Functional Redundancy of AtFtsH Metalloproteases in Thylakoid Membrane Complexes

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FtsH is an ATP-dependent metalloprotease found in bacteria, mitochondria, and plastids. Arabidopsis (Arabidopsis thaliana) contains 12 AtFtsH proteins, three in the mitochondrion and nine in the chloroplast. Four of the chloroplast FtsH proteins are encoded by paired members of closely related genes (AtFtsH1 and 5, and AtFtsH2 and 8). We have previously reported that AtFtsH2 and 8 are interchangeable components of AtFtsH complexes in the thylakoid membrane. In this article, we show that the var1 variegation mutant, which is defective in AtFtsH5, has a coordinate reduction in the AtFtsH2 and 8 pair, and that the levels of both pairs are restored to normal in var1 plants that overexpress AtFtsH1. Overexpression of AtFtsH1, but not AtFtsH2/VAR2, normalizes the pattern of var1 variegation, restoring a nonvariegated phenotype. We conclude that AtFtsH proteins within a pair, but not between pairs, are interchangeable and functionally redundant, at least in part. We further propose that the abundance of each pair is matched with that of the other pair, with excess subunits being turned over. The variegation phenotype of var1 (as well as var2, which is defective in AtFtsH2) suggests that a threshold concentration of subunits is required for normal chloroplast function. AtFtsH1, 2, 5, and 8 do not show evidence of tissue or developmental specific expression. Phylogenetic analyses revealed that rice (Oryza sativa) and Arabidopsis share a conserved core of seven FtsH subunit genes, including the AtFtsH1 and 5 and AtFtsH2 and 8 pairs, and that the structure of the present-day gene families can be explained by duplication events in each species following the monocot/dicot divergence.

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Interchangeability of Subunits in the Upper AtFtsH-Containing Band

AtFtsH1 and AtFtsH5 are the most closely related members of the AtFtsH gene family in Arabidopsis (approximately 90% amino acid identity). Based on our previous results showing that members of another closely related AtFtsH gene pair—AtFtsH2 and AtFtsH8—have redundant functions, we wanted to examine whether the same is true for AtFtsH1 and AtFtsH5. To test this hypothesis, we overexpressed AtFtsH1 in the var1-1 variegation mutant, which defines the AtFtsH5 locus (Fig. 1A; Sakamoto et al., 2002). The var1-1 allele has a nucleotide insertion in its N terminus that is predicted to generate an early stop codon; if stable, a truncated translation product would be produced lacking most of the functional domains of the protein. Taken together with the finding that AtFtsH5 mRNAs are not detectable, this suggests that var1-1 is likely a null allele (Sakamoto et al., 2002).

Figure 1A shows the results of the overexpression experiments. Full-length AtFtsH1 cDNAs were amplified by reverse transcription (RT)-PCR, cloned into a binary vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and transformed into var1-1 via Agrobacterium-mediated transformation. A number of kanamycin-resistant lines were identified in the T1 generation; all resembled wild-type plants. A representative line, designated C5W1No2, was selected for further study (Fig. 1A). Southern-blot analysis revealed that this line contains a single T-DNA insertion (Fig. 1B). This is in accord with genetic data showing that selfing of C5W1No2...
gives rise to green and variegated T2 progeny in a ratio of approximately 3:1 (data not shown). Green T2 progeny were used in the experiments described below.

Whereas the data in Figure 1A are consistent with the idea that overexpression of AtFtsH1 rescues the variegation phenotype of var1-1, it was necessary to confirm this at the level of AtFtsH1 expression. Semi-quantitative RT-PCR analyses (Fig. 1C) revealed that the transcript levels of AtFtsH1 are sharply elevated in C5W1No2 compared to var1-1. By contrast, AtFtsH2 and AtFtsH8 mRNA levels are normal in the overexpressor. Only trace amounts of the AtFtsH5 transcript are present in var1-1 (as anticipated from Sakamoto et al., 2002) and C5W1No2.

To examine protein accumulation in the overexpression plants, we performed 2-D green gel analyses of Arabidopsis thylakoid membrane proteins. Previous studies have shown that two AtFtsH-containing bands can be resolved on green gels of Arabidopsis thylakoids: upper and lower bands consisting, respectively, of AtFtsH1 and 5 and AtFtsH2 and 8 (Yu et al., 2004). Whereas both AtFtsH-containing bands are reduced in abundance in var1-1 (Fig. 2A), they are restored to normal in C5W1No2 (Fig. 2B). To confirm this finding, we performed western-immunoblot analyses on thylakoid membrane proteins fractionated by one-dimensional SDS-PAGE. To detect AtFtsH1 specifically, we generated an antibody against a peptide that is unique to this protein (see "Materials and Methods"). This antibody reacts with AtFtsH1 proteins that are expressed in E. coli, but not with E. coli-expressed AtFtsH5 or AtFtsH2 (data not shown). We detected similar levels of AtFtsH1 in wild type and var1-1, but a significant increase in C5W1No2 (Fig. 2C). Western immunoblots were also probed with an antibody that detects both AtFtsH2 and 8 (Yu et al., 2004). As illustrated in Figure 2D, the band containing AtFtsH2 and 8 is decreased in amount in var1-1, but is present at normal levels in C5W1No2. In summary, the data in Figure 2, A to C, are consistent with the idea that (1) wild-type AtFtsH complexes contain optimal levels of the 1-5 and 2-8 pairs; (2) var1-1 has a reduction in 5, but not 1, and this is matched by a reduction in 2 and 8; and (3) optimal complex levels (as found in the wild type) can form when 1 is overexpressed in var1-1; these complexes contain 1 and 2-8.

Examination of the patterns of protein expression on the 2-D green gels revealed that, with the exception of the AtFtsH-containing bands, there were no significant differences in protein accumulation among the overexpression, wild-type, and var1-1 plants (data not shown). To verify this conclusion, we generated polyclonal antibodies to representative thylakoid proteins, including the D1 protein of PSII, cytochrome b6/f Rieske protein (PetC), and the a-subunit of the plastid membrane ATP synthase. Figure 2D shows that the steady-state levels of all three proteins are similar in the three plants. This suggests that an alteration in AtFtsH protein abundance does not have major pleiotropic effects, at least in the green leaf sectors of the mutants.

Using polyclonal AtFtsH5/VAR1 and AtFtsH2/VAR2 antibodies, it was reported that VAR1 and VAR2 might interact directly (Sakamoto et al., 2003). One factor complicating these studies was the lack of isoform-specific antibodies, i.e. their AtFtsH5/VAR1...
antibody detected 1 and 5, and their AtFtsH2/VAR2 antibody detected 2 and 8. With the availability of AtFtsH1-specific antibodies, we wanted to test whether 1 interacts with the 2-8 pair, as suggested in our model of AtFtsH complex formation (Yu et al., 2004). In these experiments, we coimmunoprecipitated thylakoid membrane samples from wild-type Columbia using our AtFtsH1 or AtFtsH2/VAR2 antibodies; the latter antibodies, as mentioned earlier, detect 2 and 8. Figure 2E (lane 5) shows a western immunoblot of control thylakoid membranes: Bands corresponding to 2 and 8 (top) and 1 (bottom) can be seen. The relatively low abundance of AtFtsH1 in the membranes of wild type confirms the data in Figure 2C. In lane 4, the AtFtsH1 antibody was used to coimmunoprecipitate thylakoid proteins. The immunoprecipitates were then electrophoresed on polyacrylamide gels and subjected to western analysis, using either the VAR2 or AtFtsH1 antibodies as probes. Bands containing 1 and 2 and 8 are visible, suggesting that 1 interacts with 2 and/or 8. In complementary experiments, the VAR2 antibody was used to coimmunoprecipitate thylakoid proteins (lane 2). Again, bands corresponding to 1 and 2 and 8 were observed on the western blots. This confirms the data in lane 4. As expected, coimmunoprecipitation with preimmune sera did not give rise to bands on the western blots (lanes 1 and 3). We conclude that 1 interacts with 2 and/or 8 in AtFtsH complexes in the thylakoid.

Considered together, the data in Figures 1 and 2 show that AtFtsH1 overexpression rescues the var1-1 variegation phenotype. This suggests that AtFtsH1 and 5 are interchangeable in thylakoid membranes. The data also show that there are coordinate changes in the levels of the proteins in the upper and lower bands in wild type, var1-1, and the overexpression plants. Control of subunit abundance likely occurs posttranscriptionally, since the overexpression plants have normal levels of AtFtsH2 and 8 mRNAs, but reduced amounts of AtFtsH2 and 8 proteins. Perhaps the simplest hypothesis is that AtFtsH1 is interchangeable with AtFtsH5 in AtFtsH complexes, and that AtFtsH1 exerts its effect by stabilizing AtFtsH2 and 8 in the membrane.

One prediction of the above hypothesis is that down-regulation of AtFtsH1 protein levels in a var1-1 background should result in plants with a more severe phenotype than var1-1. A complete lack of AtFtsH1 and 5 might be lethal if these proteins are essential for viability. To test this, we transformed var1-1 with an antisense AtFtsH1 cDNA driven by the CaMV 35S promoter. A number of antibiotic-resistant T1 lines were selected and selfed. Figure 3 shows the T2 progeny from a representative T1 line. These plants have a variety of color phenotypes that range from variegations similar to var1-1 (Fig. 3A) to partially albino (Fig. 3, B–D) and completely albino (Fig. 3, E and F). PCR was used to confirm the presence of the antisense construct in the individual T2 plants (data not shown). However, we did not try to monitor changes in

Figure 3. Antisense down-regulation of AtFtsH1 expression in var1-1. A partial AtFtsH1 cDNA was cloned in reverse orientation behind the CaMV 35S promoter, and the construct was transformed into var1-1. Representative T2 plants from an antibiotic-resistant T1 plant are shown. These plants have a range of color phenotypes, from those that are similar to var1-1 (A) to those that are partially albino (B–D) or completely albino (E and F). This variability was observed in the T2 progeny of multiple independent T1 lines. Bars = 5 mm.

AtFtsH1 expression in these plants because the parental var1-1 mutants have extremely low levels of AtFtsH1 mRNAs and proteins (see Figs. 1C and 2C). Nonetheless, the phenotypes of the antisense mutants are consistent with the idea that down-regulation of AtFtsH1 in a background that lacks AtFtsH5 can be lethal.

Expression of AtFtsH1 and AtFtsH5

To examine AtFtsH1 and AtFtsH5 expression, we generated transgenic Columbia plants that express AtFtsH1 promoter-GUS and AtFtsH5 promoter-GUS fusions. Figure 4 shows that GUS activities are similar in seedlings (Fig. 4, A and C) and fully expanded older leaves in both sets of transformants (Fig. 4, B and D). Examination of other growth stages did not reveal obvious staining differences between the two constructs (data not shown). We conclude that AtFtsH1 and AtFtsH5 promoter activities are similar to one another, and that these genes are ubiquitously expressed, especially in green organs of the plants. These expression patterns resemble those of AtFtsH2 and AtFtsH8, as previously reported (Yu et al., 2004).

Interchangeability of Subunits between the Upper and Lower AtFtsH-Containing Bands

Considered together with previous results (Sakamoto et al., 2003; Yu et al., 2004), the current data suggest that AtFtsH1 forms multimeric complexes in thylakoid membranes, and that these complexes contain at least two pairs of interchangeable subunits: AtFtsH2 and 8 (lower band) and AtFtsH1 and 5 (upper band). The question arises as to whether FtsH homologs in different pairs are interchangeable. To address this
question, we tested whether overexpression of \textit{AtFtsH2} can rescue the \textit{var1} variegation phenotype, i.e. whether \textit{AtFtsH2} can substitute for \textit{AtFtsH5}. For these experiments, we generated a construct in which the full-length \textit{AtFtsH2} cDNA was placed under the control of the CaMV 35S promoter, then transformed into \textit{var1-1}. As a control, the construct was transformed into the \textit{var2-4} variegation mutant, which lacks \textit{AtFtsH2}. Figure 5, B and D, shows that \textit{AtFtsH2} transcripts are significantly elevated in both sets of transformed plants. Yet, \textit{AtFtsH2} overexpression is only able to rescue the \textit{var2-4} variegation (Fig. 5A). A failure to rescue the \textit{var1-1} variegation was observed in multiple lines (Fig. 5C).

Despite the sharply increased levels of \textit{AtFtsH2/VAR2} mRNAs in the C5W2 plants, \textit{AtFtsH2} and 8 protein levels are the same in these plants as in \textit{var1-1}, i.e. excess \textit{AtFtsH2} does not accumulate (Fig. 5E). \textit{AtFtsH1} protein levels are also similar in the two sets of plants. Perhaps the simplest hypothesis is that \textit{AtFtsH2} cannot substitute for \textit{AtFtsH5}. If so, we hypothesize that \textit{AtFtsH2} mRNAs are translated in \textit{var1-1}, since they appear to be translatable in \textit{var2-4}, but that \textit{AtFtsH2} proteins are turned over posttranslationally because there are not enough compatible partners (\textit{AtFtsH1} in \textit{var1-1}) to form stable complexes.

**OsFtsH Gene Family in Rice**

At least three different efforts are under way to sequence the rice genome (about 420 megabases in size), and draft sequences have been published from the \textit{japonica} cultivar (approximately 389 megabases; Goff et al., 2002) and from the \textit{indica} cultivar (approximately 361 megabases; Yu et al., 2002). In addition, The Institute for Genomic Research (TIGR) has recently released its third version (December 30, 2004) of annotations of pseudomolecules (virtual contigs) from the International Rice Genome Sequencing Project (IRGSP; http://rgp.dna.affrc.go.jp/IRGSP/index.html), encompassing approximately 370 megabases of the genome. This resource is easy to access (http://www.tigr.org/tdb/e2k1/osa1). To determine the \textit{FtsH}
gene complement in rice (OsFtsH genes), we searched the annotated pseudomolecule database and identified a total of nine FtsH homologs, designated OsFtsH1 through OsFtsH9 (Table I). All nine appear to be expressed insofar as each is represented in the databases by cDNAs and expressed sequence tags (ESTs).

Figure 6 shows the phylogenetic relatedness of the nine OsFtsH genes compared to the 12 Arabidopsis AtFtsH genes and four Synechocystis FtsH genes that are present in the fully sequenced genomes of the latter two organisms. Full-length protein sequences were used in the analyses. Several features can be discerned. First, for every Arabidopsis AtFtsH gene (or closely related gene pair), there is a corresponding gene (or gene pair) in rice, except for AtFtsH12. Second, there are four closely related Arabidopsis gene pairs—AtFtsH1/5, AtFtsH2/8, AtFtsH3/10, and AtFtsH7/9—of which three are localized in plastids and one in the mitochondrion. The only conserved pair is the mitochondrial pair (AtFtsH3 and AtFtsH10 in Arabidopsis, OsFtsH3 and OsFtsH8 in rice). The products of the other three Arabidopsis gene pairs are represented by single genes in rice. A unique feature of the organization of the OsFtsH gene family is that OsFtsH4 and OsFtsH5 are tandemly arrayed on the genome, suggesting they arose by a duplication event; in Arabidopsis, a single locus (AtFtsH4) corresponds to these genes. Collectively, the data in Figure 6 suggest that there is a core complement of higher plant FtsH genes and that the emergence of this core predates the monocot/dicot divergence. However, elaborations on this core occurred during the subsequent course of evolution.

Comparison of the predicted gene structures of the nine OsFtsH genes from the annotated pseudomolecule sequences with the sequences of cDNAs and ESTs revealed that the TIGR annotations of intron-exon borders are largely correct, with only a few discrepancies (data not shown). However, a comparison of the structures of the rice and corresponding Arabidopsis genes reveals a striking conservation of intron-exon borders and exon size. For instance, Figure 7 shows the structural conservation of the two sets of plastid-localized proteins in the upper and lower bands of the 2-D gels. This conservation is consistent with the idea that FtsH gene families in higher plants evolved from a common core of conserved enzymes.

Also consistent with the idea of a core are the results of comparative 2-D green gel analyses of thylakoid membranes isolated from rice (a monocot) and pea (Pisum sativum; like Arabidopsis, a dicot; Fig.

### Table I. Rice OsFtsH gene family

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Arabidopsis Homolog</th>
<th>GenBank ID</th>
<th>BAC Clone</th>
<th>Chromosome</th>
<th>Protein Size (a.a.)</th>
<th>Cellular Location</th>
<th>Expression</th>
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<td>AtFtsH1/5</td>
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<td>C</td>
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<td>C</td>
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<td>769</td>
<td>C?</td>
<td>AK060394</td>
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</table>
hypothesis is that the various FtsH proteins became
localized in Arabidopsis or rice. These include the genes in
Arabidopsis and rice. Intron-exon boundaries were confirmed by examination of
cDNA and EST sequences. Boxes indicate exons and lines represent introns. Numbers above each box refer to size of the exon (bp).
Untranslated regions were not included in the structures. Colored
introns. Numbers above each box refer to size of the exon (bp).
Untranslated regions were not included in the structures. Colored
regions correspond to various FtsH domains. Blue represents transit
peptide region, green represents ATP-binding region, and red repre-
sents zinc-binding site.

Figure 7. Structures of rice and Arabidopsis FtsH genes. Gene structures were constructed based on annotation of the Arabidopsis and rice
genomes. Intron-exon boundaries were confirmed by examination of
cDNA and EST sequences. Boxes indicate exons and lines represent introns. Numbers above each box refer to size of the exon (bp).
Untranslated regions were not included in the structures. Colored
regions correspond to various FtsH domains. Blue represents transit
peptide region, green represents ATP-binding region, and red repre-
sents zinc-binding site.

2A). Similar to Arabidopsis, upper and lower FtsH-containing bands are present in both of these species (Fig. 8A). This was confirmed by western-immunoblot
analyses of the 2-D gels using our AtFtsH1 and VAR2 antibodies, where bands corresponding to these proteins can be seen (Fig. 8B).

The western blots also reveal that streaking is
apparent toward higher M, areas of the 2-D gels of
Arabidopsis thylakoid proteins when the VAR2 anti-
body is used as a probe. These results confirm earlier data and are consistent with the idea that VAR2 partic-
ipates in oligomer formation (Yu et al., 2004). On
the other hand, similar streaking is not seen using
the AtFtsH1 antibody; we speculate this might be
because of the low abundance of this protein and a
consequent inability to detect oligomers. In a similar
vein, the streaking is not detectable on the westerns in
pea or rice using either antibody as a probe. This could
mean that pea and rice do not form oligomers or that
they are not abundant enough to be detected using
Arabidopsis antibodies.

DISCUSSION
Subunit Interchangeability: Gene Duplication
and Redundant Functions

E. coli has one FtsH gene, cyanobacteria have four
(Kaneko et al., 1996), rice has at least nine (Table I), and
Arabidopsis has 12 (Fig. 6). Why are so many FtsH
genescalled here for higher plants? These data show that
a core complement of 7 FtsH genes was in place by the
time of the monocot/dicot divergence (see Fig. 7).
Some of these core genes subsequently became duplicated in Arabidopsis or rice. These include the genes in
the upper and lower bands of the 2-D gels. One hypothesis is that the various FtsH proteins become

specialized in their activity. For instance, the chaper-
one activity of FtsH normally acts to promote the
protease activity of the enzyme (Suzuki et al., 1997).
However, it is possible the enzyme could act independently as a chaperone, as found in the Clp class of
proteases (Nielsen et al., 1997). We found that mem-
bers of the 2-8 and 1-5 duplicated pairs in Arabidopsis
are interchangeable, while proteins of different pairs
are not interchangeable. This lack of interchangeability
does not necessarily mean that the members of the two
pairs have different functions, e.g. it could simply
signify an inability to assemble efficiently. Neverthe-
less, whether the two have similar, or different, func-
tions is now under investigation.

Another hypothesis is that multicellularity required
a subspecialization of function such that it became
necessary for the various FtsH gene family members to
differ in the timing and/or cell specificity of their
expression. In this respect, it is interesting that all four
AtFtsH genes (AtFtsH1, 2, 5, and 8) have similar
expression profiles, with abundant expression in green
organs of the plant. Although our experiments were
not refined enough to allow us to detect cell-specific
differences in expression, the absolute levels of
expression of these genes vary: AtFtsH2 is the most
highly expressed (at the mRNA and protein levels),
followed by AtFtsH1 and AtFtsH5, with approximately
50% of the mRNA and protein expression levels of
AtFtsH2, and finally AtFtsH8, whose mRNA and
protein expression are about 20% that of AtFtsH2
(Sinvy-Villalobo et al., 2004). As Sinvy-Villalobo
et al. (2004) have pointed out, these expression levels
roughly correlate with the severity of phenotype
observed in mutants that have lesions in these genes:
Null mutants of AtFtsH2 and AtFtsH5 are variegated,
whereas null (T-DNA insertion) mutants of AtFtsH1
and AtFtsH8 have no obvious phenotypic changes
(Sakamoto et al., 2003; F. Yu and S. Rodermel, un-
published data).

Although we do not understand the reason for the
quantitative differences in expression among the

Figure 8. Comparative 2-D green gel analyses. A, 2-D green gels were
prepared as described in Figure 2A using isolated thylakoid membranes
from Arabidopsis, rice, and pea. The regions of each gel containing the
upper and lower FtsH-containing bands are shown. B, Western immu-
noblot analysis of 2-D gels probed with AtFtsH1 and VAR2 antibodies are
shown.
var1, there could be unequal partitioning of AtFtsH1, with some plastids receiving more of this protein than others. An alternative explanation is that all plastids receive similar amounts of AtFtsH1, but that there might be intrinsic differences in the partitioning of AtFtsH1, with some plastids receiving more of this protein than others. An alternative hypothesis is that the AtFtsH2 can form homo-oligomers, which might constitute a fraction of the AtFtsH complex pool (Sakamoto et al., 2003). This might not be unexpected because AtFtsH2 is the most abundant FtsH subunit. Regardless, how the total optimum AtFtsH pool size is attained is not known.

Mechanism of Variegation

The data in this study confirm and extend a model of variegation in which chloroplast function requires a threshold concentration of AtFtsH multimers (Yu et al., 2004). Below this threshold, a white plastid forms, while above this threshold, a normal chloroplast is produced. According to our revised model, AtFtsH complexes contain two types of subunits—AtFtsH2 and 8 and AtFtsH1 and 5. Because alterations in AtFtsH protein abundance do not influence AtFtsH transcript abundance, we propose that levels of the AtFtsH1 and 5 pair are matched with those of the AtFtsH2 and 8 pair, and that excess subunits are turned over. In cells in which the number of AtFtsH complexes is below the threshold, chloroplast differentiation is impaired, leading to the generation of white plastids. Conversely, plastids with threshold or higher levels of AtFtsH complex formation develop normal chloroplasts. We speculate that these decisions occur during the conversion of undifferentiated proplastids in the leaf meristem into mature chloroplasts early in leaf development. The process of sorting out of dividing plastids would then result in the generation of clones of white plastids and cells (white sectors) or plastids of chloroplast-containing cells (green sectors).

If our model is correct, there are at least two possibilities to explain the threshold phenomenon. One is that there is unequal partitioning of a nuclear gene product to the plastids (>100) within a cell. For instance, in the case of var1, there could be unequal partitioning of AtFtsH1, with some plastids receiving more of this protein than others. An alternative explanation is that all plastids receive similar amounts of AtFtsH1, but that there might be intrinsic differences among plastids within a developing leaf cell in a biochemical process that is required for normal chloroplast development. For instance, there might be variability in rates of D1 repair, such that not all plastids need the same amount of AtFtsH1 for this process to occur efficiently. Regardless of the precise mechanism, our model is in accord with early data showing that cells in the white sectors of var2 are heteroplastidic and contain some normal-appearing chloroplasts, as well as a large number of abnormal plastids (Chen et al., 1999). This plastid autonomy indicates that not all plastids in a given cell are equivalent in function.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana (ecotype Columbia) and pea (Pisum sativum) were grown in growth rooms under continuous fluorescent lights (about 100 μmol m⁻² s⁻¹) at 22°C. Rice (Oryza sativa sp. japonica cv Nipponbare) was grown in growth chambers under 16-h-day/8-h-night cycles at 28°C.

2-D Green Gel Analysis

Leaf tissues used in this study were collected from 3-week-old Arabidopsis plants, 3-week-old rice plants, and 2-week-old pea plants. Chloroplast membranes were isolated as previously described (Yu et al., 2004) by grinding the tissue in 0.33 M sorbitol, 10 mM EDTA, 50 mM HEPES, pH 8.0, and 0.05% bovine serum albumin using a Waring blender. The homogenate was filtered through two layers of Miracloth and centrifuged at 2,600 × g for 3 min. Crude chloroplasts were washed with 10 mM MOPS, pH 8.0, followed by centrifugation at 10,000 × g for 10 min. The pellet, which contains thylakoid membranes, was resuspended in 0.33 M sorbitol, 5 mM MgCl₂, and 50 mM HEPES, pH 8.0. Chlorophyll concentrations were measured on the resuspended membranes using 95% ethanol (Lichtenthaler, 1987). All the steps were performed at 4°C under dim light.

First-dimension green gel analysis was carried out as previously described (Yu et al., 2004). In brief, thylakoid membranes were solubilized in a mixture of detergents (0.45% octylglucoside, 0.45% decyl maltoside, 0.1% lithium dodecyl sulfate, 10% glycerol, 2 mM Tris maleate, pH 7.0), and samples corresponding to 45 μg of chlorophyll were loaded onto a 3-mm 8% polyacrylamide (native) gel (MiniProtein II; Bio-Rad, Hercules, CA). The gels were electrophoresed at 4°C at 10 mA for 90 min. For the second dimension, gel strips were cut out of the native gels and incubated in 2 × SDS sample buffer (Laemmli, 1970) at 65°C for 2 h. SDS-PAGE was then performed as described using 14% SDS-polyacrylamide gels (Xu et al., 1994).

Antibody Production, Coimmunoprecipitation, and Western Immunoblotting

To generate a specific antibody for AtFtsH1, a 15-amino acid peptide PLIQNNEILKAPSK that is specific for AtFtsH1 was synthesized, and polyclonal antibodies were generated in rabbits (Protic, Poway, CA). The antibody was used at a 1:10,000 dilution.

Nucleotide fragments corresponding to amino acids P196-G353, A51-S229, and G367-V507 of the D1 protein of PSII, the cytchrome b₅₆₃ Rieske protein (PetC), and the ATP synthase a-subunit, respectively, were amplified by PCR and subcloned into the pET15b vector (Novagen, Madison, WI). The resulting constructs were transfected and expressed in Escherichia coli BL21(DE3) (Novagen). All the expressed peptides formed inclusion bodies, which were purified and solubilized (by procedures suggested by the manufacturer) and injected into rabbits. After three injections, cleared sera were used as antibodies against each antigen. All three antibodies were used at a 1:1,000 dilution.

Coimmunoprecipitations were carried out essentially as described by Sakamoto et al. (2003). Briefly, thylakoid membranes were suspended in phosphate-buffered saline at a concentration of 0.8 mg chlorophyll/mL, then solubilized with 0.8% dodecylmaltoside. After 30-min incubation on ice, samples were diluted to 0.1 mg chlorophyll/mL and antibodies against AtFtsH1 or AtFtsH2/VAR2 were added. Sepharose-coupled protein A was
added after 4 h and the samples were incubated overnight at 4°C. Immuno-
precipitates were recovered by centrifugation at 12,000g for 15 s, then washed
three times with phosphate-buffered saline containing 0.05% dodecylmaltoside.
Finally, the samples were boiled for 5 min in 2× SDS buffer and subjected to standard SDS-PAGE.

For western immunoblotting, proteins were transferred from 12% SDS-
polyacrylamide gels to nitrocellulose membranes (Immobilon-NC, Millipore, Billerica, MA) and probed with various antibodies. The AtFtsH2/VAR2 antibody was described in Chen et al. (2000); it detects both AtFtsH2 and 8. The SuperSignal West Pico chemiluminescence kit (Pierce, Rockford, IL) was used for signal detection. The nitrocellulose membranes were stained after transfer with Ponceau S to examine loading efficiency and transfer quality.

Plasmid Construction and Arabidopsis Transformation

To generate an AtFtsH1 overexpression construct, an AtFtsH1 cDNA was amplified using hexa DNA polymerase (Invitrogen, Carlsbad, CA) with forward primer 5′-AACCTGACAGAAGAAGAAGAACGAGGGCTC-3′ and reverse primer 5′-GAACATCATGATGGACACGACTCACTG-3′ (XhoI sites underlined). The PCR product was digested with XhoI and cloned into the XhoI site of a binary vector derived from pBI111LF1C. This vector lacks the GUS gene, but contains the CaMV 35S promoter and the neomycin phosphotransferase gene. The resultant plasmid was named pBI111LF1C. To generate plasmid var1-1 plants that overexpress AtFtsH1, pBI111LFIC was mobilized into Arabidopsis var1-1 mutants using Agrobacterium tumefaciens strain LBA4404. Kanamycin-resistant plants were selected on Murashige and Skoog medium supplemented with 50 mg/l kanamycin. PCR and Southern blotting were performed to verify that the plants were transformed. Segregation of phenotypes was scored in the T2 generation; RNA and protein analyses were also performed using T2 generation plants.

To generate an AtFtsH1 antisense construct, a 1,500-bp AtFtsH1 cDNA fragment from pBI111LFIC was cut out using XhoI and SstI and then inserted into a pBI111L plasmid digested with XhoI and SstI. In this way, this AtFtsH1 cDNA fragment is under 35S promoter control in antisense orientation.

To generate an AtFtsH2/VAR2 overexpression construct, forward primer 5′-ATAGGATCCGACATGGCTCTCTCATACATC-3′ and reverse primer 5′-ATACCATCAGATGGATAGCTCATATCCTGACGG-3′ (BamHI site underlined) were used to amplify AtFtsH2/VAR2 full-length cDNA. The cloning procedure is essentially the same as the AtFtsH1 construct above, except the restriction enzyme used was BamHI.

To generate AtFtsH1 and AtFtsH5/VAR1 promoter-GUS fusion constructs, a similar approach was taken as in Yu et al. (2004). The primers for AtFtsH1 are forward primer 5′-CATAGCTCAGGAGCTGAGTGTGGTCTTCT-3′ and reverse primer 5′-CATGCTTAGCTGGACAGGAACTGAGAAC-3′ (XhoI site underlined). The primers for AtFtsH5/VAR1 are forward primer 5′-CATGATGACCACCATGATGACGAGCAG-3′ and reverse primer 5′-CATGACATGACCAACGAGGAGCAGGAGGACG-3′ (BamHI site underlined).

Manipulations of DNA and RNA

Genomic DNAs were isolated and Southern-blot analyses were performed as described in Wetzel et al. (1994). Total cell RNAs were isolated from plants using the Trizol RNA Reagent (Invitrogen). Semiquantitative RT-PCR was carried out essentially as described in Yu et al. (2004). In brief, the RNA was reverse transcribed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). The RT reaction products were diluted 10 times, and the PCR reactions were performed using 32 cycles in a total volume of 50 μL. This is within the linear range of amplification for each of the fragments examined. Gene-specific primers for AtFtsH1 were 5′-GTCTCAAGAAGCGATCGAACG-3′ and 5′-GGCTGAAAGCGCAAGAGAGGT-3′. Gene-specific primers for AtFtsH2/VAR2 were 5′-ATCTCTCATGCTTGATCATG-3′ and 5′-ACCAATTCAATTGCAGGATC-3′. Gene-specific primers for AtFtsH3/VAR1 were 5′-CAACACCCACGACACCAATAT-3′ and 5′-CCCTTACAGAAAGATATAAACAACC-3′. Gene-specific primers for AtFtsH5 were 5′-TCCCTTCCGAAATTACACAG-3′ and 5′-CCCACACTAATGGTATGGA-3′. Gene-specific primers for the Actin 2 gene were 5′-TCACAAGAGGACAGCTTCCACCTAG-3′ and 5′-ACACAAAGGTGCTACATGAAAGAAG-3′.

Functional Redundancy of AtFtsH in Thylakoid Membranes

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