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An Intracellular Signal Transduction Pathway between the Chloroplast and Nucleus Is Involved in De-Etiolation[1]

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Chloroplast development requires the coordinated expression of nuclear and chloroplastic genes. A hypothesized signal from the chloroplast couples the transcription of certain nuclear genes encoding photosynthetic proteins with chloroplast function. We have previously described an *Arabidopsis thaliana* mutant, *gun1*, which has a defect in the signal transduction pathway coupling such nuclear and plastidic gene expression. Here we show that *gun1* seedlings are also defective in establishing photoautotrophic growth. *gun1* seedlings develop normally in the dark, but, based on morphological criteria and the kinetics of chlorophyll accumulation, photosynthetic mRNA accumulation, and the differentiation of etioplasts to chloroplasts, are retarded in their ability to de-etiolate. Therefore, we propose that the *GUN1* gene plays an important role in the transition from heterotrophic to photoautotrophic growth, suggesting an important physiological role for the plastid-nucleus signaling pathway during chloroplast biogenesis.

Dicotyledonous seedling development is dramatically affected by light. The transition from heterotrophic to photoautotrophic growth, with the associated differentiation of leaves and chloroplasts, is dependent on the presence of light and developmental signals (Possingham, 1980). Chloroplast development (greening) involves the temporally regulated biosynthesis of components of the photosynthetic apparatus and the carbon reduction cycle, and requires the coordinated expression of both nuclear and chloroplastic genes. When seeds germinate in the dark, the small, colorless, undifferentiated plastids (proplastids) enlarge and develop into etioplasts with a large, internal structure termed the prolamellar body (Leech, 1976; Kirk and Tilney-Bassett, 1978). A set of light-regulated genes, mostly encoding components of the photosynthetic apparatus, are not expressed or are expressed at very low levels in the dark (Gilmartin et al., 1990; Terzaghi and Cashmore, 1995). Subsequently, when the aerial portion of the developing seedling is exposed to light, the etioplasts of cotyledons and primary leaves develop into photosynthetically functional chloroplasts. During this process, the nuclear and chloroplast-encoded photosynthetic genes become expressed at over 100 times the level at which they were expressed in the dark (Thompson and White, 1991).

In addition to light, the development of chloroplasts is also regulated by intrinsic developmental signals that control leaf differentiation. For instance, light-regulated chloroplast differentiation is restricted to the leaf and stem tissues, and within leaves, it is restricted to certain cell types: the mesophyll, bundle-sheath, and guard cells. Thus, the differentiation of proplastids to chloroplasts is related to leaf development in higher plants and must also involve positional information. Furthermore, the developmental stage of the chloroplast itself appears to regulate the expression of nuclear genes coding for chloroplast-destined proteins (Harpster et al., 1984; Mayfield and Taylor, 1984; Taylor, 1989). For example, in photooxidative mutants of maize or in a variety of plants in which chloroplast development is arrested with an inhibitor, light-regulated nuclear genes are not expressed. This observation led to a hypothesis that a signal from the chloroplast is necessary for optimal transcription of mRNAs for nuclear genes encoding chloroplastic proteins.

In a previous study (Susek et al., 1993), we obtained genetic evidence in *Arabidopsis thaliana* for such a retrograde signaling pathway between the chloroplast and the nucleus. Specifically, we isolated a series of Arabidopsis mutants that uncouple nuclear *Lhcb* (encoding the chlorophyll *a*/*b* binding proteins of PSII) and *RBCS* (small subunit of Rubisco) gene expression from the functional state of the chloroplast. The mutants, designated *gun* for genomes uncoupled, were selected for their ability to express *Lhcb1*2* (also known as *CAB3* [Susek et al., 1993] and *CAB180* [Leutwiler et al., 1986]) reporter genes under several conditions in which chloroplasts did not develop. The *gun* alleles isolated to date are all recessive and consist of at least six complementation groups, *gun1* to *gun6* (Susek et al., 1993; N. Mochizuki and J. Chory, unpublished data). These results suggest a model in which nonfunctional plastids send a signal to repress nuclear photosynthetic gene expression.

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Abbreviation: PORA, NADPH:protochlorophyllide oxidoreductase.

One might expect that mutations that disrupt important intracellular signaling pathways would cause severe physiological or morphological abnormalities, but most *gun* mutants look remarkably normal under conditions of variable daylength, light quality, or intensity, or when grown in the dark, on soil, or on plates with or without exogenous Suc. In a previous study (Susek et al., 1993), we did observe a subtle phenotypic difference between several *gun* alleles and wild-type seedlings during the transition from dark-grown to light-grown development, a process called de-etiolation. These preliminary data suggested that a critical time for coordinating the activities of photosynthetic genes in the nucleus and chloroplasts is during the establishment of photoautotrophic growth. In the present study, we examine in greater detail the greening phenotypes of the mutant allele, *gun1-1*, which shows the strongest phenotype in terms of uncoupling nuclear from plastidic gene expression. Specifically, we investigated the kinetics of greening of *gun1-1* in terms of plastid morphology, chlorophyll accumulation, and the derepression of photosynthetic gene expression. Our results suggest that the chloroplast-to-nucleus signaling pathway plays an important role in optimizing photosynthetic gene expression during the transition from heterotrophic to photoautotrophic growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The isolation of *gun1-1* was described previously (Susek et al., 1993). In the experiments described here, *Arabidopsis thaliana* seeds were surface-sterilized with 1.7% sodium hypoclorite containing 0.1% Tween 20, and washed thoroughly with sterilized water. Sterilized seeds were plated on Murashige-Skoog agar plates containing 2% Suc and B-5 vitamins. Plates were chilled at 4°C for 24 to 36 h, and then maintained at 20 to 22°C under a mixture of continuous fluorescent and incandescent lights (2×10^{16} quanta cm^{-2} s^{-1}) for 12 h to induce germination. For the dark-to-light shift experiment, plants were grown in the dark for the indicated periods following the treatment described above, then transferred to continuous white light of various fluence rates, as described below.

Pigment Analysis

Chlorophyll was extracted from 50 mg of fresh plant tissue with *N,N*-dimethylformamide for 12 h at 4°C in complete darkness. The extract was subjected to spectrophotometric measurements at 603, 647, and 664 nm. Specific chlorophyll content was calculated using the equations of Moran (1982) and normalized to the total fresh weight of tissue in each sample.

RNA Gel-Blot Analysis

Plant tissues were harvested and stored at −70°C. RNA was isolated by a modification of the guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and analyzed on RNA gel blots as described previously (Chory et al., 1991). The DNA probes used were also described previously (Chory et al., 1989).

Electron Microscopy

Wild-type and mutant seedlings were grown on Murashige-Skoog plates and harvested, fixed, and sectioned as described previously (Susek et al., 1993).

RESULTS AND DISCUSSION

gun1 Mutants Are Defective in the Transition from Etiolated to Light Growth

gun1-1 shows a nearly wild-type phenotype under most growth conditions, except upon the transition from dark-grown (etiolated) to light-grown development. Dark-grown *gun1-1* mutants were indistinguishable from wild-type etiolated seedlings, having elongated hypocotyls and small, folded cotyledons (Fig. 1). However, etiolated *gun1-1* mutants were slow or failed completely to initiate cotyledon expansion and hypocotyl hook opening when transferred to high-photon-fluence-rate white light (Fig. 1). When *gun1-1* seedlings were grown for 6 d in the dark, followed by 24 h of continuous white light, there was little development of cotyledons. In contrast, wild-type cotyledons expanded and turned green after 24 h in the light. After 10 d of dark growth followed by 24 h of light, wild-type cotyledons turned green more slowly and less synchronously, whereas in mutant seedlings, cotyledons did not turn green nor did the apical hook open. Wild-type and mutant seedlings that failed to de-etiolate during the first 36 h of light did not develop further in prolonged periods of light growth (data not shown). The length of the dark period is plotted against seedling viability for wild-type and *gun1-1* seedlings in Figure 2. Twelve percent of *gun1-1* seedlings de-etiolated in the light after 6 d of growth in the dark, whereas 63% of the wild-type seedlings were still able to de-etiolate when given the identical light treatments. Among the *gun* alleles this greening deficiency was most prominent in *gun1-1* (data not shown).

To examine whether etiolated *gun1-1* seedlings lose viability during growth in the dark or during the transition to photoautotrophic growth, we treated 8-d-old etiolated seedlings with continuous white light of three different fluence rates (high, medium, and low, i.e. 200, 60, and 10 μmol m^{-2} s^{-1}, respectively). After 1 week, the number of de-etiolated seedlings was obtained (Fig. 3). Both wild-type and *gun1-1* seedlings showed greatly restored viability when transferred to 10 μmol m^{-2} s^{-1} white light, although about 50% of *gun1-1* seedlings died. These data indicate that dark-grown *gun1-1* seedlings have the potential to de-etiolate, but are susceptible to strong light, possibly due to an inability to rapidly accumulate the pigment-protein complexes that protect the seedling against photooxidation.

A second possible explanation for the reduced viability of *gun1* mutants when transferred to high-photon-fluence-rate white light is that dark-grown mutants may exhaust

their protein reserves stored in seeds prematurely, thereby making the transition from heterotrophic to photoautotrophic growth more difficult. However, we did not observe a significant difference in the dry weight of wild-type compared with *gun1-1* seeds (data not shown); therefore, it is unlikely that the greening phenotype of *gun1-1* is caused by a shortage of seed reserves.

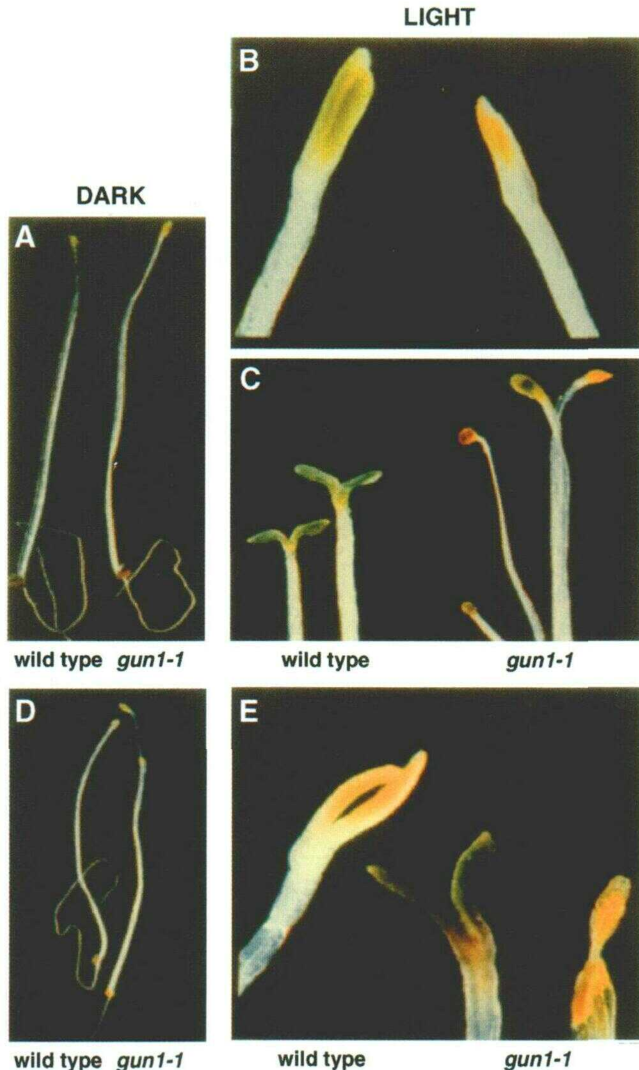


Figure 1. Wild-type and *gun1-1* seedlings grown in the light after dark growth. Transgenic pOCA107-2 (wild type) and *gun1-1* plants were germinated and grown in the dark for 6 (A, B, and C) or 10 d (D and E), then transferred to the light for 0 (A and D), 6 (B), or 24 h (C and E). After 6 d of dark growth, wild-type cotyledons showed more pigment accumulation and more cotyledon expansion and unfolding than mutant plants (B and C). In the wild-type plant on the left in B, the cotyledons have expanded and begun to turn green. After 24 h there is little development of the mutant cotyledons (C). After 10 d of dark growth, wild-type cotyledons greened more slowly and less synchronously (E), whereas mutant cotyledons did not turn green, unfold, or expand (E).

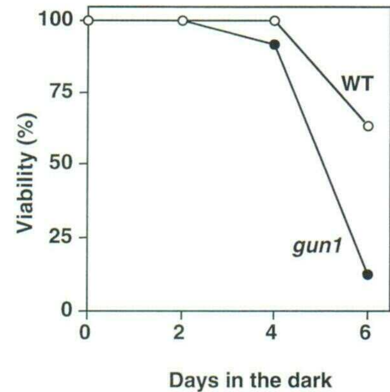


Figure 2. Reduced viability of *gun1-1* seedlings during de-etiolation. Wild-type and *gun1-1* seedlings were germinated and grown in the dark for the indicated periods, then transferred to continuous light (200 μmol m^{-2} s^{-1}). After 1 week, the number of seedlings with expanded and green cotyledons (viable) and seedlings that failed to green (dead) were counted. The viability of *gun1-1* was five times lower than that of the wild type after 6 d of dark growth. ○, Wild type (WT); ●, *gun1-1* (*gun1*).

The *gun1-1* Mutation Retards Chloroplast Differentiation during the Early Stages of Greening

The phenotypes of de-etiolating *gun1-1* seedlings suggest that chloroplast differentiation might also be affected during the early stages of greening in *gun1-1*. To distinguish between developmental and light-induced effects of *gun1-1* on de-etiolation, we examined ultrathin sections of etiolated and greening cotyledons. Figure 4 shows that wild-type and *gun1* etioplasts look identical, suggesting that *GUN1* is not required for normal plastid development in etiolated cotyledons. Three hours after transfer of wild-type seedlings to the light, plastids assumed a more rounded shape, had less organized prolamellar bodies, and began to accumulate perforated or noncontinuous stromal

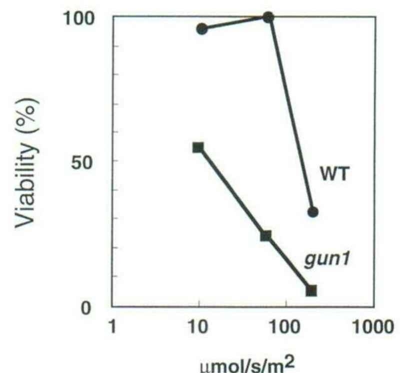
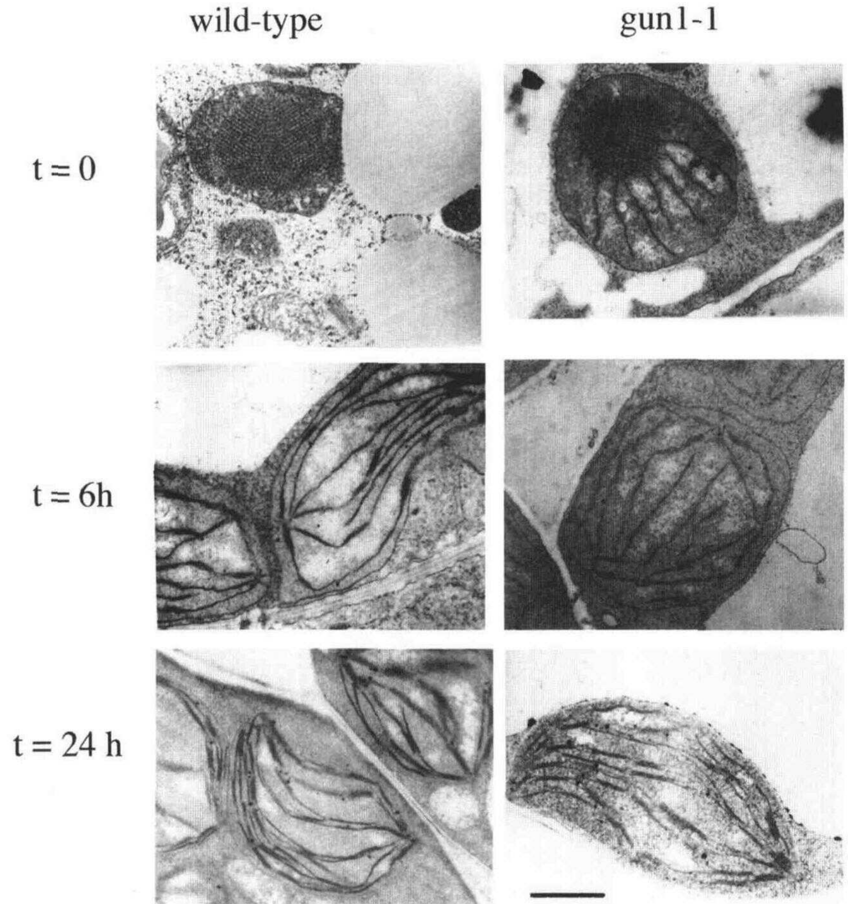


Figure 3. The viability of wild-type and *gun1-1* seedlings is dependent on the fluence rates of light. Eight-day-old, dark-grown wild-type and *gun1-1* seedlings were exposed to continuous white light of differing fluence rates for 1 week. The viability of the *gun1-1* seedlings was increased significantly in low-fluence white light. Wild-type seedlings were also more viable at lower fluences of light, although they were fully viable at higher fluence rates. ●, Wild type (WT); ■, *gun1-1* (*gun1*).

Figure 4. Electron micrograph showing the time course of the etioplast-to-chloroplast transition in wild-type and *gun1-1* seedlings. pOCA107-2 (wild type) and *gun1-1* plants were grown in the dark for 7 d, then transferred to continuous light for 0, 6, or 24 h. Scale bar = 1 μm.



lamellae (not shown). In contrast, after 3 h of light, *gun1-1* plastids still retained an amoeboid shape and had accumulated few perforated thylakoid membranes (data not shown). After 6 h in the light, wild-type plastids were lens-shaped, the stromal lamellae had few perforations, and the granal stacks were significantly developed (Fig. 4). In contrast, mutant plastids from plants grown for 6 h in the light were less developed. They contained abundant perforated stromal lamellae, but few stacked grana (Fig. 4). After 24 h in the light, *gun1* thylakoid membrane stacks were beginning to form, but there were fewer of them than in wild-type chloroplasts. After 48 h, the strains had nearly indistinguishable, fully developed chloroplasts (data not shown). This experiment is further evidence that *gun1* mutants de-etiolate more slowly than wild-type seedlings.

Recently, it has been reported that a collection of Arabidopsis mutants that de-etiolate in the dark are also delayed in greening (Lebedev et al., 1995). These mutants lack the prolamellar body typically found in etioplasts and also do not accumulate PORA, which is a major constituent of the prolamellar body. When etiolated seedlings are first exposed to light, there is a rapid turnover of PORA within the plastid (reviewed by Reinbothe et al., 1996). In addition, a cytosolic PORA precursor protein is imported into the plastid in a protochlorophyllide-dependent manner that functions best in etiolated seedlings or in chloroplasts at the end of the night cycle. Etiolated *gun1-1* seedlings develop normal etioplasts by morphological criteria, suggesting that the accumulation of PORA in plastids of *gun1-1* mutants occurs as in the wild type. It is possible, however, that *gun1-1* mutants are impaired in subsequent events associated with the etioplast-to-chloroplast transition, namely, in the kinetics of turnover of PORA or in the ability

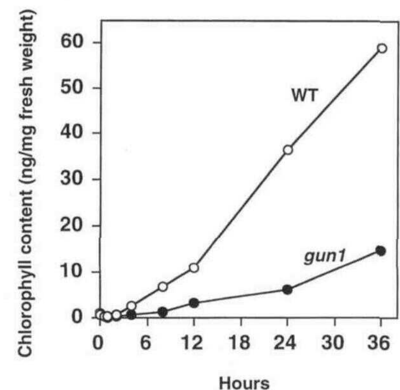


Figure 5. Chlorophyll accumulation in wild-type and *gun1-1* seedlings after prolonged dark growth. Six-day-old, dark-grown wild-type and *gun1-1* mutant seedlings were harvested at the indicated times after transfer to continuous light. The amount of extracted chlorophyll was determined by spectrophotometry and normalized to the fresh weight of seedlings. ○, Wild type (WT); ●, *gun1-1* (*gun1*).

to limit import of the PORA precursor to the developing chloroplast. This would result in *gun1* mutants having an increased susceptibility to photooxidative damage.

gun1-1 Accumulates Less Chlorophyll and Light-Regulated Nuclear mRNAs during the Early Stages of De-Etiolation

We next examined chlorophyll accumulation in *gun1-1* seedlings during de-etiolation (Fig. 5). Plants were grown in the dark for 6 d, then transferred to continuous light (60 μmol m^{-2} s^{-1}) for various times. As expected, both wild-type and *gun1-1* seedlings had undetectable amounts of chlorophyll in the dark; however, the wild type began to accumulate chlorophyll within 4 h of transfer to the light, whereas *gun1-1* had a significant lag phase (12 h) before chlorophyll accumulated (Fig. 5). After 36 h of growth in the light, the specific chlorophyll content of *gun1-1* was approximately 4-fold lower than that of the wild type. By 72 h, the specific chlorophyll content of *gun1-1* was identical to that of the wild type (data not shown).

We have shown that the *gun1-1* mutation uncouples both *Lhcb* and *RBCS* gene expression from chloroplast development while maintaining normal light/dark regulation (Susek et al., 1993). In the previous studies, we did not examine the accumulation of *Lhcb* and *RBCS* mRNAs during the initial transition from dark to light growth. Because of the results presented above, we wished to examine the accumulation of *Lhcb* and *RBCS* mRNAs during the early stages of transfer from the dark to high-fluence white light. Figure 6 shows that both wild-type and *gun1-1* seedlings had a significant increase in *Lhcb* and *RBCS* mRNA accumulation in response to light; however, the mRNA levels were approximately 4-fold lower in *gun1-1* than in the wild type, even after 36 h of exposure to light. These data are consistent with the kinetics of chlorophyll accumulation in the mutant.

CONCLUSIONS

gun1 mutations uncouple the normally coordinated expression of nuclear and chloroplastic genes that encode components of the photosynthetic apparatus. These mutations also result in delayed greening, suggesting that a critical time for coordinating nuclear and chloroplastic gene expression is during the very early stages of the transition from heterotrophic to photoautotrophic growth.

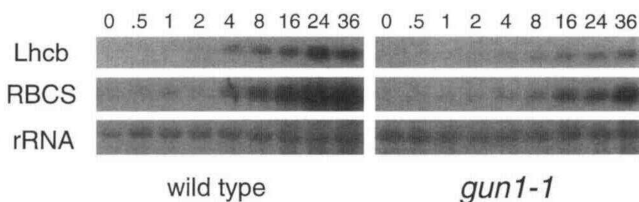


Figure 6. Induction of *CAB* and *RBCS* expression in wild-type and *gun1-1* seedlings. Six-day-old, dark-grown wild-type and *gun1-1* seedlings were harvested at the indicated times after the transition to continuous light. Approximately 3 μg of total RNA was loaded in each lane. Note that the intensity of the signals is not comparable between *CAB* and *RBCS* because the autoradiographs were exposed for different periods of time.

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