PSA3, a Protein on the Stromal Face of the Thylakoid Membrane, Promotes Photosystem I Accumulation in Cooperation with the Assembly Factor PYG7

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PSI is a large protein-pigment complex located in the thylakoid membrane in cyanobacteria, plants, and algae. Although the structure and components of PSI are well characterized, mechanisms that orchestrate its assembly are poorly understood. In this study, we discovered a novel nucleus-encoded protein, Photosystem I Assembly3 (PSA3), that is required for PSI accumulation. PSA3 is conserved among green photosynthetic eukaryotes but is lacking in cyanobacteria. Mutations in the psa3 gene cause the specific loss of PSI in Arabidopsis (Arabidopsis thaliana) and maize (Zea mays). Ribosome profiling and pulse-labeling analyses showed that chloroplast-encoded PSI subunits are synthesized at normal rates in psa3 mutants, indicating that PSA3 is involved in the biogenesis of PSI at a posttranslational step. PSA3 resides on the stromal face of the thylakoid membrane, where it is found in a complex that is slightly smaller than PSI. Structural predictions suggest that PSA3 binds a basic peptide in a manner that is sensitive to the oxidation state of Cys pairs flanking the predicted peptide binding groove. PSA3 and the previously described PSI biogenesis factor PYG7 interact in yeast two-hybrid and bimolecular fluorescence complementation assays, and they are found in thylakoid membrane complexes of similar size. These and other results indicate that PSA3 cooperates with PYG7 to promote the stable assembly of PSI, and that the Psac subunit is likely to be the primary target of their action.

PSI is one of two photosystems in the thylakoid membranes of cyanobacteria, green algae, and plants. In higher plants, PSI is an ~600 kD complex composed of two subcomplexes: the PSI core complex that transfers electrons from plastocyanin in the thylakoid lumen to ferredoxin in the stroma, and Light Harvesting Complex I (LHCI), which harvests light energy (Nelson and Junge, 2015). The PSI core complex consists of nine membrane-intrinsic subunits (PsaA, PsaB, and PsaF–PsaL) and three peripheral subunits (PsaC, PsaD, and PsaE; Mazor et al., 2015), approximately half of which are encoded in the chloroplast genome. The LHCl complex is composed of four nucleus-encoded Lhca proteins, which are integral membrane proteins that are related to the Lhcb proteins associated with PSII (Nelson and Junge, 2015). The reaction center subunits of the PSI core complex, PsaA and PsaB, form a heterodimer that binds most of the cofactors that are involved in charge separation and electron transfer (Mazor et al., 2015). Other core subunits are arranged around the PsaA/B heterodimer and participate in various functions, including the binding of LHCI and interaction with plastocyanin and ferredoxin. Remarkably, PSI includes roughly two hundred prosthetic groups, including chlorophylls, carotenoids, iron-sulfur clusters, and phylloquinones (Mazor et al., 2015).

Although the composition and structure of PSI are well understood, much remains to be learned about the biogenesis of this enormously complex particle (Yang et al., 2015a). There is evidence, however, that the assembly of PSI in photosynthetic eukaryotes is initiated by the formation of a PsaA/PsaB heterodimer in the membrane. Subsequently, PscA, PscD, and PsaE, which form the so-called “stromal ridge,” are added to the stromal side of the PsaA/PsaB dimer (Ozawa et al., 2010). In the final steps, other small PSI subunits and LHCl associate with the core (Ozawa et al., 2009; Ozawa et al., 2010; Yang et al., 2015a). Six proteins have been identified that are required specifically for PSI accumulation and that are believed to orchestrate specific steps in the assembly process. These include the plastid-encoded proteins Ycf3 and Ycf4, and the nucleus-encoded proteins Y3IP1, PYG7, PPD1, and PSI Assembly2 PSA2 (Yang et al., 2015a). Ycf3, Ycf4, and Y3IP1 associate with the stromal face of the thylakoid membrane, and they have been suggested to participate...
in the assembly of the stromal ridge (Boudreau et al., 1997; Ruf et al., 1997; Ozawa et al., 2009; Albus et al., 2010; Krech et al., 2012). PPD1 and PSA2 associate with the luminal face of the thylakoid membrane (Liu et al., 2012; Fristedt et al., 2014; Roose et al., 2014), whereas PYG7 is an integral membrane protein (Stöckel et al., 2006). A recent study of the PYG7 ortholog in Chlamydomonas reported that it is required for PSI biogenesis specifically in the presence of molecular oxygen, which led to a model in which PYG7 shields Fe-S clusters or the cysteines that bind them from oxidation during PSI assembly (Heinnickel et al., 2016). Despite this progress, the biochemical roles of these proteins in PSI biogenesis remain unknown.

Here, we describe a novel nucleus-encoded PSI biogenesis factor, PSA3, that is conserved in green photosynthetic eukaryotes but absent in cyanobacteria. Our results provide strong evidence that PSA3 cooperates with PYG7 on the stromal face of the assembling PSI complex to facilitate PSI assembly, and suggest that this function is mediated by the binding of a basic peptide in a redox-sensitive manner.

RESULTS

Identification of the psa3 Gene in Maize (Zea mays) and Functional Conservation of its Arabidopsis (Arabidopsis thaliana) Ortholog

PSA3 came to our attention during our identification of causal mutations in the Photosynthetic Mutant Library (PML), a large collection of Mu transposon-induced nonphotosynthetic maize mutants (Belcher et al., 2015). The original allele, which we named psa3-1, is recessive and confers a subtle pale green seedling phenotype (Fig. 1A). These mutants lack the PSI reaction center protein PsA but have near normal levels of core subunits of PSII, the cytochrome b$_6$f complex, the ATP synthase, and Rubisco (Fig. 1B). As expected for plants lacking the PSI reaction center, the mutant seedlings exhibit elevated chlorophyll fluorescence (Fig. 1A, bottom) and die after approximately 3 weeks of growth in soil, when seed reserves are exhausted. To find the causal mutation, we used a deep-sequencing approach to identify Mu-transposon insertions that cosegregate with the phenotype (Williams-Carrier et al., 2010). Among the handful of cosegregating insertions, an insertion mapping near the start codon of a previously unstudied gene, GRMZM2G051403 (B73 genome v3), stood out as an appealing candidate for several reasons. First, the product of its Arabidopsis ortholog (AT3G55250) had been detected in proteome analyses of purified chloroplasts (Zybailov et al., 2008), and a T-DNA insertion in this gene has been associated with a pale green seedling phenotype (Myouga et al., 2010). Second, transcriptome data showed this gene to peak in its expression in young leaf tissue in maize, coinciding with a period of active chloroplast development (Supplemental Fig. S1A). Third, the ATTED-II database (Aoki et al., 2016) showed the Arabidopsis ortholog to be coexpressed with genes involved in thylakoid biogenesis, including the PSI assembly factors PYG7 (Stöckel et al., 2006) and PSA2 (Fristedt et al., 2014; Supplemental Fig. S1B).

To validate this assignment, we recovered a second insertion in the same gene in a reverse-genetic screen of the PML mutant collection. The second allele, psa3-2, has a Mu-insertion in exon 3 (Fig. 1C) and conditions protein and pigment phenotypes similar to those of psa3-1 (Fig. 1, A and B). Furthermore, the heteroallelic progeny of complementation crosses (psa3-1/-2) show the same pigment (Fig. 1A) and protein defects (Supplemental Fig. S2) as the parental alleles. Taken together, these results provide strong evidence that GRMZM2G051403 corresponds to the psa3 gene.

The maize and Arabidopsis psa3 orthologs are referred to as Zm-psa3 and At-PSA3 below, according to the standard gene nomenclature for each species. We analyzed the phenotype of an Arabidopsis mutant with a T-DNA insertion in the third exon of At-PSA3 (Fig. 2A). Homozygous At-psa3 mutants die shortly after transplanting to soil. When grown on Murashige and Skoog (MS) medium containing 2% Suc, At-psa3 mutants exhibited a pale green, slow-growing phenotype, an elevated level of chlorophyll fluorescence, and a loss of the PsA subunit of PSI (Fig. 2, B and C). These phenotypes were restored to the wild type by expression of a transgene encoding At-PSA3 with a C-terminal 3×FLAG-tag (Fig. 2, B and C). These results strongly suggest that PSA3 function is conserved between maize and Arabidopsis.

PSA3 Is Required for the Accumulation of PSI and a Proposed PSI Assembly Intermediate

To examine the effects of PSA3 on the abundance of assembled thylakoid membrane complexes, thylakoid membranes from both the maize and Arabidopsis mutants were solubilized with n-dodecyl-$\beta$-maltoside (DDM) and resolved by blue native-polyacrylamide gel electrophoresis (BN-PAGE; Fig. 3). Two stained bands that are expected to include PSI based on previous reports were reduced in intensity in both the Zm-psa3 and At-psa3 mutants: One of these corresponds to an NDH-PSI supercomplex and the other includes comigrating PSI and PSII complexes (Fig. 3, left panels). The loss of PSI and the NDH-PSI supercomplex was confirmed by probing immunoblots of BN-PAGE gels with antibodies to the PSI core subunit PsaD (Fig. 3, right panels). A smaller PsA-containing complex was prominent in the wild-type maize sample (marked with an asterisk in Fig. 3) but absent in the Zm-psa3 mutant. It seems likely that this corresponds to a complex in tobacco that was recently proposed to be a stable intermediate in PSI assembly (Wittenberg et al., 2017). A pair of complexes with the analogous features, albeit at lower abundance, can also be seen in the Arabidopsis data. Two-dimensional BN-PAGE/sodium dodecyl sulfate (SDS)-PAGE analysis confirmed the loss of
assembled PSI in the maize and Arabidopsis psa3 mutants (Supplemental Fig. S3). Results of noninvasive fluorometric assays (Supplemental Fig. S4) showed a mild decrease in PSII activity and a near absence of PSI activity and correlate well with the abundance of PSII and PSI as inferred from the immunoblot and BN-PAGE data.

PSA3 Is Not Required for the Expression of Chloroplast Genes Encoding PSI Subunits or Assembly Factors

To investigate how PSA3 participates in PSI biogenesis, we analyzed the expression of chloroplast genes encoding PSI subunits and PSI assembly factors. An in vivo pulse-labeling assay showed that PsaA and PsaB are synthesized at normal rates in At-psa3 mutants (Fig. 4A). During a subsequent chase in the presence of an excess of nonradioactive Met, radiolabeled PsaA and PsaB decreased more rapidly in the mutants than in the wild type, indicating that they are degraded more rapidly in the mutants. Other PSI subunits are difficult to detect in pulse-labeling experiments. As an alternative approach, we monitored synthesis of the plastid-encoded subunits with a ribosome profiling method that uses high-resolution microarrays to provide a quantitative and high-resolution map of ribosome “footprints” on chloroplast mRNAs (Zoschke et al., 2013). The normalized abundance of ribosome footprints mapping to all chloroplast genes encoding PSI subunits and PSI assembly factors is similar in the wild type and Zm-psa3 mutants (Fig. 4B; Supplemental Fig. S5A). As expected based on these data, mRNAs encoding PsaA/B, PsaC, and Ycf3 are of normal size and abundance in Zm-psa3 mutants (Supplemental Fig. S5B). Taken together, these results strongly suggest that PSA3 acts posttranslationally to promote the stable accumulation of PSI.

PSA3 Is Found in Green Photosynthetic Eukaryotes and Is Predicted to Have an Acidic Groove that Binds a Peptide Ligand

PSA3 orthologs are found in land plants and in green algae (Fig. 5A) but appear to be absent in cyanobacteria and nonphotosynthetic organisms (see Phytozome
Protein Required for PSI Biogenesis

Database; Goodstein et al., 2012). Proteins with the analogous phylogenetic distribution have been grouped into the “GreenCut” gene set (Karpowicz et al., 2011), but PSA3 was not reported as a GreenCut gene. Although the protein sequence is highly conserved among vascular and nonvascular land plants, it is quite divergent in Chlamydomonas reinhardtii (Fig. 5A), and this may account for its absence from the GreenCut list. At-PSA3 was previously annotated as Pigment Defective Embryo 329 (Lloyd and Meinke, 2012). The rice ortholog (LOC_Os05g45030) is annotated as Calcium Homeostasis Regulator CHoR1 (http://rice.plantbiology.msu.edu/index.shtml), but we were unable to discern the basis for this annotation.

The maize and Arabidopsis orthologs are predicted by TargetP to have an N-terminal chloroplast-targeting peptide (Emanuelsson et al., 2007), consistent with the detection of At-PSA3 in the Arabidopsis chloroplast proteome (Zybailov et al., 2008). PSA3 orthologs lack predicted transmembrane segments and domains of known function. A structural prediction of the maize ortholog by I-TASSER (Yang et al., 2015b; Fig. 5B) suggests that the protein is largely alpha helical with a surface that is generally basic, but with a groove lined by acidic residues. I-TASSER predicts further that this acidic groove binds a peptide ligand. Strikingly, the groove is flanked on both sides by adjacent Cys pairs in angiosperms and three of these cysteines are conserved in the moss Physcomitrella patens (asterisks in Fig. 5A and yellow spheres in Fig. 5B). These features suggest the intriguing possibility that PSA3 binds a basic peptide in a manner that is regulated by the redox state of these cysteines.

PSA3 Associates with the Stromal Face of the Thylakoid Membrane

We raised a polyclonal antibody to a recombinant fragment of maize PSA3 (marked by a line in Fig. 5A).
This antibody detected a protein of the expected size (~26 kD) in wild-type maize leaf tissue whose abundance is strongly reduced in Zm-psa3 mutants (Fig. 6A), which strongly suggests that the immunoreactive ~26 kD protein is PSA3. The antibody does not cross react with Arabidopsis PSA3, so maize was used for all experiments below that relied on the antibody.

PSA3 is enriched in isolated chloroplasts in comparison with its abundance in total leaf extract (Fig. 6B), confirming its chloroplast localization. To investigate its location inside the chloroplast, chloroplasts were fractionated into thylakoid and stromal fractions (Fig. 6C, left lanes). PSA3 was enriched in the thylakoid fraction, but a substantial amount was also detected in the stroma. PSA3 was stripped from the membranes by treatment with Na₂CO₃ or NaBr, whereas the integral membrane protein HCF106 was not (Fig. 6C, middle lanes). These results indicate that PSA3 is a membrane-extrinsic protein, consistent with the fact that it lacks predicted transmembrane segments. Treatment of thylakoid membranes with proteinase K or thermolysin degraded the bulk of PSA3 but left the luminal protein OE23 intact (Fig. 6C). Both proteins were degraded when thylakoid membranes were disrupted by treatment with a low concentration of Triton X-100, although a protease-resistant fragment of PSA3 remained (Fig. 6C, asterisk). Taken together, these results demonstrate that PSA3 is bound to the stromal face of the thylakoid membrane.

### PSA3 Accumulation in Mutants Lacking PSI Assembly Factors

To gain insight into functional relationships between PSA3 and other proteins involved in PSI biogenesis, we compared the abundance of PSA3 and components of the photosynthetic apparatus in maize mutants lacking previously described PSI biogenesis factors (Fig. 7). This comparison included a mutant lacking the luminal assembly factor PSA2 (Zm-psa2; Fristedt et al., 2014), a mutant lacking the integral membrane assembly factor PYG7 (Zm-pyg7), and mutants lacking the stromal assembly factors Y3IP1 or YCF3 (Zm-ψ3ip1 and Zm-otp51, respectively; Boudreau et al., 1997; Ruf et al., 1997; Stöckel et al., 2006; Albus et al., 2010; Belcher et al., 2015). Zm-otp51 is a “surrogate” ycf3 mutant in that OTP51 is required specifically for the expression of the chloroplast ycf3 gene (de Longevialle et al., 2008; Khrouchtchina et al., 2012). A maize Zm-tab2 mutant was also included in this survey (Belcher et al., 2015). Zm-TAB2 is orthologous to Arabidopsis ATAB2 and *Chlamydomonas* TAB2, which were reported to be translational activators for chloroplast PSI genes (Dauvillée et al., 2003; Barneche et al., 2006). However, recent ribosome profiling data provided evidence that ATAB2 and Zm-TAB2 act posttranslationally to promote PSI accumulation (Belcher et al., 2015).

As expected, all of the mutants have reduced levels of PSI core subunits (PsaA, PsaC, PsaD, PsaE, PsaK, PsaL). These deficiencies are least severe in the Zm-psa3 and Zm-pyg7 mutants, correlating with the fact that the alleles analyzed are likely to be hypomorphic (see “Materials and Methods”). Subunits of other complexes, and in particular the D1 reaction center protein of PSII, were reduced to lower levels in the Zm-ψ3ip1, Zm-tab2, and Zm-otp51 mutants than in the psa3 mutant despite their similarly severe PSI defect. The basis for this differential effect on PSI is not known.

PSA3 accumulated to near normal levels in all of the mutants (except the psa3 mutant itself), demonstrating that PSA3 accumulates independently of PSI. Interestingly, PSA3 levels were reduced slightly in the Zm-pyg7 mutant (Fig. 7). However, we found that PSA3 levels varied according to genetic background (i.e. the inbred line into which the mutation was crossed), and this reduction with respect to wild-type samples was not always observed (Supplemental Fig. S6). That said, PSA3 was consistently found at lower levels in pyg7 mutants than in psa2 mutants (Supplemental Fig. S6). These results hinted that PYG7 and PSA3 may

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**Figure 4.** Synthesis of plastid-encoded PSI subunits is not reduced in psa3 mutants. A, In vivo pulse-chase analysis of chloroplast-encoded thylakoid membrane proteins. The leaves of 12-d-old Arabidopsis seedlings were preincubated with cycloheximide for 30 min, and [1^35]S-Met was then added for 20 min (t = 0). Unlabeled Met was then chased at a concentration of 1 mM (chase) and plants were harvested 1 and 1854 Plant Physiol. Vol. 174, 2017
functionally or physically interact. This view was confirmed in experiments described below.

**PSA3 Is Found in a Thylakoid Membrane Complex That Is Slightly Smaller Than PSI**

To determine whether PSA3 is bound stably to other proteins, thylakoid membranes from wild-type maize seedlings were solubilized with DDM, fractionated by BN-PAGE, and analyzed by immunoblotting with the PSA3 antibody (Fig. 8). The same blots were reprobed to detect PsaA in order to mark the position of PSI. Thylakoid membranes from Zm-psa2 and Zm-pyg7 mutants were analyzed in parallel to determine whether PYG7 or PSA2 influence the multimeric state of PSA3.

The most prominent PSA3-containing complex in wild-type samples appeared at first glance to comigrate with PSI (Fig. 8). However, by carefully aligning the images, it became clear that this complex migrates slightly ahead of PSI. This PSA3-containing complex accumulates to normal or even increased levels in psa2 mutants, providing further evidence that it is not mature PSI. Interestingly, the abundance of this complex is reduced in the Zm-pyg7 mutant, suggesting that PYG7 is required for its formation or for PSA3 to bind. In accord with this view, PYG7 is also found in a complex...
that is close in size to PSI (Stöckel et al., 2006; Yang et al., 2017). Taken together, these results suggested that PYG7 and PSA3 might be found together in the same large complex.

**PSA3 Interacts with PYG7 in Yeast and in Chloroplasts**

PSA3 is located on the stromal face of the thylakoid membrane, where it could potentially interact with PSI assembly factors or PSI subunits that are exposed to the stroma. Appealing candidates include the membrane extrinsic PSI subunits PsaC, PsaD, and PsaE, the membrane extrinsic assembly factors YCF3 and Y3IP1, and the integral membrane assembly factor PYG7. PYG7 is predicted to include a transmembrane segment near its N terminus followed by a tetratricopeptide repeat (TPR) domain and a short C-terminal tail (see Supplemental Fig. S7). The TOPCONS algorithm (Tsirigos et al., 2015) predicts that PYG7's TPR domain and C-terminal tail are on the stromal side of the membrane and this prediction was recently confirmed (Yang et al., 2017).

A yeast two-hybrid assay did not detect interactions between PSA3 and the PSI subunits PsaC, PsaD, and PsaE (Supplemental Fig. S8A). The potential for PSA3 to interact with the assembly factors YCF3, Y3IP1, and PYG7 was tested with the split ubiquitin system in yeast, which can detect interactions involving membrane-bound proteins (Pasch et al., 2005). PSA3 was used as the “prey,” and YCF3, Y3IP1, or PYG7 were tested as the “bait.” YCF3 and Y3IP1 activated the reporter even with the negative control prey (Supplemental Fig. S8B), so their interaction with PSA3 could not be assessed. However, a robust interaction was observed between PSA3 and PYG7 (Fig. 9A). This interaction was detected when the NubG ubiquitin fragment was fused to the N terminus of PSA3, but not when it was fused to PSA3’s C terminus, suggesting that PSA3’s C terminus contributes to the interaction with PYG7. In support of this view, deletion of the C-terminal 54 amino acids of PSA3 eliminated the interaction (Fig. 9A, NubG-AtPSA3ΔC).

As noted above, PSA3 is predicted to harbor a peptide-binding groove that is lined with acidic residues (see Fig. 5B). We examined PYG7 for features that might bind this groove. An appealing candidate was identified in the C-terminal “tail” that follows PYG7’s TPR domain (see Supplemental Fig. S7). This region is predicted to adopt an amphipathic alpha helix with a basic surface. However, deletion of this region did not

**Figure 6.** Immunoblots demonstrating localization of PSA3 to the stromal face of the thylakoid membrane. Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. An excerpt of an image of each blot stained with Ponceau S is shown to illustrate the total population of proteins in each lane and to serve as a loading control. The large subunit of Rubisco (RbcL) is marked. A, Immunoblot analysis of total leaf proteins from maize, demonstrating specificity of the PSA3 antibody. The size of the major protein detected in the wild type (WT; ~26 kD) corresponds to that predicted for mature Zm-PSA3. Several cross-reacting proteins accumulate to elevated levels in the mutants. The identities of these proteins are not known. B, Relative PSA3 concentration in extracts of leaf, purified mitochondria (Mito), and purified chloroplasts (CP). A blot that had been used in a previous publication (Kroeger et al., 2009) was reprobed to detect PSA3. D1 (the PsbA reaction center of PSII) served as a marker for chloroplast proteins and MDH (malate dehydrogenase) served as a marker for mitochondrial proteins. The images for the controls were reported previously (Kroeger et al., 2009) and are reproduced here with permission. C, Intrachloroplast localization of PSA3. Maize chloroplasts were hypotonically lysed and separated into a stromal and thylakoid membrane fraction. The membranes were washed with sodium carbonate or NaBr and centrifuged to recover pellet (P) and supernatant (S) fractions. Separate aliquots of the membrane fraction were treated with thermolysin or proteinase K, or with a combination of 0.1% Triton X-100 and the indicated proteases. Equivalent portions of each fraction were analyzed by immunoblotting, using antibodies to PSA3, HCF106, or OE23. HCF106 is an integral membrane protein that is largely exposed to the stroma (Settles et al., 1997). OE23 is bound to the luminal face of the thyakoid membrane. The asterisk marks a fragment of PSA3 that is resistant to protease digestion even after membrane solubilization.
prevent PYG7’s interaction with PSA3 in the split ubiquitin assay (see Fig. 9A; bait: AtPyg7ΔC-Cub).

To determine whether the PYG7-PSA3 interaction detected in yeast occurs inside chloroplasts, we used a bimolecular fluorescence complementation assay (BiFC). At-PSA3 fused to either the N- or C-terminal segments of YFP was coexpressed in Arabidopsis protoplasts with At-PYG7 fused to the complementing half of YFP. YFP fluorescence was observed as punctate signals inside chloroplasts with both sets of constructs (Fig. 9B; Supplemental Fig. S9). YFP fluorescence was not observed when PSA3 was tested against the stroma-localized PSI subunit PsaD, providing evidence that the PSA3-PYG7 interaction is not due simply to their colocalization near PSI. Interestingly, YFP fluorescence was also reconstituted in tests involving PSA3 and the stromal PSI subunit PsaC (Fig. 9B; Supplemental Fig. S9).

Figure 7. Abundance of PSA3 in maize mutants lacking various PSI assembly factors. Total leaf extract from plants of the indicated genotypes were fractionated by SDS-PAGE and analyzed by immunoblotting with antibodies to the indicated proteins. The panels come from four replicate blots, each of which was reprobed several times. The Ponceau-S stained blot below serves as a loading control and is the same blot that was probed to detect PSA3. The line marks the position where one lane was digitally removed. The remaining lanes in each panel all come from the same original digital image.

Figure 8. Immunoblot analyses of PSA3-containing complexes resolved by BN-PAGE. Thylakoid membranes from maize seedlings of the indicated genotype were solubilized with 1% DDM and resolved by BN-PAGE. A and B show experiments that were performed at separate times from independent seedling grow-outs. The major PSA3 band in the wild-type samples migrates just ahead of a clearing in the gray background that corresponds to the PSI + PSII dimer band on the stained gels. The variable abundance of PSA3 in psa2 mutants seen by BN-PAGE (compare A and B) can also be observed by denaturing immunoblot analysis (Supplemental Fig. S6) and may be a function of genetic background. A, The gel shown to the left was transferred to nitrocellulose, and the blot was probed sequentially to detect PSA3 and PsaA. B, The gel shown to the left was transferred to nitrocellulose and probed to detect PsaA. Replicate samples on the same gel were blotted to nitrocellulose and probed to detect PSA3. The line marks the position where two lanes were digitally excised. The remaining lanes in each panel all come from the same original digital image.
Both the PSA3-PYG7 and PSA3-PsaC assays produced robust YFP signal in many protoplasts (Supplemental Fig. S9). It has been proposed that PYG7 interacts with PsaC to promote PSI assembly or stability (Heinnickel et al., 2016; Yang et al., 2017). Thus, it is reasonable to speculate that PSA3, PYG7, and PsaC are all found in close proximity despite the negative result for a PSA3-PsaC interaction in the yeast two-hybrid assay. For example, if PsaC requires its [Fe-S] cluster ligands in order to adopt a conformation that can bind PSA3, the interaction would be unlikely to occur in the context of the yeast nucleus.

DISCUSSION

In this work, we identified a new PSI biogenesis factor in maize and Arabidopsis, and we provided evidence that this factor, denoted PSA3, collaborates with the previously described PSI assembly factor PYG7. We showed that PSA3 acts posttranslationally to promote PSI accumulation, that it localizes to the stromal face of the thylakoid membrane, and that it is found in a thylakoid membrane complex that is slightly smaller than PSI. The possibility that PSA3 and PYG7 might function in concert with one another was initially suggested by their highly correlated expression in Arabidopsis (mutual rank value of 1 at the ATTED-II database; Supplemental Fig. S1). The following experimental data supports this view: (1) PSA3 and PYG7 are both found in a thylakoid membrane complex that is similar in size to PSI (Fig. 8; Stöckel et al., 2006; Yang et al., 2017); (2) the association of PSA3 with this complex is disrupted in a pyg7 mutant but not in a mutant lacking the PSI assembly factor PSA2 (Fig. 8); (3) PSA3 interacts with PYG7 in a yeast two-hybrid assay (Fig. 9A); (4) a BiFC assay showed that PSA3 and PYG7 are in close proximity in vivo (Fig. 9B); (5) PSA3 interacts with PsaC, whereas the same assay did not detect proximity to a different subunit of PSI’s stromal ridge (Fig. 9B; Supplemental Fig. S9); (6) PYG7 was recently shown to interact with PsaC (Yang et al., 2017). Taken together, these results suggest that PSA3 and PYG7 cooperate to promote the stable incorporation of PsaC into PSI.

It was recently shown that the PYG7 ortholog in Chlamydomonas is required for PSI accumulation only when cells are grown in the presence of O2 (Heinnickel et al., 2016). This intriguing finding led to a model in which PYG7 protects PSI from oxidative damage. Because the assembly of PSI’s [Fe-S] clusters and their binding to the cysteines in PsaC are particularly sensitive to oxidation, it was suggested that PYG7 shields either the [Fe-S] clusters themselves or the cysteines

![Figure 9](https://example.com/image9.png)

**Figure 9.** PSA3 interacts with PYG7 in yeast and in vivo. A, Split-ubiquitin yeast two-hybrid assay for the interaction between PSA3 and PYG7. The bait vector encoded the C-terminal ubiquitin fragment (Cub) fused to the C terminus of mature At-PYG7. Prey vectors encoded the N-terminal ubiquitin fragment (NubG) fused to the C terminus or N terminus of mature At-PSA3 (AtPSA3-NubG and NubG-AtPSA3, respectively). An additional prey construct encoded PSA3 lacking its C-terminal 55 amino acids (NubG-AtPSA3 ΔC). Alg5 and NubI were used as negative and positive controls, respectively. Protein interactions were determined by the growth of yeast on selective medium (-TLHA, top). B, BiFC assay demonstrating interactions of PSA3 with PYG7 and PsaC in Arabidopsis protoplasts. Protoplasts were cotransformed with plasmids encoding the indicated fusion proteins. YFPN and YFPC are the N- and C-terminal fragments of YFP, respectively. The PSA3-PsdD interaction assays (bottom two rows) served as a negative control. Bars = 10 μm.
they bind. Our data add to this emerging model by providing evidence that PSA3 and PYG7 interact with one another and that PSA3 is in close proximity to PsaC in vivo. Although we cannot rule out a role for PSA3 and PYG7 in the postassembly stabilization of PSI, a role in de novo assembly seems more likely based on the developmental timing of their expression: The mRNAs encoding PYG7 and PSA3 peak in abundance early in maize leaf development, in a developmental zone corresponding to the onset of chloroplast biogenesis (see Supplemental Fig. S1A). In fact, their mRNAs decline to low levels in the apical regions of the leaf harboring mature, photosynthetically active chloroplasts.

The predicted structure of PSA3 has unusual features that may provide clues as to its mechanisms of action. PSA3 is predicted to bind a peptide ligand in a groove that is lined with acidic side chains (Fig. 5B). Strikingly, this groove is flanked on both sides by Cys pairs. Although closely spaced cysteines often bind metal prosthetic groups, adjacent cysteines are unusual, and where they do occur they rarely bind the same metal ligand (Miller et al., 1989; Richardson et al., 2017). Furthermore, adjacent cysteines are not known to bind [Fe-S] clusters (Roche et al., 2013). Thus, although current data imply that PSA3’s function is focused on PsaC, which binds two [4Fe-4S] clusters, it seems unlikely that PSA3’s Cys pairs bind an [Fe-S] cluster. Furthermore, it seems unlikely to be coincidental that the Cys pairs flank a predicted peptide-binding groove. Disulfide bonds between adjacent cysteines strain the protein backbone and can modulate protein conformation and activities (Park and Raines, 2001; Carugo et al., 2003). Based on these observations, we favor a model in which the oxidation state of the paired cysteines impacts interactions between a basic peptide and PSA3’s acidic groove. PsaC seems an unlikely ligand for this groove because it has a paucity of basic amino acids. PYG7, however, has a pI of approximately 9 and the C-terminal tail that follows its TPR motifs is particularly rich in basic amino acids. However, this tail is not required for the interaction between PYG7 and PSA3 in a yeast split ubiquitin assay (Fig. 9A). The identification of the ligand that binds PSA3’s acidic groove will be an important step toward elucidating PSA3’s mechanisms and presents a challenge for future experiments. However, existing data support the view that these interactions serve some aspect of PSI biogenesis that is centered on PsaC and that PYG7 and PSA3 collaborate in this process.

PSA3 orthologs are present throughout the green euukaryotes (Fig. 5A), but they appear to be absent in cyanobacteria. Cyanobacteria do, however, encode a PYG7 ortholog, which is called Ycf37. The conservation of PYG7/Ycf37 but not PSA3 in cyanobacteria seems to be at odds with our proposal that PSA3 and PYG7 collaborate. However, loss of Ycf37 has only a small effect on PSI activity in cyanobacteria (Dühring et al., 2006), contrasting with the severe PSI deficiency observed in mutants lacking PYG7 in Arabidopsis, maize, and Chlamydomonas (Fig. 7; Stöckel et al., 2006; Heinnickel et al., 2016). PSA3 may have coevolved with PYG7 in photosynthetic eukaryotes to adapt to the increase in oxidative stress resulting from partitioning photosynthesis and respiration into different compartments and from increasing atmospheric O2 (see discussion in Heinnickel et al., 2016). The PSI assembly factors PPD1, PSA2, and Y3IP1 are also specific to photosynthetic eukaryotes (Albus et al., 2010; Liu et al., 2012; Fristedt et al., 2014). The acquisition of these assembly factors correlate with structural changes to PSI during the course of evolution, including the acquisition of entirely different light harvesting systems (Yang et al., 2015a).

PSI is an extraordinarily complicated structure, and the events along its assembly pathway are largely unknown. One intermediate in PSI assembly has been reported in Chlamydomonas (Ozawa et al., 2010) and one in tobacco (Wittenberg et al., 2017). These complexes have similar properties and may represent species-specific variants of the same basic entity: They contain the stromal ridge subunits PsaC and PsaD, they lack PsAK, PsaG, and LHCs, and PsaF is either absent or loosely bound. This complex is approximately 450 kD in tobacco (Wittenberg et al., 2017), which is substantially smaller than mature PSI. BN-PAGE analysis of maize thylakoid membranes revealed what is likely to be the analogous complex, as it has a similar size and protein composition (Figs. 3 and 8, marked with asterisks; Fristedt et al., 2014). Results presented here show that both PSA3 and PYG7 are required for the accumulation of this complex (Figs. 3 and 8), consistent with the view that their activity is related to the maturation/incorporation of the PsA subunit. However, neither PSA3 nor PYG7 is stably bound to this intermediate. Instead, both PSA3 and PYG7 are found in larger particles that are similar in size to PSI: PYG7 cosediments with PSI in Suc gradients (Stöckel et al., 2006; Yang et al., 2017) and PSA3 migrates slightly ahead of PSI during BN-PAGE (Fig. 8). Two observations presented here support the view that these are one and the same complex: (1) the mutational loss of PYG7 disrupts the PSA3-containing complex, whereas the loss of a different PSI assembly factor, PSA2, does not (Fig. 8); and (2) PYG7 and PSA3 interact in yeast and in chloroplasts (Fig. 9). By contrast, PSA2 and another luminal PSI assembly factor, PPD1, are found in smaller complexes of approximately 350 kD (Fristedt et al., 2014; Roose et al., 2014).

It is currently unclear whether the complexes harboring PSA3, PYG7, and the other characterized PSI assembly factors are intermediates in PSI assembly or are associated transiently with assembling PSI components. However, the use of a panel of antibodies to each assembly factor in conjunction with a panel of mutants that lack them offers promise for elucidating the interdependencies of these complexes and, ultimately, the path to a mature PSI complex.
MATERIALS AND METHODS

Plant Materials

The maize (Zea mays) psa3 gene corresponds to gene GRMZM2G051403 (B73 genome v3). The Zm-psa3 mutant was originally identified as a PSI-deficient mutant in a large-scale immunoblot screen of mutants in the PML mutant collection (Belcher et al., 2015). Mx insertions that cosegregate with Zm-psa3-1 were identified by Mx-illuminia sequence analysis (Williams-Carrier et al., 2010). A second allele, Zm-psa3-2, was then identified in a sequencing-based reverse genetic screen of the same mutant collection. The mutant alleles were propagated by outcrossing heterozygotes to inbred lines and self-pollinating the F1 progeny to produce ears segregating homozygous mutants. Insertion alleles of the maize ortholog of Arabidopsis (Arabidopsis thaliana) PSY7 (GRMZM5G809292, B73 v3) were recovered using the analogous approaches. The insertions sites and phenotypes of the Zm-psy7 mutants are shown in Supplemental Figure S10. Zm-psa2, Zm-gsi1PI, Zm-tal02, and Zm-otf51 mutants were described previously (Khrouchtchova et al., 2012; Fristedt et al., 2014; Belcher et al., 2015). Maize seedlings were grown on soil in a growth chamber under day-night cycles: 16 h light (400 μmol photons m⁻² s⁻¹), 28°C/8 h dark, 26°C. The apical portion of the second and third leaves were harvested between 8 and 9 d after planting for protein, RNA, and ribosome profiling analysis. The chloroplast fractions used for immunoblot analysis of PSY3 localization were obtained as described previously (Williams and Barkan, 2003). The PSY3 ortholog in Arabidopsis is AT3G55250 (see http://cas-pogs.ucoregon.edu/#/pog/18720 for evidence of orthology). A line harboring a T-DNA insertion in this gene (SAIL_503_B01, Col-0) was obtained from the SALK collection (http://signal.salk.edu). The presence of the insertion was confirmed by sequencing PCR products (primers listed in Supplemental Table S1). Arabidopsis seeds were sterilized and grown on MS medium supplemented with 2% Suc and 0.8% agar, under 12-h light/12-h dark cycles, 50 μmol photons m⁻² s⁻¹ at 25°C. For complementation of the At-psa3 mutant, the At-PSA3:AtDNA was cloned into the pCAMBIA1300 vector to express AtPSA3 with a C-terminal Flag tag. Transformants were generated by the floral-dip method (Clough and Bent, 1998).

Chlorophyll Fluorescence and P700 Oxidation Measurements

Chlorophyll a fluorescence induction kinetics were monitored with a mini-PAM chlorophyll fluorimeter (Walz). After a 30-min dark adaptation, plants were illuminated with low intensity red light (0.05-0.1 μmol photons m⁻² s⁻¹) to induce minimum fluorescence (F₀). This was followed by a saturating pulse of light (8000 μmol photons m⁻² s⁻¹ for 0.8 s) to induce Fm. The steady-state fluorescence (Fₛ) was then recorded for 4 min during illumination with actinic light (50 μmol photons m⁻² s⁻¹). P700 absorbance changes at 830 nm were measured with a PAM101 chlorophyll fluorimeter (Walz) as previously described (Meurer et al., 1996). Oxidation of P700 was induced by saturating far-red light (24 μmol photons m⁻² s⁻¹).

Immunoblot, SDS-PAGE, and BN-PAGE Analysis

SDS-PAGE and immunoblot analyses were performed as described previously (Barkan, 1998). Thylakoid membranes were prepared, solubilized with 1% DDM, and fractionated by BN-PAGE as described in Peng et al. (2008). For immunoblot analysis of BN-PAGE gels, proteins in the gels were denatured by incubation in 2×SDS-urea PAGE buffer (8 M urea, 5% SDS, 20% Glycerol, 50 mM Tris-HCl, pH 6.8, 5% β-Mercaptoethanol, and 1% Bromophenol blue) for 1 h and then electrophoretically transferred to nitrocellulose.

Antibodies

Polyclonal antibodies to PetA, AtPb, AtPa, PsaD, D1, OEC2, and HCF106 were described previously (Roy and Barkan, 1998). The HCF106 and NidH antibodies were generous gifts of Rob Martienssen and Klaus Steinmueller, respectively. The other antibodies used here were purchased from Agrisera. A polyclonal antibody against Zm-PSA3 was produced in rabbits at Agrisera, using recombinant Zm-PSA3 (amino acid sequence from 110 to 269).

Analyses of Chloroplast Gene Expression

In vivo pulse-chase analysis of chloroplast-encoded proteins in Arabidopsis was performed as described in Liu et al. (2012). In brief, excised primary leaves were incubated in labeling buffer (20 μg/mL cycloheximide, 1 mM KH₂PO₄, pH 6.3, 0.1% (w/v) Tween 20) for 30 min, and 5 μL [³⁵S]-Met (> 1000 Ci/mmol, 11 μCi/mL) was added for 20 min. The radioactive buffer was removed and replaced with chasing buffer (labeling buffer supplemented with 1 mM Met) and processed for thylakoid membrane purification and SDS-PAGE after varying amounts of time (0, 1, or 2 h). Lanes were loaded on the basis of equal cpm (30,000 cpm per sample).

Protein-Protein Interaction Assays

Yeast two-hybrid analyses involving membrane-associated proteins were performed using the DUAL membrane 2 split ubiquitin system (Staglar et al., 1998; Dualsystems Biotech) according to the manufacturer’s instructions. A sequence encoding mature At-PYG7 (amino acids 60–260), and At-PYG7 ΔC (amino acids 60–273) were cloned into the pCCW-SUC vector to express bait proteins fused to Cub-LexA-VPl6. Mature At-PSA3 (amino acids 46–277) was used as the “prey” by cloning into pDsl-Ne and pDL2-xN to express fusion proteins with NubG at the N or C terminus of PSA3, respectively. Mature PSA3 with a C-terminal deletion (ΔC, 55 amino acids as marked in Fig. 5A) was also cloned into pDSL-Ne for use as prey. The bait and prey plasmids were cotransformed into the NWy32 yeast strain, and the interactions were determined by growth on agar plates with Synthetic Defined (SD) medium lacking Leu, Trp, His, and adenine. Traditional yeast two-hybrid assays were performed with the GAL4 two-hybrid system (Clontech) according to the manufacturer’s instructions. A sequence encoding mature At-PSA3 was cloned into pGBK7 (bait vector), and sequences encoding PsaC, PsaD, and PsaE were cloned into pGADT7 (prey vector). The bait and prey plasmids were cotransformed into Y2H Gold yeast strain (Clontech). The transformants were grown on agar plates with SD medium lacking Leu, Trp, His, adenine and X-a-gal.

BiFC assays were performed as described by (Walter et al., 2004), using vectors that were generously provided by Congning Lu (Chinese Academy of Sciences). Full-length cDNAs encoding At-PSA3, At-PYG7, At-PsaC, and At-PsaD were cloned into pUC-SPYNe and pUC-SPYCE, respectively. Constructs were cotransformed into Arabidopsis protoplasts, and YFP fluorescence was captured by a confocal laser-scanning microscope (LSM 510 meta; Zeiss) 16 h later.

Accession Numbers

Genes discussed in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At-PSA3 (AT3G55250), Zm-PSA3 (GRMZM2G051403), Pp-PSA3 (XP_001780245.1), Cr-PSA3 (XP_0101710248.1), Zm-PYG7 (GRMZM5G809292), Zm-Y3IP1 (GRMZM2G002165), Zm-Psa2 (GRMZM2G0021687), At-PYG7 (At1g22700), At-Y3IP1 (AT5G44650), and At-PSA2 (AT2G34860).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. PSY3 expression profiles in maize and Arabidopsis.

Supplemental Figure S2. Immunoblot analysis of core subunits of photosynthetic complexes in the heteroallelic progeny of complementation test crosses between plants harboring the Zm-psa3-1 and Zm-psa3-2 alleles.

Supplemental Figure S3. Two-dimensional BN-PAGE/SDS-PAGE separation of thylakoid membrane complexes in maize and Arabidopsis wild type and psa3 mutants.

Supplemental Figure S4. Spectroscopic assays of photosynthetic electron transport in Zm-psa3 mutants.

Supplemental Figure S5. Expression of chloroplast genes encoding PSI subunits in psa3 mutants.

Supplemental Figure S6. Immunoblot showing genotype-dependent accumulation of PSA3 in maize leaf tissue.

Supplemental Figure S7. Multiple sequence alignment of PYG7 orthologs.
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OTP51 with two LAGLIDADG motifs is required for the cis-splicing of

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