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Cis-acting elements for selective expression of two photosynthetic genes in transgenic plants

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The most abundant mRNAs in leaves of higher plants encode the small subunit (rbcS) of ribulose 1,5-bisphosphate carboxylase and the chlorophyll *a*/*b*-binding (Cab) protein. Nuclear genes for these mRNAs are expressed in an organ-specific manner and their expression is induced by light acting through phytochrome. We have used DNA sequence manipulation *in vitro* coupled with transgenic plant expression systems to define sequence determinants for regulated expression of these two photosynthetic genes. At least two *cis*-acting elements for the *rbcS* genes have been identified. A conserved sequence from the transcription start site to the 5′ boundary of the TATA box is involved in light-inducible transcription. In addition, an upstream enhancer-like element (240–280 base pairs) confers phytochrome responsiveness and organ specificity on constitutive promoters. An enhancer-like element (*ca.* 260 base pairs) in the upstream region of the wheat *Cab*-1 gene also shows similar functions.

Introduction

Light is one of the major environmental factors affecting plant development. Besides its role in photosynthesis, light triggers a diverse set of adaptive and morphological changes. One of the most dramatic effects of light is on leaf development. For example, pea seedlings germinated in the dark do not synthesize chlorophyll, their lives are unexpanded and plastic development is arrested at the stage of etioplasts. Upon illumination, chlorophyll synthesis is initiated, the leaves expand and concomitantly the etioplasts are converted to chloroplasts. During the photoconversion of etioplasts to chloroplasts there is rapid synthesis of chloroplast proteins in response to light. Most of the chloroplast proteins synthesized are encoded by nuclear genes, the most prominent of which are the small subunit (rbcS) of ribulose 1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxylyase (dimerizing), EC 4.1.1.39) and the chlorophyll *a*/*b*-binding (Cab) protein of the light-harvesting chlorophyll–protein complex. In addition to their light-inducibility, genes encoding these two proteins are expressed in an organ-specific manner, being most active in photosynthetic tissues. Our laboratory is interested in the mechanisms by which light turns on gene expression in specific tissues during plant development. We have chosen the *rbcS* and *Cab* genes as paradigms to investigate the details of the photoresponses and to define *cis*-acting elements that regulate light-inducibility and organ-specific expression. This paper summarizes recent results from our laboratory on the selective expression of the rbcS and Cab genes *in vivo* and in transgenic plants.

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Organ-specific expression of *rbcS* genes

The *rbcS* gene family of pea is composed of at least five members, which are linked and segregate as a single Mendelian unit (Polans *et al.* 1985). Our laboratory has isolated and characterized three members (*rbcS*-E9, -3A and -3C) (Coruzzi *et al.* 1984; Fluhr *et al.* 1986*b*), and Cashmore (1983) has determined the nucleotide sequences of two other members designated *rbcS*-3.6 and -8.0. Comparison of the nucleotide sequences revealed that all the five gene members are highly conserved in their coding regions but show considerable sequence differences in their 3′ untranslated portions. The 3′ sequence divergence among the gene members has allowed us to use 3′ nuclease mapping techniques to estimate their relative transcript abundance in different plant organs and also in response to light induction.

Northern blot hybridizations (Coruzzi *et al.* 1984) as well as 3′ S_1 nuclease analyses (Fluhr *et al.* 1986*b*) indicated that the *rbcS* gene family in pea is expressed in an organ-specific fashion. The highest *rbcS* transcript level occurs in leaves, where it is the most abundant mRNA. Pericarps contain about 40% of the leaf *rbcS* transcript level, petals and seeds about 10%, and stems about 5%. Little or no *rbcS* transcript is detected in roots.

Members of the *rbcS* gene family are expressed at different levels in different plant organs (Fluhr *et al.* 1986*b*). The *rbcS*-3A and -3C genes are most highly expressed in green leaves, accounting for 40 and 34%, respectively, of the total *rbcS* transcript in green leaves. The *rbcS*-8.0 gene contributes about 19% of the total leaf *rbcS* transcript, whereas the remaining 7% is accounted for by *rbcS*-E9 and -3.6 transcripts. The same pattern of specific *rbcS* transcript distribution is found in pericarps and stems. By contrast, seeds and petals contain a lower level of *rbcS*-E9, -3.6 and -8.0 transcripts than in the other organs.

Differential induction of specific *rbcS* transcripts by light

3′ S_1 nuclease analyses revealed that etiolated pea leaves contain low levels of *rbcS*-3A and -3C transcripts. These levels can be elevated to about the steady-state levels of mature green leaves after exposure to white light for 24 h. In contrast, the light-induced accumulation of *rbcS*-E9 and -8.0 transcripts in etiolated leaves shows a pronounced time lag and these transcripts are barely detectable even after 24 h of continuous illumination (Fluhr *et al.* 1986*b*). These two transcripts begin to accumulate only at a later developmental stage. The molecular basis of this differential response of specific *rbcS* transcripts to light is not known. However, it is likely to be related to the developmental stage of the leaves because all the *rbcS* transcripts show the same kinetics of accumulation when dark-adapted mature green leaves are re-exposed to light.

Effects of light quality on *rbcS* gene expression

The responses of the pea *rbcS* genes to light quality also depend on the developmental stages of the leaves (Fluhr & Chua 1986). In etiolated leaves the *rbcS* transcript levels (3A, 3C and E9) can be elevated by a flash of red light, and the enhancing effect can be reversed by far-red illumination. These results confirm and extend previous work (Tobin 1981; Sasaki *et al.* 1983; Thompson *et al.* 1983; Jenkins *et al.* 1983) that the expression of *rbcS* genes is controlled by phytochrome. On the other hand, an identical red-light flash has no apparent effect on the *rbcS* transcript abundance in mature pea leaves, although in these tissues the expression of the

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rbcS genes is induced by continuous white light. Comparison of the *rbcS* transcript levels in mature pea leaves, after continuous illumination with equal fluence rates of blue and red light, reveals that blue light is more effective. Because the enhancing effect of blue light can be nullified by far-red light the results suggest that in mature green leaves blue light also activates phytochrome in addition to a putative blue light photoreceptor, and maximal induction of the *rbcS* transcript level requires the cooperation of the two photoreceptors. Similar requirements have been noted for the induction of antocyanin synthesis in *Sorghum* seedlings (Oelmuller & Mohr 1985).

Cis-acting elements for regulated expression of *rbcS* genes

An immediate aim of our laboratory is to identify *cis*-acting elements for photoinduction of *rbcS* genes in etiolated and mature green leaves (Fluhr & Chua 1986). A related goal is to define DNA sequence elements that direct preferential expression of the *rbcS* genes in leaves and other photosynthetic tissues (Coruzzi *et al.* 1984; Fluhr *etal.* 1986*b*). As a first step we transferred into petunia two pea genomic fragments containing the *rbcS*-3A and -3C genes with 410 base pairs (bp) and 2 kilobase pars (kbp) of upstream sequence, respectively. We found that the two genes retain their responsiveness to phytochrome and blue light in the appropriate tissues of the transgenic petunia plants (Fluhr & Chua 1986). Moreover, the distribution of the pea *rbcS* transcripts in leaves, stems and roots of transgenic petunia reflects that in the pea plants. Similar phytochrome-mediated and organ-specific expression of the *rbcS*-3A and -3C genes can be recapitulated in etiolated F_1 seedlings of transgenic tobacco (R. Fluhr & N.-H. Chua, unpublished data). These results indicate that 410 bp of 5′ upstream sequence from the *rbcS*-3A gene is sufficient for the photoinduced expression by red and blue light and for organ specificity. Similar experiments with deletion mutants of the *rbcS*-E9 gene revealed that 352 bp of 5′ upstream sequence is sufficient for white light induction and organ-specific expression in transgenic petunia (Nagy *et al.* 1985).

To localize the *cis*-acting regulatory elements, a series of chimaeric genes were constructed and their expression evaluated in transgenic tobacco or petunia (Fluhr *et al.* 1986*a*). A 425 bp 5′ flanking fragment (from position −410 to +15) of *rbcS*-3A is sufficient to confer photoinducibility on the coding sequence of the bacterial chloramphenicol acetyl transferase (*CAT*) gene preferentially in leaves of transgenic plants. By the use of convenient restriction sites the active region was delimited to a 280 bp fragment (from −327 to −48) that can confer phytochrome responsiveness and leaf-specific expression on the TATA box of the constitutive 35*S* promoter of cauliflower mosaic virus (CaMV). This fragment behaves like a transcriptional enhancer because its activity is independent of its orientation with respect to the 35*S* promotor (cf. Gluzman 1983). Experiments with the *rbc*-E9 gene also uncover a similar 5′ region (−317 to −82) that confers phytochrome-induced transcription in either orientation on another constitutive promoter, the nopaline synthase promoter. This transcriptional enhancement 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 (figure 1). We note that Timko *et al.* (1986) have previously identified a 900 bp enhancer-like element (from −973 to −90) of the pea *rbcS*-3.6 gene, which confers regulated expression on the nopaline synthase promoter in transformed tobacco calluses. However, the activity of this element in transgenic plants has not been reported.

In previous work with transformed calluses as an expression system we have identified a

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3A  -329  GGCCAACATACTACAATTGAATTTTCATGGATACAATTCTTACAAAATAAAAATATCGACATAACCACCATC
E9  -329  ATCTATCTGTCAACACTAACATCGAATGTACTTATCTCATTAGTTTAAATTATTGTTTGATCATGTTTAATC

    -257  ACACATTTACACTCTTCACATGAAAAGATAAGATCAGTGAGGTAATATCCACATGGCACTGTCCTATTGGTG
    -257  CTTATACTGTTGTTAGTTTTTTCAGTTAGCTTAGTGGGCATCTTACACGTGGCATTATTA-TCCTATTGGTG

    -185  GCTTATGATAAGGCTAGCACACAAAA--TTTCAAATCTTGTGTGGTTAATATGGCTGCAAACTTTATCATTT
    -185  GCTAATGATAAGGTTAGCACACAAAACTTTTCAA-TCTTGTGTGGTTAATATGACTGCAAAGTTTATCATTT

    -115  TCACTATCTAACAAGATTGGTACTAGGCAGTAGCTAAGTACCACAATATTAAGACCATAATATTGGAAATAG
    -114  TCACAATCCAACAA-ACTGGTTCTAGGCAGTGGCCAACTACCACAATTTTAAGACCATAATATTGGAAATAG

    -43    ATAAATAAAAACATTATATATAGCAAGTTTTAGCAGAAGCTTTGCAATTCATACAGAAGTGAGAAAAATGGC
    -43    AAAAATCAATACATTATATAAAGCAAGTTTTAGTACAAGCTTTGCAATTCAACCACAAGAACTAAGAAAGTC
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FIGURE 1. Sequence comparison between the 5′ flanking regions of the pea *rbcS*-3A and -E9 genes. Sequences from the pea *rbcS*-3A (Fluhr *et al.* 1986*b*) and -E9 (Coruzzi *et al.* 1984) genes were aligned with the transcriptional start sites as reference points (filled triangles, labelled bases number 1). Identical bases are marked by asterisks. Boxes I–V are conserved among the four pea *rbcS* genes (Cashmore 1983; Coruzzi *et al.* 1984; Fluhr *et al.* 1986*b*). Box IV contains the TATA box. The 5′ upstream enhancer-like sequences of the two genes are delimited by vertical arrows. Horizontal arrows indicate sequence elements that resemble the SV40 enhancer core sequences sequence.

33 bp segment (from −35 to −2), including the TATA box, as being involved in light regulation of the *rbcS*-E9 gene (Morelli *et al.* 1985). To evaluate the role of this element in transgenic plants we assembled a chimaeric gene consisting of a 5′ upstream region (−941 to −40) from the CaMV 35*S* promoter, the −48 to −1 region of *rbcS*-3A, and the CAT coding sequence. The CAT mRNA from this transcription unit is induced 4–5 times by light in transgenic tobacco (C. Kuhlemeier, R. Fluhr, F. Nagy & N.-H. Chua, unpublished data). The sequence from the transcription start site (−1) to the 5′ boundary of the TATA box (*ca.* −48) is very well conserved between *rbcS*-3A and -E9. Our results show that this region also plays a role in light-induction in transgenic plants.

Trans-acting factors for *rbcS* genes

The 5′ upstream enhancer-like element probably interacts with one or more nuclear protein factors to regulate transcription of the *rbcS* genes. We have begun to investigate this protein–DNA interaction by the gel-electrophoresis DNA binding assay, which has been used recently to characterize prokaryotic and eukaryotic transcriptional regulatory factors (cf. Hendrickson 1985). Using poly(dI–dC) as a non-specific competitor DNA, we observed binding of the *rbcS*-3A enhancer-like element (from −327 to −48) to one or more factors present in nuclear extracts from mature pea leaves. The binding activity is unaffected by excess amounts of a CaMV 35*S* promoter fragment (from −300 to +8) but is greatly diminished

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by excess unlabelled 5′ upstream fragment from *rbcS*-3A. These results suggest that the binding activities we have observed are specific for the 5′ upstream regulatory element and that the interaction may be important for light regulation. DNA-footprinting experiments are in progress to locate the binding site more precisely.

Structure and expression of a wheat *Cab* gene

The *Cab* genes offer another interesting system to investigate mechanisms by which phytochrome regulates plant gene expression. mRNA encoding the Cab protein is a major component of the leaf mesophyll cell mRNA population (Broglie *et al.* 1981). The Cab mRNA level is very low in etiolated leaves but can be increased considerably after a brief flash of red light. The stimulating effect of red light can be reversed by far-red light (Apel 1979; Tobin 1981; Thompson *et al.* 1983; Jenkins *et al.* 1983), and far-red light alone also elicits a small increase in the Cab mRNA level (Kaufman *et al.* 1984). These modulating effects of red and far-red light are hallmarks of phytochrome regulation (cf. Tobin & Silverthorne 1985). A distinctive feature of *Cab* gene regulation by phytochrome is its very-low-fluence (VLF) response characterized by an exceptional sensitivity to very low intensities of red light. Kaufman *et al.* (1984, 1985) have shown that the *Cab* genes require a 10^4-fold lower fluence rate than the *rbcS* genes for their expression. Comparison of the sequence determinants for phytochrome responsiveness of the two photosynthetic genes may help to elucidate the molecular basis for this differential sensitivity to red light.

We isolated a wheat *Cab* gene, designated *Cab*-1 (previously referred to as *WhAB*1.6) that does not contain any intron and encodes a mRNA of 1100 nucleotides. This gene is expressed early during the development of wheat leaf but is inactive in roots (Lamppa *et al.* 1985*a*). Etiolated leaves contain negligible amounts of *Cab*-1 mRNA but the level can be increased by at least 20 times after 24 h of continuous white light (Lamppa *et al.* 1985*b*). The same level of *Cab*-1 induction can be obtained with just a 3 min pulse of red light. The red induction is largely but not completely reversed by far-red illumination, and far-red light alone also elicits a small increase in the *Cab*-1 mRNA level (Nagy *et al.* 1986*a*). These results provide evidence that the wheat *Cab*-1 gene shows a VLF response to phytochrome (Kaufman *et al.* 1984, 1985).

Cis-acting elements for regulated expression of the wheat *Cab*-1 gene

To investigate whether the wheat *Cab*-1 gene would be correctly regulated in a dicot we transferred a genomic fragment containing 4.4 kb of 5′ upstream sequence of the *Cab* gene into tobacco by using a Ti-mediated gene transfer vector. The transgenic tobacco plants were selfed and the seeds were germinated in the dark. After seven days the etiolated F_1 seedlings were subjected to various light treatments and the RNAs were analysed by 5′ S_1 nuclease protection. Our analyses showed that the wheat *Cab*-1 gene retains the VLF response to phytochrome in transgenic tobacco seedlings (Nagy *et al.* 1986*b*); moreover, it is expressed in an organ-specific manner (Lamppa *et al.* 1985*b*).

A number of chimaeric constructs and 5′ deletion mutants were analysed in an attempt to define *cis*-acting regulatory sequences of the *Cab*-1 gene. A 1.8 kbp fragment from the 5′ flanking region (position −1816 to +31) of the *Cab*-1 gene can confer phytochrome responsiveness and leaf-specific expression on the CAT coding sequence (Nagy *et al.* 1986*b*).

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On the other hand, when the CAT coding sequence is driven by the nopaline synthase (NOS) promoter, there are no significant changes in the CAT mRNA levels between dark and the different light treatments.

In a separate series of experiments a wheat *Cab*-1 gene containing 1.8 kbp of 5′ upstream sequence was found to respond correctly to phytochrome activation and be expressed at high levels in the appropriate organs of transgenic tobacco (F. Nagy, M. Boutry & N.-H. Chua, unpublished data). This gene was chosen as the wild-type from which a series of 5′ deletion mutants was generated by Bal31 nuclease digestion. The endpoints of the mutants were determined by sequence analyses. Eleven mutants with strategic breakpoints (at positions −1495, −1038, −801, −714, −654, −454, −354, −230, −180, −144 and −127) were assayed for the following properties: (1) induction of the *Cab*-1 transcript level in dark-adapted transgenic plants by white light; (2) organ-specific distribution of the transcript; and (3) phytochrome-controlled expression of the *Cab*-1 gene is etiolated transgenic seedlings. The three regulated functions are retained by all the deletion mutants including 5′ Δ-144 but not by 5′ Δ-127. In another experiment, we showed that a 5′ upstream fragment (from −354 to −90) of the *Cab*-1 gene can confer light-responsiveness on the constitutive CaMV 35*S* promoter. This fragment is also active in an inverted orientation and therefore functions like a transcriptional enhancer. Taken together, these results suggest that an important *cis*-acting regulatory element is located between −90 and −144 of the *Cab*-1 gene. Experiments are in progress to determine whether the *Cab*-1 promoter is also responsive to light.

Conclusions and prospects

Our genetic dissection of the *rbcS* genes so far has revealed at least two *cis*-regulatory regions involved in light-responsiveness. A short conserved sequence of the *rbcS* genes, extending from the transcriptional start site up to around the 5′ boundary of the TATA box, can confer photoregulated transcription in leaves of transgenic plants. In addition, there is an upstream segment (from *ca.* −320 to −90) that can confer not only light- and phytochrome-responsiveness but also organ-specificity on the CaMV 35*S* promoter and the nopaline synthase promoter. The upstream segment behaves like a transcriptional enhancer because it can activate transcription of the two heterologous promoters at two different distances from the S_1 start site; moreover, it retains its regulatory properties when placed in an inverted orientation. It is likely that regulated expression of the *rbcS* genes involves the interaction between the promoter element and the enhancer-like element upstream.

By analogy with other regulated genes (cf. Yamamoto 1985), we postulate that the *rbcS* upstream segment contains one or more sequence elements that mediate light-responsiveness. The consensus sequence of such a light-responsive element (LRE) has not yet been established but it may include the 'GT' sequence motif, which resembles the SV40 enhancer core sequence (cf. Gluzman 1983) and is located at approximately position −140 of all *rbcS* genes characterized so far (table 1). The relative affinity of the LRE and the promoter element for *trans*-acting factors may determine, in part, the time course of light induction of various genes during greening, the differential responses of these genes to light intensity, and their different steady-state transcript levels in the light. Likewise, the number of LREs in the 5′ upstream region and their locations relative to the promoter may similarly regulate the light-induced transcription rate.

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Table 1. Comparison of the 'GT' sequence motif in the −140 region of *rbcS* genes of higher plants

gene	sequence	reference
pea *rbcS*-E9	GTGTGGTTAATATG	Coruzzi *et al.* (1984)
pea *rbcS*-3A	GTGTGGTTAATATG	Fluhr *et al.* (1986*a*)
pea *rbcS*-3C	GTGTGGTTAATATG	Fluhr *et al.* (1986*a*)
pea *rbcS*-3.6	GTGTGGTTAATATG	Herrera-Estrella *et al.* (1984)
soybean *SRS*1	GTGTGGCCAATATA	Berry-Lowe *et al.* (1982)
tobacco *Ntss*23	GTGTGGATATTAAG	Mazur & Chui (1985)
N. plumbagini-folia *rbcS*-8B	GTGTGGATATTAAG	C. Poulsen (unpublished)
petunia *SSU*8	GTGTGGATATTAAA	Tumer *et al.* (1986)
petunia *SSU*11	ATGTGGCCATTAAT	Tumer *et al.* (1986)
consensus	GTGTGG$^{A}_{T}$TA$^{A}_{T}$TA$^{A}_{T}$	

We have found that expression of the *rbcS* genes in mature green leaves requires the activation of a blue photoreceptor in addition to phytochrome (Fluhr & Chua 1986). It is not known at present whether the two photoresponses are mediated by the same LRE. Similarly we need to investigate whether sequence determinants for light responsiveness and organ-specific expression are congruent.

The number of plant genes that are induced by light is not known, but is likely to include most, if not all, genes involved in photosynthesis. We now need to establish whether multiple *cis*-regulatory elements is a general feature for all light-inducible genes. Unfortunately, very few of these genes have been sequenced, and it is therefore not possible to determine if there is any sequence homology in the 5′ flanking regions. In the *Cab*-1 gene, two imperfect 'GT' sequence motifs exist in the upstream enhancer-like region centred on −120 and −180 (M. Boutry, unpublished data). However, the LRE of the *Cab* genes may have a different consensus sequence from that of the pea *rbcS* genes. We consider it unlikely that each light-inducible gene should have its own LRE, because this would lead to a multitude of *trans*-acting factors and genes encoding them. More probably the light-inducible genes will fall into a few separate classes and each class may have its distinct LRE and the cognate *trans*-acting factors. A major challenge in the future is to characterize these LREs and their sequence requirement, and elucidate the mechanisms by which these elements enhance transcription specifically in the light.

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