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)

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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 nia 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 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 #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 (GTTACCTGGGCGCTT) 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 (Asp718), 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 EcoRI–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 EcoRI or EcoRV 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 µl 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 HindIII. The 3' deletion mutants were obtained in a similar manner from plasmid pGA618, which was constructed by inserting the 2044-bp EcoRI–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. J04096).

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fragment into pGAg16. pGAg18 was opened at either Sac II site located 68 bp downstream from the ATG initiation codon in the coding region of the cabI 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 (Asp718 site) with [α-32P]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 pGAg165 to generate pGAg111-mt. pGAg165 was derived from pGAg80 (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 pGAg80. Therefore, translation started from cabI 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 (pGAg714-mt) were generated by fusing the 3' deletion mutant fragments to the 5' deletion mutant pGAg111-158 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 (22). N. tabacum shoot cultures were stably transformed by the cocultivation methods as described earlier (22).

Analysis of Gene Expression. To reduce variation of expression 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 μg of total protein was used in the analyses of cabI–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 cabI 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 cabI promoter was progressively deleted from the 5' side of the DNA fragment with BAL-31. We have constructed two plasmids (pGAg166 and pGAg167) 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 T1 plasmid, generating a translational fusion between the cabI 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 cabI 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 cabI 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 organ specificity. The other element, located between —321 and —253, appears to be a modulator that enhances the cabI promoter activity.

The Far Upstream Region Is Also Involved in cabI 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 cabI 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 cabI promoter were examined in transformed tobacco plants (Fig. 3).
Fig. 2. Characterization of the 5' deletion mutants. (A) Effects of the deletion mutants on the strength and light regulation of the cabl 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 cabl 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 cabl promoter. To examine whether these regulatory elements could function with a heterologous promoter, several 3' deletion mutants of the cabl 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 cabl–nos promoters were studied in transformed tobacco plants. When the cabl 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 cabl–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 cabl promoter upstream region, nos promoter 5' deletion −101, and CATase coding region, respectively. The end points of 3' deletion mutants of cabl promoter are indicated by arrows.
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 cab1 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 cab1 promoter specificity was nullified by deleting the 7 bp (deletion -303). This deletion also removed 4 bp from CCGTATACGTGT, which is a potential Z-DNA-forming element, indicating that this sequence is important for organ specificity and light inducibility of cab1 promoter.

DISCUSSION

The cis-acting DNA sequences required for the organ-specific and light-inducible expression of cab1 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 cab1 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 cab1 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 cab1 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 cab1 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 cab1 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) in the immediate region around this sequence were also found in the promoter of the nos gene (24).

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 the sequences indicate the end points of 3′ deletions.

The numbers indicate the end points of Z-DNA-forming sequence deletions. The potential Z-DNA-forming sequence in the promoter of Arabidopsis cab1 is shown at the top of the diagram. The promoter of nos is shown at the bottom of the diagram. The potential Z-DNA-forming sequence is shown in bold type.
are found in an inverted orientation at −1230 and −240 regions of the cabI 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 cabI 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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