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Increased rRNA Gene Activity during a Specific Window of Early Pea Leaf Development

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rRNA gene transcription rates were determined during light-mediated leaf development in *Pisum sativum*. The rate of transcription was observed to increase within 1 day of exposure to light and return to control levels 4 days after exposure. A striking similarity was observed between periods of elevated rRNA gene transcription and increased mitotic activity, suggesting a possible link between the two events.

rRNA plays a critical role in the cellular translational process and represents the most abundant transcript in most procaryotic and eucaryotic cells. In addition to the constitutive expression of rRNA genes observed in most cell types (presumably reflecting ribosome turnover rates), rRNA gene transcription rates are known to be regulated under a variety of circumstances. In plant cell suspensions and animal cell cultures a temporal correlation has been demonstrated between the rates of rRNA synthesis and cell division (for example, see reference 5; see also references in reference 11). Regulation of rRNA expression also occurs during specific developmental stages in several animal systems (12), and differences in rRNA gene transcription rates in undeveloped and fully developed pea leaves have been observed (3). rRNA synthesis is affected by light in several plant species (15), and in those species examined, phytochrome mediates at least part of this response.

In the pea cultivar Alaska, approximately 4,000 rRNA genes per haploid genome compose two arrays which are located on separate chromosomes (K. J. Piller, S. R. Baerson, N. O. Polons, and L. S. Kaufman, submitted for publication). Two major length variants occur, an 8.6-kilobase (S) and a 9.0-kilobase (L) variant, which form independent arrays on chromosomes 4 and 7, respectively (6; Piller et al., submitted). Several lines of evidence suggest that the two length variants are differentially expressed during light-mediated leaf development. After seedlings receive 3 days of light exposure, DNase I-hypersensitive sites which are not present in dark-grown seedlings appear in the S variant near the site of initiation (7). The L variant contains these hypersensitive sites under all conditions examined. Similarly, a promoter-proximal *Hpa*II site becomes demethylated and then remethylated in the S variant after 3 and 7 days of white light, respectively, whereas the comparable site in the L variant is constitutively undermethylated (18). The undermethylation of cytosine residues and the appearance of nuclease hypersensitivity adjacent to sites of transcript initiation have been associated with actively expressed rRNA gene arrays in a number of animal systems (for examples, see references 9, 13, 14, 16). Combined with the observation that nuclei isolated from light-grown seedlings have more nucleoli than those of dark-grown seedlings (L. S. Kaufman, unpublished data), the studies of Kaufman et al. (7) and Watson et al. (18) suggest that the L variant is constitutively expressed while the S variant is expressed only during specific stages of leaf development.

Relatively little is known about the regulation of gene expression in developing leaves in spite of the fact that leaf development in flowering plants represents one of the most dramatic effects of light on plant development. Furthermore, several key events occurring during leaf development, namely, cell division, cell expansion, and chloroplast development, are likely to play an important role in determining temporal patterns of gene expression within a developing leaf cell. It is therefore of interest to determine whether rRNA genes are being activated at certain stages of leaf development and, if so, what developmental processes are related to changing rRNA gene transcription rates.

Changes in rRNA gene transcription during leaf development. To examine the transcription rate of rRNA genes during the process of leaf development, nuclear run-on assays were performed using nuclei isolated from the shoot apices of pea seedlings bearing leaves at progressively more advanced stages of development. To obtain such a set of plants, seeds were planted in darkness (10), and different groups were transferred to continuous fluorescent white light (100 μmol per m^2 per s; Sylvania Cool White) on successive days (Fig. 1) and harvested 7 days after planting. In this manner seedlings of the same chronological age but possessing leaves at different stages of development could be obtained.

Nuclei were isolated from tissue at and above the third node by Percoll density centrifugation (7). All transcription assays were performed in 100-μl volumes with 10^6 nuclei as described previously (10), except that transcription assays were conducted for 30 min at 30°C. Purified transcripts obtained from identical numbers of nuclei were used for hybridization to excess filter-bound plasmid sequences. Cloned DNA sequences were immobilized on GeneScreen with a Minifold II Slot-Blot system (Schleicher & Schuell, Inc.). The plasmids used were pRRNpss1, a genomic clone representing a complete S variant isolated from the pea cultivar Alaska (Piller et al., submitted), and pAB96, a cDNA clone representing the major chlorophyll *a/b* binding protein of photosystem II in peas (1). Controls using α-amanitin and dactinomycin confirmed RNA polymerase I activity and established that transcription occurred in a template-dependent manner (data not shown). Hybridization conditions were as described previously (10), with *Escherichia coli* genomic DNA substituted as the nonspecific competitor. Hybridization was quantified by scanning den-

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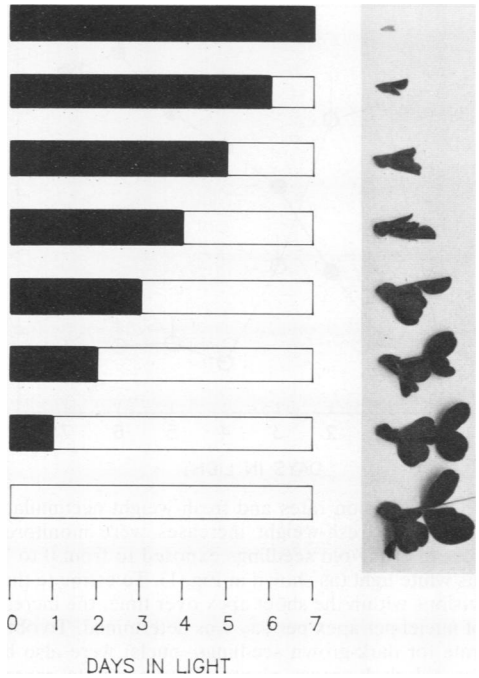

FIG. 1. Growth regimen for 7-day-old pea seedlings. Seeds were allowed to soak and were planted in darkness, and different groups were transferred to continuous fluorescent white light on subsequent days. Shoot apices (tissue at and above the third node) were harvested from each group after 7 days. Each bar represents the light treatment given to a specific experimental group; filled regions indicate time in darkness, and open regions indicate time in light. The photograph at right shows examples of shoot apices harvested for each light treatment.

sitometry or by two-dimensional beta scanning (Ambis System II; Automated Microbiology Systems) and corrected for background against pGEM4 sequences. Saturation studies confirmed that hybridization signals were proportional to the input of labeled transcripts (data not shown).

Figure 2A shows the rate of rRNA transcription as a function of days of white-light exposure. The data indicated that transcription rates increased within 1 day of light exposure, reached a maximal level between days 2 and 3 of exposure (2.75- and 2.4-fold increases, respectively), and returned to dark levels by day 4 of exposure. No increase in rRNA gene copy number per nucleus was detected by Southern analysis during the experimental period (data not shown). No change in ploidy was detected, confirming previous observations (2, 8).

Previously published data regarding DNase I hypersensitivity and cytosine methylation suggest that the increased transcription rate is due to the specific activation of the S variant. These data, combined with those of Fig. 2A, suggest that the rRNA gene array containing the S variant becomes active within 1 day of light exposure and returns to an inactive state by 4 days of exposure.

The transcription rates of the gene family encoding the major chlorophyll *a/b* binding protein of photosystem II (*Cab*) as well as the overall transcriptional activity were examined as an internal control for nonspecific transcription (*Cab* gene transcription increases during pea leaf development [3]). Purified run-on transcripts prepared from the same extracts used in the rRNA experiments were used for hybridization to pAB96 (Fig. 2B). Total trichloroacetic acid-insoluble counts were used to determine total transcriptional activity. As with rRNA, both total and *Cab* transcription rates rose sharply over the first 2 days of light exposure. Unlike the rRNA rates, total and *Cab* transcription rates did not return to dark-grown levels as leaf development pro-

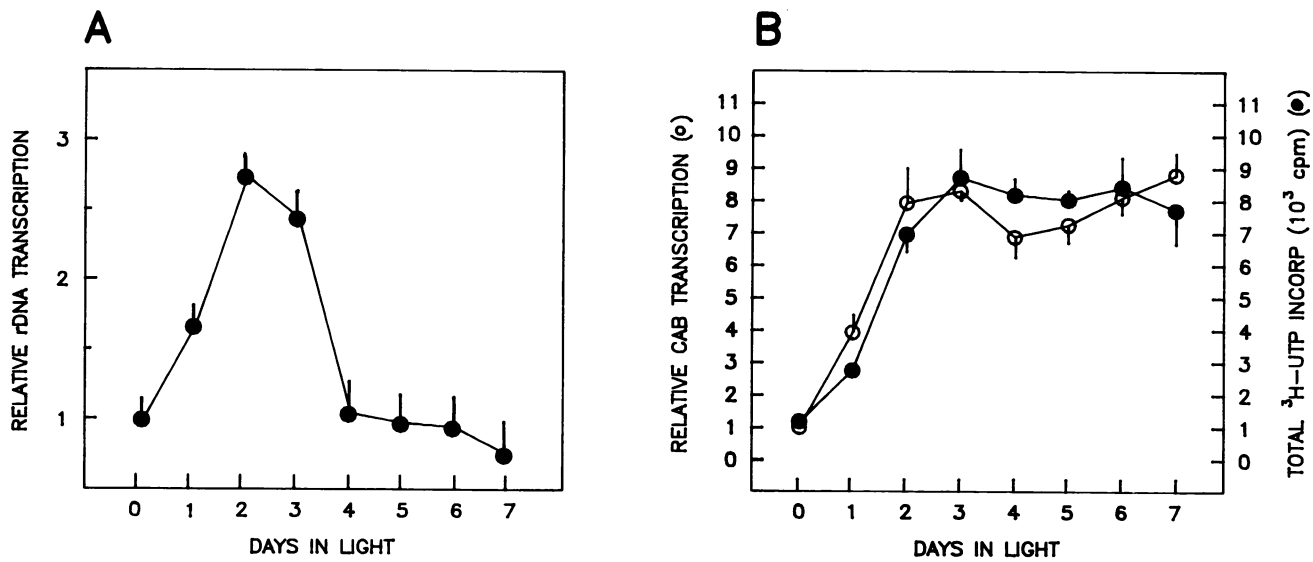

FIG. 2. Changes in rRNA gene, *Cab* gene, and total nuclear transcription during leaf development. Nuclear run-on assays were conducted as described in the text, and hybridization signals were corrected against hybridization to pGEM4. Transcription rates shown have been normalized to the rates of dark-grown seedlings (0 days in light). Each datum point represents the average of three independent experiments. The error bars represent the standard errors of the means, and where not shown, the standard error of the mean is enclosed within the datum point. (A) rRNA gene transcription during leaf development. Purified run-on transcripts were hybridized to filter-bound pRRNpss1 sequences (described in the text). (B) *Cab* gene and total nuclear transcription during leaf development. Purified run-on transcripts were hybridized to filter-bound pAB96 sequences (described in the text). Total nuclear transcription rates were determined by collection of trichloroacetic acid-precipitated run-on transcripts onto glass filter disks and then liquid scintillation counting.

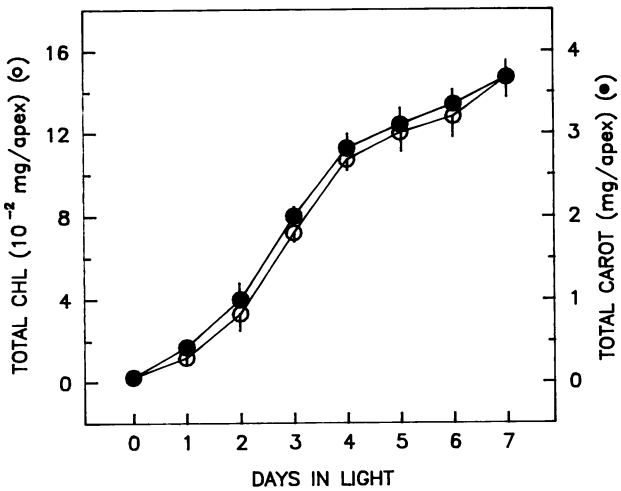

FIG. 3. Accumulation of chlorophyll and carotenoids. The total chlorophyll and carotenoid contents of shoot apices were determined for seedlings receiving from 0 to 7 days of light exposure (as shown in Fig. 1). An identical number of apices from each experimental group were ground in 80% acetone to extract membrane-associated pigments. Chlorophyll (CHL) and carotenoid (CAROT) concentrations within the extracts were determined spectrophotometrically (see the text for references). Datum points represent the average of three independent experiments. The error bars represent the standard errors of the means and where not shown are enclosed within the datum point.

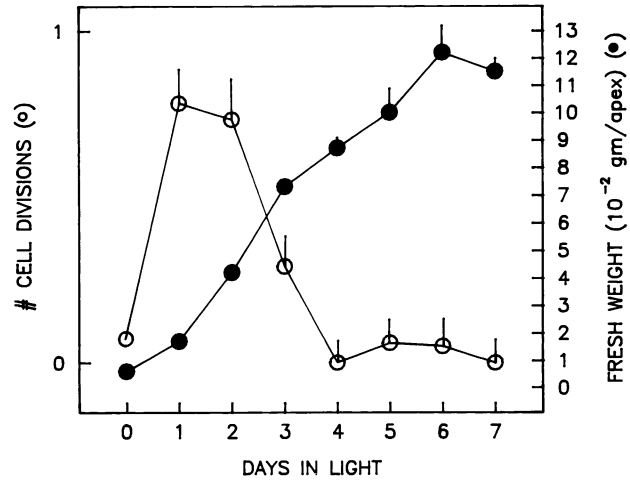

FIG. 4. Cell division rates and fresh-weight accumulation. Cell division rates and fresh-weight increases were monitored in the shoot apices of 7-day-old seedlings exposed to from 0 to 7 days of continuous white light (as shown in Fig. 1). To estimate the number of cell divisions within the shoot apex over time, the increase in the number of nuclei per apex per day was determined. To obtain a cell division rate for dark-grown seedlings, nuclei were also harvested from 6-day-old dark-grown plants. Datum points represent the averages of three independent experiments. Error bars represent the standard errors of the means and where not shown are enclosed within the datum point.

gressed. These data demonstrate that while colinearity exists among rRNA, total, and *Cab* transcription rates during the initial stages of leaf development, the overall regulation of rRNA transcription is unique after 2 days of exposure. The relationship between *Cab* and total transcription rates bears further investigation.

Correlation with cellular processes during leaf development. The processes of chloroplast development, cell expansion, and cell division require large increases in the synthesis of specific proteins and, consequently, may require an increase in the amount of ribosomes and rRNA per cell. A correlation between the developmental profiles of these parameters and elevated levels of rRNA gene transcription would be suggestive of a physiological role for the increased transcription rate.

To monitor chloroplast development, chlorophyll and carotenoid levels in apical tissues of seedlings exposed to 0 to 7 days of light as described previously were determined (17). The amounts of chlorophyll and carotenoids per apex increased approximately linearly throughout 7 days of light exposure (Fig. 3). The fresh weights of the shoot apices (a marker for cell expansion) also increased linearly through 6 days of white light, and no significant increases were observed between days 6 and 7 (Fig. 4). No obvious relationship between rRNA gene transcription and either chloroplast development or cell expansion could be inferred from these data.

To obtain a rough estimate of the rate of cell division within the shoot apex over time, the number of nuclei harvested per apex was determined and the rate of cell division was calculated by the change in the number of nuclei per day (Fig. 4). The experimental groups showing the largest increases in cell division rate also had the highest rates of rRNA gene transcription (Fig. 2A). Seedlings exposed to 1 or 2 days of light showed an increase of approximately 75% in the number of cells per apex, and seedlings exposed to 3 days of light showed an approximately 35% increase. Seedlings exposed to 4 to 7 days of white light showed rates comparable to those of dark-grown plants. The striking similarity observed between the developmental profile of rRNA gene transcription rates and mitotic activity strongly suggests that the two processes could be linked or coordinately regulated during leaf development. The large number of in vitro and in vivo studies demonstrating an increase in rRNA synthesis associated with the onset of, or the increase in, cell division further supports this notion (for a review, see reference 12).

The results presented in this study indicate that rRNA gene expression is elevated during a 3-day period, coincident with increased mitotic activity. According to the results of previous studies (7, 18), it is probable that the increased expression is due to the specific activation (and subsequent deactivation) of the S rRNA variant. As rRNA transcription, *Cab* transcription, and total nuclear transcription increase colinearly with mitosis, it is possible that some form of coordinate regulation, perhaps related to replication, is in effect (for a review, see reference 4).

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