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Targeted inactivation of the smallest plastid genome-encoded open reading frame reveals a novel and essential subunit of the cytochrome b_6f complex

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The smallest conserved open reading frame in the plastid genome, *ycf6*, potentially specifies a hydrophobic polypeptide of only 29 amino acids. In order to determine the function of this reading frame we have constructed a knockout allele for *ycf6*. This allele was introduced into the tobacco plastid genome by chloroplast transformation to replace the wild-type *ycf6* allele. Homoplasmic *Δycf6* plants display a photosynthetically incompetent phenotype. Whereas the two photosystems are intact and physiologically active, we found that the electron transfer from photosystem II to photosystem I is interrupted in *Δycf6* plants. Molecular analyses revealed that this block is caused by the complete absence of the cytochrome b_6f complex, the redox-coupling complex that interconnects the two photosystems. Analysis of purified cytochrome b_6f complex by mass spectroscopy revealed the presence of a protein that has exactly the molecular mass calculated for the Ycf6 protein. This suggests that Ycf6 is a genuine subunit of the cytochrome b_6f complex, which plays a crucial role in complex assembly and/or stability. We therefore propose to rename the *ycf6* reading frame *petN*.

Keywords: chloroplast/cytochrome b_6f/photosynthesis/plastid transformation/*ycf6*

Introduction

Within the last decade, our knowledge concerning virtually all aspects of modern biology has benefitted immensely from the power of rapidly progressing genome projects. Owing to their relatively small size, chloroplast DNAs were among the first targets of genome projects. Homology with known eubacterial genes allowed the assignment of tentative functions to most of the sequenced plastid genome-encoded reading frames. In the following years, the functions of only relatively few of the remaining open reading frames could be elucidated, illustrating a problem encountered nowadays by practically all genome projects: the difficult transition from structural to functional genomics.

The successful development of transformation technologies for chloroplasts (Boynton *et al*., 1988; Svab *et al*., 1990) has provided the basis for addressing functional aspects of plastid reading frames by reverse genetics. Null alleles for plastid genome-encoded open reading frames are constructed and then inserted into the plastid genome by chloroplast transformation to replace the endogenous intact allele. Homoplasmic transplastomic plants will entirely lack the wild-type allele and, thus, will reveal the phenotype of plants deficient for the respective gene product. In most instances, the mutant phenotype is associated with pigment deficiency or lowered photosynthetic performance (for a review, see e.g. Rochaix, 1997). Such a phenotype is, however, not necessarily indicative of a direct role of the inactivated gene in photosynthesis. Knocked-out genetic system genes involved in the expression of plastid-encoded photosynthesis genes may result in very similar phenotypes (Allison *et al*., 1996). Therefore, thorough physiological, biochemical and molecular biological analyses are required to elucidate the molecular basis for the mutant phenotype and to assign an exactly defined function to a plastid-encoded reading frame.

Currently, two model plants are nearly exclusively used in chloroplast transformation experiments: the unicellular green alga *Chlamydomonas reinhardtii* (Boynton *et al*., 1988) and the higher plant tobacco, *Nicotiana tabacum* (Svab *et al*., 1990; Svab and Maliga, 1993). Recently, both systems have also been employed for reverse genetics purposes to elucidate the functions of open reading frames encoded in the plastid genome (e.g. Monod *et al*., 1994; Takahashi *et al*., 1996; Ruf *et al*., 1997; Burrows *et al*., 1998).

Using a reverse genetics approach, we have attempted to identify the function of the smallest plastid genome-encoded conserved open reading frame, *ycf6*. We report here that tobacco plants deficient for *ycf6* are blocked in their photosynthetic electron transport chain and specifically lack the cytochrome b_6f complex. Biochemical, physiological and biophysical data indicate that the *ycf6* gene product is a novel subunit of the cytochrome b_6f complex, which is essential for the assembly and/or stability of the complex in the thylakoid membrane.

Results

Targeted deletion of the tobacco chloroplast ycf6 reading frame

Higher plant chloroplast genomes harbor in their large single-copy region a small open reading frame of unknown function, initially designated ORF29 and later renamed *ycf6* (hypothetical chloroplast reading frame No. 6). It potentially specifies a hydrophobic protein of only 29 amino acids and is found highly conserved among all photosynthetically active organisms (Figure 1A). Strongly homologous reading frames are encoded in cyanobacteria (Kaneko *et al*., 1996) and all chloroplast genomes analyzed to date, except that of the non-photosynthetic holoparasitic

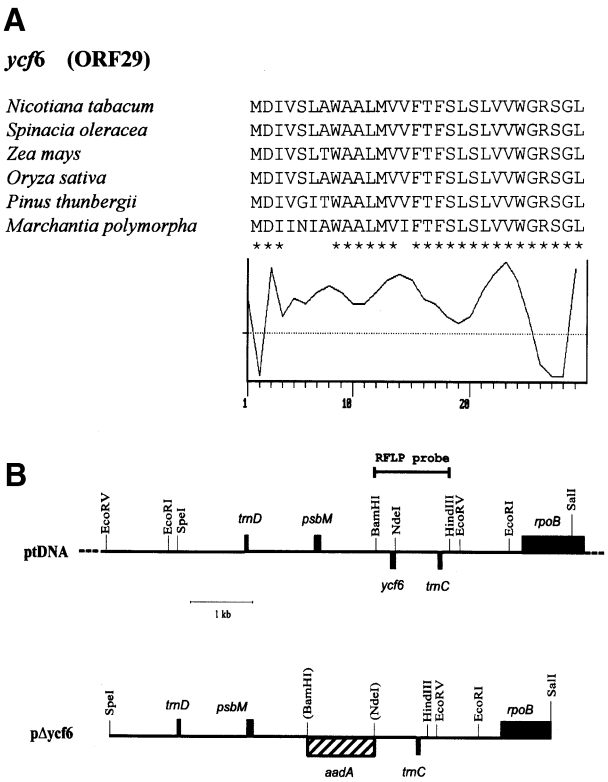

Fig. 1. Knockout of the plastid *ycf6* reading frame. (**A**) Properties of the putative Ycf6 protein. Alignment of the *ycf6*-derived amino acid sequences from higher plant species and the liverwort *Marchantia polymorpha*. Amino acid positions identical in all species listed here are denoted by asterisks. The hydropathy plot of the tobacco Ycf6 protein shows that the protein is highly hydrophobic and appears to consist of a single transmembrane domain (predicted to be largely of α-helical conformation). DDBJ/EMBL/GenBank accession Nos are: Z00044 (*N.t.*), M55297 (*S.o.*), X86563 (*Z.m.*), X15901 (*O.s.*), D17510 (*P.t.*) and X04465 (*M.p.*). (**B**) Construction of a plastid transformation vector for targeted inactivation of *ycf6*. A map of the region of the tobacco plastid genome containing *ycf6* and the map of the ptDNA targeting fragment in transformation vector pΔycf6 are shown. Genes above the line are transcribed from left to right, genes below the line are transcribed in the opposite direction. Relevant restriction sites are shown.

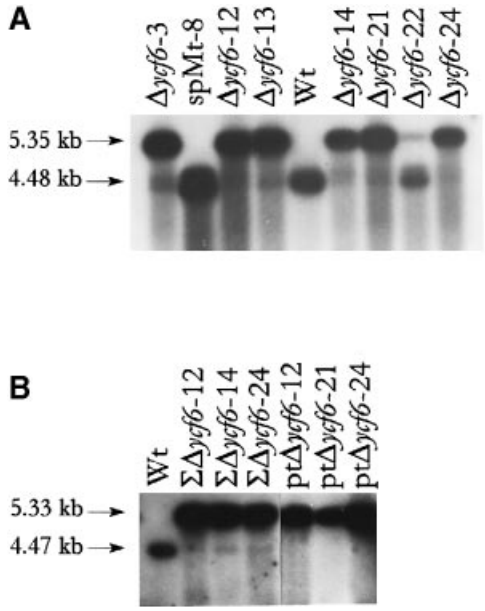

Fig. 2. RFLP analyses to test for homoplasmy of Δ*ycf6* chloroplast transformants. (**A**) Restriction analysis of total cellular DNA with *Eco*RI. Seven independently generated transplastomic lines, a wild-type control and a spontaneous spectinomycin-resistant mutant (spMt) are shown. Digestion with *Eco*RI and hybridization to a radiolabeled *Bam*HI–*Hin*dIII restriction fragment (Figure 1B) yields a 4.48 kb fragment for the wild-type genome and a 5.35 kb fragment for the transplastome. Note the presence of a weak double band of approximately wild-type size in all transplastomic lines in addition to the 5.35 kb band characteristic of the transformed plastid genome. Line Δ*ycf6-22* is still heteroplasmic, whereas all other transplastomic lines are likely to be homoplasmic. See the text for details. (**B**) Restriction analysis of total cellular DNA and purified chloroplast DNA with *Eco*RV. Digestion with *Eco*RV and hybridization to the same probe as in (A) yields a 4.47 kb fragment for the wild-type plastid genome and a 5.33 kb fragment for the transplastome. Note the appearance of a weak band of wild-type size in the three total DNA samples that is absent from the purified chloroplast DNA samples, indicating that the lines are homoplasmic and the hybridizing wild-type-like fragment is promiscuous DNA of extraplastidic origin.

plant *Epifagus virginiana* (Wolfe *et al*., 1992). The striking interspecific conservation of the reading frame in length, as well as in nucleotide and derived amino acid sequence, strongly suggests that it encodes a functionally important protein product and hence is a genuine gene. We therefore set out to determine the possible function of *ycf6* by employing a reverse genetics approach.

The position of the *ycf6* open reading frame in relation to adjacent genes in the tobacco plastid genome is depicted in Figure 1B. We constructed a null allele of *ycf6* by deleting the entire reading frame from a cloned fragment of the tobacco plastid genome (Figure 1B). A chimeric *aadA* gene conferring spectinomycin resistance was inserted to replace *ycf6* in the cloned plastid DNA (ptDNA) fragment yielding chloroplast transformation vector pΔycf6 (Figure 1B).

Plasmid pΔycf6 was introduced into chloroplasts using the biolistic protocol (Svab *et al*., 1990; Svab and Maliga, 1993). Successful chloroplast transformation results in targeted replacement of *ycf6* by the selectable marker gene *aadA* through two homologous recombination events in the flanking regions. Primary chloroplast transformants were selected on spectinomycin-containing plant regeneration medium. Out of 30 bombarded sterile tobacco leaves, we selected eight chloroplast transformants that contained the chimeric *aadA* gene in their plastid genome as shown by PCR analyses and/or restriction fragment length polymorphism (RFLP) analysis (Figure 2).

Presence of promiscuous ycf6 copies and homoplasmy of Δycf6 plastid transformants

According to our experience, subjecting primary transformants to two to three additional rounds of regeneration on selective medium is usually sufficient to rid the plants of all wild-type ptDNA copies and obtain homoplasmic lines. When we performed RFLP analyses after the third round of regeneration to test for homoplasmy, we were therefore surprised to see in all transplastomic lines, in addition to a strong band for the transplastome, a faint signal corresponding in size to the wild-type restriction fragment (Figure 2A). The presence of a hybridization signal for the wild-type ptDNA can be explained by three possible scenarios: (i) the Δ*ycf6* lines are not homoplasmic because *ycf6* is an essential gene and can not be eliminated completely; (ii) the lines are not yet homoplasmic because

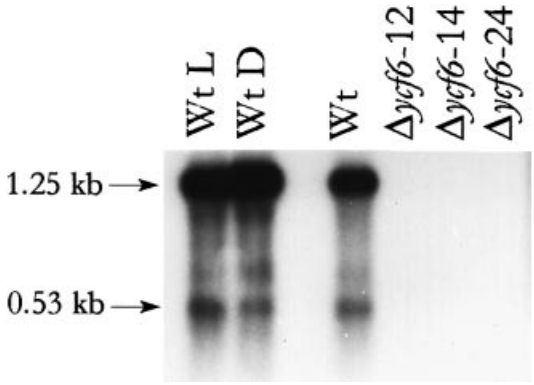

Fig. 3. Analysis of *ycf6* transcription in wild-type and Δ*ycf6* plants. Equal amounts of total cellular RNA samples were electrophoresed, blotted and hybridized to the *Bam*HI–*Nde*I restriction fragment (Figure 1B) containing the *ycf6* reading frame. Two prominent *ycf6*-containing transcripts accumulate in the light as well as in the dark. The 0.53 kb mRNA species is likely to be mature monocistronic transcript, whereas the 1.25 kb mRNA may be a precursor co-transcript with the upstream *trnC* gene. Note that the sequence recognized by the probe is present only in the wild-type plastid genome but not in the transplastomic genome (Figure 1B). The absence of any hybridization signal from the transplastomic lines provides additional proof for their homoplasmic state.

three rounds of regeneration were not sufficient for the cells to lose all wild-type genome copies; or (iii) the lines are homoplasmic and the wild-type-like hybridization signal is caused by promiscuous ptDNA present in one of the other two genomes of the plant cell. It has long been known that, in evolution, large fragments of the chloroplast DNA integrated in the mitochondrial and nuclear genomes (Stern and Lonsdale, 1982; Ayliffe and Timmis, 1992; Ayliffe *et al*., 1998). For example, the plastid *rbcL* gene is present in >30 copies in the tobacco nuclear genome (Ayliffe and Timmis, 1992). We suspected the presence of such promiscuous DNA to be the cause of the wild-type-like hybridization signals observed in our RFLPs. First indications that this could indeed be the case were that (i) the small amount of wild-type-like fragments was identical in all transplastomic lines, and (ii) upon close inspection of the wild-type-like hybridization signal, it appears not to be a single band (as one would expect for residual wild-type copies) but a double band (Figure 2A). The latter observation could suggest that at least two promiscuous copies of the *ycf6* region are present in the cell, which have diverged in evolution with respect to one of the two *Eco*RI sites. Interestingly, when we repeated the RFLP with a different restriction enzyme (*Eco*RV; Figure 2B) one of the two bands disappeared, suggesting that, at least in one promiscuous copy, the two *Eco*RV sites are also conserved. In order to provide an ultimate proof for the wild-type-like signals being derived from promiscuous DNA, we performed RFLPs with purified chloroplast DNA. This analysis revealed a single hybridizing band for the transplastome and no evidence for any residual wild-type copies (Figure 2B), strongly suggesting (i) that our Δ*ycf6* lines are homoplasmic and (ii) that promiscuous copies of the *ycf6* region are likely to be present in tobacco cells. Homoplasmy of the Δ*ycf6* plants was ultimately confirmed by our subsequent transcript analysis (Figure 3) as well as by the somatic stability of the mutant phenotype (see below).

Transcription of ycf6 in wild-type and Δycf6 plants

Having obtained *ycf6* knockout plants, we were interested in analyzing *ycf6* mRNA accumulation in wild-type and transplastomic plants. We therefore performed Northern blots using the *ycf6* coding region as a probe. As the entire *ycf6* coding region was deleted in our knockout construct, this probe should detect no mRNAs in Δ*ycf6* plants. As expected, no hybridization signals could be detected in any of the Δ*ycf6* lines (Figure 3). In contrast, the *ycf6*-specific probe detects multiple mRNA species in the wild type. The most abundant species is an ~1.25 kb transcript. In addition, there is a prominent species of ~0.53 kb size. Both transcript species are present at comparable levels in light-grown as well as in dark-adapted plants (Figure 3), suggesting that there is little (if any) light-driven transcriptional regulation of *ycf6* expression. Although we have not mapped the termini of the two transcript species, a reasonable hypothesis is that the 0.53 kb transcript represents monocistronic *ycf6* mRNA, whereas the 1.25 kb transcript is generated by co-transcription with the upstream *trnC* gene (Figure 1B).

The absence of *ycf6* transcripts from our Δ*ycf6* plants in Northern blot analysis clearly demonstrates (i) that our Δ*ycf6* lines are indeed homoplasmic and (ii) that the extraplastidic promiscuous copies of the *ycf6* region are silent and do not give rise to functional Ycf6 gene products.

Homoplasmic Δycf6 plants display a photosynthetically incompetent phenotype

Complete elimination of the *ycf6* reading frame from the chloroplast genome resulted in tobacco plants viable on sucrose-containing tissue culture medium. This observation suggests that *ycf6* is not essential for cell survival, plastid maintenance and plant development.

Shoots regenerated from homoplasmic Δ*ycf6* plants displayed a pale-green phenotype upon regeneration on spectinomycin-containing medium under standard light conditions (2000–3000 lux). When shoots were transferred to boxes and rooted on antibiotic-free medium, plants bleached out completely within a few days (Figure 4A). The pigment-deficient phenotype was much less severe under extreme low-light conditions (12–25 lux). The plants were now light-green (Figure 4B) and practically indistinguishable from wild-type plants kept under identical conditions (Figure 4C). The mutant light-sensitive phenotype turned out to be stable under non-selective conditions and the lack of somatic segregation over a period of >1 year provided additional proof for the complete absence of wild-type ptDNA copies from our Δ*ycf6* plants.

As Δ*ycf6* plants grown in low light and on sucrose were green and indistinguishable from the wild type, plants maintained under these conditions provided suitable material for physiological measurements to analyze comparatively the photosynthetic performance of the wild type and the mutant.

Δycf6 plants possess functional photosystems I and II

We first set out to test whether or not the mutant phenotype of Δ*ycf6* plants was due to a defect in photosynthetic electron transport. Photosystem II (PSII) function was

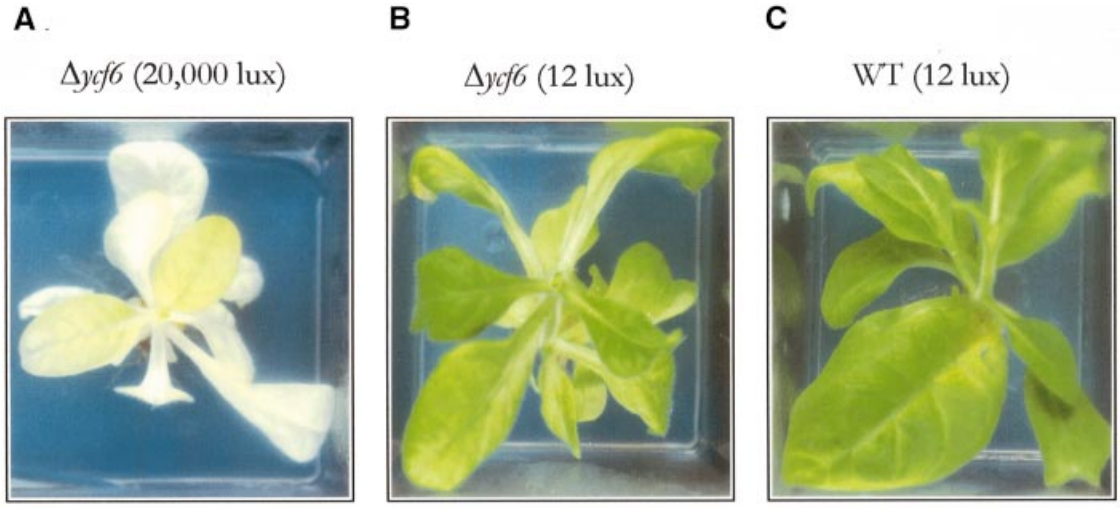

Fig. 4. Phenotype of Δ*ycf6* plants. A Δ*ycf6* plant is shown under standard light conditions (**A**) and under extreme low-light conditions (**B**). For comparison, a wild-type plant kept under identical low-light conditions is also shown (**C**).

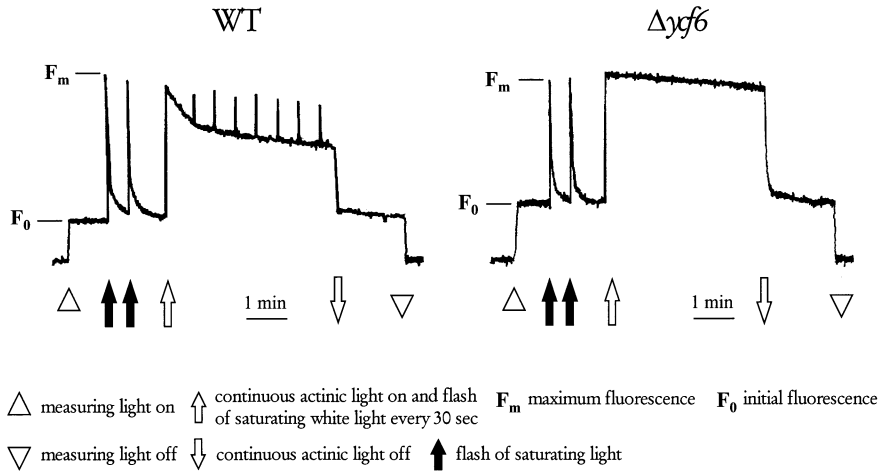

Fig. 5. Fluorescence induction as a test for PSII activity. Leaf samples from dark-adapted wild-type and Δ*ycf6* plants grown under low-light conditions were taken to measure PSII-dependent fluorescence. PSII activity is clearly present in Δ*ycf6* plants as shown by the detection of strong variable fluorescence ($F_{var} = F_m - F_0$). However, Δ*ycf6* plants show practically no photochemical quenching suggesting that, while PSII is functional, electrons accumulate in the Q_A pool and are not transferred to downstream components of the electron transport chain.

analyzed by fluorescence induction of intact leaves at room temperature. First, the minimum fluorescence F_0 was determined by exposure of dark-adapted leaves to measuring light of low intensity (Figure 5). Subsequently, maximum fluorescence F_m was obtained by illumination with a saturating light pulse that completely reduced the primary quinone-type PSII acceptor, Q_A. The ratio $(F_m - F_0)/F_m$ serves as a measure of the maximum quantum yield of PSII photochemistry. Although this ratio, and hence the maximum photochemical capacity of PSII, is slightly reduced in the mutant compared with the wild type (Figure 5), the Δ*ycf6* plants clearly possess functional PSII units capable of reducing the primary PSII acceptor Q_A.

When leaves were exposed to low-intensity actinic light, variable fluorescence ($F_{var} = F_m - F_0$) was detected for both wild-type and Δ*ycf6* plants (Figure 5). However, whereas in wild-type leaves, flashes of saturating white light superimposed onto the continuous actinic light resulted in a fluorescence rise (which reached approximately the initial value of F_m) and strong photochemical fluorescence quenching was observed, almost no photochemical quenching occurred in leaves of the Δ*ycf6* mutant (Figure 5). As photochemical quenching is dependent on the presence of oxidized PSII acceptor Q_A, these data indicate that the Q_A pool remains nearly completely reduced in the mutant. This may suggest that, in Δ*ycf6* plants, while PSII is functional, electrons accumulate in the Q_A pool and are not efficiently transferred to downstream components of the photosynthetic electron transport chain.

We next wanted to determine whether or not a defect in the other photosystem within the thylakoid membrane, photosystem I (PSI), could be the cause of the mutant phenotype of Δ*ycf6* plants. PSI function in wild-type and mutant plants was deduced from far-red spectroscopy analyses. Absorption changes at 830 nm correlate with the redox state of the reaction center chlorophyll of PSI, termed P700. In the dark, P700 is present in its reduced form (Harbinson and Hedley, 1993). Illumination of dark-adapted leaves with far-red light of 730 nm wavelength selectively excites PSI, thereby converting P700 into its oxidized form (Figure 6). Very similar absorption changes

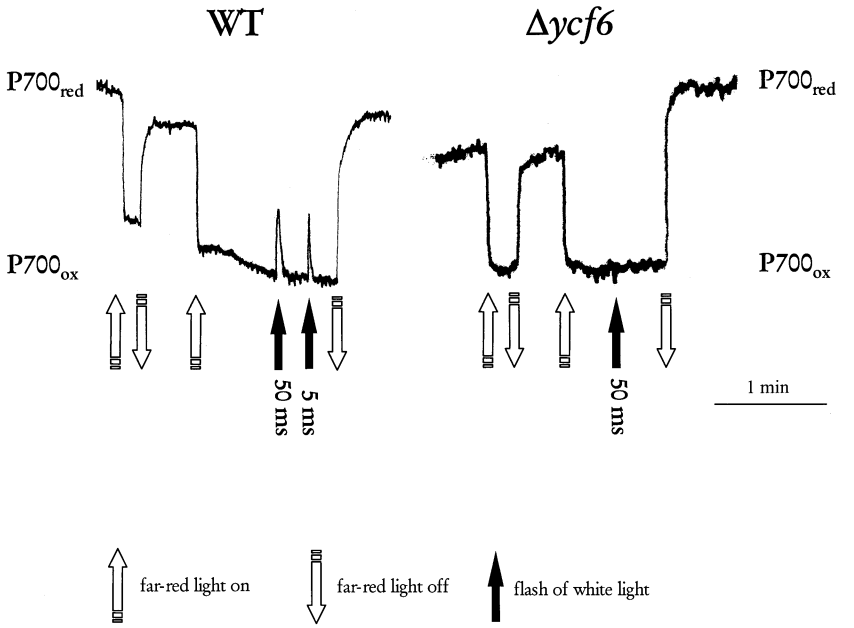

Fig. 6. Far-red spectroscopy analyses as a test for PSI function. Selective excitation of the PSI reaction center chlorophyll P700 results in a transition from the reduced to the oxidized state and hence reveals intact PSI photochemistry in both the wild type and the mutant. However, re-reduction of P700 by white-light pulses and, hence, by electrons released in PSII is only possible in the wild type, indicating that the electron transport from PSII to PSI is blocked in Δ*ycf6* plants.

were observed for wild-type and Δ*ycf6* leaves (Figure 6) demonstrating that Δ*ycf6* plants possess functional PSI.

When short pulses of white light are superimposed onto the continuous far-red light, electrons are released from PSII and transferred to PSI where they lead to a re-reduction of P700, which again can be monitored as an absorption change at 830 nm (Figure 6). Interestingly, this PSII-dependent re-reduction of P700 was observed for wild-type leaves (Figure 6) but was found to be completely absent from mutant leaves. This suggests that, while Δ*ycf6* mutants are likely to possess functional photosystems, the electron transfer from PSII to PSI is specifically blocked in the absence of *ycf6*.

Δycf6 plants specifically lack the cytochrome b_6f complex

The interrupted electron transfer from PSII to PSI in Δ*ycf6* plants can be explained by any of the following three possibilities: (i) a defect at the acceptor side of PSII; (ii) a defect at the donor side of PSI; or (iii) a defect in the cytochrome b_6f complex, a multi-protein complex of the thylakoid membrane interconnecting the two photosystems. In order to confirm the presence of intact photosystems I and II and to test the possibilities of a defective acceptor side of PSII or donor side of PSI, we performed a series of immunoblot analyses. For this purpose, we chose antibodies against protein subunits that are diagnostic for the respective complex (in that lack of the subunit is known to result in loss of the complex), as well as antibodies against proteins that are critically involved in transferring electrons from PSII to PSI (i.e. subunits located at the acceptor side of PSII and the donor side of PSI). Western blot analyses with antibodies against the D1 and D2 proteins (encoded by the plastid *psbA* and *psbD* genes, respectively) revealed that PSII accumulates in Δ*ycf6* plants at comparable levels to the wild type

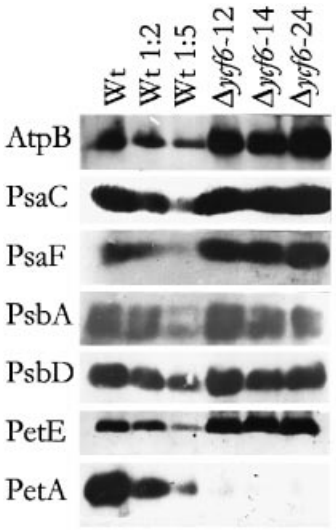

Fig. 7. Western blot analyses of thylakoid proteins from three independent transplastomic Δ*ycf6* lines and a dilution series of the wild type to test for the presence of key components of the protein complexes in the thylakoid membrane. Immunoblot analyses with antibodies against AtpB (CF1 β subunit), PsaC, the plastocyanin-docking protein PsaF and the D1 and D2 proteins (PsbA and PsbD) confirm the presence of wild-type levels of chloroplast ATP synthase, PSI and PSII in Δ*ycf6* plants. Also, antibodies against the soluble electron carrier plastocyanin (PetE) detect similar levels of plastocyanin in extracted lumenal proteins from the wild type and the mutant. In contrast, immunoblots with anti-cytochrome *f* (PetA) antibodies revealed virtually a complete absence of cytochrome *f* protein from all Δ*ycf6* lines, suggesting that the mutants lack functional cytochrome b_6f complex.

(Figure 7). Moreover, the presence of wild-type levels of the Q_A-binding protein D1 may suggest that the acceptor side of PSII is not defective in the mutant. Similarly, Western blot analyses with antibodies against the PsaC

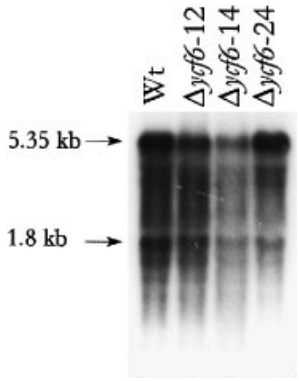

Fig. 8. RNA gel blot to detect *petA* transcripts. A *petA*-specific probe was hybridized to total cellular RNA from the wild type and three independent Δ*ycf6* lines. The mRNA accumulation pattern for *petA* has been shown earlier to be highly complex (Willey and Gray, 1990). Two major transcript species can be detected: a 1.8 kb mRNA reflecting monocistronic *petA* message and a 5.35 kb mRNA representing a polycistronic precursor transcript. Transcript pattern and accumulation are virtually identical in wild-type and Δ*ycf6* plants, suggesting that the absence of cytochrome *f* from the mutant is not due to the lack of *petA* transcription or mRNA stability.

protein and the plastocyanin-docking protein PsaF indicated wild-type levels of PSI complexes as well as an intact donor side of PSI in thylakoid membranes of the Δ*ycf6* mutant (Figure 7).

Immunoblot analysis with lumenal proteins demonstrated that plastocyanin, the soluble electron carrier transferring electrons from the cytochrome b_6f complex to the primary PSI acceptor P700, is also present at wild-type levels in Δ*ycf6* plants (Figure 7). However, when Western blots of thylakoid proteins were probed with anti-cytochrome *f* antibodies, strong accumulation of cytochrome *f* was detected in the wild type, whereas no signal was obtained for any of the homoplasmic Δ*ycf6* lines (Figure 7). Northern blot analyses showed that *petA* transcripts accumulate to wild-type levels in Δ*ycf6* plants, excluding the possibility that the absence of cytochrome *f* protein is caused by the lack of *petA* transcription or mRNA accumulation (Figure 8).

Cytochrome *f* is a key component of the cytochrome b_6f complex and is known to be essential for both electron transfer and complex assembly (Kuras and Wollman, 1994; Choquet *et al*., 1998). When cytochrome *f* is not synthesized or not targeted to the thylakoid membrane, all other subunits of the complex are highly unstable and rapidly degraded (Kuras and Wollman, 1994; Kuras *et al*., 1995). Therefore, although we could not test for the presence of other subunits of the cytochrome b_6f complex in Δ*ycf6* plants, it appears highly unlikely that any of the other known subunits of the complex accumulate in the absence of the cytochrome *f* subunit.

Western blots with antibodies against the CF1 β subunit, a key component of the chloroplast ATP synthase, also indicated that the ATP synthase is most probably intact in Δ*ycf6* plants (Figure 7). Altogether, these immunobiochemical data confirm the results of the physiological measurements and support the conclusion that the mutant phenotype of our Δ*ycf6* plants is due to the absence of electron transfer from PSII to PSI. Moreover, they strongly suggest that this block in electron transport is caused by the lack of functional cytochrome b_6f complex.

Purified cytochrome b_6f complex contains a subunit of Ycf6 size

Having assigned the *ycf6* gene product a function in the cytochrome b_6f complex of the thylakoid membrane, we were interested in defining more precisely the role of the Ycf6 gene product during the generation or maintenance of a stable cytochrome b_6f complex. The unusually small size and extremely high hydrophobicity of the putative Ycf6 protein (Figure 1A) argue against a regulatory role in the expression of plastid-encoded subunits of the cytochrome b_6f complex, or an involvement in post-translational modification of subunits of the complex. It seemed more conceivable to us that the Ycf6 protein is a structural component, i.e. an additional, previously unknown subunit of the cytochrome b_6f complex. To test this possibility we attempted to detect the Ycf6 protein within purified cytochrome b_6f complexes. Attempts to overexpress the Ycf6 protein either alone, His-tagged or as a GST fusion protein in order to raise Ycf6-specific antibodies have met with limited success (data not shown). This is not surprising, since both overexpression and antibody generation are known to be extremely difficult when the protein of interest is highly hydrophobic. As an alternative, we have employed mass spectroscopy technologies to test purified cytochrome b_6f complex for the presence of the Ycf6 protein. The complex was purified from spinach, a higher plant species particularly suitable for the isolation of highly pure cytochrome b_6f complex (Hurt and Hauska, 1981). The use of spinach rather than tobacco is unproblematic since the *ycf6*-derived amino acid sequences from tobacco and spinach are 100% identical (Figure 1A). SDS–PAGE analysis of the isolated cytochrome b_6f complex from spinach confirmed the purity of the preparation and showed the expected pattern of the seven known subunits of the complex (not shown).

Purification of the cytochrome b_6f complex requires the use of the detergent octyl glucoside, which turned out to yield very high background in electrospray ionization mass spectroscopy (ESI). We therefore used MALDI-TOF (matrix-assisted laser desorption/ionization, time of flight) mass spectroscopy as an alternative technique, which determines molecular masses with high accuracy and that, in addition, proved to be much less sensitive to octyl glucoside. The calculated molecular mass of the putative Ycf6 protein is 3169.83 Da. The MALDI-TOF spectrum was calibrated for the 2–6 kDa range with marker peptides, thereby ensuring a mass accuracy of ~0.1 Da. MALDI-TOF analysis with parameters optimized for the detection of small polypeptides within the purified cytochrome b_6f complex from spinach, also detected, in addition to peaks likely to represent the known small subunits of the complex (PetL, PetM and PetG), a prominent protein of 3169.87 Da. This value is practically identical to the theoretical molecular mass of the Ycf6 gene product (3169.83 Da). In view of the accuracy of the technique and the extremely low theoretical probability that any other protein present in the purified complex has exactly the same molecular mass, these results provide strong evidence for the Ycf6 protein being a genuine subunit of the cytochrome b_6f complex. This subunit appears to be absolutely essential and its absence from our Δ*ycf6* knockout plants may prevent the assembly of the complex or may dramatically decrease its stability.

Discussion

The cytochrome b_6f complex is a redox-coupling complex interconnecting the two photosystems in all photosynthetically active organisms. It transfers electrons from reduced plastoquinone to soluble carriers in the thylakoid lumen: plastocyanin or a *c*-type cytochrome. Whereas photoautotrophic prokaryotes and eukaryotic algae possess both types of acceptors, only the copper-containing plastocyanin is present in vascular plants. The electron transfer from PSII to PSI via the cytochrome b_6f complex is accompanied by proton translocations from the stroma to the thylakoid lumen, and hence contributes to chloroplast ATP synthesis. According to the current view, the cytochrome b_6f complex of eukaryotes consists of seven subunits: cytochrome *f* (encoded by the plastid *petA* gene), cytochrome b_6 (encoded by the plastid *petB* gene), the Rieske iron–sulfur protein (encoded by the nuclear *petC* gene), subunit IV (encoded by the plastid gene *petD*) and three smaller subunits: the PetM, PetG and PetL proteins (for a recent review, see e.g. Wollman *et al.*, 1999).

Our data presented here suggest that the cytochrome b_6f complex contains an additional, previously unknown subunit. This subunit is specified by the *ycf6* reading frame, the smallest conserved chloroplast-encoded reading frame identified to date. We have deleted the *ycf6* reading frame from the tobacco chloroplast genome and obtained mutant plants that entirely lack functional cytochrome b_6f complexes. We believe that we have generated homoplasmic Δ*ycf6* knockout plants even though the initial RFLP analyses with total cellular DNA did not give rise to a single hybridizing fragment for the mutant plastid genomes but also reproducibly showed a wild-type-like hybridization signal (Figure 2A). Nonetheless, in our opinion there is no doubt that the plants are homoplasmic. We base this conclusion on the following lines of evidence: (i) RFLP analysis with purified chloroplast DNA did not show wild-type-like hybridization signals, suggesting that the signals obtained in analyses with total cellular DNA originate from promiscuous plastid sequences residing in the mitochondrial and/or nuclear genomes; (ii) Northern blot analysis employing a *ycf6*-specific probe revealed, even upon strong overexposure, no hybridization signal for the Δ*ycf6* lines; and (iii) we maintained and propagated our Δ*ycf6* plants for over 1 year without seeing any somatic segregation (i.e. random sorting out of wild-type and mutant ptDNA copies) or appearance of dark-green, wild-type-like sectors. Homoplasmy versus heteroplasmy of transplastomic plants that apparently show low amounts of wild-type ptDNA has been discussed controversially in the past (Kofer *et al.*, 1998; Maliga and Nixon, 1998) and, in the absence of conclusive experimental data, this controversy could not be resolved. In this work we provide strong evidence that, at least in the case of the *ycf6* region of the plastid genome, the wild-type-like copies do not reside in the chloroplast but it is most likely that they are of extraplastidic origin. This finding has important implications for the experimental verification of a homoplasmic state and possibly also for the interpretation of the results from previous chloroplast transformation experiments.

The finding that *ycf6 is* a photosynthesis gene is consistent with the absence of the *ycf6* open reading frame from the plastid genome of the non-photosynthetic holoparasitic plant *E.virginiana*, which has lost practically all of its plastid-encoded photosynthesis-related genes during evolution (dePamphilis and Palmer, 1990). Two lines of evidence indicate that the Ycf6 protein is a novel subunit of the cytochrome b_6f complex: (i) deletion of the *ycf6* gene from the chloroplast genome leads to a specific loss of the cytochrome b_6f complex, whereas the two photosystems are present at wild-type levels and are physiologically fully functional (Figures 5–7); and (ii) purified cytochrome b_6f complexes contain a protein corresponding exactly in molecular mass to the predicted molecular weight of the Ycf6 protein. We therefore believe that the Ycf6 protein is a genuine subunit of the cytochrome b_6f complex and propose to rename the *ycf6* reading frame *petN*.

The Ycf6/PetN polypeptide is extremely hydrophobic and only 29 amino acids long (Figure 1A). It is therefore very likely that the protein spans the thylakoid membrane only once. The termini of the polypeptide have slightly hydrophilic properties with the N-terminus carrying a single negatively charged amino acid residue (D2) and the C-terminus containing a positively charged amino acid (R26). Thus, it seems reasonable to predict that a few amino acid residues at each terminus are exposed to the stroma and the thylakoid lumen, respectively. However, this, as well as the orientation of the protein in the thylakoid membrane, remain to be determined experimentally. Interestingly, computer searches revealed a similarity of the hydrophobic core of the Ycf6 protein (residues 5–26) to a transmembrane domain of mitochondrial Cox3 proteins (cytochrome oxidase subunit III; identity, 45%; similarity, 58%), suggesting that both proteins may employ a similar structural motif for their membrane insertion.

The seven previously identified subunits of the cytochrome b_6f complex are believed to occur in 1:1 stoichiometry (Wollman *et al.*, 1999). Our present data allow no conclusion about the stoichiometry at which the Ycf6 protein is present in relation to the other subunits of the complex. A 1:1 stoichiometry may appear likely but requires further experimentation to be confirmed.

Whereas the *ycf6* reading frame is unlinked to other photosynthesis genes in higher plant chloroplast genomes, it is part of an operon in the plastid genomes of the rhodophyte alga *Porphyra purpurea* (Reith and Munholland, 1995) and the cryptophyte alga *Guillardia theta* (Douglas and Penny, 1999). Interestingly, in these algae, *petM* is encoded in the very same operon, immediately downstream of *ycf6*. Co-transcription of the two reading frames indicates their tightly coordinated expression, which is consistent with the function of both gene products in one and the same complex. In contrast, *petM* is a nuclear gene in higher plants indicating that, in the course of evolution, the gene was transferred to the nucleus and novel regulatory mechanisms have replaced the ancestral coordinated expression of the *ycf6* and *petM* genes by co-transcription.

In conclusion, we have identified a novel and essential subunit of the cytochrome b_6f complex. This subunit is a hydrophobic polypeptide of only 29 amino acids. Its knockout leads to a complete loss of functional cytochrome b_6f complex, which, in turn, results in loss of all photosynthetic activity. We therefore conclude that the Ycf6/PetN

protein is a crucial factor for cytochrome b_6f complex assembly and/or stability.

Materials and methods

Plant material and growth conditions

Sterile tobacco plants (*N.tabacum* cv. Petit Havana) were grown on agar-solidified MS medium containing 30 g/l sucrose (Murashige and Skoog, 1962). Homoplasmic transplastomic lines were rooted and propagated on the same medium. For protein isolation and physiological measurements, wild-type and transformed plants were kept under low-light conditions (12–25 lux) to minimize photooxidative damage in the mutant chloroplasts.

Construction of a Δycf6 plastid transformation vector

The region of the tobacco chloroplast genome containing the *ycf6* reading frame was excised from a *Sal*I ptDNA clone as a *Sal*I–*Spe*I fragment corresponding to nucleotide positions 26 715–31 550 (according to Shinozaki *et al*., 1986). The fragment was ligated into a Bluescript KS vector (Stratagene, La Jolla, CA) cut with *Sal*I and *Spe*I, generating plasmid pMH8. The *ycf6* reading frame was subsequently deleted by digestion with *Nde*I and *Bam*HI. *Nde*I cuts immediately upstream of the start codon, the *Bam*HI site is located 200 nucleotides downstream of the termination codon within the 3′ untranslated region of *ycf6*. After a fill-in reaction of the recessed ends using the Klenow fragment of DNA polymerase I, a chimeric *aadA* gene conferring resistance to aminoglycoside antibiotics (Svab and Maliga, 1993) was inserted to replace *ycf6* and facilitate selection of chloroplast transformants. A plasmid clone carrying the *aadA* gene in the same orientation as *ycf6* previously, yielded the final transformation vector pΔycf6 (Figure 1B).

Plastid transformation and selection of homoplasmic transformed tobacco lines

Young leaves from sterile tobacco plants were bombarded with plasmid pΔycf6-coated 1.1 μm tungsten particles using a biolistic gun (PDS1000He; Bio-Rad, Hercules, CA). Primary spectinomycin-resistant lines were selected on RMOP regeneration medium containing 500 mg/l spectinomycin (Svab *et al*., 1990; Svab and Maliga, 1993). Plastid transformants were identified by PCR amplification according to standard protocols using the primer pair P10 (5′-AACCTCCTATAGACTAGGC-3′; complementary to the *psbA* 3′ untranslated region of the chimeric *aadA* gene) and P11 (5′-AGCGAAATGTAGTGCTTACG-3′; derived from the 3′ portion of the *aadA* coding region). Seven independent transplastomic lines were subjected to three additional rounds of regeneration on RMOP–spectinomycin to obtain homoplasmic tissue.

Isolation of nucleic acids and hybridization procedures

Total plant DNA was isolated by a rapid miniprep procedure (Doyle and Doyle, 1990). Isolation of chloroplast DNA was performed according to Triboush *et al*. (1998). Total cellular RNA was extracted using the TRIzol reagent (Gibco-BRL). Restriction enzyme-digested DNA samples were separated on 1% agarose gels and blotted onto Hybond N nylon membranes (Amersham). Total cellular RNA was electrophoresed on formaldehyde-containing 1–1.5% agarose gels and transferred onto Hybond N^+ membranes. For hybridization, [α-^{32}P]dATP-labeled probes were generated by random priming (Multiprime DNA labeling system, Amersham). A radiolabeled *Hin*dIII–*Bam*HI restriction fragment (Figure 1B) was used as probe for the RFLP analysis. A tobacco *petA*-specific probe was synthesized by radiolabeling a PCR product covering the entire coding region (obtained by amplification with primer pair P928: 5′-TGTAGAAATTTTCGGGATC-3′/P929: 5′-GCCAACAAGA-GTTTGATTC-3′). A *ycf6*-specific probe was prepared from an *Nde*I–*Bam*HI restriction fragment (Figure 1B). Hybridizations were carried out at 65–68°C in phosphate buffer (250 mM Na_2HPO_4 pH 7.2, 7% SDS) or Rapid Hybridization Buffer (Amersham). To control for equal loading, blots were stripped and rehybridized to a plastid 16S rRNA probe.

Protein isolation procedures

Thylakoid proteins from wild-type and mutant tissue were isolated according to Machold *et al*. (1979). Soluble stromal and lumenal proteins were prepared as described earlier (Ruf *et al*., 1997), except that isolated chloroplasts were used instead of total leaf material. The chloroplasts were lysed and subsequently centrifuged to remove thylakoid and envelope membranes. Purification of cytochrome b_6f complexes from *Spinacia oleracea* thylakoids was carried out according to published protocols (Hurt and Hauska, 1981).

SDS–PAGE and Western blot analyses

Isolated thylakoid or soluble proteins were separated on tricine–SDS–polyacrylamide gels (Schägger and von Jagow, 1987) and transferred to Hybond ECL nitrocellulose membranes (Amersham) using the Trans-Blot SD semi-dry transfer cell (Bio-Rad) with a standard transfer buffer (182 mM glycine, 20 mM Tris, 20% methanol, 0.05% SDS). Immunoblot detection was performed using the enhanced chemiluminescence system (ECL, Amersham).

Physiological measurements

PSII activity was determined on dark-adapted leaves from wild-type and mutant plants grown under low-light conditions (12 lux). PSII-dependent chlorophyll fluorescence was recorded at 650 nm wavelength with a pulsed amplitude modulation (PAM) fluorimeter (Walz, Effeltrich, Germany; Schreiber *et al*., 1986) under illumination of intact leaf tissue with white actinic light (flux density 94 μE/m^{2}s; pulse frequency of measuring light 1.6 kHz). For complete reduction of Q_A, leaves were exposed to pulses of saturating light (1 s; flux density 5600 μE/m^{2}s) every 30 s. The redox state of the PSI reaction center Chl P700 was monitored by following the apparent changes in absorbance of dark-adapted leaves from wild-type and mutant plants at 830 nm wavelength (Schreiber *et al*., 1988; Harbinson and Hedley, 1993). Absorbance measurements were performed with the PAM fluorimeter, using a modified emitter/detector unit. Far-red light with a peak wavelength of 730 nm was used to excite PSI selectively. To obtain complete re-reduction of PSI, leaves were exposed to white-light pulses of 4000 μE/m^{2}s.

Mass spectroscopy

MALDI was performed with a Voyager STR DE mass spectrometer (PE Biosystems). Purified cytochrome b_6f complex was co-crystallized with dihydroxybenzoic acid and analyzed in both the linear and reflector mode with an accelerating voltage of 24 and 25 kV, respectively. Measurements in the linear mode were calibrated exactly for the low-molecular-weight range (2–6 kDa) using adrenocorticotrophic hormone (18–39) and bovine insulin as internal standards. Data were confirmed by several independent measurements.

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