Activation of the heterotrimeric G protein α-subunit GPA1 suppresses the ftsh-mediated inhibition of chloroplast development in Arabidopsis

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Received 29 November 2008; revised 29 January 2009; accepted 10 February 2009; published online 16 March 2009.
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SUMMARY
Heterotrimeric G protein knock-out mutants have no phenotypic defect in chloroplast development, and the connection between the G protein signaling pathway and chloroplast development has only been inferred from pharmaceutical evidence. Thus, whether G protein signaling plays a role in chloroplast development remains an open question. Here, we present genetic evidence, using the leaf-variegated mutant thylakoid formation 1 (thf1), indicating that inactivation or activation of the endogenous G protein α-subunit (GPA1) affects chloroplast development, as does the ectopic expression of the constitutively active Gα-subunit (cGPA1). Molecular biological and genetic analyses showed that FtsH complexes, which are composed of type-A (FtsH1/FtsH5) and type-B (FtsH2/FtsH8) subunits, are required for cGPA1-promoted chloroplast development in thf1. Furthermore, the ectopic expression of cGPA1 rescues the leaf variegation of ftsh2. Consistent with this finding, microarray analysis shows that ectopic expression of cGPA1 partially corrects mis-regulated gene expression in thf1. This overlooked function of G proteins provides new insight into our understanding of the integrative signaling network, which dynamically regulates chloroplast development and function in response to both intracellular and extracellular signals.

Keywords: chloroplast development, heterotrimeric G proteins, variegation, FtsH protease, Arabidopsis.

INTRODUCTION
Chloroplasts are essential for plant cells because they not only harbor photosynthesis but also make a wide range of fundamental molecules (Neuhaus and Emes, 2000). Chloroplast development, accompanied by biosynthesis of chlorophylls, and the subsequent assembly of photosynthetic apparatus in the thylakoid membrane, is completed within a few hours of light perception (Kleffmann et al., 2007). It is known that chloroplast development is dynamically regulated by intracellular developmental cues, retrograde signaling from chloroplasts to nuclei and the ever-changing environmental conditions (Mullet, 1988; Nott et al., 2006). Although remarkable progress has been made towards understanding the molecular machinery that controls chloroplast biogenesis (Aldridge et al., 2005), the integrative network that senses intra- and extracellular signals, and subsequently transfers these signals to the machinery, remains poorly understood.

Leaf variegation mutants are useful for dissecting this signaling network, because the severity of variegation is frequently dependent upon environmental conditions and developmental stages (Sakamoto, 2003; Aluru et al., 2006). To date, a number of nuclear genes, including FtsH5/VAR1, FtsH2/VAR2, VAR3 and THF1, have been shown to control leaf variegation in Arabidopsis (Yu et al., 2007). Analysis of these mutants demonstrates that variegation is caused by abnormalities in various chloroplast functions (Sakamoto et al., 2003; Aluru et al., 2006). FtsH protease, which belongs to the ATP-dependent AAA+ protein family, and forms a...
hexameric ring structure, is the best-characterized example for explaining the mechanism of variegation in Arabidopsis (Sakamoto, 2006). FtsH subunits are encoded by type-A (FtsH1 and FtsH5) and type-B (FtsH2 and FtsH8) genes. The genes of the same type are functionally redundant, and plants defective in either type do not survive (Sakamoto et al., 2003; Yu et al., 2005).

Function of FtsH complexes has been shown to repair the photodamaged photosystem-II (PSII) reaction center D1 protein and to control chloroplast development (Lindahl et al., 2000; Bailey et al., 2002; Sakamoto et al., 2003; Zaltsman et al., 2005; Yoshioka et al., 2006). It is proposed that a threshold level of the FtsH complex is required for normal chloroplast function: chloroplast function is impaired, and then white or yellow sectors form when the complex levels fall below the threshold (Yu et al., 2007).

In eukaryotes, many extracellular signals are sensed by heptahelical cell-surface receptors, designated as G protein-coupled receptors (GPCR). These signals are then transmitted into the cell by heterotrimeric G proteins composed of α, βi and γ subunits (Neves et al., 2002). In the canonical model of G-protein signaling, G proteins are activated by binding ligands to their corresponding receptors, which subsequently catalyze the replacement of guanosine diphosphate (GDP) with guanosine triphosphate (GTP) on the Gβγ-complexed Gα subunit. Then, both the GTP-bound Gα and released Gβγ can transfer signals to downstream effectors. The signaling is switched off by the intrinsic GTP hydrolysis activity of Gα, because the GDP-bound Gα has low affinity for effectors, but high affinity for Gβγ. The duration of G-protein signal transduction can be significantly shortened by regulators of G-protein signaling (RGS), through accelerating the GTP hydrolysis of Gα (Siderovski et al., 1996; Siderovski and Willard, 2005). This G-protein signaling system, composed of GPCRs, heterotrimeric G proteins and effectors, and RGS proteins, is present in the plant kingdom (Temple and Jones, 2009). In Arabidopsis, only single canonical α (GPA1) and β (AGG1) subunits, and two γ (AGG1 and AGG2) subunits, have been identified. The other important components, GPCR and RGS, are also few (Chen et al., 2003; Gookin et al., 2008). Several downstream effectors or interactors, including a cinnbin domain-containing protein (PRN1), phospholipase Dα1 (PLDα1), prephenate dehydratase 1 (PD1) and thylakoid membrane formation 1 (THF1) have been shown to bind physically to GPA1 (Lapik and Kaufman, 2003; Zhao and Wang, 2004; Huang et al., 2006; Warpeha et al., 2006), whereas only one genetically interacting protein, a Golgi-localized hexose transporter SGB1 (suppressor of G protein β1), has been shown for AGB1 (Wang et al., 2006).

The involvement of G proteins in chloroplast development was first demonstrated by pharmaceutical studies with hypocotyl cells of the tomato aurea mutant, which is almost totally deficient in active phytochrome A (PhyA) (Neuhaus et al., 1993; Bowler et al., 1994). It was concluded that the G protein is sufficient to transduce PhyA signaling through second messages, cyclic GMP (cGMP) and Ca2+/calmodulin. Signaling pathways mediated by cGMP and Ca2+/calmodulin coordinately promote the expression of photosynthesis-related proteins, and the assembly of photosynthetic complexes. Several reports also indicated that G proteins modulate plastid gene expression, which may affect chloroplast development and function (Romero and Lam, 1993; Dingra et al., 2004; Warpeha et al., 2007). However, whether G-protein signaling plays a role in chloroplast development remains an open question. Here, using the leaf-variegated mutants thf1 and ftsh2 we provide several lines of evidence supporting the hypothesis that G proteins finely modulate chloroplast development, at least in part, via the regulation of FtsH gene expression in Arabidopsis.

RESULTS

GPA1 is a positive regulator for chloroplast development in thf1

Previous studies showed that THF1 physically interacted with GPA1 (Huang et al., 2006), and that the null thf1 mutant had a variegated phenotype in both cotyledons and leaves (Wang et al., 2004). Here, we found that the gpa1thf1 double mutant displayed a more severe leaf-variegated phenotype than the thf1 single mutant. We therefore studied whether the G-protein signaling pathway was involved in chloroplast development. As RGS proteins accelerate the deactivation of the Gα subunit, the RGS1 mutation might reduce the degree of variegation in the thf1 background. Indeed, the rgs1 thf1 plants produced fewer yellow sectors than the thf1 plants, whereas the thf1 gpa1 double mutant consistently displayed more severe variegation than thf1 at both seedling and mature plant stages (Figures 1a,b, S1). Leaf chlorophyll content was in agreement with the severity of the variegation phenotype among the mutants (Figure 1c). The rgs1 thf1 leaves had 18.9% more chlorophyll than the thf1 leaves, whereas the thf1 gpa1 leaves had only 75.8% of the thf1 level of chlorophyll. These data indicate that GPA1 is a positive regulator for chloroplast development in thf1.

Ectopic expression of cGPA1 rescues the thf1 variegation phenotype

To further study a role of G-protein signaling in chloroplast development, thf1 plants were transformed with a constitutively activated (GTPase-deficient) form of GPA1Q222L, designated as cGPA1. The results showed that ectopic expression of cGPA1 in thf1 (cGPA1/thf1) clearly rescued the variegation phenotype of thf1 in both cotyledons and leaves (Figure 2a). The chlorophyll content of the cGPA1/thf1 lines was comparable with that of wild-type (WT) plants, whereas
thf1 accumulated 83.3% of the WT chlorophyll level in leaves (Figure 2b). In addition, the ratio of chlorophyll a to b (Chl a:b) in thf1 (2.82) was also reduced to the WT level (2.50) by cGPA1 (Figure 2c). Thus, we concluded that the ectopic expression of cGPA1 was able to rescue the thf1 variegation phenotype.

Ectopically expressed cGPA1 in thf1 affects chloroplast ultrastructure and PSII activity

The observations described above prompted us to investigate the effect of cGPA1 on chloroplast development and photosynthesis in thf1. We examined the chloroplast ultrastructure in green sectors of mature leaves from 43-day-old WT, thf1 and cGPA1/thf1 plants, and in the yellow sectors of thf1 leaves, using transmission electron microscopy. In green sectors, most of the chloroplasts in thf1 and cGPA1/thf1 transgenic leaves had similar ultrastructures, including grana and stroma thylakoids, as those observed in the WT (Figure 3a,b,c). However, in cGPA1/thf1 mesophyll cells, about 20% of the chloroplasts had unusual granal stacks (Figure 3d). Such a chloroplast was not observed in both WT and thf1 leaves under our growth conditions. It is well known that PSII and the associated main chlorophyll a/b light-harvesting complex (LHCII) are predominantly localized in the stacked thylakoid membrane, which is essential for optimizing the photosynthetic machinery under various environmental conditions (Anderson, 1999). The observed change in chloroplast ultrastructure from cGPA1/thf1 leaves may indicate a role of the G-protein signaling pathway in photosynthesis under environmental stress. In yellow sectors of thf1 leaves, plastids in most mesophyll cells lacked organized thylakoid structures, and contained many membrane vesicles (Figure 3e). Interestingly, chloroplasts were present in some spongy cells that contacted with the lower epidermis in the thf1 yellow sectors (Figure 3f,g), but had fewer thylakoid membranes (Figure 3h). Taken together, our ultrastructural data support the hypothesis that G-protein signaling is involved in the regulation of chloroplast development.

A previous study demonstrated that thf1 mutants were hypersensitive to excessive light stimuli (Keren et al., 2005). To determine whether the ectopic expression of cGPA1 could rescue the sensitivity of the thf1 plant to high intensities of light, we measured the PSII maximum photochemical efficiency (Fv/Fm; Fv, variable fluorescence; Fm, maximum fluorescence) in green regions. No difference in Fv/Fm was detected among WT, thf1 and cGPA1/thf1 plants grown in normal light conditions (70–100 μmol m−2 s−1; Figure 3i, left bars). However, cGPA1/thf1 transgenic
plants were more resistant to high light intensity (1500 μmol m⁻² s⁻¹) than were thf1 plants, but were more sensitive than the WT plants (Figure 3i, middle and right bars). This result indicates that the ectopic expression of cGPA1 can partially recover PSII activity in thf1 leaves.

THF1 mutation leads to a decrease in FtsH protease that is partially recovered by the ectopic expression of cGPA1

Based on the above results, we proposed that the positive action of cGPA1 on chloroplast development and PSII activity in the thf1 background might be associated with some proteins in thylakoid membranes, where the photosynthetic complexes are localized. To test this hypothesis, we isolated thylakoid membranes from WT, thf1 and cGPA1/thf1 leaves. The membrane proteins were fractionated by SDS-PAGE (Figure 4a left panel). The result indicated that the ectopic expression of cGPA1 in thf1 obviously enhanced the density of a band with a molecular weight of approximately 71 kDa (marked with an asterisk in Figure 4a).

Quantitative analysis indicated that the relative level of the band in cGPA1/thf1 was about 60% higher than that in thf1 (Figure 4a right panel). To identify this band, the gel fragment was excised, digested with trypsin and analyzed by matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure 4b). Sequencing of the polypeptides showed that the band contained FtsH isoforms, including FtsH1 and FtsH6 (Figure 4c). This finding provided a line of important evidence to suggest that the variegation phenotype of thf1 may be attributed to a low level of the FtsH complex.

As the D1 protein, a core subunit of the PSII reaction center, is a substrate for FtsH protease, we measured the activity of FtsH protease in thf1 by examining the stability of the D1 protein. To do this, thylakoid membranes were extracted from leaves that were treated with Lincomycin (an inhibitor of protein biosynthesis in chloroplasts) for 1 h, and then subsequently exposed to high levels of light. As shown in Figure 5a, the D1 protein was more stable in thf1 than in the WT during treatment, whereas the stability of D2 protein, which is another subunit of the PSII reaction center, and is used as a control, was not affected by the THF1 mutation. This result suggests that FtsH activity is reduced in thf1.

We then investigated FtsH levels by Western blot analysis. Note that FtsH levels detected by antibodies against FtsH5 and FtsH2 represent type A and type B, respectively (Sakamoto et al., 2003). The result indicated that both type-A and type-B FtsHs were significantly reduced in thf1 (Figure 5b). Quantitative analysis showed that the relative levels of type-A and type-B FtsHs in cGPA1/thf1 accumulated to levels that were 2- and 1.35-fold that found in thf1, respectively. However, expression of THF1 was not altered in any of the ftsH single mutants (Figure 5c). Thus, these results suggest that THF1 functions as a regulator for FtsH expression.

The ectopic expression of cGPA1 enhances mRNA levels of FtsH subunits

The reduced accumulation of FtsHs in thf1 could be a result of either reduced expression or increased degradation, or of both. To verify these possibilities, the steady-state levels of mRNAs for FtsHs were analyzed by Northern blot with...
specific probes. Our data showed that expression of all four FtsH genes in thf1 was similar to that in the WT (Figure 6a), suggesting that the THF1 mutation-induced decrease in FtsH accumulation takes place at a post-transcriptional step. In contrast, the ectopic expression of cGPA1 in thf1 increased the level of each FtsH transcript. In particular, the expression of FtsH2 was 2.3-fold higher than that in the WT (Figure 6a). This result suggests that the recovered level of FtsHs in cGPA1/thf1 is at least in part attributable to an increase in FtsH transcripts.

We then investigated the stability of FtsH subunits in thf1. To do this, FtsH levels in thylakoid membranes were dynamically examined after protein biosynthesis in the cytoplasm was inhibited by cycloheximide (CHX). Immuno-blot analysis showed that 50% or less of FtsHs remained in thf1 and cGPA1/thf1, whereas about 90% of FtsHs remained in WT plants when treated with CHX for 6 h (Figure 6b). These data indicated that the ectopic expression of cGPA1 had no effect on the thf1-induced instability of FtsH complexes.

Genetically, THF1 and FtsHs act in the same pathway for leaf variegation

The critical role FtsH protease played in the thf1-induced variegation phenotype prompted us to investigate whether they functioned in the same pathway for leaf variegation. Homozygous mutants of thf1 were crossed with ftsh5 or ftsh2. New phenotypes with severely variegated or albino seedlings were observed in the F2 generation. The albino seedlings, which mimic the phenotypes of ftsh5 ftsh1 and ftsh2 ftsh8 double mutants (Zaltsman et al., 2005), were

Figure 4. SDS-PAGE profiles of thylakoid membrane proteins and MALDI-TOF MS analysis.
(a) A representative SDS-PAGE profile stained with Coomassie blue (left panel). The 71-kDa band is indicated by an asterisk. The relative levels of the 71-kDa band were normalized to the band marked with two asterisks (right panel).
(b) MALDI-TOF MS of tryptic peptides eluted from the 71-kDa band in (a). Asterisks indicate peaks matching the peptide fingerprints of FtsH peptides.
(c) A representative product ion spectra of m/z 1508.75; the sequence derived is shown as an inset.
identified as ftsh5 thf1 and ftsh2 thf1 double mutants (Figure 7a). The genotyping analysis indicated that plants with severe variegated phenotypes were homozygous for thf1, and were heterozygous for either ftsh5 or ftsh2, which was more apparent than in any of the thf1, ftsh5 and ftsh2 single mutants (Figure 7a,b). These results suggest that the THF1 mutation has a strong effect on FtsH function. To evaluate the effect of FtsH1 and FtsH8 on thf1 variegation, we made ftsh1 thf1 and ftsh8 thf1 double mutants. As shown in Figure 7c, both the ftsh1 thf1 and ftsh8 thf1 double mutants survived, and displayed phenotypes that were similar to that of the thf1 mutant. This result is in accord with previous reports showing that neither the single ftsh1 and ftsh8 mutants nor the double ftsh1 ftsh8 mutant displayed leaf variegation (Zaltsman et al., 2005).

Figure 5. The THF1 mutation leads to a decrease in FtsH accumulation, which is partially rescued by the ectopic expression of cGPA1.

(a) FtsH activity was measured by D1 stability. The D2 level was used as a control. +Linc, leaves were floated on 1 mM lincomycin solution at 20 μmol m⁻² s⁻¹ for 3 h. +Linc/HL, leaves that had been pre-treated with lincomycin were subsequently exposed to 1800 μmol m⁻² s⁻¹ irradiance; 2.0 μg of chlorophyll were loaded in each lane.

(b) Western blot analysis of FtsH proteins in 43-day-old wild-type (WT), thf1 and cGPA1/thf1 plants (left upper panel). The left lower panel shows the relative levels of type-A and type-B in thf1 and cGPA1 thf1, compared with the WT. The error bars represent ±SD. The gel stained with Coomassie blue (right panel) was shown as a loading control.

(c) Western blot analysis of THF1 in 10-day-old WT, ftsh5, ftsh2, ftsh1, ftsh2 and thf1 plants. *Non-specific band detected by the antibody.

To determine directly whether the FtsH complex is a bottleneck limiting chloroplast development in thf1, we tested if type-A or type-B subunits were able to complement the variegation phenotype of thf1. Because FtsH genes within the same type are interchangeable, the full-length cDNA of FtsH1 or FtsH8 was introduced into the thf1 mutant (Figure S2a). As expected, overexpression of either FtsH1 or FtsH8 markedly rescued the variegated or yellow cotyledons of thf1 (Figure 7d, upper panel). The leaf variegation of thf1 was also largely complemented by FtsH1 and FtsH8 (Figure 7d, lower panel). This result demonstrates that FtsH protease plays an important role in the THF1-regulated development of chloroplasts.

Chloroplast development promoted by the ectopic expression of cGPA1 is dependent on FtsH complexes

Given that the cGPA1-promoted development of chloroplasts was attributable to the elevated levels of the FtsH complex,
the variegation phenotype might also be rescued by the introduction of cGPA1 into ftsh2. The transgene cGPA1 was identified by genomic PCR (Figure S2b). Indeed, the ectopic expression of cGPA1 significantly rescued the variegation phenotype in the first pair of true leaves, where the variegation is most obvious in ftsh2 mutants (Figure 8a). Western blot analysis also showed that the transgenic lines accumulated higher levels of FtsH subunits than the ftsh2 mutants (Figure 8b). Quantitative analysis showed that the relative levels of type-A and type-B FtsHs in ftsh2 only accumulated to 3.3 and 43.7% of the WT levels, respectively, whereas in cGPA1/ftsh2 lines the levels were 10.2 and 57.5% of the WT levels, respectively. Thus, we reasoned that the type-A and type-B levels, enhanced by the ectopic expression of cGPA1, were correlated with the recovery of the severe variegation phenotype of ftsh2.

To further test the hypothesis that the cGPA1-rescued variegation phenotype was coupled to FtsH subunits, we incorporated the cGPA1 gene into the ftsh8 ftsh2 double mutant, by crossing the transgenic cGPA1/ftsh2 line with the single ftsh8 mutant. The resulting ftsh2 ftsh8 double mutants displayed white or slightly yellow seedlings (Figure 8c), whereas the presence of a single wild-type FtsH8 allele in the ftsh2 mutants gradually turned the cotyledons from yellow to green (Figure 8c). In the F2 population, the frequency of seedlings with white leaves generated from ftsh2 × ftsh8 (2.71/16) had no statistical difference from those from cGPA1/ftsh2 × ftsh8 (2.78/16), suggesting that the ectopic expression of cGPA1 is incapable of rescuing the phenotype of the ftsh2 ftsh8 double and FtsH8+/– ftsh2 mutants. A smaller than expected ratio of lethal seedlings (3/16) was also reported by Zaltsman et al. (2005), because of some aborted seeds in the siliques of F1 plants. Interestingly, we found that some of the seedlings with white leaves that segregated from cGPA1/ftsh2 × ftsh8 exhibited greener cotyledons than did those from ftsh2 × ftsh8. We genotyped seedlings with differentially greening cotyledons and found that the presence of cGPA1 was correlated with the greener cotyledons (Figure 8c). In the FtsH8+/– ftsh2 background the chlorophyll content in cotyledons increased from 0.24 to 0.61 mg g⁻¹ of fresh weight, with the ectopic expression of cGPA1. Therefore, we conclude that cGPA1-promoted chloroplast development is coupled to the FtsH complex.

Microarray analysis shows that the expression of a batch of genes in thf1 is recovered by ectopically expressed cGPA1

To better understand the molecular basis underlying cGPA1-promoted chloroplast development in thf1, we performed a microarray analysis using total RNAs extracted from 3-day-old light-grown seedlings of the cGPA1/thf1 transgenic line, the thf1 mutant and the WT. Transcriptomic comparisons were performed to find genes differentially expressed in thf1 over the WT and in cGPA1/thf1 over thf1 through the genome annotation program on the TAIR website (http://www.arabidopsis.org/portals/genAnnotation). Among the 22 743 genes represented on the Affymetrix chip, 560 genes, including 151 upregulated genes and 409 downregulated genes, were mis-regulated by at least twofold in thf1 relative to the WT (Table 1). Consistent with the role of THF1 in chloroplast development, 25.9% (106) of the 409 downregulated genes are predicted to function in plastids. This proportion is markedly higher than that predicted for whole genomic plastidic proteins, i.e. approximately 3500...
In contrast, 10.6% (16) of the 151 upregulated genes encoded proteins targeted to plastids. On the other hand, we found that 924 genes, including 369 upregulated genes and 555 downregulated genes, were mis-regulated by at least twofold in the cGPA1/thf1 seedlings compared with the thf1 seedlings (Table 1).

Table 1

<table>
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<th>Functional category</th>
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<th>thf1 Downregulated (409 genes) (%)</th>
<th>cGPA1/thf1 Upregulated (389 genes) (%)</th>
<th>cGPA1/thf1 Downregulated (555 genes) (%)</th>
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<td>106 (25.9)</td>
<td>126 (34.1)</td>
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Table 1 The ectopic expression of cGPA1 partially rescues the mis-regulated transcripts in thf1: numbers and percentages of the mis-regulated genes (by at least twofold) are shown for thf1 relative to the wild-type (WT), and for cGPA1/thf1 relative to thf1, respectively.

(about 15.4% of the genes in the array; Arabidopsis Genome Initiative, 2000). In contrast, 10.6% (16) of the 151 upregulated genes encoded proteins targeted to plastids. On the other hand, we found that 924 genes, including 369 upregulated genes and 555 downregulated genes, were mis-regulated by at least twofold in the cGPA1/thf1 seedlings compared with the thf1 seedlings (Table 1).

Among these differentially expressed genes in cGPA1/thf1, compared with thf1, interestingly 18.7% of the upregulated genes are predicted to function in plastids, whereas only 6.3% of the downregulated genes are predicted to function in plastids. These results indicate that the expression of cGPA1 in thf1 has a significant impact on the expression profiling of the genes that is directly attributable to chloroplast development. Furthermore, among these mis-regulated genes, we found that 49 (32.5%) of the genes upregulated in thf1 were downregulated in the cGPA1/thf1 line (Table S1), whereas 32 (7.8%) downregulated genes in thf1 were upregulated in the cGPA1/thf1 line (Table S2). These opposite expression

Figure 8. The ectopic expression of cGPA1 rescues the variegated leaf phenotype of ftsh2. (a) Phenotypes of 21-day-old wild-type (WT) and transgenic plants. cGPA1/ftsh2-A and cGPA1/ftsh2-B are two independent transgenic lines expressing cGPA1 in ftsh2. (b) FtsH levels in 10-day-old WT, ftsh2, cGPA1/ftsh2-A and cGPA1/ftsh2-B plants detected by immunoblot analysis (left upper panel). The relative levels of type A and type B in ftsh2 and two transgenic lines were normalized to those of WT levels (left lower panel). The right panel shows the Coomassie blue-stained gel as a loading control. (c) Genotype identified by PCR with gene-specific primers, and comparison of phenotypes of 21-day-old seedlings with (lanes 1 and 2) or without (lanes 3 and 4) the transgene of cGPA1 in the FtsH8+/– ftsh2 (lanes 1 and 3) or ftsh2 ftsh8 (lanes 2 and 4) background.
patterns detected in thf1 and cGPA1/thf1 seedlings by the microarray analysis were validated for selected genes by Northern bolt analysis (Figure 9a,b). Taken together, the results indicate that cGPA1 overexpression can partially correct the mis-regulated gene expression caused by the THF1 mutation.

DISCUSSION

G-protein signaling pathway for chloroplast development

Chloroplast development must be kept in harmony with developmental and environmental cues, as photooxidation easily takes place in developing chloroplasts. The D1 protein at the reaction center of PSII undergoes rapid renewal, in which the FtsH protease is required for D1 degradation. Certainly, a low activity of FtsH protease leads to the accumulation of damaged D1 protein, followed by oxidative stress and inhibition of chloroplast development or photosynthesis. In this study, we established a genetic linkage between the G-protein signaling pathway and chloroplast development via FtsH protease. In addition, our data also indicate that G proteins are probably involved in the regulation of photosynthesis under various environmental conditions. In general, our results support a hypothesis that G proteins are fine tuners, adjusting various cellular processes to their best status in Arabidopsis.

A role of G proteins in chloroplast development has been reported in the tomato aurea mutant (Neuhaus et al., 1993; Bowler et al., 1994). We also explored whether cGPA1 could rescue the pale-green phenotype of hy2 which is a homolog of aurea in Arabidopsis (Parks and Quail, 1991; Muramoto et al., 2005). Unexpectedly, the ectopic expression of cGPA1 was unable to rescue the pale-green phenotype of hy2 (data not shown), indicating that cGPA1 probably functions specifically in FtsH-mediated chloroplast development. This disagreement with pharmaceutical results may reflect different molecular mechanisms underlying G protein-regulated chloroplast development in plant species. The blocked chloroplast development in hy2 is attributed to the inhibition of 5-aminolevulinic acid (ALA) biosynthesis (Terry and Kendrick, 1999).

FtsH protease is regulated by G proteins and THF1

In higher plants, the way in which FtsH protease is regulated remains unknown, in spite of its critical role in chloroplast development and photosynthesis. Our several lines of
evidence showed that FtsH protease is finely controlled by THF1 and the G-protein signaling pathway. Using genetic and molecular biological approaches, we found that THF1 is an important regulator for FtsH protease. Further analysis indicated that the FtsH complex is not stable in the absence of THF1. It seems that the THF1-regulated expression of FtsH is similar to that in the single ftsh2 and ftsh5 mutants, where the coordinated decrease in FtsH accumulation is attributed to the degradation of unassembled subunits (Sakamoto et al., 2003), but not to a reduction in transcriptional levels of the other FtsH genes (Yu et al., 2004). How FtsH complexes are post-translationally regulated by THF1 remains to be further investigated.

Genetic screening for ftsh2 suppressors revealed that the balance between chloroplast protein synthesis and degradation plays an important role in chloroplast development (Park and Rodermeil, 2004; Miura et al., 2007; Yu et al., 2008). These suppressors include two subunits (ClpC2 and ClpR1) of Clp protease, SVR1 (a chloroplast-localized homolog of pseudouridine synthase), SCO1 (chloroplast elongation factor G) and FUG1 (chloroplast translation initiation factor 2). Mutations of these genes ultimately lead to a decrease in chloroplast protein accumulation. It was proposed that reduced rates of plastid protein synthesis decrease the demand for FtsH complexes in developing chloroplasts, allowing more plastids to overcome a threshold and to turn green in ftsh2 plants (Yu et al., 2008). In sharp contrast, cGPA1-promoted chloroplast development is dependent on an increase in the FtsH expression in the thf1 and ftsh2 backgrounds. In addition, fug1 has been demonstrated to reduce the photosynthetic activity of ftsh2 as a result of the suppression of protein biosynthesis in chloroplasts (Miura et al., 2007), whereas PSII activity in cGPA1/thf1 plants was higher than in thf1 plants (Figure 3i). Thus, our data reveal a new mechanism for plants to maintain protein balance in developing chloroplasts, through the upregulation of gene expression.

**A possible role of G proteins in retrograde signaling**

Retrograde signaling from chloroplasts to the nucleus has recently been shown to mediate the expression of many nuclear genes in response to a variety of intra- and extracellular changes, such as chlorophyll biosynthesis, high levels of light and pathogen attack (Marwell et al., 1999; Nott et al., 2006). However, little is known about how a signal is transferred from chloroplasts to the nuclei. Retrograde signals include reactive oxygen species (ROS), intermediates in chlorophyll biosynthesis, and redox generated by the electron transfer pathway (Nott et al., 2006; Koussevitzky et al., 2007). Kato et al. (2007) reported that retrograde signaling pathways were involved in the suppression of nuclear-encoded photosynthetic gene expression in the yellow or white sectors of variegated leaves. Interestingly, G proteins have been shown to influence cellular responses to oxidative stresses generated in chloroplasts, such as ozone and far-red irradiation in Arabidopsis (Joo et al., 2005; Wei et al., 2008). Furthermore, hydrogen peroxide was reported to promote the dissociation of the Gx subunit from the approximately 700-kDa complex (Wang et al., 2008). Chloroplasts are recognized as a primary site producing ROS, and dynamically contact the plasma membrane. As previously reported, GPA1 and THF1 interact at sites where the plastid membrane abuts the plasma membrane (Huang et al., 2006). Thus, whether G proteins are involved in retrograde signaling pathways will be an interesting topic for future studies.

**A model for G protein-mediated chloroplast development**

The genetic and molecular biological data presented here indicate that THF1 functions either upstream of or in parallel with GPA1 in the regulation of chloroplast development. In addition, THF1 was also suggested to work downstream of the GPA1-mediated glucose signaling pathway in roots (Huang et al., 2006). A plausible explanation for these different results is that G proteins often act in a tissue- or cell-specific manner, and their multiple functions are executed by different G-protein complexes (Perfus-Barbeoch et al., 2004; Chen, 2008). Although the physiological significance of G protein-mediated chloroplast development remains unclear, our data lay a solid foundation towards a complete dissection of the G-protein signaling pathway in the future.

Here, we propose a model for G protein-mediated chloroplast development via FtsH protease (Figure 10). It has been well documented that FtsH protease is a critical factor for chloroplast development in Arabidopsis (Yu et al., 2007). Results derived from this study suggest that THF1 and G proteins are the new regulators for FtsH protease. THF1 is highly conserved in oxygenic photosynthetic organisms, but its biochemical function has not yet been elucidated. THF1 is
localized in the stroma, thylakoids and the outer envelope of chloroplasts (Wang et al., 2004; Huang et al., 2006; L. Zhang and J. Huang, unpublished data). Generally, the THF1-mediated regulation of chloroplast development may be achieved via G protein-dependent and/or -independent pathways. In the G protein-independent pathway, stroma- and/or thylakoid-localized THF1 controls chloroplast development through affecting the stability of FtsH protease. In the G protein-dependent pathway, the outer envelope-localized THF1 may function as a scaffold or modulator required for the full activation or fine control of the G-protein signaling pathway. The activated form of GPA1 can transmit signals from the plasma membrane to nuclei via second messengers and/or other signaling molecules, and ultimately controls the expression of the FtsH genes needed for chloroplast development. Calcium- and cGMP-dependent pathways have been shown to play an important role in G-protein signal transduction (Neuhaus et al., 1993; Bowler et al., 1994). Whether the G protein-regulated FtsH expression is mediated by Ca\(^{2+}\) and/or cGMP signaling pathways remains to be investigated. The two pathways may crosstalk to form a complicated mechanism by which the activity of FtsH protease in chloroplasts is finely regulated at different levels of gene expression.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

The *Arabidopsis thaliana* ecotype Columbia-0 was used. The mutants used for the analyses or generation of double mutants have previously been described: *thf1* (Huang et al., 2008), *gpa1-4* (Jones et al., 2008), *gpa1-2* (Chen et al., 2003), *fth1* and *fth8* (Sakamoto et al., 2003), and *fth2* and *fth5* (Yu et al., 2004). Double mutants were identified in the F2 generation by an allele-specific PCR-based genotyping procedure. For phenotypic observation, seeds were stratified at 4°C for 4 days, and then sown onto soil in a 22°C growth chamber with a 8-h light/16-h dark cycle. Alternatively, surface-sterilized seeds were sown onto half-strength MS agar plates supplemented with 1% sucrose. The seedlings were transferred to soil once established.

**Plasmid constructions and transgenic plants**

Plasmids were constructed using the Gateway cloning system (Invitrogen, http://www.invitrogen.com). The constitutively active form of GPA1\(^{1222,11}\) was generated as described in Chen et al. (2003). FTS1 and FTS8 cDNA were cloned from the ABRC stock U19697 (FtsH1) and U14077 (FtsH8) into the pENTR SD/D-TOPO entry vector with gene-specific primers (Table S3). The fragments were then recombined into the pGWB2 destination vector (Research Institute of Molecular Genetics, Shimane University, Japan). Arabidopsis plants were transformed by Agrobacterium GV3101 with the flower-dip method (Clough and Bent, 1998). Transgenic T1 plants were screened on media supplemented with kanamycin. Transgenic lines were confirmed by RT-PCR or by PCR products amplified from genomic DNA with gene-specific primers (Table S3). Transgenic lines overexpressing cGPA1 were identified by the presence of the cGPA1 transgene or transcripts. The PCR products were further digested with the restriction enzyme Ddel, and generated three bands with 448, 360 and 67 bp.

**Chlorophyll content, chlorophyll fluorescence and transmission electron microscopy**

Chlorophyll was extracted with 80% acetone at 4°C for 24 h in darkness. Chlorophyll \(a\) and chlorophyll \(b\) were determined by the absorbance at 645 and 663 nm with a spectrophotometer (UNIC UV-2102; PCS, Shanghai, China, http://www.unico1.com.cn/e/index.asp), respectively. Chlorophyll fluorescence was measured as described by Zhou et al. (2008). Leaves of 43-day-old plants grown in short-day conditions (8-h light/16-h dark) were fixed in a solution of 4% glutaraldehyde, and then processed, embedded and viewed via electron microscopy, as described by Harris et al. (1994).

**Total RNA extraction and northern blot analysis**

Total RNA was isolated using the RNAsena total RNA isolation system (Promega, http://www.promega.com) in accordance with the manufacturer’s instructions. A 10-μg portion of total RNA was loaded in each lane, separated on formaldehyde gels and then stained with ethidium bromide to confirm equal loading of RNA. Digoxigenin-labeled DNA probes with gene-specific primers (Table S3) were used in the hybridization solution. Northern blotting was conducted as described by Huang et al. (2000).

**Thylakoid membrane preparation, immunoblot analysis and protein identification**

Thylakoid membrane was prepared according to Aro et al. (1993). Total proteins of thylakoid membranes equivalent to 2.0 μg of chlorophyll were loaded in each lane, and separated by denaturing SDS-PAGE, and then transferred to Hybond-ECT nitrocellulose membrane (Amersham Biosciences, now part of GE Healthcare, http://www.gehealthcare.com) and immunoblotted with various antibodies. The specificity of α-FtsH5 (VAR1) and α-FtsH2 (VAR2) has already been examined by Sakamoto et al. (2003). The α-THF1 was a gift from Dr Korth (University of Arkansas, http://www.uark.edu). The antibodies AS05084 and AS06146 against the D1 and D2 proteins, respectively, were bought from Agrisera (http://www.agrisera.com). Immunodetection of the protein gel blots was performed using the ECL plus western blotting detection system (Amersham Biosciences). For protein identification, bands were excised from the gels and the peptide sequence was analyzed as described by Peltier et al. (2004).

**Transcriptomic analysis of genes in cGPA1/thf1 transgenic, thf1 and WT plants**

We used 3-day-old WT (control), *thf1* and cGPA1/*thf1* seedlings for microarray analysis. Samples were collected and combined from three independent experiments grown on half-strength MS supplemented with 1% sucrose under short-day conditions. Total RNA was isolated using the TRIzol (Invitrogen) extraction procedure, and was further purified with RNaseasy minikits (Qiagen, http://www.qiagen.com). Double-strand cDNA was synthesized using a one-cycle cDNA synthesis kit (Affymetrix, http://www.affymetrix.com), followed by purification on a GeneChip sample clean-up module (Affymetrix). Biotin-labeled cRNA was prepared and hybridized.
to whole genome Affymetrix ATH1 GeneChip microarrays containing 22 746 Arabidopsis transcripts, as suggested by the manufacturer. The probe arrays were scanned using the 2500 Affymetrix GeneChip Scanner. The acquired data were analyzed using the MICROARRAY SUITE (MAS 5.0; Affymetrix), and databases of gene expression profiles were built with single-array analyses (see the Affymetrix statistical algorithms reference guide). The transcripts of the whole genome genes were classified into three categories by detection P-values: present (P < 0.04), absent (P > 0.06) and marginal (0.04 < P < 0.06). The significant difference (P < 0.0025) in the up or downregulated transcriptional level of the detected genes between thf1 and the WT or cGPA1/thf1 and thf1 was examined using Wilcoxon’s signed rank test. The genes were selected by the signal log ratio ≥1.0 (with an at least twofold variation of transcript levels) for mis-regulated genes, and were further analyzed for gene functional annotation through the TAIR gene ontology (GO) annotation download website (http://www.arabidopsis.org/tools/bulk/go/index.jsp).

ACKNOWLEDGEMENTS

The authors thank Dr S. Rodermel for providing ftsh5 and ftsh2 seeds, and Dr S. McCormick, Dr W. Briggs, Dr L. Li and Dr J. Chen for their critical review of the manuscript. This work was supported by grants from the National Key Basic Research Program of China (2007CB108800), the National Natural Scientific Foundation of China (30570131), the Bairen Project of Chinese Academy of Sciences and the Shanghai Scientific Committee (06PJ14103).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The genetic manipulation of endogenous GPA1 activity partially rescued the leaf variegation of thf1.

Figure S2. Identification of transgenic plants.

Table S1. Among the 151 genes that are upregulated (by up to twofold) in thf1, transcripts of 49 genes are downregulated, by at least twofold, in cGPA1/thf1.

Table S2. Among 409 genes that are downregulated (by up to twofold) in thf1, transcripts of 32 genes are upregulated at by least twofold in cGPA1/thf1.

Table S3. Primers used in this study.

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REFERENCES


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