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Adaptation to Fe-deficiency requires remodeling of the photosynthetic apparatus

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The molecular mechanisms underlying the onset of Fe-deficiency chlorosis and the maintenance of photosynthetic function in chlorotic chloroplasts are relevant to global photosynthetic productivity. We describe a series of graded responses of the photosynthetic apparatus to Fe-deficiency, including a novel response that occurs prior to the onset of chlorosis, namely the disconnection of the LHCI antenna from photosystem I (PSI). We propose that disconnection is mediated by a change in the physical properties of PSI-K in PSI in response to a change in plastid Fe content, which is sensed through the occupancy, and hence activity, of the Fe-containing active site in Crd1. We show further that progression of the response involves remodeling of the antenna complexes—specific degradation of existing proteins coupled to the synthesis of new ones, and establishment of a new steady state with decreased stoichiometry of electron transfer complexes. We suggest that these responses are typical of a dynamic photosynthetic apparatus where photosynthetic function is optimized and photooxidative damage is minimized in graduated responses to a combination of nutrients, light quantity and quality.
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Introduction

Fe deficiency is a serious nutritional problem for virtually all forms of life. Compared with heterotrophs, photosynthetic organisms have additional need for Fe because of the abundance of Fe in the photosynthetic apparatus, and its occurrence in many metabolic pathways within the plastid. In photosynthetic organisms, Fe deficiency is evident by development of chlorosis (chlorophyll deficiency), which is concomitant with decreased abundance of the photosynthetic machinery (Spiller *et al*., 1980; Terry, 1980; Straus, 1994). Photosystem I (PSI) appears to be a prime target, perhaps because of its high Fe content (12 Fe per PSI); cyanobacteria, for instance, change from a 4:1 ratio of PSI:PSII to a 1:1 ratio (for an overview see Straus, 1994). These organisms respond to Fe deficiency additionally by degradation of light-harvesting phycobilisomes (Guikema *et al*., 1983), and by increased expression of the 'Fe-stress-induced' gene *isiA*. The isiA protein binds chlorophyll and forms a ring of 18 molecules around a PSI trimeric reaction center (Laudenbach and Straus, 1988; Burnap *et al*., 1993; Bibby *et al*., 2001; Boekema *et al*., 2001). It is not clear whether this extra ring of chlorophyll-binding proteins functions as a light-harvesting system to compensate for decreased abundance of phycobilisomes (Bibby *et al*., 2001), or as a non-radiative dissipator of light energy (Sandstrom *et al*., 2001). The role of a non-radiative dissipator would be to protect the photosynthetic apparatus from excessive excitation under Fe deficiency, as does the PsbS subunit of PSII, which is involved in non-photochemical quenching in *Arabidopsis* (Li *et al*., 2000).

Because of the prevalence of Fe deficiency in nature and the importance of photosynthesis to global productivity, we sought to understand the molecular mechanisms involved in adaptation of the eukaryotic photosynthetic apparatus to Fe deficiency. *Chlamydomonas reinhardtii* is a choice experimental system for this purpose because manipulation of Fe nutrition is straightforward, marker genes for assessing intracellular Fe status have been described previously (La Fontaine *et al*., 2002), and there are well-developed methodologies for analyzing antenna and photosystem function *in vivo* (Bennoun and Delepelaire, 1982).

We find that Fe-deficient *Chlamydomonas* cells display responses comparable to those noted previously for vascular plants; the cells become chlorotic owing to a proteolytically-induced loss of photosynthetic components including both photosystems and the cyt b_6/f complex. The antenna protein complexes are differentially affected; specific components of LHCI are drastically reduced leading to an overall down-regulation of LHCI, whereas LHCII overall abundance remains fairly constant. Importantly, we identify distinct 'Fe-deficiency stress-induced' LHCI proteins on two-dimensional protein maps of the thylakoid membrane subproteome using a combination of mass spectrometric and immunochemical analyses (Hippler *et al*., 2001). We hypothesize that the Fe-deficiency responsive change in the type and quantity of LHCI polypeptides is part of an antenna remodeling program to bypass the light sensitivity resulting from PSI loss. We demonstrate that this 'adaptation program' is a sequential process—starting with uncoupling of the antenna from the PSI core followed by specific degradation of LHCs, induction of new LHCs, and concluding with assembly of new antenna complexes in Fe-deficient cells. We propose that the initial uncoupling of the LHCI

antenna from PSI is regulated via the PSI-K subunit of PSI, and we suggest that accumulation of PSI-K itself is influenced by the activity of Crd1, a candidate Fe-containing enzyme in chlorophyll biosynthesis and a proposed key target of plastid Fe deficiency (Chereskin and Castelfranco, 1982; Spiller *et al*., 1982; Moseley *et al*., 2000, 2002; Pinta *et al*., 2002).

Results

Progressive chlorosis and loss of photosynthetic activity in Fe-deficient Chlamydomonas

Tris-acetate-phosphate (TAP) growth medium for *Chlamydomonas* contains ~18 μM Fe, provided as a chelate with EDTA in the trace element solution (Harris, 1989). Cells inoculated into medium containing 0.1, 0.25 or 1 μM Fe grew at reduced rates and to lower final cell densities than cells grown with 18 or 200 μM Fe, which grew at similar rates (Figure 1A). Consequently we considered cells to be Fe-deficient in TAP medium containing 1 μM or less of Fe, while they were Fe replete at ⩾18 μM. Chlorosis, or reduced chlorophyll accumulation, is a hallmark of Fe-deficiency in plants (Marschner, 1995). When we measured chlorophyll accumulation in each culture, we noted a slight (20%) decrease in chlorophyll accumulation in cells grown with 1 μM Fe compared to Fe-replete cells (18 or 200 μM), and a much more severe decrease (50%) in cells grown with 0.1 or 0.25 μM Fe (Figure 1B). Interestingly, the chlorophyll content on a per cell basis in Fe-deficient cells does not drop below 50% of Fe-replete levels. Instead, at lower Fe concentrations, growth is arrested at lower densities. Thus, although the 0.1 μM Fe culture appears more chlorotic than does the one with 0.25 μM Fe, the ratio of chlorophyll per cell is about the same.

The *Fox1* gene, encoding an Fe assimilation component in *Chlamydomonas*, was used as a marker for the Fe nutritional status of the culture (La Fontaine *et al*., 2002). *Fox1* expression is induced maximally in cells grown with 1 μM Fe (Figure 1C), which is consistent with the proposed role of Fox1 in Fe assimilation; mechanisms that enhance Fe acquisition should be induced before the chlorotic response of Fe-limited cells is established. A comparison of cells grown in 0.1, 0.25, 1 or 18 μM Fe represents therefore a progressive sequence from severe Fe limitation through slight deficiency to Fe-replete conditions.

Fe is an important component of each of the photosynthetic complexes involved in electron transfer; therefore we analyzed the effects of Fe limitation on the fluorescence induction and decay kinetics (Figure 1D). Dark-adapted cells from cultures grown with 18 or 200 μM display virtually identical fluorescence rise and decay kinetics, reflecting the initial reduction of the PSII electron acceptor, Q_A, and its subsequent re-oxidation. Cells grown with 1 μM Fe display similar kinetics in the rise phase (Q_A reduction) but the fluorescence decays more slowly, indicative of a reduced rate of Q_A oxidation. This is more pronounced in cells from 0.25 μM Fe cultures than in 1 μM Fe cells, suggesting a severe block in photosynthetic electron transfer downstream of PSII. An increase in the fluorescence yield is also observed, which is diagnostic of disconnection of the light-harvesting antennae from the reaction centers (Krause and Weis, 1991). Very limited fluorescence rise, indicative of impaired PSII function, is observed in cells maintained in 0.1 μM Fe.

To analyze directly any changes in the interactions between the light-harvesting antennae (which contain the majority of the chlorophyll) and the photosynthetic reaction center, we measured the low temperature (77 K) fluorescence emission spectrum of thylakoid membranes prepared from Fe-deficient versus Fe-replete cells (Figure 2A). The fluorescence emission spectrum of a chlorophyll protein complex depends on the fate of the absorbed light energy and this, in turn, depends on the physical environment and functional interactions with other chlorophyll proteins. Fluorescence peaks at 685 and 709 nm obtained with membranes prepared from cells grown with 18 μM Fe are characteristic of LHCII attached to PSII and LHCI attached to PSI, respectively (Wollman and Bennoun, 1982). However, with samples from 1 μM Fe cells, the relative intensity of the LHCI/PSI peak increases by ~40%, and is also 'blue-shifted' by 4 nm to 705 nm. These results are best interpreted as reflecting an increased contribution to the fluorescence peak from LHCI antennae that are disconnected from PSI, as isolated LHCI antennae have a 77 K fluorescence maximum of 705 nm (Wollman and Bennoun, 1982). 'Disconnected' antennae are expected to have an increased fluorescence yield because of reduced energy transfer to the reaction center. Although a similar increase in the intensity of the LHCI/PSI peak relative to the LHCII/PSII peak is also a characteristic of the state transition, in state II the attachment of LHCII antennae to PSI causes the fluorescence λ_{max} to increase rather than decrease (Wollman and Delepelaire, 1984; Fleischmann *et al*., 1999). Therefore, we rule out the state transition as an explanation for the altered fluorescence spectrum. Yet another alternative explanation for the changed spectrum could be that the abundance of LHCI is enhanced in Fe-deficient cells. Immunoblot analysis of thylakoid membranes from Fe-deficient cells using antibodies specific to LHCI components indicates that this is not the case (data not shown).

The fluorescence emission peak attributed to LHCII/PSII is similar in 1 or 18 μM Fe, but in severely Fe-deficient cells, while the intensity of the peak is about the same, its λ_{max} is blue-shifted and appears similar to that reported for isolated LHCII antennae (Wollman and Delepelaire, 1984). We conclude that the majority of the LHCII antennae are not attached functionally to PSII in severely Fe-deficient cells.

Altered association of PSI-K with PSI

We hypothesized that the functional disconnection of the LHCI antenna in slightly Fe-deficient cells may be a consequence of a physical change in LHCI/PSI association. To probe such a change, we detergent-solubilized thylakoid membranes, isolated LHCI–PSI complexes and analyzed them for the stoichiometry of Lhca3, a LHCI subunit, relative to PSI-D (Figure 2B) or PSI-F (data not shown). We found that ~50% less Lhca3 is associated with PSI from 1 μM Fe cells compared to 18 μM Fe cells (Figure 2B). On the other hand, fractions corresponding to LHCI by itself are enriched for Lhca3 (see Supplementary data, available at *The EMBO Journal* Online). This change in the distribution of Lhca3 indicates

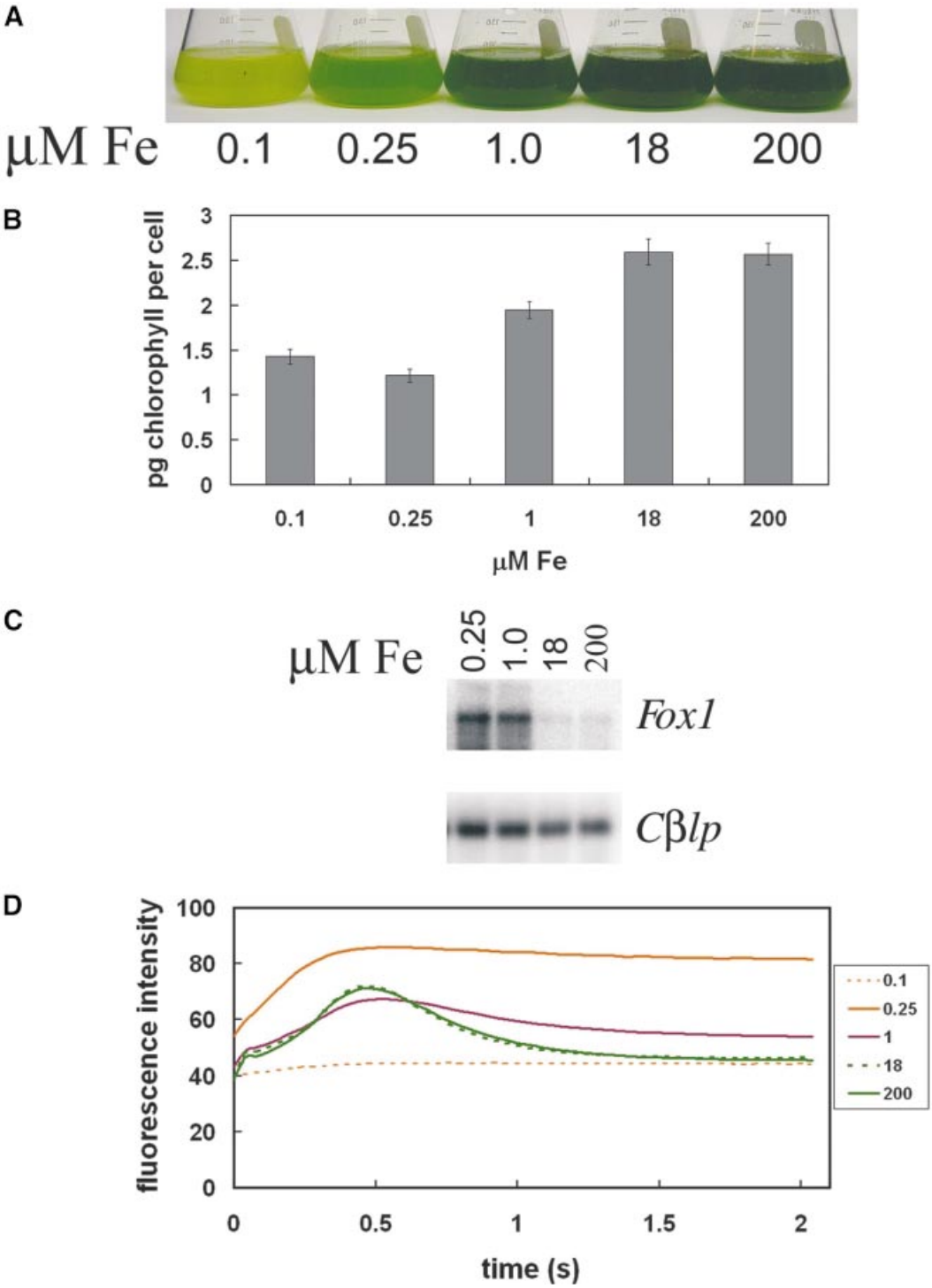

Fig. 1. (**A**) Wild-type (CC124) *Chlamydomonas* cultures grown in TAP medium containing 0.1, 0.25, 1, 18 and 200 μM of Fe, supplied as an Fe·EDTA chelate. Cultures were grown at 24°C for 3 days, with 50 μE $m^{-2}s^{-1}$ of constant light. (**B**) Estimated chlorophyll content in picograms (pg) from cells grown with the indicated concentrations of Fe. (**C**) RNA blot analysis of *Fox1* (encoding ferroxidase) expression in wild-type (CC124) cells grown with the indicated concentrations of Fe. The abundance of *Cβlp* RNA is monitored to demonstrate similar loading. (**D**) Fluorescence induction and decay kinetics from cells grown with various Fe concentrations (green solid line, 200 μM Fe; green dashed line, 18 μM Fe; purple line, 1 μM Fe; orange solid line, 0.25 μM Fe; orange dashed line, 0.1 μM Fe). Fluorescence intensity is indicated in arbitrary units.

a clear physical difference in the properties of the LHCI–PSI complex in slightly Fe-deficient cells relative to the Fe-replete situation, and it is consistent with the interpretation that the LHCI antennae are disconnected from PSI rather than that they are more abundant.

How is the change in LHCI–PSI connection mediated? We reasoned that it could occur through subunit K of PSI. The PSI-K subunit is implicated in the connection of the LHCI outer antennae to PSI through cross-linking and reverse genetic studies (Jansson *et al*., 1996; Jensen *et al*., 2000). For instance, in transgenic PSI-K-deficient plants, the long wavelength 77 K fluorescence emission signal is blue-shifted and the content of Lhca2 and Lhca3 is decreased (Jensen *et al*., 2000). Therefore, we tested for

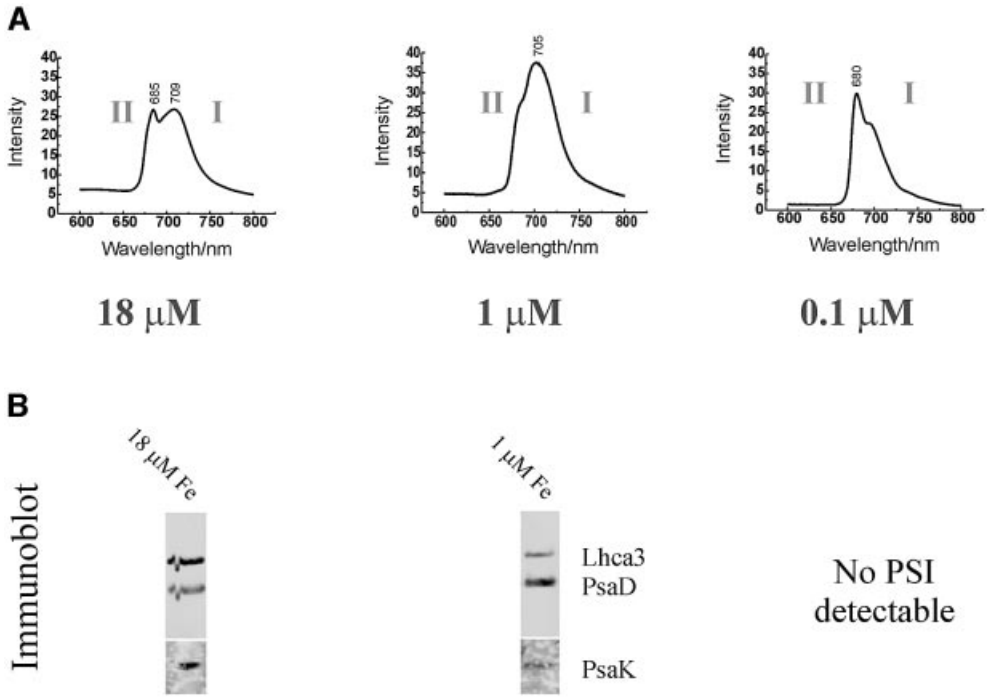

Fig. 2. (**A**) Low-temperature (77 K) fluorescence analysis of thylakoid membranes from wild-type cells grown with 18, 1 and 0.1 μM Fe. Fluorescence intensity is indicated in arbitrary units. The wavelengths (in nm) of the fluorescence peaks are indicated above the curves. Roman numerals I and II illustrate the designation of the peaks to fluorescence from PSI/LHCI and PSII/LHCII, respectively. (**B**) Immunodetection of the PSI subunits PSI-D and PSI-K, and the LHCI outer antenna subunit Lhca3 in enriched PSI/LHCI particles from cells grown with the indicated concentrations of Fe. Samples were loaded on the basis of equal protein.

the presence of PSI-K in fractions containing LHCI–PSI complexes, and found it to be reduced by 20–40% in cells grown in 1 μM Fe compared with Fe-replete cells grown at 18 μM Fe. This is compatible with an *in vivo* regulatory role of PSI-K in mediating excitation energy supply to the reaction center in response to changing nutrition or environment. In cells grown at 0.1 μM Fe, the intensity of the LHCI/PSI 77 K fluorescence peak is severely reduced, and we were not able to isolate an LHCI–PSI complex. These data, coupled with the fluorescence-induction kinetics described in Figure 1D, suggest a sequential process of adaptation to Fe-deficiency; moderate Fe-deficiency (1 μM) does not significantly affect PSII activity or PSI abundance, but causes partial disconnection of the LHCI antennae from PSI, while severe Fe-limitation (0.1 μM) results in loss of PSI and LHCI and possibly PSII, but not LHCII.

Adaptation during the transition from Fe sufficiency to Fe deficiency

In a complementary set of experiments, we monitored cell division and progressive changes in chlorophyll content and various thylakoid membrane proteins as Fe-replete cells are transferred to medium lacking Fe (Figure 3, labeled –Fe). In parallel, cells were transferred to fresh Fe-replete medium to control for the growth phase of the culture (labeled +Fe). *Fox1* mRNA is already increased 40-fold within 5 h (Figure 4) although the first cell division does not occur until 10 h after transfer (data not shown). The immediacy of the response suggests that there may be a mechanism for sensing external Fe concentration, or for sensing of an internal Fe store that is depleted rapidly prior to cell division.

The abundance of various photosynthetic complex constituents was measured by immunoblot analysis during the transition from Fe sufficiency to Fe-limited growth. The accumulation of chlorophyll-, heme- and Fe-containing complexes like PSII, PSI and Cyt b_6f (monitored according to the level of D1, PSI-A and F, and cyt *f*, respectively) is reduced to ⩽5% of the levels observed in Fe-replete cells (Figure 3B). Nevertheless, those photosynthetic complexes that are maintained must be functional, as evidenced by the growth of wild-type cells on minimal medium supplemented with only 0.1 μM Fe (Figure 8). The time-course of the loss of these complexes is similar to the time-course of Fe-deficiency-induced chlorosis, i.e. substantial changes after about 24 h. The chloroplast ATP synthase complex (CF_1-α, β, γ and ε) is unaffected by Fe limitation and was used in subsequent experiments to control for equal loading of the samples in each lane. Interestingly, the loss of ferredoxin, an abundant Fe-containing protein in the plastid, precedes the loss of photosystems and cytochromes and is nearly at the new steady-state by the end of the first day. Lhca3, which corresponds to the outer antenna, is another protein that disappears early during the transition. Lhca3 is reduced to <1% of the levels maintained in Fe-replete cells. We found that PSI-K is especially sensitive to Fe deficiency. Its abundance in total cells declined below 95% as compared with Fe-replete conditions. When purified thylakoid membranes from Fe-depleted cells are analyzed, the loss of PSI-K relative to Lhca3 or PSI-D is particularly evident

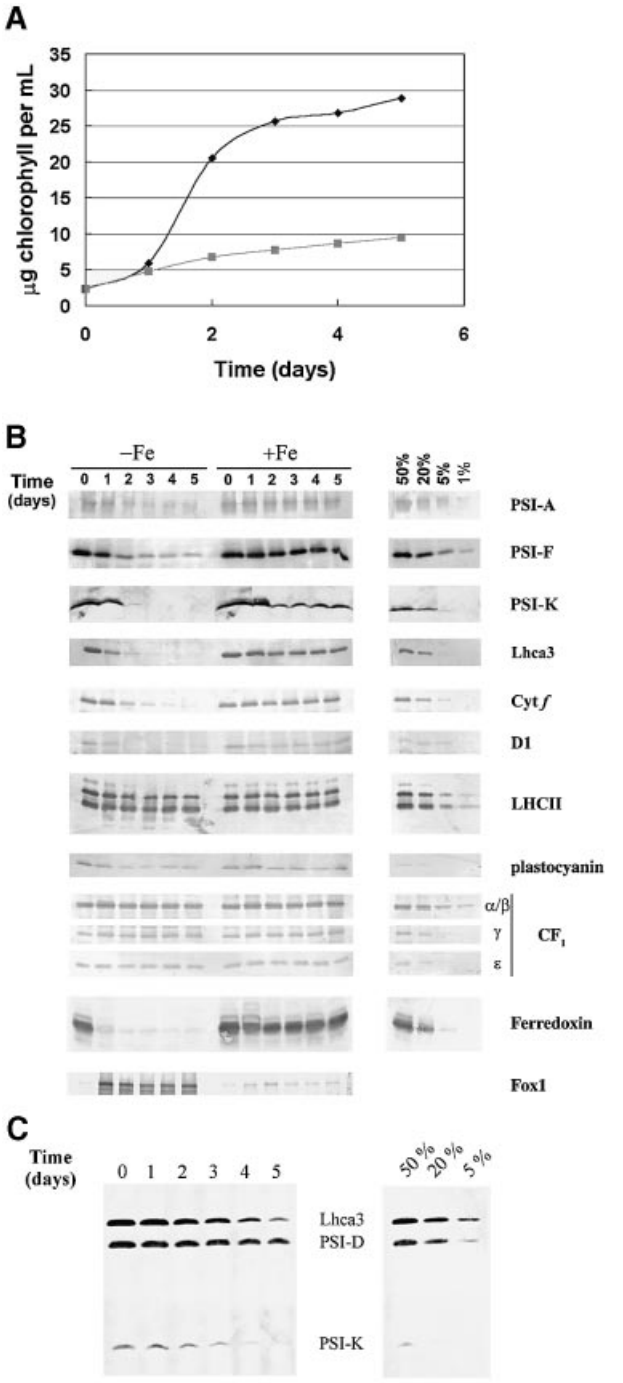

Fig. 3. (**A**) Chlorophyll concentration of wild-type (CC124) cultures grown in TAP medium with 200 μM Fe (black line) or without Fe (gray line). Both cultures were inoculated to the same initial density (1×10^6 cells/ml) from a culture containing 200 μM Fe. (**B**) Immunoblot analysis of total-cell extracts from Fe-depleted versus Fe-supplemented cells to compare the abundance of selected photosynthetic and marker proteins. Samples were normalized to contain equal amounts of ATP synthase. (**C**) Immunodetection of PSI-D, PSI-K and Lhca3 in thylakoid membranes prepared from cells during 5 days of Fe-depletion. Equal amounts of protein are added in each lane. The sample from day 0 was diluted (right hand panel) to generate a calibration curve for estimating the degree of change during the time-courses in (B) and (C).

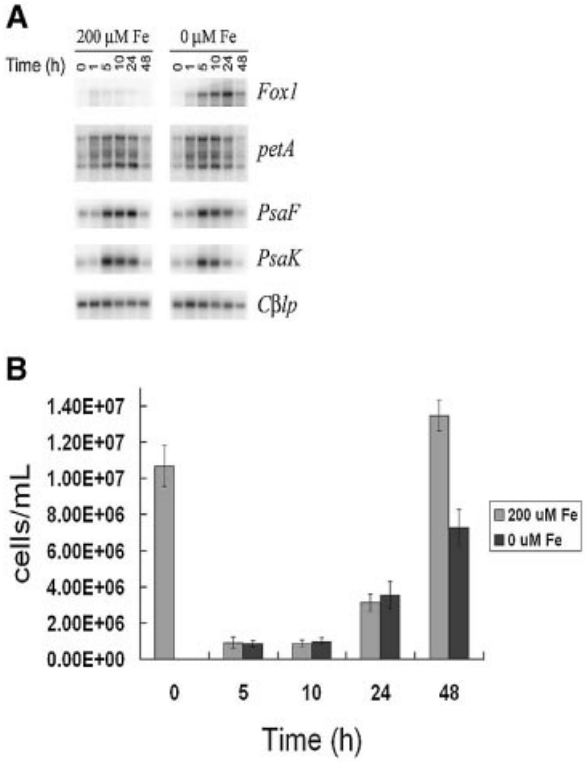

Fig. 4. (**A**) RNA blot analysis to compare *Fox1*, *PsaF*, *PsaK* and *petA* gene expression during growth in Fe-deficient versus Fe-replete medium. (**B**) Growth of the Fe-replete (200 μM Fe, light-gray bars) and Fe-depleted (no added Fe, dark-gray bars) cultures at the time points from which the RNA was prepared. Both cultures were inoculated to the same initial cell density (~1×10^6 cells/ml) from a starter culture containing 200 μM Fe.

(Figure 3C). The chronology of events is consistent with a model wherein incorporation of PSI-K into the PSI complex modulates the dynamic attachment and detachment of LHCI to PSI in response to Fe availability. On the other hand, the overall abundance of polypeptides constituting the major trimeric complexes of LHCII are not notably affected, although there are some obvious indications of changes in complex constituents based on the progressive disappearance of some lower-mobility LHCII polypeptides and the concurrent appearance of higher-mobility ones in Fe-depeleted cells (Figure 3B). Again, these changes in the composition of LHCII are evident early in the adaptive process (day 1), prior to an impact of Fe deficiency on chlorophyll accumulation.

Textbook dogma attributes Fe-deficiency chlorosis to inhibition of chlorophyll synthesis, which requires the function of Fe-containing enzymes (Taiz and Zeiger, 1998). Yet, we note that there are substantial changes in accumulation of certain chlorophyll proteins even when total chlorophyll content in the culture is only marginally affected (day 1 time point in Figure 3A), suggesting that the changes are specific regulatory events in response to perceived Fe deficiency as opposed to a necessary consequence of reduced cofactor availability for biosynthesis and accumulation (Sutton *et al*., 1987; Malnoe *et al*., 1988; Mullet *et al*., 1990). We note also that the chlorophyll content of the Fe-deficient culture increases continuously over the course of three doubling periods (Figure 3A), demonstrating that *de novo* chlorophyll synthesis does occur and is adequate to maintain chlorophyll, even in severely Fe-limited cells, at a level

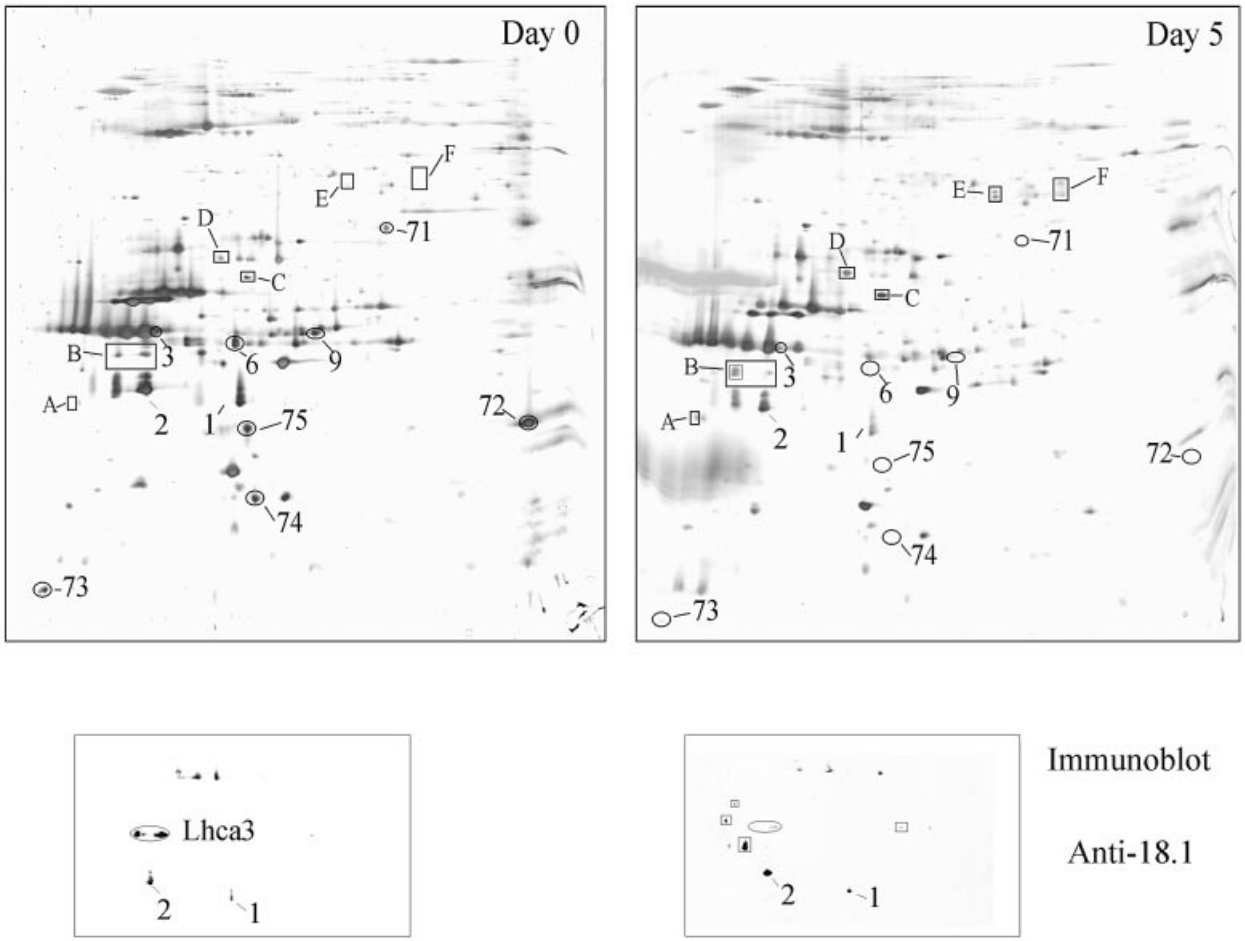

Fig. 5. (**A**) The top two panels (0 day and 5 day) represent silver-stained 2-D gels of thylakoid membranes from wild-type cells before and after 5 days of growth in Fe-deficient (0 μM Fe) medium, respectively. The labeled spots have been identified previously as follows: spots 1 and 2 correspond to the inner LHCI subunits Lhca1 and presumably Lhca4, respectively, while the labeled spots correspond to isoforms of Lhca3. (**B**) Immunodetection of LHCI subunits in thylakoid membranes separated by 2-DE as shown in (A), using an anti-serum (18.1; Bassi *et al*., 1992) which recognizes epitopes that are common to most of the LHCI subunits.

corresponding to 50% of the amount in Fe-replete cells (data not shown; Figure 1B). Most of this chlorophyll must be localized to the LHCII complexes.

Reductions in the abundance of photosynthetic complex-specific proteins are not simply due to general inhibition of protein synthesis in Fe-starved cells, since both chloroplast and nuclear gene products of the ATP synthase complex are maintained at constant levels throughout the course of three cell doublings. Also, the accumulation of Fox1 (a candidate Fe-assimilation protein) is induced ~80-fold fully within 24 h of the transition and is maintained at this level (Figure 3B) (La Fontaine *et al*., 2002). Taken together, these observations argue in favor of a highly programmed change in the stoichiometry and composition of individual complexes in the photosynthetic apparatus in cells dealing with Fe deficiency.

The reduced accumulation of some chloroplast proteins in response to Fe limitation could be a consequence of decreased transcription of the genes, inhibition of translation of specific mRNAs, or induced proteolysis of these proteins. The abundance of RNAs from the nuclear genes *PsaF* and *PsaK* encoding PSI-F and PSI-K, respectively, and the chloroplast *petA* gene encoding cyt *f* was comparable in cells grown with 200 μM Fe or in medium with no added Fe (Figure 4A and C). Furthermore, these genes display similar patterns of expression in Fe-replete and Fe-deficient medium, with RNA accumulation increasing 5- to 10-fold during log phase growth even though the accumulation of the gene products decreases in Fe-deficient cells during this period (Figure 4). We conclude that changes in the abundance of photosynthetic complex proteins are due to post-transcriptional processes.

Identification of specific Lhca proteins whose abundance is affected by Fe nutritional status

To understand the molecular changes in antenna protein complexes in Fe-deficient relative to Fe-replete cells, we employed two-dimensional gel electrophoresis (2-DE) to resolve individual subunits (Hippler *et al*., 2001). Members of individual gene families were identified subsequently by immunochemical methods. As seen in Figure 3, the major polypeptides of the trimeric LHCII

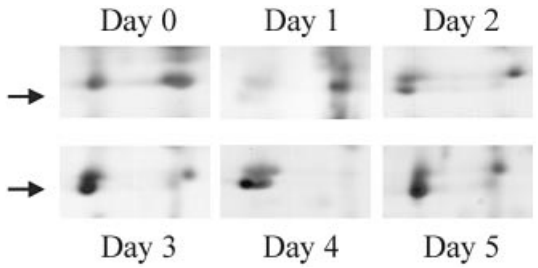

Fig. 6. Evolution of the new putative LHCI protein spot in box B during adaptation to Fe deficiency (Figure 5). Enlarged box B from silver-stained 2-DE gels of thylakoid membranes from wild-type cells before and after 1–5 days of growth in Fe-deficient (0 μM Fe) medium.

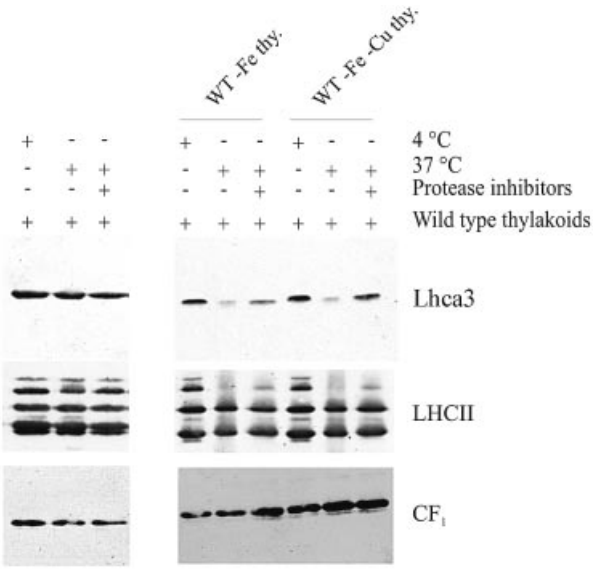

Fig. 7. Immunoblots showing the susceptibility of Lhca3 and components of the major trimeric complex of LHCII in wild-type thylakoids to proteolysis when mixed with thylakoids from Fe-deficient or Fe- and Cu-deficient cells. Chloroplast ATPase accumulation (CF_1) is shown to demonstrate equal loading and specificity of the proteolytic activity. Incubation of the thylakoids at 4°C or 37°C, with or without protease inhibitor is indicated (protease inhibitor cocktail set II; Calbiochem).

complexes are not affected significantly by Fe deficiency. Multiple differences were noted between samples prepared from Fe-replete cells versus those from cells that were Fe starved for 5 days (Figure 5). Rectangular boxes are used to mark protein spots that are induced under Fe deficiency, while those that are significantly diminished are circled. Two polypeptides, 6 and 9, which have been identified previously as components of LHCI (Hippler *et al*., 2001) and in which 6 probably corresponds to Lhca2, have disappeared after 5 days of Fe starvation. This parallels the behavior of the Lhca3 gene products (see also Figure 3B and C). Yet, there is little or no decrease in the accumulation of the LHCI inner antenna polypeptides, revealed by use of an antibody against LHCI subunits that recognizes a common epitope, such as spots 1 and 2 (Lhca1 and presumably Lhca4, respectively; identified in Hippler *et al*., 2001) (Figure 5). Most interestingly, we note the appearance of new anti-LHCI cross-reacting polypeptides, which might represent alternately-processed forms of previously described LHCI gene products, or new, Fe-deficiency specific LHCI proteins. To monitor the evolution of the Fe-deficiency induced anti-LHCI cross-reacting polypeptide in box B of Figure 5, we undertook a time-course analysis from day 1 after transfer to Fe-deficient medium to day 5 (Figure 6). These experiments show that this novel Fe-deficiency inducible spot appears faintly after 1 day and is fully present after 3 days in Fe deficiency, by which time the amount of Lhca3 is already drastically reduced. The changes in composition of the LHCI and LHCII complexes are highly specific and, we propose, particular to the Fe-deficiency response.

Induced proteolytic degradation contributes to changes in thylakoid membrane composition during adaptation to Fe deficiency

To distinguish whether the loss of photosynthetic complexes resulted from activation of a degradation machinery in Fe-deficient cells (as opposed to 'housekeeping' degradation of apoproteins or reduced translation), we mixed thylakoid membranes from Fe-deficient cells with those from Fe-replete cells (Figure 7). We found that Lhca3 and some LHCII polypeptides of the Fe-replete cells are specifically degraded relative to the α and β subunits of the coupling factor (CF_1). While translational control of the synthesis of some components of the photosynthetic apparatus during Fe depletion cannot be ruled out, this experiment indicates that a specific, membrane-associated protease is one component of the adaptive process in Fe-deficient cells. The protease activity can be solubilized by non-ionic detergents and fractionated by sucrose-density centrifugation to yield a preparation enriched in protease activity (data not shown). In the assay shown in Figure 7, the degradative activity against Lhca3 and the LHCIIs is dependent on the amount of the protease preparation added indicating that the degradation is not a property of the substrate membranes from +Fe cells but rather a property of the fractions from –Fe cells (data not shown).

Physiological function of the Fe-deficiency remodeling of chlorophyll protein complexes

Fe-deficient wild-type cells are not sensitive to high-light (600 μE $m^{-2}s^{-1}$) in spite of the reduced abundance of photosynthetic complexes (data not shown) as, for example, are photosynthetic PSI- and PSII-deficient mutants (Spreitzer and Mets, 1981; Redding *et al*., 1999). We postulate that the alterations in the composition and hence structure of the LHCs may serve to regulate excitation energy delivery to reaction centers or to dissipate excess energy and prevent photodamage. This model predicts that light-sensitive photosynthetic mutants should be 'rescued' by Fe deficiency. A suitable test strain is the *psaF*-minus mutant strain (Farah *et al*., 1995), which can grow photosynthetically in moderate light (100–200 μE $m^{-2}s^{-1}$) but not at a light intensity of 400 μE $m^{-2}s^{-1}$ (Hippler *et al*., 2000). We found that the *psaF* strain grew considerably better on minimal medium containing 0.1 μM Fe compared with 18 μM Fe under moderate light conditions (150 μE $m^{-2}s^{-1}$), although higher light intensity of 400 μE $m^{-2}s^{-1}$ remained lethal irrespective of the Fe content of the medium (Figure 8).

We conclude that changes in the light-harvesting complexes allow cells to cope with reduced photosystem function. In previous work, we had described strain *crd1*, which displayed a pleiotropic loss of PSI, LHCI and reduced LHCII under Cu-deficient conditions (Moseley *et al*., 2000). In Cu-supplemented medium, the chlorophyll protein complexes are present at normal abundance, but the 77 K long wavelength fluorescence emission is blue-shifted by >10 nm, indicative of uncoupled LHCI antenna (Moseley *et al*., 2002). This phenotype presented us the opportunity to test the role of antenna uncoupling in surviving Fe deficiency. When this mutation was introduced into the *psaF*-minus strain, the resulting *crd1/psaF* double mutant displayed enhanced growth independently of the medium Fe concentration (Figure 8). The *crd1/psaF* double mutant is resistant to light intensities where the PSI-F-deficient mutant already suffers from light stress. We attribute this effect to 'constitutively' uncoupled LHCI antenna in +Cu cells of the *crd1* mutant.

To examine the LHCI–PSI interaction in +Cu cultures of *crd1*, we solubilized thylakoid membranes with β-dodecyl-maltoside and separated them on sucrose-density gradients to isolate PSI-enriched particles. These were analyzed by high-resolution denaturing electrophoresis according to Schägger and von Jagow (1987) and compared with wild-type PSI particles isolated from Cu-sufficient and -deficient conditions (Figure 9A). Immunoblot analysis using anti-LHCI antibodies anti-14.1, anti-17.2 and anti-18.1 (Bassi *et al*., 1992; Hippler *et al*., 2001) revealed a clear depletion of these proteins in complex with PSI from the *crd1* mutant (data not shown). Yet, when whole cells are analyzed, the abundance of the LHCIs in +Cu *crd1* cells is not reduced. These findings indicate a clear physical difference in the LHCI–PSI connection in +Cu *crd1* strains, as seen already for the wild-type strain grown in 1 μM Fe.

Altered PSI-K in +Cu crd1 cells

We noted also that the PSI preparations from +Cu *crd1* cells lacked an ~5 kDa polypeptide relative to the PSI preparation from wild type. Mass spectrometry identified the protein as PSI-K (Table I). Immunoblot analysis showed that the amount of PSI-K is already clearly diminished in *crd1* thylakoid membranes as compared with wild-type membranes (Figure 9B). Thus, the deletion of Crd1 has a direct impact on the accumulation of PSI-K in the membrane. This accounts for the blue-shift of the 77 K long wavelength fluorescence emission band, since PSI-K is required for functional connection between PSI and LHCI (Jensen *et al*., 2000). The 'loosening' of the LHCI–PSI connection is apparent during the preparation of PSI–LHCI complexes by fractionation of detergent-solubilized *crd1* membranes. Immunochemical analysis (Figure 9C) reveals much lower stoichiometry of Lhca3 and complete loss of PSI-K. The PsaK mRNA levels from wild type or the *crd1* mutant are similar (data not shown), indicating that the diminution of PSI-K in the *crd1* mutant is a post-transcriptional event.

Discussion

Functional disconnection of LHCI antenna from PSI

In medium containing 1 μM Fe, chlorosis is not visually evident (Figure 1A), yet a loss of coupling between the LHCI antenna and PSI is clearly evidenced by the blue-shift of the 77 K long wavelength fluorescence emission signal from thylakoid membranes (Figure 2A). Biochemical separations of detergent-solubilized thylakoid membranes reveal a physical change in PSI and LHCI associations in these membranes relative to membranes from Fe-replete cells. Specifically, LHCI is less stably associated with PSI in Fe-deficient thylakoid membranes as evidenced by a reduced stoichiometry of Lhca3 to PSI-D in preparations of PSI–LHCI complexes

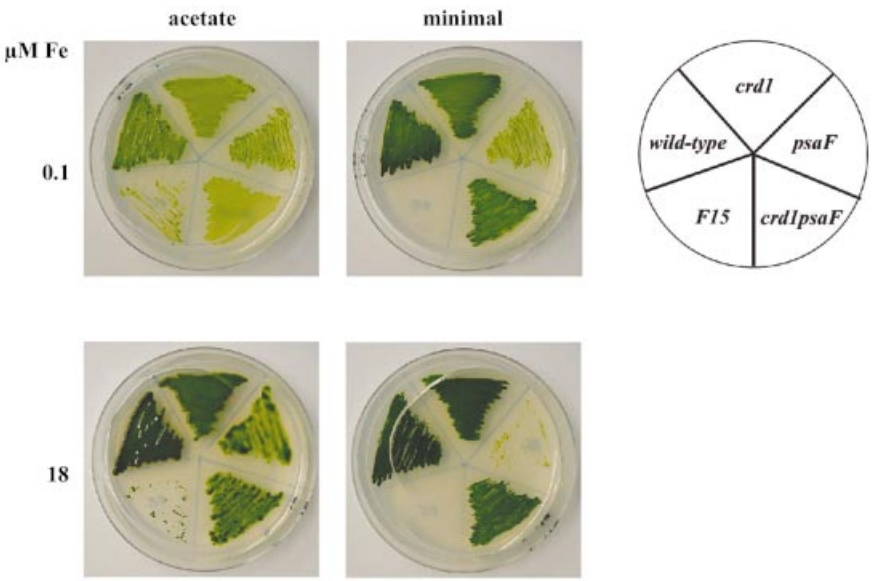

Fig. 8. Comparison of the growth of wild-type, *psaF*, *crd1*, *crd1psaF* and *F15* strains on TAP versus minimal medium, for 10 days at 25°C, with a light intensity of ~150 μE $m^{-2}s^{-1}$.

(Figure 2B). Coincident with, or perhaps even preceding, the loss of Lhca3 is loss of PSI-K (Figure 3C), which has been implicated in the connection of the Lhca3 homodimer to PSI (Jansson *et al*., 1996; Jensen *et al*., 2000). Structural analysis of PSI (Jordan *et al*., 2001) shows that PSI-K is a peripheral chlorophyll binding subunit whose location is distant from the 2-fold symmetry axis. The position within the complex is at the junction where LHCI is expected to bind, consistent with its function in eukaryotic PSI.

The occurrence of chlorophyll binding sites in PSI-K suggests to us a mechanism for Fe responsive signal transduction to the antenna reaction center complex. Specifically, we propose that the chlorophyll binding sites of PSI-K are sensitive to flux through the chlorophyll biosynthetic pathway, which in turn is affected by the activity of the Fe-requiring aerobic oxidative cyclase. This would lead to functional uncoupling between antenna and PSI in cells with reduced cyclase activity. Recently, one Fe-containing subunit of this enzyme was identified in *Rubrivivax gelatinosus* (Pinta *et al*., 2002). In chloroplasts, the homologous subunit is Crd1, which is highly conserved (for example, 44% sequence identity between *R.gelatinosus* and *A.thaliana*) and found in all organisms that synthesize Chl (or Bchl) aerobically (Moseley *et al*., 2000, 2002). *Chlamydomonas reinhardtii* contains two

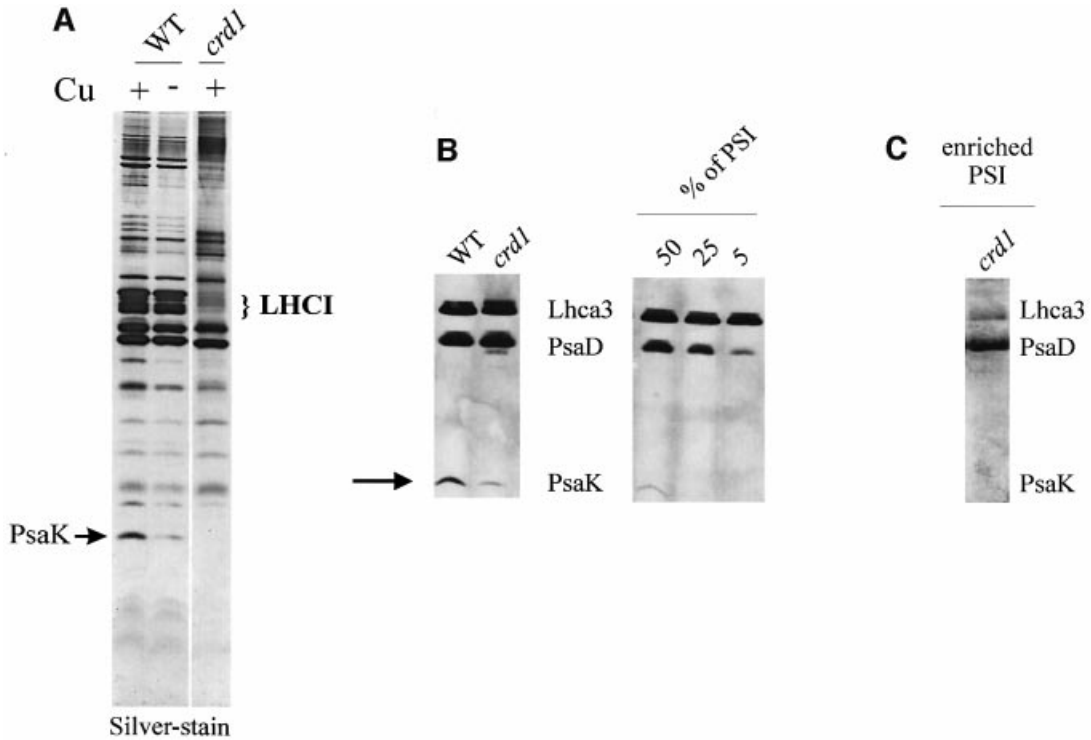

Fig. 9. (**A**) Silver-stained gel (Schägger and von Jagow, 1987) of enriched PSI/LHCI particles from +Cu (6 μM Cu) and –Cu (0 μM added Cu) wild-type and +Cu *crd1* mutant cells. The migration of the PSI-K subunit is indicated with an arrow. (**B**) Immunoblot analysis to compare the abundance of PSI-D, Lhca3 and PSI-K in thylakoid membranes from wild-type and *crd1* cells grown in TAP medium with normal Fe and Cu. The migration of PSI-K is indicated with an arrow. To determine the amount of PSI-K that is still present with the *crd1* thylakoids, we performed in parallel dilution series where the total amount of thylakoid membrane protein loaded remained the same but the amount of PSI was diminished by mixing wild-type thylakoids with thylakoids isolated from a PSI-deficient mutant. The corresponding immunoblot shows that PSI-D and PSI-K signals vanish as the amount of PSI decreases. From this experiment, we can estimate that the amount of PSI-K in *crd1* thylakoids is diminished to ~50% of the quantity found in wild type. (**C**) Immunodetection of PSI-D, Lhca3 and PSI-K in enriched PSI/LHCI particles from +Cu *crd1* cells.

Table I. Peptide masses that correspond to the *PsaK* gene product

M/z	Mass (neutral) (Da)	Delta mass (monoisotopic) (Da)	Identified as	Peptides
832.9 (+1)	831.9	0.4	L/I-Scan	51–58/59–66
822.4 (+2)	1642.8	–2.1	L/I-Scan	51–66
832.2 (+1)	831.2	–0.3	Y1-Scan (K)	51–58/59–66
801.6 (+1)	800.6	–0.9	Y1-Scan (K)	19–25
458.4 (+3)	1372.2	–1.6	Y1-Scan (K)	59–71

The silver-stained protein band was excised from SDS–PAGE fractionated wild-type PSI particles, digested in-gel by trypsin and the resulting peptides were analyzed by mass spectrometry. Peptide masses were obtained when the mass spectrometer was used in a parent ions scan mode (Wilm *et al*., 1996) searching for Y1 ions on 147 Da, (Y1-ion of Lys) and for immonium ions on Leu/Ile (86 Da). Collision-induced fragmentation of a peptide with a mass of 800.6 Da by mass spectrometry resulted in two sequence tags with amino acid sequences and corresponding fragment masses of (474.7)SSR(801) and (431.0)VV(628.0), respectively. Using peptide search (http://www.mann.emblheidelberg.de/Services/PeptideSearch/), these sequence tags identify the protein as the *PsaK* gene product of *C.reinhardtii* (Franzen *et al*., 1989).

isoforms of this protein–Crd1 and Cth1—which display a complementary pattern of expression depending on oxygen supply and Cu nutritional status. In Cu-supplemented cells, Cth1 predominates over Crd1, while in Cu-deficient cells the converse is true. Hence a *crd1* mutant is severely chlorotic in Cu-deficient conditions. On the other hand, in Cu-supplemented cells, when Cth1 is abundant, the chlorophyll content is normal (Moseley *et al*., 2000). Nevertheless, when we examined the *crd1* mutant in Cu-supplemented conditions, we noticed striking similarities to wild-type cells grown in 1 μM Fe; namely that the LHCI antenna were uncoupled from PSI (Moseley *et al*., 2002), Lhca3 was easily lost from PSI during biochemical fractionation (Figure 2B), and PSI-K was absent from purified PSI preparations (Figure 2B). The protective value of these adaptations is apparent from the enhanced growth of a PSI-F-deficient strain in the presence of the *crd1* mutation (Figure 8). On this basis, we propose that Fe-nutrition-dependent adaptation of the photosynthetic apparatus responds to the activity of the aerobic oxidation cyclase in chlorophyll biosynthesis. An attractive feature of this model is that it accommodates the long-standing dogma of a causal connection between loss of photosynthetic function in Fe-deficiency chlorosis and loss of chlorophyll biosynthetic activity.

Remodeling of the antenna

When the transition from Fe-replete to Fe-deficient state is monitored, it is clear that there are changes in the composition of LHCII (Figure 3B) and LHCI subunits (Figure 5). The disappearance of individual proteins was anticipated, but the simultaneous appearance of new proteins was unexpected. The proteins that disappear and appear have not been identified with respect to their relationship to specific Lhca/b gene products, nor is it determined whether they result from *de novo* synthesis or modification of existing proteins. In this context, we note the previous identification of differentially processed Lhc subunits in a proteome map of *Chlamydomonas* thylakoid membranes (Hippler *et al*., 2001). Since the N-terminus of the Lhca1 and Lhca4 proteins may be important in functional interactions (Schmid *et al*., 2002), differential processing may well affect the functional connections in the antenna network. The remodeling of the antenna could shift its role from light-harvesting to a more light-energy dissipative function (see Introduction).

Decreased stoichiometry of photosynthetic complexes in Fe-deficient cells from programmed degradation

As noted previously (Spiller and Terry, 1980; Terry, 1980), Fe-containing electron transfer complexes, PSII, PSI and cyt b_6/f, are reduced greatly in abundance during adaptation to Fe deficiency (Figures 1D, 2A and 3B and C). On the other hand, contrary to dogma, the ratio of chlorophyll per cell does not change more than 2-fold under Fe deficiency (Figure 1B). The main reason for this is that the major chlorophyll binding proteins in the thylakoid membrane, the LHCII proteins, remain fairly stable under Fe-deficient conditions. The adjustment of the stoichiometry of photosynthetic complexes is, therefore, not caused merely by apoprotein degradation resulting from chlorophyll deficiency (as occurs for example in chlorophyll synthesis mutants; Malnoe *et al*., 1988), but is instead a consequence of a program of adaptation involving differential chlorophyll allocation and regulated chlorophyll protein degradation. The latter is evidenced by the degradation of Lhca3 and, to a lesser extent, LHCII subunits from Fe-replete thylakoids by an activity present in Fe-deficient thylakoids (Figure 7). A similar mechanism has been invoked for adjustment of the antenna size in response to light (Lindahl *et al*., 1995; Yang *et al*., 1998). The specific degradation of LHCa subunits points toward a highly coordinated process, in which proteases are either activated or newly expressed due to Fe deficiency.

Materials and methods

Strains and culture conditions

The wild-type strain in all experiments was CC124. The *crd1* mutants were described previously (Moseley *et al*., 2000). The *psaF* mutant and the PSI-deficient strain, *F15*, were a kind gift from J.D.Rochaix. *crd1psaF* double mutant strains were generated by crosses between the *psaF* mutant and a *crd1-1::ARG7* allele. Fe-free TAP medium was made in acid-washed glassware (Quinn and Merchant, 1998) using trace elements without Cu or Fe. Cu was subsequently added from a standard solution of 100 mM $CuSO_4$, and Fe was added from a solution of 100 mM $FeSO_4$ chelated with EDTA (Moseley *et al*., 2000). Solid TAP and high-salt minimal medium were prepared using agar washed with EDTA and de-ionized water as described previously (Quinn and Merchant, 1998). Liquid cultures were grown at 20–25°C with 200–250 r.p.m. shaking, and light intensities between 50–100 μmol $m^{-2}s^{-1}$. Strains were cultured on plates at 24–25°C.

Chlorophyll determination

Cells were collected by centrifugation (14 000 r.p.m. for 5 min in a microcentrifuge) from 0.5 ml or 1.0 ml aliquots of cultures. Chlorophyll was extracted from the cells into 1 ml of 80% acetone, 20% methanol. Precipitated protein was removed by centrifugation and the chlorophyll concentration was estimated (Porra *et al*., 1989).

Fluorescence emission analysis

Room-temperature fluorescence induction kinetics were measured using an open FluorCam detector (Photon Systems Instruments, Czech Republic). Fluorescence emissions were recorded from liquid cultures of cells after dark-adaptation periods of at least 5 min using an actinic light intensity of ~60 μmol $m^{-2}s^{-1}$ for 2–5 s. Low-temperature (77 K) fluorescence emission spectra were recorded using a Fluoromax-2 spectrofluorometer (Instruments S.A. Inc., Munich, Germany) from thylakoid membranes prepared (Chua and Bennoun, 1975) and resuspended in 20 mM HEPES pH 7.5 and 60% glycerol to a density equivalent to 5 μg/ml chlorophyll.

Protein analysis

Total cell extracts were as follows: cultures were grown for 6 days in TAP medium with 200 μM supplemental Fe, cells were harvested by centifugation (5000 r.p.m. for 5 min), washed once, centrifuged (5000 r.p.m. for 5 min) and then resuspended in –Fe TAP medium. Cultures with (200 μM) or without (0 μM) added Fe were inoculated to an initial cell density of ~1×10^6 cells/ml, and grown with 250 r.p.m. shaking at 22°C, 50 μmol $m^{-2}s^{-1}$. An aliquot of cells from the starter culture harvested immediately prior to inoculation of the 0 μM and 200 μM Fe medium served as the zero time point. Cells were counted daily, and aliquots of each culture were harvested by centrifugation, resuspended to a density of ~4×10^8 cells/ml in 10 mM NaH_2PO_4 solution pH 7.0 and stored at –20°C. Immunoblot analysis of the frozen whole cells (dissolved in SDS-containing sample buffer, heated and resolved by electrophoresis) was described previously by Moseley *et al*. (2002). Antibody dilutions were: 1:1000 for anti-PSI-A, anti-PSI-F, anti-PSI-K, anti-Lhca3 (14.1), anti-Cyt *f*, anti-D1, anti-LHCII, anti-ferredoxin and anti-Fox1; 1:2000 for anti-plastocyanin; and 1:5000 for anti-CF_1. Sample loading was adjusted so that each sample contained the same amount of the α/β subunits of CF_1. Quantitation of the bands was performed using a VersaDoc imaging system and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

RNA gel blot analysis

Preparation of total RNA and hybridization was performed as described previously by Quinn *et al*. (1999). Total RNA (3 μg) was loaded in each lane. Hybridization was detected by exposure of the blots to PhosphorImager screens, and quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Isolation of PSI complexes

The isolation of PSI particles was described previously (Hippler *et al*., 1997). Protein determination in solution was done by the bicinchoninic acid method according to the manufacturer's instructions (Sigma).

Two-dimensional gel electrophoresis, immunodetection and mass spectrometry

Sample preparation for 2-DE, immunodetection and mass spectrometry of trypsin-digested protein spots was performed as described previously (Hippler *et al*., 2001).

Peptide synthesis and coupling to BSA for antibody production

Edman degradation of mature PSI-K had indicated that the N-terminus of the protein corresponded to position 26 with Ala as the first amino acid (Franzen *et al*., 1989), defining the sequence from position 1 to 25 as putative transit peptide. A tryptic peptide identified by mass spectrometry matches with the peptide sequences deduced by translation of *PsaK* from position 19 to 25, indicating two possible *in vivo* processing sites for the transit peptide of PSI-K. Thus, peptide CRSSVVVRADGFIGSST, corresponding to amino acids 19–34 of PSI-K (Franzen *et al*., 1989) was synthesized, coupled to BSA using the crosslinker MBS via the N-terminal Cys, as described previously (Hippler *et al*., 2001), and used for the production of antibodies at EUROGENTEC (Belgium).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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