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Leaf Developmental Age Controls Expression of Genes Encoding Enzymes of Chlorophyll and Heme Biosynthesis in Pea (*Pisum sativum* L.)[1]

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The effects of leaf developmental age on the expression of three nuclear gene families in pea (*Pisum sativum* L.) coding for enzymes of chlorophyll and heme biosynthesis have been examined. The steady-state levels of mRNAs encoding aminolevulinic acid (ALA) dehydratase, porphobilinogen (PBG) deaminase, and NADPH:protochlorophyllide reductase were measured by RNA gel blot and quantitative slot-blot analyses in the foliar leaves of embryos that had imbibed for 12 to 18 h and leaves of developing seedlings grown either in total darkness or under continuous white light for up to 14 d after imbibition. Both ALA dehydratase and PBG deaminase mRNAs were detectable in embryonic leaves, whereas mRNA encoding the NADPH:protochlorophyllide reductase was not observed at this early developmental stage. All three gene products were found to increase to approximately the same extent in the primary leaves of pea seedlings during the first 6 to 8 d after imbibition (postgermination) regardless of whether the plants were grown in darkness or under continuous white-light illumination. In the leaves of dark-grown seedlings, the highest levels of message accumulation were observed at approximately 8 to 10 d postgermination, and, thereafter, a steady decline in mRNA levels was observed. In the leaves of light-grown seedlings, steady-state levels of mRNA encoding the three chlorophyll biosynthetic enzymes were inversely correlated with leaf age, with youngest, rapidly expanding leaves containing the highest message levels. A corresponding increase in the three enzyme protein levels was also found during the early stages of development in the light or darkness; however, maximal accumulation of protein was delayed relative to peak levels of mRNA accumulation. We also found that although protochlorophyllide was detectable in the leaves immediately after imbibition, the time course of accumulation of the phototransformable form of the molecule coincided with NADPH:protochlorophyllide reductase expression. In studies in which dark-grown seedlings of various ages were subsequently transferred to light for 24 and 48 h, the effect of light on changes in steady-state mRNA levels was found to be more pronounced at later developmental stages. These results suggest that the expression of these three genes and likely those genes encoding other chlorophyll biosynthetic pathway enzymes are under the control of a common regulatory mechanism. Furthermore, it appears that not light, but rather as yet unidentified endogenous factors, are the primary regulatory factors controlling gene expression early in leaf development.

A large number of gene families have been characterized in plants and shown to contain one or more members whose expression is regulated in spatially and temporally distinct patterns during growth and differentiation. In addition, numerous studies have demonstrated that both endogenous factors (e.g. nutrient availability, phytohormones, etc.) and exogenous factors (e.g. light) are involved in modulating and coordinating this developmental gene expression (Goldberg, 1988; Comai et al., 1989; Edwards and Coruzzi, 1990; Gilmartin et al., 1990; Thomas, 1993). Among these, the role of light in the regulation of growth and differentiation of higher plants has received particular attention because of its obvious and pronounced effects on development. When grown in the light, plants undergo a series of temporally and spatially coordinated changes known as photomorphogenesis that leads to the eventual attainment of their natural form. During this process leaves expand, stems undergo radial enlargement, pigments accumulate within developing chloroplasts, and numerous metabolic processes associated with photosynthetically competent cells become operational. In the absence of light a radically different growth pattern emerges, leading to the formation of a seedling devoid of pigments with highly appressed, unexpanded leaves incapable of photosynthetic function. Left growing in the dark, these etiolated seedlings will eventually die.

Among the processes activated during light-induced development is the tetrapyrrole biosynthetic pathway leading to the formation of Chls, heme, and their derivatives necessary for the assembly and function of the photosynthetic apparatus. The steps of the tetrapyrrole biosynthetic pathway are well established for most organisms, and in higher plants and algae numerous studies have demonstrated that both soluble and membrane-associated enzymes located exclusively within the chloroplast are involved in the formation of Chl and plastidic heme (Beale and Weinstein, 1990).

Previous studies have also shown that Chl formation is regulated primarily at two points in the biosynthetic pathway: the synthesis of ALA and the reduction of Pchlide. ALA is the first committed precursor of all tetrapyrroles in plants and its synthesis is generally accepted as the rate-limiting step (Beale and Weinstein, 1990). In plants ALA formation requires the coordinated actions of three plastid-localized

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Abbreviations: ALA, δ-aminolevulinic acid; PBG, porphobilinogen.

enzymes and appears to be regulated by a combination of factors, including de novo enzyme synthesis and turnover (Beale, 1990), feedback inhibition by both heme and Pchlide (Beale, 1990; Beale and Weinstein, 1990), and phytochrome (Huang et al., 1989).

Enzymes catalyzing steps of the biosynthetic pathway in common to both Chl and heme formation (e.g. PBG deaminase, ALA dehydratase, coproporphyrinogen oxidase, etc.) are presumably present at cellular levels that are not rate limiting. The abundance and activity of these enzymes, however, appear to be subject to modulation by both light and cell type (Smith, 1986; Boese et al., 1991; Spano and Timko, 1991; Witty et al., 1993).

The second regulatory point is specific to the Chl branch of the pathway and occurs at the reduction of Pchlide. In most angiosperms Pchlide reduction is a light-dependent reaction catalyzed by NADPH:Pchlide reductase (Griffiths, 1974). Both NADPH:Pchlide reductase and its substrate Pchlide accumulate to high levels in the leaves of dark-grown angiosperms. In monocots both NADPH:Pchlide reductase and the mRNA encoding it appear to decrease rapidly upon exposure of etiolated leaves to light (Apel, 1981; Santel and Apel, 1981). In dicots a somewhat different situation is observed. Although light induces a decline in the levels of NADPH:Pchlide reductase protein, a corresponding decrease in mRNA is not always found (Forreiter et al., 1990; Kittsteiner et al., 1990; Benli et al., 1991; Spano et al., 1992a). The photoreduction of Pchlide and the subsequent integration of newly formed Chl into the developing thylakoid appear to be closely tied to chloroplast development and the overall process of photomorphogenesis.

At present little is known about the mechanisms and factors that control the expression of the enzymes for Chl biosynthesis and how this key biosynthetic process is integrated with the overall process of chloroplast development. The availability of cloned cDNAs and/or genomic fragments encoding various enzymes of Chl and heme biosynthesis (e.g. glutamate 1-semialdehyde aminotransferase [Grimm, 1990], ALA dehydratase [Boese et al., 1991], PBG deaminase [Witty et al., 1993], and NADPH:Pchlide reductase [Spano et al., 1992a]) made it possible to look in greater detail at how these various activities are regulated. In this study we have examined the relationship between leaf developmental age and light on the expression in pea (*Pisum sativum* L.) of members of three nuclear gene families encoding enzymes of the Chl biosynthetic pathway. We show that the expression of these gene families is tightly coordinated during development and provide evidence showing that endogenous factors related to leaf age and possibly the extent of chloroplast development are involved in this control mechanism. We also show that the effect of light on the expression of these genes is highly dependent on the stage of seedling development.

MATERIALS AND METHODS

Plant Growth Conditions

Pea seeds (*Pisum sativum* L. var Progress No. 9, W.A. Burpee and Co., Warminster, PA) were allowed to imbibe overnight (approximately 12–18 h) in distilled water at room temperature. In some experiments the embryos were immediately dissected from the seeds and the embryonic foliage leaves were carefully excised to minimize the amount of contaminating hypocotyl tissues, as shown in Figure 1. For developmental analyses, soaked seeds were planted in moistened vermiculite and grown at 25 to 28°C either in complete darkness or under a bank of cool-white fluorescent bulbs supplying 150 $\mu W/cm^2$ (8000 lux) for periods of time ranging from 1 to 14 d as specified. At each time point, the leaf tissues were collected and either processed immediately (whole cell extracts) or rapidly frozen in liquid N_2 and stored at −80°C for later use. For light-induction experiments, soaked seeds were sown in moistened vermiculite and grown in complete darkness at 25 to 28°C for periods ranging from 1 to 14 d, after which the seedlings were transferred into the light and illuminated with white light as indicated above for 24 or 48 h. All manipulations involving dark-grown seedlings were conducted in total darkness or under dim green safelights (Schiff, 1972).

Extraction of Total RNA and RNA Gel-Blot Analysis

Total RNA was prepared from pea leaf tissues as previously described (Spano et al., 1992a). The quantity of total RNA recovered was determined by spectrophotometry and equivalent amounts of total RNA isolated from the various tissues were separated on agarose-formaldehyde gels (Sambrook et al., 1989). The RNA was then transferred to nitrocellulose filters (Schleicher & Schuell) and the filters were prehybridized in 5× SSPE (1× SSPE = 0.15 M NaCl, 0.25 M NaH_2PO_4, pH 7.4, 2.5 M Na_2EDTA), 50% (v/v) deionized formamide, 5× Denhardt's medium, 0.1% (w/v) SDS, and 100 μg/mL denatured salmon sperm DNA at 42°C for 6 to 12 h. Hybridization was carried out for 12 to 16 h at 42°C in buffer containing 5× SSPE, 50% (v/v) deionized formamide, 5× Denhardt's medium, 0.1% (w/v) SDS, and 1×10^6 to 2×10^6 cpm/mL denatured ^{32}P-labeled probes. The filters were washed twice for 15 min in 0.1× SSC, 0.1% (w/v) SDS at 23°C. After washing, the filters were blotted dry, wrapped in Saran Wrap, and exposed to Kodak X-Omat autoradiographic film using Cronex intensifying screens.

For quantitative slot-blot analyses, serial dilutions of equivalent amounts of total RNA from the various tissues were denatured by formaldehyde and bound to nitrocellulose filters according to the manufacturer's protocol (Schleicher & Schuell). Aliquots of total RNA isolated from dark- and light-grown tissues were loaded on the same nitrocellulose filter, hybridized with the same ^{32}P-labeled probes, and washed under identical conditions. The amount of hybridization was determined by measuring the amount of bound radiolabeled probe using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. The maximum expression observed for a particular gene product was arbitrarily set at 1 and the relative transcript abundance is given as a percentage of that maximum expression, as shown in Figures 2 and 3.

DNA fragments used as probes in RNA gel and slot-blot analysis were purified by electrophoresis on low-melting agarose gels and labeled by random-primed synthesis (Feinberg and Vogelstein, 1983) with [^{32}P]dCTP (specific activity ≥ 600 mCi/mmol; New England Nuclear) using the Rapid-

Primed II Kit according to the manufacturer's protocol (Boehringer Mannheim). DNA fragments used as probes consisted of the following: a 691-bp *Eco*RI fragment from pPPCR700, encoding pea NADPH:Pchlide reductase (Spano et al., 1992a); a 1.3-kb *Eco*RI fragment from pALAD10, encoding the pea ALA dehydratase (Boese et al., 1991); a 1.2-kb *Eco*RI fragment from pPBGD9, encoding pea PBG deaminase (Witty et al., 1993); a 363-bp fragment encoding the pea ribulose-1,5-bisP carboxylase small subunit cloned by PCR amplification using oligonucleotide primers based on published coding sequences for *rbcS*-3A and *rbcS*-3C genes (Fluhr et al., 1986) and previously defined procedures (Spano et al., 1992b); and a 350-bp *Eco*RI fragment of the 18S rRNA from white pine (A.J. Spano, unpublished results).

Measurement of Pchlide Fluorescence Spectra

Fluorescence emission spectra were measured at 77 K using an SLM Aminco 8000C spectrofluorometer as described before (McEwen et al., 1991). The spectra were recorded as energy density per unit interval of wavelength. The integration time was 0.1 s. Fluorescence emission spectra were measured with an excitation wavelength of 440 nm and corrected for the intensity variations of the excitation light and for variation in sensitivity of the photomultiplier. All spectra shown are mean values of three to six separate measurements made on tissue from 3 to 10 different pea seedlings.

Preparation of Whole Cell Extracts and Western Blot Analysis

Whole cell extracts from various plant tissues were prepared by grinding approximately 0.5 g of tissue in a glass homogenizer containing 1 to 2 mL of 40 mM Tris-HCl, pH 8.0, and 2.0% (w/v) SDS. The homogenate was clarified by centrifugation for 10 min at 10,000*g*, and the resulting supernatant fluid was removed. Aliquots were taken for protein determination using the bicinchoninic acid method (Pierce Chemicals, Inc.) and the remaining supernatant fluid was made 5.0% (v/v) with β-mercaptoethanol and denatured for 3 min at 100°C.

Aliquots of total protein extracts were fractionated by SDS-PAGE on 12% (w/v) acrylamide gels using the buffer system of Laemmli (1970) as described by Spano and Timko (1991). Fractionated proteins were transferred to nitrocellulose filters and immunological detection was carried out using alkaline phosphatase-conjugated goat anti-rabbit second antibody as described by Spano and Timko (1991). Preparation of antiserum against the pea PBG deaminase (Spano and Timko, 1991), spinach ALA dehydratase (Boese et al., 1991), and oat NADPH:Pchlide reductase (Darrah et al., 1990) were described previously.

RESULTS

Analysis of Steady-State mRNA Levels Encoding Enzymes of Chl Synthesis during Growth in Darkness and Light

To study the effects of developmental age on the expression of genes encoding various enzymes in the Chl and heme biosynthetic pathways, pea seeds were allowed to imbibe for 12 h and either the foliage leaves of the embryo were excised or the seeds were planted and seedlings grown for various lengths of time in complete darkness or under continuous white-light illumination. After growth in darkness or light for lengths of time ranging from 1 to 14 d, the leaf tissues were collected and total RNA was isolated and analyzed by gel-blot analyses. In dark-grown seedlings the primary leaves remained unexpanded at the epicotyl throughout the 14 d of growth after imbibition (referred to as postgermination) and all tissues above the hook were harvested and analyzed together, as shown in Figure 1A. In light-grown plants there was little stem and internode elongation during the first 7 d after imbibition (d 1–7 postgermination) and the primary leaves also remained relatively tightly folded above the hypocotyl. Therefore, in a procedure similar to that used for the collection of tissues from dark-grown seedlings, leaf tissues from light-grown seedlings were collected in bulk at these early developmental stages. By approximately 8 d postgermination the majority of the light-grown seedlings exhibited significant stem and internode elongation, permitting us to differentiate among leaf pairs of different ages. In our studies we have designated the developmentally youngest leaf pair (i.e., the unfolded leaves at the shoot apical meristem) as leaf 1 and progressively older pairs of leaves as leaves 2 through 4, respectively, at each subsequent time point analyzed (i.e. 8, 10, 12, and 14 d postgermination), as shown in Figure 1C.

Equivalent amounts of total RNA isolated from leaf tissues of either dark- or light-grown seedlings of various developmental ages were fractionated on agarose-formaldehyde gels, blotted to nitrocellulose filters, and hybridized with ^{32}P-labeled probes specific for the pea ALA dehydratase, PBG deaminase, or NADPH:Pchlide reductase, as shown in Figure 1, A and C. To minimize variation in signal intensity due to differences in specific activity of the ^{32}P-labeled probes, filters containing RNAs isolated from the dark- and light-grown tissues were hybridized with the same ^{32}P-labeled probe solutions and washed under identical conditions. The steady-state mRNA levels encoding the various Chl biosynthetic enzymes and control proteins or RNAs were quantitated by slot-blot analyses using serial dilutions of equivalent amounts of total RNA from the various tissues. As in the RNA gel-blot analyses, RNA isolated from dark- and light-grown tissues was loaded onto the same nitrocellulose filter, hybridized with the same ^{32}P-labeled probes, and washed under identical conditions. The amount of hybridization was determined by measuring the amount of bound radiolabeled probe using a PhosphorImager, and the relative transcript abundances are given as percentages of the maximum expression for a particular gene, as shown in Figure 2.

The general patterns of gene expression for ALA dehydratase, PBG deaminase, and NADPH:Pchlide reductase were similar. mRNAs encoding both ALA dehydratase and PBG deaminase were detected in the foliage leaves of embryos that had been allowed to imbibe for 12 to 18 h, although the abundance of the two transcripts in these young, nonphotosynthetic tissues is quite low (approximately 1.5% of maximum expression). In contrast, no mRNA encoding the NADPH:Pchlide reductase could be detected. The presence of mRNA for ALA dehydratase and PBG deaminase (but not

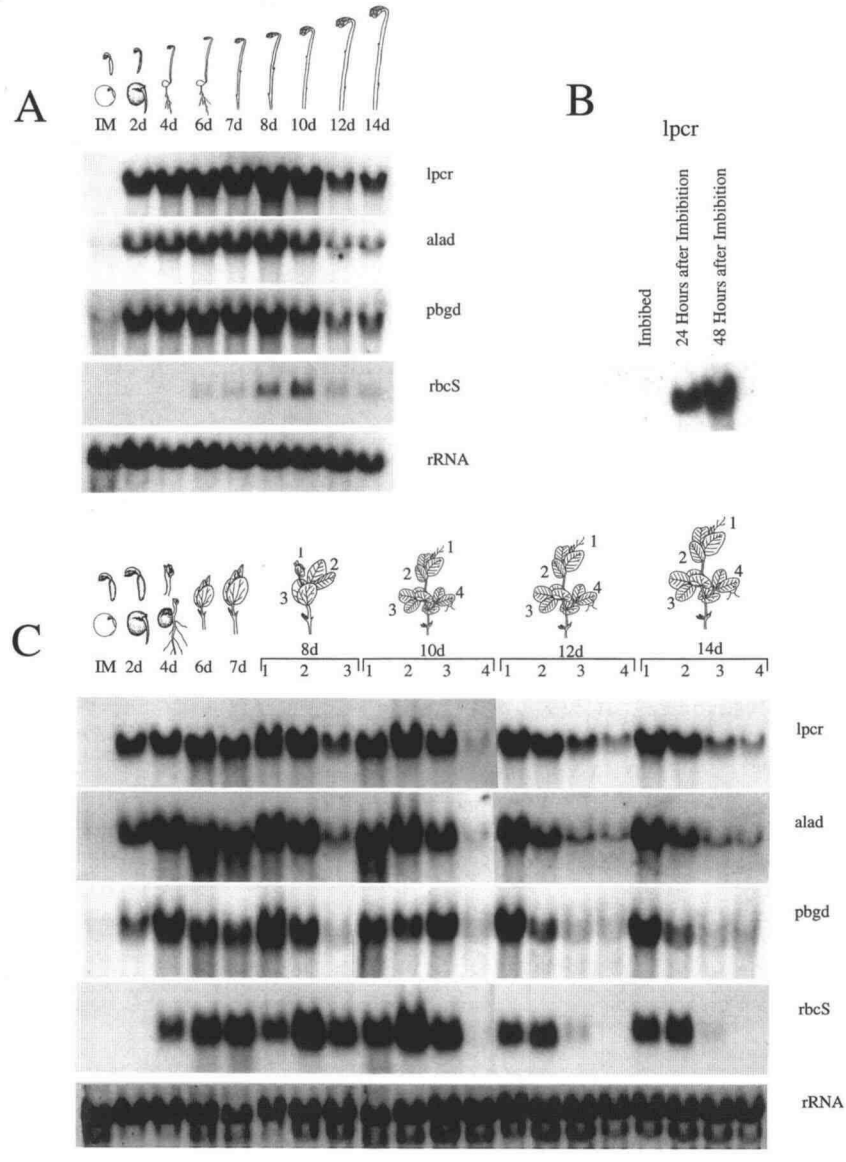

Figure 1. Gel-blot analysis of RNA isolated from pea seedling of various developmental ages grown in either light or dark. Total RNA was extracted from pea embryonic axes that had imbibed for 12 h (IM) and leaf tissues of seedlings grown for 2 to 14 d either in total darkness (A) or continuous light (B and C), labeled 2 through 14 d, respectively. In etiolated tissues, all leaves above the hook were collected as described in "Materials and Methods." In light-grown tissues leaves were pooled (d 2–7) or individual leaves were collected (d 8, 10, 12, and 14). Individual leaves were collected as shown in the diagram, with the youngest leaves designated 1 and the oldest 4. Equivalent amounts of total RNA (50 μg) isolated from etiolated leaves were denatured in loading buffer, fractionated on formaldehyde-agarose gels, blotted to nitrocellulose, and hybridized with ^{32}P-labeled probes. The filter was stripped and reprobed with the gene probes indicated.

NADPH:Pchlide reductase) at this very early developmental time is consistent with a requirement for a basal level of expression of those enzymes required in heme formation for plastidogenesis and general cellular activities.

During development in the dark, the steady-state mRNA levels for both ALA dehydratase and PBG deaminase increased 10-fold, reaching a maximum level of abundance approximately 6 to 8 d postgermination (Fig. 1A). Seedlings grown in continuous white light showed a similar developmentally programmed increase in the levels of mRNA encoding these two enzymes in the first 6 to 8 d postgermination (Fig. 1C). The steady-state levels of mRNA encoding ALA dehydratase and PBG deaminase mRNA in dark-grown tissues were approximately 1.5- and 2.5-fold greater than those observed in the light-grown tissues during this time frame for ALA dehydratase and PBG deaminase, respectively.

As the seedlings matured during growth in the light, stem and internode elongation occurred, making it possible to discriminate among leaves of different ages on these plants. As shown in Figure 1C, when ALA dehydratase and PBG deaminase mRNA levels were measured in leaves of different ages at various stages postgermination, transcript abundance was found to be highly dependent on leaf developmental age. In general, transcript abundance increased with increased age until the leaf was fully expanded, after which time mRNA levels decreased sharply. Thus, the rapidly growing youngest leaves of seedlings 8 d postgermination (Fig. 1C, leaf 1, d 8) contained high steady-state mRNA levels encoding ALA dehydratase and PBG deaminase, whereas by 14 d postgermination (Fig. 1C, leaf 4, d 14) mRNAs encoding these two enzyme activities were barely detectable.

A similar profile of developmentally regulated expression was observed for the NADPH:Pchlide reductase (Fig. 1C). Although not detectable in foliage leaves of embryos that had imbibed, mRNA encoding NADPH:Pchlide reductase accumulated rapidly in the primary leaves of dark-grown

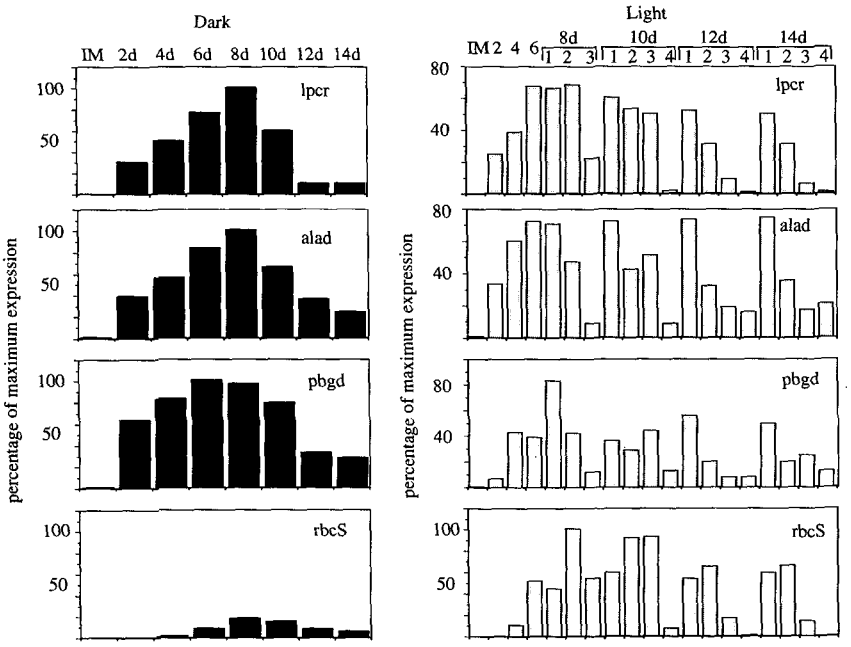

Figure 2. Histograms representing the steady-state mRNA levels encoding the enzymes of the Chl biosynthetic pathway. Total RNA was prepared from dark- (Dark) and light-grown (Light) plants of various developmental ages as described in the legend to Figure 1. Serial dilutions of the various RNA samples were blotted to a single nitrocellulose filter and the filter was hybridized with ^{32}P-labeled probes as indicated. The amount of bound probe was quantitated using a Molecular Dynamics PhosphoImager. The maximum expression observed for a particular gene product was arbitrarily set at 1 and the relative transcript abundance is given as a percentage of that maximum expression.

seedlings. Significant amounts of NADPH:Pchlide reductase message could be observed by 1 d postgermination, and by 2 d postgermination approximately 30% of the maximum level of expression in dark-grown leaf tissue was observed (Fig. 1, B and C).

Leaves of dark-grown seedlings continued to accumulate NADPH:Pchlide reductase mRNA during subsequent growth in the dark, with message levels reaching a plateau between d 6 and 8 postgermination. After this time the steady-state levels of NADPH:Pchlide reductase mRNA began to decline. It is unlikely that this decrease simply reflects the adverse physiological effects of prolonged etiolation, since not all transcripts examined exhibited a similar temporal decline (see below).

In contrast to the inverse effects of light on steady-state levels of mRNA encoding NADPH:Pchlide reductase previously reported in monocots (Apel, 1981; Kay and Griffiths, 1983), young, rapidly expanding leaves of light-grown pea seedlings accumulated significant amounts of the NADPH:Pchlide reductase mRNA. As shown in Figure 1C, the primary leaves of young, 1- to 2-d-old light-grown seedlings accumulated NADPH:Pchlide reductase mRNA as rapidly as the primary leaves of dark-grown seedlings. In later developmental stages, the youngest rapidly growing leaves of 8-d-old seedlings (leaf 1 and 2, d 8) contained higher steady-state levels of NADPH:Pchlide reductase mRNA than the analogous leaves from seedlings at later developmental ages (leaf 1 and 2, d 10 and 14, respectively). Thus, similar to the situation observed for ALA dehydratase and PBG deaminase mRNA levels, the abundance of NADPH:Pchlide reductase mRNA was found to be highly correlated with leaf developmental age, so that as the leaf expansion occurred in light-grown seedlings, mRNA levels peaked and then declined to nearly undetectable levels.

As a control for our studies, we monitored the steady-state mRNA levels of various gene families with well-characterized photoregulated and constitutive expression characteristics (Barkardottir et al., 1987) under these same developmental conditions. As shown in Figure 1, A and C, mRNA encoding the small subunit of ribulose-1,5-bisP carboxylase was nearly undetectable in the leaves of dark-grown seedlings at all developmental ages but accumulated to extremely high levels in leaves of seedlings grown in the light. In contrast, mRNAs encoding the β subunit of the mitochondrial F_1-ATPase or actin showed little change in relative abundance regardless of developmental age or light availability (data not shown). On the basis of these data it appears unlikely that the increased abundance of ALA dehydratase, PBG deaminase, and NADPH:Pchlide reductase mRNA is the result of a general increase in RNA polymerase activity during these early stages of seedling development, but rather that it occurs by a selective and coordinated increase in transcription of these gene families.

Light and Developmental Effects on Protein Levels

To determine whether the changes in the steady-state levels of mRNAs encoding ALA dehydratase, PBG deaminase, and NADPH:Pchlide reductase resulting from interaction of light and developmental age were accompanied by corresponding changes in enzyme abundance, extracts of total protein prepared from the various leaf tissues of dark- and light-grown seedlings were analyzed by western blot analysis using polyclonal antiserum directed against either NADPH:Pchlide reductase (Darrah et al., 1990; Spano et al., 1992a) or ALA dehydratase (Boese et al., 1991).

As shown in Figure 3, during the early stages of seedling development in the dark (i.e. 1–8 d postgermination), the level of NADPH:Pchlide reductase enzyme protein increased with the increased age of the leaf. At the earliest developmental stages (1–2 d postgermination) there appeared to be a slight uncoupling of the rates of mRNA and protein accu-

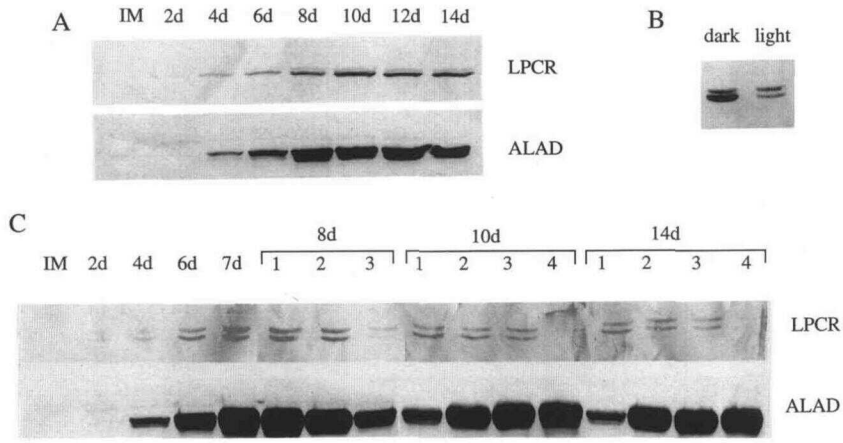

Figure 3. Western blot analysis of NADPH:-Pchlide reductase and ALA dehydratase protein levels in pea seedlings of various developmental ages grown in either light or dark. Equivalent amounts of total protein (100 μg) from leaves of various developmental stages as described in the legend to Figure 1 were fractionated by SDS-PAGE, transferred to nitrocellulose, and reacted with antibodies specific for either the NADPH:Pchlide reductase or ALA dehydratase as described in "Materials and Methods." A, Western blots of extracts from dark-grown seedlings. B, Western blots of extracts of total protein (100 μg) from the leaves of 6-d-old dark- and light-grown seedlings fractionated on the same gel for comparison of the abundance of the two immunoreactive polypeptides, M_r 36,000 and M_r 38,000. C, Western blots of extracts from light-grown seedlings.

mulation. For example, in leaves of dark-grown seedlings 1 d postgermination, the level of NADPH:Pchlide reductase mRNA was approximately 40% of the maximum level of expression, whereas only a trace amount of NADPH:Pchlide reductase protein could be immunodetected.

In contrast to the observed negative effect of increased leaf developmental age on mRNA abundance (e.g. steady-state levels of mRNAs encoding NADPH:Pchlide reductase were 8.4% of the maximum level of expression at 14 d postgermination), the level of immunoreactive protein remained high throughout the later stages of seedling growth. The levels of immunoreactive NADPH:Pchlide reductase protein were comparable among 8-, 10-, 12-, and 14-d-old dark-grown leaves. The sustained levels of NADPH:Pchlide reductase protein in dark-grown tissue likely reflect the stability of this enzyme in the ternary complex with its substrates (NADPH and Pchlide) in the absence of light.

The negative effects of light and developmental age on NADPH:Pchlide reductase protein levels could be observed most easily in our examination of enzyme protein levels in the leaves of light-grown seedlings. At the earliest stages of light-grown seedling development, increased protein levels paralleled increased mRNA levels. There was, however, a slight lag observed in protein accumulation in the dark-grown seedlings. In 8- to 14-d-old light-grown seedlings, protein levels were inversely correlated with leaf developmental age, similar to the previously noted inverse relationship between leaf developmental age and steady-state mRNA levels. Thus, as shown in Figure 3, very young, rapidly growing leaves contained the highest levels of immunodetectible NADPH:Pchlide reductase protein, whereas in the oldest or most fully expanded mature leaves (corresponding to leaf 3 and 4 in d 8 to 14 postgermination) the amount of immunodetectible NADPH:Pchlide reductase protein was substantially diminished. This dramatic decrease in immunodetectible NADPH:Pchlide reductase protein with increased leaf age likely results not only from the decreased mRNA levels (as shown above) but also from increased protein turnover in the light (Kay and Griffiths, 1983).

Consistent with our previous observations (Spano et al., 1992a), two immunoreactive forms of NADPH:Pchlide reductase having M_r values of 36,000 and 38,000 were detected in our western blots of total protein extracts from leaves of both dark- and light-grown seedlings at all developmental stages (Fig. 3). In the leaves of dark-grown seedlings, the M_r-36,000 polypeptide was consistently more abundant than the M_r-38,000 polypeptide, whereas the two polypeptides were equally present in the extracts from leaves of light-grown seedlings (Fig. 3C). The M_r-38,000 polypeptide also has a relatively higher abundance in older versus younger leaves of light-grown seedlings, and in the oldest leaves of 12- to 14-d-old light-grown seedlings the M_r-36,000 polypeptide is completely absent but the M_r-38,000 polypeptide can still be detected. The basis for these two immunological forms and their relationship to each other remain to be determined.

The effects of light and leaf developmental age on ALA dehydratase protein levels is similar but not identical to that observed for NADPH:Pchlide reductase. At the early stages of seedling development ALA dehydratase protein levels increased with increased developmental age regardless of whether the seedlings were grown in the light or darkness. As the seedlings matured the levels of protein remained high in the leaves of both dark- and light-grown plants. Even in the oldest leaves (Fig. 3, leaf 4, d 10 and 14) significant amounts of immunoreactive protein were detected despite the fact that in these leaves the level of transcript encoding ALA dehydratase was extremely low. Although the steady-state levels of ALA dehydratase mRNA were slightly higher

in the dark than in light, the protein levels were significantly lower in the dark.

Accumulation of Pchlide Is Coincident with Gene Expression during Development

During development etioplasts accumulate Pchlide fluorescing at 633 to 636 nm and 650 to 657 nm. The two peaks represent a mostly nonphototransformable short-wavelength form denoted $Pchlide_{636}$ and a completely phototransformable long-wavelength form designated $Pchlide_{657}$ (Virgin, 1981). The long-wavelength Pchlide is believed to be in a ternary complex formed by NADPH:Pchlide reductase, Pchlide, and NADPH, whereas the short-wavelength form corresponds to Chlide outside the ternary complex (Oliver and Griffiths, 1982). The accumulation of the two forms of Pchlide was measured during seedling development by their 77 K fluorescence emission spectra. Pchlide was not detectable until 24 h after imbibition (Fig. 4A), and the first detectable form was the short-wavelength form, $Pchlide_{636}$. Long-wavelength $Pchlide_{657}$ appeared approximately 48 h after imbibition and its appearance coincided with the first immunodetectable accumulation of NADPH:Pchlide reductase protein. As the leaves developed, both forms of the Pchlide accumulated to relatively high levels, as indicated by the changes in relative fluorescence intensity shown in Figure 4, A and B. The ratio of $Pchlide_{636}$:$Pchlide_{657}$ remained high in the leaves of dark-grown seedlings up to 4 d after imbibition, indicating that the major form of Pchlide at these developmental stages was the short-wavelength form (Fig. 4C). The ratio of $Pchlide_{636}$:$Pchlide_{657}$, however, decreased dramatically from a value of 3.0 at 5 d postgermination to a value of 1.4 at 8 d postgermination. The low $Pchlide_{636}$:$Pchlide_{657}$ ratios in the older leaves are indicative of the presence of large amounts of NADPH:Pchlide reductase protein at these stages and are consistent with our data on the time course of NADPH:Pchlide reductase accumulation.

Leaf Developmental Age, Not Light, Primarily Controls NADPH:Pchlide Reductase Levels

To examine further the interaction between light and leaf developmental age in the control of NADPH:Pchlide reductase gene expression, quantitative slot-blot analysis of NADPH:Pchlide reductase mRNA levels was carried out on total RNA isolated from pea seedlings grown for various lengths of time in total darkness and subsequently transferred into the light and grown for either 24 or 48 h under continuous white-light illumination. The results of these analyses are presented in Figure 5.

When 2-d-old dark-grown seedlings were transferred to light, the steady-state levels of mRNA encoding NADPH:Pchlide reductase increased in the "greening" primary leaves after 24 and 48 h of illumination. The mRNA levels in the leaves illuminated for 24 and 48 h were, however, lower than those found in the leaves of seedlings maintained for the same amount of time in constant darkness, suggesting that light slightly inhibited a developmentally controlled increase in NADPH:Pchlide reductase mRNA. Similarly, leaves of 6-d-old dark-grown seedlings illuminated

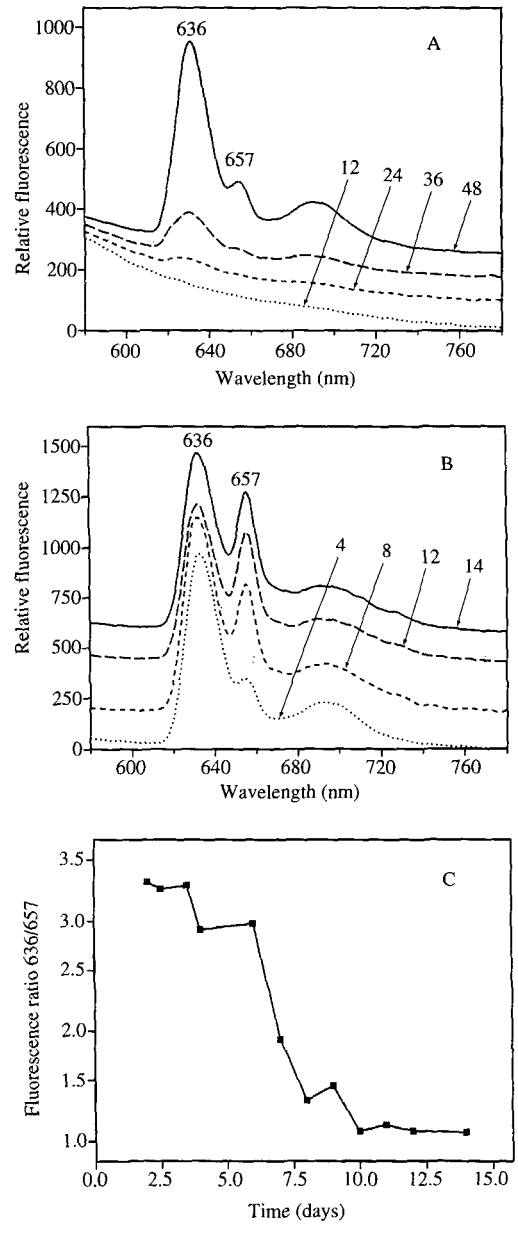

Figure 4. Low-temperature fluorescence emission spectra taken from leaves of dark-grown pea seedlings at different stages of development. Fluorescence emission spectra were measured with an excitation wavelength of 440 nm and corrected for the intensity variations of the excitation light and for variation in sensitivity of the photomultiplier. All spectra shown are mean values of three to six separate measurements made on tissue from 3 to 10 different pea seedlings. The spectra shown in A and B are displaced for better readability. A, Spectra from leaves harvested from plants 12, 24, 36, and 48 h after imbibition. B, Spectra from leaves harvested from plants 4, 8, 12, and 14 d after imbibition. C, The intensity ratio of the 636- and 655-nm fluorescence emission peaks determined from fluorescence spectra measured on pea leaves of different ages.

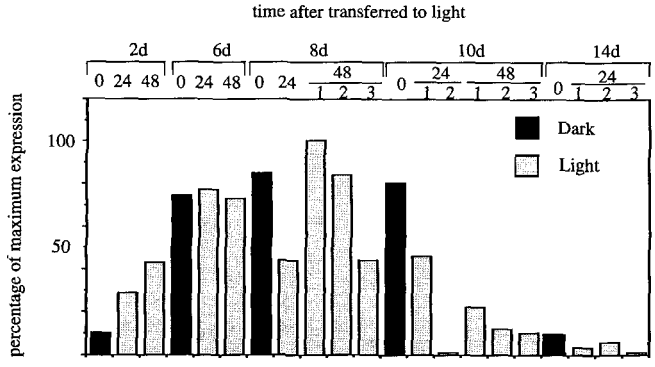

Figure 5. Effects of light on the steady-state levels of NADPH:Pchlide reductase mRNA in the leaves of dark-grown seedlings of different developmental ages. Pea seeds were allowed to imbibe for 12 h, planted in moistened vermiculite, and grown for 2 (2d), 6 (6d), 8 (8d), 10 (10d), or 14 d (14d) in total darkness. The etiolated seedlings were transferred into light and grown for either 24 or 48 h. Leaf tissues were harvested either in bulk (2d through 6d) or as individual leaves of different ages with the youngest leaf designated as 1 and the oldest as 4. Message abundance was quantitated as described in the legend to Figure 2.

for 48 h contained approximately 78% of the transcript level observed in the youngest leaves of 8-d-old dark-grown seedlings. Thus, at the earliest stages of seedling growth, developmentally controlled increases in transcript abundance compensate for any negative effect of light on transcript levels, resulting in a net increase on overall steady-state mRNA levels.

As the seedlings mature the negative interaction between light and developmental age becomes more pronounced. We have previously noted that the level of NADPH:Pchlide reductase mRNA decline with age of the leaves in 10- to 14-d-old dark- or light-grown seedlings. Consistent with this observation is the observation that when 10- or 14-d-old dark-grown seedlings are transferred to light, the combined negative effects of developmental age and light result in decreased steady-state mRNA levels in the leaves of 24 and 48-h illuminated versus dark-grown seedlings. In these experiments, more pronounced negative effects of light on NADPH:Pchlide reductase mRNA levels were correlated with increased age in individual leaf pairs excised from dark-grown seedlings at these later developmental stages.

Thus, as a result, the net increase in transcript abundance resulting from developmental effects diminishes and the negative effect of light results in a pronounced decrease in overall transcript abundance.

DISCUSSION

Dramatic changes occur during seed germination and postgermination development, and these changes are reflected in the expression of mRNA populations distinct from those observed in the embryo and mature plants (Thomas, 1993). During this developmental period, the basic architecture of the plant is established and numerous metabolic pathways become activated, including those leading to Chl and heme formation. Our studies demonstrate that the expression of gene families encoding enzymes involved in Chl formation are coordinately regulated during early seedling development. Furthermore, the expression of these genes during these early stages of development appears to be regulated primarily by endogenous factors acting independent of light, since the extent to which mRNA levels derived from the three genes increased was similar in both dark- and light-grown seedlings.

Axes of embryos that had been allowed to imbibe show different capacities for the expression of genes involved in tetrapyrrole formation. For example, mRNAs encoding both ALA dehydratase and PBG deaminase are present in such embryos at levels about 1.5% of their maximum level of expression. In contrast, mRNA encoding the NADPH:Pchlide reductase is barely detectable in these tissues. Within 24 h after imbibition, however, a significant level of NADPH:Pchlide reductase transcript can be detected. This subtle difference in timing of expression likely reflects the difference in requirement for the products of these genes. Both ALA dehydratase and PBG deaminase are involved in the synthesis of biosynthetic pathway intermediates common to Chl and heme formation, and their early expression likely reflects the immediate requirement by the cell for heme as chromophore of electron transport components and cofactor of enzymes involved in the mobilization of stored energy reserves for growth and differentiation. In contrast, gene families whose products are involved in the photosynthetic carbon fixation pathway or some other aspect of chloroplast metabolism are expressed later in development. For example, even as late as 4 d postgermination no *rbcS* transcript is detectable in the leaves of dark-grown seedlings and only about 10% of maximum expression is found in the leaves of light-grown seedlings (see below).

Evidence for a basal level of expression of some, but not all, of the enzymes unique to the Chl branch of the tetrapyrrole biosynthesis pathway comes from our observation that Pchlide accumulation can be detected almost immediately after imbibition. Interestingly, it is the short-wavelength form of the molecule, $Pchlide_{636}$, that accumulates first, with the long-wavelength form, $Pchlide_{657}$, not being detectable until 48 h after imbibition. Furthermore, the high ratio of $Pchlide_{636}$:$Pchlide_{657}$ in the leaves of dark-grown seedlings during the first 5 d postgermination indicates that the majority of the Pchlide formed at these early developmental stages is not bound in the ternary complex of NADPH:Pchlide reductase, Pchlide, and NADPH. The $Pchlide_{636}$:$Pchlide_{657}$ ratio decreased dramatically as NADPH:Pchlide reductase levels increased during leaf development, with the $Pchlide_{636}$:$Pchlide_{657}$ ratio reaching its lowest level coincident with maximum NADPH:Pchlide reductase mRNA and protein accumulation approximately 8 d postgermination. The molecular basis and physiological relevance of the lag between the accumulation of Pchlide and the ability of the plant cell to form the long-wavelength photoreducible ternary complex remains to be determined.

There is now substantial evidence indicating that a complex interaction exists between the nucleus and chloroplast genomes during development (Taylor, 1989). Although the basis of this interaction is still under investigation, it is clear that a high degree of regulation and coordination of plastid and nuclear gene expression occurs as undifferentiated plastids develop into photosynthetically competent chloroplasts

(Deng and Gruissem, 1987; Rapp and Mullett, 1991; Susek and Chory, 1992). As leaves grow and subsequently reach their maximum level of expansion, chloroplast maturation also reaches completion and the need for continued Chl synthesis decreases dramatically. It has been previously shown that the capacity for Chl synthesis both in vivo and in isolated organelles is related to plastid age, with mature or fully developed chloroplasts being less biosynthetically active than younger, less developed plastids (Castelfranco and Jones, 1975; Fuesler et al., 1984; Gomez-Silva et al., 1985; Beale and Weinstein, 1990). Here we have shown that expression of the genes encoding enzymes of Chl and heme formation also declined rapidly during leaf development. In fact, in the oldest leaves NADPH:Pchlide reductase mRNA levels were only 2% of those found in the youngest leaves of the same seedling. A similar trend was also observed for the levels of ALA dehydratase and PBG deaminase messages, although the extent of the decrease was much less, with the oldest leaves containing about 30% of the steady-state levels found in the youngest leaves of the same seedling.

There also appears to be a decrease in the capacity of mature plastids to import proteins from the cytosol. In wheat, for example, the import of photosynthesis-related proteins into plastids is light independent and declines during plastid maturation (Dahlin and Cline, 1991). Since all of the enzymes involved in Chl and heme formation examined thus far, including the three enzymes investigated here, are encoded in the nuclear genome, synthesized in the cytoplasm as higher mol wt precursors, and posttranslationally imported into etioplasts or developing chloroplasts, the decreased protein and mRNA abundance may also reflect feedback control resulting from a diminished capacity of mature plastids to import these proteins.

Light-independent developmental regulation has been observed in members of other nuclear and chloroplast gene families involved in the formation of the photosynthetic apparatus in plants. For example, activation of *rbcS* and *cab* gene expression in the first few days after germination and growth in total darkness has been observed in several dicot species (Walden and Leaver, 1981; Fiebig et al., 1990; Wanner and Gruissem, 1991; Brusslan and Tobin, 1992). In dark-grown monocots, similar developmental control has been observed for the *rbcS* and *cab* genes, but in these species leaf expansion proceeds at approximately the same rate in the light or dark (Dean and Leech, 1982; Mayfield and Taylor, 1984; Lamppa et al., 1985). In maturing barley and wheat seedlings, accumulation of *rbcS* and *cab* mRNA was found to decrease with increased age of the seedling, but could be stimulated by subsequent illumination (Lamppa et al., 1985; Barkardottir et al., 1987; Rapp and Mullet, 1991). Developmentally controlled light-independent changes in chloroplast gene transcription have also been observed that occur temporally and spatially concomitant to changes in expression of nuclear genes for photosynthesis-related proteins (Deng and Gruissem, 1987; Baumgartner et al., 1989; Fiebig et al., 1990; Rapp and Mullet, 1991). These studies indicate that the initial expression of chloroplast genes and some nuclear genes encoding photosynthesis-related proteins is coordinated with leaf development and that this regulation is independent of light.

The negative effect of light on NADPH:Pchlide reductase gene expression and protein accumulation in monocots is well documented (Apel, 1981; Santel and Apel, 1981; Schulz and Senger, 1993). The effects of light on NADPH:Pchlide reductase gene expression and protein accumulation in dicots and gymnosperms have been less clearly defined (Forreiter et al., 1990; Kittsteiner et al., 1990; Benli et al., 1991; Spano et al., 1992a, 1992b; Forreiter and Apel, 1993). In the leaves of grasses, such as barley and oat, a broad developmental gradient exists within a single leaf (Dean and Leech, 1982; Mayfield and Taylor, 1984; Lamppa et al., 1985), making it difficult to define the effects of light on NADPH:Pchlide reductase gene expression independent of leaf age. In the experiments described here we have been able to distinguish between light and developmental effects on the control of gene expression by carefully analyzing leaves of various developmental stages (ranging from the foliage leaves of embryos to the mature leaves of 14-d-old seedlings) from light- and dark-grown plants. We showed that early in leaf development light has only a small negative effect on the NADPH:Pchlide reductase gene expression, and that this negative light effect was overcome by developmentally programmed increases in gene expression. As the seedling matured, however, the effects of light on gene expression appeared to become more pronounced in the older leaves. In seedlings grown under continuous white light, NADPH:Pchlide reductase gene expression was essentially independent of light control and both mRNA and protein accumulated to high levels.

Our studies demonstrate that the expression of the genes encoding ALA dehydratase, PBG deaminase, and NADPH: Pchlide reductase and likely those genes encoding other enzymes of tetrapyrrole synthesis are regulated to a greater extent by developmental factors than by light. These findings provide a groundwork for a broader investigation of the endogenous physiological and molecular genetic factors operating to coordinate the developmental expression of the various gene families coding for the Chl and heme biosynthetic enzymes.

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