Organ-Specific and Light-Induced Expression of Plant Genes

ROBERT FLUHR, CRIS KUHLEMEIER, FERENC NAGY, NAM-HAI CHUA

Light plays a pivotal role in the development of plants. The photoregulation of plant genes involves recognition of light quality and quantity by phytochrome and other light receptors. Two gene families, *rbc*S and Cab, which code for abundant proteins active in photosynthesis, the small subunit of ribulose bisphosphate carboxylase and the chlorophyll a/b binding protein, show a 20- to 50-fold increase in transcript abundance in the light. Analyses in calli and transgenic plants of deletions of the *rbc*S gene and of chimeric constructions has allowed localization of two regions involved in light-induced transcription. One element is confined to a 33–base pair region surrounding the TATA box. In addition, an enhancer-like element contained within a 240–base pair fragment can confer phytochrome-induced transcription and organ specificity on nonregulated promoters.

THE DEVELOPMENT OF A HIGHER PLANT FOLLOWS A BROAD and plastic pathway. A plant can adjust internode length, alter leaf shape, realign leaves and stems, change its pigment content, and switch from vegetative to reproductive phase, all in response to one environmental cue—the quality and intensity of ambient radiation. Many of the photomorphogenic changes that take place during the plant's lifetime are triggered by alterations in gene expression. Some of these alterations can be monitored within a few minutes or hours and it is these relatively rapid events that lend themselves to analysis by molecular techniques.

The photomorphogenic process can be divided into light reception, signal transduction, and selective induction of gene expression (*1*). Only one photoreceptor, phytochrome, has been isolated thus far (*2*). This pigment exists in two forms: P_R and P_{FR}. In the dark, phytochrome is found mainly as P_R but it can be converted to the physiologically active P_{FR} form by a pulse of red light. P_{FR}, in contrast, can be reconverted to P_R by far-red illumination. In addition to phytochrome, other photoreceptors have been described; the most prominent ones absorb in the blue to ultraviolet region (*3*). Whereas nothing is known about transduction of the light signal and the intervening reactions leading to gene expression, light-regulated genes have been studied in great detail. Here we review the relevant literature and then present our recent experiments on the *cis*-acting elements that mediate the regulated expression of one of these genes.

Light Induction

Since light exerts such wide-ranging effects on various aspects of plant development, it is expected that the activities of a large number of plant genes are modulated by light [reviewed in (*3*)]. More than 40 enzymes have been reported thus far to increase in activity upon illumination of etiolated plants (plants germinated and grown in the dark) (*3, 4*). In 1978, Apel and Kloppstech extracted messenger RNA's (mRNA's) from etiolated and irradiated barley leaves and compared their in vitro translation products (*5*). With the aid of monospecific antibodies to the chlorophyll a/b-binding (Cab) protein, they showed that the level of translatable Cab mRNA is low in etiolated barley leaves but can be elevated by brief illumination with red light. The elevation by red light, however, could be reversed by far-red light, demonstrating for the first time that phytochrome regulates the translatable Cab mRNA level. Similar phytochrome control of translatable mRNA content was subsequently demonstrated for the ribulose bisphosphate carboxylase small subunit (*rbc*S) genes in *Lemna* (*6*) and in etiolated pea seedlings (*7*). These studies have been extended with complementary DNA (cDNA) probes to show that phytochrome increases the transcript abundance of the *rbc*S and Cab genes and also genes encoding at least ten other, as yet unidentified, polypeptides (*8*).

In higher plants, the *rbc*S and Cab transcripts are encoded by multigene families whose members show extensive sequence homology in their coding regions (*9*). To monitor phytochrome responses of individual members of a gene family, we have used gene-specific probes for pea *rbc*S genes and a wheat Cab gene to estimate the abundance of specific transcripts by S1 nuclease protection assays. We demonstrated that the expression of at least three members (3A, 3C, and E9) of the pea *rbc*S gene family (*10*) and of one wheat Cab gene (*11*) is controlled by phytochrome. Similar gene-specific probes have been used to demonstrate phytochrome control of individual *rbc*S genes in *Lemna* by dot-blot hybridization (*12*).

Although the *rbc*S and Cab transcripts, together with a number of unidentified transcripts, increase in abundance with a pulse of red light, the fluence rate dependence of these genes differs markedly (*13*). For example, the pea Cab mRNA is elevated by a low fluence rate of red light, whereas an increase of the *rbc*S mRNA content requires at least four orders of magnitude higher light fluence rate. Whether different light fluence rate requirements reflect alternative mechanisms of light activation remains to be elucidated.

The *rbc*S and Cab mRNA's belong to a group of transcripts that are present in low amounts in etiolated tissues and increase in abundance after red light activation of phytochrome. The opposite expression pattern is shown by the protochlorophyllide reductase mRNA (*14*) and mRNA for phytochrome itself (*15*), the transcript level being high in the dark and decreased by red light. It is intriguing how light can have such opposite effects on gene expression.

The photocontrol of mRNA abundance may be brought about by changes in the transcription rate or transcript stability or both. Gallagher and Ellis (*16*) compared transcription rates in nuclei

The authors are at Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021-6399.

isolated from etiolated pea leaves and from leaves greened by illumination with white light. They showed that white light-induced increases in *rbc*S and Cab mRNA's can be accounted for mostly by an increase in the transcription rates. This study has been refined by Silverthorne and Tobin (*17*), who showed that the transcription rate of *rbc*S and Cab genes in *Lemna* is increased by red light and that the enhancing effect can be abrogated by far-red light. Similar phytochrome control of transcription rate has also been observed for the barley Cab gene (*18*).

Previous work on phytochrome regulation of gene expression has been carried out mainly with etiolated plants (*3*). The use of nonchlorophyllous tissues avoids possible screening effects of red light and any complications that may arise from activation of photosynthesis. While mature green plants grown under a diurnal light-dark cycle do show light-induced gene expression, the photoresponses appear to be more complicated than those in etiolated tissues. When light-grown pea plants are placed in the dark for 3 days, their *rbc*S transcripts decline by at least 20 times (*10*). In contrast to etiolated plants, the *rbc*S transcripts in the dark-adapted, green plants are not elevated by a brief illumination with red light, but they can be restored to their initial level by continuous illumination with white light for 6 to 12 hours. We have shown that the effective wavelength for the white-light induction of the *rbc*S transcripts is in the blue region (*10*). Moreover, the enhancing effect of blue light can be reversed by far-red illumination. Similarly, in light-grown soybean seedlings, the light-activated transcription rate of *rbc*S genes can be decreased rapidly by a single far-red pulse (*19*). A slow response to blue light has been shown in a tobacco suspension culture, in which the level of translatable *rbc*S mRNA increases only after 6 to 12 days of continuous illumination (*20*).

On the basis of these results, we have proposed that the photoresponses of *rbc*S gene expression depend on the developmental stage of the pea leaves (*10*). Induction of *rbc*S mRNA in etiolated seedlings (that is, in immature non-green tissue) is clearly and exclusively a phytochrome-mediated response. In mature green tissues, the phytochrome level is reduced to a few percent of the level in etiolated tissues (*15*). Moreover, the remaining "green" phytochrome is immunologically distinct from the "etiolated" phytochrome (*21*). We suggested that regulation of *rbc*S mRNA levels in green plants is mediated by a blue photoreceptor in addition to phytochrome. A similar requirement for the cooperation between the two photoreceptors has been suggested for anthocyanin synthesis in etiolated seedlings of *Sorghum vulgare* (*22*). Whether these two photoresponses also operate to induce the transcription of Cab and other photoregulated genes in mature green tissues is not yet known.

Expression of *rbc*S Genes in Transformed Calli

Since changes in gene expression pattern appear to underlie many light-induced developmental events, the *rbc*S and Cab genes were used as paradigms to investigate the sequence of events leading to light-activation of transcription. To identify DNA sequence elements that mediate the photoresponses, tumor-inducing (Ti) vector systems were used to transfer mutant gene constructs into plant cells (*23*). RNA analysis revealed that the pea *rbc*S-E9 gene with 1052 bp of 5′ upstream sequence is expressed in transformed petunia cells (*24*). The *rbc*S-E9 transcript is 20 to 50 times more abundant in light-grown than in dark-adapted calli. Pea *rbc*S-E9 transcripts isolated from transformed petunia calli retain the authentic 5′ ends, appear to be spliced correctly, and are polyadenylated at the same site as in peas. Similar light-regulated expression has been reported for another pea *rbc*S gene, designated SS3.6 in tobacco calli (*25*), and a soybean *rbc*S gene (*26*) in soybean calli. In these experiments *rbc*S upstream regions were fused to bacterial coding sequences; it is not known whether transcription of these chimeric *rbc*S genes initiated at the correct site.

The retention of light-inducible expression of the pea *rbc*S-E9 gene in petunia calli argues for the use of this expression system to evaluate the transcriptional properties of hybrid and mutant gene constructs. In the first series of experiments, we showed that the *rbc*S-E9 5′ flanking fragment, from position −1052 to −2, was sufficient to confer light-inducible expression on a heterologous coding sequence (*27*). To localize 5′ sequence elements required for maximal expression and light-inducibility, we constructed a series of 5′ deletion mutants and an internal deletion mutant missing a putative CAAT box. We found that a mutant that retains only 35 bp of 5′ sequence is still responsive to light stimulation. These results, and the observation that the 5′ flanking fragment (−1052 to −2) is sufficient for light-regulated expression, led us to conclude that a 33-bp sequence, between −35 and −2, is involved in light-inducibility of the *rbc*S-E9 gene. Similar 5′ deletions of another *rbc*S pea gene (SS3.6) fused to bacterial coding sequences showed that a −90 deletion mutant retained a low level of light inducibility (*28*).

Expression in Transgenic Plants

Although transformed petunia or tobacco calli serve as a convenient assay system for foreign *rbc*S genes and their derivatives, the system is nevertheless subject to severe limitations. First, the elevation of *rbc*S mRNA after illumination of dark-adapted calli requires at least 5 to 10 days, depending on the species (*27, 28*). It is not known to what extent this slow induction mimics the much more rapid photoinduction in etiolated leaves. During this time, developmental changes in the structure of callus cells take place. Therefore, the effects of light and cellular differentiation on *rbc*S gene expression cannot be resolved. Second, a pulse of red light has no effect on *rbc*S mRNA levels in calli, thus precluding experiments on phytochrome-mediated responses (*11*). Third, calli transformed by oncogenic Ti vectors contain unusually high amounts of phytohormones, which may alter the photoresponses of the foreign *rbc*S genes (*29*). Fourth, calli grow heterotrophically, requiring a carbon source such as sucrose, which is known to suppress chloroplast development (*30*). Finally, it is impossible to evaluate organ or cell-type–specific expression of the introduced genes, since the transformed calli are nonmorphogenic.

These problems associated with transformed calli can be obviated if the expression of foreign genes is assessed in transgenic plants. Accordingly, we repeated our gene transfer experiments using a "disarmed" Ti vector, which does not interfere with plant regeneration (*23*). We found that the expression of the transferred *rbc*S-E9 gene is light-inducible and organ-specific (that is, high in leaves, low in stems, and almost undetectable in roots) in both transgenic tobacco and petunia (*31*). Therefore, the foreign *rbc*S-E9 gene with 1052 bp of 5′ upstream sequence contains all the requisite *cis*-acting regulatory elements for light-inducible expression in the appropriate organs. Moreover, the −352 deletion, which showed at least a tenfold decrease in expression in calli (*27*), exhibited wild-type activity in transgenic petunia plants (*31*). Thus, there is a major discrepancy between results obtained with transformed calli and transgenic plants. The reason for this difference may lie in the limitations of the calli system. For example, because of the elevated phytohormone levels additional upstream sequences may be necessary for full light induction of *rbc*S genes in transformed calli. Whatever the explanation for this discrepancy, it is obvious that for the reasons stated above, experiments on light-induction of intro-

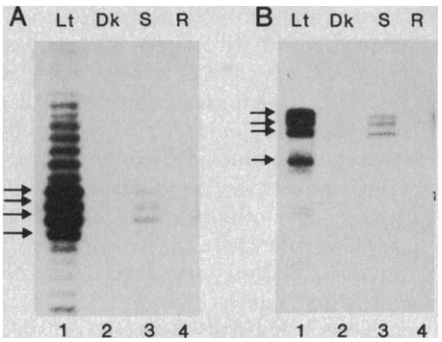

Fig. 1. Quantitative S1 nuclease analysis of the regulated expression of an endogenous *rbc*S gene and the pea *rbc*S-3A gene in transgenic *Nicotiana tabacum*. Total RNA was isolated from leaves (lane 1), stems (lane 3), and roots (lane 4) of plants grown in 16 hours light–8 hours dark cycles, and from leaves (lane 2) of plants placed in the dark for 3 days. (Panel A) The endogenous *N. tabacum rbc*S gene was probed using 3 μg of total RNA and a synthetic 68-base oligonucleotide extending from nucleotides (nt) 971 to 1038 of the tobacco gene NtSS23 (*74*). With this probe, the transcriptional start site of the tobacco NtSS23 gene was determined to be around 57 nt upstream of the initiation codon (*74*). (Panel B) The transferred gene from pea, *rbc*S-3A, was probed with a gene-specific 3′ end Hind III–Hinc II fragment (*10*) using 10 μg of total RNA. Hybridization conditions for the oligonucleotide probe were 42°C in a solution containing 1*M* NaCl, 60 m*M* sodium phosphate, 1 m*M* EDTA (*p*H 7.0), and for the 465-bp Hind III–Hinc II fragment were 37°C in a solution of 50% formamide, 0.4*M* NaCl, 2 m*M* EDTA, and 20 m*M* Pipes (*p*H 6.8). S1 nuclease digestion was performed as reported (*10*). Major S1 signals are indicated by arrows. Multiple bands of one nucleotide spacing are inherent to S1 techniques and represent either heterologous termini or incomplete protection at the termini. RNA was extracted from 0.5 to 5.0 g of tissues according to Kirk and Kirk (*75*), except that 0.5 m*M* aurintricarboxylic acid was added to the extraction medium as an inhibitor of ribonuclease (*76*). The 68-base oligonucleotide was synthesized with an Applied Biosystems Synthesizer (Model 380A). Abbreviations: Lt, light; Dk, dark; S, stems; R, roots.

duced genes should be carried out with transgenic plants rather than transformed calli.

We have characterized two other members (3A and 3C) of the pea *rbc*S gene family. The high-level expression of the *rbc*S-3A and -3C genes as compared to the *rbc*S-E9 gene, and their regulation by phytochrome (*10*), render them attractive for investigations on the molecular mechanisms of phytochrome response. When the pea *rbc*S-3A and *rbc*S-3C genes were transferred into petunia, we were able in both cases to reconstruct in immature buds and etiolated leaves of transgenic petunia phytochrome-mediated changes in the transcript level. In mature transgenic petunia, we found that the *rbc*S-3C gene is modulated by a blue photoreceptor in addition to phytochrome, as is the case in mature pea seedlings. These experiments show that the molecular mechanism of gene regulation by phytochrome and blue light is conserved among these plant species, opening the way for defining the phytochrome and blue photoresponses in molecular terms.

In addition to *rbc*S genes, pea and petunia Cab genes have been introduced into transgenic plants (*32, 33*). In the case of the pea gene, 400 bp of upstream region were sufficient to direct light-regulated and organ-specific expression of a bacterial coding sequence. The mechanisms for light regulation and phytochrome-mediated transcription appear to be conserved between monocots and dicots. A wheat Cab gene retains photoinducible expression in leaves of both transgenic petunia and tobacco (*34, 35*). We have constructed a chimeric gene comprised of a 1.8-kb 5′ flanking fragment of the wheat Cab gene fused to the coding sequence of chloramphenicol acetyltransferase (CAT). The level of the Cab-CAT transcript in transgenic petunia is elevated by red light and repressed by far-red light, demonstrating phytochrome control of the wheat Cab regulatory sequences in petunia (*36*).

Clonal Variation in Expression of Foreign Genes in Transgenic Plants

Early studies of chromosomal translocations in *Drosophila* showed that the activities of genes were sometimes drastically altered when transferred to new chromosomal positions (*37*). Therefore, it was not surprising to find that the activity of genes introduced into *Drosophila* by P element transduction (*38, 39*) or transferred into mice (*40*) was also influenced greatly by their chromosomal insertion sites. In these transgenic organisms, the expression level of the inserted gene varies from one clone to another and the variation is not correlated with the gene copy number. To examine this phenomenon in plants, individual transgenic petunia containing one or two copies of the pea *rbc*S-E9 gene were analyzed (*31*). When the transcript level of this gene was compared to that of a linked NOS-nptII gene, a 50-fold variation in activity was obtained. None of the clones exhibited expression in inappropriate tissues, as has been reported for the rabbit β-globin gene in transgenic mice (*41*). A 200-fold variation in transcript level was reported for the expression of a chimeric gene, comprising the petunia Cab 5′ flanking fragment fused to the coding sequence of octopine synthase (OCS), in transgenic petunia (*33*). The Cab-OCS chimeric gene was linked to the nopaline synthase (NOS) gene and the two promoters were

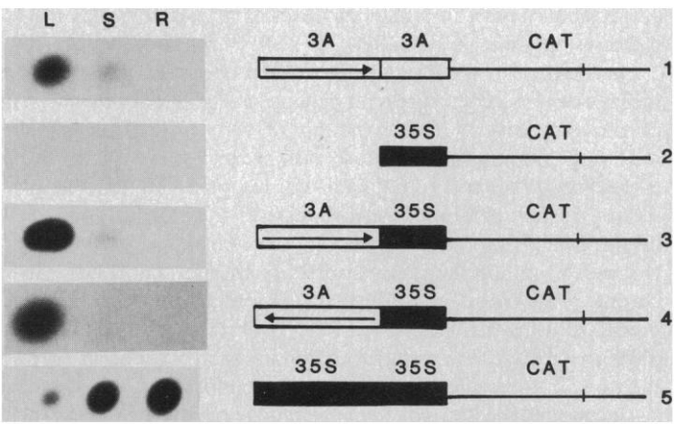

Fig. 2. Upstream enhancer-like sequence element in the pea *rbc*S-3A gene that directs organ-specific expression. CAT activity was assayed essentially as described (*77*). Protein (5 μg) was incubated in a volume of 46.5 μl with 1 nmol of [^{14}C]chloramphenicol (0.05 μCi) for 1 hour at 37°C. Thin-layer chromatograms were autoradiographed overnight. The 3-acetyl-chloramphenicol spots are shown. The reporter gene was the bacterial CAT gene fused to the *rbc*S-E9 3′ polyadenylation sequence (*27*). The following elements were placed upstream: (1) *rbc*S-3A upstream region from −410 to +15; (2) CaMV 35*S* promoter from −46 to +8 (*49*); (3) *rbc*S-3A upstream region from −327 to −48, fused to CaMV 35*S* promoter from −46 to +8; (4) as (3) but with the *rbc*S-3A element in the reversed orientation; (5) full CaMV 35*S* promoter fragment from −941 to +8 (*54*). The low abundance of many major leaf proteins, for example ribulose-1,5-bisphosphate carboxylase, in stems, and their absence in roots, biases the determination of CAT activity on a total soluble protein basis. The product from a constitutively expressed gene will make up a larger portion of the soluble protein fraction in stems and roots than in leaves. Therefore, the ratio of CAT activities seen in construct 5 is what is expected from a constitutively expressed gene. Similar results were reported by Simpson *et al.* (*34*). These hybrid genes were cloned into the polylinker region of pMON145, transferred to *Agrobacterium tumefaciens* GV3111SE, and transgenic *N. tabacum* var. *xanthi* plants were obtained after leaf disc transformation (*23*). The results shown for each construct were based on analysis of three or more independent transgenic clones. Abbreviations: L, leaves; S, stems; R, roots; 35S, 35*S* promoter fragment from CaMV; CAT, chloramphenicol acetyltransferase.

separated by about 10 kb. Jones *et al.* (*33*) found that the OCS and NOS enzymatic activities varied independently among the petunia transgenic clones. The cause for clonal variation among transgenic organisms is unknown. It may be due to insertion into a heterochromatic region (*37*), stochastic DNA methylation (*42–45*), or the inclusion of prokaryotic vector sequences (*41, 46*).

Despite certain problems associated with position effects, the definitive test of tissue-specific expression of *cis*-acting genetic elements can be carried out only in transgenic animals or plants. Deletion analysis of genes has shown that specific expression in transgenic mice requires 213 bp of the 5′ flanking region of the elastase I gene (*47*) and only 48 bp of 5′ flanking sequence of the human β-globin gene (*46*). In transgenic plants, upstream regions of 352 bp (*rbc*S-E9) (*31*), 410 bp (*rbc*S-3A) (*10*), or 400 bp (Cab-AB80) (*32*) have been shown to be sufficient for photoregulation and organ-specificity.

An Enhancer-Like Element for Regulated Expression

Figure 1 shows the expression in transgenic tobacco of a pea genomic fragment containing the *rbc*S-3A gene with only 410 bp of 5′ flanking sequences. The pattern of expression of the transferred pea gene (Fig. 1B) closely mimics the expression of the endogenous tobacco gene (Fig. 1A) with respect to light-inducibility and organ-specificity. The *rbc*S-3A transcript level based on total RNA is approximately the same as that found in pea plants. Similar results were obtained with a deletion mutant of the *rbc*S-E9 gene with its end point at position −352 (*31*). These results suggest that 350 bp of *rbc*S sequence upstream of the S1 nuclease-defined start site is sufficient for light-induced expression in the appropriate organs.

An *rbc*S-3A 5′ flanking fragment, from −410 to +15, is sufficient to direct organ-specific expression of the bacterial chloramphenicol acetyltransferase (CAT) coding sequence (Fig. 2, lane 1). Moreover, we found that the CAT mRNA is regulated by light. Previous analysis with the *rbc*S-E9 gene showed that 5′ deletion from position −352 to −35 has little effect on the already low expression level in transformed calli (*27*). In contrast, a similar deletion leads to a drastic decrease in expression level of the *rbc*S-E9 and *rbc*S-3A gene in transgenic plants (*48*). Therefore, we decided to investigate the function of the deleted upstream fragment (−327 to −48) independently by making use of the CaMV 35*S* promoter, which is insensitive to light (*49*). The 35*S* promoter is active in all three organs examined, but its activity in leaves is the lowest on a total protein basis (Fig. 2, lane 5). Deletion of the promoter to position −46 reduces its transcription rate by at least 20 times (*49*), such that little CAT activity (Fig. 2, lane 2) or RNA (Fig. 3B, lanes 1 and 2) is detected. This truncated 35*S* promoter was used to test the function of the *rbc*S upstream fragment. When the *rbc*S-3A gene upstream fragment, from −327 to −48, which does not include the TATA box region (Fig. 4), is fused to the truncated 35*S* promoter followed by the CAT coding sequence, there is a preferential increase of the CAT enzymatic activity in leaves of the transgenic plants (Fig. 2, lane 3). These results demonstrate that the *rbc*S-3A 5′ upstream fragment is able to direct organ-specific expression of the truncated 35*S* promoter.

Cochran and Weissmann (*50*) constructed mosaic promoters with the 5′ upstream region of the herpes simplex virus thymidine kinase gene and the rabbit β-globin TATA box. They found that the mosaic promoters retain the transcription start site of the β-globin gene. These results are consistent with the notion that the transcription start site is fixed by the position of the TATA box (*51*). To see whether the *rbc*S-3A 5′ upstream fragment has any effect on transcription initiation of the 35*S* promoter, we performed 5′ S1 nuclease protection assays using RNA preparations from transgenic tobacco plants containing either construct 3 or 5 (Fig. 2). Figure 3A shows that the S1 start site for the hybrid construct 3A-35*S*-CAT (Fig. 2, construct 3) is approximately six nucleotides upstream of that of the full 35*S* promoter (*49*).

The stability of the CAT activity prevented us from using this enzymatic assay to compare the transcription activity of the 3A-35*S*-CAT hybrid construct (Fig. 2, construct 3) between light-grown and dark-adapted tissues. Therefore, we measured transcript levels directly by 3′ S1 nuclease protection assays. Figure 3B shows that this *rbc*S-3A upstream fragment increases CAT transcript level in the light (compare lanes 2 and 4) but not in the dark (compare lanes 1 and 3). The effect is reversible since the light CAT mRNA level is reestablished upon illumination. It should be emphasized here that when the CAT gene is driven by a constitutive promoter, such as the NOS (*25*) or CaMV 35*S* (*10, 27, 49*) promoters, the CAT transcript levels are not influenced by light. Therefore, the accumulation of CAT transcript in the light (Fig. 3) must reflect an increase in the transcription rate.

Since many upstream regulatory elements of animal and yeast genes can function in a bidirectional manner (*52*), we examined the effect of the 3A upstream fragment in the opposite orientation. Indeed, this fragment also confers light-induced transcription (Fig. 3B, lanes 5 and 6) and organ-specific expression (Fig. 2, lane 4) when placed in an inverted orientation in the hybrid construct. Therefore, the *rbc*S-3A upstream element has the properties expected of a light-inducible transcription enhancer (*52*). Experiments with the *rbc*S-E9 gene also demonstrate that a similar 5′ upstream region (−317 to −82) can confer light-induced transcription in either orientation on another constitutive promoter, the nopaline synthase promoter (Figs. 3C and 3D). This enhancement in tran-

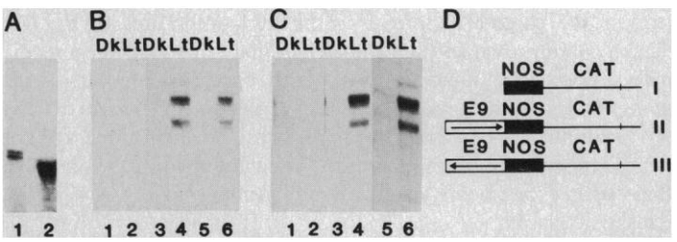

Fig. 3. Light-induced transcription conferred by *rbc*S upstream enhancer-like elements. Total RNA was extracted from leaves of transgenic plants maintained in a 16 hours light–8 hours dark cycle and harvested in the middle of the light period (panel A, lanes 1 and 2; panel B, lanes 2, 4, and 6; panel C, lanes 2, 4, and 6) or from plants kept in the dark for 3 days (panels B and C, lanes 1, 3, and 5). (Panels A and B) The chimeric genes depicted in Fig. 2 were transferred to tobacco and the transcripts were analyzed by quantitative S1 digestion. (Panel A, lane 1) Construct 3, 20 μg RNA; (lane 2) construct 5, 1.5 μg RNA, analyzed with a uniformly labeled probe extending from inside the CAT-coding sequence to 46 nt upstream of the 35*S* transcription start site (*49*). (Panel B, lanes 1 and 2) Construct 2; (lanes 3 and 4) construct 3; (lanes 5 and 6) construct 4. The CAT transcripts were analyzed by quantitative S1 digests with 20 μg of total RNA and a 642-bp gene-specific probe derived from the 3′ end of the *rbc*S-E9 gene (*53*). (Panel C) The chimeric genes depicted in panel D were transferred to petunia. (Lanes 1 and 2) Construct I; (lanes 3 and 4) construct II; (lanes 5 and 6) construct III. The CAT transcripts were analyzed as in panel B. (Panel D) The chimeric genes were assembled as follows: the nopaline synthase promoter fragment was isolated from pMON145 (*24*) as an Sst II–Pst I fragment extending from −150 to +33 and cloned in front of the CAT-coding sequence fused to the *rbc*S-E9 3′ polyadenylation sequence (*27*). The *rbc*S-E9 upstream region was isolated as a Bal I–Hinc II fragment, which extends from −82 to −317 (*53*), and inserted in front of the nopaline synthase promoter in both orientations. For each construct, the results were based on analysis of at least three independent clones. Abbreviations: Lt, light; Dk, dark; NOS, nopaline synthase promoter; CAT, chloramphenicol acetyltransferase.

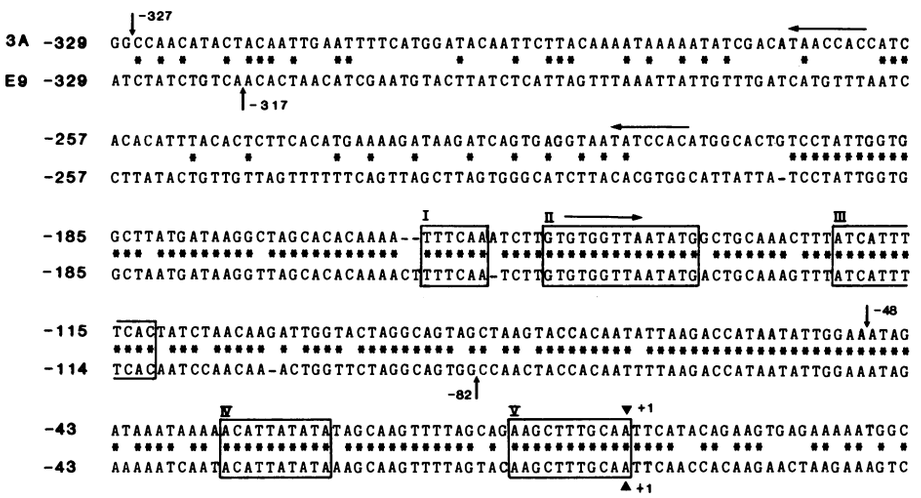

Fig. 4. Sequence comparison between the 5′ flanking regions of the pea *rbc*S-3A and E9 genes. Sequences from the pea *rbc*S-3A (*78*) and E9 (*53*) genes were aligned with the transcriptional start sites as reference points (filled triangles, labeled bases number 1). Identical bases are marked by asterisks. Boxes I through V are conserved among the four pea *rbc*S genes (*25, 53, 78*). In addition, sequences resembling Box II are also found in *N. tabacum* (*74*), *N. plumbaginifolia* (*79*), and soybean *rbc*S sequences (*13*) at similar positions with respect to the S1 start sites. Box IV contains the TATA box. Boundaries of the 5′ upstream enhancer-like sequences of the two genes are indicated by vertical arrows. Arrows overline sequence elements that resemble the SV40 enhancer core consensus sequence.

scription rate is detected primarily in leaves of the transgenic plants. These findings are consistent with the high degree of sequence conservation in the relevant regions of the two genes (Fig. 4). We note that the two enhancer-like elements are almost identical in their sequences until position −196; beyond that, the sequences diverge. Whether the divergent segment, from −196 to −330, is needed for one or more of the regulated functions remains to be established. Timko *et al.* (*28*) were the first to report the effects of a 900-bp fragment (from −973 to −90) from another *rbc*S gene (SS3.6) in either orientation on the expression of a nopaline synthase promoter-CAT chimeric construct. They found that the CAT activity is higher in calli grown continuously in the light than in calli grown continuously in the dark, showing that the ~900-bp fragment behaves like an enhancer element in the light-grown calli. In addition to the limitations that apply to transformed calli in general (see above), these experiments are further complicated by the fact that the dark-grown and light-grown calli are maintained on media with very different sucrose and cytokinin concentrations. It is hard to assess how much each of these variables contributes to the observed difference in CAT enzyme activity. Whether this fragment can function as an enhancer in rapid light induction is not known, since such experiments would require analysis of RNA levels in transgenic plants.

We have reported previously that the *rbc*S-3A gene is under phytochrome control both in pea and in transgenic petunia plants (*10*). To see if this effect could be mediated by the upstream enhancer-like region, we germinated seeds of a selfed transgenic tobacco plant containing the hybrid 3A-35S-CAT gene (Fig. 2, construct 3) in the dark and tested for phytochrome response. Figure 5 shows that the transferred hybrid gene responds to red-far-red treatment in a manner similar to the endogenous tobacco genes. Similar phytochrome regulation was also obtained with transgenic petunia plants containing a chimeric CAT gene driven by the *rbc*S-E9 enhancer-like element. Thus, a 240- to 280-bp fragment from either the *rbc*S-3A or the *rbc*S-E9 upstream region can confer phytochrome-induced transcription in addition to organ-specificity and white light regulation. We do not yet know whether these three regulatory phenomena are mediated by the same or overlapping sequence elements. Analysis of further deletions and mutations is needed to localize the regulatory elements more precisely and to determine whether light induction (blue photoresponse and phytochrome-mediated response) and organ-specific expression are mediated by the same element or elements. An inspection of seven *rbc*S genes points to a small conserved sequence GTGTGG$^{AAAA}_{TTTT}$ previously noted by us (*53*) at 150 bp upstream from the transcriptional start site (*54*). The similarity of this *rbc*S sequence to the SV40 core enhancer (*55*) and to the control sequence in yeast Ty-elements (*56*) lends support to the suggestion of its biological significance. However, this element probably does not function independently in mediating regulated expression since similar sequences are found in the CaMV 35*S* promoter (*54*), which is insensitive to light (*27*).

Conclusions and Prospects

Consideration of the results presented in this article, together with those published elsewhere (*24–28*), indicates that regulated transcription of *rbc*S genes is mediated by at least two, and maybe more, elements in the 5′ flanking region. A short conserved sequence surrounding the TATA box region is involved in light regulation in transformed calli (*27, 28*). This sequence from the *rbc*S-E9 TATA box region, and a similar promoter sequence from the *rbc*S-3A gene, can also confer photoregulated transcription in leaves of transgenic plants when constitutively expressed 5′ flanking regions of the CaMV 35*S* and 19*S* promoter are placed upstream (*57*). Independent of this promoter element, an upstream sequence can confer not only light- and phytochrome-responsiveness, but also organ-specific expression on two different heterologous promoters.

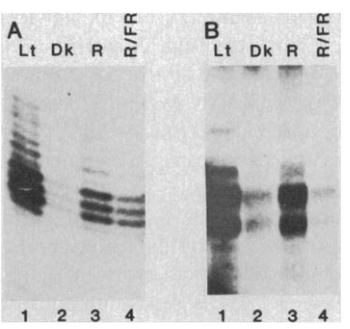

Fig. 5. The upstream enhancer-like element of *rbc*S-3A confers phytochrome-regulated transcription on a heterologous promoter. F_1 seeds from one of the selfed transgenic *N. tabacum* plants containing the 3A-35S-CAT hybrid gene (Fig. 2, construct 3) were surface-sterilized and germinated in the dark on plates containing Murashige and Skoog medium and sucrose (*23*). On the eighth day, plates were subjected to either continuous illumination with white light for 24 hours or to the light treatments specified, and then returned to the dark for 24 hours before RNA extraction. (Panel A) Quantitative S1-nuclease digests of 20 μg of total RNA using the *N. tabacum* 5′ probe specific for the endogenous *rbc*S gene (*74*). (Panel B) Quantitative S1 nuclease digest of 40 μg of total RNA using the *rbc*S-E9 3′ end probe specific for the chimeric CAT transcripts. (Lane 1) Continuous white light (1200 μE m^{-2} sec^{-1} for 24 hours); (lane 2) no light treatment; (lane 3) 3 minutes red light (R) (40 μE m^{-2} sec^{-2}); (lane 4) 3 minutes of red light followed by 12 minutes of far-red treatment (R/FR) (40 μE m^{-2} sec^{-1}).

Since this element functions in a bidirectional manner and at two different distances (positions −46 and −150) from the S1 nuclease-defined start site, it has the expected properties of a transcription enhancer. In analogy to other regulatory elements (*52*), we propose that this enhancer-like sequence contains one or more light-responsive elements (LRE's). Regulated transcription of *rbc*S genes may be the result of synergistic interaction between the promoter and the LRE's. Regulatory elements located both in the promoter region and elsewhere have also been reported for insulin and immunoglobulin genes (*58–60*).

The *rbc*S genes in higher plants are expressed most abundantly in leaves, moderately in stems, and at low levels in roots (*53*). Even in leaves, the expression is specific to certain cell-types; the *rbc*S polypeptide is detected only in mesophyll and guard cells but not in epidermal cells (*61*). These observations suggest that the *rbc*S genes may be active only in chloroplast-containing cells and that the organ-specific expression of these genes is simply a reflection of the proportion of this cell-type in the various organs. Here, we show that the enhancer-like elements of the two pea *rbc*S genes also confer organ-specific expression in transgenic plants. However, these analyses will have to be refined in the future. Specifically, it will be important to know whether these elements direct expression in the appropriate cell types of leaves and stems. The development of in situ hybridization of mRNA in tissue sections with cDNA probes and immunocytochemical staining of tissue sections with antibodies to CAT or other reporter gene products will extend the analyses to the cell level.

Further experiments are also needed to delimit the boundaries of this element and to define its critical nucleotides. With regard to the latter, the conserved GTGTGG$^{AAAA}_{TTTT}$ sequence, found in all *rbc*S genes characterized to date, may be an important component of the LRE. Based on results obtained with other regulated genes (*62*), we propose that light-induced transcription of *rbc*S involves the binding of one or more *trans*-acting factors (*63*) to the promoter element and to the LRE. The affinity of these two elements for the factors may determine, in part, the time course of light induction of various genes during greening, the differential responses of these genes to light intensities, and their different steady state transcript levels in the light. The activity of the LRE of pea *rbc*S genes appears to depend on the developmental stage of the plant. In etiolated leaves, a red light pulse triggers *rbc*S gene transcription that continues in the dark over a period of several days (*10, 64*). In contrast, the expression of *rbc*S genes in mature leaves is suppressed after the plants are returned to the dark (*10, 16, 65*). The mechanisms by which transcription is sustained in the dark after a red light trigger in immature leaves, but reversibly activated by light in mature leaves, are not known.

An important area of future research will be the identification of the intermediate steps leading from activation of the photoreceptor to induction of gene expression. The most rapid changes in light-induced transcription are monitored 20 to 30 minutes after illumination (*15*). Photoresponses, such as the leaf closure of *Oxalis oregana* (*64*), the opening of stomata (*66*), and the chloroplast rotation in the green alga *Mougeotia* (*67*), can be observed less than a minute after application of the light stimulus. These physiological changes most likely do not involve gene expression. The rapidity of such responses suggests modification of preformed factors rather than their de novo synthesis. It is possible that the transduction chain of these rapid events shares common pathways with those leading to gene activation. In the perception of light by vertebrates, GTP-binding proteins and cGMP-phosphodiesterase are implicated in the initial events of phototransduction (*68*). In other well-studied animal systems, phospholipid turnover, changes in Ca^{2+} levels, protein phosphorylation, and ubiquitination (*69*) have been shown to play a role in signal transduction. There is good evidence for a role of Ca^{2+} and calmodulin in mediating the phytochrome-regulated chloroplast rotation in *Mougeotia* (*67*). However, there is no direct experimental evidence linking the Ca^{2+}-calmodulin system to regulation of gene expression in plants. One can speculate that Ca^{2+}-regulated protein kinases (*70*) reversibly modify DNA binding proteins to increase their affinity for the LRE's. Such *trans*-acting factors are expected to be differentially distributed in etiolated versus green tissues. Their sequence-specific binding to the LRE may be monitored by deoxyribonuclease I footprinting (*71*), gel retardation assays (*72*), or exonuclease III protection assays (*73*). Functional characterization of the factors, however, would require the development of a soluble in vitro system that retains regulated transcription of light-responsive genes. The structural and functional analysis of these *trans*-acting factors will be an important step toward the elucidation of the complete sequence of events that enables light to regulate the expression of plant genes.

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