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Light-independent developmental regulation of *cab* gene expression in *Arabidopsis thaliana* seedlings

(endogenous regulation/phytochrome)

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ABSTRACT **We found a transient increase in the amount of mRNA for four nuclear genes encoding chloroplast proteins during early development of *Arabidopsis thaliana*. This increase began soon after germination as cotyledons emerged from the seed coat; it occurred in total darkness and was not affected by external factors, such as gibberellins or light treatments used to stimulate germination. Three members of the *cab* gene family and the *rbcS-1A* gene exhibited this expression pattern. Because timing of the increase coincided with cotyledon emergence and because it occurred independently of external stimuli, we suggest that this increase represents developmental regulation of these genes. Further, 1.34 kilobases of the *cab1* promoter was sufficient to confer this expression pattern on a reporter gene in transgenic *Arabidopsis* seedlings. The ability of the *cab* genes to respond to phytochrome preceded this developmental increase, showing that these two types of regulation are independent.**

Regulation of the *cab* genes encoding the chlorophyll *a*/*b*-binding proteins (cab) of photosystem II has been studied extensively in higher plants. Phytochrome increases *cab* gene transcript accumulation in many etiolated monocots and dicots (1), and the rate of *cab* gene transcription has been shown to be under red/far-red control in some cases (2–4); *cab* gene transcription is also positively regulated by blue light (5, 6). When plastid development is blocked, cab mRNA fails to accumulate, indicating that a functioning chloroplast is required for *cab* gene expression (7). The *cab* genes are also regulated by circadian rhythms (8–10).

The *cab* genes can be subject to endogenous control during different stages of plant development. During the first days of seedling growth in the mustard species *Sinapsis alba*, *cab* genes become competent to respond to phytochrome, and at this time cab mRNA is detectable in the dark (11). Similarly, cab mRNA levels increase in soybean cotyledons before emergence from the soil and then decrease as the cotyledons senesce (12). Later in plant development, after bolting has occurred, *cab* gene expression is reduced in the rosette leaves of *Arabidopsis* (13). The levels of plant hormones change during plant development, and both cytokinin and abscisic acid have been shown to affect *cab* gene expression. Cytokinin can increase the amount of cab mRNA by a posttranscriptional mechanism (14), whereas abscisic acid has been shown to decrease both cab and rbcS (ribulose-bisphosphate carboxylase/oxygenase small subunit) mRNA levels (15–17).

Arabidopsis thaliana has become a model system to understand the regulation of gene expression in plants (18). We have isolated three *cab* genes from *Arabidopsis* (19) and shown them to be under phytochrome control (20). In the process of studying competence to respond to phytochrome in dark-grown *Arabidopsis*, we observed a transient accumulation of cab mRNA in dark-grown seedlings. The results presented here characterize this endogenous regulation that occurs during early seedling development. Because regulation of *cab* genes in response to external stimuli has been widely studied, the present findings provide an important perspective to research in this area.

MATERIALS AND METHODS

Growth Conditions. *A. thaliana* ecotype Columbia and a transgenic derivative of this line, cab(+14)B (21), were used in all experiments. Seeds were sown onto Whatman no. 1 filters, which were placed on GP medium [MS salts (GIBCO)/3 mM MES/Benomyl at 20 mg/liter (Cooke Laboratory Products, Portland OR)/0.7% Phytagar (GIBCO), pH 5.7 with or without 2% sucrose] under a dim-green safelight. GA_{4+7} (Abbott) was included in the GP medium at a concentration of 150 μM. The imbibing seeds were stored for 2 days at 4°C in metal cannisters sealed with black light-proof sealing tape. Cannisters were warmed to room temperature, and seedlings were exposed to white light for 15 min. This 15-min white-light treatment marked the beginning of the time course. When the 15 min of white light was omitted, the time course commenced when plates had been warmed to room temperature. To ensure total darkness, plates were returned to cannisters, sealed with light-proof tape, and placed into a light-tight box in a light-tight room that was maintained at 25°C. At indicated intervals, plates were removed in total darkness, and then seedlings were harvested into liquid N_2 under a dim-green safelight. All safelights were tested by a germination assay and did not induce germination beyond the dark level. Light treatments for phytochrome experiments were 1 min of saturating red or 1 min of saturating red immediately followed by 10 min of saturating far-red. Light sources have been described (22). After these treatments, seedlings were returned to complete darkness for 4 hr before harvest into liquid N_2. To produce seeds that have higher germination levels in the dark, plants were grown under fluorescent lights (23).

Isolation of Total RNA. RNA was extracted by using a modified procedure of that described in ref. 24. When RNA was isolated from seedlings grown for 24 hr or less, 600 seeds were used; for older seedlings 300 seeds were used. Frozen tissue was ground in liquid N_2 by using a small mortar and pestle for 30 sec and then transferred to a 1.5-ml microcentrifuge tube using a chilled Teflon spatula. One hundred and fifty microliters of extraction buffer (50 mM Tris, pH 8.3/150 mM NaCl/10 mM EDTA/1% lauryl sarcosine) and 150 μl of PIC (phenol/isoamyl alcohol/chloroform, 24:1:24, equilibrated with 100 mM Tris, pH 8.3/10 mM EDTA) were added to the frozen tissue. Tissue was homogenized for 30 sec by using a Vortex mixer or by using an Omni 1000 homogenizer

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Abbreviation: nt, nucleotide(s).

with a 5-mm grinding tip (Omni International, Waterbury, CT). The aqueous phase was reextracted with PIC and then precipitated using 0.1 vol of 3 M sodium acetate, pH 5.2, and an equal volume of isopropanol. DNA was removed by two sequential LiCl precipitations. The first precipitation was in a total volume of 1.5 ml (1125 μl of 10 mM Tris, pH 8.3/10 mM EDTA plus 375 μl of 8 M LiCl) for 16 hr on ice, whereas the second precipitation was done in half that volume for 6 hr on ice. Pellets were washed with 80% (vol/vol) EtOH, dried, and resuspended in 10 mM Tris, pH 8.3/1 mM EDTA. Yields were typically >30 μg.

Probes for RNase Protection. Two probes used in this study, cab1 and cab1–tms2 have been described (21). The cab2 and cab3 RNA probe was made by PCR with the following primers: upstream, 5′-GGGAATTCAAATCCAATGAGTA-GAGAGAT-3′; and downstream, 5′-GGGGATCCTCA-CACGGCCGCTTCCGAGGA-3′. The upstream primer begins at −150 nucleotides (nt) from the transcription start of cab3, whereas the downstream primer is from a region (+100 nt) identical in both cab3 and cab2. This PCR product was cloned into pGEM4Z at the *Eco*RI and *Bam*HI sites (pGEM-cab3,2) and digested with *Eco*RI to produce a T7 RNA polymerase template. This RNA probe spans a dinucleotide difference between the cab2 and cab3 genes located at −30 nt in the upstream untranslated region of the transcripts. The longer protected fragment (≈150 nt) corresponds to the cab3 transcript, whereas the smaller protected fragment (≈130 nt) corresponds to the cab2 transcript. A GG dinucleotide is cleaved when the RNA probe protects the cab2 transcript, and when the amount of RNase T1 (which cleaves at guanine residues) is increased by 3-fold no more cab2 fragment is released, indicating that measurements of both cab3 and cab2 transcripts are accurate.

The *ubq3* sequence and template DNA were from J. Callis (University of California, Davis). The ubq3 RNA probe, which spans the 3′-untranslated region and is specific to the ubq3 transcript, was made by using the following PCR primers: upstream, 5′-TCCTATCGATTTCGTTT-3′; and downstream, 5′-GATGAAAAACTTGAAGTGGGGATC-CGG-3′. The upstream primer is located 100 nt upstream from the end of the cDNA, and the downstream primer is located 90 base pairs (bp) downstream from the end of the cDNA. The PCR fragment was cloned into pBluescriptKS(+) at the *Cla* I and *Bam*HI sites (pubq3ds) and digested with *Xho* I to produce a T7 RNA polymerase template. This probe protects a 103-nt fragment.

The rbcS-1A RNA probe is also from the 3′-untranslated region and is specific to the rbcS-1A transcript. This probe was made by using the following primers: upstream, 5′-GGGGATCCGTTAATTTCCCTTTGCTTTTC-3′; and downstream, 5′-GGGAATTCGAATCCGATAGAATAT-GTCTC-3′. The upstream primer is located immediately past the termination codon, whereas the downstream primer is located 150 bp downstream of the termination codon (25). This PCR fragment was cloned into pBluescriptSK(+) at the *Eco*RI and *Bam*HI sites [pSK(+)ssu-1A] and digested with *Xba* I to produce a T7 RNA polymerase template. The rbcS-1A RNA probe protects a fragment of ≈150 nt.

RNase Protection Analysis. RNA probes were synthesized by using Promega T7 RNA polymerase and buffer as described by the manufacturer, except that 80 μCi of [α-^{32}P]UTP (800 Ci/mmol, Amersham; 1 Ci = 37 GBq) was used. RNase protections were done by using 5 μg of total RNA and 10 μg of tRNA according to the protocol of ref. 26, except that the final 70% wash was omitted. In the early time course experiment from 0 to 24 hr (see Fig. 1), samples contained 15 μg of total RNA and 10 μg of tRNA. Protected fragments were electrophoresed on 8% sequencing gels, and radioactivity in protected bands was quantified by scintillation counting. The ubq3 RNA probe protects a family of bands that can be seen in Fig. 1; for quantitation, the highest molecular weight of these was used.

RESULTS

***cab* Gene Expression Shows Both Endogenous and Phytochrome Regulation During Etiolated Seedling Growth.** The levels of cab1, originally named cab140 (19), mRNA and the effect of phytochrome action on these levels were determined over a 9-day period and are shown in Fig. 1. Germination was initiated by a 15-min white-light treatment, and, at the indicated times after this treatment, etiolated *Arabidopsis* seedlings received 1 min of red light, 1 min of red light immediately followed by 10 min of far-red light, or were left untreated. Seedlings were returned to the dark for 4 hr before transcripts were quantified by RNase protection. A ubiquitin gene (*ubq3*) served as a constitutive control that was not regulated by phytochrome.

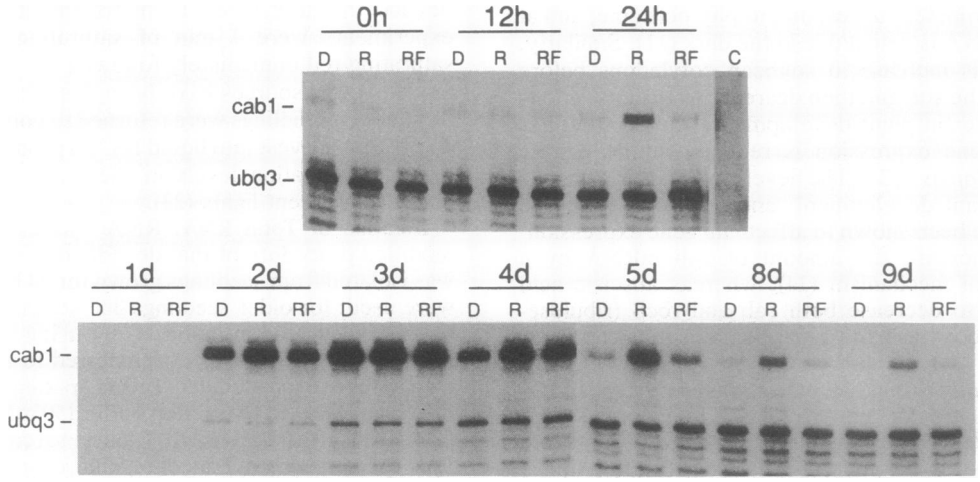

FIG. 1. RNase protection analysis of cab1 and ubq3 transcript levels during early seedling development. At indicated times (h, hr; d, days) etiolated seedlings received the following light treatments: D, no light; R, 1 min of red light; RF, 1 min of red light immediately followed by 10 min of far-red light. Seedlings were returned to darkness for 4 hr before RNA was extracted for analysis. (*Upper*) Samples contain 15 μg of *Arabidopsis* RNA and 10 μg of tRNA; exposure time was 18 hr. C, the control lane, contains only 10 μg of tRNA. (*Lower*) Samples contain 5 μg of *Arabidopsis* RNA and 10 μg of tRNA; exposure time was 6 hr. The *Upper* and *Lower* probes were labeled to different specific activities.

After seeds were sown, they were imbibed for 2 days in the dark at 4°C and then warmed to room temperature. At this time (0 hr) they contained low, but detectable, amounts of the cab1 transcript both in the dark and after a red-light treatment. The control lane does not contain *Arabidopsis* RNA, and at a comparable exposure no band can be seen at the position of the fragment that was protected by the cab1 RNA probe. This low level of cab1 mRNA seen in imbibed seeds indicates that little cab1 mRNA is retained from embryo development. After 12 hr of growth in darkness (Fig. 1, lane 12h D) cab1 transcript levels were still low. But after 24 hr, when radicles had emerged from the seed coat, cab1 RNA was induced by red light (lane 24h R), and the effect of red was reversed by far-red light (lane 24h RF), demonstrating that the *cab1* gene was under phytochrome control. At 24 hr the cab1 transcript was at a low, but detectable, level in the dark (lane 24h D).

After 2 days of dark growth, the cotyledons had emerged from the seed coat, and there was an ≈50-fold increase in the dark cab1 mRNA level compared with day 1. The ubq3 mRNA level also increased but only by 3-fold. Phytochrome further induced cab1 transcript accumulation above the dark level (Fig. 1, lane 2d R). At day 3, the hypocotyls had begun to lengthen, and the cotyledons were closed and unexpanded. The cab1 mRNA levels were at their highest level in the dark, and red light did not stimulate a measurable increase in the cab1 message (lanes 3d D and R). After day 3 and continuing through day 9, cab1 transcript levels decreased in the dark. Phytochrome regulation remained, but the red-light-induced level of the cab1 transcript decreased in older seedlings. The hypocotyls continued to lengthen during this period, and cotyledons remained closed until day 7 when they unfolded but did not expand.

The ubq3 mRNA increased steadily during etiolated seedling growth, reaching a maximum at day 8 and remaining at this level through day 9. The ubq3 mRNA did not peak at day 3, suggesting that the transient increase seen for the cab1 mRNA was not due solely to a general increase in RNA polymerase II activity. Fig. 2 shows that other members of the *cab* gene family, *cab2* and *cab3*, formerly named *cab165* and *cab180*, respectively (19), as well as the *rbcS-1A* gene, also showed a developmentally regulated pattern of mRNA expression in etiolated seedlings. The cab2 and cab3 mRNA levels were substantially lower than the cab1 mRNA level, and a longer exposure was required for detection, as can be seen by comparing intensities of the ubq3 signals. The rbcS-1A mRNA level was already high after 24 hr of dark growth; it peaked at day 2, one day earlier than the cab1 transcript, and then declined similarly to the cab1 mRNA. Control lanes without *Arabidopsis* RNA showed no background bands at the positions of cab3, cab2, and rbcS-1A (data not shown). Thus, we have observed light-independent endogenous control of three *cab* genes and one member of the *rbcS* gene family in etiolated *Arabidopsis* seedlings.

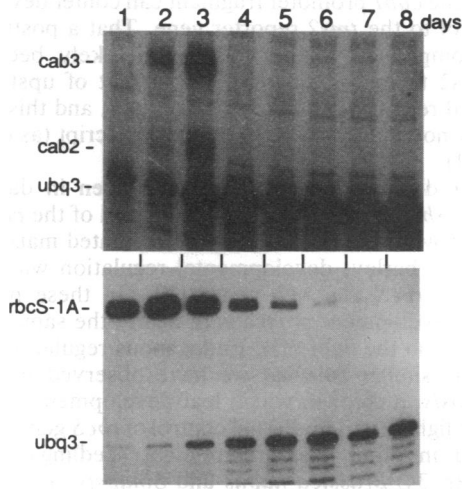

FIG. 2. RNase protection analysis of cab3, cab2, and rbcS-1A transcript levels during early seedling development. At 24-hr intervals over an 8-day period, dark-grown seedlings were harvested for RNase protection analysis. All samples contain 5 μg of *Arabidopsis* RNA and 10 μg of tRNA; exposure times were 13 hr for cab3 and cab2 and 2 hr for rbcS-1A.

A Circadian Rhythm Is Not Involved in the Dark Expression Pattern. We tested the possibility that the 15 min of white light used to stimulate germination could have acted as a *zeitgeber* for circadian control. To verify that mRNA levels were not oscillating on a 24-hr cycle, cab1 mRNAs were assayed 2 to 3 times daily during a 5-day period of dark growth. Fig. 3 shows that the cab1 mRNA levels followed a steady increase and decrease; therefore, the endogenous regulation of *cab1* gene expression seen during early seedling development is not circadian. This result also suggests that imbibed seeds are not yet capable of responding to a *zeitgeber*.

Endogenous Regulation Occurs Independently of the Light Treatment Used to Stimulate Germination. We also considered the possibility that the 15 min of white light used to trigger germination could have induced the high levels of *cab* gene expression seen in the dark. We therefore used two alternative treatments to stimulate germination and examined the subsequent pattern of *cab* gene expression during dark growth. In one experiment, germination was stimulated by 150 μM GA_{4+7} (27). In a parallel experiment, seeds with high levels of dark germination were used (23). Fig. 4 *A* and *B* show that both sets of seedlings showed an endogenous increase in the level of cab1 mRNA during growth in darkness, similar to that seen when 15 min of white light was used to stimulate germination (Fig. 1). The approximately one-half-day delay in the initial increase of cab1 mRNA probably results from the nonuniform germination that occurs under these alternative germination conditions. In the experiment shown in Fig. 4*B*, the seedlings grown from seeds with high levels of dark germination showed an unusually high level of cab1 transcript.

Developmental Regulation Occurs with Sucrose. To see if an external carbon source would affect *cab* gene expression in the dark, seedlings were grown on standard medium/2% sucrose. Sucrose can induce the expression of the petunia *CHS-A* gene in *Arabidopsis* (28). Fig. 4*C* shows that the effect of sucrose was to alter the timing of the transient increase in cab1 mRNA. The maximum level of cab1 mRNA occurred at day 4 rather than at day 3, and although a decline in this level was still observed, at day 8 cab1 transcripts were 3-fold more abundant in sucrose-grown seedlings than in seedlings grown without sucrose. The ubq3 mRNA levels were not affected by sucrose. That sucrose did not abolish the light-independent increase of cab mRNA levels in etiolated *Arabidopsis* seedlings and even contributed to the maintenance of higher levels

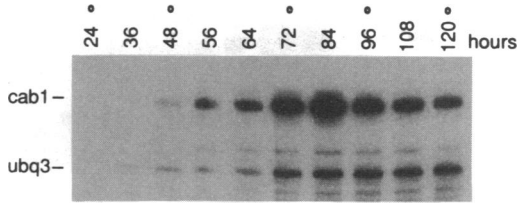

FIG. 3. RNase protection analysis of cab1 and ubq3 transcript levels from dark-grown seedlings harvested over a 5-day time course. RNA was isolated from dark-grown seedlings harvested at the indicated times and subjected to RNase protection analysis. Circles above hours indicate days.

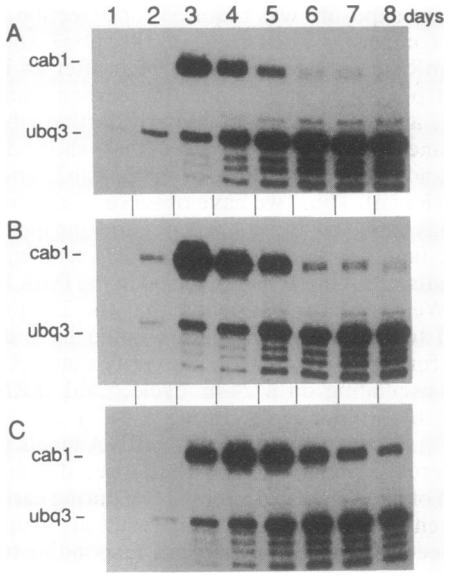

FIG. 4. RNase protection analysis of cab1 and ubq3 transcript levels from dark-grown seedlings grown under different conditions. (*A*) Seeds were sown onto medium containing 150 μM GA_{4+7} to stimulate germination and did not receive a 15-min white-light treatment. (*B*) Seeds with high levels of dark germination were sown onto normal medium and did not receive a 15-min white-light treatment. (*C*) Seeds were sown onto medium containing 2% sucrose and did receive a 15-min white-light treatment. RNA was isolated from dark-grown seedlings at the indicated times and subjected to RNase protection analysis.

of cab mRNA in older seedlings, differs from the observations made in maize bundle-sheath protoplasts, where sucrose repressed *cab* gene expression (29).

The *cab1* Promoter Is Sufficient to Confer Responsiveness to Endogenous Cues. We have used a *cab1–tms2* transgenic line, cab(+14)B (21), to see whether the *cab1* promoter could confer developmental regulation upon a reporter gene. The *cab1–tms2* fusion contains 1.34 kilobases (kb) of the *cab1* promoter, including the initial 14 bp of the cab1 transcript. Transgenic seedlings were harvested at 24-hr intervals over an 8-day period of dark growth. The cab1–tms2 transcript was quantified by RNase protection analysis and is shown in Fig. 5, along with cab1 and ubq3 mRNAs. The cab1–tms2 message appeared at day 2, as did the endogenous cab1 message, peaked at day 3, and then returned to a low level at day 4. This expression pattern paralleled that of the native *cab1* gene, but the amount of transgene transcript produced was substantially less. Thus, 1.34 kb of the upstream region of the *cab1* gene is sufficient to confer responsiveness to endogenous cues in etiolated *Arabidopsis* seedlings.

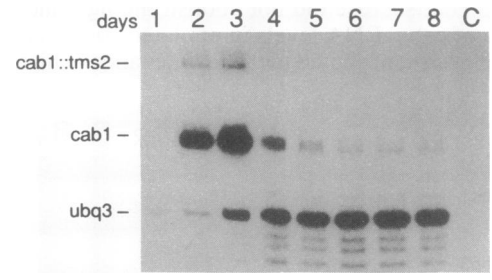

FIG. 5. RNase protection analysis of cab1–tms2, cab1, and ubq3 transcript levels from a dark-grown transgenic *Arabidopsis* line. Dark-grown cab(+14)B transgenic seedlings that harbor the full-length (1.34 kb) *cab1* promoter fused to the *tms2* gene were harvested at the indicated times. RNA was isolated and subjected to RNase protection analysis.

DISCUSSION

We have observed a transient increase in mRNA levels for three members of the *cab* gene family and for the *rbcS-1A* gene in *Arabidopsis* seedlings grown in complete darkness. A 50-fold increase in cab1-transcript levels occurs between 24 and 48 hr of etiolated growth; cab1 mRNA levels peak 24 hr later and then decrease. A transcript encoding ubiquitin increases only 3-fold between 24 and 48 hr and does not decrease; thus the pattern seen for the *cab* and *rbcS* genes is not solely due to a general increase and decrease in transcription. The initial increase in cab transcripts coincides with the emergence of the cotyledons from the seed coat. Furthermore, the pattern of expression occurs independently of light exposure, exogenous gibberellins, and sucrose. Thus, we suggest that we are observing an internal, developmental regulation of cab and rbcS mRNAs. Others (Andree Dedonder, personal communication; William Kubasek and Fred Ausubel, personal communication) have also seen high levels of cab and rbcS mRNAs in dark-grown *Arabidopsis*.

This developmental regulation of the *cab* genes can be distinguished from phytochrome regulation. This difference is most clearly illustrated by the temporal separation of these two types of regulation: the ability to respond to phytochrome occurs before the onset of developmental regulation and continues after the dark levels have decreased. Additionally, phytochrome regulation continues to be observed over and above the pattern of expression in the dark, again suggesting that these two types of regulation are independent. Previously, the high levels of cab mRNA observed in the dark were thought to signal competence to respond to phytochrome (11), but we now have shown that these two controls of *cab* gene expression are separable.

We have also demonstrated that a 1.34-kb fragment of the *cab1* gene can confer developmental regulation to a reporter gene in etiolated seedlings. Deletion analysis of this fragment in transgenic *Arabidopsis* seedlings will allow us to find regions of the *cab1* promoter that respond to the internal signals. Because we observed phytochrome control independently of developmental control in etiolated *Arabidopsis*, these developmental elements probably will be separate from the elements that confer responsiveness to phytochrome.

We do not yet know whether this developmental regulation is occurring at the transcriptional or posttranscriptional level. It is likely that a transcriptional component is involved because the *cab1* promoter fragment can confer developmental control to the *tms2* reporter gene. That a posttranscriptional component is involved is less likely because the cab1–tms2 transcript contains only 14 nt of upstream untranslated region from the *cab1* promoter, and this length is probably not enough to stabilize the transcript (as discussed in ref. 21).

Similar developmental control was seen in dark-grown maize for *rbcS* genes (30), and a high level of the *cab-1* gene transcript was detected in 8-day-old etiolated maize (31). In dark-grown barley, developmental regulation was also observed for *rbcS* and *cab* genes (32). In these monocots, leaf-cell development proceeds to nearly the same extent in the dark as in the light (33). Endogenous regulation in these species is similar to what we have observed in etiolated *Arabidopsis*, a dicot in which leaf development strictly depends on light. Similar internal control of *rbcS* genes has been observed in other dark-grown dicot seedlings, including cucumber (34) *Brassica napus* and *Sinapsis alba* (35), with peaks in *rbcS* accumulation occurring at 2–3 days after sowing, close to the time that we observed the highest levels of cab and rbcS transcript accumulation in *Arabidopsis*. High levels of rbcS1, rbcS2, and rbcS3A transcripts have been detected in 7-day-old dark-grown tomato as well (36). Like *Arabidopsis*, the other dicot species mentioned above do not

develop leaves in the dark. These examples illustrate that the extent of leaf development in the dark does not correlate with the developmental regulation of cab and rbcS transcripts in etiolated seedlings.

Transcription of plastid genes has also been observed to follow a similar developmental pattern. In the mustard *Sinapsis alba*, many plastid genes are transcribed in the dark, with *trnK* showing the same timing of increase and decrease as seen for *cab* in *Arabidopsis* (35). In barley, general plastid transcription reaches a peak in the same leaf sections that show peaks of rbcS and cab transcripts (32). Thus, the early light-independent developmental program seems to involve both the plastid and the nucleus. This developmental program could be important for preparing the seedling for the rapid biogenesis of the photosynthetic apparatus when the cotyledons emerge from the seed coat 2 days after sowing. Rapid synthesis of the light-harvesting chlorophyll proteins could help the plant protect itself from photo-oxidative damage that occurs when newly synthesized chlorophyll is not incorporated into the photosynthetic apparatus (1).

The increased amounts of cab mRNA seen in soybean cotyledons before they emerge from the ground (12) could resemble the developmental regulation described here, but the plants used in these experiments were grown in light, which can penetrate the soil and stimulate *cab* gene expression.

Sucrose has been reported to repress *cab* gene expression in a transient assay with maize bundle-sheath protoplasts (29), but we found that sucrose does not repress *cab* gene expression during etiolated seedling growth in *Arabidopsis*. It is likely that sucrose affects mature bundle-sheath protoplasts differently than etiolated seedlings. We observed that higher levels of cab1 mRNA were maintained in older seedlings when sucrose was included in the medium. This fact suggests that the decline of cab mRNA seen at day 4 without sucrose is, in part, metabolic, due to a decline in the limited storage reserves provided by the small *Arabidopsis* seed.

Our findings are important because this developmental regulation must be taken into account when studying other aspects of gene regulation in *Arabidopsis*. Further, this endogenous regulation of *cab1* gene expression seen in etiolated *Arabidopsis* during early seedling development provides a simple experimental system in which to study mechanisms of internal control.

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