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The 5′-proximal region of the wheat Cab-1 gene contains a 268-bp enhancer-like sequence for phytochrome response

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We have previously reported that the expression of the wheat Cab-1 gene is subject to phytochrome regulation and a 1.8-kb 5′ upstream sequence of this gene is sufficient for the regulated expression. To delineate sequences for the phytochrome response we analyzed a series of 5′ deletion mutants as well as chimeric gene constructs comprising different sequences of the Cab-1 upstream region in transgenic tobacco seedlings. We found that a deletion mutant containing a 357-bp 5′ upstream sequence still exhibits maximal levels of phytochrome-regulated expression. A 268-bp enhancer-like element, located between −89 and −357, is responsible for the phytochrome response of the Cab-1 gene; sequences upstream from −357 to −843 and downstream from −124 to +1100 are probably not involved. Finally, we show that the Cab-1 mRNA stability is not regulated by phytochrome.

Key words: chimeric genes/transgenic tobacco/transcription regulation/transcript stability

Introduction

Higher plants have developed several signal transducing systems to sense changes in environmental conditions. One such system, which responds to fluctuations in ambient light quality and fluences, has as its receptor a pigment known as phytochrome (Shropshire and Mohr, 1983). This photoreceptor can exist in two interconvertible forms: P_R which absorbs red light and P_{FR} which absorbs far-red light. In the dark, phytochrome is synthesized as P_R, the physiologically inactive form. Upon irradiation with red light, it is rapidly activated to P_{FR} which initiates a large number of developmental events from seed germination to flowering. The developmental programmes triggered by P_{FR} can be blocked if the red light is followed immediately by far-red light which converts P_{FR} back to the inactive P_R. This photoreversibility, i.e. activation by red light and reversion by far-red light, is the hallmark of phytochrome action.

The morphogenetic consequences elicited by phytochrome most likely involve changes in gene expression. Recent studies have indicated that plant genes can be regulated by phytochrome in diametrically opposite manners. Whereas mRNA levels for the small subunit of ribulose-1,5-bisphosphate carboxylase (rbcS) (Tobin, 1981) and the chlorophyll *a/b*-binding protein (Cab) (Apel and Kloppstech, 1978) are increased by red light and decreased by far-red light, the reverse is true for the mRNA levels of phytochrome (Colbert *et al.*, 1983) and protochlorophyllide reductase (Apel, 1981; Batschauer and Apel, 1984). Irrespective of the mode of regulation, accumulated evidence indicates that transcriptional control is likely to be a major component (Silverthorne and Tobin, 1984; Mosinger *et al.*, 1985). Phytochrome control of plant gene expression is further complicated by the great diversity of fluence responses (Kaufman *et al.*, 1986). For example, the Cab genes are exquisitely sensitive to red light and require 1000 times less fluence rate for activation as compared with the rbcS genes (Kaufman *et al.*, 1984).

Our laboratory is interested in delineating the sequence of events leading from light reception by phytochrome to the activation of target gene transcription. So far, we have focused primarily on the terminal step of the signal transduction pathway, namely the identification of *cis*-acting DNA sequences that mediate phytochrome-induced transcription. To explore the molecular basis for the different fluence requirement, we have investigated the pea rbcS genes (Coruzzi *et al.*, 1984; Fluhr *et al.*, 1986b) and the wheat Cab-1 gene (Lamppa *et al.*, 1985) as examples of genes showing low fluence and very low fluence

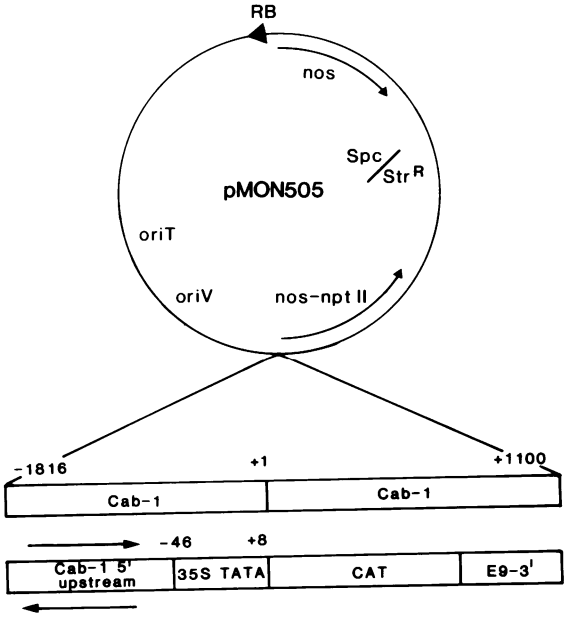

Fig. 1. A schematic diagram of the pMON505 binary vector with the inserted chimeric genes. (1) A 2.9-kb wheat genomic fragment containing the entire wheat Cab-1 gene as a *ClaI*–*ClaI* insert which includes 1816-bp 5′ upstream sequences, the coding sequence and the 3′ non-coding region. 5′ Deletion mutants of the wheat Cab-1 gene were cloned into the same vector as discussed in Materials and methods. (2) Different 5′ upstream regions of the Cab-1 gene were fused in two orientations to a chimeric gene containing the 35S TATA box, the bacterial CAT gene followed by the polyadenylation sequence of the pea rbcS–E9 gene. Details of the 35S–CAT–rbcS–E9 construction were given in Kuhlemeier *et al.* (1987). The orientations of the inserted genes relative to the *nos–nptII* gene are shown. The binary cloning vector pMON505 has been described in detail by Horsch and Klee (1986). RB, Right-hand border; nos, nopaline-synthase gene; nos–nptII, nopaline-synthase–neomycin phosphotransferase II chimeric gene; spc/str R, Tn7 transposon; oriT, oriV, replication origins from RK2; CAT, chloramphenicol-acetyltransferase; Cab-1, chlorophyll *a/b* binding gene of wheat.

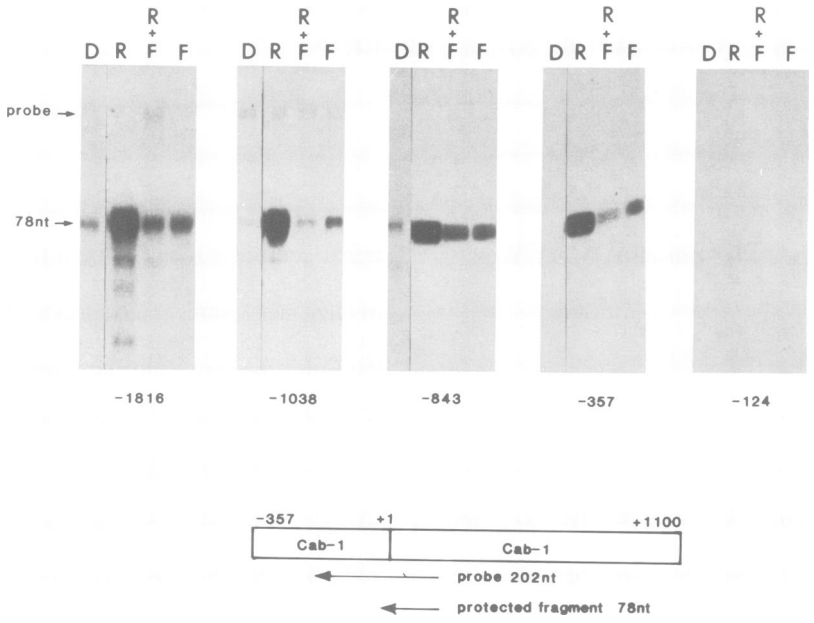

Fig. 2. Phytochrome responses of 5′ deletion mutants of the wheat Cab-1 gene in transgenic tobacco seedlings. Transgenic tobacco plants were grown in a glasshouse and selfed after flowering. Mature seeds were collected, surface sterilized and germinated in the dark on MS medium for 10 days. After illumination with red (3 min; 100 μE M-2 sec+1), far-red (10 min 30 μE M-2 sec-1), or red followed immediately by far-red light the samples were returned to the dark for 24 h before RNA isolation. RNAs were analyzed by 5′ S1 nuclease protection using a 202-nucleotide single-stranded probe as described in Materials and methods. Twenty-five microgams of RNA was used for the −1816, −1038, −843 and −352 mutants while 100 μg RNA was analyzed for the −124 mutant. D, Dark; R, red-light; R and F, red light followed by far-red light; F, far-red light alone. The positions of the 202-nucleotide probe and the 78-nucleotide protected fragment are indicated by arrows.

responses, respectively. For the rbcS genes, we have identified a 250−280-bp 5′ upstream fragment that can function as an enhancer-like element to confer phytochrome-responsive transcription on a heterologous promoter (Fluhr *et al.*, 1986a). In the case of the wheat Cab-1 gene we have found that 1.8 kb of 5′ upstream sequence is sufficient for full phytochrome response in transgenic tobacco seedlings (Nagy *et al.*, 1986b). Simpson *et al.* (1986a) have also reported that a 2.5-kb 5′ upstream sequence of a pea Cab gene can confer phytochrome response to a chimeric gene in transgenic tobacco plants. We have extended our analysis of the Cab-1 gene and we show here that the *cis*-regulatory sequence for phytochrome responsiveness resides in a 268-bp DNA fragment located in the 5′-proximal region of the Cab-1 gene. This fragment can potentiate phytochrome-dependent transcription from the cauliflower mosaic virus (CaMV) 35S promoter in a bidirectional manner. Finally, we show that the Cab-1 transcript stability is not modulated by phytochrome.

Results

Phytochrome response of 5′ deletion mutants

We have previously shown that 1.8 kb of 5′ upstream sequence of the wheat Cab-1 gene is sufficient for its phytochrome response in transgenic tobacco seedlings (Nagy *et al.*, 1986b). To define *cis*-acting DNA sequences that mediate this response we generated a series of 5′ deletion mutants from the Cab-1 gene by either *Bal*31 nuclease digestion or cleavage at convenient restriction sites. Mutants with breakpoints at −1816, −1494, −1254, −1038, −843, −714, −654, −563, −460, −357 and −124 were introduced into tobacco using the binary vector pMON505 (Horsch and Klee, 1986) and *Agrobacterium tumefaciens* containing the 'disarmed' plasmid GV3111-SE (Figure 1) (Rogers *et al.*, 1986). For each construct at least eight independent, transgenic tobacco clones were raised and the Cab-1 transcript level was assayed by 5′ S1 nuclease protection (data not shown). Among the eight transgenic clones, three that expressed high Cab-1 transcript levels in young leaves were selected. These clones were selfed and the F_1 seedlings used for phytochrome experiments.

Figure 2 shows that the Cab-1 transcript level of the −1816 construct is low in the dark but can be elevated 10−15 times by a brief irradiation with red light. The enhancing effect of red light can be abrogated by far-red light while far-red light alone promotes a small increase in the Cab-1 transcript level over the dark control. The characteristic responses of the Cab-1 gene to red and far-red light are still maintained in mutants −1494, −1254, −1038, −843, −714, −654, −563, −460 and −357, but the results of only three mutants are shown (Figure 2). Note that the Cab-1 transcript level of the −357 mutant at 18 h after a flash of red light is comparable with that of the −1816 construct which is taken as the wild type in these experiments. Further deletion of 233 bp from −357 to −124 reduces the Cab-1 transcript to an undetectable level. These results show that 357 bp of 5′ upstream sequence is sufficient for phytochrome response, as well as for maximal expression of the Cab-1 gene in transgenic tobacco seedlings.

An upstream enhancer-like element for phytochrome-inducible transcription

We have previously demonstrated that the expression of the full 35S promoter of CaMV is insensitive to light (Nagy *et al.*, 1986a). Furthermore, the activity of the promoter can be decreased 20 times by deletion of 5′ upstream sequences to position −46 (Odell *et al.*, 1985).

Under our assay conditions, the −46 mutant of the 35S promoter does not show any detectable signal for 10 independent transgenic clones examined (Figure 3, panel 1). A 911-bp frag-

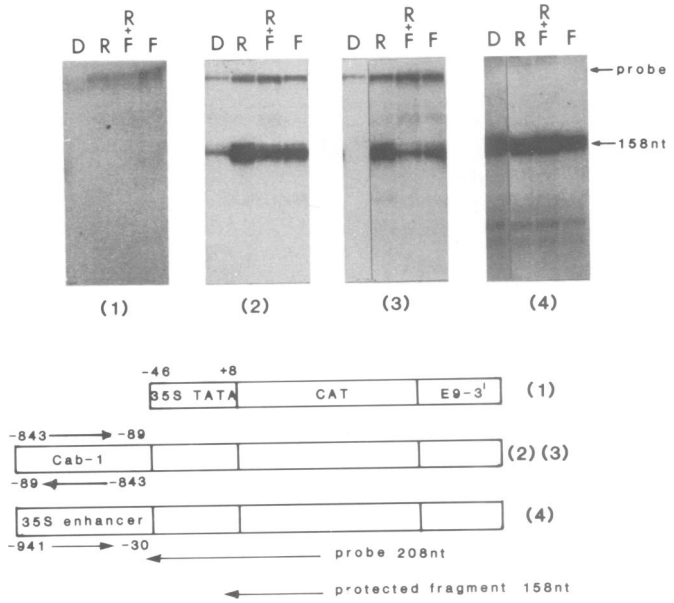

Fig. 3. A 754-bp fragment in the 5′ upstream region of the Cab-1 gene can confer phytochrome responsiveness to a heterologous promoter. F_1 seedlings of transgenic tobacco containing different chimeric gene constructs were germinated in the dark for 10 days as described in Figure 2. RNA samples were analyzed by 5′ S1 nuclease protection using a 208-nucleotide single-stranded probe as described in Materials and methods. Fifty micrograms of RNA per lane were used for **panels 1–3** while 10 μg RNA per lane was used for **panel 4**. For abbreviations see Figure 2. The positions of the 208-nucleotide probe and the 158-nucleotide protected fragment are indicated by arrows.

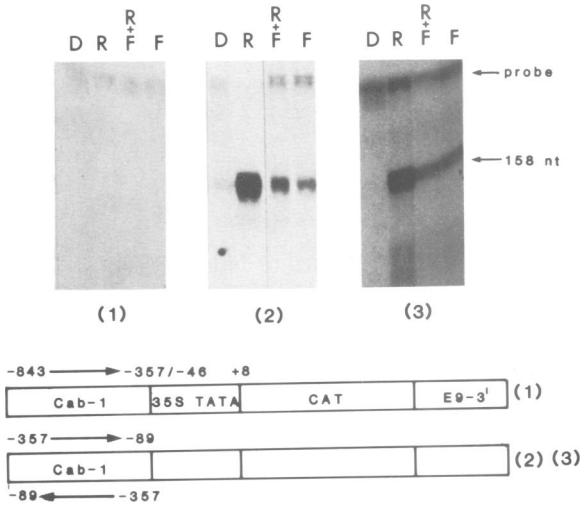

Fig. 4. The phytochrome-responsive element of the Cab-1 gene is localized in the 5′-proximal region from −357 to −89. Experiments to analyze phytochrome responsiveness of these chimeric gene constructs were carried out as described in Figure 2. Each lane contained 50 μg total RNA. The positions of the 5′ S1 probe and the 158-nucleotide protected fragment are indicated by arrows. For abbreviations see Figures 1 and 2.

ment from the 5′ upstream region of the 35S promoter elevates the transcript level from the 35S TATA box construct by at least 15 times (10 independent clones) (Figure 3, panel 4). In addition, the transcription of this chimeric construct is not regulated by phytochrome, confirming previous results (Fluhr and Chua, 1986). The low expression level and its insensitivity to phytochrome renders the −46 mutant of the 35S promoter a suitable vehicle to assay for phytochrome-regulated transcriptional activity of any DNA fragments placed upstream.

Figure 3, panel 2 illustrates that a 754-bp fragment from the 5′ upstream region of the Cab-1 gene can also potentiate transcription from the −46 mutant of 35S. Eight out of 10 transgenic clones containing this construct are active, and among these, the transcript level is at least 5-fold lower than that obtained with the 35S enhancer (Figure 3, panel 4). More importantly, the transcription is responsive to phytochrome as shown by the red induction and far-red reversal (Figure 3, panel 2). The 754-bp Cab-1 fragment can also stimulate transcription when placed in the inverted orientation. However, only two out of 10 transgenic clones are active whereas the remaining eight do not express any detectable transcript. Nevertheless, transcription of the chimeric construct in the two active clones is still regulated by phytochrome (Figure 3, panel 3). Taken together, our results show that the 754-bp fragment from −843 to −89 of the Cab-1 gene can potentiate phytochrome-responsive transcription from a heterologous promoter. We note that the Cab-1−35S chimeric constructs (panels 2 and 3 in Figure 3) are more responsive to far-red light than the −843 mutant (Figure 2).

To localize the active region of the Cab-1 5′ upstream fragment (−843 to −89) we made use of a convenient *Eco*RV site at −357 to divide the fragment into proximal (−89 to −357) and distal (−357 to −843) subfragments. These subfragments were fused in either orientation to the −46 mutant of the 35S promoter. In each of the constructs depicted in Figure 4, 10 independent transgenic clones were assayed for their response to phytochrome activation. We found that the distal subfragment is inactive in all the 10 clones tested, showing no detectable transcript level. On the other hand, the proximal subfragment can confer phytochrome-inducible transcription from the 35S promoter in an orientation-independent fashion. For construct 2, seven out of 10 transgenic clones were active and for construct 3, five out of 10 transgenic clones (Figure 4). Representative results are shown in Figure 4. We emphasize that we have never recovered any transgenic clone that exhibits constitutive expression, i.e. detectable transcription but not regulated by phytochrome. These results clearly demonstrate that *cis*-acting sequences for phytochrome response of the Cab-1 gene are located between −89 and −357.

The nucleotide sequence of the Cab-1 region from +85 to −843 is shown in Figure 5.

Sequences downstream of −124 are not involved in phytochrome regulation

Analysis of the 5′ deletion mutants suggests that all the positive elements are located upstream of −124. Since the −124 mutant does not produce any measurable transcript, we do not know whether there are any negative elements for phytochrome responses located downstream of −124. We also do not know whether phytochrome regulates the stability of the Cab-1 transcript. To address these questions we assembled three chimeric constructs (Figure 6) and examined their expression in transgenic tobacco seedlings. For each construct shown in Figure 6, seven independent transgenic clones were examined. Construct 1 comprises the Cab-1 promoter (−124 to +30) fused to the CAT coding sequence. A 35S enhancer element is placed downstream of the poly(A) addition sequence to elevate the expression level. This transcription unit produces a chimeric transcript containing the CAT mRNA coding sequence and 30 nucleotides of the 5′ end of Cab-1 mRNA. Figure 6, panel 1 shows that the level of this chimeric transcript is not affected

●-843
AAGCAGGGAGCTAATTAATTGGTGGTCACGAATCCACTTGGTCTTCCTATCCACACATGTTTCACTGGTT
●-714
TGCCCGTGGGTTCCGCCGTACCTCTAGTGTTTTTCAGGGGTTGGACAATCACAAGAGGCAAAGAAACACA
●-654
TATTTACCTCTAGTATTTTTCAGGGAATGGACAATCACAAGAGGCAAAGAAACACATATTTACTCTTGTA
GTCATGTCATGTGTGTACTGACCGTATGAAAAAATTTCTGGAACGATCAACGCCAGATAACAAATTTTTT
●-563
TTCTCAAACGGAGACAAAATATTTGCCTAATCAGTTAATTAAGCATAAGAGAATTATTCAGTTAGTTAAT
●-460
GAAAAATCAGAAAAAAAACACGACACAAACCTATCACACGCGCATGCAACCCCATAAAAGCACGATCACT
●-357
CCGACAATCAAACCCAACAGGCAAAGAAAACATGCAAGACGATGCAAGTGGCACGATCAGCCAGATATCG
AATTTGTTGGGCAAGCGCCCAGTGTGATGTACGTTGGGAAAACTTGCAAGAGGATGCGACCAAATGAACT
GGTAAACCATCCCGTGAGCGTGGCCTACACATTTTAAGCCAGCGGTCTCTTTCGACTTGTCTTACAAAAG
CTGGTCCAGTCACGAGCCTTAGCCCTAACCATAGCCACAAGTACATCTCATCCATTTAAGGCCTCTGCGT
●-124
GCACCAATGGCATCCAAGCTGCAGATTTCTTTTCACCACCGTCTCTCTTGTCAGCTGTCACCAAGCACCA
ACCAATTAATTTCTCTATATTCTTGTTCCCTCAAGGCTCAAGCTCTTTAAATAGCTCCATACAATCTCCT
CTTAAACCATCTCCACACACTCAAGCCACACTATTGGAGAACACACAGGGACAACACACCATAAGTGCAG
▲+1

M A A T T
CGCA ATG GCG GCC ACC ACC

Fig. 5. Nucleotide sequence of the 5′ upstream region of the wheat Cab-1 gene from −843 to +85. The transcription start site is determined by S1 nuclease protection and indicated by an arrow. The presumptive TATA and CAAT boxes at −25 and −72 respectively are underlined. The breakpoints of the 5′ deletion mutants are marked by filled circles. The single-letter code for the first five amino acids is given above each codon. An SV40 enhancer-like sequence is indicated by a dashed line. The sequence of the Cab-1 coding and 3′ non-coding region has been published recently (Lamppa *et al.*, 1985).

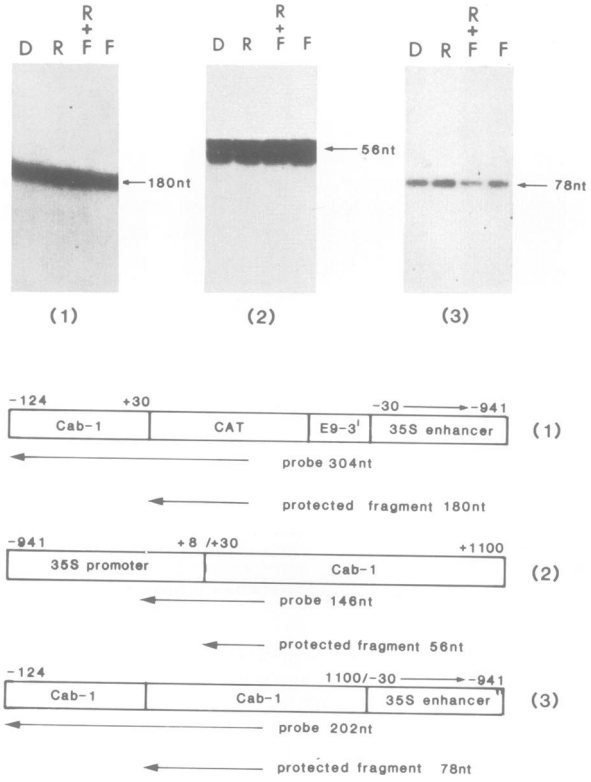

Fig. 6. Sequences of the CAB-1 gene downstream from −124 are not responsive to phytochrome regulation. Chimeric gene constructs containing different regions of the Cab-1 gene [(1) −124 to −30; (2) +30 to +1100; (3) −24 to +1100] were tested for phytochrome-induced gene expression using transgenic tobacco seedlings as described in the legend to Figure 2. RNA samples were analyzed by 5′ S1 nuclease protection experiments. For experimental details see Materials and methods. Each lane contained 25 μg of total RNA. The positions of the protected fragments (180, 56 and 78 nucleotides in chimeric gene constructs 1, 2 and 3 respectively) are indicated by arrows. For abbreviations see Figures 1 and 3.

by red or far-red light. These results indicate that the Cab-1 promoter region from −124 to +30 does not contain any negative elements for phytochrome response, and the stability of the Cab-1−CAT chimeric mRNA is not regulated by phytochrome.

To see if the Cab-1 transcript itself is sensitive to phytochrome we used the constitutive CaMV 35S promoter (Odell *et al.*, 1985) to drive the expression of the Cab-1 coding sequence. Figure 6, panel 2 shows that the Cab-1 transcript is expressed equally in the dark or upon exposure to red and far-red light. These results strongly suggest that the Cab-1 coding sequence does not contain any regulatory elements and that the Cab-1 mRNA stability is insensitive to phytochrome.

The chimeric mRNA produced by construct 2 contains, at its 5′ end, eight nucleotides from the 35S transcription unit. It could be argued that this small piece of 5′ leader RNA could modify the response of the Cab-1 mRNA to red and far-red light. We therefore assembled a third construct in which the 35S enhancer fragment was placed at the 3′ end of the −124 mutant to increase the transcript level (Figure 6, construct 3). This transcription unit produces the authentic Cab-1 mRNA and its level is essentially unaffected by red and far-red light. Together, these results provide conclusive evidence that there is no phytochrome control of the Cab-1 mRNA stability.

Although all of the transgenic clones containing the chimeric genes (Figure 6) are active, there is a 20-fold variation in the transcript level among them. These results are consistent with previous reports (Nagy *et al.*, 1985; Jones *et al.*, 1986; Poulsen *et al.*, 1986) that the expression of transgenes is influenced by their chromosomal positions. We note that in chimeric constructs 1 and 3 the 35S upstream fragment is able to potentiate transcription when placed at the 3′ end of the transcription unit (Figure 6). To our knowledge this is the first report that a plant upstream regulatory element is still active when relocated downstream of the TATA box, indicating that the 35S element functions as a transcriptional enhancer.

Discussion

In this paper we have attempted to define *cis*-acting elements of the wheat Cab-1 gene that are responsive to phytochrome induction by two complementary lines of investigation. In the first, we analyzed phytochrome responses of a series of 5′ deletion mutants retaining varying amounts of upstream sequences from −124 to −1816. We found that the −357 mutant still exhibits full phytochrome response whereas little or no expression is seen with the −124 mutant. Two conclusions may be drawn from these results: (i) sequences upstream of −357 of the Cab-1 gene are not required for phytochrome response; and (ii) a 233-bp fragment between −124 and −357 contains regulatory elements for expression and/or phytochrome induction.

The above conclusions are reinforced independently by experiments with chimeric gene constructs in which various Cab-1 upstream fragments were evaluated for the ability to potentiate transcription from a truncated CaMV 35S promoter. We found that the proximal subfragment (−89 to −357) of the 5′ upstream region is active in this assay whereas the distal subfragment (−357 to −843) is inactive. More importantly, the proximal subfragment retains its activity when placed in the inverted orientation suggesting that it has a property expected of a transcription enhancer. In both cases (Figure 3 and 4) transcription is regulated by phytochrome as revealed by the red induc-

tion and far-red reversal. These results, together with those obtained with the 5′ deletion mutants, firmly establish that sequences for phytochrome response of the Cab-1 gene are located between −89 and −357. All our chimeric genes do not contain the putative CAAT sequence yet they still respond to phytochrome, suggesting that the CAAT sequence is dispensable for phytochrome regulation of the Cab-1 gene.

The transcript level of the various chimeric gene constructs (Figures 3 and 4) is ~5 times lower than that of the Cab-1 deletion mutants (Figure 2). This difference could be due to differences in the half-life of the respective transcripts or to reduced transcription rates of the chimeric constructs.

Although the −124 mutant of the Cab-1 gene is inactive it does not imply that there are no regulatory elements located downstream of this breakpoint. Negative regulatory elements could be present but might only be uncovered when the transcription level is elevated by positive elements (Kuhlemeier *et al.*, 1987). To test this possibility we increased the transcription level of the −124 mutant with a constitutive CaMV 35S enhancer. The Cab-1 transcript from this chimeric construct is identical to the authentic Cab-1 transcript. We found that the Cab-1 transcript level is not affected by red and far-red light. These results provide direct evidence that phytochrome does not regulate the Cab-1 transcript stability. It follows, therefore, that phytochrome regulation of the Cab-1 gene expression is largely, if not entirely, at the transcriptional level.

A 280-bp fragment from the 5′ upstream region (−48 to −327) of the pea rbcS-3A gene has been shown to confer phytochrome-inducible transcription on a heterologous promoter (Fluhr *et al.*, 1986a). Comparison of the nucleotide sequence of the rbcS-3A and Cab-1 enhancer-like fragments reveals no obvious homology. We note that the two genes differ with respect to their fluence responses; the Cab-1 gene is more sensitive to red light than the rbcS-3A gene. It is possible that the expression of the two genes is mediated by different *cis*-acting elements.

A 247-bp 5′ upstream fragment of a pea Cab gene has been reported by Simpson *et al.* (1986b) to confer white light inducibility on a heterologous promoter. Moreover, this fragment is active when placed in the inverted orientation. However, whether the same upstream fragment would mediate phytochrome induction has not been addressed. Our experiments reported here differ from those of Simpson *et al.* (1986) in two important aspects: (i) the developmental stage of the plant material (mature plant versus etiolated seedlings) and (ii) the quality and the duration of the light treatment (continuous white light versus red light pulse). There is evidence at least for the rbcS genes that photoresponses of gene transcription are modified by developmental stages of the plant materials (Fluhr and Chua, 1986). Preliminary results from our laboratory indicate that transcriptional regulation of the Cab-1 gene is also subject to developmental modulation. Experiments are in progress to delineate sequence requirements for phytochrome response in etiolated seedlings and white light induction in green, mature plants.

Materials and methods

Isolation of 5′ deletion mutants

A 2.4-kb *Sal*I−*Sal*I fragment containing the wheat Cab-1 promoter region and a part of the coding sequence was isolated from the genomic clone whAB1.6 (Lamppa *et al.*, 1985) and subcloned into a pEMBL8+ vector (Dente *et al.*, 1983). Progessive deletions were made from the vector *Hin*dIII site using *Bal*31 nuclease according to Barnes *et al.* (1983). The deletions were sized by gel electrophoresis and their exact breakpoints were determined by dideoxy sequencing as described by Biggins *et al.* (1983). Eleven deletion mutants with the following breakpoints −1816, −1494, −1254, −1058, −843, −714, −654, −563, −460, −357 and −124 were chosen for further manipulations. Plasmids containing these deletion mutants were cleaved with *Bst*XI−*Cla*I and the resulting fragments carrying the deleted Cab-1 promoter region from +30 (*Bst*XI) to the various deletion breakpoints were cloned into a modified pMON505 binary vector as follows. A pMON505 plasmid containing the entire whAB1.6 genomic clone as a *Cla*I−*Cla*I insert was digested with *Bst*XI and partially with *Cla*I. After digestion, the original Cab-1 promoter fragment from +30 to −4000 (*Bst*XI−*Cla*I) was removed and replaced by the *Bst*XI−*Cla*I fragments carrying the deleted promoter mutants from +30 to the various deletion endpoints.

Ti-mediated gene transfer

pMON505 binary cloning vectors containing different chimeric genes (Figures 1−4 and 6) were transferred by triparental crosses into a 'disarmed' *A. tumefaciens* (GV3111SE) in which all phytohormone biosynthetic genes and the TL-DNA right border from the endogenous pTiB6535E plasmid have been deleted. *A. tumefaciens* cells containing different chimeric gene constructs were co-cultured with leaf discs of *Nicotiana tabacum* SR1 on a medium containing 100 μg/ml kanamycin, 500 μg/ml carbenicillin and phytohormones for plant regeneration (Horsch *et al.*, 1985). After root formation, transgenic plantlets were transferred to soil and grown to flowering in a glasshouse.

Preparation of RNA and 5′ S1 nuclease protection assays

RNA was extracted from transgenic tobacco seedlings according to Kirk and Kirk (1985) except that 0.5 mM aurin tricarboxylic acid was added as an inhibitor of RNase. The transcript level of the transgenes was determined by 5′ S1 nuclease protection assay (Berk and Sharp, 1977; Weaver and Weissmann, 1979). RNA isolated from transgenic seedlings carrying the intact wheat Cab-1 gene or its 5′ deletion mutants (see Figures 2 and 5) was hybridized with a 202-nucleotide single-stranded probe. To obtain this probe a *Pst*I−*Xho*I fragment of the Cab-1 gene (from −124 to +368) was subcloned into a modified pEMBL vector in which the original linker region was removed and replaced by an *Eco*RI−*Pst*I−*Xho*I−*Hin*dIII linker region. Single-stranded DNA was isolated according to Dente *et al.* (1983) and used as a template to label the complementary strand. The 'hot' strand was synthesized by polymerase I large fragment in the presence of ^{32}P-labeled dTTP and a mixture of unlabeled dGTP, dCTP, dATP nucleotides using a 16-mer oligonucleotide as primer. After chasing with cold dTTP the DNA was digested with *Pst*I and the labeled strand (202 nucleotides) was separated on a 6% urea−polyacrylamide gel. The isolated strand was hybridized with the appropriate amount of total RNA (for details see figure legends) in a solution (10 μl) containing 80% formamide, 0.4 M NaCl, 2 mM EDTA and 20 mM Pipes (pH 6.8) for 12 h at 52°C. After hybridization the reaction mixture was diluted to 150 μl with a solution containing 0.5 M NaCl, 30 mM NaOAc (pH 4.6), 1 mM $ZnSO_4$, 20 μg/ml of denatured salmon sperm DNA, 1000 U/ml S1 nuclease (BRL) and incubated at room temperature for 90 min. Single-stranded DNA fragments (78 nucleotides) protected from S1 nuclease digestion were sized in 6% sequencing gels and visualized by autoradiography. 5′ S1 nuclease protection assays to analyze the transcript level of different chimeric constructs containing the 35S TATA−CAT−E9−3′ hybrid gene cassette (Figures 3 and 4) were performed according to Kuhlemeier *et al.* (1987). The 35S TATA−CAT cassette was subcloned into pEMBL8+. The complementary strand was synthesized using a CAT sequence-specific 16-mer oligonucleotide as primer in a system described previously for the Cab-1 5′ S1 probe. The 208-nucleotide probe was gel purified and hybridized with 50 μg total RNA in a solution (10 μl) containing 50% formamide, 0.4 M NaCl, 2 mM EDTA and 20 mM Pipes (pH 6.8) for 12 h at 37°C. S1 nuclease digestion and further manipulations were carried out as described previously for the Cab-1 5′ S1 nuclease protection assay. Transcript levels of the Cab-1−CAT−E9−3′−35S enhancer and 35S−Cab-1 chimeric genes (for details see Figure 5, 1 and 2 respectively) were also determined by 5′ S1 nuclease protection assays. Probes for these 5′ S1 nuclease protection experiments were isolated as follows. The Cab-1−CAT and the 35S−Cab-1 cassette were subcloned in pEMBL, described previously, and labeled single-stranded DNAs were synthesized using the CAT or Cab-1 gene-specific 16-mer oligonucleotides as primers. The labeled 304- or 146-nucleotide single-stranded probes were purified and hybridized with 25 μg of total RNA in a solution (10 μl) containing 80% formamide, 0.4 M NaCl, 2 mM EDTA and 20 mM Pipes (pH 6.8) for 12 h at 52°C. S1 nuclease digestion was carried out as described previously for the Cab-1 5′ S1 nuclease protection assay.

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