Immunophilin AtFKBP13 Sustains All Peptidyl−Prolyl Isomerase Activity in the Thylakoid Lumen from *Arabidopsis thaliana* Deficient in AtCYP20-2[†]

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ABSTRACT: The physiological roles of immunophilins are unclear, but many possess peptidyl−prolyl isomerase (PPIase) activity, and they have been found in all organisms examined to date, implying that they are involved in fundamental, protein-folding processes. The chloroplast thylakoid lumen of the higher plant *Arabidopsis thaliana* contains up to 16 immunophilins (five cyclophilins and 11 FKBPs), but only two of them, AtCYP20-2 and AtFKBP13, have been found to be active PPIases, indicating that the other immunophilins in this cellular compartment may have lost their putative PPIase activities. To assess this possibility, we characterized two independent *Arabidopsis* knockout lines lacking AtCYP20-2 in enzymological and quantitative proteomic analyses. The PPIase activity in thylakoid lumen preparations of both mutants was equal to that of corresponding wild-type preparations, and comparative two-dimensional difference gel electrophoresis analyses of the lumenal proteins of the mutants and wild type showed that none of the potential PPIases was more abundant in the AtCYP20-2 deficient plants. Enzymatic analyses established that all PPIase activity in the mutant thylakoid lumen was attributable to AtFKBP13, and oxidative activation of this enzyme compensated for the lack of AtCYP20-2. Accordingly, sequence analyses of the potential catalytic domains of lumenal cyclophilins and FKBPs demonstrated that only AtCYP20-2 and AtFKBP13 possess all of the amino acid residues found to be essential for PPIase activity in earlier studies of human cyclophilin A and FKBP12. Thus, none of the immunophilins in the chloroplast thylakoid lumen of *Arabidopsis* except AtCYP20-2 and AtFKBP13 appear to possess prolyl isomerase activity toward peptide substrates.

Cyclophilins and FKBPs[1] (FK506 binding proteins) are two ubiquitous families of immunophilins, receptors of the immunosuppressive drugs cyclosporin A and FK506, respectively (*1*). Several hundred immunophilins have been identified in living organisms. However, the cellular functions of most of these proteins remain obscure. Despite the lack of sequence similarities between the two families, both of them include enzymes possessing peptidyl−prolyl *cis/trans* isomerase (PPIase) activity (*2*−*4*). *Cis/trans* isomerization of polypeptide chains around proline residues may impede correct protein folding. Accordingly, PPIase activity of several immunophilins has been shown to be important for catalysis of protein folding in vivo (*4*−*6*). A number of immunophilins have also been characterized as multifunctional proteins and active components of receptors (*7*), chaperone complexes (*8*), membrane channels (*3*), and membrane pores (*9*), but in many cases there is no evidence that they have isomerase activity. Analysis of the *Arabidopsis thaliana* genome has revealed 52 immunophilins, the largest set of immunophilins identified in any organism to date (*10*). Only a small fraction of the plant immunophilins has been studied and characterized, but some of those that have been investigated have been shown to be important for plant growth and development, as reviewed in refs *10*−*12*. Deletion of three complex immunophilins, cytosolic AtCYP40 (*13*), nuclear AtFKBP72 (*14*), and cytosolic AtFKBP42 (*15*), has resulted in diverse developmental defects in *Arabidopsis*. These proteins have been suggested to associate with protein chaperone complexes, and no evidence has been acquired that they have isomerase activity, or that such activity is required to fulfill their biological functions. For instance, AtFKBP42 has been found to be inactive as a PPIase (*15*).

Bioinformatic and proteomic studies have predicted or identified up to five cyclophilins and 11 FKBPs in the chloroplast thylakoid lumen of *Arabidopsis*, making immunophilins the largest group of enzymes in this compartment, raising questions about why this tiny cellular compartment

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[1] Abbreviations: FKBP, FK506 binding protein; PPIase, peptidyl−prolyl *cis/trans* isomerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis.

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contains so many of these proteins (*10*, *12*, *16*−*19*) and prompting speculations that the protein (un)folding activities of these potential isomerases are one of the central functions of the lumen (*16*, *17*). However, recent mapping of isomerase activities in the thylakoid lumen of *Arabidopsis* has demonstrated that all of the PPIase activity can be attributed to just two immunophilins: AtCYP20-2 and AtFKBP13 (*20*). Thus, the roles of the immunophilins in the thylakoid lumen of plant chloroplasts remain to be elucidated, and a key issue (addressed in the study presented here) is how many of them have PPIase activity.

Two cyclophilins, TLP40 (*21*, *22*) and TLP20 (*23*), possessing PPIase activity have been isolated from the chloroplast thylakoid lumen of spinach. TLP40 was characterized as a complex multifunctional cyclophilin that regulates the activity of a thylakoid membrane protein phosphatase (*21*, *22*), while TLP20 was shown to be the most active PPIase in the spinach thylakoid lumen (*23*). However, the PPIase activities of both TLP40 and TLP20 have only been assayed with a single synthetic peptide substrate. This so-called A-substrate, which contains an alanine residue preceding a proline residue (*21*, *23*), is suitable for analyzing the PPIase activity of cyclophilins but not ideal for FKBPs, which preferentially catalyze the isomerization of peptides with a prolyl preceded by a bulky hydrophobic residue such as leucine (L-substrate) (*24*, *25*). A recent study of PPIase activities in the *Arabidopsis* thylakoid lumen, in which both A- and L-substrates were used, found that all PPIase activity was attributable to AtCYP20-2 (a homologue of spinach TLP20) and AtFKBP13, the latter being the major contributor for both substrates, while AtCYP38 (a homologue of spinach TLP40) did not have detectable PPIase activity (*20*). Furthermore, the activity of AtFKBP13 was significantly repressed by the thiol-reducing agent DTT. This finding is consistent with the previously reported redox regulation of recombinant AtFKBP13 (*26*) and supports a recently proposed paradigm: that modulation of the redox states of proteins in the chloroplast thylakoid lumen plays a major role in the regulation of enzymatic activities in this compartment (*27*). In addition, the level of AtCYP20-2, the only cyclophilin found to be active as a PPIase in the thylakoid lumen of *Arabidopsis*, was significantly increased when *Arabidopsis* plants were exposed to high light (*28*) or low temperature (*19*), suggesting that this PPIase plays a functional role in acclimation responses.

In the study presented here we combined reverse genetic, quantitative proteomic, and enzymological approaches to investigate the PPIase activity and immunophilins in the thylakoid lumen of *Arabidopsis* plants lacking AtCYP20-2. We found that the abundance of none of the lumenal immunophilins increased in the AtCYP20-2 deficient plants, but they retained wild-type levels of PPIase activity, which appeared to be due to compensatory increases in the activity of AtFKBP13 via oxidative activation. Finally, sequence analyses of all lumenal cyclophilins and FKBPs confirmed the conclusion drawn from our experimental findings that most immunophilins in the chloroplast thylakoid lumen of *Arabidopsis* are not active PPIases.

EXPERIMENTAL PROCEDURES

Plant Material. A. thaliana plants homozygously carrying T-DNA insertions in the *AtCYP20-2* gene (locus At5g13120) and wild-type (ecotype Columbia-0) plants were grown hydroponically (*29*, *30*) under 8/16 h light/dark photoperiods, with a light intensity of 150 μmol of photons m^{-2} s^{-1}, except in high-light experiments in which the light intensity was 800 μmol of photons m^{-2} s^{-1}. Plants intended for further analyses of thylakoid lumen by 2D separations were cultivated on GM-agar plates, transferred to soil after 10 days, and harvested after 7 weeks.

The mutant lines were SALK_009552 and SALK_129937 [obtained from the Salk Institute collection (*31*) and hereafter referred to as *AtCYP20-2a* and *AtCYP20-2b*, respectively]. Plants that were homozygous for the T-DNA insertion were identified by PCR analysis using the *AtCYP20-2* gene-specific primers 5′-TTT GCA CAG GTA AAT ATG CTT CAT AGG AT-3′ (forward) and 5′-AGA TCG GAC TTT AAA TAC AAA TTG CTC GT-3′ (reverse) and the T-DNA left border specific primer 5′-TGG TTC ACG TAG TGG GCC ATC G-3′. RNA was isolated from samples of the plants using a Qiagen RNeasy plant mini kit according to the manufacturer's instructions. Reverse transcription PCR was performed using the following primers, based on sequences in the *AtCYP20-2*, *AtCYP20-3*, and *AtCYP38* coding regions: 5′-CCT CCT TCA CAC GTC TTA TAC CAA-3′ (forward) and 5′-TGT CTG TCT CTT GTT CCT CTA TCA G-3′ (reverse) for *AtCYP20-2*; 5′-GTC TTC TAT GCA GAT GGT TCA CAC T-3′ (forward) and 5′-AAG ATT CCA GGT CCA GTA TGC TTC-3′ (reverse) for *AtCYP20-3*; 5′-TTA GTG TCG TCA ATT CCT CCA GAT-3′ (forward) and 5′-AGC CAT CAA CCA TAT CCT CTT CTA T-3′ (reverse) for *AtCYP38*. PCR products were separated in 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

Isolation of Thylakoid Lumen Proteins. Soluble proteins from the thylakoid lumen were isolated as in ref *16*, except that for lumen samples subjected to chromatographic separations the Yeda-press buffer was changed to 20 mM Tricine, pH 7.8, 5 mM $MgCl_2$, and 100 mM sucrose. The chlorophyll and protein concentrations were determined as in ref *32*, following extraction in 80% acetone, and according to ref *33* with bovine serum albumin as a standard, respectively.

Chromatographic Fractionation of Lumenal PPIases. Lumenal proteins from wild-type and *AtCYP20-2a* mutant plants were separated by applying samples, acquired as described above, to a Resource Q strong anion-exchange column (GE Healthcare) equilibrated with 20 mM Tricine, pH 7.8, and eluting with a bilinear NaCl gradient in 20 mM Tricine, pH 7.8, buffer (0−0.25 M NaCl over 25 min, then 0.25−1 M NaCl over 5 min, followed by 1 M NaCl isocratically held for 4 min; flow rate 1 mL/min throughout). The void volume was also collected, and the proteins therein were separated using a Resource S strong cation-exchange column (GE Healthcare), with the same column equilibration and elution protocols as for the Resource Q column.

Peptidyl−*Prolyl cis/trans Isomerase Assays.* PPIase activities toward each of two peptide substrates were measured in coupled reactions with chymotrypsin as described by ref *2*. The peptide substrates *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide (A-substrate) and *N*-succ-Ala-Leu-Pro-Phe-*p*-nitroanilide (L-substrate) (both from Bachem) were each dissolved in trifluorethanol, with 400 mM LiCl to increase the proportion of the *cis* isomer (*25*). The reactions were started by adding the peptide substrate to a mixture of the protein

sample and 64 μM chymotrypsin in 0.1 M Tricine, pH 7.8 at 10 °C. The change in absorbance at 390 nm due to the release of *p*-nitroaniline was monitored using a Perkin-Elmer Lambda 25 spectrophotometer, and the extinction coefficient of 13400 M^{-1} cm^{-1} (*24*) was used for kinetic calculations. Reaction rates were derived by nonlinear curve fitting to a first-order rate equation using Origin 7.5 software. The enzymatic activity of each sample was calculated as the difference between the rates of catalyzed and spontaneous reactions. Reductive inhibition of PPIase activity was measured by calculating the difference in the activities of the lumenal proteins assayed before and after incubation in the presence of 2 mM DTT for 30 min at 22 °C. Inhibition of PPIase activity by FK506 was assayed after incubating the protein samples with 1−10 μM FK506 for 60−90 min on ice.

SDS−PAGE and Immunoblotting. Proteins were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) in 15% acrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were blocked with milk overnight and then treated according to the manufacturer's recommended protocol for the ECL Plus kit (GE Healthcare). Polyclonal antiserum to AtCYP20-2 (raised against a synthetic peptide, Cys-Gly-Gln-Leu-Pro-Met-Ser-Glu-Ala, corresponding to the C-terminus of the protein) was kindly provided by Dr. P. G. N. Romano. Polyclonal antisera to AtFKBP13 and AtCYP38 were generated against synthetic peptides corresponding to amino acids 17−31 and 211−225 of the respective mature proteins.

Fluorescent Labeling of Lumenal Proteins. Prior to labeling the lumenal samples were spin-dialyzed using Microsep 10 K Omega concentrators (Pall Life Sciences) with water to reduce their salt concentrations to less than 10 mM. Proteins were precipitated by adding 4 volumes of acetone and incubating the resulting mixtures at −20 °C for 2 h. The samples were sedimented in a microcentrifuge (10000*g* for 10 min) and then solubilized in 4.6 M urea, 2.2 M thiourea, 5.1% CHAPS (w/v), and 30 mM Tris-HCl, pH 8.5. The proteins were labeled using the fluorescent cyanine dyes developed for GE Healthcare's 2D-DIGE system, following the manufacturer's recommendations. Internal standard samples were prepared by pooling all protein samples included in this study (i.e., three samples representing each of four biological replicates) and were minimally labeled with Cy2. Individual protein samples were labeled with either Cy3 or Cy5, in an equimolar mixture, to minimize dye bias effects. Cyanine dyes were reconstituted in 99.8% anhydrous DMF and added to labeling reactions in a ratio of 100 pmol of CyDye to 25 μg of protein. The labeling mixture was incubated on ice in darkness for 30 min, after which time the reaction was terminated by adding 10 nmol of lysine and incubating for a further 10 min on ice. Three protein samples labeled with different dyes were then pooled, and the appropriate volume of rehydration buffer [4.6 M urea, 2.2 M thiourea, 5.1% CHAPS (w/v), 0.11 M DTT (w/v), 0.9% pH 3−10 Pharmalyte carrier ampholytes (v/v) (GE Healthcare)] was added to the final volume of 350 μL prior to IEF.

2D Gel Separation. Samples were separated by isoelectric focusing in the first dimension and SDS−PAGE in the second dimension, as described in ref *16* with a minor alteration of the slab gel gradient to $T = 10-18\%$. Images of the separated, Cy2-, Cy3-, and Cy5-labeled proteins were acquired using a Thyphoon 9400 imager (GE Healthcare) at excitation/emission wavelengths (nm) of 488/520, 532/580, and 633/670, respectively. All of the gels were scanned at 1000 μm resolution. Images were cropped to remove areas extraneous to the gel image using ImageQuant v5.2 (GE Healthcare) prior to image analyses.

Image Analyses. The acquired images were analyzed using DeCyder v4.0 software (GE Healthcare). The Cy2, Cy3, and Cy5 images from all of the gels were imported and processed in an automated fashion using the DeCyder batch processor. The number of spots for each codetection was set to 2500. Following gel-to-gel matching of spots, the statistical significance of differences in the abundance of the corresponding proteins between mutant and wild-type samples was assessed by Student's *t*-test evaluation of differences in the log-standardized signals from the gels using the DeCyder BVA software module. The apparent masses and p*I*s of the proteins were estimated by the DeCyder BVA software using identified proteins of known masses as references.

Staining of Gels, Trypsin Digestion, and Mass Spectrometric Analyses. Gels were silver stained according to ref *34*, and the spots of interest previously highlighted by the DeCyder software were manually excised from the gels and then digested in-gel, using sequencing-grade modified trypsin (Promega) essentially according to the procedure described in ref *35*. Electrospray ionization tandem mass spectra were acquired using an API Q-STAR Pulsar i (Applied Biosystems, Foster City, CA) hybrid mass spectrometer equipped with a nanoelectrospray ion source (MDS Protana, Odense, Denmark). Collision-induced decomposition of selected peptide ions was performed using the instrument settings recommended by Applied Biosystems with manual control of the collision energy.

RESULTS

AtCYP20-2 Arabidopsis Mutants and Their Characteristics. To assess the functional significance of the AtCYP20-2 protein, we used a reverse genetics approach. Two independent *Arabidopsis* lines with T-DNA insertions in the *AtCYP20-2* gene (locus At5g13120) were obtained from the Salk Institute collection (*31*) and named in this study *AtCYP20-2a* and *AtCYP20-2b* (SALK_009552 and SALK_129937, respectively). *AtCYP20-2a* has a T-DNA insertion in the third intron, while the insert in *AtCYP20-2b* is located in the first intron of the gene (Figure 1A). Homozygous plants with the disrupted *AtCYP20-2* gene were obtained by self-crossing and verified by PCR analysis of genomic DNA with two gene-specific primers and a T-DNA-specific primer as shown in Figure 1B. To assess the expression of *AtCYP20-2* at the transcriptional level, total RNA was isolated and subjected to semiquantitative reverse transcription PCR. No *AtCYP20* transcript was detected in *AtCYP20-2a* samples, but a product was found, at ca. 40% of wild-type levels in *AtCYP20-2b* samples (Figure 1C; quantification data not shown). Notably, the forward primer for the reverse transcription PCR (see Experimental Procedures) was situated in the second exon of *AtCYP20-2*, which is downstream of T-DNA insertion in *AtCYP20-2b*. The *AtCYP20-2* mutants were further characterized at the protein content level by immunoblotting. The analyses of the total

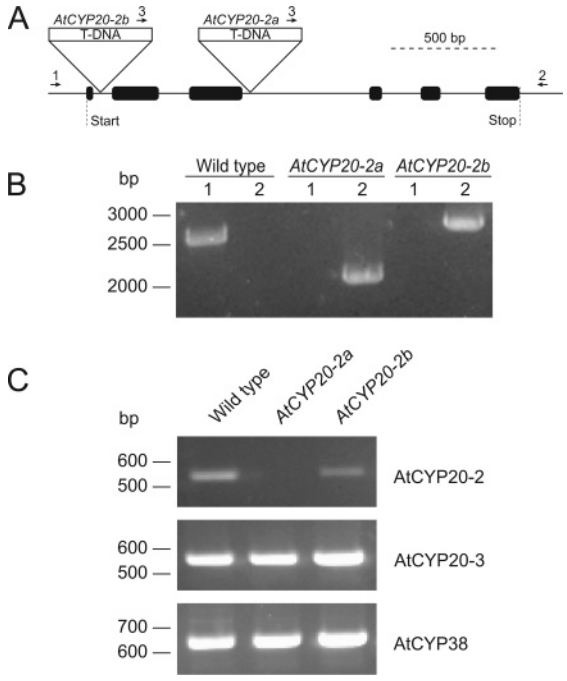

FIGURE 1: Characterization of homozygous *AtCYP20-2a* and *AtCYP20-2b Arabidopsis* mutants. (A) Localization of two different T-DNA insertions within the At5g13120 gene. Exons and introns are presented by broad and thin segments, respectively. Positions of two gene-specific primers (1 and 2) and a T-DNA-specific primer (*3*) are indicated by arrows. (B) Ethidium bromide-stained gel with the PCR products obtained from genomic DNA isolated from wild type or *AtCYP20-2a* and *AtCYP20-2b* mutants, as indicated. PCR was performed using gene-specific primers 1 and 2 (lane 1) or a combination of a gene-specific (2) and a T-DNA-specific primer (3) (lane 2). (C) Representative ethidium bromide-stained gels with the products of reverse transcription PCR of mRNA isolated from the wild type and the *AtCYP20-2* mutants. The PCR products were obtained using primers specific for *AtCYP20-2*, *AtCYP20-3*, and *AtCYP38*, as indicated.

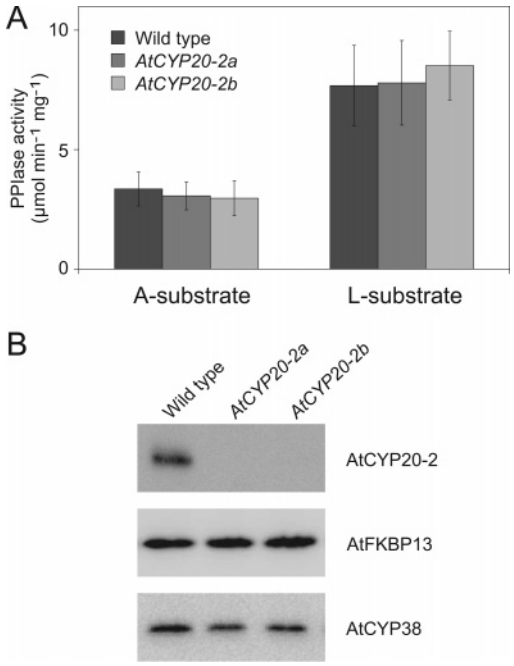

FIGURE 2: Effect of the absence of AtCYP20-2 on the total PPIase activity in the *Arabidopsis* thylakoid lumen. (A) Total PPIase activity in the thylakoid lumen from the wild type and *AtCYP20-2* mutants (as indicated), measured with A- and L-substrates and expressed in enzymatic units. (B) Representative immunoblotting of lumenal immunophilins AtCYP20-2, AtFKBP13, and AtCYP38 in wild-type *Arabidopsis* and two *AtCYP20-2* mutants.

leaf extracts revealed that the *AtCYP20-2b* mutant expressed 10−20% of AtCYP20-2, as compared to the wild type (data not shown). However, no AtCYP20-2 was detected in the thylakoids of either mutant line with the anti-AtCYP20-2 antibodies in the linear range of the immunoresponse (Figure 2B). This finding demonstrates that accumulation of the mature AtCYP20-2 in the thylakoid lumen is abolished in both mutant lines. AtCYP20-2 translated in the *AtCYP20-2b* mutant is presumably not imported into the thylakoid lumen due to corruption of the N-terminal chloroplast transit peptide by the T-DNA insertion (see Figure 1A). Neither of the two mutants lacking the AtCYP20-2 protein showed any obvious phenotypic deviations with respect to growth rate, flowering, chlorophyll content, or biomass under standard growth conditions (data not shown). The level of AtCYP20-2 has previously been found to increase in high-light cultivation conditions (*28*). Therefore, we exposed both AtCYP20-2-deficient mutants to high light, but this treatment did not reveal any apparent phenotypic differences in comparison with the wild type (data not shown).

We also examined the effects of the absence of AtCYP20-2 on total PPIase activity in the thylakoid lumen. For this purpose we isolated thylakoid lumenal proteins from wild-type *Arabidopsis* and the mutants and assayed their enzymatic activity toward both A- and L-substrates. To our surprise, despite the absence of AtCYP20-2 no obvious reduction in the total PPIase activity in the mutants was found, as compared with the wild type (Figure 2A). The protein that appeared most likely to be able to compensate for reductions in isomerase activity due to the loss of AtCYP20-2 was AtFKBP13, the most active PPIase in the *Arabidopsis* thylakoid lumen (*20*). However, immunoblotting analysis of the wild-type and mutant thylakoid proteins with AtFKBP13-specific antibodies did not detect any significant differences in the abundance of this immunophilin (see Figure 2B for representative blots). Western analyses also confirmed the absence of AtCYP20-2 in both mutants and the similarity of levels of AtCYP38 in the mutant and wild-type plants (Figure 2B). Therefore, we concluded that loss of the PPIase activity in the *AtCYP20-2* mutants was compensated either by upregulation of the expression of other PPIases in the thylakoid lumen of *Arabidopsis* (*16*, *17*, *19*) or by increase in the activity of AtFKBP13.

Comparative Proteomic Analyses of Thylakoid Lumen from the Mutants and Wild Type. In an attempt to identify the protein(s) responsible for the compensation of PPIase activity in the absence of AtCYP20-2, and to analyze secondary changes in protein abundance in the mutants, we used "difference gel electrophoresis" (DIGE). This technique involves minimal labeling by fluorescent dyes and is excellent for protein quantification after 2D separation. Thylakoid lumen preparations from four replicates of each mutant line and wild type were used in these experiments. Representative fluorescent images of differentially labeled proteins in the two-dimensionally separated preparations from (i) the wild type and *AtCYP20-2a* and (ii) the wild type and *AtCYP20-2b* are shown in panels A and B of Figure 3, respectively. In these cases, wild-type proteins were labeled with the fluorescent dye Cy3, while the mutant samples were labeled

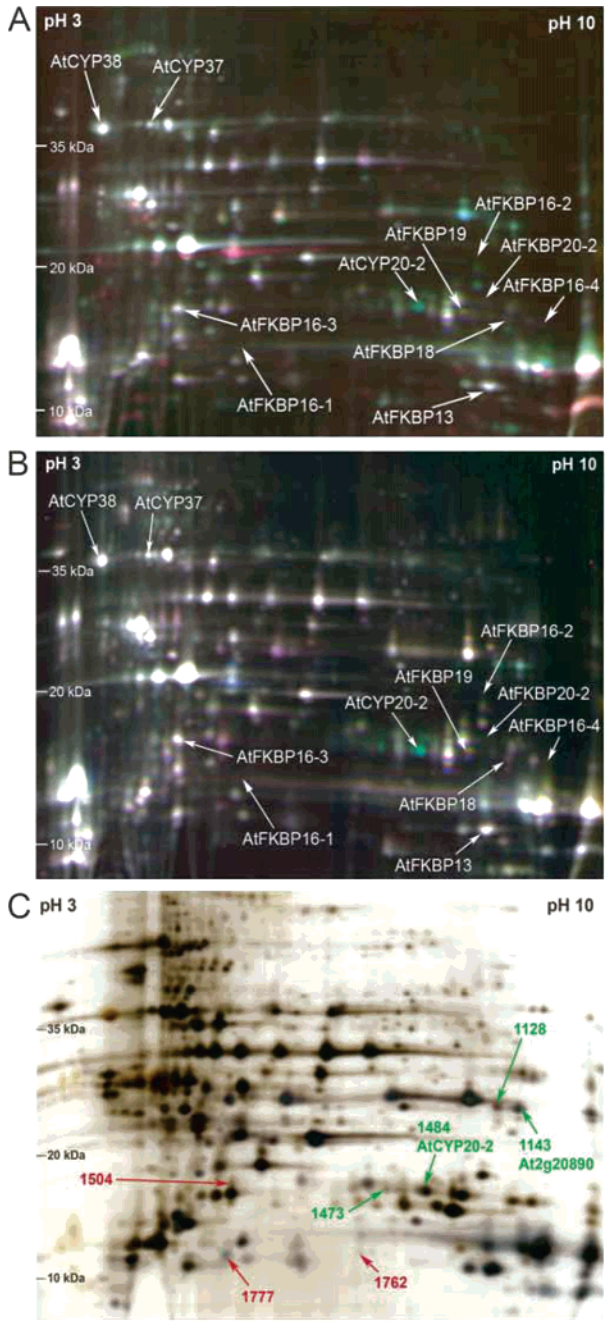

FIGURE 3: Protein expression changes in the thylakoid lumen of two *AtCYP20-2* mutants. (A, B) Representative 2D gels showing differential protein expression in the *AtCYP20-2a* (A) and *AtCYP20-2b* (B) mutants compared to the wild type. Prior to the electrophoretic separation, the protein samples from the wild-type and mutant strains were labeled with the fluorescent dyes Cy3 and Cy5, respectively. Consequently, a protein that is expressed more strongly in the wild-type lumen produces a green signal, while a protein that is more abundant in the mutant lumen appears red. White spots indicate no change in expression. The immunophilins previously identified in the *Arabidopsis* thylakoid lumen (*10*, *12*, *16*, *17*) are labeled, and their positions are marked with arrows. None of these immunophilins, except AtCYP20-2, demonstrated apparent changes in expression in the mutants as compared with the wild type (see Table 1 and Supporting Information, Table 1). (C) A representative silver-stained 2D gel with indications of the proteins that showed more than 2-fold statistically significant changes in expression in both *AtCYP20-2* mutants, as compared to the wild type. The positions of three proteins upregulated in the mutants are shown in red, and those of four proteins downregulated in the mutants (including AtCYP20-2) are shown in green.

with the Cy5 dye. Proteins less abundant in the respective mutants than in the wild type are seen as green spots, while proteins that are more abundant in the mutants are in red. White spots indicate proteins that did not differ in abundance between the mutant and wild-type samples. After gel-to-gel matching of protein spots, in total 199 protein spots were detected in all gels and included in the statistical analyses. The criteria we applied to consider a protein spot to be up- or downregulated were that (i) there had to be at least 2-fold absolute differences in their abundance between wild-type samples and samples of both mutants and (ii) the differences in abundance had to exceed the 95% confidence intervals according to Student's *t*-tests of the data. These stringent criteria were chosen to minimize the number of false positive spots detected. The proteins that met these criteria are shown in Table 1, together with the lumenal immunophilins that were unambiguously identified by MS in the 2D gels. The total dataset for the experiments can be obtained from Supporting Information, Table 1.

Our first aim was to investigate whether the absence of AtCYP20-2 changed the abundance of any other cyclophilins or FKBPs in the thylakoid lumen. The positions of the cyclophilins and FKBPs previously reported to be present in the *Arabidopsis* thylakoid lumen (*16*, *17*) are marked in Figure 3A,B. These proteins were identified by matching our gels with published gels and additional MS analyses. The candidate immunophilins are listed in Table 1. None of these potential PPIases showed a statistically significant change in abundance in the thylakoid lumen as a consequence of the absence of AtCYP20-2. Thus, compensation for the loss of PPIase activity in the thylakoid lumen of the *AtCYP20-2* mutants, described above, was not a result of increased amounts of other cyclophilins or FKBPs.

We also examined whether any other lumenal proteins were affected by the absence of AtCYP20-2. As indicated in Figure 3C and summarized in Table 1, the sizes of only three protein spots (1143, 1473, and 1128), besides the expected AtCYP20-2 spot (1484), decreased in the mutants. The downregulated protein corresponding to spot 1143 was identified as a recently reported TFH1 (At2g20890) protein suggested to play a crucial role in vesicle-mediated thylakoid membrane biogenesis (*36*). This protein was suggested to be localized on the stromal side of the thylakoids (*36*, *37*) and, thus, is unlikely to be involved in PPIase activities in the thylakoid lumen. Two protein spots (1762 and 1504) increased according to our stringent threshold criteria for differentially expressed proteins, and another two (1128 and 1777) marginally failed to meet one of them. The former was more than 2-fold less abundant in both mutants than in the wild type, but its abundance in the *AtCYP20-2a* line fell just outside the 95% confidence interval of its abundance in the wild type (Student's *t*-test, $P = 0.068$), while the latter met the 95% confidence criterion but was only upregulated 1.8-fold in the *AtCYP20-2a* line. Despite several attempts to identify the proteins corresponding to spots 1473, 1128, 1762, 1504, and 1777 by MS after in-gel digestion, we were not able to identify any of them with confidence. Failure to identify the proteins corresponding to these positions in 2D gels has also been reported in several previous proteomic studies of *Arabidopsis* thylakoid lumen (*16*, *17*) and may be due to the MS identification procedures being substantially less sensitive than the DIGE technique.

Table 1: Summary of Protein Expression Changes Caused by Knocking Out AtCYP20-2[a]

master spot no.	protein name	gene locus	exptl p*I*	exptl mass (kDa)	AtCYP20-2a/AtCYP20-2b times protein change	AtCYP20-2a/AtCYP20-2b Student's *t*-test (*P*)
707	AtCYP38	At3g01480	5.3	37.3	−1.0/1.1	0.980/0.300
680	AtCYP37	At3g15520	5.3	34.7	1.0/1.2	0.930/0.160
1511	AtFKBP19	At5g13410	8.2	18.1	−1.2/1.0	0.130/0.690
1492	AtFKBP16−3	At2g43560	5.5	18.4	1.0/1.1	0.860/0.280
1826	AtFKBP13	At5g45680	8.6	14.1	1.1/1.1	0.810/0.310
1484	AtCYP20−2	At5g13120	7.8	18.5	−22/−22	0.000/0.000
1143	THF1	At2g20890	8.9	24.5	−2.4/−2.4	0.036/0.008
1473	not identified		7.4	18.6	−4.8/−4.7	0.001/0.000
1128	not identified		8.6	24.6	−2.1/−2.4	0.068/0.014
1762	not identified		7.1	15.0	2.1/3.8	0.014/0.004
1504	not identified		5.9	18.5	2.1/2.0	0.008/0.007
1777	not identified		5.8	14.8	1.8/2.0	0.043/0.005

[a] The relative changes in protein expression were assayed by 2D-DIGE. The lumenal cyclophilins and FKBPs presented in the upper lines were unambiguously identified by MS in both the mutant and wild-type plants. Protein spots with significantly changed abundance in the mutants *AtCYP20-2a* and *AtCYP20-2b*, compared to the wild type, are presented in the lower lines. The complete set of the data on 199 protein spots that were detected in all gels is presented in Supporting Information, Table 1.

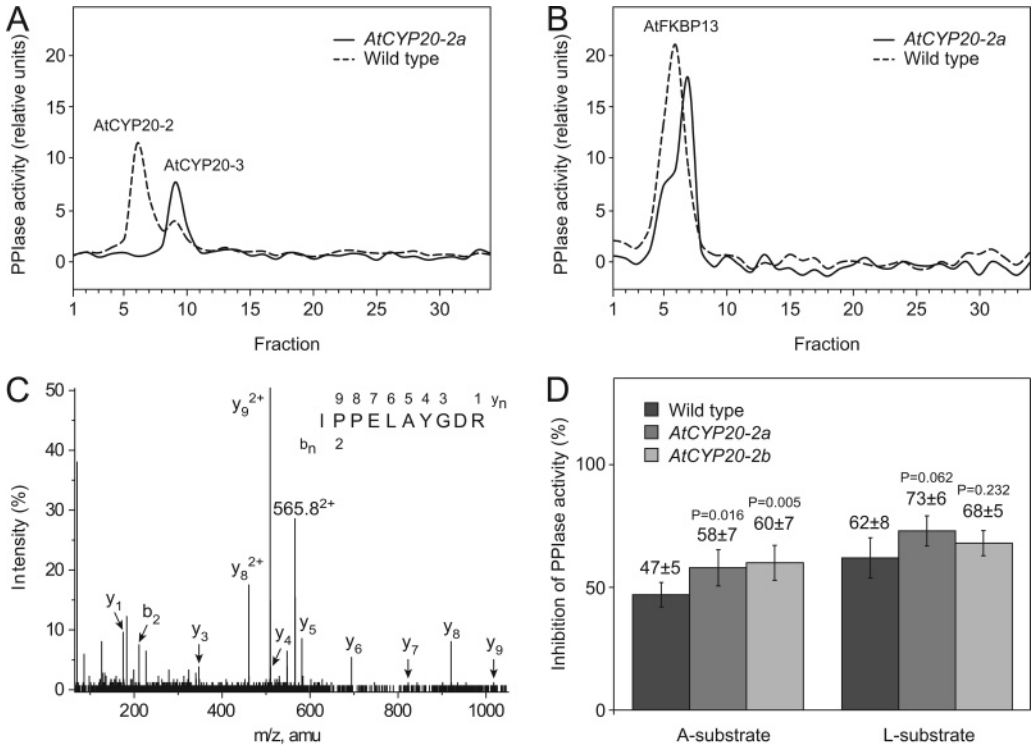

FIGURE 4: Identification of AtFKBP13 as the only active lumenal PPIase in the AtCYP20-2-deficient mutant *AtCYP20-2a*. (A, B) Fractionation of the lumenal PPIase activity toward the A-substrate by anion-exchange and cation-exchange chromatography, respectively. The profiles of PPIase activity are shown by dashed lines for the wild type and solid lines for the *AtCYP20-2a* mutant. The immunophilins corresponding to the activity peaks according to ref *20* are labeled. (C) Collision-induced fragmentation spectrum and sequencing of a tryptic peptide from AtFKBP13 found in fraction 7 of the cation-exchange separated *AtCYP20-2a* mutant lumen proteins [see (B)]. The signal of a doubly charged peptide ion is indicated at *m*/*z* 565.8. The sequence of the peptide (corresponding to amino acids 172−181 in the initial translation product of AtFKBP13) is shown, and the major detected b-ions (N-terminal fragments) and y-ions (C-terminal fragments) are indicated. (D) Inhibition of the total PPIase activity (as measured with the A- and L-substrates) in the thylakoid lumen from wild type and AtCYP20-2 mutants by DTT. The inhibition of PPIase activity due to the DTT treatment (represented in percentages of the initial activity) was more pronounced in the lumen proteins from the mutants, and for the activity toward the A-substrate the difference between the mutants and wild type was statistically significant (see *P*-values above the bars).

Identification of AtFKBP13 as the Only Active PPIase Present in the Lumen of AtCYP20-2 Mutants. To identify the enzyme(s) responsible for maintaining PPIase activity in the *AtCYP20-2* mutants at wild-type levels, we subjected thylakoid lumen proteins from both the *AtCYP20-2a* and wild-type lines to successive separations by anion- and cation-exchange FPLC. It was shown previously that the anion-exchange column binds lumenal cyclophilins AtCYP20-2 and AtCYP38 as well as the stromal cyclophilin AtCYP20-3 present in the lumenal samples as a contamination, while the cation-exchange column binds AtFKBP13 (*20*). The corresponding peaks of wild-type PPIase activity toward the A-substrate are indicated by dashed lines in Figure 4A,B. In the case of the separated *AtCYP20-2a* mutant lumen no isomerase activity peak corresponding to AtCYP20-2 was detected, as shown by the solid line in Figure 4A (the single peak of activity was produced by the contaminating stromal AtCYP20-3). All of the lumenal PPIase activity detected in

the mutant was recovered in a single peak that eluted from the cation-exchange column (Figure 4B). The fractions with the activity peak contained only one major protein band of about 14 kDa, which was excised and subjected to in-gel digestion and subsequent MS analyses of the peptides. Figure 4C shows a collision-induced fragmentation spectrum of a tryptic peptide from this protein, which allows reading of a 10 amino acid long sequence corresponding to AtFKBP13. Therefore, AtFKBP13 was the only active PPIase detected in the lumen of the *AtCYP20-2a* mutant, and we propose that activation of AtFKBP13 compensated for the lack of AtCYP20-2. This hypothesis is indirectly supported by the slight shift of the AtFKBP13 activity peak to the right (see Figure 4B), indicating an increase in the isoelectric point of this protein that could be due to the regulatory oxidation of its cysteine residues.

We recently found that the activity of AtFKBP13 toward both A- and L-substrate peptides can be significantly inhibited by the reduction of intramolecular thiol links within it by DTT (*20*). Furthermore, thiol-mediated redox regulation of the activity of recombinant AtFKBP13 has been well characterized at the molecular level (*26*). Therefore, to assess the possibility that the absence of AtCYP20-2 is compensated by oxidative activation of AtFKBP13, we measured the PPIase activity in the lumen samples from the mutants and wild-type plants before and after addition of 2 mM DTT. The results of these experiments, shown in Figure 4D, demonstrate that DTT induced greater reductions in PPIase activity toward the A-substrate in the two mutants than in the wild type; the difference was small but statistically significant ($P = 0.016$ and $P = 0.005$ for *AtCYP20-2a* and *AtCYP20-2b*, respectively). The inhibition of isomerase activity toward the L-substrate was also more pronounced in the mutants than in the wild-type plants, although the differences were not statistically significant in this case (Figure 4D). These findings show that PPIase activity was more strongly inhibited in the mutants than in the wild type and suggest that loss of AtCYP20-2 in the mutants was compensated by oxidative activation of AtFKBP13.

We also found that FK506, a specific inhibitor of FKBPs, had a stronger inhibitory effect on PPIase activity in lumen preparations from the mutant lines than in corresponding wild-type preparations (data not shown). These findings corroborate the hypothesis that FKBPs (AtFKBP13) are largely responsible for maintaining PPIase activity in the mutant lumen. However, this effect could not be quantified due to contamination by stromal cyclophilin AtCYP20-3, which is generally present in lumen samples at varying levels, accounting for 5−12% and 0.5−1.5% of the total PPIase activities toward the A- and L-substrates, respectively (*20*).

Analysis of Lumenal Immunophilins for the Presence of Residues Crucial for PPIase Activity. According to our results, only 2 of up to 16 immunophilins identified or predicted to be present in the thylakoid lumen of *Arabidopsis* possess PPIase activity. To explore the structural basis for the absence of this activity in the other lumenal immunophilins, we aligned the sequences of their domains corresponding to the PPIase-catalyzing domains in homologous human proteins and mapped the amino acid residues that have been experimentally proven to be important for the isomerase activity of human immunophilins.

In human FKBP12, which has been extensively studied by mutational analyses, residues Asp-37, Arg-42, Trp-59, His-87, and Phe-99 have been found to be the most important for PPIase activity (*38*−*40*). Figure 5 displays an alignment of human FKBP12 and all *Arabidopsis* FKBPs targeted to the lumen (including AtFKBP17-1, AtFKBP17-2, and AtFKBP17-3, although their presence in the lumen awaits experimental confirmation). AtFKBP13 appears to be the most highly conserved lumenal FKBP, since all of the residues important for PPIase activity are conserved in it except His-87, which is substituted by Ala. His-87 is rather variable among members of the FKBP group (*41*), and mutational studies of human FKBP12 have shown that substantial PPIase activity can be retained following some substitutions of this residue. For instance, substitution by alanine resulted in only a 23% reduction in activity. Another important residue that is missing in all lumenal FKBPs except AtFKBP13 is Trp-59, which has been proposed to be the docking site for proline in the substrate (*42*). All lumenal FKBPs, except AtFKBP13, contain at most two of the five important residues, of which Asp-37 and Phe-99 seem to be the most highly conserved (Figure 5). The results of the sequence analysis support our experimental data showing that all of the lumenal FKBPs except AtFKBP13 have little or no isomerase activity. Recent studies of recombinant AtFKBP20-2 (*43*) have found it to have sufficient PPIase activity to detect in assays with very high concentrations of the protein but too low (500-fold lower than that of AtFKBP13) to detect in samples of biological material with native levels of the protein. These findings are consistent with our conclusion that significant degeneration of the PPIase activities of lumenal FKBPs, including AtFKBP20-2, has occurred.

Site-directed mutagenesis studies of human cyclophilin A have revealed that residues His-54, Arg-55, Phe-60, Gln-111, Phe-113, Trp-121, and His-126 are extremely important for the PPIase activity of this enzyme and that Arg-55, Phe-60, and His-126 are the most essential, substitution of these residues causing 99% losses of activity (*44*). In Figure 6, all of the chloroplast cyclophilins of *Arabidopsis* are aligned with human cyclophilin A. The most highly conserved of these proteins are the stromal AtCYP20-3 and the lumenal AtCYP20-2, both of which possess all of the above-mentioned residues that are important for isomerase activity (*45*). In addition, both of these enzymes exhibit PPIase activity. All other lumenal cyclophilins lack most of the residues required for the enzymatic activity, which agrees well with our experimental data on the absence of their PPIase activity in the *Arabidopsis* thylakoid lumen preparations.

DISCUSSION

Immunophilins comprise the largest family of auxiliary enzymes found in the thylakoid lumen of plant chloroplasts in proteomic analyses (*16*−*18*, *46*). The physiological functions of cyclophilins and FKBPs in the thylakoid lumen are unknown, but these functions are assumed to include chaperone activities and catalytic involvement in the folding of proteins that are inserted into the thylakoid membrane and translocated into the thylakoid lumen (*10*−*12*, *17*). Protein folding processes in the chloroplast thylakoid lumen are known to be similar, in some respects, to those in

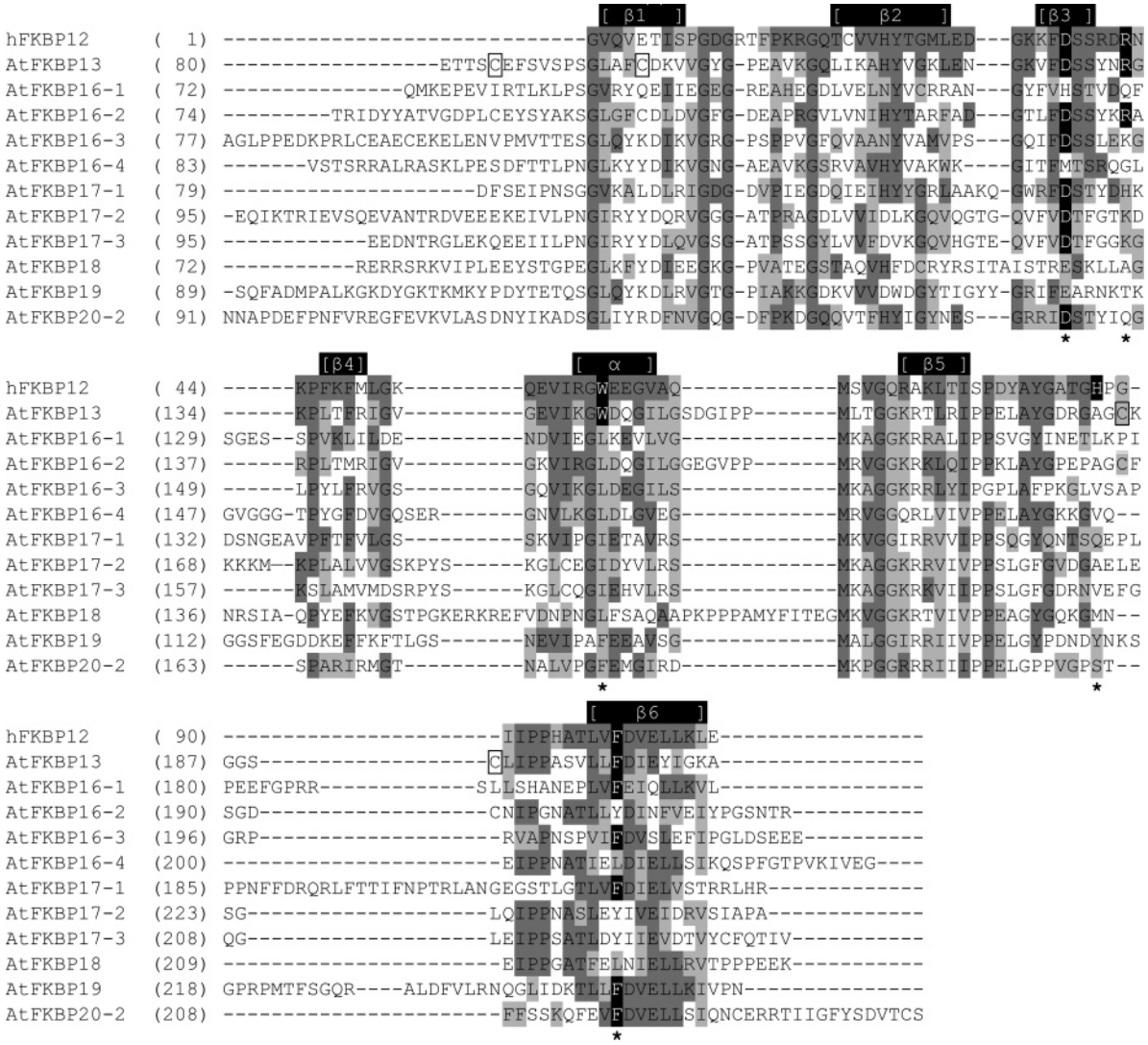

FIGURE 5: Alignment of potential catalytic domains of FKBPs from the thylakoid lumen of *A. thaliana* with the human FKBP12. The elements of secondary structure as annotated in http://www.expasy.ch/uniprot/P62942 are outlined above the alignment. The amino acid residues crucial for PPIase activity of FKBP12 (hFKBP12), as determined by site-directed mutagenesis (*38*−*40*), are enclosed in black-bordered boxes and labeled with asterisks below the alignments. Residues identical, or similar, to those in human FKBP12 are shaded dark and light gray, respectively. AtFKBP13, the most highly conserved of all lumenal FKBPs, lacks only one of the five critical amino acid residues. All of the other lumenal FKBPs contain no more than two of these crucial amino acids. The four cysteine residues involved in the control of PPIase activity of AtFKBP13 are boxed.

bacterial periplasm. Unlike the cytoplasm and chloroplast stroma, for example, which are rich in ATP-dependent chaperones, bacterial periplasm contains ATP-independent PPIases (*47*, *48*), as does the chloroplast thylakoid lumen (*16*−*18*, *46*). Several PPIases in the periplasm of *Escherichia coli* have been shown to be involved in the folding of outer membrane proteins (*47*, *48*). Furthermore, *E. coli* remained viable if any one of these PPIases was deleted, and null mutation of two PPIases, *ppiD* and *surA*, appeared to be the minimum change in its complement of the proteins that was lethal for the bacterium (*6*). Indeed, in later work genetic inactivation of all four periplasmic isomerases in *E. coli* resulted in a strain that was viable, albeit with reduced growth rates and increased susceptibility to certain antibiotics (*49*). Moreover, chaperone PPIases in the periplasm of *E. coli* have been found to be functionally redundant (*47*), and the biological (chaperone) function of one of them, SurA, was not affected by deletion of both of its PPIase catalytic domains (*50*). In this respect it should be mentioned that the specific functions of PPIases are not generally well understood. Our present work and earlier studies (*20*, *23*) of PPIase activities in the thylakoid lumen of spinach and *Arabidopsis* chloroplasts have revealed a number of unexpected findings. First, only a small fraction of lumenal cyclophilins and FKBPs possess detectable PPIase activity toward conventional substrate peptides. This implies that most of the immunophilins in the thylakoid lumen have evolved as proteins with highly specific functions and interacting partners. Second, the PPIase activity in the thylakoid lumen is redundant; knockout of an active cyclophilin AtCYP20-2 is apparently compensated by an increase in the activity of AtFKBP13, while this finding can be unambiguously proven only by the study of the double AtCYP20-2 and AtFKBP13 mutant. Third, cellular homeostasis probably requires stable PPIase activity in the thylakoid lumen: the total activity measured in the lumen of *AtCYP20-2* mutants was indistinguishable from that of the wild-type plants.

Two independent knockout lines that were deficient in the active PPIase AtCYP20-2 showed no phenotypic difference

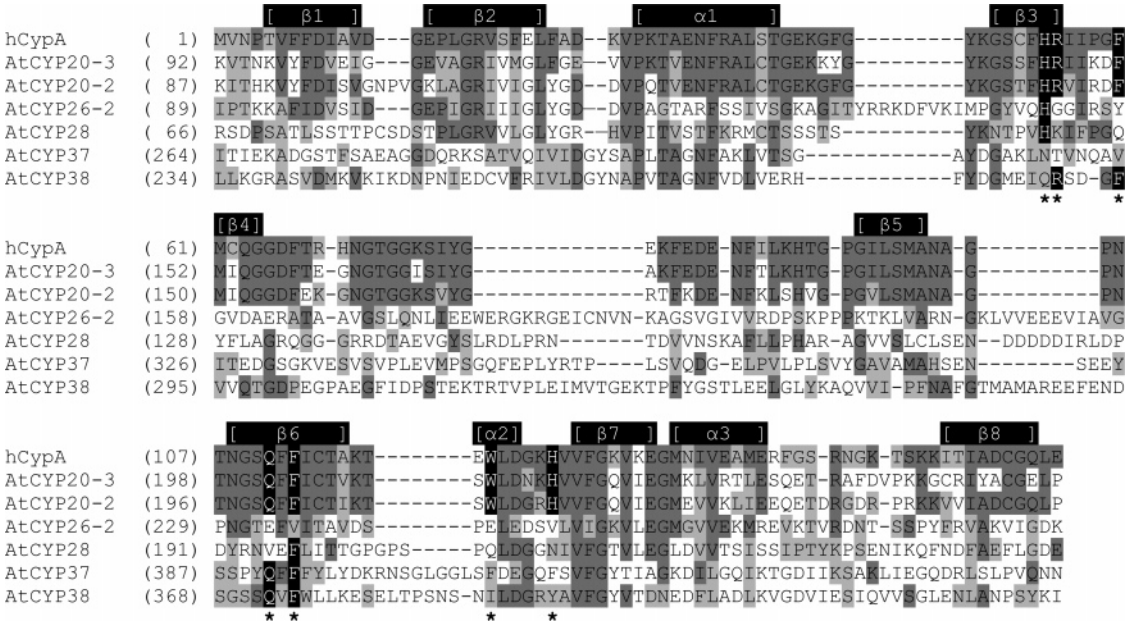

FIGURE 6: Alignment of potential catalytic domains of chloroplast cyclophilins from *A. thaliana* with the human cyclophilin A. The secondary structure elements are shown above the alignment according to http://www.expasy.ch/uniprot/P62937. The amino acid residues crucial for cyclophilin PPIase activity, as determined by site-directed mutagenesis of the human cyclophilin A (hCypA) (*44*), are enclosed in black-bordered boxes and labeled with asterisks below the alignments. Residues identical, or similar, to the human cyclophilin A are shaded dark and light gray, respectively. The stromal AtCYP20-3 and the lumenal AtCYP20-2 are the only two chloroplast cyclophilins possessing all seven residues important for the isomerase activity.

from the wild-type plants, and the PPIase activity in their thylakoid lumen proved to be equal to that of the wild type. We first hypothesized that this was due to the accumulation of some other active PPIases. To assess this possibility, we compared the protein complements of thylakoid lumen preparations from the mutant and wild-type plants by quantitative proteomic analyses using 2D-DIGE methodology. Unexpectedly, the mutants demonstrated no statistically significant changes in amounts of immunophilins previously identified in the *Arabidopsis* lumen (*16*−*19*), apart from the anticipated disappearance of AtCYP20-2. However, chromatographic fractionation of the *AtCYP20-2a* mutant lumen and subsequent analysis of the profile of PPIase activity in the obtained fractions provided clues regarding the mechanism that compensated for the loss of its PPIase activity. The only active PPIase found in the thylakoid lumen of the *AtCYP20-2a* mutant was AtFKBP13, suggesting that increased activity of this highly redox-sensitive enzyme (*20*) compensated for the lack of AtCYP20-2. The relative inhibition of PPIase activity by DTT, a thiol reducing agent that inactivates AtFKBP13, was more pronounced in the mutants than in the wild type. These findings imply that AtFKBP13 was in a more oxidized and active state in the *AtCYP20-2* mutants than in the wild type, suggesting that oxidative activation of AtFKBP13 compensated for the lost PPIase activity in the AtCYP20-2-deficient mutants. However, the definite and final statements on redundancy and compensation of PPIase activity in *Arabidopsis* thylakoid lumen should wait for isolation and characterization of the AtCYP20-2 and AtFKBP13 double mutant.

Oxidative activation of the PPIase activity of AtFKBP13 via the reversible formation of two disulfide bridges by two pairs of cysteine residues has been intensively studied in the recombinant protein (*26*, *51*). However, the enzymes catalyzing this process in the lumen in vivo are still unknown, although the amounts of these regulatory proteins are probably very low, which precludes their identification by standard proteomic techniques. One of the candidate oxidant enzymes is cytochrome c_{6A}, which was discovered through its interaction with AtFKBP13 in a yeast two-hybrid assay (*52*) but has never been identified in proteomic studies, as reviewed in ref *53*. It was recently proposed that cytochrome c_{6A} might catalyze the formation of disulfide bridges in thylakoid lumen proteins in a single-step disulfide exchange reaction, with subsequent transfer of the reducing equivalents to plastocyanin (*54*). Using the DIGE technique, we found significant changes in the amounts of a few minor proteins in the lumen of *AtCYP20-2* mutants but were unable to identify them with our MS-based methods. Intriguingly, however, one of the protein spots found to be upregulated in the lumen of both *AtCYP20-2* mutants may correspond to cytochrome c_{6A} according to its predicted molecular mass and isoelectric point (spot 1777; see Figure 3C; Table 1).

The sequence analyses of 11 FKBPs and 5 cyclophilins identified or predicted to be present in the thylakoid lumen of *Arabidopsis* revealed AtFKBP13 and AtCYP20-2 to be the most highly conserved lumenal immunophilins in this plant. All of the others showed massive losses of the amino acid residues required for PPIase activity. These findings are remarkably consistent with experimental evidence that AtFKBP13 and AtCYP20-2 are the only two lumenal enzymes that have detectable PPIase activity in thylakoid lumen preparations (*20*). In a recent study of another lumenal immunophilin, AtFKBP20-2, the PPIase activity of the recombinant enzyme was estimated to be 500-fold lower than that of AtFKBP13 (*43*), indicating that it would be impossible to detect in current assays using preparations isolated from plants. Taken together, these observations suggest that the vast majority of immunophilins in the thylakoid lumen have diverged into proteins with highly specialized functions. We

hypothesize that their isomerase activity has been either lost or fine-tuned to unique interacting partners. Further, At-FKBP13 and AtCYP20-2 avoided this differentiation, possibly fulfilling the need for general folding catalysts with broader substrate specificity. This is consistent with the earlier findings of AtCYP20-2 upregulation in *Arabidopsis* plants exposed to increased light irradiances (*28*) or to cold stress (*19*). Nevertheless, the present work shows that the PPIase activity of AtCYP20-2 is redundant. The compensation for the lost PPIase activity in the *AtCYP20-2* knockout mutants appears to proceed via oxidative activation of the redox-regulated AtFKBP13 without any statistically significant changes in the abundance of this protein or other immunophilins detected in the chloroplast thylakoid lumen by means of quantitative proteomic analyses.

SUPPORTING INFORMATION AVAILABLE

Table 1 presenting the quantitative data on the relative abundance of 199 protein spots found in all gels of the thylakoid lumen of two *AtCYP20-2* knockout mutants in comparison with the wild-type plants and the results of statistical analyses of these quantitative data. This material is available free of charge via the Internet at http://pubs.acs.org.

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