The establishment of photosynthetic protein complexes during chloroplast development requires the influx of a large number of chloroplast proteins that are encoded by the nuclear genome, which is critical for cytosol and chloroplast protein homeostasis and chloroplast development. However, the mechanisms regulating this process are still not well understood in higher plants. Here, we report the isolation and characterization of the pale green Arabidopsis pga1-1 mutant, which is defective in chloroplast development and chloroplast protein accumulation. Using genetic and biochemical evidence, we reveal that PGA1 encodes AtFtsH12, a chloroplast envelope-localized protein of the FtsH family proteins. We determined a G703R mutation in the GAD motif of the conserved ATPase domain of AtFtsH12 in the gpg1-1 viable hypomorphic allele of the essential gene AtFtsH12. In de-etiolation assays, we showed that the accumulation of photosynthetic proteins and the expression of photosynthetic genes were impaired in pga1-1. Using the FNRctp-GFP and pTAC2-GFP reporters, we demonstrated that AtFtsH12 was required for the accumulation of chloroplast proteins in vivo. Interestingly, we identified an increase in expression of the mutant AtFtsH12 gene in pga1-1, suggesting a feedback regulation. Moreover, we found that cytosolic and chloroplast proteostasis responses were triggered in pga1-1. Together, taking advantage of the novel pga1-1 mutant, we demonstrate the function of AtFtsH12 in chloroplast protein homeostasis and chloroplast development.

In the chloroplast, the physical and chemical processes of photochemistry and carbon assimilation are mediated by multiple photosynthetic protein complexes such as Photosystem II (PSII), Photosystem I (PSI), Cytochrome bc1, and ATP synthase for light reactions, and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) for carbon reactions. As a consequence of endosymbiosis, many photosynthetic protein complexes are composed of subunits encoded by both photosynthesis-associated nuclear genes (PhANGs) and genes in the chloroplast genome (1). During chloroplast development, the expression of many PhANGs is activated by light signaling (2). PhANG transcripts are translated in the cytosol and their protein products are subsequently translocated into the chloroplast through the TOC/TIC system (translocon at the outer/inner envelope membrane of chloroplasts) (3). In the chloroplast, photosynthetic genes in the chloroplast genome are predominantly transcribed by the plastid-encoded RNA polymerase (PEP), and the rpo genes encoding PEP core subunits are transcribed by the nucleus-encoded RNA polymerase (NEP) (4). Thus, the establishment of functional photosynthetic protein complexes during chloroplast development is a critically important and highly complex process, requiring the coordination of both anterograde signaling from the nucleus to control chloroplast gene expression and retrograde signaling from the chloroplast to regulate nuclear gene expression (1, 2, 5–8).

The chimeric composition of photosynthetic protein complexes poses an enormous challenge for maintaining protein homeostasis (proteostasis) and numerous chaperone and protease systems are involved in the regulation of proteostasis in the cytosol and in the chloroplast (6, 9, 10). In the cytosol, the disruption of chloroplast gene expression or protein import leads to cytosolic protein stress (11, 12). In vascular plants, the heat shock transcription factors are involved in the cytosol protein-stress response, similar to heat shock responses in yeast and mammalian cells (13, 14). In the chloroplast, protein quality responses were observed in WT Arabidopsis treated with lincomycin to block chloroplast translation (15) or in the var2 mutant with impaired photosystem II repair cycle (16). Numerous chaperones and proteases are also present in the chloroplast (16). For example, stroma chaperones such as HSP90 and HSP70 are important components of the protein quality control systems (17). In addition, the serine protease Clp is the main proteolytic machinery in the chloroplast stroma (18). In Chlamydomonas, the conditional depletion of chloroplast ClpP1 induced the expression of nuclear genes involved in plastid protein quality control such as chloroplast HSP70 and an involvement of ClpP1 in chloroplast-unfolded protein stress response was proposed (19, 20).
In eukaryotes, FtsH family of ATPases associated with diverse cellular activities (AAA+) proteins plays key roles in protein quality control in different organelles (21). In Arabidopsis, AtFtsH3, AtFtsH4, and AtFtsH10 are targeted into mitochondria and are involved in the assembly or stability of the oxidative phosphorylation complexes (22–24). The thylakoid FtsH complexes, consisting of AtFtsH1, VAR1/AtFtsH5, VAR2/AtFtsH2, and AtFtsH8, are heterohexamers involved in the PSII repair cycle and chloroplast development (22, 25–29). In the chloroplast envelope, AtFtsH12 forms the heteromeric AtFtsH12–FtsH1 (FtsH inactive) complexes with several members of the FtsH protein family and interacts with the TIC complex and the plastid NAD-dependent Malate Dehydrogenases (30–32). Null mutations of components of the AtFtsH12–FtsH1 complexes, such as AtFtsH12, FtsH11, FtsH12, FtsH4, FtsH5, or plastid NAD-dependent Malate Dehydrogenase, give rise to embryonic lethal phenotypes, indicating the essential nature of these proteins (3, 30, 31, 33). The ATPase activity of the AtFtsH12–FtsH1 complex was proposed to provide the driving force for chloroplast protein translocations (30). This is similar to the yeast mitochondrial FtsH homolog YME1, which uses the energy of ATP hydrolysis to drive the translocation of substrates into the proteolytic domain (34).

In this work, we report a chloroplast development mutant pale green Arabidopsis 1-1 (pga1-1), which showed defects in the accumulation of photosynthetic proteins and photosynthetic gene expression. Map-based cloning and molecular complementation confirmed that PGA1 encodes AtFtsH12. Biochemical evidence showed that AtFtsH12 is a part of large complexes. Furthermore, AtFtsH12 is required for the accumulation of photosynthetic proteins and photosynthetic gene expression during de-etiolation. Moreover, the defective accumulation of chloroplast proteins in pga1-1 induced cytosolic and chloroplast proteostasis responses. Our results demonstrate that AtFtsH12 plays important roles in chloroplast protein accumulation and cytosol-chloroplast protein homeostasis.

Results

The identification of the pale green Arabidopsis 1-1 mutant

To dissect the molecular mechanisms underpinning chloroplast development, we have systematically isolated and characterized different categories of leaf color mutants in Arabidopsis, including virecent (35), variegation (36), and pale green Arabidopsis (pga) mutants. The pga1-1 mutant showed a characteristic pale-green leaf color phenotype in both juvenile and adult stages, and a reduced plant stature, compared with the WT (Figs. 1, A and B and S1). Consistent with the pale-green appearance, the chlorophyll content in rosette leaves of 2-week-old pga1-1 was reduced to ~30% of that in the WT (Fig. 1C). Next, we checked the steady-state accumulation of photosynthetic proteins in 2-week-old pga1-1 plants (Fig. 1D). The PSII reaction center subunit D1 and the thylakoid FtsH complex subunit VAR2/AtFtsH2 were reduced to about 50% of those in the WT, while RbcL and RbcS, the large and small subunits of RuBisCO, respectively, accumulated to ~25% of those in the WT (Fig. 1D). Interestingly, the levels of light-harvesting antenna complex (LHC) subunits, such as LhcB2 for PSII and LhcA1 for PSI, showed the strongest reductions in pga1-1, to only ~10% of those in the WT (Fig. 1D).

Next, we analyzed the accumulation of photosynthetic protein complexes with blue native PAGE (BN-PAGE). Thylakoid membranes isolated from WT and pga1-1 were solubilized with 1% n-dodecyl-β-D-maltoside or 2% digitonin (Fig. S2, A and B). Overall, 1-D BN-PAGE revealed that major complexes, such as PSI supercomplexes, PSI, PSII dimers, and monomers, as well as LHClI trimers, still accumulated in pga1-1, albeit to significantly reduced levels compared with the WT (Fig. S2, A and B), and 2-D denaturing BN-PAGE further confirmed the general reduction of photosynthetic protein complexes and subunits (Figs. 1E and S2C). In addition, soluble proteins from 4-day-old WT and pga1-1 seedlings were resolved on 1-D BN-PAGE, and the accumulations of RuBisCO and PEP complexes were also significantly reduced in pga1-1 (Fig. 1F).

Collectively, these data establish that PGA1 is required for chloroplast development and the accumulation of photosynthetic proteins and complexes.

PGA1 encodes AtFtsH12

To identify the PGA1 locus, a map-based cloning approach was employed, using a F2 population derived from a cross between pga1-1 and the Landsberg erecta (Ler) ecotype. Initial mapping was performed with a DNA pool from 95 pga1-1 individuals using molecular markers distributed on five chromosomes, and the PGA1 locus was located to the long arm of chromosome 1, close to the marker F5A18#1 (Fig. S3). Fine mapping using 285 pga1-1 individuals further placed PGA1 between markers T8K14#1 and F19K16#1 (Fig. 2A). Genomic DNA sequencing of genes in this region identified a G to A missense mutation in the coding region of AT1G79560, converting the 703rd amino acid residue from glycine to arginine (G703R) (Fig. 2B). AT1G79560 encodes AtFtsH12, a member of the chloroplast FtsH protein family (22). AtFtsH12 protein contains several distinct domains including an N-terminal chloroplast transit peptide, two putative transmembrane domains TM1 and TM2, an ATPase domain, and a conserved HExxH motif for the zinc-dependent proteolytic domain M41 (G703R) (Fig. 2A). AT1G79560 encodes AtFtsH12, a member of the chloroplast FtsH protein family (22). AtFtsH12 protein contains several distinct domains including an N-terminal chloroplast transit peptide, two putative transmembrane domains TM1 and TM2, an ATPase domain, and a conserved HExxH motif for the zinc-dependent proteolytic domain M41 (Figs. 2B, S4 and S5). The G703R mutation was located in the GAD (Glycine-Alanine-Aspartate/Glutamate) motif, which is part of the ATPase domain and is well conserved in FtsH homologs (Figs. 2, B and C and S4) (37). The predicted 3-D structure of the ATPase domain of AtFtsH12 showed that the conserved Gln703 is in an α-helix near the ATP-binding pocket, resembling its counterpart Gly484 in yeast Yme1 (Figs. 2C and S4) (34).

To confirm that PGA1 is AtFtsH12, we complemented the pga1-1 mutant with two constructs. One vector contained the full-length AtFtsH12 cDNA driven by the constitutive UBQ10 promoter (pLBOQ10:AtFtsH12), while the other included the full-length AtFtsH12 genomic region fused with GFP-coding
sequences at the 3’ driven by its native promoter (pAtFtsH12:gAtFtsH12-GFP) (Fig. 2, D and E). Independent pga1-1 pUBQ10:AtFtsH12 and pga1-1 pAtFtsH12:gAtFtsH12-GFP transgenic lines showed WT-like green leaf color and plant stature, indicating that pga1-1 can be rescued by AtFtsH12 and that AtFtsH12-GFP is functional in planta (Fig. 2, D and E). To detect the AtFtsH12 protein, we raised a polyclonal antibody against the loop domain between the two transmembrane domains TM1 and TM2 of AtFtsH12 (Fig. 2B). In pga1-1 pAtFtsH12:gAtFtsH12-GFP lines, the AtFtsH12-GFP fusion protein (~130 kDa) was readily detected using either an anti-GFP antibody (Fig. S6A) or the anti-AtFtsH12 antibody (Fig. S6, B and C). The endogenous AtFtsH12 (~100 kDa) was also detected by the anti-AtFtsH12 antibody (Fig. S6, B and C). Together, these results demonstrate that the mutation in AtFtsH12 causes the pga1-1 phenotype, and PAGI encodes AtFtsH12.

Null mutants of AtFtsH12 were previously reported as embryonic lethal (30, 31, 33). Thus, the viable pga1-1 represents a novel hypomorphic allele of AtFtsH12 and provides a valuable material to dissect AtFtsH12 functions. In addition, when pUBQ10:AtFtsH12 or pAtFtsH12:gAtFtsH12-GFP was transformed into WT or pga1-1, ~20% of hygromycin-resistant T1 transgenic plants showed different degrees of albino leaf coloration and abnormal leaf development in rosette leaves (Fig. S7A). We examined the transcript and the protein levels of AtFtsH12 in T1 albino transgenic lines. Surprisingly, AtFtsH12 transcripts were highly accumulated in the white tissues, but AtFtsH12 protein level was greatly reduced compared with those in WT (Fig. S7, B and C). This suggests that the albino transgenic plants may not arise from reduced AtFtsH12 mRNA levels due to cosuppression. Alternative possibilities, such as poisonous effects of overexpressed AtFtsH12, may be responsible for this phenotype. Together with the pale-green phenotype of pga1-1, these results indicate that AtFtsH12 plays essential roles in chloroplast development during vegetative growth.

**AtFtsH12 forms large protein complexes on the chloroplast envelope**

AtFtsH12 is predicted to contain a chloroplast transit peptide, and we used two methods to determine the subcellular localization of AtFtsH12. First, leaf mesophyll protoplasts were...
isolated from the *pga1-1 paAtFtsH12:gAtFtsH12-GFP* lines, and ring-like AtFtsH12-GFP signals nicely surrounding the chlorophyll fluorescence were observed, indicating that AtFtsH12-GFP is targeted to the chloroplast and is likely localized to the chloroplast envelope membrane (Figs. 3A and S6D). Interestingly, some GFP signals were also observed in the cytosol (Figs. 3A and S6D). Immunoblotting using the anti-GFP antibody revealed a protein band (~37 kDa) in *pga1-1 paAtFtsH12:gAtFtsH12-GFP* (Fig. S6A). Therefore, the cytosol GFP signals may derive from degraded AtFtsH12-GFP fragments that were not recognized by the anti-AtFtsH12 antibody against the loop structure of AtFtsH12 (Figs. 2B and S6C).

As an alternative approach, we fractionated intact chloroplasts from WT plants into Thy (thylakoids), Env1 (mixed thylakoid and envelope membranes), Env2 (mostly envelope membranes), and Str (stroma) as described (35). Equal amounts of proteins from these fractions were analyzed with immunoblotting against different marker proteins, including VAR2/AtFtsH2 for thylakoids, TOC34 (Translocase of chloroplast 34) for envelope, and RbcL for stroma respectively, confirming the identities of these fractions (Fig. 3B). AtFtsH12 was mainly detected in Env2, as well as a minor presence in Env1 using the anti-AtFtsH12 antibody, supporting that AtFtsH12 is associated with the chloroplast envelope (Fig. 3B).

Next, we checked whether AtFtsH12 forms protein complexes like other Arabidopsis FtsH proteins (22, 24, 26). Immunoblotting of the 2-D SDS-PAGE gel showed that AtFtsH12 was present in high molecular weight complexes that were significantly larger than known photosynthetic protein complexes, such as PSII supercomplexes and PSI-NDH supercomplexes (Fig. 3C) (38). These large AtFtsH12 complexes are consistent with previously reported 2-MDa AtFtsH12 complexes that are associated with the TIC complexes (30).

**The feedback regulation of AtFtsH12 gene expression by the *pga1-1* mutation**

In immunoblotting analyses, we consistently observed that AtFtsH12*G703R*, the mutant form of AtFtsH12, accumulated to a higher level in *pga1-1* than the level of AtFtsH12 in the WT (Figs. 4A and 6, B and C). The increased accumulation of

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**Figure 2. PGA1 encodes AtFtsH12.** A, map-based cloning of the PGA1 locus. The PGA1 was mapped between molecular markers T8K14#1 and F19K16#1 using 285 individual plants. In this region, a G703R mutation in AT1G79560/AtFtsH12 was identified. B, schematic representation of protein domains in AtFtsH12. C, the GAD motif is highly conserved in FtsH homologs, including 12 AtFtsHs from *Arabidopsis thaliana*, ScYME1 from *Saccharomyces cerevisiae*, HsYME1L from *Homo sapiens*, and TmFtsH from *Thermotoga maritima*. D, complementation of *pga1-1* with *pUBQ10:AtFtsH12*. E, complementation of *pga1-1* with *pAtFtsH12:gAtFtsH12-GFP*. Representative 2-week-old plants are shown in (D) and (E). The scale bars represent 0.5 cm. ATPase, the ATPase domain; cTP, chloroplast transit peptide; GAD, the Gly-Ala-Asp motif in the ATPase domain; Loop, the loop domain between TM1 and TM2; M41, Zinc-dependent protease M41 domain; TM, putative transmembrane domains.
AtFtsH12G703R was restored to the WT level in pga1-1 pUBatFtsH12Q10:AtFtsH12 and pga1-1 pAtFtsH12G703R:AtFtsH12-GFP, respectively (Figs. 4A and S6, B and C). Interestingly, the transcript level of AtFtsH12G703R was also elevated in pga1-1 revealed by RT-qPCR (Fig. 4B). In contrast, the transcript levels of PhANGs such as LhcB2.2 and RbcS were significantly reduced (Fig. 4B). However, the transcript levels of chloroplast genome-encoded photosynthetic genes such as psbA and rbcL in pga1-1 were comparable to those in the WT (Fig. 4B). Using BN-PAGE, we observed that the G703R mutation did not affect the formation of AtFtsH12 complexes, and AtFtsH12G703R complexes also accumulated to a markedly higher level in pga1-1 than the level of AtFtsH12 complexes in the WT, indicating that AtFtsH12G703R could be assembled into large complexes effectively (Fig. 4C).

To establish the genetic basis for this feedback regulation, heterozygous pga1-1 mutants (pga1-1+/+) were examined. pga1-1+/+ showed WT-like phenotypes, and the accumulation of AtFtsH12 protein in pga1-1+/+ was similar to that of the WT (Fig. S8C). These findings suggest that pga1-1 is a recessive mutant, and the feedback regulation of AtFtsH12 is not activated in pga1-1 heterozygotes.

### AtFtsH12 is required for chloroplast development and the accumulation of cytosol-translated chloroplast proteins

To further analyze the accumulation of photosynthetic proteins in pga1-1, we used a de-etiolation assay, which enables the determination of photosynthetic protein levels in a time course during illumination (29). The protein level of AtFtsH12 increased in the WT upon 24-h illumination, indicating that AtFtsH12 is induced during de-etiolation (Figs. 5A and S9). Interestingly, the accumulation of the mutant form of AtFtsH12 was not elevated in etiolated pga1-1 seedlings compared with that of the WT form of AtFtsH12 in etiolated WT seedlings, in contrast to the findings with 2-week old light-grown WT and pga1-1 seedlings (Figs. 5A and 4A). Upon 24-h light treatment, the level of the mutant form of AtFtsH12 was still comparable in pga1-1 compared with that of the WT form of AtFtsH12 in the WT (Fig. 5A). In addition, the accumulation of chloroplast genome-encoded photosynthetic proteins, such as RbcL and D1, as well as nuclear genome-encoded photosynthetic proteins RbcS and LhcB2, were also strongly induced during de-etiolation in the WT (Fig. S5A). In stark contrast, the accumulation of these proteins was greatly compromised in pga1-1 during de-etiolation, and the defective accumulation of photosynthetic proteins in pga1-1 was recovered in complementation lines (Fig. 5A). At the mRNA level, PEP-dependent transcripts such as rbcL and psbA accumulated to much lower levels in pga1-1 compared with those in the WT after 24-h illumination, suggesting that abnormal accumulation of photosynthetic proteins was associated with defective chloroplast gene expression (Fig. 5B). However, the NEP-transcribed gene rpoB was only modestly affected in pga1-1 during de-etiolation (Fig. 5B). The expression of pTAC2, a nuclear gene encoding an accessory subunit of PEP involved in the assembly of PEP complexes (39), was not affected in pga1-1 (Fig. 5B). Interestingly, the rpoTp gene, encoding the NEP, was upregulated in pga1-1 (Fig. 5B).

To observe cytosol-translated chloroplast protein accumulation in vivo during de-etiolation, we introduced a p35S:FNR<sub>cyt</sub>-GFP chloroplast-localized GFP reporter line into pga1-1 (40) to generate pga1-1 p35S:FNR<sub>cyt</sub>-GFP. As the expression of FNR<sub>cyt</sub>-GFP was driven by the constitutive 35S promoter, signals of FNR<sub>cyt</sub>-GFP were observed in etioplasts.
PGA1/AtFtsH12 functions in chloroplast protein accumulation

(at 0 h) and chloroplasts (24-h illumination) in the WT background (Fig. 5C). In contrast, the accumulation of FNR_{ctp}-GFP signals was severely reduced in etioplasts and chloroplasts in pga1-1 (Fig. 5C). In addition, we observed that protochlorophyllide (0 h) and chlorophyll (24 h) fluorescence in pga1-1 were also severely reduced compared with those in the WT (Fig. 5C). Moreover, the sizes of etioplasts (0 h) and chloroplasts (24 h) were smaller in pga1-1 than those in the WT (Fig. 5D). Average mesophyll cell area in cotyledons was also significantly reduced in pga1-1 compared with that in the WT (Fig. 5E). However, the average number of chloroplasts per cell was comparable in pga1-1 and WT (Fig. 5F). These data suggest that the partial loss of AtFtsH12 function leads to defective chloroplast development and reduced accumulation of chloroplast proteins during de-etiolation.

Next, we investigated the accumulation of FNR_{ctp}-GFP in protoplasts isolated from rosette leaves of WT p3SS:FNR_{ctp}-GFP and pga1-1 p3SS:FNR_{ctp}-GFP, respectively (Fig. 6). In WT p3SS:FNR_{ctp}-GFP, strong FNR_{ctp}-GFP signals were exclusively observed in the stroma of chloroplasts (Fig. 6). In contrast, FNR_{ctp}-GFP showed much reduced signals in the chloroplast in pga1-1 p3SS:FNR_{ctp}-GFP protoplasts, while an aberrant accumulation of FNR_{ctp}-GFP signals in the cytosol was also observed in pga1-1 p3SS:FNR_{ctp}-GFP protoplasts (Fig. 6). These findings suggest that AtFtsH12 is required for the accumulation of cytosol-translated chloroplast proteins in the chloroplast.

To investigate how AtFtsH12 modulates the accumulation of PEP complexes, we introduced pTAC2-GFP fusion gene driven by the native pTAC2 promoter (PropTAC2:pTAC2-GFP) into the pga1-1 background by genetic crossing. In 4-day-old cotyledons, signals of chlorophyll autofluorescence were much weaker in pga1-1 PropTAC2:pTAC2-GFP lines than in the ptac2-5 PropTAC2:pTAC2-GFP complementation lines (Fig. S10), suggesting that chloroplasts in pga1-1 were underdeveloped. Signals of pTAC2-GFP were also much lower in the pga1-1 PropTAC2:pTAC2-GFP lines than in the ptac2-5 PropTAC2:pTAC2-GFP lines (Fig. S10). These data demonstrate that AtFtsH12 is involved in the accumulation of the pTAC2 subunit of the PEP complex.

The pga1-1 mutation triggers cytosolic and chloroplast protein stress responses

In contrast to the canonical bacterial FtsH harboring a linker region of ~70 amino acid residues between the two transmembrane domains, AtFtsH12 contains a longer linker region with ~260 amino acid residues, which are highly conserved in AtFtsH12 homologs from Chara braunii, Klebsormidium nitens, Marchantia polymorpha, and Physcomitrella patens (Figs. 2B and S1A). Based on the topology of AtFtsH12, this loop is predicted to be located in the intermembrane space between the outer and inner envelopes (30–32). Yeast two-hybrid assay showed that the loop domain of AtFtsH12 can interact directly with the N-terminus of LhcB2 precursor protein (Fig. S11, B and C). When the chloroplast import apparatus was disrupted and LhcB2 precursors were overaccumulated in the cytosol, the expression of ctHSP70, coding for a cytosol-localized HSP70, was induced in response to cytosolic protein stress (11).

We reasoned that the compromised accumulation of PhANG products in chloroplast may induce a similar response in pga1-1. At the transcript level, we found that both ctHSP70 and cpHSP70 coding for a chloroplast-localized HSP70 were upregulated in pga1-1 compared with the WT (Fig. 7A). In addition, the expression of HsfA2, encoding the Heat Shock Transcription Factor A2 (HsfA2) involved in the
Figure 5. AtFtsH12 is required for chloroplast protein homeostasis and chloroplast development. A, the accumulation of photosynthetic proteins and AtFtsH12 in WT, pga1-1, pga1-1 pAtFtsH12:gAtFtsH12-GFP, and pga1-1 pUBQ10:AtFtsH12 before and after 24-h de-etiolation. Protein loading was normalized to equal fresh tissue weight and confirmed by CBB-stained PVDF membranes. B, RT-qPCR analyses of the steady-state transcript levels of psbA, rbcL, rpoB, pTAC2, and rpoTp in WT and pga1-1 before and after 24-h de-etiolation. Relative transcript levels with respect to those in the WT were calculated using the 2−ΔΔCt method, and PP2A was used as the reference gene. Data are means ± s.d. of four biological replicates. ns not significant, *0.01 < p < 0.05, ***0.0001 < p < 0.001, and ****p < 0.0001. C, the accumulation of GFP in chloroplasts of WT p35S:FNRctp-GFP and pga1-1 p35S:FNRctp-GFP before and after 24-h de-etiolation. The scale bars represent 10 μm. GFP and chlorophylls (protochlorophyllide at 0 h) were detected by confocal microscopy. Exposure times in milliseconds (ms) were labeled in order to compare fluorescent signal intensities in different genotypes. D, changes of chloroplast area during de-etiolation for 24 h. Three replicates containing 150 chloroplasts were analyzed (n = 50 chloroplasts in each replicate) for each genotype. E, cell area after 24-h de-etiolation. Three replicates containing 60 cells were analyzed (n = 20 cells in each replicate) for each genotype. F, average number of chloroplasts along the 2-D cell wall after 24-h de-etiolation (n = 40 cells were counted in each genotype). ns not significant, and ****p < 0.0001. CBB, Coomassie Brilliant Blue.
PGA1/AtFtsH12 functions in chloroplast protein accumulation

chloroplast-unfolded protein response (cpUPR) (15, 16), was also upregulated in pga1-1 (Fig. 7A). Consistent with transcript levels, ctHSP70 and cpHSP70 proteins accumulated to higher levels in pga1-1 (Fig. 7B). The ectopic induction of ctHSP70 and cpHSP70 was reversed in pga1-1 pLB-Q10:AtFtsH12 lines (Fig. 7B). Chloroplast proteases are also involved in maintaining proteostasis during cpUPR (10). The Clp complex is a major protease system involved in the degradation of chloroplast proteins (18). We found that the accumulation of ClpP1 was increased in pga1-1 compared with that in the WT, but ClpP3 level was not significantly altered (Fig. 7B). The increase of ClpP1 protein level is consistent with the upregulation of its transcripts (Fig. 7A). Next, we analyzed the accumulation of Clp complexes in WT and pga1-1 (Fig. 7C). In contrast to a greatly reduced level of RubisCO in pga1-1, the levels of Clp complexes including the core complex and the P ring complex were not affected (Fig. 7C). These findings suggest that Clp complexes are maintained in pga1-1, despite defective chloroplast development.

To probe the genetic interaction between AtFtsH12 and components in chloroplast protein stress response, we generated double mutants of pga1-1 and clpr1-2 (SALK_088407), a T-DNA insertion allele of ClpR1, encoding a nonproteolytic subunit of the Clp complex (41, 42). clpr1-2 displayed a distinct virescent leaf color phenotype and a smaller plant stature (Fig. 7D). In pga1-1 clpr1-2 double mutant, we observed a dramatic reduction of rosette leaf sizes compared with the respective single mutants (Fig. 7D). Quantitative analysis revealed that the average sizes of the first pair of rosette leaves in pga1-1 and clpr1-2 was ∼40% and ∼80% of that in the WT, respectively (Fig. 7E). In contrast, the average size of the first pair of rosette leaves in pga1-1 clpr1-2 was only ∼10% of that in the WT, significantly smaller than the expected leaf size if the genetic interaction between pga1-1 and clpr1-2 is additive (0.8 × 0.4 = 0.32) (Fig. 7E) (43). These findings suggest a synergistic genetic interaction between PGA1/AtFtsH12 and ClpR1 in regulating plant development.

Together, our data show that a hypomorphic mutation in PGA1/AtFtsH12 leads to the induction of cytosol and chloroplast protein stress responses, and chloroplast protein accumulation and degradation coordinately regulate chloroplast and plant development.

**Discussion**

FtsH proteins are conserved membrane-localized, ATP-dependent AAA+ family members that play essential roles in protein quality control in both prokaryotes and eukaryotes (21, 44). The Arabidopsis thaliana genome encodes 12 AtFtsH proteins that are localized to the membrane systems of
chloroplasts and mitochondria (22). Despite the tremendous progress in the elucidation of AtFtsH functions, much remains to be learned about the regulation of organelle functions by AtFtsH proteins. We have been investigating the regulation of chloroplast development by the thylakoid FtsH complexes, using the var2 leaf variegation mutant which is defective in VAR2/AtFtsH2 (45, 46). Extensive characterizations of var2 genetic modifier mutants established that the cytosol-chloroplast proteostasis can regulate leaf variegation and chloroplast development (29, 47). In this work, we reported the isolation of the pale green Arabidopsis 1-1 (pga1-1) mutant. We confirmed that pga1-1 was caused by a point mutation in PGA1/AtFtsH12, encoding a member of the AtFtsH family proteins (22). AtFtsH12 is an essential gene in Arabidopsis and previous work has shown that null alleles of AtFtsH12 are embryonic lethal (30–32), thus the hypomorphic

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**Figure 7.** The pga1-1 mutation triggers cytosolic and chloroplast protein stress responses. **A**, RT-qPCR analyses of steady-state transcript levels of chTHSP70, cpHSP70, HsfA2, ClpP1, ClpP3, ClpR1, rpoB, rpoTP, and pTAC2 in 2-week-old WT and pga1-1. Relative transcript levels with respect to those in the WT were calculated using the $2^{\Delta\Delta Ct}$ method, and Actin2 was used as the reference gene. Data are means ± s.d. of four biological replicates. ns not significant, *0.01 < p < 0.05, **0.001 < p < 0.01, and ****p < 0.0001. **B**, the accumulation of chTHSP70, cpHSP70, ClpP1, and ClpP3 in 2-week-old WT, pga1-1, and pga1-1 pUBQ10:AtFtsH12. Total proteins were extracted, and protein loading was normalized to equal fresh tissue weight and confirmed by CBB-stained PVDF membranes. **C**, the accumulation of Clp complexes in 2-week-old WT and pga1-1. Soluble proteins were extracted and resolved on 1-D BN-PAGE (left panel). The 1-D gel immunoblotting was performed using anti-ClpP1 and anti-ClpP3 antibodies (right panel). The Core complex and P ring complexes were indicated according to the migration of RuBisCO (indicated by the asterisk) (65). **D**, phenotypes of 2-week-old WT, pga1-1, clpr1-2, and pga1-1 clpr1-2. The scale bar represents 0.5 cm. **E**, relative leaf areas of the first pair of rosette leaves of 2-week-old WT, pga1-1, clpr1-2, and pga1-1 clpr1-2 (n ≥ 29 individuals for each genotype). The average leaf area of the WT was defined as 1.0. ****p < 0.0001. BN-PAGE, blue native PAGE; CBB, Coomassie Brilliant Blue; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.
pga1-1 allele offers a unique and valuable genetic resource for the investigation of AtFtsH12 functions. Interestingly, pga1-1 contains a G703R mutation in the conserved GAD motif of the ATPase domain (Fig. 2C). A similar glycine to arginine mutation (G433R) in the GAD motif of VAR2/AtFtsH2 was also reported in the var2-19 mutant, which causes a leaf variegation phenotype in Arabidopsis (37). These findings on one hand show that the conserved glycine is important for AtFtsH12 and VAR2/AtFtsH2 functions but on the other hand indicate that the Gly to Arg mutations likely do not abolish protein functions entirely. This raises a tempting implication that a similar Gly to Arg genetic manipulation may provide a strategy to bypass the lethal phenotype of other essential proteins containing the conserved ATPase domain. Unexpectedly, the G703R mutation in AtFtsH12 does not affect the assembly and the size of AtFtsH12 complexes in pga1-1 (Fig. 4C), which exists in large protein complexes in agreement with previous reports (30). Together with published results, our findings suggest that the G703R mutation leads to a reduced but not completely abolished AtFtsH12 activity (34, 37).

In addition to the AAA+ ATPase domain, many FtsH proteins also contain a conserved HExxH M41 metalloprotease domain (Fig. S5). The proteolytic activities of FtsH family members have been demonstrated in a number of cases (48–50). However, despite the presence of the conserved HExxH sequences in AtFtsH12, the conserved histidine769 in the proteolytic motif M41 is dispensable for chloroplast and plant development (30). Similarly, the conserved histidine488 is also dispensable for VAR2/AtFtsH2 in chloroplast development (51). It had been proposed that ATP-dependent FtsH proteins not only mediate proteolysis but also the insertion of proteins into membranes and assembly of protein complexes, acting like a chaperone (52–54). The participations of FtsHs in protein transport and assembly into membranes have been reported in Escherichia coli and Arabidopsis (30, 55). Similar to its homologs function as a chaperone in bacteria, it was proposed that AtFtsH12, together with other AtFtsHi members, may provide driving forces to pull chloroplast protein precursors through envelope membranes by the hydrolysis of ATP with the ATPase domain (30). It is also speculated that AtFtsH12 may have similar functions in organelar membrane protein quality control as its homologs. Whether AtFtsH12 acts on misfolded or unassembled proteins in the chloroplast envelope requires further investigation.

Several lines of evidence support the notion that AtFtsH12 is required for the accumulation of chloroplast proteins. Firstly, we determined that the steady levels of many nuclear-encoded and plastid-encoded photosynthetic proteins were reduced significantly in pga1-1 compared with the WT (Fig. 1D). Moreover, the accumulation of most photosynthetic protein complexes was also reduced in pga1-1 (Fig. 1, E and F). Overall, the most dramatic impact of the loss of AtFtsH12 seems to be on the accumulation of LHCs and their subunits. Consistently, we observed that the loop domain between the transmembrane domains TM1 and TM2 of AtFtsH12 was able to interact with LhcB2 in yeast two-hybrid assays, suggesting a direct functional link between the two proteins (Fig. S11). Alternatively, as the most abundant membrane proteins in the chloroplast, LHC protein accumulation may be more sensitive to disturbed chloroplast protein homeostasis (56). Secondly, the accumulation of photosynthetic proteins was examined in de-etiolation assays. The pga1-1 mutant showed a greatly compromised photosynthetic protein accumulation during de-etiolation (Fig. 5A). Thirdly, we monitored the accumulation of two chloroplast GFP marker proteins in the chloroplast in the pga1-1 mutant. FNRcpr-GFP driven by the 35S constitutive promoter and pTAC2-GFP driven by its native promoter were not efficiently accumulated in the chloroplast in pga1-1 (Figs. S10 and 5C). In addition, we observed some GFP signals that did not overlap with chlorophylls in protoplasts of pga1-1 p35S:FNRcpr-GFP (Fig. 6). The compromised accumulation of chloroplast proteins and GFP reporters in pga1-1 might be caused by various mechanisms, including gene transcription, protein translation and translocation, stability, assembly and so on.

The aberrant induction of AtFtsH12 transcripts in pga1-1 indicates the possible operation of feedback regulation of AtFtsH12 by its translation product and the WT form of AtFtsH12 may be part of a pathway that represses its own expression (Fig. 4A). Alternatively, it is also plausible that the induced AtFtsH12 expression represents a compensating response to the defective chloroplast protein accumulation and chloroplast development in pga1-1. However, this feedback regulation of the accumulation of AtFtsH12 in pga1-1 was not observed during the 24-h de-etiolation assay, suggesting that this mechanism may be regulated by developmental stages or growth conditions (Figs. 4A and 5A). An unexpected observation in pga1-1 is the activation of genes involved in the control of proteostasis. In contrast to the repressed expression of PhaNGS, transcript levels of ctHSP70, cpHSP70, and HsfA2 were markedly increased in pga1-1 (Fig. 7A). These genes are involved in protein quality control, and their upregulations were also observed in lincomycin-induced or var2-mediated cpUPR (15, 16). The upregulation of ctHSP70 and HsfA2 may represent a response to the stress caused by the over-accumulation of cytosol-translated chloroplast proteins in pga1-1. Compared with low levels of photosynthetic proteins, the relatively higher levels of Clp and cpHSP70 indicated an additional mechanism to maintain proteostasis in the chloroplast (Fig. 7). Taking advantage of the nonlethal pga1-1 allele, we were able to show a synergistic interaction between pga1-1 and clp1-2, providing genetic evidence for the cooperation of AtFtsH12 and the Clp complex in regulating plant development. However, the molecular mechanism behind this synergistic interaction is still not clear. How these chaperon and protease systems cooperate in the cytosol and in the chloroplast to maintain proteostasis needs to be further explored.

Overall, it is possible that defects in chloroplast protein accumulation or chloroplast development in pga1-1 are able to generate signals that repress the expression of both nuclear and chloroplast photosynthetic genes, while activating the expression of genes involved in maintaining protein homeostasis in both the cytosol and the chloroplast, implying a
complex regulatory network. Future work with pga1-1 and additional mutants and components in this network will shine more light on this critical process.

**Experimental procedures**

**Plant materials and growth conditions**

The WT *A. thaliana* and the pga1-1 mutant are Columbia ecotype. The pga1-1 mutant was identified from an ethylmethanesulfonate mutagenesis pool and backcrossed with the WT for five times. The *ptac2-5* *pTAC2-GFP* line is a complementation line of the T-DNA insertion mutant *ptac2-5* (SAIL_244_G05, CS811416) in the *pTAC2* gene, transformed with a *pTAC2-GFP* fusion gene driven by its native promoter (from our group’s unpublished data). The WT *p35S:FNRctp-GFP* line is kindly provided by Dr Ralf Bernd Klösgen. After surface sterilization, Arabidopsis seeds were stratified at 4°C for 2 days and grown on commercial soil mix (Pindstrup) or on half-strength Murashige and Skoog medium (1/2 MS) containing 1.0% Bacto Agar. The plant growth room was maintained at 22°C under continuous light with a light intensity of ~100 μmol photons m⁻² s⁻¹. In the climate chamber, plants were grown under 12-h day/12-h night cycle. For the de-etiolation assay, seeds were germinated on 1/2 MS for 3 days in the dark at 22°C before transferring under light for 24 h.

**RNA manipulations and RT-qPCR**

Total RNAs were extracted from 2-week-old seedlings or from de-etiolated seedlings grown on 1/2 MS, using the Trizol RNA reagent (Invitrogen) according to the manufacturer’s instruction. Reverse transcription reactions were performed with 1.0 μg total RNA using a PrimeScript Reverse Transcription Kit (Takara) and a mixture of oligo (dT)₁₈ and random hexamer primers. RT-qPCR was carried out using the FastStart Essential DNA Green Master (Roche) on the CFX96 Real-Time PCR System (Bio-Rad). The relative transcript levels of each gene were analyzed using the comparative 2⁻ΔΔCt method (57), and *ACT2* or *PP2A* were used as the reference gene. To ensure the reproducibility of RT-qPCR, three or four biological replicates were performed. All primers used in this study are listed in Table S1.

**Plasmid constructions and plant transformation**

To complement *pga1-1*, two binary vectors, *pLBQ10:AtFtsH12* and *pAtFtsH12gAtFtsH12-GFP*, were constructed. The full-length cDNA of *AtFtsH12* was amplified using primers *FtsH12 utrF* and *FtsH12 stcR* for *pLBQ10:AtFtsH12*. The full-length genomic DNA of *AtFtsH12* (*gAtFtsH12*) was amplified using primers *FtsH12 F* and *FtsH12 R* for *pAtFtsH12gAtFtsH12-GFP*. PCR products were digested with *BamHI* and cloned into a modified *pCambia1300* vector containing the *UBQ10* promoter and a *pCambia1300-GFP* vector, respectively. WT and homozygous *pga1-1* plants were transformed by the floral dip method (58). Transgenic lines were screened on 1/2 MS plates containing 25 mg l⁻¹ hygromycin.

**Protein 3-D structures prediction**

The PDB file of AtFtsH12 (alphafold.com/entry/Q9SAJ3) was obtained from the AlphaFold Protein Structure Database (59). The 3-D structure of ATP molecule (DB00171) was obtained from the DrugBank Online (60). The molecular docking between AtFtsH12 and ATP was performed with the Autodock software (https://autodocksuite.scripps.edu/autodock4/) and visualized with PyMOL 2.5.

**Preparation of anti-AtFtsH12 polyclonal antibodies**

To express the loop domain of AtFtsH12, the cDNA fragment corresponding to the predicted loop region (amino acid residues 175–432) of AtFtsH12 was amplified using *FtsH12 gF* and *FtsH12 gR*, and cloned into *pet28a* with Gibson Assembly Cloning Kit (NEB), to generate *pet28a-AtFtsH12-Loop*. The recombinant AtFtsH12-Loop protein was expressed in *E. coli* BL21 (DE3) and purified with nickel-chelate affinity chromatography. The purified AtFtsH12-Loop protein was used as an antigen to produce anti-AtFtsH12 polyclonal antibodies.

**Yeast two-hybrid assays**

The yeast two-hybrid assay was performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech). The coding regions for the loop domain (amino acid residues 175–432), the ATPase domain (amino acid residues 456–736), and the M41 domain (amino acid residues 756–961) of AtFtsH12 were cloned into the prey vector pGAD7T. The coding region for the N-terminus of LhcB2 (amino acid residues 1–117) was cloned into the bait vector pGBK17. The transformed clones were screened using plates containing double dropout SD medium lacking leucine and tryptophan and further using quadruple dropout SD medium lacking adenine, histidine, leucine, and tryptophan. The quadruple dropout SD medium plates supplemented with 20 mg l⁻¹ X-α-Gal were used for high stringency.

**Chloroplast fractionation, total protein extraction, and immunoblotting**

The chloroplast fractionation was performed as described (35), and the protein was loaded based on equal protein concentrations. Total protein extraction from 2-week-old leaves or de-etiolated seedlings was performed based on equal fresh tissue weight as described (29). For immunoblotting, protein samples were separated with 12% SDS-PAGE containing 8.0 M urea and transferred to PVDF membranes (0.22 μm, Millipore). Immunoblotting was performed with standard procedures, and signals were detected using Clarity Western ECL Substrate (Bio-Rad). The antibodies used in this study were listed in Table S2.

**Preparation of crude chloroplast membranes, soluble stromal proteins, and BN-PAGE**

Preparation of crude chloroplast membranes and BN-PAGE were performed as described (29). Briefly, for 1-D BN-PAGE, crude membranes solubilized with the 25BTH20G buffer (25 mM Bis–Tris–HCl pH7.0 and 20% glycerol) containing 1% n-dodecyl-β-D-maltoside (w/v) or 2% digitonin (w/v) were
resolved on 3 to 10% gradient native PAGE. As the chlorophyll content in pga1-1 was reduced to ~30% of that in the WT (Fig. 1C), the loading of thylakoids is based on the difference between their chlorophyll contents (corresponding to 6.0 μg chlorophylls from pga1-1 and 20.0 μg chlorophylls from the WT sample). For 2-D SDS-PAGE, gel lanes were excised from the 1-D BN-PAGE and denatured in 2× SDS sample buffer at room temperature and was resolved on 12% SDS-PAGE containing 8.0 M urea. The AtFtsH12 complexes on 2-D gel were detected with immunoblotting using the AtFtsH12 antibodies. The PEP complex in pga1-1 was analyzed with 3 to 10% BN-PAGE according to (62). For detecting Clp complexes, 2-week-old seedlings of WT and pga1-1 were homogenized in liquid nitrogen. Total soluble proteins were extracted with the 25BTH20G buffer and resolved on 3 to 10% BN-PAGE gel.

Confocal microscopy

To observe the localization of AtFtsH12-GFP, protoplasts were prepared from 4-week-old rosette leaves of pga1-1 pAtFtsH12gAtFtsH12-GFP lines, using the enzyme solution (20 mM MES pH5.7, 0.4 M Mannitol, 20 mM KCl) containing 1.5% cellulase R10 (w/v) and 0.4% macerozyme R10 (w/v) to remove cell walls (63). Mesophyll protoplasts of pga1-1 pAtFtsH12gAtFtsH12-GFP were collected directly by centrifugation at 100g for 2 min. To monitor the fluorescence of pTAC2-GFP and FNRRCP-GFP, cotyledons or isolated protoplasts were used directly for confocal microscopy imaging. In order to compare fluorescent signal intensities in different genotypes, GFP or chlorophyll autofluorescence images were acquired with the same exposure time (for example, 600 milliseconds for GFP and 20 milliseconds for chlorophyll in Fig. S10). GFP fluorescence and chlorophyll autofluorescence were monitored with a spinning disk confocal microscope (Revolution-XD, Andor). Confocal images were processed and the chloroplast area, cell area, numbers of chloroplasts, and first pair of rosette leaf area were determined using ImageJ (https://imagej.nih.gov/ij/) (64).

Statistical analyses

Scatter plots showed mean ± SD, including measurements of chlorophyll, RT-qPCR, and quantification of chloroplast area, cell area, numbers of chloroplasts, and first pair of rosette leaf area. Significance analyses were performed using a one-way or two-way ANOVA analysis, using GraphPad Prism 8 (https://www.graphpad.com/scientific-software/prism/).

Data availability

All data presented are contained within the article.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflict of interest with the contents of this article.

Abbreviations—The abbreviations used are: AAA, ATPases associated with diverse cellular activities; BN-PAGE, blue native PAGE; cpUPR, chloroplast-unfolded protein response; LHC, light-harvesting antenna complex; NEP, nucleus-encoded RNA polymerase; PEP, plastid-encoded RNA polymerase; PSI, Photosystem I; PSII, Photosystem II; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

References
PGA1/AtFtsH12 functions in chloroplast protein accumulation


