expression of Nuclear Genes Involved in Chlorophyll Biosynthesis¹

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Mutants with defects in the cytochrome (cyt) b₆/f complex were analyzed for their effect on the expression of a subgroup of nuclear genes encoding plastid-localized enzymes participating in chlorophyll biosynthesis. Their defects ranged from complete loss of the cyt b₆/f complex to point mutations affecting specifically the quinone-binding Q₉ site. In these seven mutants, light induction of the tetrapyrrole biosynthetic genes was either abolished or strongly reduced. In contrast, a normal induction of chlorophyll biosynthesis genes was observed in mutants with defects in photosystem II, photosystem I, or plastocyanin, or in wild-type cells treated with 3-(3’-dichlorophenyl)-1,1-dimethylurea or 2,5-dibromo-3-methyl-6-isopropyl benzoquinone. We conclude that the redox state of the plastoquinone pool does not control light induction of these chlorophyll biosynthetic genes. The signal that affects expression of the nuclear genes appears to solely depend on the integrity of the cyt b₆/f complex Q₉ site. Since light induction of these genes in Chlamydomonas has recently been shown to involve the blue light receptor phototropin, the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Christoph F. Beck (beck@uni-freiburg.de).

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suggested that the integrity of a photosynthetic complex, rather than its activity in electron transfer, could be the source of the signals that control nuclear gene expression. While a large collection of data is available on the genes whose expression is perturbed in response to alterations in the chloroplast (Biehl et al., 2005; Leister, 2005), the site of signal generation within the chloroplast have not yet been identified.

In the analysis presented here, we focused on the role of the cytb$_{6/f}$ complex in signaling toward the nucleus and present an analysis of seven mutants defective in this complex. In all of these mutants, the light induction of a subset of chlorophyll biosynthetic genes was either abolished or strongly diminished, while the expression of these genes was not affected when photosynthetic electron flow and the redox state of the plastoquinone pool were altered by inhibitors or by mutations in other photosynthetic complexes.

RESULTS

Mutants Defective in the Cytb$_{6/f}$ Complex

The mutants analyzed and their relevant characteristics are summarized in Table I. They include two mutants isolated in a screen aimed at the identification of genes that control light sensitivity (P1-15 and P2-26). These two strains are as well as five mutants ($\Delta$petA, mcd1-F16, tca1-693, petD-PWYE, petC-$\Delta$1, and clpP1-AUU) described before have defects in photosynthesis, i.e. they do not grow photoautotrophically and thus require acetate for growth. In addition, they are light sensitive, i.e. they grew on acetate-containing plates irradiated with a fluence rate of 20 $\mu$E m$^{-2}$s$^{-1}$ of white light but not when irradiated with a fluence rate of 80 $\mu$E m$^{-2}$s$^{-1}$ or 500 $\mu$E m$^{-2}$s$^{-1}$ in the case of the P1-15 mutant. The P1-15 and P2-26 strains displayed fluorescence induction kinetics typical for cytb$_{6/f}$ mutants, i.e. a continued rise in fluorescence up to levels attained in the presence of 3-(3′4′-dichlorophenyl)-1,1-dimethylurea (DCMU; Fig. 1A). In P2-26, the maximum level of fluorescence obtained in the absence of DCMU was slightly higher than that seen in the presence of the herbicide, indicating a complete block in cytb$_{6/f}$ electron transfer. The higher level in the absence of DCMU may be explained by a full oxidation of plastoquinone, acting as a quencher of fluorescence (Joliot et al., 1998). Mutant P1-15, in contrast, resembles leaky cytb$_{6/f}$ mutants (see, for example, figure 4 in de Vitry et al., 1999), with a lower level of fluorescence in the absence

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<td>$\Delta$psbD</td>
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Rieske Fe-S protein, leading to a complete absence of this essential subunit. In the strain we have used, the latter mutation was combined with clpP1-AUU, a mutation reducing accumulation of the ClpP protease, resulting in the stabilization of a cyt3/f subcomplex lacking the Rieske protein (Majeran et al., 2000). The absence of cyt f was demonstrated by immunoblot analysis for mutant P2-26 and confirmed for mutants ΔpetA, mcd1-F16, and tca1-693 (Fig. 1B). Traces of Rieske protein could be observed in some of the mutants depending on the individual experiment, resulting from incomplete degradation when other subunits are missing (O. Vallon, unpublished data). In contrast, both cyt f and Rieske proteins accumulated normally in the petD-PWYE mutant, as described before (Zito et al., 1999). Also included in this analysis was the mutant stt7, which is defective in the protein kinase that is required for the phosphorylation of the major light-harvesting protein (LHCII), an essential requirement for state transitions to occur (Depe`ge et al., 2003). This mutant has normal levels of cyt3/f (Fig. 1B), grows photoautotrophically, and is not light sensitive. Mutant AP6 has a defect in the PCY gene for plastocyanin (PC, Fig. 1B) and was used as a control. We observed a reduced level of PC in P1-15, which was shown to be caused by a second site mutation since it separated from the cyt3/f defect in crosses. This defect was not linked to the regulatory phenotype of mutant P1-15 (data not shown).

Mutants with Defects in the Cyt3/f Complex Exhibit Deregulation of Genes Involved in Chlorophyll Synthesis

A number of Chlamydomonas reinhardtii genes involved in chlorophyll biosynthesis (HEMA, GSA, ALAD, CPXI, CHLD, CHLI1, and CTH1) have been shown to be induced in dark-adapted cultures by a shift from dark to light of a moderate fluence rate (40 μE m-2 s-1; Vasilieuskaya et al., 2004). The expression of five of these nuclear genes was analyzed at the RNA level in those mutants that either lacked the cyt3/f complex or possessed strongly reduced levels. In all cases, the patterns of mRNA accumulation following the shift to light were clearly different from that of the wild type, exhibiting a lack of light-induced accumulation (Fig. 2). In some mutants, mRNA levels for genes GSA, ALAD, and CHLI1 in the dark or after shift to light remained below the level of detection. In the P1-15 mutant, some residual light-induced mRNA accumulation was observed but this was distinctly lower than in the wild-type strain. In contrast to the five genes of tetrapyrrole biosynthesis, HSP70B, a nuclear gene encoding a chloroplast-localized chaperone (Schroda et al., 1999), exhibited normal light induction in the mutants. Also, expression of CBP, encoding a Gα-like protein (von Kampen et al., 1994) and used as a loading control, appeared not to be affected by defects in the cyt3/f complex. Thus, expression of a subset of nuclear genes encoding enzymes required for chlorophyll synthesis was specifically...
deregulated in mutants defective in the cytb/f complex, i.e. showed no up-regulation in the light.

Genetic Analysis of Cytb/f Mutants

In *C. reinhardtii*, a haploid organism, second site mutations manifest themselves phenotypically and may falsely contribute to the phenotype of a first site mutation. It could be envisioned that mutations affecting gene regulation tend to accumulate over time in cytb/f mutants, provided they enhance their survival under storage conditions. To address this question, mutants with defects in the cytb/f complex were crossed with wild-type strains and random spores were picked among the progeny and scored for defective photosynthesis, light sensitivity, and light induction of chlorophyll biosynthesis genes (Table II). If the deregulation of the genes observed was due to a mutation different from that affecting the cytb/f complex, independent segregation of the phenotypes acetate requirement/light sensitivity (ac<sup>2</sup>) and no light regulation of chlorophyll biosynthesis genes (lrc<sup>2</sup>) would be expected (unless the two lesions were closely linked).

For the chloroplast-born mutation ΔpetA, 93% of the progeny was acetate requiring and light sensitive, as expected from the largely uniparental transmission of chloroplast genes (Harris, 1989). Four each of the

### Figure 2

Expression of selected genes of chlorophyll biosynthesis and HSP70B in mutants lacking cytb/f complex components. A, RNA-blot analysis, details of which are given in “Materials and Methods.” Prior to light exposure, cultures were incubated in the dark for 20 h (D). Then, cultures were shifted to light (fluence rate 40 μE m<sup>−2</sup> s<sup>−1</sup>) for 1 h (L1) and 2 h (L2). At these time points samples were taken for RNA extraction. For RNA-blot analyses, 10 μg of total RNA were hybridized with probes specific for the genes indicated. The constitutively expressed *CBLP* gene, encoding a Gβ-like protein (von Kampen et al., 1994), served as a loading control. B, Quantitative evaluation of RNA-blot data from at least three experiments. The relative induction of each gene with SEM was normalized using the signal for the *CBLP* gene. The induction ratios given were determined by dividing the values of the L2 samples by those of the D samples.
ac− mutant and ac+ progeny were tested for their ability to induce chlorophyll biosynthesis genes upon dark-to-light shift using RNA blots. To ensure that truly recombinant progeny was tested, as opposed to unmutated parental gametes that would have survived the zygote selection process, mt− clones were chosen for the ac+ progeny, and mt+ clones for the ac− progeny. In the eight clones tested, strict correlation was observed between the photosynthesis defect/light sensitivity and absence of gene induction by light (Table II).

Crosses of the four nuclear mutants showed an approximately equal distribution of wild-type and ac− phenotypes (Table II), as expected for segregation of single nuclear mutations. As above, four randomly picked clones of each phenotypic class were analyzed for the lrc phenotype and here again, perfect correlation was observed between the ac and lrc phenotypes (Table II). In spite of the small number of progeny analyzed, the fact that this result was observed for five independent mutants carrying mutations in two distinct genomes clearly shows that the defect in light induction of the chlorophyll biosynthesis genes is a direct consequence of cytb6/f deficiency.

Light Induction of Genes Involved in Chlorophyll Synthesis Is Not Affected by Other Types of Photosynthesis Mutations or by Inhibitors of Electron Transport

The deregulation phenotype of mutants defective in the cytb6/f complex raised the question whether interruption of photosynthetic electron transport by itself is sufficient to cause the alteration in gene expression observed. To address this question we made use of mutants defective in different steps of electron transport. A ΔpsbD mutant, defective in PSII, showed a wild-type pattern of gene expression (Fig. 3). Also mutant AP6, which lacks PC (Fig. 1B) and thus, in the absence of the alternative electron carrier cyt c6 that only is present when cells are deprived of copper (Merchant and Bogorad, 1986), is unable to transport electrons from the cytb6/f complex to PSI, showed light induction of the genes assayed (Fig. 3). The slight delay in light induction in the PC mutant was not observed with other PC-defective strains (data not shown). Lastly, mutant ΔpsaB, defective in PSI, was also not affected in the regulation of the genes assayed. We conclude that a block in photosynthetic electron transport by itself is not sufficient to prevent light induction of the nuclear genes analyzed. Importantly, while in PSI mutants the plastoquinone pool is rapidly oxidized during the dark-to-light shift, the reverse will occur in PSI and PC mutants where, just like in cytb6/f mutants, the plastoquinone pool will be overreduced. Thus, the deregulation phenotype of cytb6/f mutants cannot be attributed to an indirect effect on the redox state of the plastoquinone pool during illumination.

In accordance with these observations, the application of the PSI inhibitors DCMU and the phenolic herbicide dinoterb did not affect gene expression patterns (Fig. 3). These compounds interact with the Qb binding site of the PSI complex and prevent reoxidation of the primary quinone acceptor by the plastoquinone pool.

More surprisingly, 2,5-dibromo-3-methyl-6-isopropyl benzoquinone (DBMB), which inhibits the oxidation of plastoquinol at the Qb site of the cytb6/f complex (Roberts et al., 2004), did not influence the light induction of the chlorophyll biosynthesis genes (Fig. 3). DBMB binds at the Qb site of cytb6/f, where plastoquinol is reoxidized, suggesting that plastoquinol binding and turnover at this site is not necessary for light induction to occur. To rule out any direct effect of light on the electron transfer chain, the PSI mutant was treated with DCMU. Here again, light induction was observed both at 3 μM DCMU (Fig. 3) and 6 μM DCMU (data not shown), in spite of the fact that inactivation of both photosystems would obviously prevent any light-induced redox changes in the plastoquinone pool and cytb6/f complex.

Analysis of Mutants with Discrete Defects in Cytb6/f Subunits or in Cytb6/f Signaling

These observations suggested that the abrogation of light induction of the chlorophyll biosynthesis genes was due to the lack of accumulation of the cytb6/f complex, rather than to the absence of electron transport through the complex. We therefore analyzed mutants that accumulated the cytb6/f complex, but carried more discrete defects preventing electron transfer. The petC-Δ1 mutant (de Vitry et al., 1999) is devoid of Rieske Fe-S protein, hence of a functional
Q₀ site, but accumulates almost normal levels of cyt f and other core subunits of the complex, at least when combined with a mutation that attenuates ClpP (Majeran et al., 2000). This strain clearly exhibited the same deregulated phenotype seen for the mutants that lack the cytb₆/f complex (Fig. 4). We also analyzed the petD-PWYE mutant, which carries three substitutions in subunit IV and assembles the complex (including the Rieske protein), but is unable to bind plastoquinol in the Q₀ pocket and therefore to transfer electrons (Zito et al., 1999). This mutant also exhibited no light induction of the five genes analyzed (Fig. 4). The petD-PWYE mutant was crossed to wild type, and the phenotype of the progeny was analyzed as described above. Again, a perfect correlation was observed between the ac² and lrc² phenotypes (Table II; Fig. 5).

Together, these results indicate that normal light induction of chlorophyll biosynthesis genes not only requires the assembly of a complete cytb₆/f complex, but also that its Q₀ site is able to bind quinones. The Q₀ site is also known to regulate state transitions, activating the LHClI kinase in a redox-dependent manner. In Chlamydomonas, the Stt7 kinase has been shown to be required for this process (Dépège et al., 2003). In Arabidopsis, mutation of the orthologous gene Stn7 (Bellaflora et al., 2005) not only impairs state transitions, but also affects expression of nuclear genes, as does inactivation of the paralogous gene Stn8 (Bonardi et al., 2005). We therefore analyzed the Chlamydomonas stt7 mutant and found that it exhibited a pattern of gene expression similar to that of the wild-type strain (Fig. 4), indicating that the Stt7 kinase is not involved in light induction of chlorophyll biosynthesis genes.

DISCUSSION

The light induction of the five chlorophyll biosynthesis genes analyzed here has recently been shown to be mediated via the blue light receptor phototropin (Im et al., 2006). The fact that induction of these genes observed after a shift from dark to light is markedly diminished in strains with reduced levels of phototropin implies an essential role of this photoreceptor in the regulation of these genes. This signaling pathway is envisioned to act at the level of activation of transcription or, alternatively, via stabilization of the transcripts. For one gene (GSA), a partial characterization of the blue light signaling pathway has suggested the participation of a heterotrimeric G-protein and phospholipase C, an increase in the cytosolic Ca²⁺ concentration, and activation of calmodulin as well as of a calmodulin-dependent...

![Figure 3. Effect on gene expression patterns of mutations affecting PSII, PC, PSI, and of inhibitors that block electron transfer from PSII to the cytb₆/f complex in wild-type cells. All cultures were incubated in the dark for 20 h (D). Cultures of the 4A+ strain and the four mutants were then exposed to white light for one (L1) and 2 h (L2). For inhibitor studies, dinoterb (final concentration 30 μM), DCMU (final concentration 3 μM), and DBMIB (final concentration 1.5 μM) were added to cultures 40 min prior to shift to light. Samples were processed as described in the legend of Figure 2. Note that DBMIB caused overaccumulation of HSP70B mRNA in the dark and in the light, an observation that was not pursued.](image)

![Figure 4. Expression of genes in mutants with defects in cytb₆/f subunits or in state transitions. Mutant cultures, after incubation for 20 h in the dark (D), were shifted to light (40 μE m⁻² s⁻¹) for one (L1) and 2 h (L2). Samples were processed as described in the legend of Figure 2. Strain clpP1-AUU is a parent of the strain petC-D1 clpP1-AUU, and is used to show that the attenuation of ClpP is not responsible for an impairment in light induction.](image)
kinase (Im et al., 1996). Here we show that mutants lacking a functional cytb6/f complex are unable to carry out this light-induction process. This suggests that the light activation of these nuclear genes is in part controlled by signals originating from the chloroplast, where the gene products reside. However, none of the mechanisms hitherto described as mediating retrograde chloroplast-to-nucleus signaling (Beck, 2005; Nott et al., 2006) appear able to explain our results.

Intermediates of the tetrapyrrole biosynthetic pathway are known to participate in the regulation of chlorophyll biosynthesis genes in particular at the level of transcript accumulation. In Arabidopsis, Mg-Protoporphyrin IX accumulates in photodamaged chloroplasts, leading to repression of LHCBI expression (Strand et al., 2003). The light-mediated activation of HEMA1 and, to a minor degree that of GSA, was impaired by chloroplast-damaging treatments such as the application of the phytoene desaturase-inhibitor norflurazon, by far-red light pretreatment, or by inhibitors of plastid protein synthesis (Kumar et al., 1996; McCormac and Terry, 2004). In contrast, the C. reinhardtii HEMA gene is induced by the feeding of Mg-Protoporphyrin IX (Vasileuskaya et al., 2005), which also activates expression of three nuclear HSP70 genes (Kropat et al., 1997; Vasileuskaya et al., 2004). It could be hypothesized that lack of light induction in cytb6/f mutants is related to a defect in the accumulation of Mg-protoporphyrin IX, or its release from the chloroplast. However, no link can be made at present between cytb6/f activity and the metabolism of tetrapyrroles. We note that HSP70B is normally induced in cytb6/f mutants, which suggests that the mechanism of tetrapyrrole signaling is not impaired.

We have also examined the possibility that reactive oxygen species, produced in the cytb6/f mutants upon exposure to light, would prevent the induction of the chlorophyll biosynthetic genes. Exogenous addition of hydrogen peroxide (2 mM) did not prevent the light induction of the genes assayed; neither did singlet oxygen generated by treatment with methylene blue in the light (Anthony et al., 2005), suggesting that reactive oxygen species are unlikely to play a role in this deregulation (data not shown).

Redox control has been invoked as a major player in the field of chloroplast-to-nucleus signaling in higher plants (for review, see Fey et al., 2005) as well as in green algae (Escoubas et al., 1995; Maxwell et al., 1995; Durnford and Falkowski, 1997). This is based mostly on experiments where the redox state of the plastoquinone pool was manipulated by illumination in the presence of DCMU or DBMIB, as well as by changes in the light regime. For a number of nuclear genes, the transcript levels have been shown to vary in a manner consistent with long-term regulation of photosystem stoichiometry and light-harvesting capacity. However, the chlorophyll biosynthesis genes that we have studied have not been identified as redox controlled in these studies. Here, we show that treatment with DCMU and DBMIB do not prevent Chlamydomonas wild-type cells from inducing the chlorophyll biosynthesis genes (Fig. 3), in spite of the fact that they will change drastically, and in opposite ways, the redox state of the plastoquinone pool (fully oxidized with DCMU, fully reduced with DBMIB).

Figure 5. Analysis of lrc phenotypes among progeny from a cross between the petD-PWYE mutant and wild type. The data represent one example for the analyses performed with progeny from all crosses listed in Table II. The analyses were done as described in the legend of Figure 2. Above the columns, the phenotype of the strain analyzed with respect to acetate requirement and mating type is listed.
Moreover, PSII mutants on the one hand, and PSI as well as PC mutants on the other hand, do undergo changes in the redox state of the plastoquinone pool and of the high potential chain of the cyt b6/f complex (cyt f and Rieske protein) when illuminated, but in opposite directions. Still, all showed normal light induction of the chlorophyll biosynthesis genes (Fig. 3). It could be argued that cyclic electron transport, which is still carried out in the presence of DCMU or in PSI mutants (Finazzi et al., 1999), can lead to reduction of the plastoquinone pool in response to light. But we have observed light induction in a DCMU-treated PSI mutant, where no redox change can be brought about by illumination (Fig. 3). Thus, we conclude that changes in the redox state of the plastoquinone pool play no role in the light induction of the chlorophyll biosynthesis genes. In this respect, the signaling we describe differs markedly both from the putative redox control of nuclear genes mentioned above, and from state transitions.

State transitions govern light-energy distribution between photosystems and the balance between linear and cyclic electron transport, and are redox controlled (for review, see Wollman, 2001). Cytb6/f plays a central role in this process: cyt b6/f mutants are unable to perform this reorganization because they fail to activate the protein kinase responsible for LHCII phosphorylation. The kinase has been suggested to be physically associated with the cyt b6/f complex. The complete loss of state transitions in the petD-PWYE mutant clearly demonstrated the involvement of the QO site in kinase activation (Zito et al., 1999). Based on inhibitor studies (Finazzi et al., 2001), a dynamic model has been proposed where activation of the kinase requires oscillation of the Rieske protein between its proximal and distal positions. The protein kinase Stt7 is required for LHCII phosphorylation since strains with defects in the STT7 gene are unable to undergo state transitions (Fleischmann et al., 1999). Here we show that the Stt7 kinase is not involved in the control of the chlorophyll biosynthesis genes, since an stt7 mutant exhibited a wild-type pattern of gene expression (Fig. 4). This does not necessarily mean that the signaling process under study has no component in common with state transitions, because the Stt7 kinase could simply be a downstream component of a cascade. However, the fact that the control of signaling is different, redox in one case, light in the other, argues for distinct mechanisms.

Still, the fact that mutants with defects in the QO site (petD-PWYE and petC-D1) are completely unable to induce the chlorophyll biosynthesis genes (Fig. 4), in spite of having an assembled (or partially assembled) cyt b6/f complex, indicates a requirement for a functional QO site. How do we account for a light-signaling process that is at the same time dependent on the presence of a functional QO site, and independent on changes in its occupancy?

We examined the possibility that the cyt b6/f complex itself senses light. A chlorophyll molecule is present in the cyt b6/f complex (Stroebel et al., 2003), which in principle could act as a light sensor if its excited state could be transduced into a stable modification/conformational change of the complex. Its phytyl chain lies in the vicinity of the QO site, which led us to consider the possibility that its conformation or presence was affected in QO site mutants. However, a series of petD mutants lacking this chlorophyll molecule (kindly provided by F. Zito) still showed induction of the chlorophyll biosynthesis genes by light, ruling out this hypothesis. In contrast, a mutant carrying a petD deletion mutation was defective in induction (data not shown). No mutants are available that affect the other pigment of the complex, β-carotene, but the fact that its excited states are extremely short lived does not make it an attractive candidate for a signaling role.

We thus would like to propose another type of mechanism whereby an essential component of a light-dependent signal-generating system would interact with the cyt b6/f complex and be stabilized/activated by this interaction. Light could act directly on the associated signaling component, if it was to harbor a chromophore. Alternatively, and possibly more likely, it could just be part of a light-signaling cascade in which phototropin serves as a light receptor. Not much is known about how phototropin regulates gene expression (Huang and Beck, 2003; Im et al., 2006). It can be hypothesized that the pathway that leads to activation of the chlorophyll biosynthesis genes has a branch located in the thylakoid membrane, which could act as a sensor of the state of the photosynthetic apparatus.

Our results are not in line with the assumption that physiological states conditioned by photosynthetic mutations represent the principal trigger for different modes of plastid signaling and nuclear response (Maiwald et al., 2003; Ihnatowicz et al., 2004; Biehl et al., 2005). Rather, the identification of a role for the cyt b6/f complex in plastid signaling suggests that components of the photosynthetic machinery themselves may be involved. The results thus provide new routes to analyze signaling by the chloroplast. One of the principal questions to be addressed will have to focus on the molecular nature of the signal generated and its subsequent transduction to the cytosol/nucleus.

**MATERIALS AND METHODS**

**Algal Strains**

*Chlamydomonas reinhardtii* strain 4A+ (mt+) and mutant stt7 were obtained from J.-D. Rochaix (University of Geneva). Strain 4A− (mt−), a wild-type strain that is near isogenic to 4A+ was generated by backcrossing to 4A+ (Dent et al., 2005). Mutants P1-15 (CAL007.01.20) and P2-26 (CAL011.01.03) were generated in the 4A− strain as described previously (Dent et al., 2005). In these two mutants, the phenotypes acetate requirement/light sensitivity were not linked to zeocin resistance, suggesting that these phenotypes were caused by separate mutational events (Dent et al., 2005; data not shown). Mutants defective in PSI (Ap(3b)D), PC (Ap(3b)D), PSII (ApD), cyt b6/f (ApD), mcd1-F16, iso1-639, petD-PWYE, and petC-D1ColdP6-AUU, or state transitions (stt7), have been described before (Table 1).

**Culture Conditions**

Strains were grown heterotrophically or phototrophically in Tris-acetate phosphate (TAP) media or photoautotrophically in minimal media.
Germinated by irradiation and random spores were spread on TAP agar plates were incubated in the light for 24 h and then in the dark for at least 4 d. photoautotrophically in minimal plates. All plates were scored for cell growth into subcultures of 50 mL each, and incubation continued in the dark. For measurements of fluorescence induction at room temperature, dark-adapted cells were transferred to a 1 mL cuvette placed in a home-built chamber for 16 h in the light. Genomes of mt and mt were mixed, incubated for 1 h, and then plated on TAP agar (4%) medium. These plates were incubated in the light for 24 h and then in the dark for at least 4 d. Zygotes adhering to the agar after nonmated cells had been scraped off were germinated by irradiation and random spores were spread on TAP agar plates (1.5%). For testing acetate requirement and light sensitivity, the progeny of random spores was irradiated with 80 μE m⁻² s⁻¹ or 500 μE m⁻² s⁻¹ on TAP or photoautotrophically in minimal plates. All plates were scored for cell growth and color after 10 to 14 d of incubation.

Immunoblot Analyses
Cells were sedimented by centrifugation (3,000 ×g for 5 min) and resuspended in 0.1 M diethanolamine/0.1 M NaClO₄. After adding 0.66 volumes of 5% SDS/30% Suc, they were added. In cases where the lysates were too viscous, samples were sonicated. Homogenization of the suspensions was achieved by rapid shaking at room temperature for 20 min. The protein concentration was determined by the method of Lowry et al. (1951). Peroxidase-conjugated anti-rabbit serum (Sigma-Aldrich) was used to detect the primary antibodies. For signal detection we used the enhanced chemiluminescence system (Amersham Biosciences).

RNA Gel-Blot Analyses
RNA extraction, electrophoretic separation of RNA, and hybridizations were performed as described previously (von Gromoff et al., 1989). Ten micrograms of total RNA per lane were separated on formaldehyde-containing agarose gels under denaturing (0.1% SDS) conditions. The proteins were transferred into nitrocellulose (TAP-N) medium at a density of 1 × 10⁵ cells/ml and incubated for 16 h in the light. Genomes of mt and mt were mixed, incubated for 1 h, and then plated on TAP agar (4%) medium. These plates were incubated in the light for 24 h and then in the dark for at least 4 d. Zygotes adhering to the agar after nonmated cells had been scraped off were germinated by irradiation and random spores were spread on TAP agar plates (1.5%). For testing acetate requirement and light sensitivity, the progeny of random spores was irradiated with 80 μE m⁻² s⁻¹ or 500 μE m⁻² s⁻¹ on TAP or photoautotrophically in minimal plates. All plates were scored for cell growth and color after 10 to 14 d of incubation.

Probes for Hybridization
The probes used for detection of HSP70B and HEMA transcripts have been described previously (Schroda et al., 2001; Vasilievskaya et al., 2004). Probes used to detect mRNAs of the other genes were a 1.6 kb EcoRI, Smal cDNA fragment for GSA (Matters and Beale, 1994), a 1.7 kb cDNA fragment for ALAD (Matters and Beale, 1995), a 1.4 kb DNA fragment for CHLH1 (H subunit of Mg-chelatase; Chekounova et al., 2003), and a 459 bp PCR fragment from genomic DNA for CHLJ1 (I subunit of Mg-chelatase). For a loading control, CBLP encoding a GASP-like protein (von Kampen et al., 1994) was used as a probe.

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