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A cytoplasmically inherited barley mutant is defective in Photosystem I assembly due to a temperature sensitive defect in ycf3 splicing.

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Abstract

A cytoplasmically inherited chlorophyll-deficient mutant of barley (*Hordeum vulgare* L) termed CL3, displaying a *viridis* (homogeneously light-green colored) phenotype has been previously shown to be affected by elevated temperatures. In the present paper, biochemical, biophysical and molecular approaches were used to study the CL3 mutant under different temperature and light conditions. The results lead to the conclusion that an impaired assembly of photosystem I under higher temperatures and certain light conditions is the primary cause of the CL3 phenotype. Compromised splicing of *ycf3* transcripts, particularly at elevated temperature, resulting from a mutation in a noncoding region (intron 1) in the mutant *ycf3* gene results in a defective synthesis of Ycf3, which is a chaperone involved in PSI assembly. The defective PSI assembly causes severe photoinhibition and degradation of PSII.
Introduction

Chlorophyll (Chl) deficient mutants have been extensively utilized to study development and functions of chloroplasts (e.g., Gustafsson 1942; von Wettstein 1961; Taylor et al., 1987; von Wettstein et al., 1995; Leon et al., 1998). Most of the Chl-deficient mutants reported previously originate from nuclear gene mutations. This is not surprising since most of the genes necessary for plastid development and pigment biosynthesis have migrated from plastids to the nucleus during evolution of plants (Hess and Börner, 1999). Previously, some cytoplasmically inherited Chl-deficient mutants selected from a family carrying a mutator genotype in barley (*Hordeum vulgare* L) have been described (Prina 1992, 1996). Mutants viable under field conditions have been selected in order to facilitate their multiplication, hybridization and, later on, selection of nonmutator type, genetically stable genotypes. Due to their breeding behavior, those mutants were called cytoplasmic lines (CLs) (Prina 1996). One of these lines, termed CL3, exhibits a *viridis* (homogeneously light-green colored) phenotype, which is much more pronounced at elevated temperatures (Prina et al., 1996). Wild type (WT) barley seedlings become chlorotic at growth temperatures in excess of 32ºC (Smillie et al., 1978). Some temperature-sensitive mutants, which were previously described, showed heat sensitivity for chloroplast development at temperatures lower than those producing chlorosis in wild type (WT) and also, a decrease of photosystem I (PSI) and photosystem II (PSII) activities (Smillie et al., 1978). In all these mutants, the genes affected were nuclear encoded. Hence, CL3 is the first temperature-sensitive chloroplast gene mutant described in higher plants. Chlorosis is a syndrome that can result from primary deficiencies at many different points in the chloroplasts. Not surprisingly, mutants in chlorophyll biosynthesis are chlorotic, but so are mutants e.g. in carotenoid biosynthesis or in the function of either of the photosystems. In the latter cases, chlorosis is primarily a result of reactive oxygen species that are generated as a result of inefficient radical scavenging or due to faulty electron transfer processes. Since reactive oxygen species cause damage many chloroplast components, a primary mutation at any point of the electron transport chain can cause damage to other sites of the chain and to pigment biosynthesis, thereby causing generation of even more reactive oxygen and exacerbated damage. Hence, it can be difficult to determine the primary cause of chlorotic phenotypes.
In the present paper, biochemical, biophysical and molecular approaches were used to study the responses of the CL3 mutant to different temperatures and light conditions. The results show that an impaired assembly of PSI causes the CL3 phenotype and results in photodamage to the entire photosynthetic apparatus. Compromised and temperature-dependent splicing of ycf3 transcripts resulting from a mutated noncoding region (intron 1) in ycf3 is the primary cause of the phenotype.

Results

The *viridis* phenotype of the CL3 mutant is more pronounced at higher temperatures

Figure 1 shows WT and CL3 barley seedlings growing at two different temperatures and light qualities 6 days after germination. The permissive and optimal temperature was chosen at 18°C and 32°C as the restricted temperature for stress treatment. The light environments were provided by either a combination of fluorescent and incandescent light or solely by fluorescent light, at 100 µmol photons m\(^{-2}\) s\(^{-1}\) in both growth chambers. At 18°C the mutant showed a slightly pale phenotype (Fig. 1A and 1C), which was more pronounced in fluorescent light than in the combination of fluorescent and incandescent light. At 18°C the CL3 plants would develop and set seeds in spite of the mutation. At 32°C the *viridis* phenotype of CL3 was much more pronounced, and none of the plants survived (Fig. 1B and 1C), but again the phenotype was much more severe in fluorescent light (Fig. 1D) than in the combination light (Fig. 1B).

Unless otherwise noted, all experiments reported below were carried out with seedlings 6 days after germination, grown in the mixed light, i.e. plants corresponding to Fig. 1A and 1B.

Pigment contents

To quantify Chl content and to investigate to what extent the contents of other pigments were altered, barley seedlings were grown for 6 days after germination at the two temperatures mentioned above under a combination of fluorescent and incandescent light, and pigments were quantified by HPLC (Table I). Chl content was slightly
reduced in WT leaves at 32°C and the CL3 mutant grown at 18°C as compared to WT at 18°C, but reduced to about 24% of WT levels in the mutant at 32°C.

Xanthophyll-cycle pigments (violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z), determined relative to Chl) were increased 2-fold in CL3 compared to WT at both temperatures. Temperature did not have a significant effect on the VAZ-Chl ratio. However, the de-epoxidation state of V+A+Z was increased in CL3 already at 18°C, and at 32°C more than 50% of the xanthophyll cycle pigment pool was partially or completely de-epoxidated.

Effects of growth temperature on chloroplast ultrastructure in the CL3 mutant

Transmission electron microscopy revealed striking differences in the ultrastructure of plastids from CL3 plants grown at 32°C as compared to those grown at 18°C under fluorescent light only (Fig. 2A-D). In the mutant, at 32°C mesophyll cells had small plastids of abnormal appearance, with a rather elongated shape containing "clumps" of electron-dense material as well as an increased number of plastoglobuli. Numerous thylakoids and grana-like structures with swollen thylakoids were found in most of the plastids (Figs. 2A and B).

CL3 plants grown at 18°C had more developed and elongated chloroplasts (Fig. 2C) with both stacked and unstacked thylakoid membranes. Frequently, thylakoid membrane organization was altered in particular at the periphery of the chloroplast, and swollen stacked thylakoids were observed (Fig. 2D). In contrast, no significant differences in chloroplast ultrastructure were observed between WT plants grown at different temperatures (data not shown).

The severe effect on chloroplast ultrastructure observed in plants grown at standard light conditions of 100 μmol photons m⁻² s⁻¹ prompted us to investigate ultrastructure of chloroplasts from plants grown under low-light conditions (10 μmol photons m⁻² s⁻¹). Chloroplasts of both genotypes showed an essentially normal development of the internal membrane system with an increase in the fraction of the appressed thylakoid membranes as compared to those which had developed at 100 μmol photons m⁻² s⁻¹ (Fig. 2E-H). In CL3 plants, however, the chloroplasts were enlarged and displayed a somewhat rounded shape (Fig. 2E). Frequently, an enlarged thylakoid luminal space was discernible, mainly in stroma thylakoids and on the borders of grana stacks.
Additionally, numerous "vesicle-like" structures appeared in these chloroplasts (Fig. 2E and F).

**Fluorescence analyses**

To assess whether Chl deficiency in the CL3 mutants was related to defects in pigment synthesis or loss in specific Chl-containing proteins, 77 K Chl fluorescence emission spectra of thylakoid suspensions from 6 days after germination seedlings were acquired (Fig. 3). Three major features are observed in the 77 K fluorescence emission spectra: A fluorescence band peaking at about 685 nm corresponds to emission from the PSII core antenna complex CP43 and LHCII. A further, usually less pronounced maximum at 695 nm is ascribed to the combined emissions of the PSII reaction center and the core antenna complex CP47. The most pronounced peak in the 77 K fluorescence emission spectra is observed at about 735 nm and originates from PSI emission. When normalized at 685 nm, a considerable decrease of the PSI-peak relative to PSII was observed for the CL3 mutant, being more pronounced for 32°C-grown plants than for 18°C-grown plants. Moreover, a pronounced 5-nm blue-shift of the long wavelength PSI-peak was observed.

The 77 K fluorescence data could indicate that primarily PSI is affected by the CL3 mutation. To further investigate this indication and to determine how PSI deficiency would affect overall photosynthetic efficiency and electron transport, room temperature Chl fluorescence was analyzed using a pulse amplitude modulation (PAM) fluorometer. The maximum photochemical efficiency of PSII in the dark-adapted state, $F_{v}/F_{m}$, was 0.812 and 0.755 for WT and CL3 mutant leaves, respectively, when the plants were grown at 18°C. When plants were grown at 32°C, $F_{v}/F_{m}$ of WT plants was only slightly diminished (to 0.777) but the value for the CL3 mutant was drastically decreased to 0.361 - indicating serious malfunction of PSII.

The response of the photochemical quenching parameter ($qP$) to increasing actinic light (AL) intensity for plants grown at different temperatures is shown in Fig. 4A. WT and the CL3 mutant plants responded in a dramatically different manner: WT leaves grown at either temperature showed an almost identical - gradual - decrease of $qP$ with increasing AL intensities. In contrast, the 18°C-grown CL3 plants displayed a dramatic decrease of $qP$ already at moderate AL intensities, well below the growth light level,
and CL3 plants grown at 32°C showed no photochemical quenching (i.e., photosynthetic activity) at all.

The light-response of non-photochemical quenching (quantified as NPQ) in WT and CL3 mutant plants grown at different temperatures is shown in Fig. 4B. The largest capacity for NPQ build-up with increasing AL intensity was observed in 18°C grown WT plants whereas it was only slightly diminished in 32°C grown WT plants and 18°C grown CL3 plants. CL3 plants grown at 32°C, however, displayed a drastically diminished capacity to develop NPQ (Fig. 4B). Moreover, the observed NPQ in the latter plants corresponded almost entirely to the very slowly or non-relaxing photoinhibitory quenching (data not shown).

Proteins of the photosynthetic apparatus

The fluorescence data indicate that both PSI and PSII are affected in the CL3 mutant depending on the experimental conditions. To further investigate these responses, the amounts of different thylakoid proteins were determined by immunoblotting. Analyses of the D1 reaction center subunit of PSII and the major light-harvesting complex II (LHCII) are presented in Fig. 5. When the plants were grown at 18°C only little difference between WT and CL3 plants with respect to D1 and LHCII content was observed. In contrast, these proteins were considerably decreased in amount or even absent in the mutant when grown at 32°C. Absence of qP, considerably lowered Fv/Fm, and severe photoinhibition in these seedlings suggest that the disappearance of the D1 protein may result from an severely over-reduced intersystem electron transport chain. To test this notion, the effects exerted by fluorescent light only were compared with those of a mixture of fluorescent and incandescent light of the same light intensity (100 µmol photons m⁻² s⁻¹) (Fig. 5). Incandescent light is exciting PSI more efficiently (due to its higher proportion of far-red light) and therefore this light mixture should potentially reduce over-reduction of the intersystem electron transport chain and excitation pressure on PSII. Indeed, under mixed light conditions, the D1 and LHCII proteins were observed in CL3 seedlings at approximately similar levels as in the WT, even under growth at 32°C.

To circumvent the effects of severe photoinhibition we only used plants grown under mixed illumination for the analysis of PSI proteins (Supplemental Fig. S1). PsaC and PsaE were absent or almost undetectable in CL3 plants at both temperatures. However,
PsaD was nearly undetectable in the mutant grown at 32°C, but when CL3 plants were grown at 18°C it appeared as a weak band after three days following germination and its level further increased at days 6 and 9. To more accurately quantify the amount of PSI subunits in the CL3 mutant, immunoblotting was carried out with different dilutions of thylakoids from the 6 days after germination seedlings grown at 18°C (Supplemental Fig. S2). This analysis shows that the amount of PsaA/B and PsaD in CL3 corresponded to approximately 10-25% of the WT levels. PsaE and PsaC could be detected, but the amounts were lower, particularly for PsaC which was present at less than 3% of the WT level. Immunoblots with antibodies against PsaF and LHCI subunits are shown in Supplemental Fig. S3. PsaF is essentially absent in CL3 at both temperatures whereas little effect on LHCI proteins was seen. To determine whether the PSI deficiency is elicited at the transcriptional or post-transcriptional level, RNA was isolated and transcript levels for psaC, psaD and psaE were determined by Northern blotting. No significant differences in the transcript levels were found between the mutant and WT, neither at 18°C nor at 32°C (data not shown).

DNA sequences of putative candidate genes

The specific primary defect in PSI assembly and the secondary photoinhibitory effects on PSII suggested that either one of the plastid encoded PSI subunits or a factor required for PSI assembly could be mutated. We therefore sequenced the plastid genes psaA, psaB and psaC in the CL3 mutant and the sequences were identical to the published barley plastome (NC_008590).

The lack of direct effect on PSI genes prompted us to investigate the chloroplast genes ycf3 or ycf4, which have been reported to be involved in PSI assembly (de Longevialle et al., 2008; Naver et al., 2001; Boudreau et al., 1997). The corresponding fragments of plastid DNA from CL3 mutant and WT were amplified and sequenced. The sequence of ycf4 was found to be identical in mutant and WT, indicating that the CL3 phenotype was not related to ycf4. The complete ycf3 sequence in barley consists of three exons (124 bp, 230 bp and 159 bp long) split by two introns (759 bp and 723 bp). The coding sequence of the ycf3 gene recently published for barley (NC_008590) is identical to our WT sequence. However, two point mutations were found in intron 1 of the ycf3 gene.
These mutations are a T→C substitution at nucleotide 528 and a T insertion between nucleotides 150 and 151.

**ycf3 transcripts**

To assess the effects at the mRNA level of the mutations in intron 1 of ycf3, total RNA was isolated from WT and mutant grown at 18°C and 32°C and analyzed by RT-PCR (Fig. 6). In the WT, a band of about 500 bp, corresponding to the intron-less fully spliced transcript (513 bp) was observed at both temperatures. However, two bands were observed in CL3 grown at 18°C, one of 513 bp corresponding to the intron-less transcript, and another one between 1.2 kb and 1.5 kb which, according to sequencing, corresponded to a larger processing intermediate of ycf3 that contained the three exons plus additionally intron 1 (1.272 kb) (Supplemental Fig. S5). Surprisingly, when seedlings were grown at 32°C, the band corresponding to the fully spliced transcript was absent in CL3 and the major band observed corresponded to the immature transcript of 1.272 kb. In silico FOLD analysis of the RNA sequence of intron 1 (Zuker and Stiegler, 1981) showed that the WT RNA is predicted to have a stable structure at both temperatures, whereas the CL3 RNA has a different structure and is predicted to change considerably between the two temperatures (Supplemental Fig. S6).

Analysis of the ycf3 cDNA also revealed an editing site in exon 2 similar to one previously observed in maize (Ruf et al., 1994; Ruf and Kössel 1997). It was also observed that editing in exon 2 had already occurred in the intron 1-containing transcript of ycf3 from CL3 as previously observed in intron 1-containing transcript of ycf3 from maize (Ruf and Kössel 1997) (Supplemental Fig. S5). Interestingly, nucleotide 44 in exon 1, which is the other editing site in maize (Ruf et al., 1994; Ruf and Kössel 1997), was already a T in barley.

To confirm that the inefficient splicing of ycf3 transcript was a specific effect and not a general problem with mRNA processing, other transcriptional units containing introns were analyzed. Northern blots for atpF and petB transcripts showed no significant alterations in the transcript patterns of these two genes between CL3 mutant and WT (data not shown).

**Ycf3 protein**
To assess the amount of Ycf3 protein in WT and CL3 we made an immunoblot with an antibody raised against a barley Ycf3 peptide (Fig. 7). Ycf3 was present in CL3 at 18°C but almost absent at 32°C. There appeared to be a higher amount of the chaperone in the mutant at 18°C compared to the WT, however, this could be an effect of protein overloading as the gel was loaded on a chlorophyll basis (Supplemental Fig. S7).

**Discussion**

The mutant described here showed a dramatic phenotype at the restrictive temperature, and even at low light intensity the plants could not survive at 32°C beyond the seedling stage. The plants that had been grown under those conditions for 6 days after germination were pale green but not obviously affected in growth (Fig. 1B). At the permissive temperature of 18°C the CL3 mutant plants survive and they eventually set viable seed. Six days after germination the mutant plants did not show any visible symptoms except for a slight chlorophyll deficiency (Fig. 1A). Closer inspection showed that even at the permissive conditions, the plants had an impaired PSI and severely decreased capacity for photosynthesis (Figs. 3 and 4). The amount of PSI in the thylakoids was severely decreased, especially at the higher temperature (Figs. S1 and S2). Analysis of fluorescence also strongly indicated that PSI was primarily affected in the mutant. In general, PSI is known to be much more stable, also at high temperatures, than PSII (Al-Khatib and Paulsen, 1999; Takeuchi and Thornber, 1994; Berry and Björkman, 1980), but in the CL3 mutant PSI was more affected than PSII. The biochemical, spectroscopic and physiological phenotype resembles that described for several other PSI mutants, where the amount of PSI is decreased and a secondary damage to PSII and other thylakoid proteins is observed especially at higher light intensity and with light that predominantly excites PSII (Haldrup et al., 2000, 2003).

PSI is a multi-subunit pigment-protein complex embedded in the thylakoid membranes of chloroplasts and cyanobacteria. PSI catalyses light induced electron transfer from plastocyanin (and/or cytochrome c in certain cyanobacteria) to ferredoxin - ultimately leading to NADP reduction (see e.g. Jensen et al., 2007 for a review). PSI is composed of at least 19 protein subunits in higher plants (Jensen et al., 2007, Nelson and Yocum 2006). PsaA, PsaB, PsaC, Psal and PsaJ proteins are chloroplast encoded, while the other subunits are encoded by nuclear genes. Psal and PsaJ are not essential...
for PSI function and assembly (Hansson et al., 2007, Xu et al., 1995), and we have therefore not investigated them in this study. PsaA and PsaB form the reaction center complex around which all other subunits are assembled. PsaC, PsaD and PsaE are components of a stromal peripheral domain, which interacts with the intrinsic membrane proteins PsaH, PsaI, PsaL, and PsaO (Jensen et al., 2007, Nelson and Yocum 2006). The peripheral subunits are essential for PSI function, whereas the H, I, L, and O subunits are not. It is generally assumed that the initial event in PSI assembly is the formation of a heterodimeric PsaA/PsaB core complex, followed by association of PsaC, which is required for subsequent binding of PsaD and PsaE (Choquet and Vallon 2000; Chitnis, 2001; Antonkine et al., 2003; Fromme et al., 2001). In addition, PsaD was found to be essential for the stable binding of PsaC and PsaE (Li et al., 1991, Naver et al., 1995) and Arabidopsis mutants deficient in PsaD cannot assemble stable PSI (Haldrup et al., 2003, Ihnatowicz et al., 2007). While PsaC and PsaD seem to be essential for accumulation of stable PSI this is not the case for the other small subunits. Thus, although the PsaF subunit is essential for PSI function in plants, inactive PSI complexes lacking PsaF were present in Arabidopsis plants with downregulated PsaF expression (Haldrup et al., 2000). Plants with decreased content of PsaF or PsaD had a low PSI activity and hence a strong tendency to overreduction of the intersystem chain, which made PSII highly susceptible to photodamage (Haldrup et al., 2000, 2003, Ihnatowicz et al., 2004). PSI with impaired function can also generate reactive oxygen, which may directly damage PSII (Tjus et al., 2001). In either case, the compromised photosynthesis will make it difficult for the plant to generate the energy required for PSII repair thereby further exacerbating PSII photodamage. Other PSI mutants, e.g. when PsaE, PsaH or PsaN are absent, still have a functional PSI, albeit with a decreased activity (Haldrup et al., 1999, Naver et al., 1999, Pesaresi et al., 2002, Ihnatowicz et al., 2007). In such cases the plants are able to respond with a change in PSI/PSII ratio and/or relative antenna size of the photosystems and thereby limit redox imbalance and oxidative damage to either photosystem.

CL3 plants germinated under permissive conditions had some intact PSI complexes, but they accumulated very slowly and the PsaC, PsaE and PsaF subunits were clearly substoichiometric compared to PsaA/B and PsaD (Figs. S1-S3). This observation implies the presence of PSI complexes containing PsaA, PsaB, and PsaD but devoid of PsaC. This is a surprising finding. In the cyanobacterium Anabaena it has been found that inactivation of the psaC gene results in accumulation of PSI devoid of PsaC (Mannan et
al, 1991). Whether the complexes contained PsaD was not reported. In contrast, *Chlamydomonas reinhardtii* with an inactivated *psaC* gene does not accumulate PSI, presumably because the entire complex becomes unstable in the absence of PsaC (Takahashi et al., 1991). The barley plants investigated in the present study seem to have a response different from *C. reinhardtii* since PsaC appears not to be essential for accumulation of PSI, even though accumulation is inhibited substantially. Nevertheless, it should be born in mind, that PSI devoid of PsaC must be inactive, since PsaC carries the ultimate electron acceptors in PSI, iron sulfur clusters $F_A$ and $F_B$, which are indispensable.

Given the specific effect of the CL3 mutation on PSI it seemed that the site of the mutation would be either in PSI itself or in a gene encoding a factor required for function of PSI. The PsaA, PsaB and PsaC subunits are all required for PSI function, but in the CL3 mutant transcripts are present in normal amounts for all three proteins and they do not contain any differences from the WT transcripts. This caused us to look at two plastid genes that are known to encode factors specifically required for PSI assembly, i.e. *ycf3* and *ycf4* (Naver et al., 2001; Boudreau et al., 1997). While *ycf4* did not contain any differences in CL3 compared to WT, we observed a dramatic difference with *ycf3*. In the CL3 plants the *ycf3* gene had two mutations in the non-coding intron 1 sequence and somehow this difference interfered with splicing of the first intron. Thus, the CL3 plants contain highly decreased amounts of the correct *ycf3* transcript at the permissive conditions and undetectable amounts at the restrictive conditions (Fig. 6). Sequences of *ycf3* homologues are conserved from cyanobacteria to higher plants: they consist of three exons and two group II introns in the latter (Maier et al., 1995). Group II introns have a ribozymic activity and adopt a conserved secondary structure (Michel and Ferat, 1995; Chu et al., 2001). In wild type tobacco, *ndhB* (a group II intron gene) pre-mRNA is blocked at high temperature (Karcher and Bock, 2002). One of the alternatives the authors proposed is that an aberrant RNA secondary structure formation of the intron at high temperature indirectly causes the splicing defect. In silico FOLD analysis of the RNA sequence of intron 1 from WT and mutant showed that CL3 RNA is susceptible to change at different temperatures (Supplemental Fig. S6) and that temperature change in the mutant leads to disruption of the RNA secondary structure required for splicing the intron, especially at 32°C.

Interestingly, an Arabidopsis mutant deficient in splicing of intron 2 of *ycf3* has been described (de Longeviale et al., 2008). The primary defect is in a nuclear gene *OTP51*
which is specifically required for splicing of this particular intron. The *otp51* mutant does not contain detectable Ycf3 and PSI.

In spite of the decreased amount of *ycf3* transcript at the permissive temperature, the amount of Ycf3 protein was not affected (Fig. 7). However, at the higher temperature the protein was almost missing in CL3. This result explains the presence of a functional PSI at 18ºC in the mutant and the incomplete assembly of PSI at the restrictive temperature.

In *ycf3*-deficient *C. reinhardtii* the PSI proteins PsaA, PsaD, PsaC and PsaF were undetectable (Boudreau et al., 1997). Surprisingly, the Ycf3 deficient thylakoids still contained detectable PsaE, but this protein must have been associated with non-PSI proteins in the membranes (Boudreau et al., 1997). In contrast, in CL3 mutant, PsaA could be observed while PsaE was highly reduced. A tobacco mutant with a completely deleted *ycf3* gene displayed a light sensitive phenotype, lacked PSI and was photosynthetically inactive (Ruf et al., 1997).

For *C. reinhardtii* it has been demonstrated that the Ycf3 protein is required for PSI assembly, acting as a chaperone that interacts with the PSI subunits PsaA and PsaD (Naver et al., 2001). These authors also generated a temperature sensitive mutant with an altered Ycf3 protein, which did not accumulate PSI when grown above a restrictive temperature. The conditional Ycf3 mutant allowed Naver et al. (2001) to generate intact PSI in *C. reinhardtii* lacking Ycf3, and they could exclude that Ycf3 plays any role in PSI stability. As discussed above, PSI in plants can assemble in the absence of most small subunits, and only PsaC and PsaD are absolutely required for accumulation of PSI complexes. Hence, Ycf3 would likely be involved in integrating one or both of these subunits in the PSI complex, and this agrees with the observed interaction of Ycf3 with PsaA and PsaD (Naver et al., 2001). It should be pointed out that the studies of Naver et al. (2001) did not exclude that Ycf3 may interact with PsaC as well.

**Conclusion**

Despite several studies it is still not clear how the Ycf3 protein assists the assembly of PSI. The tobacco and *C. reinhardtii* mutants lacking Ycf3 do not assemble PSI, but in the present study, some PSI assembly took place, especially of PsaD whereas PsaC assembly appeared to be almost completely inhibited. Given the total absence of PSI in *ycf3* null mutants, the PSI present in the CL3 mutant is due to the presence of Ycf3
protein. Immunoprecipitation has shown that Ycf3 interacts with PsaD, although it could not be excluded that Ycf3 also interacts with other PSI subunits (Naver et al., 2001). The present study indicates that Ycf3 has a specific role in assembly of PsaC with the PSI complex.

**Materials and Methods**

**Plant material and growth conditions**

Seedlings of two-rows spring barley (*Hordeum vulgare* L cytoplasmic line 3 (CL3) (Prina 1996) and MC182 (accession number of the Institute for Genetics, INTA) as wild type (WT) control, were grown at 18°C or 32°C with a 16 h-photoperiod. Two models of growth chambers were used: a Sanyo-type (with fluorescent tubes only) and a Conviron-type chamber (with fluorescent tubes and incandescent lamps) and in both cases the light intensity was 100 μmol photons m⁻² s⁻¹. In one of the experiments, seedlings were cultivated under low light conditions (incandescent light of 10 μmol photons m⁻² s⁻¹). All the experiments were carried out on primary leaves and unless otherwise noted the leaves were from 6 days after germination seedlings grown in the Conviron chamber with a mix of incandescent and fluorescent light at 100 μmol photons m⁻² s⁻¹.

**Pigment analyses**

Pigment analyses were performed as described previously (Lokstein et al., 2002): Leaf tissue was frozen in liquid nitrogen and stored at -80°C until extraction. Pigments were analyzed by HPLC on a Waters chromatography system (600 E gradient module, 717 autosampler, 996 diode array detector) using a Waters Spherisorb ODS2 Analytical Column (5 μm particle size; 4.6 × 250 mm). Gradients consisted of acetonitrile:water:triethylamine (900:100:1, solvent A) and ethyl acetate (solvent B) with 100% A for 16 min, 66.7% A/ 33.3% B for 6 min, 59.7% A/ 40.3% B for 12 min, 33.3% A/ 66.7% B for 0.2 min, 100% B for 2.5 min, and 100% A for 4.3 min. The flow rate was 1 mL min⁻¹. Pigments were identified by their specific retention times and
absorption spectra and quantified by comparison to authentic standards using
photodiode-array detection at 440 nm.

Transmission electron microscopy

Small samples (about 2 mm thick) of first-leaf blades were fixed overnight in 2.5%
glutaraldehyde in 0.1 M phosphate buffer pH 7.2 at 4°C, post-fixed in 1% osmium
tetroxide in water for 2 h at 4°C, dehydrated in a graded ethanol-acetone series and
embedded in Spurr’s resin. Semi-thin sections (1 μm thick) and ultrathin sections for
light and transmission electron microscopy respectively, were obtained with an
ultramicrotome (Reichert-Jung, Vienna, Austria). Semithin sections were stained with
1% Toluidine Blue O (Sigma) and examined in an Axioskop 2 Zeiss photomicroscope.
Ultrathin sections were mounted on grids, stained with lead citrate followed by uranyl
acetate, and examined in a Zeiss EM109T transmission electron microscope.

Fluorescence analyses

In vivo Chl fluorescence was measured using a pulse amplitude modulation (PAM)
fluorometer (FMS 2, Hansatech) with attached leaves as previously described (Lokstein
et al., 2002). Fluorescence parameters were calculated as follows: 

\[ \frac{F_{\text{m}}}{F_{\text{m}} - F_{\text{0}}} = \frac{F_{\text{m}} - F_{\text{0}}}{F_{\text{m}}} \]

is the maximum photochemical efficiency of PSII, in the dark-adapted state. Nonphotochemical quenching was quantified as 

\[ \text{NPQ} = \left( \frac{F_{\text{m}}}{F_{\text{m}}'} \right) - 1 \]

Photochemical quenching was quantified as 

\[ \text{qP} = \left( \frac{F_{\text{m}} - F_{\text{m}}'}{F_{\text{m}}'} \right) \]

Photon flux densities (PFDs) were measured and adjusted using a quantum sensor (Li-Cor, LI-189A; Lincoln, NE, USA).

77 K fluorescence emission spectra were recorded using a Fluorolog FL-112
spectrofluorometer with a 1680 emission double monochromator (Jobin-Yvon,
Longjumeau, France). Thylakoid membranes were suspended in 66% (v/v) glycerol/20
mM Tricine, pH 7.8 to a final Chl concentration of 2.5 µg mL\(^{-1}\) and immediately
dropped into liquid nitrogen. Frozen pearls were transferred to a Dewar vessel mounted
in the fluorometer. Excitation was at 440 nm.

DNA isolation, PCR and sequencing
Total DNA was extracted from leaves following a standard protocol (Fang et al., 1992). PCRs for sequencing ycf3 gene were performed using 20 ng of DNA, 2X Taq buffer, 1 mM MgSO4, 0.32 µM of each primer, 0.32 mM dNTP mix, and 2.5 U Platinum Taq Pfx (Invitrogen). Following denaturation at 94°C for 5 min, the reaction mixtures were heated to 94°C for 15 s, 55°C for 30 s and 68°C for 1 min, in 30 cycles. PCR fragments were cloned into pCR4 Blunt-TOPO (Zero Blunt TOPO PCR Cloning Kit for sequencing, Invitrogen). Three independent colonies of each genotype were sequenced. Sequence data alignments (Chenna et al., 2003) were performed using the ClustalW WWW Service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). PCRs for amplifying the cDNA, were performed using 20 ng of DNA, 1X Taq buffer, 3mM MgCl2, 0.25 µM of each primer, 0.25 mM of deoxynucleotide triphosphate mix, and 1 U Taq DNA polymerase (Invitrogen). The sequence of ycf3 gene was amplified by PCR with primers designed on the basis of published information of the chloroplast DNA sequence of wheat (Triticum aestivum) (GenBank NC_002762). A fragment of 2 kb was amplified using primer P1, 5’-TTATTCAAATTCAAGCGCTTCGTA-3’ and primer P6, 5’-ATGCCTAGATCCCGTGTAAATGGAA-3’. Four internal primers were designed for sequencing, P0, 5’-CTAAGTTTCAAACCCTAATTTTTAT-3’; P2, 5’-TTTTTTTAGTTGTATCGACCCAGTCGC-3’; P3, 5’-GCCCGGAAATAATATTCCAAAGCCT-3’; P4, 5’-CACCAATGAATTCTATTAATGCTA-3’.

RNA isolation and analysis

Total RNA was isolated from leaves with Trizol (Invitrogen). RNA for reverse transcription was digested with DNase RQ1 (Promega). Complementary DNA (cDNA) was synthesized with SuperScript III reverse transcriptase using the antisense primer P1, according to the manufacturer’s instructions (Invitrogen). Products of reverse transcription were amplified by PCR as described above and cloned into pCR2.1-TOPO Vector (TOPO TA Cloning kit, Invitrogen). Three independent colonies of each genotype were sequenced.

Thylakoid membrane isolation and immunoblotting
Thylakoid membranes were isolated according to Guiamét et al., 2002. Thylakoid proteins were separated by SDS-PAGE and immunoblotted with several antibodies to determine the presence or absence of different polypeptides. Solubilized thylakoids of each sample corresponding to the same amount of Chl were electrophoresed in 13% (w/v) SDS-polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes and probed with rabbit antibodies raised against PSI core complex (recognizing PsaA/B, C, D, E, L), PsaF, Lhca1, Lhca3, Lhca4, Lhcb1 and Lhcb2, LHCCI (kindly provided by J.J. Guiamét, INFIVE, Universidad de La Plata, Argentina) and D1 (kindly provided by Prof. A. Barkan, University of Oregon, USA). The immunodetection procedures were performed with the ECL Western Blotting Analysis System (Amersham) according to the manufacturers’ description and using horseradish peroxidase-conjugated donkey-anti-rabbit secondary antibody. The polyclonal antibody against barley Ycf3 was produced in rabbits immunized with a synthetic peptide with the sequence EAMRLEIDPYDRSYC coupled to keyhole limpet hemocyanin (GenScript Corp., Piscataway, NJ). Immunodetection of Ycf3 was accomplished with the Super Signal West Dura Extended Duration Substrate (Pierce) according to the manufacturers’ description and using horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody.

**Supplemental Material**

Supplemental Figure S1. Immunoblot analysis of thylakoid protein extracts from WT and CL3 seedlings with antisera raised against PSI. Equal amounts of chlorophyll (1 μg) were loaded per lane. Thylakoid homogenates were extracted from seedlings of 3, 6 and 9 days after germination grown at 18°C or at 32°C under a mixture of fluorescent and incandescent light (100 μmol photons m⁻² s⁻¹). FNR: ferredoxin:NADP+ reductase.

Supplemental Figure S2. Immunoblot analysis of dilutions of thylakoid protein extracts from WT and CL3 seedlings with antisera raised against PSI. Lanes 1 and 2 were loaded with 1 μg chlorophyll of WT and CL3 thylakoid proteins, and lanes 3 to 8 were loaded with dilutions of the WT thylakoid extracts as indicated. Thylakoids were extracted from 6 days after germination seedlings grown at 18°C under a mixture of fluorescent and incandescent light (100 μmol photons m⁻² s⁻¹).
Supplemental Figure S3. Immunoblot analyses of thylakoid protein extracts from WT and CL3 seedlings with antisera raised against the polypeptides indicated on the left side. Equal amounts of chlorophyll (1 μg) were loaded per lane. Thylakoid homogenates were extracted from seedlings 6 days after germination grown at 18ºC or at 32ºC under a mixture of fluorescent and incandescent light (100 μmol photons m⁻² s⁻¹).

Supplemental Figure S4. DNA sequence alignment of ycf3 gene from WT, CL3 and wheat. Exons are shown in yellow, mutations are highlighted in red. △: editing site C→T

Supplemental Figure S5. Sequence alignment of ycf3 gene DNA from WT and CL3 intron 1-containing ycf3 cDNA. Exons 1 and 2 are shown in yellow, mutations are highlighted in red, exon 3 is shown green and the editing site is highlighted in blue.

Supplemental Figure S6. RNA FOLD prediction of ycf3 intron 1: A, WT 18ºC; B, CL3 18ºC; C, WT 32ºC; D, CL3 32ºC. The prediction was carried out by calculating minimum free energy structures (Zuker and Ziegler, 1981) using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi, University of Vienna).

Supplemental Figure S7. SDS-PAGE gel stained with Coomassie Blue corresponding to immunoblot against Ycf3 protein (Fig. 7). Thylakoid protein extracts from WT and CL3 seedlings were loaded on a chlorophyll basis of 5 μg. Thylakoid homogenates were extracted from seedlings 6 days after germination grown at 18ºC or at 32ºC under a mixture of fluorescent and incandescent light (100 μmol photons m⁻² s⁻¹).

Acknowledgements
Drs. A. Barkan and J.J. Guiamét are thanked for providing antibodies against LHCII and D1.
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Table I: Pigment contents and composition in barley 6 days after germination WT and CL3-mutant leaves grown at 100 μmol photons m⁻² s⁻¹ under a mixture of fluorescent and incandescent light at different temperatures. The data are the means of at least 15 measurements (±SD).

<table>
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<th></th>
<th>Chl a+b (µg/cm²)</th>
<th>VAZ (µg/cm²)</th>
<th>Z+0.5A/NEOX (µg/cm²)</th>
<th>Neoxanthin (µg/cm²)</th>
<th>Lutein (µg/cm²)</th>
<th>B-carotene (µg/cm²)</th>
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<tbody>
<tr>
<td>WT 18°C</td>
<td>50.0 ± 7.6</td>
<td>1.5 ± 0.2</td>
<td>0.03 ± 0.01</td>
<td>1.4 ± 0.2</td>
<td>3.6 ± 0.6</td>
<td>2.3 ± 0.4</td>
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<tr>
<td>CL3 18°C</td>
<td>42.8 ± 4.7</td>
<td>2.7 ± 0.4</td>
<td>0.08 ± 0.01</td>
<td>1.4 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>WT 32°C</td>
<td>35.6 ± 4.2</td>
<td>1.4 ± 0.2</td>
<td>0.04 ± 0.01</td>
<td>1.0 ± 0.2</td>
<td>3.2 ± 0.3</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>CL3 32°C</td>
<td>10.3 ± 2.2</td>
<td>1.7 ± 0.2</td>
<td>0.38 ± 0.07</td>
<td>0.3 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>0.3 ± 0.1</td>
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**Figure legends**

Figure 1. WT and CL3 mutant barley seedlings 6 days after germination at different light quality (100 μmol photons m⁻² s⁻¹) and temperature environments. A, 18°C under a mixture of fluorescent and incandescent light. B, 32°C under a mixture of fluorescent and incandescent light. C, 18°C under fluorescent light only. D, 32°C under fluorescent light only.

Figure 2. A and B: Chloroplast ultrastructure of 6 days after germination CL3 seedlings grown at 100 μmol photons m⁻² s⁻¹ fluorescent light 32°C. C and D: Chloroplasts of CL3 seedlings grown at 100 μmol photons m⁻² s⁻¹ fluorescent light 18°C. E and F: Chloroplast ultrastructure of 6 days after germination CL3 seedlings grown at 10 μmol photons m⁻² s⁻¹ incandescent light at 18°C. G and H: Chloroplasts of WT seedlings grown at 10 μmol photons m⁻² s⁻¹ incandescent light at 18°C.

e: stroma; g: grana; pc: cell wall; * and arrows indicate swollen thylakoids; m: electrodense material; pg: plastoglobuli. Scales: A, B, C and G, 0.5 μm; D, F and H, 0.2 μm; E 0.8 μm

Figure 3. 77 K fluorescence spectra obtained with thylakoids from 6 days after germination seedlings grown at 100 μmol photons m⁻² s⁻¹ at 18°C and 32°C under a mixture of fluorescent and incandescent light. The spectra were normalized at 685 nm.

Figure 4. A, Photochemical quenching and B, non photochemical quenching of 6 days after germination seedlings grown at 100 μmol photons m⁻² s⁻¹ at 18°C and 32°C under a mixture of fluorescent and incandescent light.

Figure 5. Immunoblot analyses of thylakoid protein extracts from WT and CL3 seedlings with antisera raised against D1 and LHCII. Equal amounts of chlorophyll (1 μg) were loaded per lane. Thylakoid homogenates were extracted from seedlings of 3, 6 and 9 days after germination grown at 18°C or at 32°C under two different light environments at 100 μmol photons m⁻² s⁻¹

Figure 6. Analysis of ycf3 transcripts by RT-PCR. RNA was isolated from 6 days after germination seedlings grown at 100 μmol photons m⁻² s⁻¹ under a mixture of fluorescent and incandescent light. The size of the fragments in bps is indicated in lane 1. WT
grown at 18°C, lane 2; WT grown at 32°C, lane 3; CL3 grown at 18°C, lane 4 and CL3 grown at 32°C, lane 5.

Figure 7. Immunoblot analysis of the amount of Ycf3 protein in thylakoid protein extracts. Thylakoid homogenates were extracted from seedlings 6 days after germination grown at 18°C or at 32°C under a mixture of fluorescent and incandescent light (100 μmol photons m\(^{-2}\) s\(^{-1}\)). Equal amounts of chlorophyll (5 μg) were loaded per lane. A corresponding Coomassie stained gel is shown in Supplemental Fig. S7.
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