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# Plastocyanin Is Indispensable for Photosynthetic Electron Flow in *Arabidopsis thaliana**

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**Plastocyanin is a soluble copper-containing protein present in the thylakoid lumen, which transfers electrons to photosystem I. In the chloroplast of the flowering plant *Arabidopsis thaliana*, a cytochrome $c_6$-like protein is present, which was recently suggested to function as an alternative electron carrier to plastocyanin. We show that Arabidopsis plants mutated in both of the two plastocyanin-coding genes and with a functional cytochrome $c_6$ cannot grow photoautotrophically because of a complete block in light-driven electron transport. Even increased dosage of the gene encoding the cytochrome $c_6$-like protein cannot complement the double mutant phenotype. This demonstrates that in Arabidopsis only plastocyanin can donate electrons to photosystem I *in vivo*.**

Plants use light energy to drive electron and proton transport across the thylakoid membrane, resulting in the synthesis of NADPH and ATP and involving photosystem I (PSI)[1] and II (PSII), cytochrome $b_6/f$, and the plastid ATPase. Plastocyanin transfers electrons from cytochrome $b_6/f$ to PSI (1), and in cyanobacteria and some algae, plastocyanin can be replaced by the heme protein cytochrome $c_6$ (2, 3). The familiar idea that in higher plants only plastocyanin operates as a mobile electron donor to PSI has recently been challenged. *Arabidopsis thaliana* lines in which both of the plastocyanin-coding genes, *PetE1* and *PetE2*, had been silenced by RNA interference (RNAi) were viable, whereas plastocyanin-RNAi lines that in addition lacked a cytochrome $c_6$-like protein did not survive (4). From these data, it was concluded that in higher plants neither plastocyanin nor cytochrome $c_6$ is essential for plant growth and development but that both proteins can transport electrons from cytochrome $b_6/f$ to PSI.

Plastocyanin-null mutants have been identified and characterized in several cyanobacterial and algal species (2, 5–7). In these organisms, cyanobacterial cytochrome $c_6$ can replace plastocyanin in plastocyanin-null genotypes (5) as well as in wild-type cultures grown under conditions that result in copper deficiency (8). Accordingly, Chlamydomonas cells mutated for plastocyanin only perform photosynthesis when cytochrome $c_6$ expression is induced by making the cells deficient for copper (3). In this work, we show that Arabidopsis plants mutated in both of the two plastocyanin-coding genes and with a functional cytochrome $c_6$ cannot grow photoautotrophically because of a complete blockade in light-driven electron transport. This demonstrates that in Arabidopsis only plastocyanin can donate electrons to photosystem I *in vivo*.

## EXPERIMENTAL PROCEDURES

*Plant Propagation and Mutant Isolation*—The *En*-mutagenized *A. thaliana* (ecotype Columbia 0) population and growth of plants under short and long day conditions have been described previously (9, 10). *pete1-1.1 pete2-1.1* plants were propagated on solid MS medium (11) containing 2% sucrose. Transposon insertions within *PetE1* and *PetE2* were identified by screening the *En*-tagged population using gene-specific primers in combination with *En*-specific primers followed by hybridization with a gene-specific probe as described previously (12). Positive lines were confirmed by sequencing PCR-amplified insertion sites. The stable frameshift mutants *pete1-1.1* and *pete2-1.1* were identified according to Varotto *et al.* (13), and the double mutant *pete1-1.1 pete2-1.1* was generated by crossing the single mutants.

*Complementation of the pete1 pete2 Double Mutant*—The *PetE2* and *Atc6* cDNAs were introduced by recombination cloning (Gateway, Invitrogen) into the plant expression vector pJAN33, placing them under the transcriptional control of the Cauliflower Mosaic Virus $^{35}$S promoter. In addition, a genomic fragment containing *PetE2* and 1000 and 500 bp of DNA 5′ and 3′ to the gene, respectively, were introduced by recombination cloning into the vector pP001VS-GW. Flowers of *pete1-1.1/pete1-1.1 PetE2/pete2-1.1* plants were transformed according to Clough and Bent (14). Plants were transferred to the greenhouse, and seeds were collected after 3 weeks. At least 200 independent transgenic plants were selected on the basis of their resistance to kanamycin (pJAN33) or to the herbicide Basta (pP001VS-GW). The presence of the respective transgenes in double homozygous mutant plants was confirmed by PCR using primers that specifically allowed the amplification of the transgenes and of the endogenous *PetE2* and *pete2-1.1* alleles. Successful complementation of the double mutation was confirmed by measurements of growth and of chlorophyll fluorescence.

*Immunoblot and Chlorophyll Fluorescence Analyses*—Total proteins were isolated from 6-week-old plants as described previously (15). For denaturing PAGE analysis, 40 $\mu$g of total proteins determined by using the Bio-Rad protein assay based on the Bradford dye-binding procedure (16) were loaded for each genotype. Decreasing amounts of wild-type proteins (28 and 16 $\mu$g) were loaded in parallel lanes (0.7× WT and 0.4× WT). For immunoblot analyses, proteins were transferred to Immobilon-P membranes (Millipore, Eschborn, Germany) and incubated with antibodies specific for the large subunit of Rubisco (polyclonal chicken anti-RbcL raised against a peptide target conserved in all of the known Type I RbcL proteins (Agrisera, Vännäs, Sweden)), plastocyanin (polyclonal antibody raised against the mature form of the spinach plastocyanin expressed in *E. coli*, obtained from Ralph Bernd Klösgen

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[1] The abbreviations used are: PSI, photosystem I; PSII, photosystem II; RNAi, RNA interference; $F_V/F_M$, maximum quantum yield; $\Phi_{II}$, effective quantum yield; 1 − qP, the fraction of $Q_A$ (the primary electron acceptor of PSII) present in the reduced state; WT, wild type; MIPS, Munich Information for Protein Sequences.

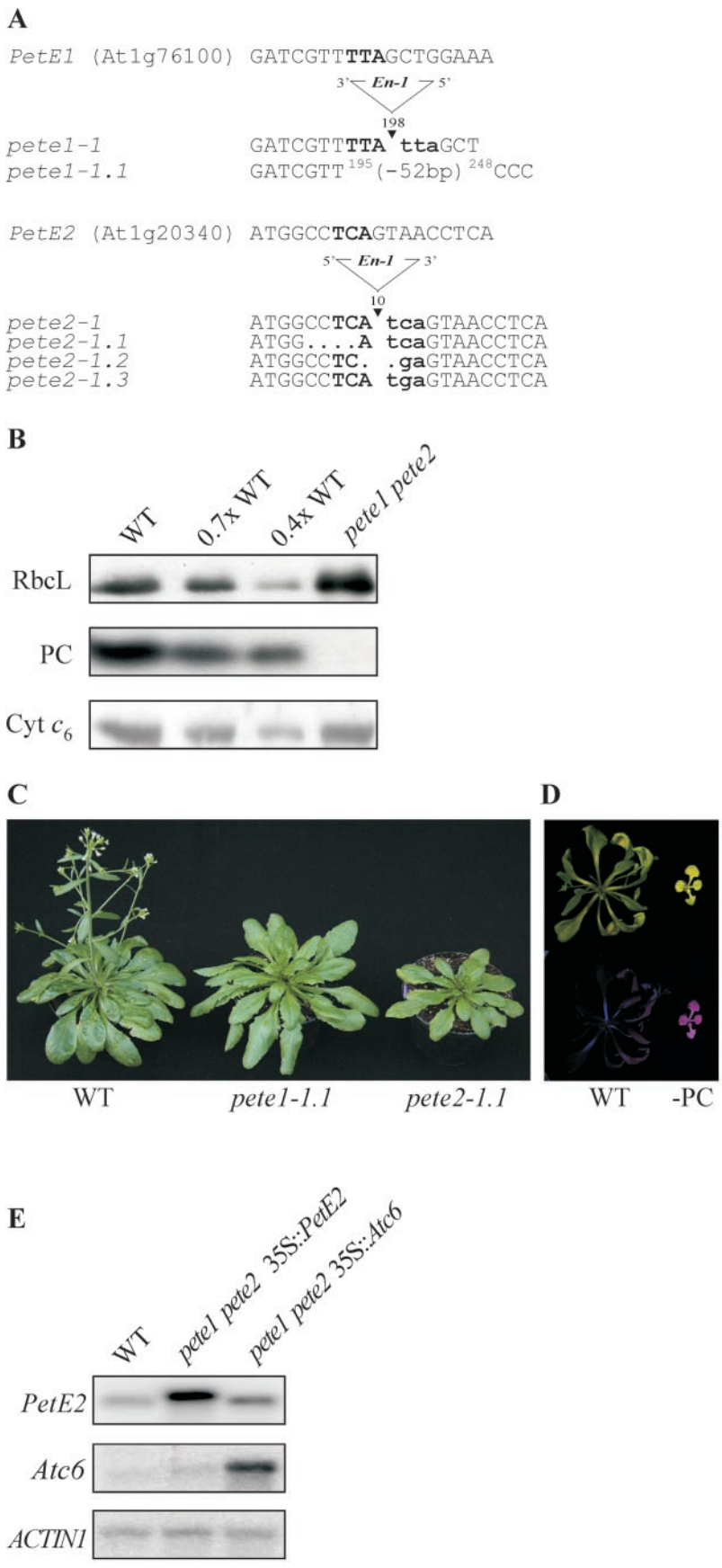


FIG. 1. **Isolation and characterization of mutants for plastocyanin in *A. thaliana*.** *A*, mutations in the loci *PetE1* and *PeteE2*. *En*-transposon insertions were located in the unique exons of *PetE1* and *PeteE2*, respectively. The proteins encoded by the two genes are highly homologous, exhibiting in their mature forms (after cleavage of the chloroplast transit peptide) a similarity/identity score of 93 and/or 83%. DNA sequences of the transposon donor sites from independent germinal revertants were obtained by PCR. Footprints left at each locus after *En* excision are indicated by *boldface lowercase letters*, whereas *bold uppercase letters* indicate the target site in the wild-type gene. Note that in *pete1-1.1* a 52-bp segment was deleted upon transposon excision, whereas a −1-bp frameshift occurred in *pete2-1.1*. *B*, immunoblot analysis of plastocyanin mutant and WT plants. 40 $\mu$g of total leaf proteins from WT and double-mutant plants were loaded (*lanes WT* and *pete1 pete2*) together with decreasing amounts of WT proteins (*lanes 0.7× WT* and *0.4× WT*). Replicate filters were immunolabeled with antibodies raised against the large subunit of Rubisco (*RbcL*), plastocyanin, and cytochrome $c_6$ (*Cyt* $c_6$). *C*, phenotypes of WT plants and of the single mutants *pete1-1.1* and *pete2-1.1*. Arabidopsis plants (8 weeks old) were grown in a greenhouse under long day conditions. The double mutant *pete1-1.1 pete2-1.1* does not survive under greenhouse conditions (data not shown). *D*, WT and *pete1-1.1 pete2-1.1* plants (*−PC*) grown on sucrose-containing MS medium and illuminated with white light (*top*) or UV light (*bottom*). *E*, Northern analysis of *PetE2* and *Atc6* transcripts in transgenic *pete1-1.1 pete2-1.1* lines. Aliquots (30 $\mu$g) of total RNA from WT and from *pete1-1.1 pete2-1.1* plants transformed with $^{35}$S::*PetE2* or $^{35}$S::*Atc6* constructs were hybridized with cDNA probes specific for *PetE2* and *Atc6*, respectively. To control for variation in loading, the blots were then probed with an *ACTIN1* cDNA fragment.

(Martin-Luther-Universität Halle-Wittenberg, Germany)), and cytochrome $c_6$ (polyclonal antibody raised against the mature form of the Arabidopsis cytochrome $C_6$ expressed in *E. coli* (see also Ref. 4), obtained from Sheng Luan (University of California, Berkeley, CA)). Signals were detected using the Enhanced Chemiluminescence Western blotting kit (Amersham Biosciences) and quantified using the Lumi Analyst 3.0 (Roche Applied Science). *In vivo*, Chl *a* fluorescence of single leaves was measured using the PAM 101/103 device (Walz, Effeltrich,

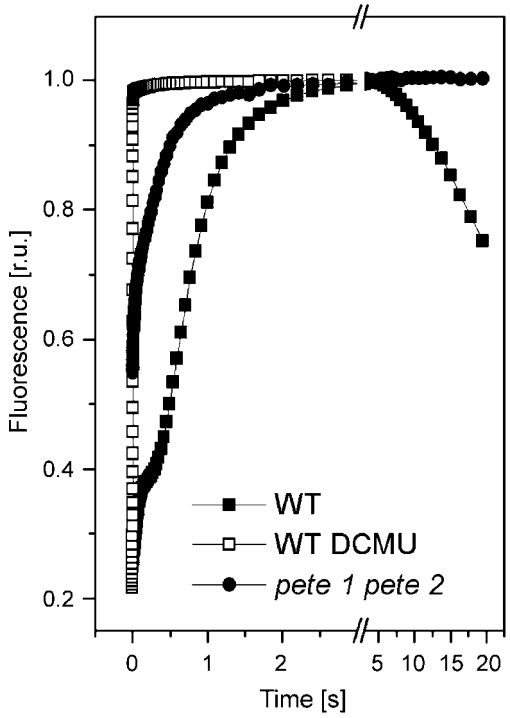


FIG. 2. **Kinetics of fluorescence emission in leaves of wild-type and *pete1 pete2* mutant plants.** Light intensity was 80 $\mu$mol photons·m$^{-2}$·s$^{-1}$ (~50 photons/photosystem/s). 3-(3,4-Dichlorophenyl)-1-1-dimethylurea (*DCMU*) (10 $\mu$M) was added by vacuum infiltration. Chlorophyll fluorescence is given as relative units (*r.u.*)

Germany) as described previously (12). Pulses (800 ms) of white light (6000 $\mu$mol photons·m$^{-2}$·s$^{-1}$) were used to determine the maximum fluorescence ($F_M$) and the ratio $(F_M - F_o)/F_M = F_V/F_M$. A 20-min illumination with actinic light (FL101/E; Walz, Effeltrich, Germany) at a rate of 80 $\mu$mol photons·m$^{-2}$·s$^{-1}$ was used to drive electron transport between the two photosystems before measuring the effective quantum yield of PSII ($\Phi_{II}$) and photochemical quenching ($qP = (F_{M'} - F_S)/(F_{M'} - F_{o'})$). Fluorescence induction kinetics were measured with a home-made fluorometer (17). Actinic light was provided by an array of green light emitting diodes (HLMP CM15, 520 nm, Hewlett-Packard). Light was concentrated on a leaf spot of ~20 mm$^2$, and fluorescence was detected on the opposite side of the sample. Emission was recorded at discrete times with a maximum resolution time of 30 $\mu$s.

## RESULTS AND DISCUSSION

In *A. thaliana*, we have isolated transposon insertion mutants for both plastocyanin-coding genes *PetE1* (MIPS accession number At1g76100) and *PetE2* (MIPS accession number At1g20340). Starting from the unstable insertion mutants, stable null alleles of both genes, *pete1-1.1* and *pete2-1.1*, were generated by selecting germinal revertants with frameshift mutations (Fig. 1*A*). The double mutant *pete1-1.1 pete2-1.1* was obtained by crossing single mutants and genotyping F2 progenies. In *pete1-1.1 pete2-1.1* plants, no plastocyanin was detectable by immunoblot analysis, whereas wild-type amounts of the cytochrome $c_6$ polypeptide were found (Fig. 1*B*).

In both *pete1-1.1* and *pete2-1.1* plants, growth was affected (Fig. 1*C*). The plastocyanin double mutants did not survive when grown on soil but could be propagated in axenic culture on medium supplemented with sucrose. However, heterotrophically grown double mutants had a substantially reduced growth rate and displayed a high chlorophyll fluorescence phenotype upon illumination with UV light (Fig. 1*D*). This last observation indi-

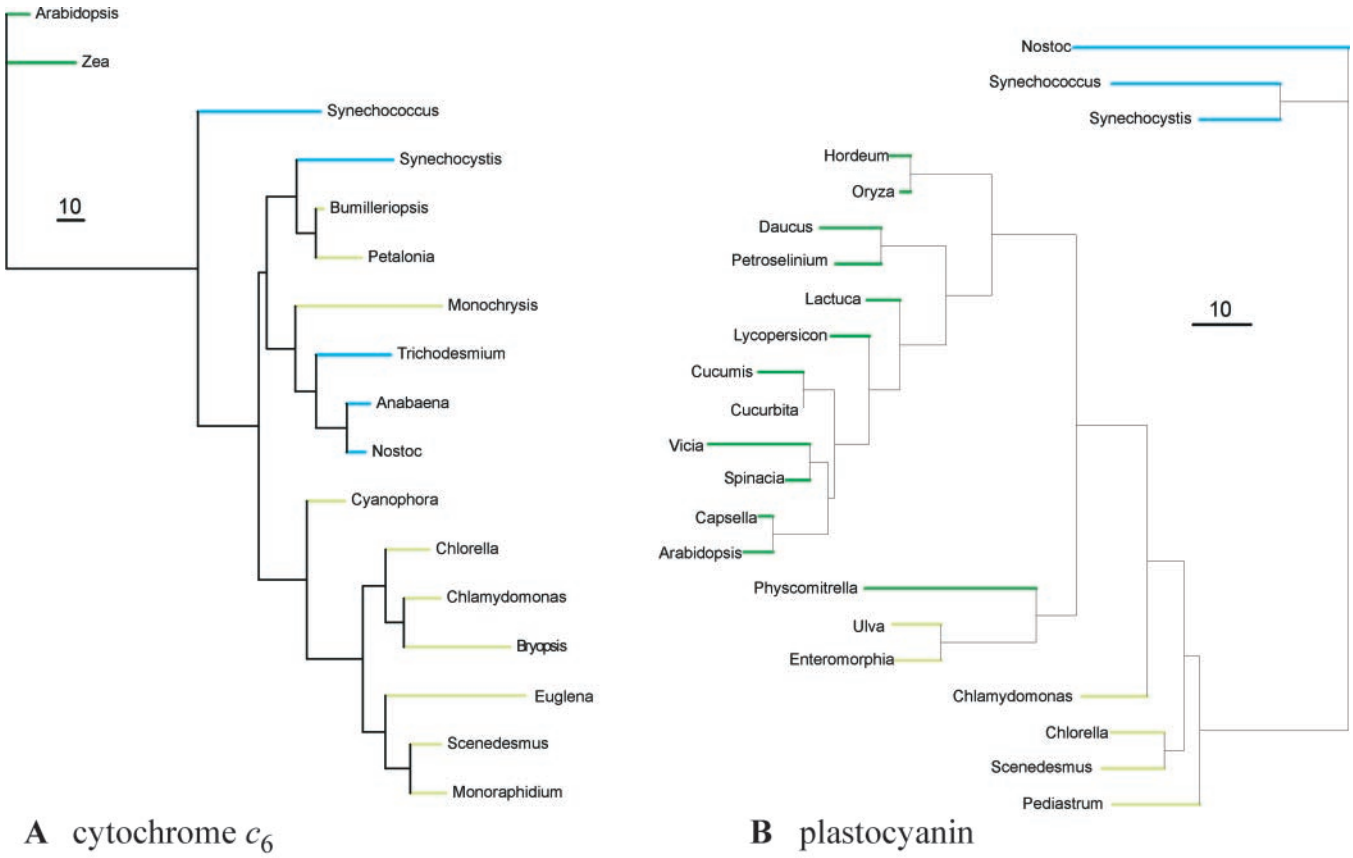


FIG. 3. **Maximum likelihood phylogenetic trees based on an alignment of prokaryotic, algal, and plant cytochrome $c_6$ (*A*) and plastocyanin (*B*).** *Dark green* branches indicate plant sequences, whereas algal and cyanobacterial sequences are indicated in *light green* and *blue*, respectively. Trees were constructed by protein maximum likelihood with PROTML (MOLPHY) (24) using the JTT-F matrix with the neighbor-joining tree of ML distances as the starting topology and RELL bootstrapping ($10^4$). Branch lengths reflect the estimated number of substitutions/100 sites.

cated that photosynthetic electron flow was blocked.

Parameters of chlorophyll fluorescence induction were determined to characterize the electron flow in the plastocyanin double null mutant. Although the maximum ($F_V/F_M$) quantum yield was decreased by ~35% in *pete1-1.1 pete2-1.1* plants compared with the wild type ($F_V/F_M$: 0.54 ± 0.04 *versus* 0.81 ± 0.01), the effective quantum yield ($\Phi_{II}$) was close to zero in the double mutant with respect to the wild type ($\Phi_{II}$: 0.04 ± 0.01 *versus* 0.74 ± 0.02), again pointing to a severe perturbation in electron flow. In addition, the fraction of $Q_A$ (the primary electron acceptor of PSII) present in the reduced state was drastically increased (1 − qP: 0.94 ± 0.01 *versus* 0.06 ± 0.02), implying that electron flow through PSII is still occurring while the block exists at a later electron transfer step.

Consistent with these data is the comparison of the fluorescence induction curves measured in the *pete1-1.1 pete2-1.1* mutant and wild-type plants (Fig. 2). Whereas the PSII photochemical rate measured in the *pete1-1.1 pete2-1.1* line was close to that of the WT (data not shown), the area above the fluorescence induction curve (a parameter proportional to the size of the PSII acceptors; Refs. 18 and 19) was largely reduced in this mutant. This finding suggests that the size of PSII electron acceptor pool was largely decreased by the mutation. The area was nevertheless larger than the one measured in the WT in the presence of DCMU (to block the reoxidation of $Q_A$), confirming that the effect of the mutation was localized downstream of PSII. As shown in Fig. 2, the mutant was blocked in a high fluorescence state, whereas in the WT, the fluorescence yield started to decrease after approximately 10 s of continuous illumination. This decrease is at least partly attributable to the activation of the Benson Calvin cycle (20). Its absence in the mutant gives a further indication that electron transfer is blocked. Moreover, the mutant did not display the plateau observed in the WT in the 20–100-ms time range during continuous illumination. This plateau has been recently ascribed to the reduction of PSI acceptors (see Ref. 17 for discussion). Its absence is consistent with a block of electron flow located downstream of the cytochrome $b_6/f$. Taken together, these results strongly strengthen the interpretation of the non-photoautotrophic mutant phenotype as the consequence of a complete block in light-driven electron transport, which seems to occur as expected at the level of the PSI donor side.

To test whether increased doses of cytochrome $c_6$ can replace plastocyanin in the *pete1 pete2* double mutant, the Arabidopsis cDNA for cytochrome $c_6$ was fused to the $^{35}$S promoter of cauliflower mosaic virus and introduced into the *pete1 pete2* line. In all of the $^{35}$S::*Atc6 pete1 pete2* individuals analyzed, *Atc6* mRNA was detected at high levels (Fig. 1*E*). However, unlike the case of the plastocyanin-overexpressing $^{35}$S::*AtPetE2* construct that served as a positive control, $^{35}$S::*Atc6* could neither reverse nor reduce the effects of the double mutation, indicating that cytochrome $c_6$ cannot replace plastocyanin *in vivo*. Also, cytochrome $c_6$ expressed from T-DNA constructs containing the *Atc6* gene under the transcriptional control of its natural promoter had no effect on the phenotype of the *pete1 pete2* double mutant.

The data concerning the photosynthetic behavior of the *pete1 pete2* double mutant are in conflict with the findings of Gupta *et al.* (4). Their oxygen evolution reconstruction assays indicate that Arabidopsis cytochrome $c_6$ can function along with plastocyanin in photosynthetic electron transport (4). But does Arabidopsis cytochrome $c_6$ actually donate electrons to PSI *in vivo*? Unlike the case in Chlamydomonas, cytochrome $c_6$ expression in *A. thaliana* is constitutive and is not up-regulated by plastocyanin depletion due to copper deficiency (4). Moreover, cytochrome $c_6$ of higher plants compared with that of prokaryotes or algae may have acquired novel functions as suggested from phylogenetic analyses (Fig. 3). The plastocyanin sequences from Arabidopsis and several other plant species branch from algal sequences, indicating a continuous evolution from the cyanobacterial endosymbiont to the plastocyanin of modern chloroplasts. On the contrary, the cytochrome $c_6$-like sequences of higher plants form a distinct clade, which is relatively distantly related to both algal and cyanobacterial proteins. This indicates that higher plant cytochrome $c_6$ has diversified in sequence perhaps in association with the acquisition of function(s) different from transferring electrons to PSI.

The RNAi-based silencing of the genes for plastocyanin as well as the combination of plastocyanin-RNAi and *Atc6* knockout was performed in the Wassilewskija ecotype of *A. thaliana* (4), whereas the *pete1 pete2* double mutant (this study) was generated in the Columbia 0 background. However, it seems improbable that intraspecific variation in photosynthetic functions could account for the difference in response. A second possibility can explain the contrasting results. Residual plastocyanin expression can take place during RNAi because of leakiness in gene silencing (21). Thus, the plastocyanin-RNAi lines may have retained enough plastocyanin to allow a residual level of photosynthesis.

If cytochrome $c_6$ cannot replace plastocyanin, at least in Columbia 0, what might its function be under normal physiological conditions? The protein might play a regulatory role rather than quantitatively transferring electrons to PSI, or in light of the results of recent proteomics analyses of the thylakoid lumen (22, 23), cytochrome $c_6$ could be involved in one of the diverse functions of the thylakoid lumen, including cross-talking with immunophilins with which it can interact in the yeast two-hybrid experiments (4).

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