

Identification of upstream regulatory elements involved in the developmental expression of the *Arabidopsis thaliana cab1* gene

(photosynthetic gene/organ specificity/light inducibility/transformation/multiple regulatory elements)

SAM-BONG HA AND GYNHEUNG AN

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340

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ABSTRACT We studied cis regulatory elements controlling the light-dependent organ-specific expression of *Arabidopsis thaliana* chlorophyll a/b binding protein gene (*cab1*) by stably transforming tobacco plants using a tumor-inducing (Ti) plasmid vector system. The results from the 5' and internal deletion analyses indicate that there are at least three cis-acting elements that are involved in the light-dependent developmental expression of *cab1* gene. Two such elements are located at the immediate upstream regulatory region and the other element is located at the further upstream region. The 1120-base-pair (bp) DNA fragment containing the immediate and far upstream region can confer light-inducible organ specificity on the truncated *nas* promoter. However, deletion of the 39-bp DNA fragment at the immediate upstream regulatory region from this hybrid promoter resulted in a nonfunctional promoter, revealing that the 39-bp region is important for the *cab* promoter specificity. Further analyses of this region suggest that a potential Z-DNA-forming sequence (ATACGTGT) is involved in light-dependent developmental expression of the *cab1* gene. Two additional Z-DNA-forming sequences (ACA-CATAT) that are inverted repeats of this sequence are also found in the upstream region where the additional regulatory elements are expected.

Expression of many photosynthetic genes is highly specific to green tissues (1, 2) and the primary regulation of these genes occurs at the transcriptional level (3-7). Studies with ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) gene indicate that several hundred base pairs of the DNA sequence preceding the coding region carry cis-acting elements, which are necessary for promoter regulation (8). In pea *rbcS-3A* gene this region is composed of multiple regulatory elements. Within the upstream enhancer region two light-responsive elements (box II and III) and their homologous sequences have been identified, with which trans-acting factors interact (9, 10). It has been suggested that positive and negative regulatory elements are involved in the control of the *rbcS* gene expression (9, 11).

Few studies have been made to define the cis regulatory elements involved in organ-specific expression of other photosynthetic genes. Expression of chlorophyll a/b binding protein (*cab*) gene, which is another family of photosynthetic genes, is also specific to green tissues. However, the expression pattern of the *cab* gene in plants is different from that of the *rbcS* under certain physiological conditions. For example, responses to light quality and diurnal rhythm are different between these two genes (2, 12). Sequence comparison of *rbcS* and *cab* upstream regions reveals no obvious homology, indicating that the expression of these two genes may be mediated by different regulatory mechanisms. Studies on wheat and pea *cab* genes indicate that an \approx 250-base-pair (bp)

DNA fragment carrying the 5' control region of the genes contains enhancer- and silencer-like properties (13, 14). In this study we conducted an extensive series of promoter analyses on the *Arabidopsis thaliana cab1* gene and identified the cis regulatory elements involved in organ specificity of the *cab* gene.*

MATERIALS AND METHODS

Strains. *Escherichia coli* strain MC1000 (15) was used as host for routine cloning experiments. *Agrobacterium tumefaciens* strain LBA4404, which carries a helper tumor-inducing (Ti) plasmid, pAL4404 (16), was used for maintenance of the binary Ti plasmids and transformation of tobacco plants. *Nicotiana tabacum* L. cv. Xanthi was maintained as sterile shoot cultures.

Generation of Deletion Mutants. To facilitate generation of deletion mutants, we have constructed pGA617 by inserting a synthetic oligonucleotide (GGTACCTCGAGGCT) at the *Ssp* I site of pUC19. Both strands of the nucleotide were chemically synthesized by a silica-based solid-phase method combined with proton-activated nucleoside phosphoramidites using an Applied Biosystems (Foster City, CA) 380A DNA synthesizer. The oligonucleotide contains restriction enzyme sites of *Kpn* I (*Asp*718), *Xho* I (*Ava* I), and *Stu* I. Similarly, pGA616 was constructed from pUC18 and the same synthetic oligonucleotide. To generate 5' deletion mutants, the 2044-bp *Eco*RI-*Sac* I fragment carrying 1396 bp of the 5' control region and 648 bp of the coding region was inserted into the multiple cloning site of pGA617. The resulting plasmid, pGA619, was linearized by opening at the unique *Eco*RI or *Eco*RV site located at 1396 bp or 253 bp, respectively, upstream from the ATG initiation codon and digested with an exonuclease, BAL-31, in solution (600 mM NaCl/12 mM CaCl₂/12 mM MgCl₂/20 mM Tris-HCl, pH 8.0) at 30°C. The reaction was stopped at 1-min intervals by removing a portion of the mixture and adding it into an Eppendorf test tube containing 0.1 ml of 0.3 M sodium acetate (pH 7.0), 0.1 ml of chloroform, and 0.1 ml of phenol. After mixing vigorously, the test tube was centrifuged for 2 min and the aqueous phase was precipitated with 2 vol of ethanol. DNA was dissolved in 10 μ l of medium-salt buffer (50 mM NaCl/10 mM MgCl₂/50 mM Tris-HCl, pH 8.0), digested with *Stu* I, and self-ligated with T4 DNA ligase. Ligated DNA was introduced into *E. coli* MC1000, and the structures of the 5' deletion mutants were analyzed by digesting DNA prepared from the ampicillin-resistant colonies with *Xho* I and *Hind*III. The 3' deletion mutants were obtained in a similar manner from plasmid pGA618, which was constructed by inserting the 2044-bp *Eco*RI-*Sac* I

Abbreviations: Ti, tumor inducing; CATase, chloramphenicol acetyltransferase.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04098).

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fragment into pGA616. pGA618 was opened at either *Sac* II site located 68 bp downstream from the ATG initiation codon in the coding region of the *cabl* gene or *EcoRV* site in the 5' control region and digested with BAL-31 as described above. The deletion end points were determined by the Maxam-Gilbert DNA sequencing method after labeling the deletion end point (*Asp*718 site) with [α - 32 P]dGTP and DNA polymerase large fragment (17).

Transformation of Plants. DNA fragments containing deletion-mutant promoters were placed in front of a reporter chloramphenicol acetyltransferase (*cat*) gene in a binary expression vector (18). The *Xho* I-*Sac* I fragment carrying the 5' deletion mutants was inserted into the multiple cloning site of the pGA655 to generate pGA711-mt. pGA655 was derived from pGA580 (19) by inserting an *Xho* I linker into the filled-in *Xba* I site. This modification shifted the reading frame of the *Sac* I to +2 relative to that of pGA580. Therefore, translation started from *cabl* would continue through the linker region and *cat* gene, producing a fusion protein that is an active chloramphenicol acetyltransferase (CATase) (20). A set of internal deletion mutants (pGA714-mt) were generated by fusing the 3' deletion mutant fragments to the 5' deletion mutant pGA711-158 carrying the CCAAT and TATA box regions but lacking the entire upstream sequences. These molecules were transferred into *A. tumefaciens* LBA4404 by the freeze-thaw method (19, 21), and the structures of the transferred molecules were examined by the rapid screening procedure (21). *N. tabacum* shoot cultures were stably transformed by the cocultivation methods as described earlier (22).

Analysis of Gene Expression. To reduce variation of expressions in transformed plants due to the position effect, at least 10 independently transformed shoots of each deletion mutant were picked and further maintained in dark and light for 3 weeks. Only three plants were analyzed for the 5' deletion mutant -563. Plants were pooled and assayed for CATase activity as described (19). Two or three plants of each deletion mutant were regenerated from the transformed shoots exhibiting representative CATase activities and used for the analysis of organ specificity. Five micrograms of total protein was used for CATase assay in all experiments, except that 15 μ g of total protein was used in the analyses of *cabl-nos* hybrid promoters. The reaction was carried out at 37°C for 20 min.

RESULTS

Fig. 1 shows the 1396-bp DNA sequences of the 5' control region that confers organ-specific and light-inducible expression of the *cabl* gene (14). To identify cis regulatory elements involved in the promoter specificity, sets of deletion mutants were generated *in vitro* and the effects of the mutations were examined in transformed tobacco plants.

At Least Two Regulatory Elements Are Located in the Immediate Upstream Regulatory Region. The 2044-bp *EcoRI*-*Sac* I fragment containing the *cabl* promoter was progressively deleted from the 5' side of the DNA fragment with BAL-31. We have constructed two plasmids (pGA616 and pGA617) by modifying pUC plasmids to facilitate generation of deletion mutants as described in *Materials and Methods*. From the more than 100 mutants obtained by this system, 12 that were spaced at about 100-bp intervals were selected for further studies. Each 5' deletion mutant was cloned into the promoter-expression vector based on Ti plasmid, generating a translational fusion between the *cabl* and *cat* coding regions.

The constructed molecules were transferred into tobacco cells by means of the *Agrobacterium* cocultivation method and transformed shoots were further grown in light and dark. As shown in Fig. 2A, the 5' deletions down to -321 did not significantly affect the strength and light inducibility of the

cabl promoter. However, a deletion to -253 reduced the promoter strength by a factor of at least 10 but retained the light inducibility. Further deletion to -158, which removed all of the region upstream of the consensus CCAAT and TATA box regions, abolished the promoter activity.

To identify elements involved in developmental regulation of the *cab* gene, plants were regenerated from transformed shoots that displayed an average level of the mutant promoter activity. The results in Fig. 2B demonstrated that the organ specificity of the *cabl* promoter was retained in all of the 5' deletion mutants except for the deletion -158, which did not show promoter activity in any of the organs. As observed in young shoots, the promoter strength of the mutant -253 was significantly reduced in leaf and stem. These results suggest that the DNA sequence between -321 and -158 contains at least two elements that control the promoter activity. One of these elements, which is located between -253 and -158, is essential for the promoter specificity. The other element, located between -321 and -253, appears to be a modulator that enhances the *cab* promoter activity.

The Far Upstream Region Is Also Involved in *cabl* Promoter Specificity. To further understand the organization of cis regulatory elements in the upstream control region, internal deletion mutants lacking the upstream region of CCAAT box were generated as described below. Eleven 3' deletion mutants with end points located between -766 and -161 of the *cabl* promoter were obtained by using the method described for the 5' deletion mutants. These mutants were then joined to the 5' deletion mutant 711-158, in which the upstream sequence of the CCAAT box region is absent. The effects of these internal deletions on organ specificity of the *cabl* promoter were examined in transformed tobacco plants (Fig. 3).

GAATTCATCAACAAAT

1380 TACTCTCAATCACACTCCTATAGAAAACGGTTAAGCTATCATTACATGTCTAGTTGGT

1320 TTTACTCAGCCCTAGAAGTGTGTTTATGTCATCACTTTCCACGAAGCACAATTTTCTT

1260 TTTTACAATCACTAGACCTCAGCGCTCACACATATGCTTTAGAGCACATTTCAAACCTT

1200 GAACTATAAAAGCTGTTAACACTAATACACTATGCGTCTTTTTTGTCCAAACACTTTT

1140 GATCCATTATTAGGAGACACTCCACTTAGAAAGATTTTCTAATCTTTGGTCAACTAGGA

1080 AGTTCAGGTTTTTCTAAACAGAAATTCATTTCCACAAGTAATTAATTTATAAGGAAATG

1020 AATAGAGAAATCAAATCATTGAAGAACAATAAATATAGATTCAAGGTCAGGCTAAGAA

960 AATATTCCTGAAGCTCAAAAAGAGTTTTCTCTCACATTATAGAAATGGCCTTTACTTTC

900 AACATTTTCCACCTATCCACATTTGGTCAGAACATTTTAACTACTTGGGATCAATTT

840 TCCGGTTGAAATGGGTTGGTGAATATCCGGTTCAGTTATATGGTGGCCGTTGGAATTGG

780 CTTATTAGTTGTGCGCGTTGTTGAAGCCGTTGGTATGGTAAAGGAGAAAGCAGACTTTGG

720 GCTATGAGTCTATGACCATGACTCGTGATATGGAGCTGCTTATGACCCCTGACCATCAC

660 CTTGATCTGGTGGATCCAATGTTTTCTTCTCTCTAATAAATATATGGTCAATACA

600 GGTGCTAATTAAGATGGTAATAATTTCTTATGTTCTCTGGTAAAGTTTGATTCATTTCC

540 GTAGTTTTAGATAATCTTATTTCCATACATAAATTTTATAGTTTATCTACTTTGTCTTT

480 ATGTTTTATCTCTAGCCCAAGAGTTATTATTATTATCAGAAGAAGAAAAAAGAAAGCA

420 **TATATACAAAAGGTTTAAATAAATGTATTATACAAGGCAATTTATCCAATTTTTTTTGT**

360 TTGGTTTACATTGATGCTCTCAGGATTTTCATAAGGATAGAGAGATCTATTGGTATACGG

300 TCACGTGATGAGTGGGTTTTCGCAATCCATGAAACGCACCTAGATATCTAAACACAT

240 ATCAATGGCAATCTCGAAGTCCGAGCCATTAACCACGTAAGCAACAAACAATCTAAA

180 CCCCATAAATAATCTGACTAGCCAAATGACCACTCAGAGATTGATTTTCAAGATAAG

120 ACAGTATTTAGATTTCTGTAT**TATATATAGCGAAAATCGCATCAATCAACCAACCCCAT**

60 TTCTTGGCTTACAACAACAAATCTTAAACGTTTTACTTTTGTGCTGCACACTCAACCTTA

ATG GCC GCC TCA ACA ATG GCT CTC TCC TCC CCT GCC TTC GCC GGT

FIG. 1. DNA sequence of the 5' control region of *Arabidopsis cabl* gene. The ATG initiation codon, TATA box, CCAAT box, and potential Z-DNA-forming sequences are in bold type. The 5' deletion end points are indicated above the sequence and the 3' end points are indicated below the sequence with arrowheads.

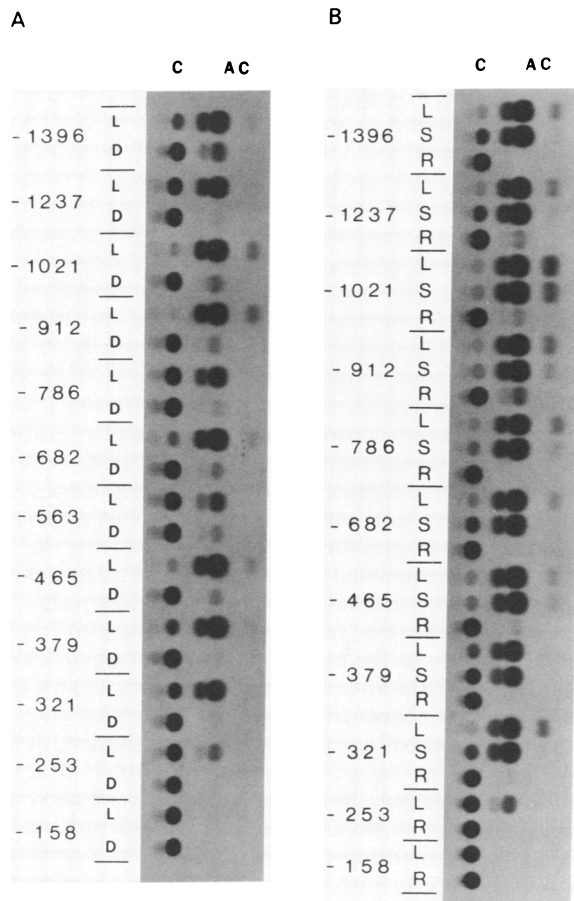


FIG. 2. Characterization of the 5' deletion mutants. (A) Effects of the deletion mutants on the strength and light regulation of the *cab1* promoter are shown as CATase activity. Pools of at least 10 (3 for the mutant -563) independently transformed tobacco shoots grown under light (L) or dark (D) were analyzed. (B) Effects of the deletion mutants on the organ specificity of *cab1* promoter are shown by CATase activity in leaf (L), stem (S), and root (R). AC, acetylchloramphenicol; C, chloramphenicol.

All of the internal deletion mutants exhibited organ-specific expression of the gene in green tissues, although bigger deletions (-518, -589, -766) decreased the promoter strength by a factor of about 2-3. The promoter activity of all of the deletion mutants was also light dependent (data not shown). These results suggest that at least one regulatory element is located at the further upstream region between -1396 and -766, whereas an additional element may be located at the -500 region.

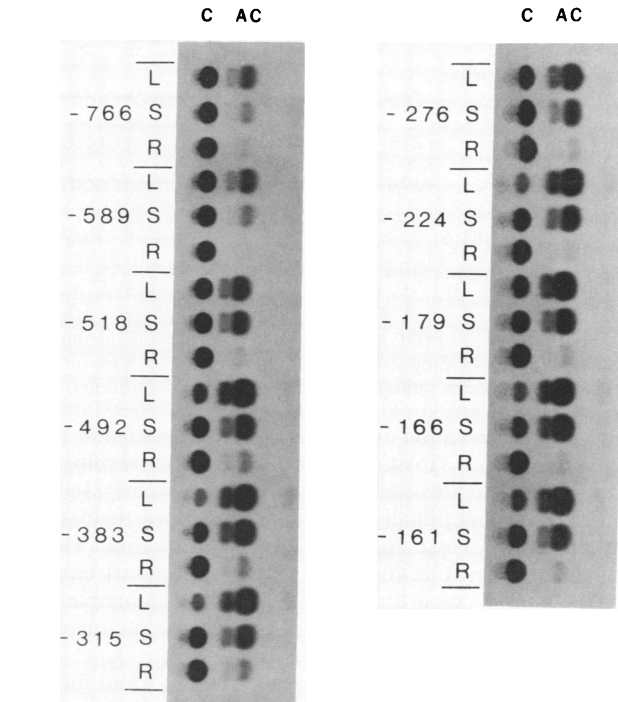


FIG. 3. Organ specificity of internal deletion mutants. Numbers indicate the end points of 3' deletion mutants that were joined to the 5' deletion mutant -158 for the construction of internal deletion mutants. Effects of the deletion mutants are shown as CATase activity of leaf (L), stem (S), and root (R). AC, acetylchloramphenicol; C, chloramphenicol.

The *cab* Upstream Regulatory Elements Function with a Heterologous *nos* Promoter. Analyses of 5' and internal deletion mutants demonstrated that there are multiple elements at the 5' control region regulating organ specificity of the *cab1* promoter. To examine whether these regulatory elements could function with a heterologous promoter, several 3' deletion mutants of the *cab1* promoter were connected to the defective *nos* promoter 5' deletion mutant -101, in which the upstream essential region was removed (23). The characteristics of these hybrid *cab1-nos* promoters were studied in transformed tobacco plants. When the *cab1* 3' deletion mutants -166, -179, -224, and -276 were linked to the *nos* -101 mutant, the hybrid promoters were organ-specific (Fig. 4) and light-inducible (data not shown). However, the bigger internal deletion mutants, -315 and -383, were unable to form an active promoter. Therefore, at least one regulatory element controlling the promoter specificity should be located at the 39-bp (between -315 and -276)

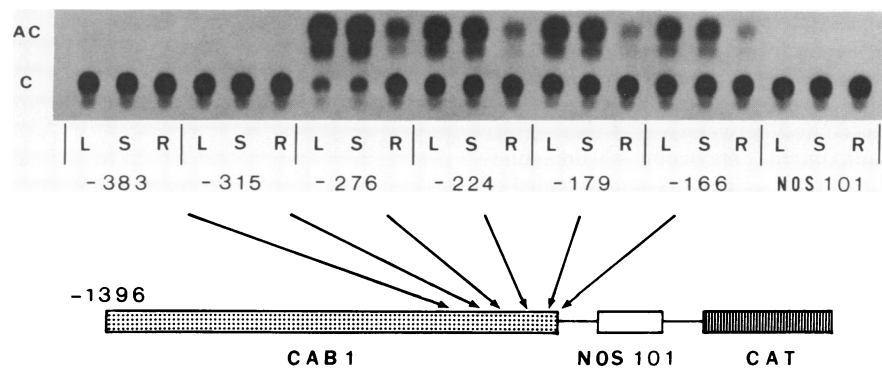


FIG. 4. Organ specificity of *cab1-nos* 101 hybrid promoters. Effects of the hybrid promoters are shown as CATase activity of leaf (L), stem (S), and root (R). Dotted, open, and striped boxes represent *cab1* promoter upstream region, *nos* promoter 5' deletion -101, and CATase coding region, respectively. The end points of 3' deletion mutants of *cab1* promoter are indicated by arrows.

L D R	-318		-158
+ - -	-258	GATCTATT CGTATACGTGT CACGTCATGAGTGGGTGTTTCGCCAATCCATGAAACGCACC...GCCAATAGC	
+ - -	-260	GATCTATT CGTATACGTGT CACGTCATGAGTGGGTGTTTCGCCAATCCATGAAACGCA.....GCCAATAGC	
+ - -	-275	GATCTATT CGTATACGTGT CACGTCATGAGTGGGTGTTTCGCC.....GCCAATAGC	
+ - -	-276	GATCTATT CGTATACGTGT CACGTCATGAGTGGGTGTTTCGC.....GCCAATAGC	
+ - -	-296	GATCTATT CGTATACGTGT CAC.....GCCAATAGC	
- - -	-303	GATCTATT CGTATACGCCAATAGC	

FIG. 5. Characterization of the immediate upstream regulatory region. The relative promoter activity of the deletion mutants in light-grown leaf (L), dark-grown leaf (D), and root (R) is shown at the left-hand side. Potential Z-DNA-forming sequence and CCAAT box are in bold type. An arrow indicates the 8-bp Z-DNA-forming sequence whose inverted repeats are also found in the other upstream regions. The numbers shown to the left of sequences indicate the end points of 3' deletions.

region. The observation that the far upstream region (between -1396 and -315) cannot function with a heterologous *nos* promoter indicates that the regulatory element(s) in this region may be functionally different from other regulatory elements located close to the CCAAT box region. These results also suggest that the DNA sequences downstream of the *cabl* CCAAT box are also involved in light-inducible and organ-specific expression of the *cab* gene by interacting with the upstream regulatory elements.

A Potential Z-DNA-Forming Sequence Is Important for the *cab* Promoter Specificity. To further understand the structure of the upstream elements at the 39-bp region, six additional mutants were made by fusing 15–60 bp of the immediate upstream region to the DNA sequence downstream from -158 (Fig. 5). The results in Fig. 5 show that the smallest DNA fragment that confers the light-dependent expression in leaf is 22 bp. This construct exhibited approximately one-fourth to one-fifth of wild-type promoter activity. Further deletion of the DNA fragment to 15 bp abolished the promoter activity. Therefore, at least one regulatory element that is involved in the *cabl* promoter specificity was nullified by deleting the 7 bp (deletion -303). This deletion also removed 4 bp from CGTATACGTGT, which is a potential Z-DNA-forming element, indicating that this sequence is important for organ specificity and light inducibility of *cabl* promoter.

DISCUSSION

The cis-acting DNA sequences required for the organ-specific and light-inducible expression of *cabl* gene were studied by introducing deletion mutants into tobacco plants and then analyzing their effects on the regulation of gene expression. A summary of the results is shown in Fig. 6. Our results indicate that there are multiple cis regulatory elements involved in organ specificity and light inducibility of the *Arabidopsis cabl* promoter as found in the 5' control region of a pea *rbcS* gene (9). Two of these elements are located in the immediate upstream region from the CCAAT box of the *cabl* promoter. One element, which is located between -158 and -253, was sufficient for promoter specificity, but another element, which is located between -253 and -321, was required for the maximum expression of the gene. Existence of additional regulatory elements was revealed in the further upstream region by the observation that deletion of the immediate upstream regulatory region from the *cabl* promoter did not affect the promoter activity. In this far upstream region, at least one regulatory element is located between -1396 and -766. This region conferred, at the reduced level, an organ-specific expression of the gene when it was connected to its own CCAAT and TATA box region. Addition of the -500 region restored the promoter to full strength in green tissues, indicating that there may be an additional element at the -500 region.

The *cabl* upstream control region containing far upstream elements and immediate upstream elements conferred organ specificity and light inducibility on a heterologous *nos* promoter. However, the far upstream regulatory elements alone were unable to form a functional promoter with the *nos* promoter, suggesting that the far upstream elements are promoter-specific modulators. Alternatively, the *nos* CCAAT and TATA box region may not be able to form an active promoter with the upstream regulatory region when it is placed at a far distance, since addition of a 200-bp DNA fragment to the sequence between the CCAAT box and upstream elements of *nos* promoter destroyed the promoter function (24).

Further studies on the immediate upstream regulatory element showed that the position of the regulatory element involved in organ-specific and light-inducible regulation of *cabl* promoter coincides with a stretch of 11 alternating purine and pyrimidine residues (CGTATACGTGT), which is a potential Z-DNA-forming sequence, located at the -300 region. Similar Z-DNA-forming sequences (ACACATAT)

		plasmid	activity		
			L	D	R
A		711-1396	++	-	-
		711-321	++	-	-
		711-253	+	-	-
		711-158	-	-	-
B		714-166	++	-	-
		714-276	++	-	-
		714-315	++	-	-
		714-492	++	-	-
		714-766	+	-	-
C		716-166	+	-	-
		716-276	+	-	-
		716-315	-	-	-
D		849-258	+	-	-
		849-296	+	-	-
		849-303	-	-	-

FIG. 6. Summary diagram of deletion analyses. A schematic representation of the *Arabidopsis cabl* promoter and its deletion mutants is shown on the left. Closed circles indicate the regulatory element of 8-bp Z-DNA-forming sequence and its inverted repeats. Squares indicate CCAAT boxes (C) and TATA boxes (T) of *cabl* (open) or *nos* (striped) promoter. Internal deletions are indicated by broken lines. L, light-grown leaf tissues; D, dark-grown leaf tissues; R, roots.

are found in an inverted orientation at -1230 and -240 regions of the *cab1* promoter where the additional regulatory elements are expected. DNA sequence comparison of the different *Arabidopsis cab* promoters revealed that another *cab* promoter also carries a potential Z-DNA-forming element with a similar sequence to the *cab1* element. The presence of potential Z-DNA-forming sequences in plant promoter regions is a common phenomenon (25). The functional analyses of *nos* promoter strongly indicate that a Z-DNA-forming element located at an immediate upstream region is essential for the promoter activity (21, 24). Interestingly, cereal storage protein genes share a potential Z-DNA-forming sequence (YATRCATGT, where Y = pyrimidine and R = purine), about 200-300 bp upstream from the transcription initiation site, which trans-acting factors interact with (26).

The cis-acting elements involved in the light-dependent organ-specific *cab* gene expression are different from the box II and III elements of the pea *rbcS* gene. Therefore, the molecular mechanisms regulating the expression of these photosynthetic genes appear to be different from each other. We were, so far, unable to obtain a deletion mutant that exhibited only either organ specificity or light inducibility, suggesting that the cis-acting elements responsible for both regulations are identical or, if not, tightly linked. Detailed analyses of the upstream cis regulatory element are necessary to answer these questions.

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