

Iron deficiency cause changes in photochemistry, thylakoid organization, and accumulation of photosystem II proteins in *Chlamydomonas reinhardtii*

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Abstract A trace element, iron (Fe) plays a pivotal role in photosynthesis process which in turn mediates the plant growth and productivity. Here, we have focused majorly on the photochemistry of photosystem (PS) II, abundance of proteins, and organization of supercomplexes of thylakoids from Fe-depleted cells in *Chlamydomonas reinhardtii*. Confocal pictures show that the cell's size has been reduced and formed rosette-shaped palmelloids; however, there is no cell death. Further, the PSII photochemistry was reduced remarkably. Further, the photosynthetic efficiency analyzer data revealed that both donor and acceptor side of PSII were equally damaged. Additionally, the room-temperature emission spectra showed the fluorescence emission maxima increased due to impaired energy transfer from PSII to PSI. Furthermore, the protein data reveal that most of the proteins of reaction center and light-harvesting antenna were reduced in Fe-depleted cells. Additionally, the supercomplexes of PSI and PSII were destabilized from thylakoids under Fe-deficient condition showing that Fe is an important element in photosynthesis mechanism.

Keywords Blue native gel electrophoresis · Fe deficiency · Light-harvesting complexes · Photochemistry · Photosystem II · Thylakoid organization

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Introduction

Iron is a relatively abundant micronutrient in the earth's crust, which often chronically limits to photosynthesis in the ocean and on land also. Iron is present predominantly in the poor form of insoluble complexes, such as Fe(III) ferric oxides in oxygen-rich surface water and neutral to alkaline soil. The soluble form of Fe(II) is very limited in soil, and the low bioavailability of iron complexes creates a major obstacle for photosynthetic organisms (Glaesener et al. 2013). Fe deficiency in photosynthetic organisms is evident by the development of chlorosis (loss of chlorophyll), which is accompanied by loss of photosynthetic machinery and inhibition of photosynthetic electron transport reactions (Spiller and Terry 1980).

It is well known that Fe is a cofactor in photosystem (PS) II, PSI, the cytochrome (Cyt) b6/f complex, and Cyt c6 (Yadavalli et al. 2011, 2012a). Since PSI is the prime target because of its relatively high Fe content (12 Fe per PSI) and for instance, changes from 4:1 ratio of PSI:PSII to 1:1 under Fe deficiency in cyanobacteria (Straus 2004). Further, Fe deficiency causes a decrease in the number of PSI complex, resulting in a bottleneck in the photosynthetic electron flow (Strzepek and Harrison 2004). Most of the cyanobacteria species express the auxiliary light-harvesting proteins which increase the cross section of PSI to balance a similar level of electron throughput with a smaller Fe investment to avoid the oxidative stress in the cell (Chauhan et al. 2011). In cyanobacteria, the alteration in the pigment–protein complex and appearance of an additional pigment–protein complex around PSII (IdiA: Iron deficiency induced protein) protect PSII at the acceptor side against damage (Michel et al. 1996; Michel and Pistorius 2004) and formation of an iron stress-induced protein A (IsiA) (Boekema et al. 2001; Chauhan et al. 2011). Fe

deficiency induces a large drop in PSI/PSII unit ratio by expression of *isiA* gene product CP43' that forms 18 subunits around the trimeric PSI core (Andaluz et al. 2006).

Fe deprivation in the green alga *Chlamydomonas* (*C.*) *reinhardtii* results in remodeling of PSI, changes in the LHCI polypeptide composition, and decreased the efficiency of energy transfer between the light-harvesting and reaction center (RC) (Moseley et al. 2002; Naumann et al. 2005). We have also reported that Fe deficiency induces rapid reduction of the levels of photosynthetic pigments due to a decrease in chlorophyll synthesis. Chlorophyll is important not only as a light-harvesting pigment but also has a structural role, particularly in the pigment-rich light-harvesting complex (LHC)I subunits. The reduced level of chlorophyll molecules inhibits the formation of large PSI–LHCI supercomplexes, further decreasing the photosynthetic efficiency in *C. reinhardtii* (Yadavalli et al. 2012a). Furthermore, in *Dunaliella salina*, a photoautotrophic green alga, a homolog protein is induced by Fe deficiency. In which Fe deprivation induced increased cell size and formation of Tidi-LHCI antenna system, coupled to the reaction center of PSI (Varsano et al. 2006).

In lettuce plants, the effect of different degrees of iron deficiency in leaf disks, the photooxidation/reduction kinetics of P700, and the chlorophyll content was severely affected. Besides a strong decline in active P700, measurements of the dark rereduction of oxidized P700 (P700+) after the termination of FR-light have shown that the different path-ways of cyclic electron flow around PSI were largely modified under iron-deficient conditions (Msilini et al. 2013a, b). During Fe deprivation in rice, the PSI core proteins PsaC and PsaD decreased by about 50 %, and PsaE was completely degraded. Among the LHCI subunits, Lhca1 and Lhca2 decreased by 40 and 50 %, respectively, whereas Lhca3 and Lhca4 were completely degraded. The dissociation of LHCI subunits may be due to increased levels of reactive oxygen species, which is suggested by the increased activity of superoxide dismutase (Yadavalli et al. 2012a, b). Also, in higher plants, Fe deficiency leads to decrease in the abundance of photosynthetic proteins, electron transport chain (Andaluz et al. 2006), PSI level (Timperio et al. 2007), and PSII quantum yield (Msilini et al. 2011). Further, in *C. reinhardtii*, Fe deprivation associated with antenna proteins in the two photosystems is differentially affected, although LHCI overall abundance and composition remain fairly stable (Naumann et al. 2005).

It is clear that Fe deprivation leads to pronounced changes in PSI but has less affect on PSII. Thus, in this study, we have focused mainly on Fe deficiency in PSII from *C. reinhardtii*. The present study focuses on changes in the morphology, functional aspects, thylakoid organization, and protein profile of the PSII core and LHCI complexes.

Materials and methods

Growth and culture conditions

Cells of wild-type *C. reinhardtii* strain CC125 (obtained from the *Chlamydomonas* resource center, University of Minnesota, USA) were grown following the standard procedures (Subramanyam et al. 2006; Subramanyam et al. 2010). An initial culture of *C. reinhardtii* cells was harvested and washed twice with Fe-free TAP medium and then inoculated into Fe-free TAP media of 1.0 L culture flask. Cells were grown under both control (Fe sufficient) and Fe-deficient conditions with continuous illumination (30–40 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Thylakoid membranes were isolated from lysed cells according to Fischer et al. (1997) and resuspended in buffer containing 200 mM sorbitol, 5 mM Tris-HCL (pH 7.5), and 5 mM CaCl_2 .

Confocal microscopy

Chlamydomonas reinhardtii cells from both iron-sufficient and iron-deficient media were subsequently immobilized with molten agar at room temperature as described earlier (Neelam and Subramanyam 2013). All the cells were captured using (Leica Microsystems, Heidelberg) with an equal magnification of 10 μm .

Fluorescence measurements

The room-temperature fluorescence emission spectra were measured as reported previously by Msilini et al. (2013a). The fluorescence spectra were measured with a Perkin-Elmer LS55 spectrofluorimeter. The excitation and emission spectral widths were fixed at 6 nm, respectively. Chl fluorescence was excited at 436 nm. The Chl content of the samples was adjusted to 10 $\mu\text{g mL}^{-1}$.

The fast OJIP fluorescence transient measurements

Chlorophyll fluorescence transients were measured with the Plant Efficiency Analyzer (PEA) at room temperature. In the Handy PEA fluorescence instrument (Hansatech Instruments Ltd., UK), activating light is provided by an array of three high-intensity light-emitting diodes, which are focused onto the cell surface to provide uniform illumination. The diodes provide red light with a peak wavelength of 650 nm and an intensity of 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, which is readily absorbed by the chloroplasts. 1 ml of actively growing cells was taken from both control (+Fe) and iron-deficient (–Fe) cells. Then each cell suspension was kept in dark for 30 min at room temperature. 10 μM of Diuron (DCMU) was mixed with iron-sufficient (+Fe) and iron-deficient (–Fe) cells,

respectively. The fluorescence measurements were taken after 30 min of dark incubation as described in (Antal et al. 2006; Kodru et al. 2015). The fluorescence transient at 20 μ s is designated F_0 , and the maximum fluorescence yield (F_m) occurs around 200 ms. The difference between F_0 and F_m is known as variable fluorescence (F_v). The ratios F_v/F_0 and F_v/F_m are used to assess photosynthetic efficiency. O-J-I-P kinetics were visualized using Biolyzer HP 3 software (for calculating JIP test parameter that quantifies energy flow through PSII) developed by Bioenergetics Laboratory, University of Geneva, Switzerland.

Blue Native gel electrophoresis

Thylakoid membranes were isolated from control (Fe sufficient), Fe-deficient cells, and repelled as mentioned above. First dimension of blue native gel separation was carried out in 4–12 % gradient gel by solubilization of thylakoids in 1 % *n*-dodecyl β -D-maltoside (DM) along with the protease inhibitors 1 mM Aminocaproic acid (ACA), 1 mM benzamide hydrochloride, and 1 mM PMSF; the gel with 50 mM ACA was run at 4 $^{\circ}$ C with increasing voltage 35, 55, 75, 120, 150, and 200 V as described in references (Schägger and von Jagow 1991; Madireddi et al. 2014).

Immunoblots

Thylakoid membranes isolated from both iron-sufficient and iron-deficient cells were separated on SDS-PAGE. Electrophoresis was performed on 12 % resolving and 5 % stacking gel of polyacrylamide. An equal amount of chlorophyll content was loaded on to each lane. Proteins were separated by electrophoresis and transferred to PVDF membrane, incubated with primary antibodies, and detected, as described by Towbin et al. (1979) for identification and quantification of polypeptides contained in the PSII–LHCII supercomplex. Primary antibodies dilutions were PsbA, 1:5000; PsbB, 1:5000; PsbO, 1:5000; PsbP, 1:5000; and LHCII complex proteins Lhcb1, 1:5000; Lhcb2, 1:5000; CP29, 1:10,000; CP26, 1:5000; and Lhcbm5, 1:10,000. All antibodies were purchased from Agrisera. Subsequently, membrane was incubated with the HRP-linked secondary antibody with dilution of 1:10,000, purchased from Cell signaling technology and for D1 antibody, the antichick secondary antibody was used. The images were then detected by chemiluminescence.

Results

Growth curve and morphological changes observed from confocal images (Fig. 1a) show control (+Fe) and iron-deficient (–Fe) cells grown under optimal growth

conditions for 6 days. The control (+Fe) cells exhibited optimal growth, whereas iron-deficient (–Fe) cells showed slow growth pattern. Control (+Fe) cells were found to be active and high optical density in log phase but it exhibited slow growth rate in iron (–Fe)-deficient cells. It is evident from the results that iron (–Fe)-deficient cells showed reduced growth when compared to control (+Fe) cells (Fig. 1a). The confocal images were obtained from autofluorescence generated from chlorophyll in *C. reinhardtii* cells. The chlorophyll was excited at 488 nm, and the emission range was in between 600–700 nm. The control (wt) cells reveal normal shape. However, the cells were appearing in rosette shape (Fig. 1b) called as palmelloids in Fe-deficient conditions. This indicates a characteristic feature of algae when grown under adverse conditions (Neelam and Subramanyam 2013). In control, the size of the cells is measured as 10–12 μ m, whereas in Fe-deficient the cell size is measured as 4–6 μ m which indicates the size of the cells decreased under Fe-deficient condition. The cells were in a dividing state which was grouping together. These cells grown in Fe stress resemble the electrical double layers.

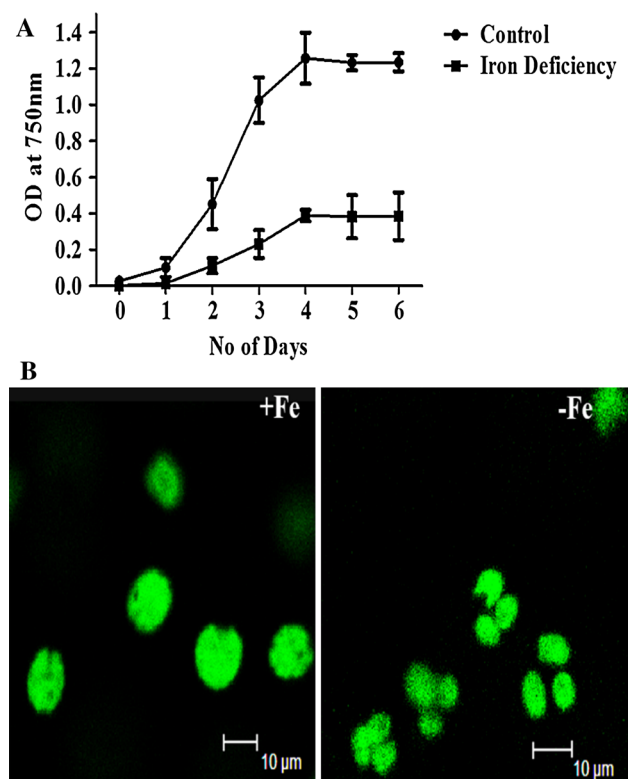


Fig. 1 a Growth curve of *C. reinhardtii* (wt) cells grown under control (+Fe) and iron-starved (–Fe) conditions for 6 days O.D at 750 nm. b Confocal microscopy images of *C. reinhardtii* showing altered cell size and cell morphology as forming palmelloid when grown under control (+Fe) and Fe-starved conditions. All images were taken equal magnification of 10 μ m

Analysis of fluorescence emission spectra

The RT fluorescence emission spectra recorded from isolated thylakoids of wt cells showed the typical peak at 683 nm and a broad shoulder at 710–740 nm which corresponds with the previous report (Ferroni et al. 2011). The 683 nm peak originated mainly from PSII, while the long wavelength emission is assigned to PSI–LHCI with the contribution of vibrational satellites of PSII (Ferroni et al. 2011). The RT fluorescence emission spectra of thylakoids from four-day grown cells in Fe-deficient medium showed a significant increase of maximum fluorescence at 683 nm than control (without Fe). This indicates the energy transfer from PSII to PSI is impaired. Fe was supplemented to the Fe-deficient cells after four days of growth (equal to the Fe concentration in +Fe medium) and further the cells were grown for 32 h and the RT fluorescence emission spectrum was measured from isolated thylakoids. The fluorescence emission almost reached the control (wt) level indicating that photosynthesis (excitation electron transport) was restored like in control (Fig. 2).

Analysis of transient Chl a fluorescence

The transient Chl a fluorescence is sensitive to various abiotic stresses, which can be analyzed by the JIP test. When dark-adapted photosynthetic cells were exposed to a 1-s pulse of strong saturating light, the transient Chl a fluorescence shows complex kinetics referred to as the OJIP transient. The first step was the rise in fluorescence from the O (F_o) to the J level in about 2 ms. The second step consists of an increase in Chl a fluorescence intensity to the I level in about 30 ms; this was followed by a slow increase in fluorescence to the P peak (F_m). It is generally accepted

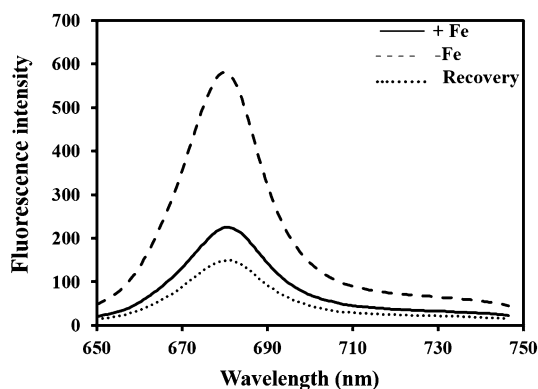


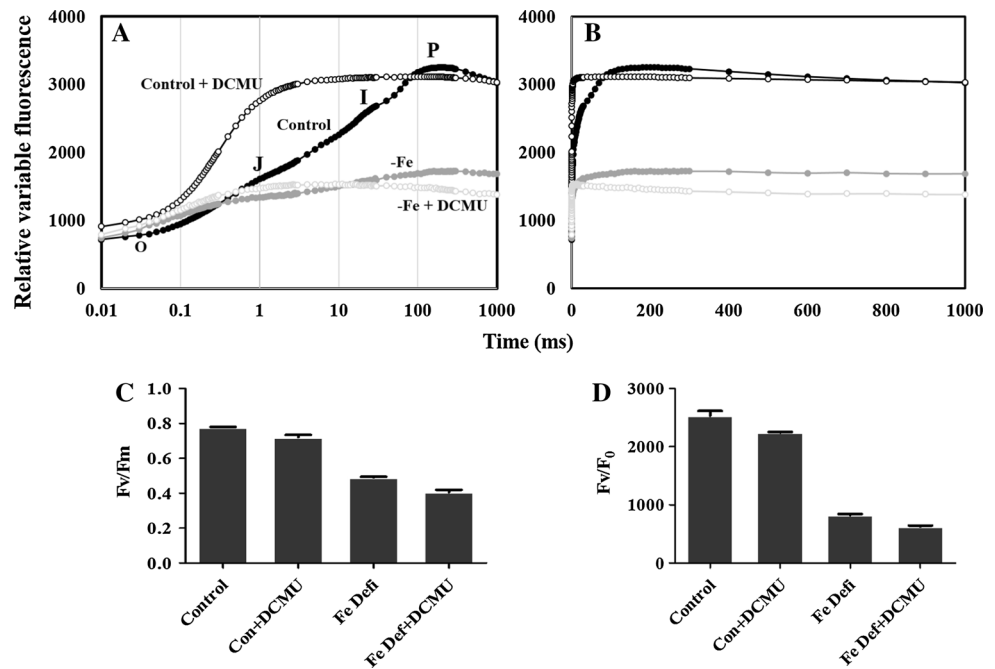
Fig. 2 Room-temperature fluorescence emission spectra of the *C. reinhardtii* cells grown under control (+Fe), iron deprivation (–Fe), and repelled (+Fe) conditions. Measurements were performed with equal Chl concentration of 10 $\mu\text{g/ml}$. Each curve is the average of 3 different measurements from three independent samples and obtained identical spectra

that the OJ phase reflects the reduction of Q_A^- while JI could represent a further closure of PSII reaction centers with their accumulation in $Q Q_B^{2-}$ stage (Kodru et al. 2015). The IP phase reflects a further reduction of the PQ pool due to a decrease in its reoxidation, as the electron carriers beyond *Cyt b_6/f* become reduced. Schansker et al. (2005) used methylviologen and dibromothymoquinone treatments in pea leaves to show that the IP phase is related to PSI activity.

The fluorescence data show significant differences during the time course, and at the three phases of OJIP transients between the control and Fe-deficient cells (Fig. 3a, b). In control cells, the three phases of OJIP are very clear and have attained maximum fluorescence indicating the efficient electron transport in the Z-scheme, which constitutes PSII, *Cyt b_6/f* , and PSI in a sequential order. The initial fluorescence level F_o decreased at 24 h under iron deficiency (data not shown), however after 72 h of growth, it recovered to the control level (Supplementary Fig. 1). A decline in F_o has also been reported in lettuce under iron-deficient condition (Msilini et al. 2011). The onset of Fe deficiency caused the F_v/F_o and F_v/F_m values to increase, followed by a decrease after prolonged Fe deficiency (Fig. 3). Initially, after 24 h under iron deficiency PSII acceptor side limitation, incomplete Q_A^- reduction at OJ phase, very little accumulation of $Q_A^- Q_B^{2-}$ and PQ pool reduction at JI phase, and limitation in further reduction of PQ pool and impairment of PSI activity were found. The electron transport beyond QA can be blocked by DCMU. DCMU-treated control cells (+Fe) depict that the P level was close to the F_m value of untreated cells (Fig. 3a). However, in the DCMU-treated Fe-deficient cells, the P values were reduced. This indicates that in Fe-deficient cells (in the absence of DCMU) at the maximum of the peak in the 0.2–2 s time range (P) not all PSII reaction centers were closed, and thus the F_v/F_m and F_v/F_o were low (Fig. 3c, d). This, however, does not necessarily mean that the maximum quantum yield of PSII was very much reduced in Fe deficiency.

In addition to damage to PSII, reduced electron flow through PSII may occur as a consequence of the retardation of the transfer of electrons from the reaction center to the PQ pool. This is due to the immediate onset of stress conditions and thereby the cells were unable to cope with the Fe deficiency. However, the three phases of OJIP transient can be seen with a slight reduction in I peak (Fig. 3a). The kinetics of OJIP transient after 48 h show that cells adapted to the Fe-deficient conditions and I peak almost absent, but J and P peaks are still clear which indicates a limitation of PQ pool reduction (data not shown). After 72 h under iron-deficient conditions, the phases of the curve were no longer distinguishable;

Fig. 3 **a** Chla fluorescence transients (OJIP) curves from (wt) control (+Fe), control with DCMU (3-(3,4-dichlorophenyl)-1,4-dimethyl urea) for 30 min in darkness, with 10 μ M concentration. Logarithmic time scale of *C. reinhardtii* cells with and without Fe; **b** the same on a linear scale; **c** F_v/F_m ; **d** F_v/F_0 . Excitation of samples at 3000 μ mol photons $m^{-2} s^{-1}$ 650 nm light. The experiments were repeated three times from different cultures



complete loss of I and P peaks indicates lower concentrations of Q_A^- , Q_B^{2-} , and PQH₂, as well as impairment of PSI and PSII under Fe-deficient conditions.

Characterization of supercomplexes from thylakoid membrane

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is one of the efficient tools to analyze the membrane supercomplexes. Hence, BN-PAGE was used for the separation of thylakoid membrane complexes from wt (containing Fe) and iron deficiency of *C. reinhardtii* grown under optimal growth conditions. By solubilization with a mild detergent like β -DM, we could separate the supercomplexes of *C. reinhardtii* as reported earlier (Madireddi et al. 2014). The results obtained from wt were similar to that of our previous observation (Madireddi et al. 2014) where the PSI–LHCI, PSII–LHCII, PSII RCC dimer, ATP synthase, Rubisco, Cyt b6f, PSI monomer, PSII RCC, LHC trimers, and LHC monomers were separated. However, there were significant changes in abundance of supercomplexes in isolated thylakoids from Fe deficiency (Fig. 4). The major supercomplexes PSII–LHCII and PSI–LHCI were completely destabilized (Fig. 4 lane 2) in Fe-deprived conditions. Also, PSII reaction center dimer, ATP synthase, RUBISCO, and Cyt b6f complexes were not assembled in Fe-deficient cells. Interestingly, the PSI and PSII monomers significantly increased. There are 2 LHCII trimers separated in wt; surprisingly, one of the trimer (LHC 3a) content has been increased, whereas, LHC 3b content has been decreased. The recovery by the addition of Fe to cells

induced supercomplexes assembly similar to the control cells, which indicates that Fe is very important for the stabilization of supercomplexes and for the efficient performance of photosynthesis.

Further, the BN-PAGE gels were placed on second dimension (SDS-PAGE) to separate the individual proteins. The wild-type supercomplexes were separated into individual proteins of phenotypic in nature (data not shown). Surprisingly, both PSII–LHCII and PSI–LHCI protein accumulation were decreased under Fe-deficient conditions. Interestingly, the protein content of LHC monomers is not much affected. Though the LHCs are present, they were not forming supercomplexes with PSI and PSII. Hence, the excited electron transfer is impaired.

Reaction center protein profile of PSII changes in Fe deficiency

Since there are remarkable changes in supercomplex arrangements, one would expect their protein content to be altered. We separated the thylakoid proteins of both wt and Fe-deficient thylakoids on SDS-gels and transferred those proteins onto PVDF membrane. These blots were probed with different antibodies of PSII core and LHCII. Figure 5a, depicts the core proteins of PSII core antenna, CP43 and CP47 are significantly diminished their content. Further, reaction center proteins of D1 and D2 content also decreased. Among the water oxidation complex subunits, PsbO content has been reduced significantly. However, PsbP is not changed in Fe-deficient conditions (Fig. 5a).

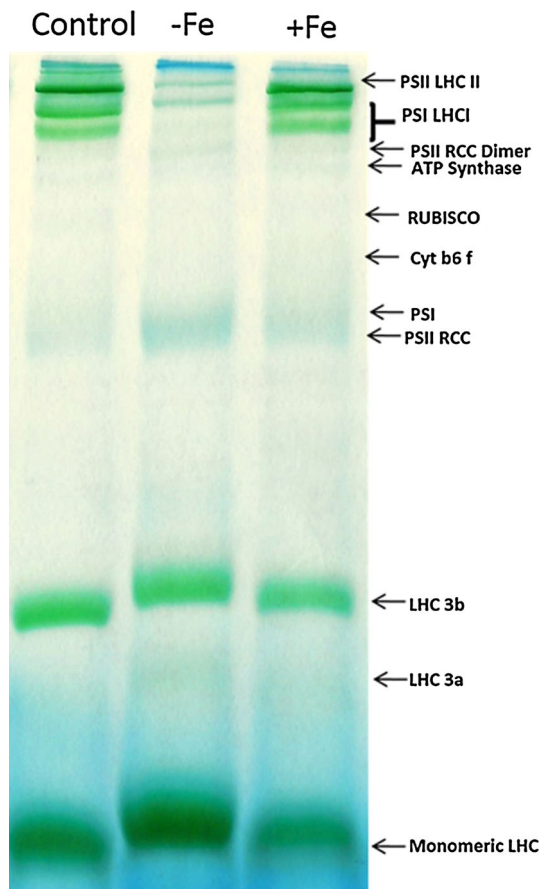


Fig. 4 Blue native PAGE of thylakoid membranes isolated from control (+Fe), iron-deprived (–Fe), and iron-replenished cells after deprivation (+Fe) were solubilized with 1 % β -DM and separated by BN-PAGE, complexes are identified according to previous literature. Equal amount of chlorophyll (20 μ g) was loaded into each lane

Light-harvesting complexes protein profile change in PSII

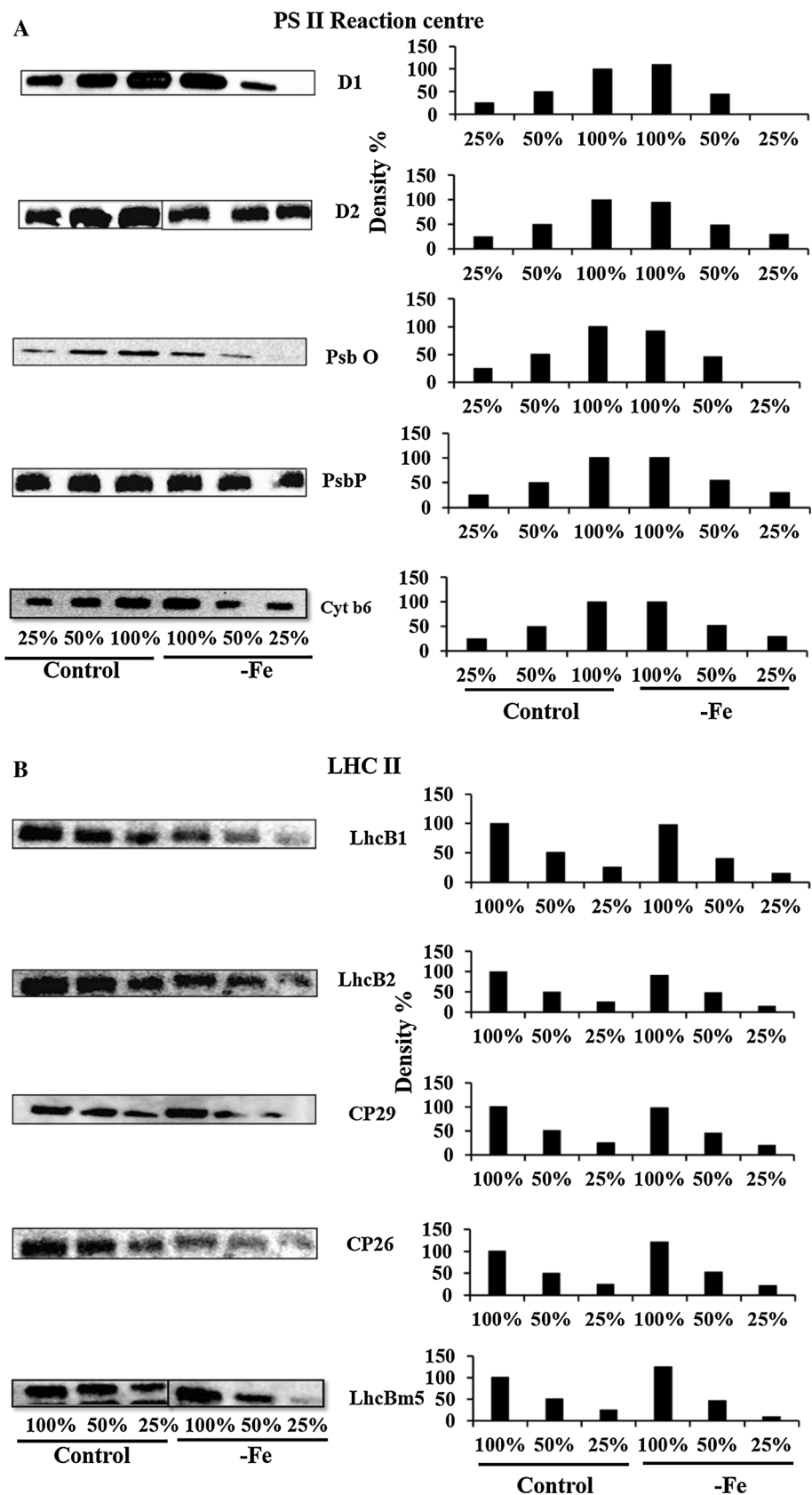
LHCII is the most abundant membrane protein complex on earth. It is well known that it participates in the efficient harvesting of sunlight and transferring excitation energy to the core complex in the photosynthesis process. Here, we have checked the abundance of LHCII proteins under Fe-deficient condition to understand the level of harvesting energy. Interestingly, the major LHCII trimer subunits (Lhcb1 and Lhcb2) were reduced, whereas the minor subunits CP26 and LhcBm5 were not significantly changed. However, CP29 and other minor subunits have remarkably changed under Fe deficiency (Fig. 5b). These results indicate that changes in LHCII protein level would hamper the efficiency of light-harvesting capacity and reduce the transfer of light energy to the reaction center.

Discussion

Iron is very well known as an essential element for plant growth and nutrition, but the effect of Fe deficiency at the photosynthetic level, particularly PSII is not well understood. Previously, we have shown that Fe deficiency induces severe damage to the PSI which leads to decreased electron transport in *C.reinhardtii* and rice (Yadavalli et al. 2012a, b). Iron is an essential element for Chl biosynthesis, pigment–protein complexes, and hence these are the first target toward iron deficiency. When the cells were grown under Fe-deficient conditions, the cells were observed as clumps which have a peculiar characteristic feature as cells assemble into a rosette-shaped structure (Fig. 1b). This kind of phenomenon is known as palmelloid which we have also observed in salt stress (Neelam and Subramanyam 2013). It is known that *C.reinhardtii* is a unicellular algae with an average diameter of 10 μ m which has been decreased in size of palmelloids in Fe-deficient condition. The previous reports suggested the palmelloid formation in *C. reinhardtii* is either due to flagellar loss/malfunction or progressive membrane gelatinization after its interaction with the environment (Lüring and Beekman 2006). In *C. applanata*, under low pH of 3.4 excess mucilage production enables sticking of palmelloid colonies together that results in large clumps (Visviki and Santikul 2000). Our results indicate that the palmelloid formation in Fe deficiency may be due to cell-wall abnormality.

Interestingly, the cell viability is the same as in control indicates even after four days of growth (Fig. 1a) but the cell size has been decreased. These data are an agreement with the palmelloid formation; at this stage the cell size has been reduced which indicates the photosynthetic activity was reduced. After being grown for 72 h under Fe-deficient conditions, the first stage of inhibitory action was detected as a relative increase of the amplitude of the OJ phase of the Chl fluorescence transient with a corresponding decline of JI, which agrees well with the treatment of pea leaves with dibromothymoquinone (DBMIB). DBMIB is an artificial quinone which inhibits the reoxidation of plastoquinol by binding to the *Cytb₆/f* complex, causing the IP phase to disappear (Schansker and Strasser 2005). The JI phase was previously shown to be complementary to O–J, the sum of the amplitudes of OJ and JI representing the complete reduction of Q_A (Kodru et al. 2015). The OJ phase is related to the initial reduction of Q_A (Kodru et al. 2015) and its enhancement was shown to be associated with a slower rate of electron transfer between Q_A and Q_B . JI phase declined in Fe deficiency as compared to control, and similar results have been obtained when *C. reinhardtii* cultures were deprived of sulfur (Antal et al. 2006), and

Fig. 5 Immunoblots of PSII and LHC proteins content from control (+Fe) and Iron-deficient (Fe) cells. Equal chlorophyll was loaded with different dilutions (25, 50 and 100 %) as a positive control and was separated by SDS gel electrophoresis. **a** PSII core proteins D1, D2, OEC (PsbO, PsbP), and Cyt b6; **b** major LHCII proteins LhcB1, LhcB2, CP29, CP26, and Lhcbm5 are detected. The adjacent bar diagram were developed by Image J software to show the quantity of protein present



limitation of Fe in lettuce (Msilini et al. 2011) suggesting that Fe and S deprivation might function similarly, through their effects on the Fe–S clusters of the electron transport chain. This is also confirmed by the addition of DCMU to cells which showed a decline of JIP phases under Fe deficiency. Also, *C. reinhardtii* belongs to the C₃ type and the contribution of PSI activity to F_o was about 30 %; the deviation in F_o , which is observed in Fig. 3, is most likely due to both PSI and PSII activity. By 72 h of Fe deficiency, the level of I peak of Fe-stressed cells approaches 90 % of F_m . There is also some deviation of F_m from the control value, possibly due to the cumulative effects of Fe deficiency on the entire photosynthetic electron transport chain. The decline in F_v/F_m together with the amplitudes of the O–J and I–P phases of the curves indicates that the OEC failed to provide electrons for PSII to reduce adequately the quinone acceptors of the photosystem in the absence of Fe, thus decreasing the maximal fluorescence yield. Also, the P phase was remarkably altered even in DCMU-treated cells indicating that acceptor side of PSII was also damaged. Thus, these results could suggest that the effects of Fe deficiency cause impairment of PSII, both acceptor and donor side of PSII. Also, these results were in agreement with the RT fluorescence emission data that increase in fluorescence maximum at 683 nm, indicating the energy transfer block from Chl a to PSII (Fig. 2). If there is a block in excitation energy transfer from Chl a to the reaction center, obviously the PSII fluorescence will increase. This was well supported by the previously published work on UV-B effect on cyanobacteria *Spirulina platensis*, considering this increase is due to uncoupling of energy transfer from Chl a to PSII which induced enhanced emission (Kolli et al. 1998). However, the addition of Fe to control cells shows the restoration of photosynthetic function within 32 h. This is confirmed by fluorescence emission, blue native gel, and immunoblot analysis of PSII core and LHCII subunits.

Further, supercomplexes of PSI and PSII from thylakoids have been equally disorganized. The monomeric forms of PSI, PSII, and LHC's were existing but to some extent the content of supercomplexes has been increased (Fig. 4). Because of Fe deficiency, an organization of supercomplexes was not formed besides addition of Fe; it has been restored after 32 h. Thus, Fe plays an important role in the formation or organization of supercomplexes. Under Fe-deficient conditions the Chl content was reduced. However, it is well known that iron limitation induces leaf chlorosis due to the decrease of Chl content. Subsequently, a pronounced iron deficiency resulted in a reduction of light-harvesting complexes content in both PSI and PSII (Timperio et al. 2007; Yadavalli et al. 2012a, b). We also reported that the detergent-solubilized thylakoids were separated on a

sucrose gradient, which showed that PSI supercomplexes were subverted. The destabilization of supercomplexes in Fe deficiency may be due to less Chl and protein biosynthesis. It is clear that if the Chl biosynthesis or protein synthesis is slowed down or retarded, it may obviously cause disorganization of supercomplexes of PSI and PSII which attributes to hampered photochemistry.

Further, the protein accumulations of PSII were characterized to see the abundance of proteins in Fe deficiency. In which most of the reaction center proteins of PSII were impaired except the PsbP (Fig. 5a). It is expected that excitation energy transfer from PSII to PSI is hampered due to less accumulation of reaction center proteins. Additionally, the LHCII proteins (Lhcb1, Lhcb2, CP26, CP29, and LhcBm5) were significantly reduced in Fe-depleted cells which indicate that light-harvesting capacity was minimized (Fig. 5b). It is well known that accumulation of PSI–LHCI proteins was declined due to lack of required amount of Fe. Plants change their antenna size depending on the light environment to optimize light-harvesting capacity (Anderson and Andersson 1988). PSI appears to be the first target of iron deficiency (Moseley et al. 2002), perhaps because of its high Fe content as the electron transfer involves 12 iron atoms in three Fe–S centers. As a consequence, PSI can be less tolerant than PSII in case of iron stress because any iron deficiency may have a site of action on the acceptor side of PSI where the majority of Fe–S centers were localized. Photoheterotrophic *Chlamydomonas* (CC424 mt-) to iron starvation showed decreased photosynthetic proteins, while respiratory proteins were increased suggests that the prioritization of respiration over photosynthesis in iron-deficient condition (Naumann et al. 2007). Also, when the *C. reinhardtii* cells grown under Fe deficiency in both photoheterotrophically (acetate) and photoautotrophically (CO₂), the accumulation of proteins was different. From these results, we demonstrate that photoheterotrophically grown cells show less accumulation of proteins, but there is no significant change in proteins while cells were grown photoautotrophically. In the presence of acetate, iron-limited *Chlamydomonas* cells maintain high growth rates by suppressing photosynthesis and increasing the respiration, while phototrophic cells maintain efficient photosynthetic systems, but still lose overall photosynthetic activity at the beginning of iron deficiency, which is delayed in phototrophic cells may be due to the increased iron content (Terauchi et al. 2010). Hence, the above data are very well in agreement with our studies that under photoheterotrophic conditions the photosynthesis is vulnerable to Fe deficiency. In our case, the PSII also damaged remarkably apart from PSI when Fe is depleted. Hence, the PSII photochemistry is impaired.

In conclusion, the cell viability is similar in both control and Fe-deficient cells. However, the cell size has been reduced. Further, the complete depletion of Fe affects the photochemistry of PSII, which indicates that the electron transport from Q_A to Q_B is impaired. Also, it suggests that both acceptor and donor side of PSII has been damaged, which is concomitant with the decreased abundance of both reaction center antenna and core proteins. Also, the differential degradation or damage of LHCII proteins observed may be due to loss of chlorophyll biosynthesis. Further, the organization of supercomplexes was disturbed due to lack of Fe and hence, Fe may be required for the organization of the thylakoid supercomplexes.

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