The PSI-D subunit of photosystem I is a hydrophilic subunit of about 18 kDa, which is exposed to the stroma and has an important function in the docking of ferredoxin to photosystem I. We have used an antisense approach to obtain Arabidopsis thaliana plants with only 5–60% of PSI-D. No plants were recovered completely lacking PSI-D, suggesting that PSI-D is essential for a functional PSI in plants. Plants with reduced amounts of PSI-D showed a similar decrease in all other subunits of PSI including the light harvesting complex, suggesting that in the absence of PSI-D, PSI cannot be properly assembled and becomes degraded. Plants with reduced amounts of PSI-D became light-stressed even in low light although they exhibited high non-photochemical quenching (NPQ). The high NPQ was generated by up-regulation of Fd oxidoreductase catalyzing the further transfer of electrons from plastocyanin on the lumenal side to ferredoxin (Fd) on the stromal side. In linear electron transport an Fd: NADP$^+$-oxidoreductase catalyzes the further transfer of electrons from Fd to NADP$^+$, generating NADPH. PSI is a large multisubunit complex and the core complex consists of up to 15 subunits in eukaryotes, denoted PSI-A to PSI-O. However PSI-M is absent in higher plants. In addition, the PSI reaction center complex contains the primary electron donor P700 (a Chl a dimer), the acceptors A$_0$ (Chl a), A$_1$ (phyloquinone), and the three [4Fe-4S] clusters F$_{A}$, F$_{B}$, and F$_{X}$. The core complex of PSI binds ~100 chlorophylls and 20 carotenoids and is surrounded by additional light harvesting complexes (LHCI). LHCI is comprised of four subunits, Lhca1 to Lhca4, which are normally present in similar amounts. A search of the Arabidopsis genome has revealed two additional genes with high similarity to the other Lhca proteins, but Lhca5 and Lhca6 have so far not been found in the thylakoid membrane (1). The subunits of PSI are all transmembrane, except for PSI-C, -D, -E, and -N; PSI-C, -D, and -E are localized on the stromal and PSI-N on the luminal side. The composition and structure of PSI has been described in a number of recent reviews (2–5). PSI-D associates with the PSI complex, and this association can occur spontaneously and may not be dependent on any cofactors (11). However Ycf3, a protein involved in the assembly of PSI, has been shown to interact with PSI-D (12). After binding to the PSI complex, the transit peptide is proteolytically removed.

Arabidopsis thaliana Plants Lacking the PSI-D Subunit of Photosystem I Suffer Severe Photoindihbition, Have Unstable Photosystem I Complexes, and Altered Redox Homeostasis in the Chloroplast Stroma*

Anna Haldrup‡, Christina Lunde, and Henrik Vibe Scheller
From the Plant Biochemistry Laboratory, Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Received for publication, May 15, 2003, and in revised form, June 4, 2003
Published, JBC Papers in Press, June 6, 2003, DOI 10.1074/jbc.M305106200

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 45-35-28-33-68; Fax: 45-35-28-33-33; E-mail: anna@kvl.dk.

‡ This work was supported by the Danish National Research Foundation and the Danish Agricultural and Veterinary Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* The abbreviations used are: PSI, photosystem I; asD, antisense psaD; CF$_{1}$, catalytic subunit of the chloroplast ATP synthase; Chl, chlorophyll; Fd, ferredoxin; LHC, light harvesting complex; NDI, NAD(P)H dehydrogenase; NPQ, non-photochemical quenching; qE, energy dependent quenching.

This paper is available on line at http://www.jbc.org

Photonsystem I (PSI)$^1$ catalyzes the light-induced transfer of electrons from plastocyanin on the luminal side to ferredoxin (Fd) on the stromal side. In linear electron transport an Fd: NADP$^+$-oxidoreductase catalyzes the further transfer of electrons from Fd to NADP$^+$, generating NADPH. PSI is a large multisubunit complex and the core complex consists of up to 15 subunits in eukaryotes, denoted PSI-A to PSI-O. However PSI-M is absent in higher plants. In addition, the PSI reaction center complex contains the primary electron donor P700 (a Chl a dimer), the acceptors A$_0$ (Chl a), A$_1$ (phyloquinone), and the three [4Fe-4S] clusters F$_{A}$, F$_{B}$, and F$_{X}$. The core complex of PSI binds ~100 chlorophylls and 20 carotenoids and is surrounded by additional light harvesting complexes (LHCI). LHCI is comprised of four subunits, Lhca1 to Lhca4, which are normally present in similar amounts. A search of the Arabidopsis genome has revealed two additional genes with high similarity to the other Lhca proteins, but Lhca5 and Lhca6 have so far not been found in the thylakoid membrane (1). The subunits of PSI are all transmembrane, except for PSI-C, -D, -E, and -N; PSI-C, -D, and -E are localized on the stromal and PSI-N on the luminal side. The composition and structure of PSI has been described in a number of recent reviews (2–5). PSI-D associates with the PSI complex, and this association can occur spontaneously and may not be dependent on any cofactors (11). However Ycf3, a protein involved in the assembly of PSI, has been shown to interact with PSI-D (12). After binding to the PSI complex, the transit peptide is proteolytically removed.
and the mature PSI-D undergoes a conformational change making the association between PSI-D and the surrounding PSI subunits more tight (13). Compared with cyanobacteria, the mature eukaryotic PSI-D has an N-terminal extension of about 30 amino acid residues rich in alanine and proline. Higher plant PSI-E has a similar Ala-Pro rich N terminus of about 30 amino acid residues rich in alanine and proline. The mature eukaryotic PSI-D has an N-terminal extension unique to higher plant PSI-D has been shown to be particularly important for the stable binding of PSI-C (14).

Chemical cross-linking of PSI in the presence of ferredoxin consistently yields a product of PSI-D and Fd (7, 16, 17). This interaction is not dependent on the presence of other PSI subunits and has even been shown with isolated PSI-D and Fd (18). These observations suggest that PSI-D has an important function in the docking of Fd. The PSI-C, -D, and -E subunits surround an indentation formed by PSI-A and -B, and this indentation has been proposed to make up the binding site for ferredoxin (6). Ferredoxin is a small (approximately 10 kDa), acidic protein and the binding to PSI is believed to be mediated by electrostatic interactions (19, 20).

For example, PSI-D has been investigated in Arabidopsis either by cosuppression, antisense, or RNA interference, or by using insertional knockout plants. The advantage of a suppression approach is that very low amounts of protein can be obtained even if the protein is encoded by two genes as is the case for PSI-D, PSI-E, and PSI-H (21, 22). The genes should, however, exhibit a certain homology. In Arabidopsis, PSI-D is encoded by two homologous genes, psd1 and psd2. Plants with 50% reduction of the stromal PSI-E have been characterized and they exhibited a decreased quantum yield of PSII, a higher level of QA reduction and higher sensitivity toward photoinhibition, even at low light intensities (23). The levels of PSI-C, -D, -H, and -L were reduced in these plants (23, 24). Plants without the stromal PSI-C have not been obtained because of the difficulties in down-regulating chloroplast-encoded proteins. Here we describe the first time the effect of reducing the content of the PSI-D subunit in vivo in higher plants.

**EXPERIMENTAL PROCEDURES**

A. thaliana (L.) Heynh. Ectotype Columbia were used for all experiments. Plants were grown in compost in controlled environment Arabidopsis chambers (Percival AR-60 I, Boone, IA) at a photosynthetic flux of 100–120 μmol of photons m⁻² s⁻¹, 20 °C, and 70% humidity. The photoperiod was 12 h for plants used for transformation and 8 h for plants used for biochemical and physiological analysis, to suppress the induction of flowering. All biochemical and physiological experiments were performed on plants used for biochemical and physiological analysis, to suppress the induction of flowering. Here we describe the first time the effect of reducing the content of the PSI-D subunit in vivo in higher plants.

**Vector Construction and Plant Transformation—**The A. thaliana psd2 cDNA (GenBankTM accession numbers AJ245907, Atg03130, and EST307C9T7) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH) and was used as a template for PCR amplification using primers 5'-CGGCTAGCTGCAATCACTGGCGCGG-3' and 5'-GCCGTCAATTCAAATCAAGATGTTTCC-3' containing the Spel and SalI restriction sites. The 651-bp fragment was cloned in antisense orientation between the enhanced CaMV 35S promoter and 3SS terminator. The insert was confirmed by nucleotide sequencing. Subsequently, a fragment containing the E35S promoter followed by the antisense psd2 and the 3SS terminator was excised with XbaI and ligated into the binary pZP111 vector (25). Because of the high identity it was expected that this construct would down-regulate both psdD genes. The vector construct was transformed into the Agrobacterium tumefaciens strain C58 (26). Plasmid integrity in A. tumefaciens cultures used for plant transformation was verified by PCR analysis of each. Arabidopsis plants were transformed according to Clough and Bent (27) using 0.005% Silwett L-77 as surfactant for 5 min. Seeds were germinated on MS medium containing 2% sucrose, 50 mg⁻¹ kanamycin sulfate, and 0.8% agar for 2 weeks and transgenic Arabidopsis plants were selected and transplanted to compost.

**Fluorescence Measurements—**Determination of conventional fluorescence parameters was performed with a PAM 101–103 fluorometer (Walz, Effeltrich, Germany) using a standard setup (28). Measurements were performed on wild-type plants and plants with 10 and 40% PSI-D under photon flux density ranging from 0 to 300 μmol of photons m⁻² s⁻¹. The NPQ was calculated as: \( (F_{m} - F)/F_{m} \), the photosynthetic quenching \( q_{P} \), was calculated as: \( (F_{m} - F)/F_{m} \), the quantum yield of \( (F_{m} - F)/F_{m} \), was calculated as: \( (1-q_{P}) \). The level of excess light energy was calculated as \( (1-q_{P}) \). Light absorption × light absorbed by PSI × photon flux density (29). Efficiency of PSI photochemistry was also measured in plants under growth light conditions followed by recovery in the dark for 40 min. A saturating flash was given every 10 min as outlined in Haldrup et al. (30).

**Chlorophyll Determination—**Total chlorophyll (Chl) and Chl a/b ratio were determined in 80% acetone according to Lichtenthaler (31). To determine the total amount of chlorophyll per cm², leaves were extracted in 80% boiling ethanol and the concentration determined according to Lichtenthaler (31).

**Immunoblot Analysis—**The amount of residual PSI-D in each individual transgenic plant was determined by immunoblotting using crude extracts (30). When thylakoids were isolated from pooled plants with determined amounts of PSI-D, the final concentration of PSI-D in the thylakoid preparation was determined by immunoblotting. Isolated thylakoids from dark-adapted plants or crude extracts from plants harvested after a 4-h light period (for Fd and Fd:NADP⁺-oxidoreductase activity) were analyzed in similar immunoblotting procedures using antibodies raised in rabbits. Antibodies against phosphothreonine were obtained from New England Biolabs. All antibodies were detected using a chemiluminescent detection system (ECLTM, Amersham Biosciences) according to the manufacturers instructions. Antibodies were the kind gift of Drs. Peter Nixon, Imperial College of Science, London, UK (26), Stefan Jansson, University of Umeå, Sweden (Lhca and Lhcb), Hans-Erik Aakerlund and Marie Eslund, Lund, Sweden (zeaxanthin epoxidase and violaxanthin de-epoxidase), Torill Hundal, Linkoping University, Sweden (D1), Sabeeha Merchant, University of California, Los Angeles, CA (cytochrome f and CF₆), and Krishna Niyogi, University of California, Berkeley, CA (PsbS). All samples for immunoblotting were loaded on a chlorophyll basis in an amount empirically determined to give a linear response. This amount varied between 0.1 and 1.0 μg of Chl depending on the antibody used. Immunoblotting analysis of each thylakoid preparation was repeated 2 or 3 times and the results varied ±5–10%.

**Low Temperature Fluorescence Measurements—**The fluorescence spectra at 77 K were recorded from 650 to 800 nm using an excitation wavelength of 435 nm and a bifurcated light guide connected to the spectrophotometer. Intact leaves were dark-adapted for 3 min before measurements.

**NADP-malate Dehydrogenase Assay—**Light activation of the chloroplast NADP-malate dehydrogenase was used to monitor the redox state of the acceptor side of PSI. Leaf extraction and NADP-malate dehydrogenase activity measurements were carried out essentially as described by Scheibe and Stitt (32). Plants with 15% PSI-D left and wild-type plants were harvested in liquid nitrogen after 15 min, 2 h, and 4 h of illumination in the growth chamber.

**Light Reflection and Light Transmission—**Light reflection and light transmission through the leaf was determined as described in Lunde et al. (33).

**RESULTS**

The Two Genes psd1 and psd2 and the Corresponding Proteins PSI-D1 and PSI-D2 Are Very Similar—The two genes encoding PSI-D are highly homologous and share 87% identity at the DNA level. At the protein level, PSI-D1 and PSI-D2 show 95% identity when the full-length aprotoproteins are compared, and 98% identity for the mature protein (Fig. 1). Alignment of PSI-D1 and PSI-D2 revealed that the C-terminal region important for the binding of ferredoxin is highly conserved between the two proteins and that the amino acid substitutions and deletions are present in the N-terminal extension unique to eukaryotic PSI-D.
Construction of Arabidopsis Plants Lacking PSI-D—The two genes encoding PSI-D share 21 combinations of completely identical stretches of 22 nucleotides. The introduction of *psaD2* in antisense orientation was therefore expected to efficiently down-regulate the expression from both genes (34, 35). The original Arabidopsis lines obtained after transformation with the *psaD2* construct were selfed, the seeds produced were plated on kanamycin-containing plates, and kanamycin-resistant plants were transferred to compost. Leaf extracts of the T1 plants were analyzed by immunoblotting and various amounts of residual (5–100%) PSI-D could be detected. In 30% of all kanamycin-selected T1 plants, a down-regulation of PSI-D was observed: 25% had 20–60% PSI-D left and 5% had only 5–10% PSI-D left. The detection limit is about 3% of wild-type levels. Transformants grown under normal conditions in soil had a clear phenotype that correlated well with the residual amount of PSI-D (Fig. 2). Plants with 5% PSI-D grew extremely slowly compared with wild-type, but under optimal growth conditions they survived longer than the wild-type because of the impaired growth. Plants with as little as 5% PSI-D left were not able to set seeds, but plants with 20–60% PSI-D eventually set seeds, so several generations could be obtained from these plants. The number of seeds in these plants was, however, highly reduced.

The Amount of PSI Is Reduced to the Same Extent as PSI-D—In plants with 30–40% PSI-D left, the P700 per 1000 Chl was 0.46 ± 0.07 compared with 1.70 ± 0.04 for wild-type plants (Table I), which is equivalent to a Chl/P700 ratio of 589 ± 14 and 2192 ± 364, respectively. In agreement with the lower P700 content, plants with 60 and 20% PSI-D also had a decreased Chl a/b ratio of 2.49 ± 0.06 and 2.11 ± 0.27, respectively, whereas in wild-type plants the ratio was 2.65 ± 0.06 (Table I). The amount of PSI-A/B was reduced to the same extent as PSI-D as well as all other PSI subunits (Fig. 3A) and the four Lhca proteins (Fig. 3B). The PSI-E protein was partly degraded indicating proteolytic degradation. Thus, the Arabidopsis plants with 20% PSI-D contain only 20% of PSI and Arabidopsis plants with 60% PSI-D contain only ~60% PSI. The remaining PSI appears to contain a full complement of all subunits, including PSI-D. Hence, the antisense PSI-D plants are in fact PSI-reduced plants. The number of seeds in these plants, however, was, highly reduced.

Plants with Low Levels of PSI-D Contain Less Chlorophyll—The leaves from antisense plants with decreased PSI-D were thinner, and showed a paler green pigmentation compared with wild-type leaves. Thus, in plants with 20% PSI-D, the paler appearance reflected a 50% lower chlorophyll content per leaf area (Table I). The transmission and reflection of white light was measured to determine the amount of absorbed light.
Antenna Function—Judged on Chl a/b ratio, Chl/P700 ratio, and immunoblotting, the amount of PSI correlates very well with the amount of PSI-D indicating that little, if any, PSI is present, which does not contain all subunits. If this is the case, the present PSI complexes should be fully functional and thereby contain a normal antenna. To test this, we measured 77 K (Fig. 4). When PSI is excited with far-red light, most of the photons are absorbed by long wavelength chlorophylls in LHCl. If the organization of LHCl were changed it would have resulted in a change in the emission spectrum, most likely a blue shift. However, the peak shape and position was identical in plants with low levels of PSI-D and wild-type plants, confirming that the AsD plants are not affected in the organization of the LHCl antenna and that the residual PSI complexes in the plants are fully functional. The decreased magnitude of the 734 nm peak reflects the lower PSI content in the AsD plants. This confirms that the residual PSI complexes in the PSI-D down-regulated plants are fully functional.

NPQ Is Increased in the Absence of PSI-D, and the Plastoquinone Pool Is Overreduced—Plants with low amounts of PSI-D exhibited very high NPQ compared with the wild-type (Fig. 5A). NPQ has several components (36): qE, energy-dependent fluorescence quenching; qT, caused by the state transitions from state 1 to state 2; and finally qI, representing photoinactivation of PSII. Usually, the major component of NPQ is qE-type quenching, which is dependent on the ability to build up a high transthylakoidal pH, a functional xanthophyll cycle, and the presence of PsbS (37). The increased NPQ indicates that the plants with reduced PSI-D try to avoid photoinhibition by dissipating the excess light energy safely as heat. However, despite the increased NPQ, the plants showed substantial overreduction of the PSII acceptors (determined as 1−qP) even at low and moderate light intensities (Fig. 5B). To determine the extent to which antisense PSI-D plants were able to compensate for the low level of photochemistry by increasing NPQ, the amount of excess energy generated by PSII (29) was calculated (Fig. 5D). The fraction of incoming light absorbed by PSII was estimated from the total amount of light absorbed by the leaves and the PSII/PSI ratio. The PSII/PSI ratio was estimated to be 1.5 and 3.75 in wild-type and asD (40% PSI) plants, respectively (38). In the wild-type plants 52% of the incoming light is absorbed (wild-type, 0.87–0.60; asD, 0.59–0.79 = 0.47). The asD plants are very sensitive to photoinhibition (Fig. 5B) and above 500 μmol m−2 s−1 the quantum yield of PSII is close to zero (results not shown). The level of excess excitation energy was therefore only calculated in the light range where photochemistry occurs. The plants with low PSI-D show a much higher production of excess excitation energy under low and moderate light intensities and at 100 μmol m−2 s−1, the light intensity to which the plants are adapted, the asD plants have to cope with a 4-fold higher production of excess excitation energy. This clearly shows that...
the asD plants are unable to up-regulate NPQ sufficiently to compensate for the reduced photochemistry.

**The Content of Proteins Involved in qE and PSI—Photo-inhibition Is Changed in PSI-D-deficient Plants**—Immunoblotting was performed to elucidate the changes leading to the significantly increased NPQ. Violaxanthin de-epoxidase converts violaxanthin to zeaxanthin and zeaxanthin epoxidase converts zeaxanthin to violaxanthin. The level of violaxanthin de-epoxidase was up-regulated 200%, whereas the zeaxanthin epoxidase was reduced to 50% of wild-type levels (Fig. 4C), suggesting that the high NPQ was generated by changing the ratio between the two enzymes, thereby shifting the xanthophyll cycle toward producing more zeaxanthin and thereby increasing \(q_E\). PsbS is also necessary for \(q_E\) and was 170% higher in the asD plants. A similar increase was observed for NAD(P)H dehydrogenase (NDH-I), which is an enzyme suggested to be involved in cyclic electron transport. Thus, three major adjustments have occurred that will contribute to the higher level of NPQ. The amount of Lhcb1 and Lhcb2 was slightly decreased in plants with reduced levels of PSI-D (Fig. 4B) and the phosphorylation level of LHCII was 480% higher than wild-type levels (Fig. 4C). The amounts of cytochrome \(f\), ATPase, Fd, and Fd:NADP\(^+\)-oxidoreductase were unchanged compared with wild-type on a chlorophyll basis. However, because the level of PSI was much lower in plants with 20 or 60% PSI-D, the amount per PSI was greatly increased.

**Plants without PSI-D Exhibit Chronic PSII Photoinhibition**—The lower content of PSI in the asD plants leads to over-reduction of the plastoquinone pool and the plants are therefore likely to suffer from PSII photoinhibition. The efficiency of PSII photochemistry is a measure of the functionality of PSII, and plants with low amounts of PSI-D exhibited a decreased \(\Phi_{PSII}\) even after 16 h of dark adaptation (Fig. 5C, 0 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) photon flux density). In the light, \(\Phi_{PSII}\) was very low, but \(\Phi_{PSII}\) in the light is affected by many factors including \(q_E\) quenching and reduced acceptors. To determine the extent of photoinhibition, \(\Phi_{PSII}\) was determined during the time after the actinic light was switched off (Fig. 6). Although a large part of the \(\Phi_{PSII}\) was restored quickly, a substantial depression of \(\Phi_{PSII}\) persisted in the dark and therefore was concluded to be because of photoinhibition. As mentioned above, even the 16-h dark period was insufficient for complete recovery of PSII from the photoinhibition. The photoinhibition of PSII may be an expected result of the increased excitation pressure, which will increase the risk of chlorophyll triplet formation in PSII. During photoinhibition, the D1 protein is phosphorylated, and this prevents degradation of D1 until the repair process can take place (39). An enhanced phosphorylation level of D1 was not detected after dark adaptation (Fig. 3C), but a mobility shift was observed when protein extracts from illuminated leaves were used, indicating that during the light period the D1 in PSI-D-reduced plants is more phosphorylated than in wild-type plants suggesting a net accumulation of damaged PSII waiting for repair.

**The Redox Conditions in the Stroma**—Because plants with low content of PSI-D suffered severely from photoinhibition, had a very slow growth, and a high phosphorylation level of LHCII, we considered that the less efficient electron transfer to ferredoxin would affect the redox conditions on the stroma side. An alteration in stroma redox conditions under illumination would be reflected in the activation level of NADP\(^+\)-malate dehydrogenase because this enzyme contains redox-sensitive disulfide bonds and is activated by reduction via the ferredoxin-thioredoxin light activation system (40, 41). After 15 min of light exposure the activation level of NADP\(^+\)-malate dehydrogenase was similar in wild-type and plants having 10–15% PSI-D left, but during prolonged light exposure the NADP\(^+\)-malate dehydrogenase activity was lower in plants with reduced levels of PSI-D. This suggests that the redox conditions in the stroma are more reduced in PSI-D-reduced plants, and it suggests that the PSI-D reduces the redox conditions on the stroma side.

**Experimental Procedures.**—The leaves were dark-adapted for 1 h prior to the measurements. The content of PSI-D left, but during prolonged light exposure the NADP\(^+\)-malate dehydrogenase activity was lower in plants with reduced levels of PSI-D. This suggests that the redox conditions in the stroma are more reduced in PSI-D-reduced plants, and it suggests that the PSI-D reduces the redox conditions on the stroma side.
malate dehydrogenase activity steadily decreased to 58% of wild-type after 2 h and 35% after 4 h (Fig. 7). This means that the redox level of thioredoxin was normal in the beginning of the light period, but became increasingly oxidized as compared with the wild-type during the light exposure. The most likely explanation for this is that the progressively higher level of photoinhibition results in lower photosynthetic activity in lower photosynthetic activity and consequently also in a lower reduction level of thioredoxin.

**DISCUSSION**

Both *psaD* Genes Were Efficiently Down-regulated by the Antisense Approach—We have succeeded in making transgenic *Arabidopsis* plants with varying amounts of *PSI-D* from 5 to 60% of wild-type *PSI-D* and thereby obtaining an efficient tool for investigating the role of the PSI-D polypeptide *in vivo*. Total lack of PSI-D is likely to be lethal because no antisense plants completely lacking PSI-D were recovered, and antisense plants with ~5% PSI-D were barely able to survive (Fig. 2). Antisense suppression and related techniques such as cosuppression and RNA interference seem to function through the formation of double stranded RNA, which is broken down to fragments of 22–25 nucleotides. These fragments directly degrade the degradation of single stranded RNA with a 100% match. The two *psaD* genes share many identical 22-nucleotide stretches and this explains why the down-regulation of both genes was successful. Recently, Andersson *et al.* (35) have simultaneously down-regulated all 8 *Lhcb1* and *Lhcb2* genes in *Arabidopsis*. The eight genes all share identical stretches of 22 nucleotides. No effect was observed with genes such as *Lhcb3*, which is very similar, but does not contain any completely conserved stretches of 22 nucleotides.

Comparison of the amino acid sequences of PSI-D from several species shows that the C-terminal part is highly conserved, especially in a region containing many basic residues. The termini of PSI-D in the structural model of *Synechococcus* PSI are not in contact with other subunits (42). However, the stabilizing effect of the extended N terminus found in higher plant PSI-D indicates that the N terminus is in contact with intrinsic subunits. PSI-D in barley has been found to cross-link to PSI-H (7), which is an integral membrane protein located near PSI-I and PSI-L. Therefore, it has been suggested that the N-terminal domain of PSI-D exerts its stabilizing effect through interaction with PSI-H (14). Data showing a destabilization of PSI in the absence of PSI-H is in good agreement with this (21). The presence of two *psaD* genes raises the question of whether the two proteins have the same function. The PSI-D proteins are identical in the highly conserved C-terminal region and the differences are only found in the variable N-terminal region (Fig. 1) (43). Based on this, the two proteins are likely to have the same function in the PSI complex. However, it is possible that the two genes respond to different signal transduction pathways, thus allowing the plant cell to respond more flexibly to environmental stimuli. In *Nicotiana sylvestris* the genes for PSI-D1 and PSI-D2 are differentially expressed during leaf development (44).

**Plants with Down-regulated PSI-D Contain No Partially Assembled PSI Complexes—**Plants down-regulated in PSI-D had a lower Chl *a/b* and P700/Chl ratio (Table I) and contained less of all other PSI and LHCl proteins as judged by immunoblotting (Fig. 3, A and B) and low temperature fluorescence (Fig. 4). We therefore conclude that only fully functional PSI complexes with PSI-D accumulate. In contrast, a PSI-D knock-out mutant in *Synechocystis* accumulated wild-type levels of PSI-A/B (9). A similar discrepancy between prokaryotes and eukaryotes has been observed in mutants lacking PSI-C. Knock-out of the *psaC* gene resulted in the accumulation of a non-functional PSI complex (45), whereas a corresponding *Chlamydomonas* mutant showed a rapid turn-over of the PSI-A/B core subunits and no accumulation of PSI (46). Interestingly, in *Synechocystis*, the PSI complex without PSI-D is completely inactive with ferredoxin as acceptor, but is able to reduce flavodoxin at low rates (47). Plants do not contain flavodoxin and therefore the lethality of a complete down-regulation is not unexpected. In the absence of PSI-D in *Arabidopsis* the incomplete PSI complex is either rapidly degraded or it is possible that a feedback regulation in the absence of PSI-D lower the synthesis of other PSI subunits. As the PSI-E subunit becomes degraded in down-regulated PSI-D plants, it seems likely that at least some parts of the PSI are rapidly degraded (Fig. 3A). Considering the present knowledge about PSI assembly, the most obvious conclusion is that in the absence of PSI-D, the PSI-C protein cannot be properly assembled with PSI and this causes a degradation of the incorrectly assembled complexes.

Plants without PSI-D Exhibit Chronic PSII Photoinhibition—Severe PSII photoinhibition was evident already under low light conditions in the plants with reduced amounts of PSI-D, and even after 16 h of darkness the PSII quantum yield (*φ*psii) did not recover to wild-type levels (Fig. 5C). The photoinhibition of PSII is likely to be a result of the increased PSII excitation pressure, which will increase the risk of chlorophyll triplet formation and the generation of reactive oxygen species. The *Chlamydomonas* F8 mutant is defective in the splicing of *psaA* mRNA and has only 10% of the wild-type PSI level (48). This mutant is still able to fix CO2 (20% of wild-type) and grow photoautotrophically, although at a reduced rate (49, 48). The mutant, like the psi-d-less plants, shows increased sensitivity to photoinhibition under high light conditions (50).

With 10–20% PSI and 100 μmol m−2 s−1 of light, the level of photosynthesis in asD plants should be able to sustain plant growth, because normal *Arabidopsis* plants can easily adapt to one-fifth the usual light intensity. However, the NADP⁺-malate dehydrogenase measurements show that significant photosynthesis might only occur in the beginning of the light period in the PSI-D-less plants (Fig. 7). The activation level of NADP⁺-malate dehydrogenase is tightly linked to the redox state of thioredoxin (51). Apparently, photoinhibition is so pronounced after a few hours in light, that the electron transport rate is no longer able to maintain the redox power required to

---

**Fig. 7.** Activation level of NADP⁺-malate dehydrogenase. Leaves from wild-type (WT) plants and from plants with 10–20% PSI-D were harvested 15 min, 2 h, and 4 h after the onset of light in the growth chamber. The activity of NADP⁺-malate dehydrogenase was determined before and after fully activating the enzyme by exposing the protein extract for 40 min to 0.25 mM dithiothreitol and pH 9.
activate the thioredoxin system. Thioredoxin is one of the main switches for the initiation of carbon assimilation and the reduction level of thioredoxin has been reported to vary from 1 to 20% in darkness to around 90% in the light (51–53). In vivo regulation via changes in the thiol disulfide redox state has been strongly implicated in the light-dependent modulation of several chloroplast enzyme activities, including a number of Calvin cycle enzymes (54) and the chloroplast ATP synthase (CF1) (55). Hence, CO₂ fixation will be severely impaired in a Calvin cycle enzymes and the chloroplast ATP synthase regulation via changes in the thiol disulfide redox state has been reported to vary from 1 to 20% in darkness to around 90% in the light (51–53). Thioredoxin is one of the main switches for the initiation of carbon assimilation and the redistribution of the excitation energy caused by movement of LHClII from PSII to PSI is also regulated via thioredoxin. Phosphorylation of LHClII by a redox-regulated kinase is correlated with state transitions and the LHClII kinase is known to be inactivated by reduced thioredoxin under high light conditions (57). The less reduced thioredoxin in the antisense plants can therefore explain the higher level of phosphorylated LHClII. Fig. 3C clearly shows that LHClII is heavily phosphorylated in plants with 20 or 60% PSI-D left. The expression of LHClII genes and phosphorylation of LHClII correlate suggesting that the signal transduction pathways for these responses share components (58). This idea was confirmed in plants with low levels of PSI-F where LHClII was more phosphorylated as in the asD plants and the amount of LHClII was also increased (30). However, even though the antisense PSI-D plants resemble PSI-F mutants in many ways, e.g. less reduced thioredoxin level (results not shown), slow growth rate, high level of LHClII phosphorylation, and susceptibility to photoinhibition under normal growth conditions, the amount of LHClII was decreased in the asD plants (Fig. 3B). A decrease in antenna size may be expected in excess light (37) but the different response in antisense PSI-F and PSI-D plants could suggest that other factors than the thioredoxin reduction state and the activity of LHClII kinase determine expression of the LHClII genes. Future studies should reveal if the different response is controlled at the transcriptional or post-transcriptional levels.

The phosphorylation level of D1 was identical in wild-type and asD plants when dark-adapted (Fig. 3C), whereas the mobility shift of D1 observed during the light period (Fig. 3D) indicates increased phosphorylation of D1 in the light in the asD plants. Phosphorylation of D1 has been found to be stimulated by moderately thiol-reducing conditions (59), but our results are more indicative of a correlation with light or the redox level of the plastoquinone pool.

High NPQ in Plants with Reduced PSI-D Is a Response to Inefficient Electron Transport—As the amount of PSI is much lower than the amount of PSII, the plastoquinone pool becomes overreduced when PSI-D is reduced, and the steady state fluorescence is highly increased (Fig. 5B). In response to the perturbation in electron transport, the plants exhibit a high NPQ even at very low light intensities. The high NPQ is not surprising in light-stressed plants, but raises the question how plants permanently up-regulate qE under normal growth conditions. At high ΔpH, the light-harvesting complexes of PSII become protonated and bind zeaxanthin, causing the dissipation of absorbed light energy as heat (60, 61). A prerequisite for a permanently high qE is therefore that a large proton gradient across the thylakoid membrane is maintained. Cyclic electron transport via Fd-dependent plastoquinone reduction, which is dependent on the PsbS protein, has recently been shown to be very important for maintaining the proton gradient (62). Pseudocyclic electron transport also participates in maintaining a high ΔpH when electrons are donated by PSI to O₂ in a non-enzymatic reaction (63). The level of NDH-I is up-regulated in the antisense plants (Fig. 3C). NDH-I is part of the chloroplast NAD(P)H dehydrogenase complex, which appears to be involved both in cyclic electron transport and in chlororespiration (64–66). The up-regulation of NDH is likely to play a photoprotective role, because a similar increase has been observed in barley under photoinhibitory conditions (67). In line with this, more severe photodamage of PSII has been observed after high light treatment in tobacco plants with a defective NDH (68). However, despite the up-regulation of NDH, and possibly also of Pgr5, it is unlikely that the actual rate of cyclic electron transport is increased because the low PSI content and the oxidized stroma will limit cyclic electron transport around PSI. Pseudocyclic electron transport will also be limited, because of the low amount of PSI and the readily available oxidized ferredoxin. Hence, neither cyclic electron transport nor pseudocyclic electron transport is likely to be responsible for the large ΔpH that must be present in the antisense plants.

Normally CF₁ of the ATP synthase is activated under conditions when carbon fixation is possible, i.e. in the light. However, as the oxidation of thioredoxin in plants with 10–20% PSI-D might turn off the ATP synthase, this will allow the plants to maintain a proton gradient despite the reduced electron transport (69). Another essential component of the qE quenching is the PsbS protein, which is a member of the light harvesting protein family (70). The functional mechanism of PsbS is not understood but it has been suggested either to act as a sink, accepting excess excitation energy and releasing it as heat or, alternatively, to be involved in the reorganization of the LHC into a state of quenching (71). Despite the lack of mechanistic details, a positive correlation between the amounts of PsbS and qE has been shown (72), and the up-regulation of PsbS is likely to be a photoprotective response to the light stress experienced by the PSI-D-less plants.

CONCLUSION

The PSI-D protein is essential for photoautotrophic growth in higher plants. Low amounts of PSI-D cause the plants to grow much more slowly. Without PSI-D, PSI cannot be properly assembled and becomes degraded, which results in a reduced amount of PSI relative to PSII. The plants are severely photoinhibited in PSI-D at normal growth conditions as a result of increased excitation pressure. The lack of PSI-D also affects the redox state of thioredoxin. During the normal light cycle thioredoxin becomes increasingly oxidized and this might be fatal for the PSI-D-less plants, because thioredoxin is one of the main switches for the initiation of CO₂ assimilation and photoprotection. Thus, it is not the low amount of PSI per se that is almost lethal to the plant, because 10–20% PSI should be enough for reasonable plant growth. Instead, the direct cause of damage and decreased growth is the inability of the plants to down-regulate the PSI levels accordingly. Hence, it can be predicted that the asD plants would grow much better if they were in a mutant background with less efficient PSII or smaller light harvesting complexes.

Acknowledgments—We thank Prof. Birger Lindberg Møller for valuable discussions and Maria Jensen and Lis Hansen for technical assistance.

REFERENCES
1. Jansson, S. (1999) Trends Plant Sci. 4, 236–240
2. Chitnis, P. R. (2001) Annu. Rev. Plant Physiol. Plant Mol. 52, 593–628
3. Freymann, P., Jordan, P., and Krauss, N. (2001) Biochim. Biophys. Acta 1507, 5–31
4. Scheller, H. V., Jensen, P. E., Haldrup, L., Lunde, C., and Knoetzel, J. (2001) Biochim. Biophys. Acta 1507, 41–60
5. Jensen, P. E., Rosgaard, L., Haldrup, A., and Scheller, H. V. (2003) Physiol. Plant. in press
Plants Lacking PSI-D Are Chronically Photoinhibited

6. Jordan, P., Fromme, P., Witt, H. T., Klukas, O., Saenger, W., and Krauss, N. (2001) Nature 411, 899–916
7. Andersen, B., Koch, B., and Scheller, H. V. (1992) Physiol. Plant. 84, 154–161
8. Jansson, S., Andersen, B., and Scheller, H. V. (1996) Plant Physiol. 112, 409–412
9. Chitnis, P. R., Reilly, P. A., and Nelson, N. (1989) J. Biol. Chem. 264, 18381–18385
10. Lotan, O., Cohen, Y., Michaeli, D., and Nechushtai, R. (1993) J. Biol. Chem. 268, 16185–16189
11. Cohen, Y., and Nechushtai, R. (1992) FEBS Lett. 302, 15–17
12. Naver, H., Boudreau, E., and Rochaix, J. D. (2001) Plant Cell 12, 2731–2745
13. Minaí, L., Cohen, Y., Chitnis, P. R., and Nechushtai, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6338–6342
14. Naver, H., Scott, M. P., Andersen, B., Møller, B. L., and Scheller, H. V. (1995) Plant Physiol. 95, 19–26
15. Li, N., Zhao, J., Bryant, D. A., and Golbeck, J. H. (1995) Biochemistry 34, 7863–7872
16. Merati, G., and Zanetti, G. (1987) FEBS Lett. 215, 37–40
17. Zilber, A. L., and Malik, R. (1988) Plant Physiol. 88, 810–814
18. Pandini, V., Alverti, A., and Zanetti, G. (1999) Biochemistry 38, 10707–10713
19. Lagoutte, B., Hanley, J., and Botta, H. (2001) Plant Physiol. 126, 307–316
20. Bottin, H., Hanley, J., and Lagoutte, B. (2001) Biochem. Biophys. Res. Commun. 287, 830–836
21. Naver, H., Haldrup, A., and Scheller, H. V. (1999) J. Biol. Chem. 274, 10784–10789
22. Scheller, H. V., Lunde, C., Haldrup, A., and Jensen, P. E. (2003) in Functional Characterization of the Photosynthetic Apparatus in Arabidopsis thaliana (Leister, D., ed) The Haworth Press, Binghamton, in press
23. Varotto, C., Pesaresi, P., Meurer, J., Oelmüller, R., Steiner-Lange, S., Salamin, F., and Leistner, D. (2000) Plant J. 22, 115–124
24. Pesaresi, P., Lunde, C., Jahns, P., Tarantino, D., Meuer, J., Varotto, C., Hirtz, R. D., Saave, C., Scheller, H. V., Salamin, F., and Leistner, D. (2002) Planta 219, 940–948
25. Hagduksiewicz, P., Svah, Z., and Maliga, P. (1994) Plant Mol. Biol. 25, 898–904
26. Zambryski, P., Joos, H., Genetello, C., Leemann, J., Van Montagu, M., and Schell, J. (1983) EMBO J. 2, 2143–2150
27. Clough, S. J., and Bent, A. F. (1998) Plant J. 16, 735–743
28. Haldrup, A., Naver, H., and Scheller, H. V. (1999) Plant J. 17, 689–698
29. Kate, C. M., Hikosaka, K., Hirotsu, N., Makino, A., and Hirose, T. (2003) Plant Cell Physiol. 44, 318–325
30. Haldrup, A., Simpson, D., and Scheller, H. V. (2000) J. Biol. Chem. 275, 31211–31218
31. Lichtenthaler, K. H. (1987) Methods Enzymol. 148, 350–362
32. Schreibe, R., and Stitt, M. (1988) Plant Physiol. Biochem. 26, 473–481
33. Lunde, C., Jensen, P. E., Resgaard, L., Haldrup, A., Gilpin, M. J., and Scheller, H. V. (1999) Plant Physiol. 119, 89–94
34. Waterhouse, P. M., Wang, M. B., and Lough, T. (2001) Nature 411, 834–842
35. Andresson, J., Wentworth, M., Walters, R., Howard, C., Ruban, A., Horton, P., and Jansson, S. (2003) Plant J. in press
36. Krause, G. H., and Weiss, E. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 313–349
37. Niogi, K. K. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 333–359
38. Walters, R. G., and Horton, P. (1994) Planta 195, 245–256
39. Baena-González, E., Barbero, R., and Aro, E.-M. (1999) Planta 205, 196–204
40. Ashton, A. R., Trevanion, S. J., Carr, P. D., Verger, D., and Ollis, D. L. (2000) Plant Physiol. 119, 439–444