A Thylakoid Membrane Protein Harboring a DnaJ-type Zinc Finger Domain Is Required for Photosystem I Accumulation in Plants*5

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Background: Photosystem I is a large protein/pigment assembly required for photosynthesis.

Results: The PSA2 protein harbors a DnaJ-type zinc finger domain, is required for Photosystem I accumulation, and is found in a Psag-containing complex in the thylakoid lumen.

Conclusion: PSA2 promotes Photosystem I biogenesis via interaction with a Psag-containing complex in the thylakoid lumen.

Significance: These findings elucidate the biogenesis of the photosynthetic apparatus.

Photosystem I (PSI) is a large pigment-protein complex and one of the two photosystems that drive electron transfer in oxygenic photosynthesis. We identified a nuclear gene required specifically for the accumulation of PSI in a forward genetic analysis of chloroplast biogenesis in maize. This gene, designated psa2, belongs to the “GreenCut” gene set, a group of genes found in green algae and plants but not in non-photosynthetic organisms. Disruption of the psa2 ortholog in Arabidopsis likewise resulted in the specific loss of PSI proteins. PSA2 harbors a conserved domain found in DnaJ chaperones where it has been shown to form a zinc finger and to have protein-disulfide isomerase activity. Accordingly, PSA2 exhibited protein-disulfide reductase activity in vitro. PSA2 localized to the thylakoid lumen and was found in a ~250-kDa complex harboring the peripheral PSI protein Psag but lacking several core PSI subunits. PSA2 mRNA is coexpressed with mRNAs encoding various proteins involved in the biogenesis of the photosynthetic apparatus with peak expression preceding that of genes encoding structural components. PSA2 protein abundance was not decreased in the absence of PSI but was reduced in the absence of the PSI assembly factor Ycf3. These findings suggest that a complex harboring PSA2 and Psag mediates thiol transactions in the thylakoid lumen that are important for the assembly of PSI.

Photosystem I (PSI) is one of the two photosystems that drive oxygenic photosynthesis and is among the most complex macromolecular structures known in nature (1). In land plants, the PSI core complex consists of at least 16 different polypeptides (PsaA–P), three Fe-S clusters, and at least 173 chlorophyll molecules; this core associates with a peripheral light-harvesting complex consisting of four additional proteins (Lhca–Lhca4) and numerous pigments (2). The biogenesis of PSI in chloroplasts is further complicated by the fact that the genes encoding its subunits are distributed between the nuclear and plastid genomes: PsaA, PsaB, PsaC, Psal, and Psal are encoded in the chloroplast, whereas the remaining subunits are nucleus-encoded (3). In higher plants, PSI biogenesis is coupled to leaf development. This process necessitates the coordinated assembly of subunits and prosthetic groups at the thylakoid membrane where PSI is situated. Several factors have been described that are required for the assembly of PSI but that are not found in the mature complex; these include the chloroplast-encoded proteins Ycf3 and Ycf4 (4–6) and the nucleus-encoded proteins Y3IP1 (7), Pyg7 (8), and PP1 (9,10). Additional proteins mediate the association of prosthetic groups with PSI subunits (11–14). Despite this progress, many aspects of PSI biogenesis remain poorly understood (3, 15).

To thoroughly characterize the nuclear gene complement required for the biogenesis of the photosynthetic apparatus, our groups are taking (i) a large scale forward genetic approach to identify nuclear genes required for photosynthesis in maize (16, 17) and (ii) a large scale reverse genetic analysis of genes that are conserved in photosynthetic organisms but absent from non-photosynthetic organisms, the so-called “GreenCut” gene set (18, 19).

EXPERIMENTAL PROCEDURES

Mutant Isolation and Plant Growth—The maize psa2 mutation is a recessive allele conditioning a pale green, non-photosynthetic phenotype that arose in maize lines harboring active Mu transposons. Heterozygotes were outcrossed to inbred lines, and the F1 progeny were then self-pollinated to recover ears segregating homozygous mutants. DNA was isolated from
mutant individuals from each of four F2 ears and analyzed by 
\textit{Mu} Illumina sequencing to map all of the \textit{Mu} insertions in each 
plant (16). An insertion in GRMZM2G021687 was found in all 
four mutant individuals; subsequent gene-specific PCR showed 
this insertion to be absent from closely related +/+ ears. maize 
seeds used for phenotypic analysis were grown in a growth 
chamber in 16-h light (120 \mu mol of photons m^{-2} s^{-1}), 8-h dark 
cycles. For exposure to high light conditions, illumination was 
increased to 800 \mu mol of photons m^{-2} s^{-1}.

Three tDNA insertions in the \textit{Arabidopsis} \textit{psa2} ortholog 
(AT2G34860) were obtained: \textit{At-psa2-1} (GABI_475C12 Col 
background) and \textit{At-psa2-2} (GABI_314G07 Col background) 
came from the GABI-Kat collection, and \textit{At-psa2-3} (SK32878 
Col background) came from the Sasaki collection. Plants 
were grown in a growth chamber under long day conditions 
(16-h light, 8-h dark) at 120 \mu mol of photons m^{-2} s^{-1}. For 
growth on sucrose-containing synthetic medium, seeds were 
surface-sterilized and plated on Murashige and Skoog agar 
medium containing 0.8% sucrose.

\textbf{Analysis of Chloroplast Gene Expression}—The expression of 
all chloroplast genes in maize \textit{psa2} mutants was analyzed with a 
ribosome profiling method that provides a genome-wide and 
quantitative readout of chloroplast ribosome positions \textit{in vivo} 
(20). The method uses high resolution microarrays to compare 
the positions and abundance of “ribosome footprints” in 
mutant and wild-type samples. The results report differences in 
both mRNA abundance and ribosome occupancy for every 
chloroplast gene.

\textbf{Chloroplast Isolation and Fractionation}—4-week-old \textit{Arabi-
dopsis} or 2-week-old maize plants were used for the prepara-
tion of chloroplasts and thylakoid membranes. 2 g of \textit{Arabi-
dopsis} leaf tissue or the leaves from three maize seedlings were 
collected either after 4 h of dark or after 3 h of illumination (120 
\mu mol of photons m^{-2} s^{-1}). Chloroplasts, thylakoid mem-
branes, and subthylakoid fractions were isolated as described 
previously (21, 22). The luminal fraction was recovered as the 
supernatant after treating isolated thylakoid membranes with 
0.2% Triton X-100 for 2 min on ice followed by centrifugation at 
140,000 \times g for 10 min.

\textbf{Antibodies}—An antibody specific for \textit{PSA2} was produced by 
immunization of rabbits with a recombinant protein correspond-
ing to amino acids 87–186 of \textit{Arabidopsis} \textit{PSA2} (Agrisera 
AS132654). Antibodies against \textit{D1}, \textit{D2}, \textit{Lhcb1}, \textit{Lhca1}, \textit{Lhca2}, 
and \textit{CP43} were obtained from Agrisera. Antibodies against \textit{CF1} 
were generated by immunization with the purified \textit{CF1} com-
plex from spinach (23). \textit{PsaA-} and \textit{PsaF-specific} antibodies were 
kindly provided by Dr. Jean-David Rochaix (University of 
Geneva). \textit{PsaG-} and \textit{PsaK-specific} antibodies were kindly pro-
vided by Dr. Poul Erik Jensen (University of Copenhagen). Anti-
body to the Rubisco large subunit (\textit{RbcL}) was a gift from Dr. 
Steve Rodermel (Iowa State University). Antibodies to \textit{PsdA} 
and cytochrome \textit{f} were described previously (24, 25). Antibody to 
\textit{PPD1} was generously provided by Johnna Roose and Terry 
Bricker (Louisiana State University).

\textbf{Protein Analyses}—Blue native polyacrylamide gel elec-
rophoresis (BN-PAGE) of thylakoid membranes was performed 
as described (26). Prior to electrophoresis, thylakoid mem-
branes were solubilized by incubation on ice (10 min) in 20% 
(w/v) glycerol, 25 mM BisTris-HCl, pH 7.0, 0.75% (w/v) n-dode-
cyl \textit{\beta}-\textit{d}-maltoside (DM) at a chlorophyll concentration of 2 
mg/ml. For immunoblot assays, proteins were separated by 
SDS-PAGE in the presence of 6 M urea or by BN-PAGE, elec-
trophoretically transferred to polyvinylidene difluoride (PVDF) 
membranes (Immobilon, Millipore), and then probed with the 
antibodies described above. Antibodies were detected with 
horseradish peroxidase-conjugated secondary antibody and 
the SuperSignal WestPico HRP detection kit (Thermo 
Scientific).

For immunoprecipitation, chloroplasts from 3-week-old 
\textit{Arabidopsis} plants were resuspended in solubilization buffer 
(20 mM Tricine-NaOH, pH 7.8, 5 mM MgCl$_2$, 100 mM sucrose, 
10 mM DM) and incubated on ice for 15 min. Particles were 
removed by centrifugation at 4 °C at 14,000 \times g for 20 min. 
Pierce cross-link magnetic beads were coupled to \textit{PSA2} anti-
body according to the manufacturer’s instructions (Pierce 
Crosslink Magnetic IP/Co-IP kit 88805). Immunoprecipita-
tions were performed by incubating the antibody-coupled 
beads with 400 \mu l of solubilized chloroplasts at a protein 
concentration of 2 \mu g/\mu l for 60 min with slow rotation at room 
temperature. The beads were washed, and the bound proteins 
were eluted according to the manufacturer’s instructions.

\textbf{Chlorophyll Fluorescence Analyses}—Plants were dark-adapted for 
20 min before each experiment. Fluorescence parameters 
were measured using a JTS-10 light-emitting diode spectrom-
eter (Bio-Logic Scientific Instruments) and calculated as 
follows: \(F_F/F_m = (F_m - F_0)/F_m\) where \(F_v\) is the calculated variable 
fluorescence, \(F_m\) is the maximal fluorescence measured imme-
diately after the saturating pulse, and \(F_0\) is the initial fluores-
cence of dark-adapted tissues. Data from different samples 
were normalized with JTS10 spectrophotometer software (Bio-
Logic) using default parameters. The kinetics of PSI photooxi-
dation were measured on detached leaves in absorbance detect-
tion mode. P700 was oxidized using 10-ms actinic flashes of 
far-red light-emitting diodes and a 705-nm interference filter. 
Baseline absorbances were obtained without actinic illumina-
tion and subtracted.

\textbf{Assaying Protein-disulfide Reductase Activity}—The ability of 
\textit{PSA2} to catalyze the reduction of disulfide bonds was tested 
with an assay for insulin precipitation that is often used as a 
proxy for protein-disulfide isomerase activity (27). Reactions 
contained 0.13 mM insulin (Sigma-Aldrich), 0.1 mM BSA, 0.1 mM 
sodium phosphate, pH 7.0, 2.5 mM EDTA, and a 1 \mu M concen-
tration of either \textit{PSA2} or \textit{RBF1}. Reactions were initiated by 
adding dithiothreitol to a final concentration of 100 \mu M. Insulin 
precipitation was monitored by the increase in absorbance at 
650 nm (27). \textit{RBF1}, a ribosome-binding protein that contains 
no cysteines in the mature sequence (21), was used as a control.

\textbf{RESULTS}

The \textit{GreenCut Gene psa2} Is Required for the Accumulation of 
Photosystem I in Maize and \textit{Arabidopsis}—The \textit{psa2} gene was 
identified during the systematic analysis of mutants in the 
Photosynthetic Mutant Library, a large collection of chloroplast-
defective mutants that arose in maize lines with active \textit{Mu} 
transposons (17). The homozygous mutants are pale green (Fig. 
1A) and die after the development of three leaves as is typical of
Lumenal Protein Required for Photosystem I Biogenesis

non-photosynthetic mutants in maize. These phenotypes were tentatively ascribed to a specific loss of PSI based on an initial immunoblot survey that probed the abundance of one core subunit of PSI, Rubisco, photosystem II (PSII), the cytochrome \(b:f\) complex, and the chloroplast ATP synthase. To identify the insertion responsible for these phenotypes, we used a deep sequencing method to sequence \(T\)DNA insertion sites, and the sequences flanking each insertion are shown below. The insertion in \(At-psa2-1\) is 39 base pairs upstream of the start codon and is followed by a nine-base pair deletion. The insertion in \(At-psa2-3\) is 799 base pairs downstream of the start codon and is followed by a deletion of five base pairs. The deleted nucleotides are shown in bold, underlined italic font.

FIGURE 1. Maize and Arabidopsis \(psa2\) mutants. A, maize \(psa2-1\) mutant. Plants were grown for 8 days in soil. The position of the \(Mu\) insertion is shown above with the nine-base pair target site duplication underlined. This insertion is 52 base pairs upstream of the start codon. B, Arabidopsis \(psa2\) mutants. Triangles mark \(T\)DNA insertion sites, and the sequences flanking each insertion are shown below. The insertion in \(At-psa2-1\) is 39 base pairs upstream of the start codon and is followed by a nine-base pair deletion. The insertion in \(At-psa2-3\) is 799 base pairs downstream of the start codon and is followed by a deletion of five base pairs. The deleted nucleotides are shown in bold, underlined italic font. Plants were grown at a photon flux density of 120 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (16-h light, 8-h dark) for 21 and 14 days (soil and sucrose, respectively). Scale bars, 1 cm.
and 300 kDa. This pattern matches that reported for Arabidopsis ppd1 mutants, which specifically lack PSI due to a defect in PSI assembly (9). The upper band corresponds in position to monomeric PSI (9). The lower band comigrates with a small fraction of PsaA; although the band at this position is often described solely as PSII monomer, a PSI-related complex has been detected previously at this position (34). Immunoblots of BN-PAGE-resolved thylakoid membrane complexes were probed to detect subunits of PSII (D1), the cytochrome b6f complex, and the stromal portion of the thylakoid ATP synthase (CF1). The complexes harboring each of these proteins were not detectably altered in psa2 mutants (Fig. 3B).

Finally, we monitored several photosynthetic parameters by measuring chlorophyll fluorescence kinetics of intact leaves of psa2 mutants in both maize and Arabidopsis. Photooxidation of P700 was monitored in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea to block electron transfer from PSII. P700 was severely impaired in the mutants but less so in dark-adapted plants (Fig. 4A). To monitor PSI activity, the fluorescence rise between F0 and Fm was monitored (Fig. 4B). The Fv/Fm value was only slightly affected in dark-adapted mutant plants but showed a decrease under normal light conditions, indicating that PSII is light-sensitive in psa2 mutants. The decrease in PSII activity became more severe with increasing light intensity (Fig. 4C), suggesting that PSII is subject to increased photoinhibition in psa2 mutants. Increased photoinhibition has been observed previously in mutants with defects in PSI (9).

These results demonstrate that the PSA2 orthologs in maize and Arabidopsis are required specifically for the accumulation and activity of PSI. It should be noted that AT2G34860 has been annotated as “embryo sac developmental arrest 3” due to a supposed role in embryo development (35). However, other than the seedling lethality that is an expected outcome of a defect in photosynthesis, we did not detect any defects in the development or inheritance of the mutants we analyzed.

PSA2 Localizes to the Thylakoid Lumen—We generated a polyclonal antibody to recombinant At-PSA2 lacking its predicted N-terminal targeting sequence. The antibody recognized a protein of the expected size (611 kDa) in leaf extracts from wild-type maize and Arabidopsis plants; this protein was reduced more than 4-fold in the maize and Arabidopsis psa2 mutants (Fig. 5A), providing strong evidence that it is PSA2.
The residual accumulation of PSA2 in the maize mutant is not surprising given that Mut insertions in 5′-untranslated regions are typically hypomorphic alleles. PSA2 was below the limit of detection in the Arabidopsis At-psy2-1 mutant; however, the At-psy2-2 allele conditioned a less severe phenotype than do At-psy2-1 and At-psy2-3 alleles (Fig. 1B), indicating that At-psy2-1 is not a null allele. PSA2 accumulated to near normal levels in non-allelic maize mutants lacking PSI, indicating that PSA2 accumulation is not coupled to that of PSI (Fig. 5B). Interestingly, however, PSA2 was absent in the Arabidopsis apo1 mutant (Fig. 5C), which lacks PSI due to a defect in the expression of the chloroplast gene encoding the PSI assembly factor Ycf3 (36, 37). This finding suggests a potential functional relationship between PSA2 and Ycf3 in PSI assembly.

PSA2 was detected in two independent proteomic analyses of thylakoid membranes in Arabidopsis, the latter of which reported its enrichment specifically in non-appressed membranes (28, 38). We confirmed that PSA2 is localized to non-appressed membranes by probing immunoblots of leaf extracts, intact chloroplasts, and chloroplast subfractions with the PSA2 antibody (Fig. 6A). PSA2 lacks predicted transmembrane domains and had been proposed to localize to the thylakoid lumen based on a predicted luminal targeting sequence (28). To test this possibility, soluble proteins in the thylakoid lumen were released by treating thylakoids with a low concentration of the thylakoid lumenase cytochrome b$_{6}$f complex (26). To test this possibility, soluble proteins in the thylakoid lumen were released by treating thylakoids with a low concentration of the thylakoid lumenase cytochrome b$_{6}$f complex (26). 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concentration of Triton X-100 (Fig. 6B). PSA2 and the soluble lumenal protein plastocyanin were both enriched in the supernatant, whereas an integral thylakoid protein (LHCB1) and a protein bound to the lumenal face (OEC33) remained with the membrane pellet (Fig. 6B). The lumenal targeting sequence of PSA2 includes a conserved “twin arginine” motif (Fig. 2), suggesting that it is translocated via the cpTAT pathway (39). In support of this view, PSA2 failed to accumulate in maize hcf106 mutants, which lack a component of the TAT targeting machinery (Fig. 6C). Translocation via the TAT pathway is consistent with the fact that PSA2 harbors a predicted zinc finger, the assembly of which in the stroma would necessitate translocation across the thylakoid membrane in a folded state.

Another PSI biogenesis factor, PPD1, was shown previously to localize to the thylakoid lumen (9). Therefore, we considered the possibility that PSA2 mediates its effects via an effect on PPD1 abundance. However, PPD1 accumulated to elevated levels in psa2 mutants (Fig. 6D), suggesting the existence of a compensatory mechanism that increases PPD1 expression in the absence of PSA2 or PSI.

**FIGURE 5.** PSA2 abundance in mutants lacking PSI. A, characterization of a PSA2 antibody. Immunoblots of maize (left) or Arabidopsis (right) chloroplast extract were probed with a polyclonal antibody raised to At-PSA2. The panels below show the same blots probed to detect Lhcb1 as a loading control. B, PSA2 abundance in maize mutants lacking the cytochrome b6f complex or PSI. Total leaf extracts from maize seedlings of the indicated genotypes were analyzed by probing immunoblots with anti-PSA2 antibody. Zm-pyg7 mutants have an insertion in the maize ortholog of the Arabidopsis PSI biogenesis gene PYG7. sgp184 has an insertion in a gene encoding a novel PSI biogenesis factor (R. Williams-Carrier and A. Barkan, manuscript in preparation). C, pet2 and pet3 mutants specifically lack the cytochrome b6f complex (25). Replicate blots were probed with antibodies to the PSI core subunits PsAD and PsA or the ATP8 subunit of the ATP synthase. A portion of the Ponceau S-stained blot used for the AtpB and PsaD probings is shown below. D, PSA2 and the soluble protein plastocyanin were both enriched in the supernatant (Sup) recovered after gentle solubilization of thylakoid membranes were purified, and equivalent amounts of protein (15 μg/sample) were analyzed by immunoblotting using antibodies against the indicated proteins. Lhcb1, PsA, and RbcL serve as markers for the indicated thylakoid membranes.

**FIGURE 6.** PSA2 is localized to the thylakoid lumen. A, intrachloroplast localization of PSA2. Proteins from Arabidopsis leaf, chloroplasts (Cp), thylakoid membranes (Thyl), stroma, appressed membranes (App), and non-appressed membranes (Non-app) were purified, and equivalent amounts of protein (15 μg/sample) were analyzed by immunoblotting using antibodies against the indicated proteins. B, separation of thylakoid membrane-bound and lumenal proteins. Proteins from Arabidopsis chloroplasts (Cp), thylakoid membranes (Thyl), and the pellet and supernatant (Sup) recovered after gentle solubilization of thylakoid membranes were purified, and equivalent amounts of protein (15 μg/sample) were analyzed by immunoblotting using antibodies against the indicated proteins. OEC33, a subunit of the oxygen evolving complex, is bound to the luminal face of the thylakoid membrane. Plastocyanin (PC) is a soluble protein in the thylakoid lumen. PsaA is an integral thylakoid protein. C, immunoblot analysis of PSA2 in maize mutants with defects in the cpTAT and cpSec thylakoid targeting pathways. D, immunoblot analysis of PPD1 in psa2 mutants. Total leaf extract from maize seedlings of the indicated genotypes were analyzed by probing immunoblots with anti-PSA2 antibody. A replicate blot probed with antibody to the photosystem II protein D2 serves as a loading control.
FIGURE 7. Analysis of multimeric complexes harboring PSA2. A, immunoblot analyses of BN-PAGE resolved thylakoid membrane protein complexes from Arabidopsis. Immunoblots were probed with antibodies to PSA2, subunits of PSI (PsaA, PsaG, and PsaK), Lhca1 (light-harvesting protein of PSI), D1 (core protein of PSI), and Lhcb1 (light-harvesting protein of PSII). B, immunoblot analyses of BN-PAGE-resolved thylakoid protein complexes from maize. Blots were probed with the indicated antibodies. The maize psa2 mutant was included as a control. C, immunoblot analysis of two-dimensionally resolved thylakoid membrane complexes in Arabidopsis. Thylakoid membranes were solubilized with DM and resolved by BN-PAGE (first dimension) followed by SDS-PAGE (second dimension). Replicate blots were probed with the indicated antibodies. PSII-LHCII, PSII-light-harvesting assemblies; PSI-M, monomeric PSI; PSI-D, PSI dimer; PSI-I, PSI monomer; LHCII-T, trimeric light-harvesting complex II.

PSA2 Associates with a PSI-related Subcomplex That Includes PsaG—To determine whether PSA2 associates with mature PSI and/or with complexes containing subsets of PSI subunits, immunoblots of BN-PAGE-resolved, DM-solubilized thylakoid membranes from Arabidopsis (Fig. 7A) or maize (Fig. 7B) were probed with the PSA2 antibody. The antibody detected one major PSA2-containing complex in both species at roughly 250 kDa. PSA2 was not detected at the position of the stained band corresponding to mature PSI. Furthermore, immunoblot analyses showed that PSA2 did not comigrate with the core PSI subunits PsaA and PsaD. However, PSA2 did comigrate with PsaG, a peripheral subunit that links Lhcb1 to the PSI core (2, 40). Immunoblot analyses of Arabidopsis extract that had been fractionated in two-dimensional gels (BN-PAGE followed by SDS-PAGE) corroborated these findings (Fig. 7C). Furthermore, the luminal PSI assembly factor PPD1 also comigrated with PSA2 in these gels. These results show that PSA2 does not associate in a stable manner with the PSI complex and suggest that it may instead be found in a complex harboring PsaG and PPD1.

To test whether PSA2, PsaG, and PPD1 are found in the same complex, the PSA2 antibody was used for coimmunoprecipitation assays with DM-solubilized thylakoid membranes from Arabidopsis (Fig. 8A). To reduce background, several assays were repeated with lysate that had been precleared by incubation with beads lacking antibody (Fig. 8B). The antibody to PSA2 coimmunoprecipitated PsaG but did not detectably coprecipitate PsaA, PsaK, or PPD1. Furthermore, PsaG was substantially depleted from the extract after immunoprecipitation, suggesting that a large fraction of PsaG is found in a complex together with PSA2. Taken together, the BN-PAGE and coimmunoprecipitation data provide strong evidence that PSA2 is found in a complex together with PsaG and that this complex lacks many other PSI components. Although PSA2

FIGURE 8. Analysis of proteins that coimmunoprecipitate with PSA2. DM-solubilized thylakoid membrane proteins were subjected to immunoprecipitation with PSA2 antibody or with antibody to the stromal ribosome assembly factor RBF1. The immunoprecipitates were separated by SDS-PAGE, and specific proteins in the input, supernatant (Sup), and immunoprecipitate (Beads) were detected by probing immunoblots with the indicated antisera. A, assays performed without preclearing lysate by incubation with beads lacking antibody. B, assays performed with lysate that had been precleared with beads lacking antibody. The preclearing step reduced the background observed for PsaA and PPD1 in A.
Lumenal Protein Required for Photosystem I Biogenesis

![Graph showing protein-disulfide reductase activity of recombinant PSA2.](image)

**FIGURE 9.** Protein-disulfide reductase activity of recombinant PSA2. The reduction of disulfide bonds in insulin was monitored by an increase in absorbance at 650 nm, which results from insulin precipitation. The reaction was initiated by the addition of dithiothreitol to a final concentration of 100 μM. The ribosome-binding protein RBF1 served as a negative control. All reactions contained BSA. Values represent the mean of three independent assays, each with three replicates. Error bars indicate one S.D.

Results presented here show that PSA2, a highly conserved protein found specifically in photosynthetic eukaryotes, localizes to the thylakoid lumen and is required for the accumulation of PSI. We provide evidence that PSA2 is found in a complex harboring the PSI subunit PsaG and that it does not associate stably with mature PSI or influence chloroplast gene expression. Although the *Arabidopsis* PSA2 ortholog AT2G34860 was previously assigned the name embryo sac development arrest 3 (35), we did not detect any defect in the inheritance of the mutant alleles used in our study. Instead, our results strongly suggest that PSA2 promotes the assembly of PSI. A role in PSI assembly rather than (or in addition to) PSI homeostasis is supported by (i) the fact that PSA2 was required for PSI accumulation even after prolonged dark adaptation (Fig. 3A), (ii) the loss of PSA2 in a mutant lacking the PSI assembly factor Ycf3 (Fig. 5C), and (iii) the coexpression of PSA2 with proteins known to function in the assembly of the photosynthetic apparatus. *Arabidopsis* coexpression data can be viewed at the

ATTED-II database (29), which reports seven genes that are directly connected with PSA2 in a coexpression network. Five of these genes are of known function; all are involved in chloroplast biogenesis and do not function directly in photosynthesis: CCB1, which is required for assembly of the cytochrome b6f complex (42); HCF136 and LPA3, which are involved in PSII assembly (43–45); At5g51110, which is orthologous to a Rubisco assembly factor in maize (46); and GC1, which is required for chloroplast division (47). Analogous coexpression databases are not available for maize. However, transcriptome data for maize seedling leaf segments representing a gradient of photosynthetic development (48) show that maize *psa2* exhibits peak expression in a “chloroplast biogenesis” zone early in leaf development similar to known thylakoid biogenesis factors and distinct from genes encoding structural components of the photosynthetic apparatus, the expression of which peaks at a later stage (Fig. 10).

PSA2 is a small protein that consists almost entirely of a heat shock protein DnaJ, cysteine-rich domain (InterPro ID IPR001305) (Fig. 2A). Three previously characterized proteins in plants likewise consist of a stand-alone domain of this type, and all localize to chloroplasts: BSD2 (31), LQY1 (32), and CYO1/SCO2 (30, 33). BSD2 localizes to the stroma and mediates Rubisco assembly (31), whereas LQY1 and CYO1 are thylakoid membrane proteins that are involved in the assembly or maintenance of membrane-embedded photosynthetic complexes (30, 32, 33, 49). The cysteine-rich domains in both DnaJ and LQY1 have been shown to bind two zinc moieties and to form a zinc finger (32, 50, 51). The four zinc-coordinating motifs characteristic of this domain family (CXXCXGXG) are conserved in PSA2 orthologs (Fig. 2), suggesting that PSA2 likewise harbors a zinc finger. This domain in DnaJ has been demonstrated to have two biochemical activities. (i) It prevents protein aggregation (50, 51), and (ii) it has protein-disulfide isomerase activity (41). The chloroplast proteins CYO1/SCO2 and LQY1 also exhibit protein-disulfide isomerase activity in *vitro* (30, 32). That PSA2 likewise mediates thiol transactions is supported by our finding that recombinant PSA2 catalyzed the reduction of disulfide bonds in *vitro*. PSA2, like BSD2, LQY1, and CYO1/SCO2, lacks the other domains characteristic of DnaJ chaperones and is therefore unlikely to act as a cochaperone with Hsp70. It seems likely, therefore, that PSA2 mediates its effects on PSI biogenesis by preventing inappropriate protein-protein interactions and/or by influencing the formation of specific disulfide bonds in the thylakoid lumen. PSA2 may, for example, interact with lumen-exposed segments of PSI subunits that harbor cysteine residues. PsaG, the one PSI subunit we detected in the ∼250-kDa complex harboring PSA2, lacks cysteines. However, Lhca1 has two conserved cysteine residues on the luminal side of the membrane, and these are in close proximity to PsaG in the PSI structure (52). Thus, a plausible hypothesis is that the PSA2-PsaG complex influences the formation of disulfide bonds involving these cysteines in Lhca1. It is important to note that the *psa2* mutant phenotype is much more severe than is the phenotype resulting from the loss of PsaG (40, 53). This implies that the role of PSA2 goes beyond facilitating the assembly of PsaG into PSI. Examination of the complete composition of the PSA2 complex by mass spectrom-
et will be an important next step toward defining the precise functions of PSA2.

PSI is among the most complex membrane-embedded assemblies in nature, so it is to be expected that its biogenesis involves numerous accessory factors. Proteins that promote PSI assembly can be divided in two functional classes: (i) proteins involved in cofactor synthesis or attachment and (ii) proteins that assist the assembly of the complex by interacting with PSI subunits (3). Two chloroplast-encoded proteins (Ycf3 and Ycf4) and three nucleus-encoded proteins (Y3IP1, Pyg7/Ycf37, and PPD1) have been shown previously to fall into the latter category (4, 5, 7–9, 54). All of these proteins interact with subunits of the core PSI complex (3). By contrast, PSA2 is found in a multimeric complex that includes PsaG. PsaG is incorporated into PSI at a late stage of assembly and is replaced more frequently than are proteins in the PSI core (55, 56). The N and C termini of PsaG are exposed to the thylakoid lumen (57), providing a potential point of contact with PSA2. PsaG influences the dynamics of the interactions between plastocyanin and PSI (58) and has been proposed to undergo a conformational change in response to light quantity/quality that influences the rate of electron transfer to ferredoxin (40). Thus, in addition to a role for PSA2 in the buildup of PSI during chloroplast development, it is plausible that PSA2 acts in mature chloroplasts to regulate PSI activity by influencing the interactions of PsaG with the core complex. Our coimmunoprecipitation data (Fig. 8) indicate that a substantial fraction of PsaG is found in a PSA2-containing complex. Perhaps this represents a “storage” complex that is poised to donate PsaG to PSI.

Several observations suggest a functional link between PSA2 and the PSI assembly factor PPD1. First, PPD1, like PSA2, localizes to the thylakoid lumen (9). Second, the accumulation of PsaG is especially sensitive to the partial loss of PPD1 (10). Finally, PPD1 is found in a complex that comigrates with the PSA2-PsaG complex in native gels (Fig. 7C). Although our coimmunoprecipitation experiments did not detect an interaction between PPD1 and PSA2 (Fig. 8B), it remains possible that these two proteins function in the same biochemical processes or pathway.

The thylakoid lumen harbors a rich collection of proteins that function in the assembly, repair, and regulation of photosynthetic complexes (59). These include LQY1, the zinc finger of which is predicted to be lumenal (32), and PPD1, which promotes PSI assembly via interaction with luminal loops of PsA and PsaB (9). PSA2 and PPD1 are the only PSI assembly factors known to reside in the thylakoid lumen (3, 9). They are also the only known PSI assembly factors to be present in photosynthetic eukaryotes but not in cyanobacteria (3, 9). PSI in plants includes several subunits that are not found in cyanobacteria, including PsaG (60, 61). It will be interesting to explore whether the evolution of eukaryote-specific PSI assembly factors like PSA2 and PPD1 was driven by distinct lumenal environments in plants and cyanobacteria and/or by the need to incorporate novel subunits into the more elaborate PSI found in eukaryotes.

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REFERENCES

1. Nelson, N., and Ben-Shem, A. (2004) The complex architecture of oxygenic photosynthesis. Nat. Rev. Mol. Cell Biol. 5, 971–982
2. Amunts, A., Drory, O., and Nelson, N. (2007) The structure of a plant photosystem I supercomplex at 3.4 Å resolution. Nature 447, 58–63
3. Schöttler, M. A., Albus, C. A., and Bock, R. (2011) Photosystem I: its biogenesis and function in higher plants. J. Plant Physiol. 168, 1452–1461
4. Krech, K., Ruf, S., Masduki, F. F., Thiele, W., Bednarczyk, D., Albus, C. A., Tiller, N., Hasse, C., Schöttler, M. A., and Bock, R. (2012) The plastid genome-encoded Ycf4 protein functions as a nonessential assembly factor for photosystem I in higher plants. Plant Physiol. 159, 579–591
5. Boudreau, E., Takahashi, Y., Lemieux, C., Turmel, M., and Rochaix, J. D.
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24608–24616

43. Cai, W., Ma, J., Chi, W., Zou, M., Guo, J., Lu, C., and Zhang, L. (2010) Cooperation of LPA3 and LPA2 is essential for photosystem II assembly in Arabidopsis. Plant Physiol. 154, 109–120

44. Plücken, H., Müller, B., Grohmann, D., Westhoff, P., and Eichacker, L. A. (2002) The HCF136 protein is essential for assembly of the photosystem II reaction center in Arabidopsis thaliana. FEBS Lett. 532, 85–90

45. Meurer, J., Plücken, H., Kowallik, K. V., and Westhoff, P. (1998) A nuclear-encoded protein of prokaryotic origin is essential for the stability of photosystem II in Arabidopsis thaliana. EMBO J. 17, 5286–5297

46. Feiz, L., Williams-Carrier, R., Belcher, S., Montano, M., Barkan, A., and Stern, D. (2014) RAF2, a novel Rubisco biogenesis factor in maize. Plant J., in press

47. Maple, J., Fujiwara, M. T., Kitahata, N., Lawson, T., Baker, N. R., Yoshida, S., and Mollier, S. G. (2004) GIANT CHLOROPLAST 1 is essential for correct plastid division in Arabidopsis. Curr. Biol. 14, 776–781

48. Li, P., Ponnala, L., Gandotra, N., Wang, L., Si, Y., Tausta, S. L., Kebrom, T. H., Provart, N., Patel, R., Myers, C. R., Reidel, E. J., Turgeon, R., Liu, P., Sun, Q., Nelson, T., and Bruntell, T. P. (2010) The developmental dynamics of the maize leaf transcriptome. Nat. Genet. 42, 1060–1067

49. Muranaka, A., Watanabe, S., Sakamoto, A., and Shimada, H. (2012) Arabidopsis cotyledon chloroplast biogenesis factor CYO1 uses glutathione as an electron donor and interacts with PSI (A1 and A2) and PSII (CP43 and CP47) subunits. J. Plant Physiol. 169, 1212–1215

50. Szabo, A., Korszun, R., Hartl, F. U., and Flanagan, J. (1996) A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. EMBO J. 15, 408–417

51. Bancki, B., Liberek, K., Wall, D., Wawrzynów, A., Georgopoulos, C., Bertoli, E., Tanfani, F., and Zylicz, M. (1996) Structure-function analysis of the zinc finger region of the DnaJ molecular chaperone. J. Biol. Chem. 271, 14840–14848

52. Amunts, A., Toporik, H., Borovikova, A., and Nelson, N. (2010) Structure determination and improved model of plant photosystem I. J. Biol. Chem. 285, 3478–3486

53. Varotto, C., Pesaresi, P., Jahns, P., Lessnick, A., Tizzano, M., Schiavon, F., Salamini, F., and Leister, D. (2002) Single and double knockouts of the genes for photosystem I subunits G, K, and H of Arabidopsis. Effects on photosystem I composition, photosynthetic electron flow, and state transitions. Plant Physiol. 129, 616–624

54. Naver, H., Boudreau, E., and Rochaix, J. D. (2001) Functional studies of Ycf3: its role in assembly of photosystem I and interactions with some of its subunits. Plant Cell 13, 2731–2745

55. Zygaldo, A., Robinson, C., Scheller, H. V., Mant, A., and Jensen, P. E. (2006) The properties of the positively charged loop region in PSI-G are essential for its “spontaneous” insertion into thylakoids and rapid assembly into the photosystem I complex. J. Biol. Chem. 281, 10548–10554

56. Ozawa, S., Onishi, T., and Takahashi, Y. (2010) Identification and characterization of an assembly intermediate subcomplex of photosystem I in the green alga Chlamydomonas reinhardtii. J. Biol. Chem. 285, 20072–20079

57. Rosgaard, L., Zygaldo, A., Scheller, H. V., Mant, A., and Jensen, P. E. (2005) Insertion of the plant photosystem I subunit G into the thylakoid membrane. FEBS J. 272, 4002–4010

58. Zygaldo, A., Jensen, P. E., Leister, D., and Scheller, H. V. (2005) Photosystem I lacking the PSI-G subunit has a higher affinity for plastocyanin and is sensitive to photodamage. Biochim. Biophys. Acta 1708, 154–163

59. Järv, S., Gollan, P. J., and Aro, E. M. (2013) Understanding the roles of the thylakoid lumen in photosynthesis regulation. Front. Plant Sci. 4, 434

60. Fromme, P., Melkozernov, A., Jordan, P., and Krauss, N. (2003) Structure and function of photosystem I: interaction with its soluble electron carriers and external antenna systems. FEBS Lett. 555, 40–44

61. Kargul, J., Nield, J., and Barber, J. (2003) Three-dimensional reconstruction of a light-harvesting complex I-photosystem I (LHCI-PSI) supercomplex from the green alga Chlamydomonas reinhardtii. Insights into light harvesting for PSI. J. Biol. Chem. 278, 16135–16141

62. Järv, S., Suorsa, M., Paakkari, V., and Aro, E. M. (2011) Optimized native gel systems for separation of thylakoid protein complexes: novel super- and mega-complexes. Biochem. J. 439, 207–214

63. Callari, S., Kouril, R., Kereiche, S., Boekema, E. J., and Croce, R. (2009) Functional architecture of higher plant photosystem II supercomplexes. EMBO J. 28, 3052–3063

64. Joliot, P., and Joliot, A. (2005) Quantification of cyclic and linear flows in plants. Proc. Natl. Acad. Sci. U.S.A. 102, 4913–4918

65. Voelker, R., and Barkan, A. (1995) Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. EMBO J. 14, 3905–3914