Cosuppression of Photosystem I Subunit PSI-H in Arabidopsis thaliana

EFFICIENT ELECTRON TRANSFER AND STABILITY OF PHOTOSYSTEM I IS DEPENDENT UPON THE PSI-H SUBUNIT

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Photosystem I (PSI) is a pigment-protein complex that mediates the light-driven electron transport across the thylakoid membrane from the soluble electron donor, plastocyanin, to the soluble electron acceptor, ferredoxin. PSI from plants contains 13 different subunits of which three are only found in plants, namely PSI-G, PSI-H, and PSI-N. The remaining 10 subunits are shared between cyanobacteria and plants. PSI-H is an intrinsic membrane protein of 10 kDa that is a subunit of photosystem I (PSI). PSI-H is one of the three PSI subunits found only in eukaryotes. The function of PSI-H was characterized in Arabidopsis plants transformed with a psaH cDNA in sense orientation. Cosuppressed plants containing less than 3% PSI-H are smaller than wild type when grown on sterile media but are similar to wild type under optimal conditions. PSI complexes lacking PSI-H contain 50% PSI-L, whereas other PSI subunits accumulate in wild type amounts. PSI devoid of PSI-H has only 61% NADP⁺ photoreduction activity compared with wild type and is highly unstable in the presence of urea as determined from flash-induced absorbance changes at 834 nm. Our data show that PSI-H is required for stable accumulation of PSI and efficient electron transfer in the complex. The plants lacking PSI-H compensate for the less efficient PSI with a 15% increase in the P700/chlorophyll ratio, and this compensation is sufficient to prevent overreduction of the plastocyanin pool as evidenced by normal photochemical quenching of fluorescence. Nonphotochemical quenching is approximately 60% of the wild type value, suggesting that the proton gradient across the thylakoid membrane is decreased in the absence of PSI-H.

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1 The abbreviations used are: PS, photosystem; LHC, light harvesting complex; Chl, chlorophyll.
thermore, PSI-H is required for interaction with PSI-L, stabilization of F_{X}, and the overall stability of the PSI complex.

**EXPERIMENTAL PROCEDURES**

**Plant Material—Arabidopsis thaliana L. cv. Colombia ecotype 0** were grown in a Percival Arabidopsis chamber at 20 °C, 70% relative humidity, and illumination of 100 μmol m^{-2} s^{-1}. Wild type plants used for transformation were grown with a 12-h photoperiod, whereas transformants were grown with an 8-h photoperiod to keep plants at the vegetative state as long as possible. Transformants were germinated on MS medium (Sigma) containing 2% sucrose, 50 mg/liter kanamycin, and 0.9% agar and transferred to soil after approximately 4 weeks.

**Vector Construction and Arabidopsis Transformation—** The psaH cDNA from Arabidopsis was obtained from the Arabidopsis Biological Resource Center (Columbus, OH) as estat12892. A segment corresponding to the entire coding region was polymerase chain reaction amplified and cloned into the BamHI and StuI sites of the binary vector pBIN121 (18). In this way the GUS gene of pBIN121 was replaced by the full-length psaH in sense orientation. The vector was transformed into Agrobacterium tumefaciens strain C58 (19). Arabidopsis was transformed by vacuum infiltration according to Bechtold et al. (20).

**Southern Blot Analysis—** Arabidopsis genomic DNA was isolated with the DNeasy kit (Amer sham, UK). The DNA (10 μg) was digested with BamHI, and the resulting fragments were separated by agarose gel electrophoresis and transferred onto nylon membrane (Boehringer Mannheim). The membranes were hybridized with a digoxigenin-labeled psaH polymerase chain reaction fragment in DIG Easy hybridization buffer according to the DIG System manual (Boehringer Mannheim). The membrane was washed in 0.5% SSC containing 1% SDS at 65 °C.

**Immunoblotting and Thylakoid Preparations—** All plant extracts and fluorescence measurements were carried out with plants grown in soil. Only fully expanded rosette leaves of comparable size from nonflowering plants were used for these analyses. Whole leaf extracts and thylakoids were prepared and analyzed by immunoblotting as described previously (14). Chl a/b ratio was determined according to Lichtenthaler (21). The thylakoid membrane was determined from absorption transients at 834 nm (see “Urea Treatment of Thylakoids and Flash-induced Absorbance Measurements”).

Thylakoids were solubilized by applying decylmaltoside to a final concentration of 0.15% to a sample adjusted to a final concentration of 100 μg Chl/ml. The solubilized thylakoids were centrifuged at 300 × g for 3 × 1 min, and the white pellet of starch was discarded.

**NADP+ Photoreduction Measurements—** NADP+ photoreduction activity was determined as described by Kjær and Scheller (22) from the absorbance change at 340 nm in a 0.5-ml reaction mixture containing 20 mM Tricine (pH 7.5), 7 mM MgCl₂, 40 mM NaCl, 2 mM sodium ascorbate, 60 μM 2,6-dichlorophenolindophenol, 0.5 mM NADP⁺, 50 mM ferredoxin, NADP⁺ oxidoreductase, 2 μM plastocyanin, 0.0125–5 μM ferredoxin, and solubilized thylakoids equivalent to 5 μg of Chl. Plastocyanin, ferredoxin, and NADP⁺ oxidoreductase were purified from barley.

**Urea Treatment of Thylakoids and Flash-induced Absorbance Measurements—** Flash-induced absorbance transients at 834 nm were determined as described previously (9, 23). The cuvette contained solubilized thylakoids (100 μg Chl/ml), 2 mM sodium ascorbate, and 60 μM 2,6-dichlorophenolindophenol. Solid urea was then added to a final concentration of 6.5 M, and the cuvette was incubated at room temperature and subjected to flash-induced absorbance measurements at different time points during the incubation. Apart from actinic flashes, the sample was kept dark during incubation.

**Fluorescence Quenching Analysis—** In vivo chlorophyll fluorescence was measured at room temperature using a PAM-101/103 fluorometer (Walz, Effeltrich, Germany). 12-week-old plants were dark-adapted for 30 min prior to the measurements. The initial fluorescence in the dark-adapted state (F₀) was determined using a very dim red light modulated at 1.6 kHz. The maximal level of fluorescence (F₅₀) was induced by an 800-ms pulse of saturating white light (>4000 μmol m⁻² s⁻¹). The maximum quantum yield of PSII was estimated in dark-adapted leaves as (F₅₀ - F₀)/F₅₀. The steady state fluorescence was measured during illumination at approximately 5–10 and 100 μmol m⁻² s⁻¹ with a KL1500 halogen lamp (Schott). During actinic illumination, the measuring light was modulated at 100 kHz. The fluorescence was allowed to stabilize over 10 min, and maximum fluorescence yield F₅₀ was then determined by giving a second 800-ms pulse. Finally, actinic light was turned off, and minimum fluorescence in the light-adapted state (Fₒ) was determined. The photochemical and nonphotochemical quenching coefficients were calculated as qₙ = (F₅₀ - F₀) / F₅₀ and qₑ = 1 - (F₅₀ - F₀)/(F₅₀ - Fₐ) (24), where qₑ indicates photochemical fluorescence quenching and qₙ indicates nonphotochemical fluorescence quenching.

**RESULTS**

**Cosuppression of the PSII-H Polypeptide—** A total of 26 transgenic plants (T₁) with kanamycin resistance and containing the psaH cDNA were produced. Leaf extracts of the T₁ plants were analyzed by immunoblotting, but no substantial down-regulation of PSII-H was observed in any of the plants. The original 26 T₁ plants were self-fertilized, and the seeds were reselected on kanamycin. Five to ten T₂ plants from each T₁ plant were analyzed by immunoblotting of leaf extracts. PSII-H was undetectable in the plants designated 15.2 and 20.2 (detection level, ~3% of wild type level) and was substantially down-regulated in plants 20.5, 3.2, and 3.4 (Fig. 1A). T₂ progeny of plant 15.2 was not down-regulated, whereas 75% of the progeny of plant 20.2 lacked PSII-H (Fig. 1B). Further, 10% of the progeny of 3.4 and 5% of the 3.2 and 20.5 progeny lacked PSII-H (Fig. 1B). Progeny from the remaining T₁ plants did not show substantial down-regulation of PSII-H and were not further analyzed.

Genomic DNA of T₂ progeny of T₁ plants numbered 3, 15, and 20 was digested with BamHI, which cleaves at the 5’ end of the inserted psaH. The blot was hybridized with a psaH-specific probe (Fig. 2). The blot shows that wild type plants have two copies of psaH that are also found in all the transformants. Several Arabidopsis EST sequences are available that clearly contain psaH sequence, and these are easily divided into two groups. Sequence alignment shows that Arabidopsis has two expressed psaH genes with coding sequences that are 97% identical and that encode proteins with identical amino acid sequences (data not shown). Southern blot analysis showed that the T₂ generation of the transformants contained 2–4 copies of inserted psaH in addition to the two wild type genes (Fig. 2). Because of the variation in down-regulation, all plants were analyzed by immunoblotting at the time they were used for other experiments. However, the initial analysis of leaf extracts of small plants was confirmed by immunoblotting of three different thylakoid preparations of the same plants performed within 1 week to 4 weeks. Thus, cosuppression of the PSI-H subunit seemed stable throughout the life of individual plants despite the variation between plants (data not shown).

**Plants Lacking PSI-H Grow Poorly on Agar Medium and Have Diminished Nonphotochemical Quenching—** Growth and development of plants lacking PSI-H were similar to wild type plants when grown in soil. In contrast, the plants clearly

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**FIG. 1. Identification of plants lacking PSII-H by immunoblotting.** Total leaf protein equivalent to 1.5 μg of Chl was loaded in each lane of the gel. The blots were developed with antibodies against PSII-H and PSII-F. A, analysis of T₁ plants 3.2 and 3.4 were progeny of T₁ plant 3, 20.2–20.5 were progeny of the T₁ plant 20, and 15.2 were progeny of the T₁ plant 15. B, examples of analysis of T₂ progeny of the T₁ plants shown in A. Due to variation in the development of the blots, the content of PSII-H and PSII-F in each sample should only be compared with the wild type (WT) sample from the same blot.
The subunits that are close to PSI-H, were probed with antibodies directed against PSI-H, the isolated thylakoids were subjected to immunoblot analysis to analyze the polypeptide composition of a PSI complex lacking PSI-H. These numbers are significantly different (p < 0.05). No significant change in qP, which reflects the steady state redox level of quinone QA in PSII and thus reflects the redox state of the plastoquinone pool, was observed in plants lacking PSI-H. However, qN, which largely reflects energy-dependent quenching due to the proton gradient across the thylakoid membrane was decreased to about 60% of wild type levels.

PSI fluorescence emission spectra determined on leaves at 77 K were identical in plants lacking and containing PSI-H (25). Furthermore, the functional antenna size of PSI is unaffected by the lack of PSI-H as evidenced by measurements of P700 absorption changes in leaves illuminated with varying intensity of far red light (25).

Absence of PSI-H Affects Binding of PSI-L to the PSI Complex—The Chl/P700 ratio (\(\pm\) S.E., n = 7–14) was 696 ± 32 in wild type thylakoids and 606 ± 17 in thylakoids from plants devoid of PSI-H. These numbers are significantly different (t test, p < 0.05) and indicate that plants devoid of PSI-H compensate for the deficiency by increasing the PSI/PSII ratio. To analyze the polypeptide composition of a PSI complex lacking PSI-H, the isolated thylakoids were subjected to immunoblotting. The blots were probed with antibodies directed against: PSI-C, -A/B, and -N and Lhca1, but this was not consistently observed and was not correlated with the content of PSI-H. Similar blots were incubated with antibodies against PSI-D, -F, -H, -I/J, and -L. The PSI-I/J antibody recognizes both PSI-I (upper band) and PSI-J (lower band).

Electron Transfer Rate Is Decreased in the Absence of PSI-H—To analyze the importance of PSI-H for electron transfer, NADP+ photoreduction was determined. At standard assay conditions of 3 \(\mu\)mol ferredoxin, NADP+ reduction of thylakoids from plants devoid of PSI-H was only 61% of wild type NADP+ reduction (Fig. 5). The analyzed thylakoids contained only intact PSI complexes with respect to the electron acceptor composition as shown by flash photolysis (see Fig. 6A; 0 h of urea treatment). Therefore, the observed decrease in NADP+ reduction of PSI lacking PSI-H must be explained by a corresponding decrease in forward electron transfer rate. As the ferredoxin concentration became rate-limiting for the measured NADP+ reduction at a ferredoxin concentration below 1 \(\mu\)mol, the difference between thylakoids without PSI-H and wild type disappeared (Fig. 5). Thus, the second order rate constant for ferredoxin reduction appears to be unchanged in PSI lacking PSI-H.

**TABLE I**

| 5–10 \(\mu\)mol photons | 100 \(\mu\)mol photons |
|--------------------------|-----------------------|
| Wild type PSI-H-less     | Wild type PSI-H-less  |
| \(\Phi_p\)               | \(\Phi_p\)             |
| 0.79 ± 0.01              | 0.80 ± 0.01           |
| 0.07 ± 0.01              | 0.77 ± 0.01           |
| 0.02 0.91               | 0.03 0.91             |
| \(q_p\)                 | \(q_p\)               |
| 0.89 ± 0.03              | 0.94 ± 0.01           |
| 0.91 ± 0.02              | 0.92 ± 0.01           |
| 0.01 0.26               | 0.01 0.15             |
| \(q_N\)                 | \(q_N\)               |
| 0.16 ± 0.02              | 0.26 ± 0.01           |
| 0.10 ± 0.01              | 0.15 ± 0.02           |

**FIG. 2.** Southern blot analysis of transformants (T2). Total DNA from wild type and transformants (designated by numbers) was digested with BamHI, which cleaves at the 5’ end of the inserted psaH clone, electrophoresed, transferred to nylon membrane, and hybridized with a psaH probe. T2 plants have two to four copies of psaH in the genome in addition to the two wild type (WT) genes. Sizes of marker DNA are shown in kb (lane M).

**FIG. 3.** Phenotype of plants lacking PSI-H (20.2) compared with wild type (WT). Plants (6–7 weeks old) were grown on Murashige and Skoog (MS) medium containing 2% sucrose, at 20 °C and 70% humidity for an 8-h photoperiod at 100 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\).

**FIG. 4.** Immunoblot analysis of accumulation of PSI subunits in wild type and plants lacking PSI-H. Thylakoids were prepared from different plants lacking PSI-H (20.2.2, 20.5.9, 3.2.9, and 3.4.17), and wild type (WT) and samples of protein equivalent to 0.5 \(\mu\)g of Chl were loaded on SDS gels. The blots were incubated with antibodies against PSI-D, -F, -H, -I/J, and -L. The PSI-I/J antibody recognizes both PSI-H (upper band) and PSI-J (lower band).
sum of exponential decays with the characteristic time constants. For a discussion of recombination rates see Refs. 26 and 27.

Both PSI complexes containing and lacking PSI-H were intact before the addition of urea as evidenced by the observation that the 30-ms phase was the single component of the absorbance decay (Fig. 6A). Urea was then added to the samples to a final concentration of 6.5 M, resulting in a slow dissociation of the PSI complex. After 1 h of urea treatment, the 1-ms phase was visible in the wild type PSI complex and the 50-μs phase in the complex without PSI-H (Fig. 6B). The 50-μs signal increased in PSI without PSI-H to become the major component after 2 and 4 h of urea treatment (Fig. 6, C and D). The curve traces for the wild type PSI showed increasing amounts of the $F_X$ to P700$^-$ 1-ms phase. However, faster recombinations indicating the destruction of $F_X$ were not observed (Fig. 6, B, C, and D).

Urea treatments were carried out with three samples of thylakoids from each type of plant. The absorption decay components calculated for each time point were averaged and plotted as a function of time (Fig. 6E). The sum of the wild type components was adjusted to 100%, and the phases of the components was normalized according to this. The 30-ms component ($F_A/F_{RH}$ to P700$^-$) of the wild type increased with the duration of urea treatment and was replaced by the 1-ms phase (Fig. 6E). However, in the absence of PSI-H, the 30-ms component decreased about twice as fast as in the wild type. Thus, PSI-C with $F_A/F_{RH}$ is released approximately twice as fast from PSI when PSI-H is absent compared with the release of PSI-C in the wild type samples. The 1-ms component in the samples lacking PSI-H replaced the 30-ms phase for a short period but was then replaced by the 50-μs phase ($A'_1$ to P700$^-$). This fast component was essentially absent in the wild type. Thus, $F_X$ is much less stable in the absence of PSI-H. After 4 h of urea treatment the sum of the three decay components in the absence of PSI-H was only 21% of the wild type. The remaining 79% represents urea-degraded PSI complex where no P700 absorption changes could be detected with the time resolution of the measurement. Thus, prolonged urea treatment of PSI lacking PSI-H leads to inactivation of electron acceptors earlier than $F_X$ or possibly damage to P700 itself.

**DISCUSSION**

The cosuppression strategy in *Arabidopsis* was successful and yielded plants without detectable PSI-H. This results has enabled us to investigate the function of the PSI-H subunit *in vivo* as well as *in vitro*. Although the approach was successful, the frequency of substantial down-regulation in the transformed plants was low, and no plants lacking PSI-H were obtained in the T1 generation. It has previously been reported that down-regulation of LHCl in an antisense approach was not observed, although the mRNA level was reduced to extremely low levels (28). On the other hand, down-regulation of the PSI-N subunit was very efficient in a similar cosuppression and antisense approach (14), and the successful down-regulation of the LHCl subunit Lhca4 by an antisense approach was recently reported (29). Possibly, the difficulty in obtaining down-regulation of PSI-H is related to the presence of two expressed copies of *psaH* in *Arabidopsis*. In contrast, PSI-N is expressed from a single gene. Gene knock-out by homologous recombination is an excellent way of studying the role of PSI subunits in cyanobacteria. However, in plants this technique is not yet straightforward. The present results show that PSI subunits can be efficiently down-regulated in plants by cosuppression. Thus, this approach can be very useful in dissecting the role of individual components of PSI.

During growth on sterile medium, plants without PSI-H showed pronounced stunting of growth and yellowing of leaves (Fig. 3). Surprisingly, however, no difference in visual appear-
Forward Electron Transport Is Decreased in Plants Lacking PSI-H—Steady state electron transport is clearly decreased in PSI lacking PSI-H. The decrease does not appear to be due to heterogeneity of PSI in the absence of PSI-H because there is no difference at low ferredoxin concentration. The interaction between ferredoxin and PSI is kinetically complex (12, 30). Ferredoxin forms a complex with PSI in both the reduced and oxidized form. Intracomplex electron transfer is heterogeneous and takes place with several different time constants. At ferredoxin concentrations below the dissociation constant for the PSI-ferredoxin complex, the limiting factor for electron transfer is the second order rate constant. Thus, this rate constant appears to be unaltered in PSI lacking PSI-H. At higher ferredoxin concentration, intracomplex electron transfer may become limiting, and the lower rate in the absence of PSI-H suggests that intracomplex electron transfer is affected. This may be due to different rate constants of transfer or to different relative contributions of the different time constants. The situation in PSI lacking PSI-H may resemble the situation in cyanobacterial PSI where the rate of ferredoxin reduction is much lower than in plant PSI, at least in a large fraction of complexes (30). In principle, the difference at high ferredoxin concentration could also be due to another limiting step in electron transfer. However, we find this unlikely. Forward electron transfer from P700 to Fy/Fz is very fast compared with the rates of NADP⁺ reduction, and the interaction between plastocyanin and PSI appears to be largely governed by PSI-F and PSI-N. Nevertheless, further detailed investigations of different steps of electron transport will be necessary to determine the precise mechanism by which PSI-H affects electron transport. A likely explanation is that lack of PSI-H perturbs the binding of PSI-D and PSI-C, causing a less productive binding of ferredoxin.

Plants Lacking PSI-H Accumulate More PSI—The plants devoid of PSI-H have compensated by synthesizing more PSI as evidenced by the 15% lower Chl/P700 ratio. This compensation is sufficient under optimal conditions, where lack of PSI-H had little impact on plant growth. The lower efficiency of PSI might be expected to lead to overreduction of the electron transport components connecting PSI and PSII. However, again the compensation seems sufficient because no significant increase in qP was observed. Furthermore, the size of the functional PSI antenna is identical in plants with and without PSI-H, at least in state 1 (25). Plants lacking PSI-H show less nonphotochemical quenching of fluorescence, which may indicate a smaller transthyllakoidal proton gradient. A smaller proton gradient could result from less overall electron transport. However, because the plants do not exhibit differences in growth, we do not think that this is likely. Alternatively, the less efficient reduction of ferredoxin may lead to altered redox levels in the stroma with a resulting decrease in cyclic electron transport and therefore a decrease in proton pumping. Finally, it may be imagined that lack of PSI-H lead to altered permeability of the membrane for protons.

The lower scope for PSI activity and the lower nonphotochemical quenching in the absence of PSI-H may have little significance under optimal and constant growth conditions. However, under photoinhibitory conditions it may be predicted that plants lacking PSI-H would suffer more severely from overreduction of plastocyanine. Other stress condition such as low light intensity or conditions where the demand for ATP is increased may also be expected to lead to more severely affected plants.

PSI-H Stabilizes PSI—A substantial decrease in stability of PSI was observed in thylakoids from plants without PSI-H. The relative instability resembles the situation in cyanobacteria, where urea has a much faster and stronger effect on PSI than in plants. We have hypothesized that PSI-H interacts with the N-terminal extension of PSI-D which is important for the high stability of plant PSI (8). The lower stability of the PSI complex lacking PSI-H is therefore in good agreement with the hypothesis. Future experiments with in vitro reconstitution in the presence or absence of PSI-H should allow us to test the hypothesis further and investigate the interaction of PSI-H with PSI-D and other subunits in detail. The instability of Pφ and earlier acceptors was a surprising result because PSI-H is unlikely to be directly involved in coordinating electron acceptors. Possibly, lack of PSI-H leads to a general instability of the PSI complex in the presence of urea and a progressive disintegration of the entire complex. Although the instability of PSI was easily observed in the in vitro experiments, no severe disintegration of the PSI complex appears to take place during normal growth because all electron acceptors were functional in the thylakoids lacking PSI-H. However, the partial lack of PSI-L suggests a lower stability of the complex also under in vivo conditions. Possibly, more severe disintegration of PSI could occur under certain stress conditions, e.g. heat stress. Because PSI-L content is decreased, it can be speculated whether the role of PSI-H in electron transport is mediated through PSI-L. We find this unlikely because PSI-L is relatively far removed from the site of interaction of the soluble electron transfer proteins (4). However, experiments with plants lacking only PSI-L will be required to address this issue.

Conclusion—In summary, PSI-H has been shown to be not important for LHCl interaction with PSI (25) but to be essential for efficient electron transfer of PSI and for stability of the PSI complex. The cyanobacterial PSI complex is dissociated eight times faster in the monomeric than in the trimeric form as shown by urea treatment of monomers and trimers (4, 31). Plant PSI is a monomer that is more stable upon urea treatment than the trimeric cyanobacterial PSI (8). The peripheral antenna in cyanobacteria consists of extrinsic phycobiliproteins. In contrast, plants have adopted a different antenna consisting of membrane intrinsic Chl a/b-binding proteins, i.e. LHCl and LHClI. It can be speculated that the trimeric structure of PSI was abandoned as LHCl became associated with PSI in plants, and this resulted in a need for stabilizing the now monomeric PSI complex. PSI-H may have evolved simultaneously, fulfilling the role as a stabilizing factor.

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