Coordination of Plastid Protein Import and Nuclear Gene Expression by Plastid-to-Nucleus Retrograde Signaling

Tomohiro Kakizaki², Hideo Matsumura, Katsuhiro Nakayama, Fang-Sik Che, Ryohei Terauchi, and Takehito Inaba*

The 21st Century Centers of Excellence Program, Cryobiofrontier Research Center, Iwate University, Morioka, Iwate 020–8550, Japan (T.K., K.N., T.I.); Iwate Biotechnology Research Center, Kitakami, Iwate 024–0003, Japan (H.M., R.T.); and Department of Environmental Biology, Faculty of Bioscience, Nagahama Institute of Bioscience and Technology, Nagahama, Shiga 526–0829, Japan (F.-S.C.)

Expression of nuclear-encoded plastid proteins and import of those proteins into plastids are indispensable for plastid biogenesis. One possible cellular mechanism that coordinates these two essential processes is retrograde signaling from plastids to the nucleus. However, the molecular details of how this signaling occurs remain elusive. Using the plastid protein import2 mutant of Arabidopsis (Arabidopsis thaliana), which lacks the aToc159 protein import receptor, we demonstrate that the expression of photosynthesis-related nuclear genes is tightly coordinated with their import into plastids. Down-regulation of photosynthesis-related nuclear genes is also observed in mutants lacking other components of the plastid protein import apparatus. Genetic studies indicate that the coordination of plastid protein import and nuclear gene expression is independent of proposed plastid signaling pathways such as the accumulation of Mg-protoporphyrin IX and the activity of ABA INSENSITIVE4 (ABI4). Instead, it may involve GUN1 and the transcription factor AtGLK1. The expression level of AtGLK1 is tightly correlated with the expression of photosynthesis-related nuclear genes in mutants defective in plastid protein import. Furthermore, the activity of GUN1 appears to down-regulate the expression of AtGLK1 when plastids are dysfunctional. Based on these data, we suggest that defects in plastid protein import generate a signal that represses photosynthesis-related nuclear genes through repression of AtGLK1 expression but not through activation of ABI4.

Plastids are a diverse group of organelles that perform essential metabolic and signaling functions within all plant cells. It is generally believed that plastids originated from a unicellular photosynthetic bacterium that was taken up by a eukaryotic host cell (Dyall et al., 2004). During evolution, most of the genes encoded by the bacterial ancestor have been transferred to the host nuclear genome; for example, the plastid genome of Arabidopsis (Arabidopsis thaliana) encodes fewer than 100 open reading frames (Martin et al., 1998). Consequently, plastid biogenesis is dependent on the import of nuclear-encoded plastid proteins (Keegstra and Cline, 1999; Soll and Schleiff, 2004; Kessler and Schnell, 2006; Inaba and Schnell, 2008; Jarvis, 2008), the genes for which must be expressed at an appropriate level. For example, many of the photosynthesis-related nuclear genes that are required for chloroplast biogenesis are induced via photoreceptors, such as phytochrome, in response to light quality and quantity (Terzaghi and Cashmore, 1995), so that the photosynthesis-related proteins will be available for import into developing chloroplasts. Other types of plastids, according to their specific metabolic functions, need other sets of nuclear-encoded proteins. Therefore, the expression of specific sets of nuclear genes and the import of their translation products are indispensable for plastid differentiation.

After they have been imported into plastids, nuclear-encoded plastid proteins combine with plastid-encoded proteins to form functional multiprotein complexes, such as photosystems and metabolic enzymes. Because plastids need a distinct set of proteins in specific amounts dictated by their functional and metabolic states, it is also critical for plastids to give feedback information to the nucleus, so that nuclear gene expression can be adjusted appropriately. This fine-tuning mechanism is important for determining which nuclear-encoded proteins are imported, and consequently it regulates the differentiation of proteins.
plastids in a tissue- and developmental state-dependent manner.

To accomplish such regulation, plastids send signals (called plastid signals) to the nucleus via multiple signal transduction pathways. To date, three distinct plastid processes—tetrapyrrole biosynthesis, plastid gene expression, and organellar redox reactions—have been shown to give rise to plastid signals (Nott et al., 2006). These plastid signals have been studied using specific inhibitors and controlled light conditions. For example, tetrapyrroles may act as a plastid signal that is induced by norflurazon, an inhibitor of carotenoid synthesis. Genetic studies have identified several genomes uncoupled mutants (gun1 to gun5) that exhibit norflurazon-insensitive LHCB expression, and many of these mutants have lesions in the tetrapyrrole biosynthetic pathway (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003). However, there are conflicting reports on the mechanism of norflurazon action (La Rocca et al., 2001; Strand et al., 2003; Mochizuki et al., 2008; Moulin et al., 2008). Another plastid process that affects nuclear gene expression is plastid gene expression: inhibition of plastid protein synthesis by lincomycin generates a plastid signal that suppresses the expression of photosynthesis-related genes in the nucleus (Mulo et al., 2003). This signaling pathway is only active during the first 3 d of seedling development (Beck, 2005). In contrast, a redox retrograde signaling pathway seems to play an important role in light acclimation of mature plants (Beck, 2005). Thus, alteration of the functional state of the plastid by inhibitors or by light conditions generates a wide variety of plastid signals, depending on the developmental stage of the plant.

Analysis of mutants lacking plastid protein import receptors has suggested that plastid biogenesis requires the coordination of plastid protein import with nuclear gene expression and that this coordination is likely to involve retrograde signaling from plastids to the nucleus. The protein import receptors of plastids are composed of two distinct families of GTP-binding proteins (Kessler and Schnell, 2006). One of these, the atTOC159 family, consists of four genes in Arabidopsis—aTOC159, aTOC132, aTOC120, and aTOC90 (Bauer et al., 2000; Hillbrunner et al., 2004)—and appears to comprise a set of functionally distinct receptors. The plastid protein import2 (ppi2) mutant, which lacks the most abundant receptor, atTOC159, exhibits a severe albino phenotype. The mutant fails to accumulate representative photosynthesis-related proteins, even though nonphotosynthetic proteins are accumulated normally (Bauer et al., 2000). This difference in accumulation is in part attributable to the ability of atTOC159 to recognize photosynthesis-related, but not nonphotosynthetic, proteins as its substrates (Smith et al., 2004). An intriguing observation is that photosynthesis-related nuclear gene expression also seems to be compromised in ppi2. For example, two representative photosynthesis-related nuclear genes, LHCB and small subunit of Rubisco (SSU), appear to be downregulated at the mRNA level in ppi2, whereas two nonphotosynthetic genes, atTOC34 and chorismate mutase1, are unaffected (Bauer et al., 2000). When an artificial substrate of atToc159, preSSU-GFP, was expressed in ppi2 under the control of a constitutive promoter, the expression of preSSU-GFP was no longer down-regulated and ppi2 accumulated a significant amount of the unprocessed protein in the cytosol (Smith et al., 2004). These observations suggest that plastid protein import and nuclear gene expression are tightly coordinated to regulate the flow of nuclear-encoded plastid proteins in response to the functional and developmental states of the plastids. However, the molecular mechanism that coordinates protein import and nuclear gene expression remains elusive.

In this study, we examine the role of plastid protein import in the regulation of nuclear gene expression. Using mutants defective in plastid protein import, we show that such defects cause down-regulation of photosynthesis-related nuclear gene expression. This regulation appears to be mediated by a plastid signaling pathway that may involve both GUN1 and AtGLK1 and is distinct from the ABA INSENSITIVE4 (ABI4) pathway. We propose a novel mechanism by which plastids regulate nuclear gene expression according to the rate of plastid protein import and the functional state of plastids.

RESULTS

Expression of Photosynthesis-Related Nuclear Genes Is Specifically Impaired in ppi2 Plants

It has been shown that atToc159 is a membrane-bound GTPase and appears to play a critical role in the import of nuclear-encoded photosynthesis-related proteins (Kessler et al., 1994; Schnell et al., 1994; Bauer et al., 2000; Smith et al., 2004). Therefore, ppi2-1 (originally referred to as ppi2; Bauer et al., 2000), which lacks the atToc159 protein import receptor, fails to accumulate major photosynthesis-related proteins (e.g. LHCP and SSU) but not nonphotosynthetic plastid proteins (Bauer et al., 2000). Interestingly, mRNA accumulation for a couple of nuclear-encoded photosynthesis-related proteins was also shown to be compromised in the ppi2-1 mutant (Bauer et al., 2000). This observation suggests that plastid protein import and nuclear gene expression are tightly coordinated to regulate the flow of nuclear-encoded plastid proteins in response to the functional and developmental states of the plastids.

To further pursue the link between plastid protein import and nuclear gene expression, we expanded our analysis of expression in the ppi2-1 mutant to include a dozen nuclear-encoded photosynthesis-related and nonphotosynthetic plastid proteins. Total proteins were extracted from wild-type (Wassilewskija [Ws]) and ppi2-1 leaves, resolved by SDS-PAGE, and probed with antibodies against various proteins (Fig. 1A;
Supplemental Fig. S1). As a control, the membranes were also probed with antibody against actin. As shown in Figure 1, A and B, the levels of all of the photosynthesis-related proteins examined were decreased in ppi2-1 to less than 20% of the level in the wild type, supporting the idea that atToc159 is responsible for the import of photosynthesis-related proteins. To further investigate the mechanisms that regulate the accumulation of photosynthesis-related proteins in ppi2-1 plastids is correlated with their mRNA expression level in the nucleus.

We next examined the accumulation of protein (Fig. 1C; Supplemental Fig. S1) and mRNA (Fig. 1D) of nonphotosynthetic plastid proteins to investigate whether the down-regulation of nuclear-encoded plastid proteins in ppi2-1 is specific to photosynthesis-related proteins. Five proteins exhibiting various activities were chosen for the analysis. As shown in Figure 1, C and D, all of the proteins except the carboxyl transferase α-subunit of acetyl-CoA carboxylase (ACC-CTα) accumulated to normal or higher levels in the mutant compared with the wild type. Real-time PCR analysis revealed that the mRNA accumulation for these proteins was not down-regulated.
in the ppi2-1 mutant (Fig. 1D). The discrepancy between the levels of ACC-CTα mRNA and ACC-CTα protein might be an indirect effect of the slightly reduced expression of the plastid-encoded β-subunit (ACC-CTβ) in ppi2-1 (Fig. 1D).

These results were further confirmed by examining the phenotype of an additional atTOC159 mutant (referred to as ppi2-2), a T-DNA insertion mutant in the Columbia (Col-0) background from the GABI-Kat collection (Supplemental Fig. S2). In this mutant, which has no detectable atTOC159, the expression profile of nuclear genes encoding plastid proteins was similar to that of ppi2-1, except for a slight decrease in IEP37 expression (Fig. 2A). Taken together, these data indicate that the ppi2 mutation compromises the accumulation of nuclear-encoded photosynthesis-related proteins and that the decrease in protein accumulation is strongly correlated with the down-regulation of nuclear-encoded gene expression.

Impact of the Protein Import Defect on the Nuclear Transcriptome in the ppi2 Mutant

The lack of atToc159 in Arabidopsis results in a severe albino, seedling-lethal phenotype, as highlighted by analysis of the ppi2-1 mutant (Bauer et al., 2000). This phenotype implies that the defect in atToc159 causes dramatic changes in nuclear gene expression in the ppi2-1 mutant. Therefore, to investigate the global impact of the atToc159 defect on nuclear-encoded gene expression, we performed SuperSAGE (for Super Serial Analysis of Gene Expression) analysis using wild-type and ppi2-1 plants. SuperSAGE is a powerful tool to examine global gene expression profiles quantitatively (Matsumura et al., 2003). As summarized in Supplemental Figure S3, 126,238 tags (29,238 unique tags) and 204,218 tags (43,580 unique tags) were identified from wild-type and ppi2-1 cDNAs, respectively. To identify genes corresponding to each tag sequence, The Arabidopsis Information Resource 7 gene annotation database was queried with 26-bp tags using the BLASTN program. Then, the 2,000 genes most significantly affected by the ppi2-1 mutation (1,000 each of up- and down-regulated genes) were classified based on the Gene Ontology terms (http://www.arabidopsis.org/tools/bulk/go/index.jsp; Supplemental Tables S1 and S2). Among the down-regulated genes, approximately 30% encoded proteins destined for chloroplasts or plastids (Fig. 1E). In contrast, only approximately 5% of the up-regulated genes encoded proteins targeted to chloroplasts or plastids (Fig. 1E). Given the fact that only 10% to 15% (approximately 3,500) of nuclear genes are predicted to encode plastid proteins, it appears that this class of genes is preferentially down-regulated in the ppi2-1 mutant. These data also suggest that there is a tight coordination between plastid protein import and the nuclear transcriptome and that this regulation is critical for proper accumulation of plastid proteins and plastid biogenesis.

Figure 2. Expression profiles of nuclear-encoded plastid proteins in a mutant allelic to ppi2-1 and in other mutants defective in plastid protein import. A, Quantitative analysis of nuclear-encoded gene expression in the ppi2-2 mutant by real-time PCR (Col-0 background; allelic to ppi2-1). B, Immunoblot analysis of plastid proteins in the ppi2-2 mutant. C, Quantitative analysis of nuclear-encoded genes in the attoc132 attoc120+/- mutant by real-time PCR. D, Immunoblot analysis of plastid proteins in the attoc132 attoc120+/- mutant. E, Quantitative analysis of nuclear-encoded genes in the attic20-I mutant by real-time PCR. F, Immunoblot analysis of plastid proteins in the attic20-I mutant. In each case, transcript levels (A, C, and E) were analyzed by real-time PCR and normalized to the transcript levels of ACTIN2. The expression level in the wild type was set to 1. Each bar, plotted here on a log scale, represents the mean of at least three independent samples. Error bars represent 1 sd. Asterisks above the bars indicate significant differences (P < 0.05) between the wild type and the respective mutant, as determined by Student’s t test. The ppi2-2, attic20-I, and Col-0 plants were grown on plates. attoc132 attoc120 +/- and Ws were grown on soil.
Defects in Plastid Protein Import Result in Down-Regulation of Photosynthesis-Related Genes in the Nucleus

The correlation between protein import defects and the down-regulation of photosynthesis-related nuclear genes was further examined using additional mutants: attoc132 attoc120+/- and tic20-I. The attoc132 attoc120+/- mutant has fewer atToc132/120 protein import receptors, which, unlike atToc159, are specific for nonphotosynthetic proteins (Ivanova et al., 2004; Kubis et al., 2004). The tic20-I mutant lacks a putative channel component of the protein translocation machinery at the inner envelope membrane of chloroplast. In this mutant, the import of both nonphotosynthetic and photosynthesis-related proteins is compromised (Teng et al., 2006). As shown in Figure 2, C and E, the expression of two photosynthesis-related nuclear genes, OE23 and LHCB1, was impaired in both mutants. However, the effect on the expression of the nonphotosynthetic genes IEP37 and pyruvate dehydrogenase E1α subunit (E1α) was relatively moderate (Fig. 2, C and E). The accumulation of each protein was proportional to the mRNA expression level (Fig. 2, D and F). These results suggest that plants with reduced import of nonphotosynthetic proteins also specifically down-regulate the expression of photosynthesis-related nuclear genes.

Since all of these mutants exhibit pale or albino phenotypes in the early stages of cotyledon development (Bauer et al., 2000; Ivanova et al., 2004; Teng et al., 2006), it was possible that the arrest of plastid development at the cotyledon stage led to the severe growth defects in the adult stage. That is, the down-regulation of nuclear gene expression in these mutants could be a developmental effect rather than a direct effect of defects in protein import. To obtain more evidence that the inhibition of plastid protein import results directly in the down-regulation of nuclear gene expression, we took advantage of atTOC159-silenced plants. These plants have a transgene expressing sense atTOC159 from a constitutive promoter (Bauer et al., 2002), and most of them look normal during the early stages of growth. However, as the plants grow older, some of their leaves become variegated due to cosuppression of the endogenous atTOC159 gene (Fig. 3, B–D). Given the late onset of the variegated phenotype (Fig. 3C), it was assumed that normal proplastid-to-chloroplast differentiation had been completed during the cotyledon stage. We analyzed mRNA and protein accumulation in green versus albino sectors of these plants (Fig. 3, C and D, arrows and arrowheads). In the albino sectors, the accumulation of nuclear-encoded photosynthesis-related proteins was specifically impaired, and this observation was in part attributable to the down-regulation of photosynthesis-related nuclear gene expression (Fig. 3, E and F). In contrast, neither mRNA nor protein accumulation for the nonphotosynthetic proteins, E1α and IEP37, was significantly affected.

Altogether, these data indicate that defects in protein import into chloroplasts result in the down-regulation of photosynthesis-related nuclear gene expression regardless of the import substrates of the defective import apparatus. They also suggest that defects in protein import into plastids, such as those caused by the ppi2 mutation, induce the generation of plastid signal that suppresses the expression of photosynthesis-related genes in the nucleus.
Plastid Mg-Protoporphyrin IX Levels May Not Be Correlated with the Repression of Photosynthesis-Related Nuclear Genes in *ppi2*

The down-regulation of nuclear gene expression in response to defects in plastid protein import suggests that there are retrograde signaling pathways that coordinate plastid protein import and nuclear gene expression. It has been proposed that intermediates in the tetrapyrrole synthesis pathway act as retrograde signals, and several *GUN* genes have been identified that are involved in the synthesis of tetrapyrrole and in the signaling activities of pathway intermediates (Vinti et al., 2000; Mochizuki et al., 2001; Larkin et al., 2003; Strand et al., 2003). According to one scenario, the accumulation of Mg-protoporphyrin IX (Mg-ProtoIX) results in the repression of photosynthesis-related nuclear genes when chloroplasts are damaged by norflurazon treatment (La Rocca et al., 2001; Strand et al., 2003). Although other reports show that the level of Mg-ProtoIX is not correlated to the level of *LHCB* expression (Mochizuki et al., 2008; Moulin et al., 2008), it is still of interest to test whether *GUN* genes and tetrapyrroles are responsible for the down-regulation of photosynthesis-related nuclear genes in plants defective in plastid protein import.

We first examined the expression of *GUN4*, a key regulator of Mg-Protoporphyrin IX synthesis (Larkin et al., 2003), in the *ppi2-2* mutant. We switched from *ppi2-1* to *ppi2-2* for these experiments, as the *ppi2-2* mutant is in the Col-0 background and can be used for crossing with other mutants in the same background. Real-time PCR analysis revealed that the expression of *GUN4* in 4-week-old *ppi2-2* was approximately 30% that in the wild type (Fig. 4A). Furthermore, *GUN4* protein was dramatically decreased in 4-week-old *ppi2-2* mutant (Fig. 4B). We observed similar results in other plastid protein import mutants (Fig. 2, D and F). Because *GUN4* stimulates Mg-chelatase activity (Larkin et al., 2003), these results suggest that the synthesis of Mg-Protoporphyrin IX would also be significantly impaired in these mutants. Consistent with this idea, the level of Mg-Protoporphyrin IX in the *ppi2-2* mutant was much lower than that in wild-type plants (Fig. 4C). Therefore, the down-regulation of photosynthesis-related nuclear genes in *ppi2* mutants is unlikely to be caused by hyperaccumulation of Mg-Protoporphyrin IX in the mutant.

**Coordination of Plastid Protein Import and Nuclear Gene Expression Requires Novel Components Other Than ABI4**

Given our results indicating that *GUN4* and the level of Mg-Protoporphyrin IX may not be involved in the down-regulation of photosynthesis-related nuclear genes in *ppi2*, we next focused on the GUN1 protein, a pentatricopeptide repeat protein in plastids. GUN1 has been proposed to act as an intermediate between *GUN4* and ABI4 and is positioned downstream of well-characterized plastid signals such as redox state and plastid gene expression signals (Koussevitzky et al., 2007). This raises the possibility that in the import mutants, some plastid signal other than Mg-Protoporphyrin IX could act to repress nuclear gene expression via GUN1. Indeed, the *ppi2-2* mutant has normal levels of *GUN1* expression (Fig. 4A); therefore, the mutant would not be expected to be impaired in this step of plastid signaling. We next examined the effect of a *gun1* mutation on nuclear gene expression in *ppi2*. We attempted to generate a double mutant of *gun1-101* and *ppi2-2* (*gun1-101 ppi2-2*). Interestingly, when the progeny of a *gun1-101 ppi2-2/+ (gun1-gun1;atTOC159/attoc159)* line were examined, we were unable to recover albino (*gun1-101 ppi2-2*) plants. Instead, we found that ap-
approximately 25% of the seeds in *gun1-101 ppi2-2/+* siliques were aberrantly shaped (Supplemental Fig. S4). These data suggest that the *gun1-101 ppi2-2* double mutant has defects in embryogenesis and is not viable. When developing embryos were examined by light microscopy, every embryo in *gun1-101 ppi2-2/+* siliques appeared to be normal until the late heart stage (Fig. 5, A and B). Subsequently, about 25% of the embryos started to twist or bend backwards at the walking stick embryo stage (Fig. 5, C and D). Thus, these phenotypes are likely to be associated with the lethality of the *gun1-101 ppi2-2* double mutant.

To further examine the role of GUN1 in the down-regulation of nuclear gene expression in *ppi2*, we recovered *gun1-101 ppi2-2* embryos from ovules containing late heart stage embryos, cultured them on a basal medium, and obtained seedlings of *gun1-101 ppi2-2* (Fig. 5F). The embryo-rescued *gun1-101 ppi2-2* double mutant seedlings appeared to be more sickly than embryo-rescued *ppi2-2* single mutant seedlings (Fig. 5, compare E and F). To characterize the gene expression profile of the double mutant, we measured the mRNA and protein accumulation of nuclear-encoded plastid proteins. Although the accumulation of photosynthesis-related proteins in the double mutant was not increased compared with the *ppi2-2* mutant (Fig. 5H), derepression of two photosynthesis-related genes (*LHCB1* and *GUN4*) in the *gun1-101 ppi2-2* mutant was observed (Fig. 5G). These data suggest that GUN1 may play a role in coordinating nuclear gene expression and plastid protein import.

To further clarify whether the down-regulation of nuclear gene expression in *ppi2* mutants is mediated by GUN1-ABI4 or some other, novel pathway, we examined the role of ABI4 in the regulation of gene expression in *ppi2*. It has been proposed that ABI4 acts as a negative regulator of photosynthesis-related nuclear gene expression and that the competitive binding of ABI4 to the G-box in response to plastid signals inhibits the induction of *LHCB* expression (Koussevitzky et al., 2007). We examined the accumulation of *ABI4* mRNA in 3-d-old seedlings and rosette leaves by real-time PCR analysis (Fig. 6A). Interestingly, the accumulation of *ABI4* transcripts was much higher in *ppi2-2* seedlings compared with the wild type. This may be explained by the retarded growth of *ppi2-2* plants, in that 3-d-old mutant seedlings are less mature than wild-type ones and *ABI4* expression normally declines with age. Con-

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Analysis of the *gun1-101 ppi2-2* double mutant obtained by embryo rescue. A to D, Embryogenesis of the *gun1-101 ppi2-2* double mutant viewed with a light microscope with a differential interference contrast unit. A, Early heart stage. B, Late heart stage. C, Walking stick stage. D, Mature embryo stage. E and F, Visible phenotypes of *ppi2-2* (E) and *gun1-101 ppi2-2* (F) plants obtained by embryo rescue. Seedlings were cultured for 2 weeks. Bars = 1 mm. G, Quantitative analysis of gene expression in embryo-rescued (ER) *ppi2-2* and *gun1-101 ppi2-2* seedlings (2 weeks old). The mRNA levels were analyzed by real-time PCR and normalized to the levels of *ACTIN2*. The expression level in wild-type seedlings (1 week old) was set to 1. Each bar, plotted here on a log scale, represents the mean of at least three independent samples. Error bars represent 1 SD. Asterisks above the bars indicate significant differences (P < 0.05) between *ppi2-2* and *gun1-101 ppi2-2*, as determined by Student’s t test. H, Accumulation of plastid proteins in embryo-rescued seedlings (2 weeks old). Total protein extracts were resolved by SDS-PAGE and probed with antisera against the proteins indicated to the left.
sistent with the previous observation that the expression of \textit{ABI4} is restricted to developing embryos and seedlings (Finkelstein et al., 1998; Soderman et al., 2000), we could not detect \textit{ABI4} transcripts in either wild-type or \textit{ppi2-2} rosette leaves (Fig. 6A).

Because \textit{ABI4} is postulated to act as a negative regulator in plastid signaling pathways, the hyper-accumulation of \textit{ABI4} in \textit{ppi2} led us to speculate that this may account for the mutant’s decreased expression of photosynthesis-related nuclear genes. To assess the role of \textit{ABI4} in the down-regulation of nuclear gene expression in \textit{ppi2}, we generated \textit{abi4 ppi2} double mutants. We employed two different \textit{abi4} alleles (Fig. 6B). The \textit{abi4-1} mutant has a 1-bp deletion, leading to a frameshift, and has been confirmed to be a loss-of-function mutant (Finkelstein et al., 1998). We also identified another mutant, a T-DNA insertion mutant referred to as \textit{abi4-2} (Supplemental Fig. S5), from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). \textit{ABI4} transcripts were barely detectable in both mutants (Fig. 6C), confirming that they both appear to be loss-of-function mutants. When \textit{ppi2-2} was crossed with \textit{abi4-1}, the resulting \textit{abi4-1 ppi2-2} double mutant seedlings showed expression of photosynthesis-related nuclear genes (such as \textit{LHCB1}, \textit{SSU1A}, and \textit{GUN4}) that was at least as low as that of \textit{ppi2-2} single mutant seedlings (Fig. 6D). In the case of \textit{LHCB1} and \textit{GUN4}, the repression was more extreme in \textit{abi4-1 ppi2-2} than in \textit{ppi2-2}. Similar results were obtained from another double mutant, \textit{abi4-2 ppi2-2} (Fig. 6E).

Taken together, these data suggest that the down-regulation of nuclear gene expression in \textit{ppi2} is mainly mediated by a novel pathway that is independent of \textit{ABI4}. Instead, this pathway is likely to involve \textit{GUN1} and transcription factors other than \textit{ABI4}.

\textbf{AtGLK1 May Coordinate Plastid Protein Import and Nuclear Gene Expression}

Recently, it has been suggested that the binding of \textit{ABI4} to G-boxes prevents the binding of G-box bind-
ing factors (GBFs), leading to the down-regulation of nuclear gene expression (Koussevitzky et al., 2007). In this model, the activities of positive (GBFs) and negative (ABI4) factors are key regulators of photosynthesis-related nuclear gene expression. Our results (presented above) suggested that there may be other positive and negative regulators in addition to ABI4 and GBFs. To identify such potential regulators, we surveyed a data set obtained by SuperSAGE for transcription factors that are significantly down-regulated (less than 25% of wild-type level) in ppi2-1. We reasoned that such transcription factors might act as positive regulators of photosynthesis-related nuclear gene expression in response to plastid signals in the wild type. As shown in Table I, we identified 16 transcription factor genes that are suppressed in ppi2-1 (P < 0.05, Fisher's exact test). Intriguingly, AtGLK1, a transcriptional activator that interacts with GBF1 and GBF3 (Tamai et al., 2002), was identified among these transcription factors. The GLK gene family is also known to affect chloroplast biogenesis (Fitter et al., 2002). To examine whether the expression of AtGLK1 is associated with the down-regulation of nuclear gene expression in mutants defective in plastid protein import, we examined the expression level of AtGLK1 in various mutants by real-time PCR analysis (Fig. 7, A and B). The ppi2, attoc132 attoc120+/, and attic20-I mutants all showed decreased AtGLK1 expression. Because Arabidopsis has a functionally redundant gene, AtGLK2 (Fitter et al., 2002), we examined the expression of AtGLK2 in ppi2 and confirmed that the expression of AtGLK2 was also impaired (Fig. 7C). In contrast, the expression of G-box-binding factors, such as GBF1 and HY5, was unaffected or up-regulated in the mutant (Fig. 7C).

We next investigated the link between AtGLK1 and known plastid signaling pathways. A recent paper reported that GLK genes are responsive to norflurazon and lincomycin treatments (Waters et al., 2009). When wild-type plants were treated with norflurazon, AtGLK1 expression was significantly down-regulated (Fig. 7B). In contrast, the level of AtGLK1 in the gun1-101 mutant treated with norflurazon was not decreased to the same level as in norflurazon-treated wild-type plants (Fig. 7D). A similar result was obtained for the gun1-101 ppi2-2 double mutant. The gun1-101 ppi2-2 double mutant exhibited a much higher AtGLK1 expression level compared with the ppi2-2 single mutant (Fig. 5G). Thus, GUN1 may act as a negative regulator of AtGLK1 expression when plastids are dysfunctional, leading to subsequent down-regulation of photosynthesis-related genes in the nucleus.

To further confirm that AtGLK1 is indeed a positive regulator in the plastid signaling pathway that coordinates plastid protein import and photosynthesis-related nuclear gene expression, we generated transgenic plants overexpressing AtGLK1 in a ppi2-2 background. Interestingly, these plants had pale green cotyledons and mature leaves (Fig. 8A), unlike the albino phenotype typical of ppi2 mutants. Likewise, the chlorophyll content was partially restored in these transgenic plants (Fig. 8B). To compare the effects of AtGLK1 overexpression with those of the abi4 mutation (Fig. 6, D and E), we isolated mRNA from 7-d-old seedlings of the overexpression lines. As expected, the level of AtGLK1 in these seedlings was more than 10-fold higher than in the wild type (Fig. 8C). In addition, the expression of some photosynthesis-related genes in the AtGLK1 overexpression lines was partially restored from the ppi2-2 phenotype (Fig. 8C). Notably, all of the LHCb genes examined were significantly up-regulated in the overexpression line. Although the function of AtGLK1 is still subject to debate (Savitch

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*Tag represented as a 22-bp sequence excluding the Nall site (CATG).
et al., 2007; Waters et al., 2009), our data seem to support the proposal that AtGLK1 coordinates the expression of photosynthetic genes. In contrast to the gun1-101 ppi2-2 double mutant, the increased photosynthetic gene expression was correlated with the restoration of protein accumulation in the transgenic line (Fig. 8D). The accumulation of translocon components (Toc and Tic proteins) was not affected, indicating that the recovery of photosynthesis-related proteins was not due to an unexpected up-regulation of protein import capacity.

Taken together, these data suggest that AtGLK1 may act as a positive regulator in a plastid-to-nucleus signaling pathway that coordinates plastid protein import and nuclear gene expression in response to the functional state of plastids. Furthermore, GUN1 down-regulates the expression of AtGLK1 when plastids are dysfunctional, leading to the suppression of nuclear-encoded photosynthesis-related genes.

DISCUSSION

Plastid protein import and the expression of nuclear-encoded plastid proteins are both integral parts of plastid biogenesis. However, the mechanism by which these two processes are coordinated remains to be characterized. In this study, we examined the link between plastid protein import and nuclear gene expression. As summarized by the model in Figure 9, we found two important links between the two processes. First, we provided empirical evidence that plastid dysfunction caused by defects in plastid protein import generates a plastid signal. Analysis of mutants lacking plastid protein import receptors revealed that inhibition of plastid protein import results in down-regulation of nuclear gene expression (Figs. 1 and 2). We also observed up-regulation of atTIC110 expression in the ppi2-1 mutant (Fig. 1D), and this may be an attempt by the plant to compensate for protein import defects. Second, we showed that the plastid signal generated by import defects acts through suppression of GLK1 expression but not activation of ABI4 (Fig. 9). The abi4 ppi2 double mutant did not exhibit restoration of LHC region expression compared with the ppi2 single mutant. In contrast, the gun1 ppi2 mutant showed recovery of both AtGLK1 and LHC B expression. Thus, suppression of GLK1 expression is mediated by the activity of GUN1 (Fig. 9). Overall, these data suggest that plastid dysfunction caused specifically by defects in plastid protein import may act as a signal to coordinate plastid protein import and the expression of nuclear-encoded photosynthetic proteins.

The protein import receptors of plastids are composed of two distinct families of GTP-binding proteins. The function of these proteins has been elucidated mostly by genetic studies, in which it has been assumed that the substrate proteins of each receptor are likely to be specifically decreased in
mutants for that receptor. This is apparent in the case of the ppi2 mutants, which lack atToc159: atToc159 appears to be a receptor for photosynthesis-related proteins, and ppi2-1 fails to accumulate them (Bauer et al., 2000; Smith et al., 2004). Likewise, proteome analysis of an attoc132 single mutant coincides well with the conclusion from biochemical studies that atToc132 is a receptor for nonphotosynthetic proteins (Ivanova et al., 2004; Kubis et al., 2004). However, our results underscore the complexity of the regulation of plastid biogenesis by plastid protein import.

As shown in Figure 2C, significant reduction of atToc132/atToc120 receptor abundance in the attoc132 attoc120+/- mutant results in an unexpected decrease in photosynthesis-related proteins, apparently due to repression of photosynthesis-related nuclear genes. Although the atToc132/atToc120 receptor preferentially binds nonphotosynthetic proteins, it also binds photosynthesis-related proteins, albeit more weakly (Ivanova et al., 2004). Feedback regulation of photosynthesis-related nuclear genes in response to the lack of atToc132/atToc120 may reduce the competition of
photosynthesis-related proteins for these binding sites, allowing plastids to preferentially incorporate essential nonphotosynthetic proteins. Thus, the regulation of nuclear gene expression based on the status of plastid protein import is likely to be a strategy for plastids to maintain essential functions under stress.

Down-regulation of nuclear gene expression is caused by specific signals from plastids. However, the primary signaling molecules that are generated by protein import defects remain to be characterized. It has been proposed that the accumulation of Mg-ProtoIX may act as a signaling molecule derived from plastids when plants are treated with norflurazon. However, we could not detect any accumulation from plastids when plants are treated with norflurazon. Although plastid signals generated by norflurazon and lincomycin have been shown to act through the GUN1-ABI4 pathway, they may also act through the GUN1-GLK1 pathway.

According to existing literature and our results here, plastid signaling pathways activated by redox, norflurazon, lincomycin, and defects in protein import converge at GUN1 in chloroplasts (Fig. 9). Downstream of GUN1, however, the signaling pathway for protein import defects utilizes GLK1 but not ABI4 (Fig. 9). It is not clear whether the redox, norflurazon, and lincomycin pathways are also mediated by GLK1. A recent paper reported that the expression of AtGLK genes is sensitive to norflurazon and lincomycin (Waters et al., 2009). Consistent with this observation, we confirmed that the expression of AtGLK1 is repressed by norflurazon (Fig. 7B). Thus, we favor the model that the norflurazon signaling pathway utilizes both ABI4 and GLK1. The next question is how GUN1 discriminates between different upstream plastid signals and is able to send the signal generated by defects in protein import specifically to the GLK1 pathway. We speculate that additional components act together with GUN1 to specify downstream pathways. For example, distinct factors may associate with GUN1 in response to different signals. It is also possible that GUN1 is modified differently depending on the type of signal, allowing GUN1 to associate with different downstream components.

Although AtGLK1 appears to be important for the induction of photosynthesis-related nuclear genes, the expression of those genes in ppi2-2 lines overexpressing AtGLK1 was not fully restored to the wild-type level (Fig. 8C). This suggests that additional factors are involved in the plastid-to-nucleus signaling pathway. One possibility is that a negative regulator, like ABI4, which is more abundant in mutant (Fig. 6A), keeps the genes partially repressed even in the presence of an excess of a positive factor (AtGLK1). Recently, HY5 has been identified as a negative regulator of photosynthesis-related nuclear genes when plastid biogenesis is arrested by lincomycin treatment (Ruckle et al., 2007). In this study, we observed a remarkable induction of HY5 in the ppi2-1 background (Fig. 7C). It is notable that HY5 acts as a negative regulator in the plastid signaling pathway, whereas it functions as a positive regulator in photomorphogenetic pathways (Öyama et al., 1997). Thus, it is not surprising if the induction of HY5 is associated with the decrease of photosynthesis-related nuclear gene expression in the ppi2 mutant. According to our SuperSAGE analysis, there are many other transcription factors up- or down-regulated in ppi2. The combinatorial interplay between these transcription factors may play critical roles in fine-tuning photosynthesis-related nuclear gene expression in response to the functional state of the plastids.

In summary, we demonstrated that plastid protein import and nuclear gene expression are tightly coordinated and that this coordination may be in part mediated by GUN1 and the transcription factor AtGLK. In contrast to other transcription factors that have been identified so far in retrograde signaling, AtGLK may act as a positive regulator of photosynthesis-related nuclear gene expression. These results provide
evidence that plastid signaling pathways constitute a complex signaling network composed of positive and negative regulators. Further identification of signaling components should provide insight into the signaling network that coordinates nuclear and plastid functions.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All experiments were performed on Arabidopsis (Arabidopsis thaliana) accessions Col-0 and Ws. The T-DNA insertion lines (Supplemental Table S3) were obtained from GABI-Kat (Russo et al., 2003), the collections of the Salk Institute Genome Analysis Laboratory (Alonso et al., 2003), and the Syngenta Arabidopsis Insertion Library (Sessions et al., 2002). Plants were grown on soil (expanded vermiculite) or on 0.5% agar medium containing 1% Suc and 0.5 mM Murashige and Skoog salts at pH 5.8. To synchronize germination, all seeds were kept at 4°C for 2 d after sowing. Plants were grown under continuous white light (80 μmol m⁻² s⁻¹, unless specified) at 22°C and 50% relative humidity in a growth chamber (LHP-350S; NK system). The plants grown on soil were watered with Hyponex (Hyponex Japan) diluted 1,000-fold in water.

PCR were performed with the RNA templates prior to cDNA synthesis to normalize to that of 18S ribosomal RNA or ACTIN2 listed in Supplemental Table S4. The transcript level of each gene was determined by RT-PCR for ABI4 that were used for real-time PCR (Supplemental Table S4).

RT-PCR for ABI4

Homozygous abi4-1 and abi4-2 plants were identified by genomic PCR using specific primers (Supplemental Tables S3 and S5). Total RNA was extracted from 3-d-old seedlings of Col-0, abi4-1, and abi4-2. From 500 ng of RNA for each sample, single-stranded cDNA was synthesized using an oligo(dT) primer and PrimeScript reverse transcriptase (TaKaRa). RT-PCR analysis was performed with the same gene-specific primers for ABI4 and ACTIN2 that were used for real-time PCR (Supplemental Table S4).

Antibodies and Immunoblotting

Rabbit polyclonal antibody against GUN4 was produced using a NusA-GUN4 fusion protein as the antigen (Supplemental Fig. S6). Antibodies against AtTic310 (Inaba et al., 2003, 2005), SSU (Inaba et al., 2003), LHCP (Payan and Cline, 1991), ACC-CTn (Kozaki et al., 2000), and LSU, OHE3, POR, Ela, Hsp93, and IEP37 were described elsewhere. Proteins were detected using chemiluminescence reagents with a luminoimage analyzer (AE-6972C; ATTO). For some experiments, the signal was quantified using image acquisition software (CS Analyzer; ATTO).

SuperSAGE Library Construction and Tag Sequencing

To obtain the SuperSAGE libraries, we followed the original SuperSAGE protocol described by Matsumura and colleagues (Matsumura et al., 2003; Terauchi et al., 2008) with some modification. Total RNA was isolated from the third to sixth leaves ofWs and the ppi2-2 mutant using RNAiso reagent (TaKaRa). Biotinylated cDNAs from each sample was bound to streptavidin-coated magnetic beads and digested with NlIII. The streptavidin-bound cDNA was washed and divided into two portions in separate tubes. Linkers containing a binding site for EcoP15I (a type III restriction enzyme) but different sites for PCR primers were ligated to the NlIII cleavage site at the 5’ ends of the bead-bound cDNA fragments in each pool. Both pools of cDNAs were digested with EcoP15I to release SuperSAGE tags from the beads, then the released tag pools were combined and linker-flanked ditags were formed by blunt-end ligatation. Following amplification of the ditags by linker-specific PCR, the SuperSAGE library was directly sequenced with a large-scale pyrosequencing method (454 Life Sciences). The sequences of the linkers and PCR primers are listed in Supplemental Table S6.

SuperSAGE Data Analysis and Tag-to-Gene Assignment

To identify the genes from which tags were obtained, each 26-bp tag was annotated against The Arabidopsis Information Resource 7 database using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The data used in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession numbers GSM3088557 and GSM3088558.

Quantitation of Mg-ProtoIX

Mg-ProtoIX separation and quantitation were done as described previously (Strand et al., 2003) with slight modifications. Leaf material was homogenized in 0.67 mL of 0.1 M potassium phosphate buffer (pH 7.8) with a Bead Smash 12 (Wakenyaku) homogenizer and centrifuged at 10,000g for 3 min at 4°C. The residue was resuspended in 0.67 mL of acetone:0.1 mM NH₄OH (8:2, v/v) and then homogenized and centrifuged. The residue was resuspended again in methanol:0.1 mM NH₄OH (9:1, v/v), and the same procedures described above were repeated. The collected supernatants were mixed and centrifuged prior to HPLC analysis on a Capcell Pak-C18 column (Shiseido). The extracts were eluted with a linear gradient of solvent A (30% [w/v] acetonitrile and 2.5 mM tetraethyl ammonium phosphate, pH 7.0) to solvent B (90% [w/v] acetonitrile). Column eluent was monitored by fluorescence detection (420-nm excitation, 595-nm emission), and Mg-ProtoIX was identified and quantified using chemically synthesized standards (Frontier Scientific). Fifty microliters each of standard solutions of Mg-ProtoIX from 10⁻¹ to 10⁻³ M were injected into the HPLC system; the 10⁻¹ M solution was the limit for preparing the calibration line in the used system.

Embryo Rescue

For embryo-rescue experiments, immature ovules (containing late heart stage embryos) were excised from developing siliques and cultivated on a basal medium (1× Murashige and Skoog salts, 3% Glc, 0.55 mM myoinositol, 5 mM thiamine hydrochloride, and 0.8% agar) under continuous light as described (Baus et al., 1986).

Microscopy

To observe embryo development, ovules were dissected from siliques of appropriate ages and cleared in chloral hydrate:water:glycerol (8:2:1) overnight. Differential interference contrast images were obtained using an Axio Imager A1 microscope (Zeiss).

Construction of Vector for OverExpression of AtGLK1 and Plant Transformation

A full-length cDNA of AtGLK1 was amplified by PCR using primers that specifically anneal with AGLK1_F_Spel, 5′-ACTAGTACAATGGA-GATTGATCGATG-3′; GLK1_R_Nhel, 5′-CCATCCCATCGCTTCTTCTG-3′ and subcloned into the SpeI and Nhel sites of the binary vector pCAMBIA1303. The GLK1/PCAMBIA1303 plasmid was transformed into Agrobacterium tumefaciens (GV3101) by electroporation and was introduced into ppi2-2/+ plants by the floral-dip protocol (Clough and Bent, 1998).

Chlorophyll Extraction and Quantification

Seven-day-old seedlings (20–40 mg fresh weight) were ground in liquid nitrogen with a mortar and pestle. The resulting powder was suspended in
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Immunoblot analysis of plastid proteins in ppi2 and wild-type plants.

Supplemental Figure S2. Analysis of the T-DNA insertion allele (ppi2-2) of atToc159.

Supplemental Figure S3. Overview of SuperSAGE analysis.

Supplemental Figure S4. Characterization of the gun1-101 ppi2-2 double mutant.

Supplemental Figure S5. Schematic diagram of the T-DNA insertion site in AB14.

Supplemental Figure S6. Production of antibody against recombinant GUN4 protein.

Supplemental Table S1. Annotation of down-regulated tags in ppi2-1.

Supplemental Table S2. Annotation of up-regulated tags in ppi2-1.

Supplemental Table S3. Primers used for genotyping of T-DNA insertion lines.

Supplemental Table S4. Gene-specific primers used in real-time PCR.

Supplemental Table S5. CAPS marker for genotyping of ab14-1.

Supplemental Table S6. Sequence of the primers and linkers used for SuperSAGE library construction.

ACKNOWLEDGMENTS

Part of this work was initiated in Prof. Danny Schnell’s laboratory (University of Massachusetts, Amherst), and we thank Prof. Schnell for his kind support and discussion. Antibodies were kindly provided by Drs. Felix Kessler (atToc75), Alice Barkan (OE23), Ken Cline (LHCP), Doug Randall (University of Massachusetts, Amherst), and we thank Prof. Schnell for his generous support.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GSM308557 and GSM308558.


Arabidopsis Cor15am is a chloroplast stromal protein that has cryoprotective activity and forms oligomers. Plant Physiol 144: 513–523