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Localization and conditional redundancy of regulatory elements in *rbcS-3A*, a pea gene encoding the small subunit of ribulose-bisphosphate carboxylase

(transgenic plants/cis-acting elements/light regulation/plant development/*Pisum sativum*)

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ABSTRACT **Expression of the pea *rbcS-3A* gene, one of a family of genes encoding the small subunit of ribulose-bisphosphate carboxylase [EC 4.1.1.39], is regulated by light and is restricted to chloroplast-containing cells. We analyzed the effects of light and development on *rbcS-3A* expression in transgenic plants. Two highly conserved sequences ("boxes" II and III) around nucleotide position −150 (relative to the transcription initiation site, +1) are required for *rbcS-3A* expression. The so-defined positive elements overlap with previously identified negative light-regulatory elements. In the case of box II, which has sequence similarity to the core enhancer motif of simian virus 40, a GG→CC transversion is sufficient to abolish expression. The effect of mutations in boxes II and III can only be measured when sequences upstream of −170 are removed, because sequences both 5′ and 3′ of −170 can direct light-regulated and organ-specific expression. This implies that there is a redundancy of cis-acting elements in the 5′ noncoding region of *rbcS-3A*. However, we show that the sequences upstream of −170 are dispensable only in the mature leaves of a green plant. In contrast, in the young, expanding leaves at the top of a green plant, as well as in seedlings, the distal elements are required for high-level expression. Therefore, redundancy is not absolute, and the requirements for *rbcS-3A* expression change during plant development.**

The pea *rbcS-3A* gene is a member of the gene family that codes for the small subunit of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], the key enzyme in photosynthetic carbon assimilation. The expression of this gene and other *rbcS* genes is regulated by at least three parameters: tissue type, light conditions, and stage of development (for reviews see refs. 1 and 2). The *rbcS-3A* transcript levels are highest in leaves, lower in stems, and below the limits of detection in roots (3, 4). When pea plants are put in the dark, *rbcS-3A* transcript levels decline rapidly, but they return to previous levels when the plants are reexposed to light (4). In etiolated seedlings, red light induces *rbcS-3A* expression, whereas far-red light can reverse the effect, indicating that the response is mediated by the photoreceptor phytochrome. In mature green plants, red light is ineffective and the data suggest that a blue-light receptor acts in concert with phytochrome (5). Therefore, the wavelength requirements for induction change during plant development.

Gene-transfer experiments demonstrated that a 280-base-pair (bp) enhancer-like element in the upstream region (at positions −330 to −50 relative to the transcription start site; see Fig. 1*a*) can confer both light induction and organ specificity upon a reporter gene (6). *In vitro* protein-binding experiments identified a protein factor, GT-1, that binds to two conserved sequences, boxes II and III, in the −150 region. Two additional binding sites, boxes II* and III*, with sequence homology to boxes II and III were detected further upstream, around position −220 (8). These boxes have strong sequence homology to the constitutive enhancer elements from simian virus 40 (SV40) and adenovirus, but detailed *in vivo* experiments showed that they can function as negative light-regulatory elements (LREs) (7). For example, when three copies of box II (TGTGTGGTTAATATG) are placed between the constitutive enhancer and promoter of the 35S gene of cauliflower mosaic virus, expression of a downstream reporter gene is turned off in the dark, but not in the light.

Sequences in the vicinity of box II not only play a role as negative LREs but also are important for turning on transcription. Analysis of 5′ deletion mutants showed that there is a steep drop in transcript levels when sequences between −166 and −149 are removed (7). This indicates that important enhancer elements are present in this area. However, the precise location of these positive elements has not been determined.

Our goal is to precisely identify the cis-acting elements and trans-acting factors that mediate the complex regulation of the *rbcS-3A* gene. In order to assess the effect of mutations on light induction, tissue specificity, developmental regulation, and, most important, their interrelationships, the use of intact plants as the experimental material is a necessity. Techniques have been developed for the efficient introduction of foreign DNA into plant cells, and plants containing the foreign DNA can be regenerated in large numbers (9–11). By using such transgenic plants, a large set of mutations can be evaluated for their effects in each organ, under various experimental conditions and throughout the life cycle of the plant.

In this report we delineate more precisely the sequences needed for regulated expression of the *rbcS-3A* gene. We show that mutations in boxes II and III abolish transcriptional activity, implying that positive and negative transcriptional elements physically overlap. Further, we demonstrate that sequences upstream and downstream of nucleotide −170 are capable of directing organ-specific and light-dependent transcription. Finally, we exploit the full potential of the transgenic plant system to elucidate a remarkable developmental specificity of the regulatory elements in the *rbcS-3A* upstream region.

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Abbreviations: SV40, simian virus 40; LRE, light-regulatory element; CAT, chloramphenicol acetyltransferase; 35S promoter, cauliflower mosaic virus DNA element that promotes transcription of 35S RNA.

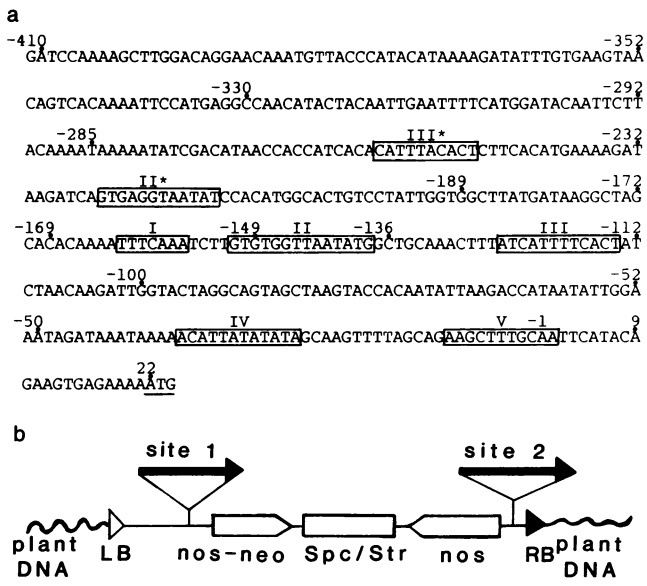

FIG. 1. Nucleotide sequence of the *rbcS-3A* upstream region and schematic representation of the structure of the transferred DNA (T-DNA) after integration into the tobacco genome. (*a*) Boxes I–V are regions of sequence identity between pea *rbcS* genes. Boxes II* and III* have homology to boxes II and III, respectively (6, 7). (*b*) In all experiments except those described in Fig. 3 *b* and *c*, the wild-type *rbcS-3A* gene containing 410 bp of upstream region and 385 bp of 3′ noncoding DNA ("reference gene") was inserted into the polylinker (site 1). The mutant genes ("test genes") were inserted into site 2, between the nopaline synthase gene (nos) and the right border of the T-DNA (RB), always in the orientation shown. All test genes have a 3′ tail derived from the *rbcS-E9* gene. For the experiments of Fig. 3 *b* and *c*, no reference gene was present and the *rbcS-3A*/35S/CAT chimeric genes (where 35S represents the 35S RNA promoter of cauliflower mosaic virus and CAT represents the sequence encoding chloramphenicol acetyltransferase) were inserted into site 1. LB, left border of T-DNA; RB, right border of T-DNA; nos-neo, nopaline synthase-neomycin phosphotransferase (plant selectable marker); Spc/Str, bacterial selectable marker. This figure was adapted from figure 1 of ref. 7.

MATERIALS AND METHODS

Gene Cloning and Transfer. All constructs were derivatives of the T-DNA vector pMON200 (12). Details of the gene manipulations are available on request. Substitution mutations (see also legend of Fig. 2) were constructed by the site-directed mutagenesis method of Inouye and Inouye (13).

The level of expression of the *rbcS-3A* gene in transgenic tobacco plants is susceptible to position effects (7). To quantitate transcript levels of mutated genes, the reference gene system described by Kuhlemeier *et al.* (14) was used (the vector is depicted in Fig. 1*b*). The reference gene consists of the intact *rbcS-3A* gene containing 410 bp of upstream sequence and 385 bp of 3′ noncoding DNA. The mutated genes ("test genes") were separated from the reference gene by 5 kbp of DNA of non-plant origin. The pseudowild-type *rbcS-3A* gene differs from the reference gene in that it has a 3′ end derived from the pea *rbcS-E9* gene. In addition, it has two small linker insertions at −50 and +15 (7). The internal deletion of the *rbcS-3A* gene (construct 3 in Fig. 4) was made in the pseudowild-type background by deleting nucleotides −162 through −117, which removes boxes I, II, and III (7). Due to the partial homology between reference and test genes at their 3′ ends, the transcripts of the two genes can be quantitated simultaneously by nuclease S1 analysis using a single probe (4). The probe, derived from the 3′ end of the *rbcS-E9* gene, protects 231 nucleotides of the test-gene transcripts and 160 nucleotides of the reference-gene transcripts.

Constructs were transferred into the "disarmed" *Agrobacterium tumefaciens* strain GV3111 SE and subsequently introduced into *Nicotiana tabacum* cv. *xanthi* by the leaf-disk procedure (10). Selection was carried out as described (7).

Nuclease S1 Protection and Chloramphenicol Acetyltransferase (CAT) Assays. Total RNA was prepared from 0.5–3 g of plant material by a small-scale procedure that does not require ultracentrifugation (14). Nuclease S1 protection analysis of 3′ termini was performed with an end-labeled single-stranded probe derived from the *rbcS-E9* gene (4). S1 analysis of the 5′ end of the 35S promoter–CAT reporter gene was done with a uniformly labeled probe prepared by primer extension (7). CAT activity was determined by the method of Gorman *et al.* (15) with slight modifications (6).

Light Induction and Plant Analysis. Light-induction experiments were performed with either mature green plants or etiolated seedlings. For the experiments with mature green plants, F_1 seeds were surface-sterilized and germinated on Murashige–Skoog (MS) medium in the presence of kanamycin at 200 μg/ml. Six to ten seedlings were transferred to soil and, when they had 10–15 leaves, were transferred to the dark for 3 days. A sample of leaves from the middle of the plant was taken under dim green "safe" light and immediately frozen on dry ice. Subsequently, the plants were returned to bright white light for 24 hr, after which period another sample was taken. For the experiments with etiolated seedlings, F_1 seeds were surface-sterilized and plated on MS medium solidified with 0.7% Bacto-Agar, without antibiotics. Approximately 250 seeds were plated per 90-mm Petri dish and 2 or 3 dishes were used for one RNA preparation. Within 40 hr after plating, the Petri dishes were transferred to complete darkness. When the seedlings were 7 days old, half of the plates were exposed to bright white light for 24 hr while the other half were kept in darkness. Light and dark samples were thus collected at approximately the same time. Entire seedlings were uprooted with care so as not to transfer agar and were immediately frozen on dry ice.

To determine the effect of leaf age on *rbcS-3A* expression (see Fig. 4), transgenic plants were grown in soil until they had 20–25 leaves. Samples were collected early in the afternoon. For the first sample, leaves at the top of the plant were pooled to give sufficient material to obtain at least 100 μg of total RNA (0.5–1 g of leaves). In the case of the −166 mutant, leaves 2, 3, 4, 5, 7, 9, 11 (the largest leaf), 13, 16, 18, 20, 22, and 24 were subsequently collected. The top leaves, the largest leaf in the middle of the plant, and the lowest leaf that was not visibly senescing were harvested from the transgenic plants containing the pseudowild-type *rbcS-3A* gene or the internal deletion.

RESULTS

Localization of Positive Cis-Acting Elements. Previous analysis of 5′ deletion mutations of *rbcS-3A* demonstrated that the 5′ boundary of a positive element required for expression is located between −166 and −149 (7). Downstream of −166 there are three short sequences, boxes I–III, that are conserved among the five pea *rbcS* genes (4). Boxes II and III have a high degree of sequence similarity to mammalian enhancers and, in addition, serve as binding sites for nuclear protein GT-1 (8). With this in mind, we decided to create small substitution mutations in boxes I–III. In doing such experiments, it is important to choose the proper sequence context. Two considerations have to be taken into account. First, the *rbcS-3A* gene, like many other genes, is susceptible to position effects in transgenic tobacco plants. To be able to quantitate the expression of a mutated gene independently of positional variation, we previously designed an agrobacterial

vector containing an unmodified *rbcS-3A* gene as an internal standard ("reference gene" or "intact *rbcS-3A* gene"). The mutated genes ("test genes") have 3′ ends derived from the pea *rbcS-E9* gene and are inserted into the same vector. By virtue of the partial homology at the 3′ ends, the transcripts of reference and test genes can be visualized and quantitated by nuclease S1 protection with a single probe (see refs. 4 and 14 and *Materials and Methods* for details). Thus, the transcripts of the intact *rbcS-3A* gene serve as a reference to calibrate the transcript level of the mutated gene. By this method, the influence of genomic position on the transgene can be virtually eliminated (7, 14). Second, the mutations must be analyzed in a minimal sequence background. Previously, when we assayed mutations in the presence of the distal part of the *rbcS-3A* gene (positions −410 to −171), no effects could be measured (7). Therefore, we made use of the single *Nhe* I restriction site, at position −175, to remove the distal sequences. In addition it must be noted that the effects of these mutations were assayed in the presence of the *rbcS-3A* promoter and coding region. Replacement of these sequences by a reporter gene severely reduced expression, indicating that cis elements indispensable for high expression are located downstream of −50 (7).

Fig. 2 shows that substitution of box I is without apparent effect, since the S1 signal from this mutant was approximately equal to that of the intact *rbcS-3A* reference gene. However, interruption of either box II or box III virtually abolishes expression. Only in the case of box III mutants could a low level of expression (<5%) be detected in some of the transgenic plants.

Box II has sequence homology to the SV40 core enhancer motif, GTGGWWWG (where W = A or T). The SV40 sequence has been analyzed extensively by site-specific mutagenesis and transfection assays (16, 17). Those studies made it clear that in the SV40 core enhancer, two adjacent guanine nucleotides are essential for transcriptional activity. Methylation-interference experiments using the *rbcS-3A* upstream region indicated that the two guanines in box II are critical for the binding of nuclear protein GT-1 (8). Therefore, we decided to change the two guanines in box II to cytosines and assess the effect of this mutation on the expression of the −175 construct. As in the case of the SV40 enhancer, a 2-bp GG→CC transversion in the −175 mutant abolished activity (Fig. 2, lane 5).

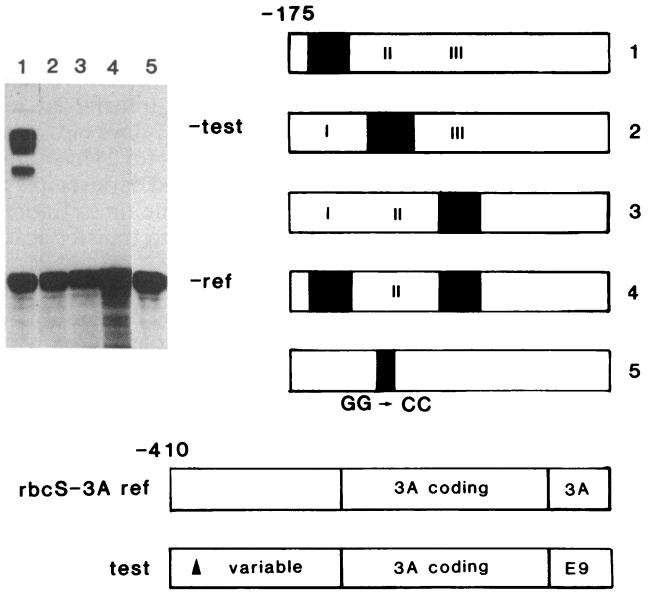

FIG. 2. Disruption of either box II or box III abolishes *rbcS-3A* expression. Precise substitution mutations were created by oligonucleotide-directed mutagenesis as described (7). The mutant DNAs were cut with restriction endonucleases *Bam*HI and *Nhe* I to remove upstream sequences from −410 to −175. The mutated genes were inserted into site 2 of the derivative of pMON200 (see Fig. 1*b*). Structures of reference and test genes are shown at the bottom. The reference gene consists of *rbcS-3A* with 410 bp of upstream DNA and 385 bp of 3′ noncoding DNA. All test genes were truncated at −175 and had a 3′ tail derived from *rbcS-E9*. Lane 1, box I (TTTCAAAT) was replaced by AGAATTCA; lane 2, box II (TGTGGTTAATAT) was replaced by ATCTAAGAGCTC; lane 3, box III (TCATTTTC) was replaced by CTCGAGAA; lane 4, box I and box III were replaced by AGAATTCA and CTCGAGAA, respectively; lane 5, the guanines in box II at positions −147 and −146 were replaced by cytosines. An average of seven transgenic plants were analyzed individually for nuclease S1 protection with a probe from the *rbcS-E9* 3′ end. This single-stranded probe protects 231 nucleotides of the test gene and 160 nucleotides of the reference gene. Twenty micrograms of total RNA was used per reaction.

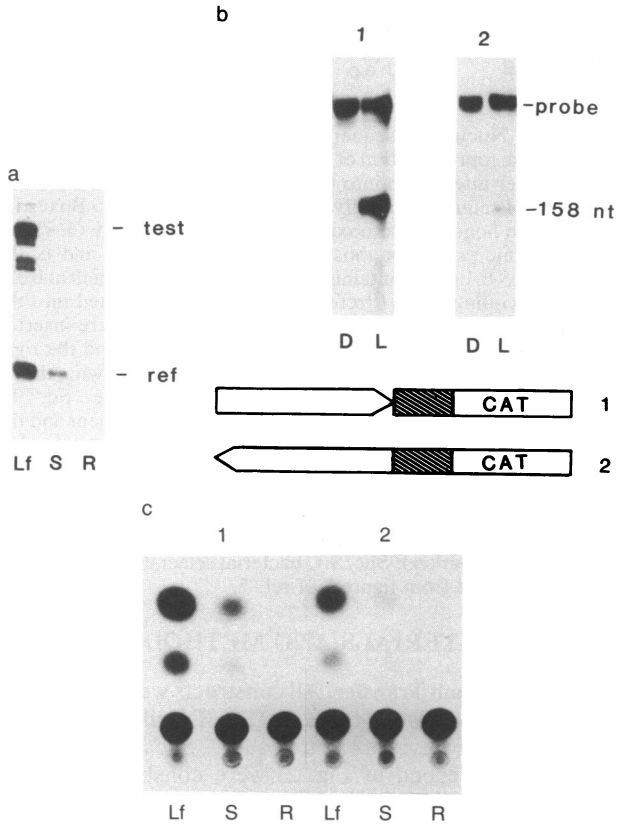

FIG. 3. Redundancy of cis-acting elements conferring light regulation and organ specificity. (*a*) The 5′ −166 deletion of the *rbcS-3A* pseudowild-type gene was cloned into the derivative of pMON200 having an intact *rbcS-3A* reference gene. Twenty micrograms of RNA from leaves (Lf), stems (S), or roots (R) was subjected to 3′ nuclease S1 analysis. The probe was as in Fig. 2. Three transgenic plants were analyzed individually; one example is shown. (*b*) Lanes 1: the *Bam*HI–*Nhe* I fragment containing nucleotides −410 to −171 of the *rbcS-3A* upstream region was fused in the normal orientation to the reporter gene. This reporter gene consisted of the 35S promoter (−46 to +6; hatched bar) fused to the CAT gene followed by the *rbcS-E9* 3′ tail. The chimeric gene was cloned into the pMON200 polylinker and introduced into tobacco as described (7). Lanes 2: as lane 1, but with the *rbcS-3A* −410 to −171 fragment in the opposite orientation. Fifty micrograms of total RNA was subjected to quantitative 5′ nuclease S1 analysis using a uniformly labeled single-stranded probe specific for the 35S promoter–CAT reporter gene. Nine transgenic plants were analyzed individually for each construct; representative examples are shown. D, dark; L, light; nt, nucleotides. (*c*) Transgenic plants containing constructs depicted in *b* were analyzed for organ-specific expression. Five micrograms of soluble protein was analyzed for CAT activity.

Redundancy of Cis Elements for Light Induction and Organ Specificity. The 5′ deletion mutant retaining 166 bp of upstream DNA is fully expressed and inducible by light (7). Mutations in either box II or box III abolish expression. However, when the same substitution mutations, or even a complete deletion of boxes I–III, were created in the *rbcS-3A* gene containing 410 bp of upstream region, no effects on transcript levels could be measured (7). These results indicated that there are redundant enhancer elements further upstream.

Here, we demonstrate directly that sequences upstream of −171 as well as sequences downstream of −166 are capable of mediating regulated expression and that there is a redundancy of cis-acting elements for both light induction and organ specificity. Fig. 3*a* shows that the −166 construct also directs organ-specific expression, since the transcript was most abundant in leaves, less abundant in stems, and absent in roots. To assess the activity of upstream sequences, the region from −410 to −171 was fused in two orientations to a 35S promoter–CAT reporter gene. This distal upstream fragment confers both light induction and organ specificity upon the reporter gene (Fig. 3 *b* and *c*). For the normal and reverse orientation, transgenic plants were analyzed individually and the results shown are from a representative individual of each. The −410 to −171 fragment is functional in either orientation, but the level of expression is lower for the reverse orientation.

Expression of the −166 Deletion Mutant During Development. In a tobacco plant, the shoot forms from the apical meristem; hence, the upper leaves are the youngest, the middle leaves are mature and fully expanded, and further down are the older, senescing leaves. Fig. 4, panel 1, shows the S1 analysis of individual leaves from a transgenic tobacco plant carrying the −166 deletion mutant with the intact *rbcS-3A* gene as the internal standard. Expression of the reference gene, on a per μg of total RNA basis, was rather uniform throughout the plant; only in the older, senescent leaves was there a decrease in transcript level. The −166 mutant was expressed to the same level as the reference gene in the fully expanded leaves in the middle of the plant and also in the older leaves at the bottom. In the young top leaves, however, the expression of the −166 mutant was decreased by a factor of 3–5 relative to the reference gene. In transgenic plants that contained the pseudowild-type *rbcS-3A* gene (*rbcS-3A* with 3′ end derived from *rbcS-E9*) or the internal deletion of boxes I–III, expression in the upper leaves was not affected (Fig. 4, panels 2 and 3).

Since the expression of the −166 mutant, which contains only proximal elements, was reduced in immature tissues, we searched for additional parameters that might be of influence. When the −166 mutant was analyzed in etiolated seedlings, expression was still induced by light (Fig. 5). However, the level of expression was diminished by a factor of 3–5 compared to the reference gene, which has both distal and proximal elements. The reduction of expression was caused by the deletion and was not due to the position of the gene in the T-DNA vector, since the pseudowild type, when located in the same position, was fully expressed (Fig. 5, lanes 5–8). Therefore, elements that are redundant in mature green plants are necessary for full expression in etiolated seedlings. This phenomenon was not restricted to the −166 mutant but also appeared in constructs that contained only boxes II and III (data not shown). The internal-deletion mutant, in which boxes I–III had been removed, was not significantly affected (Fig. 5, lanes 9–12).

DISCUSSION

The upstream region of the pea *rbcS-3A* gene contains at least four sequences (boxes II, III, II*, and III*) that serve as

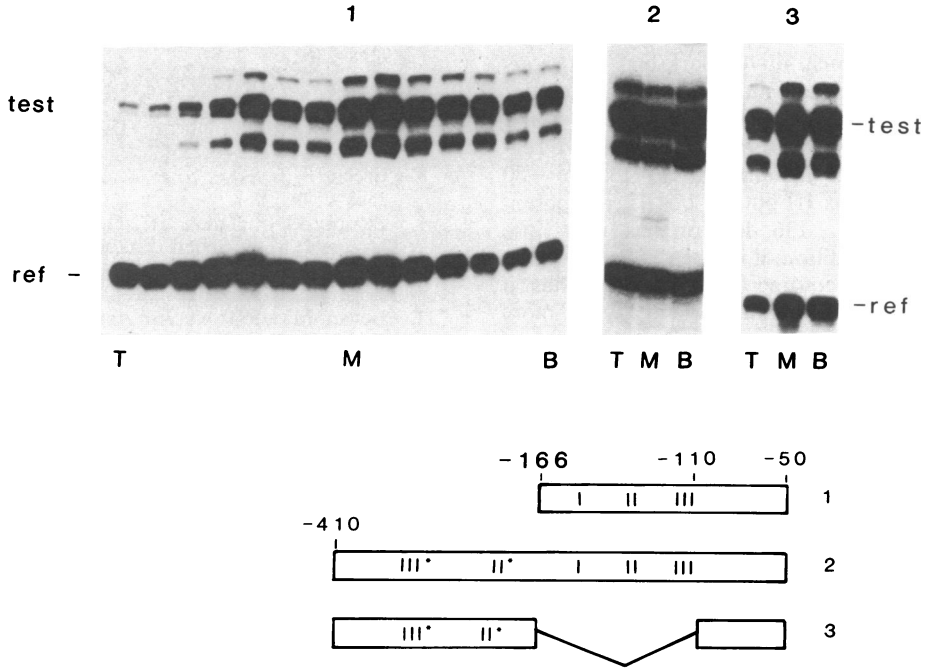

FIG. 4. Leaf age influences the expression of the −166 mutant. The three constructs analyzed all contained the intact *rbcS-3A* reference gene and either the −166 deletion of the pseudowild-type gene (construct 1), or the complete pseudowild-type gene with 410 bp of upstream region (construct 2), or the complete pseudowild-type gene with the internal deletion from −162 through −117, which removes boxes I–III (construct 3). Plants were grown in a growth chamber or in the greenhouse until they had 20–25 leaves. Individual leaves were collected, total RNA was isolated, and 20 μg was subjected to 3′ nuclease S1 analysis. Panel 1 shows analysis of individual leaves from the young top leaves (T), to the largest leaf in the middle of the plant (M), down to the oldest leaf at the bottom (B). Panels 2 and 3 show analysis of the top leaves, a leaf from the middle, and a leaf at the bottom of plants containing the pseudowild-type and the internal deletion, respectively. Four, three, and two individual plants were analyzed for the −166 construct, pseudowild type, and internal deletion, respectively.

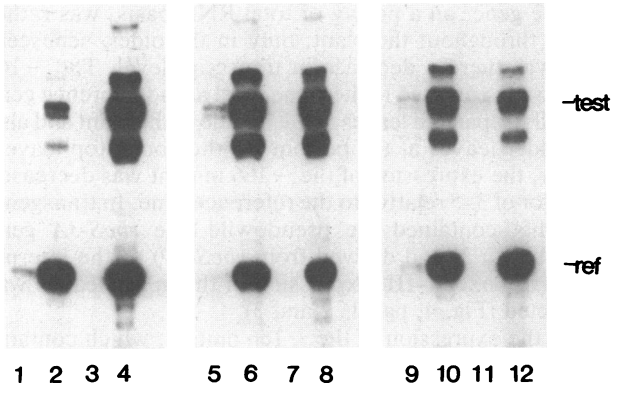

FIG. 5. The −166 mutant has reduced expression in etiolated seedlings. The same constructs as in Fig. 4 were analyzed for expression in etiolated seedlings. Lanes 1–4, −166 mutant; lanes 5–8, pseudowild type; lanes 9–12, pseudowild type with internal deletion of boxes I–III; lanes 1, 5, and 9, etiolated dark; lanes 2, 6, and 10, etiolated light; lanes 3, 6, and 11, mature green, dark; lanes 4, 8, 12, mature green, light. Twenty micrograms of total RNA was subjected to 3′ nuclease S1 analysis using the probe derived from the *rbcS-E9* gene. Seedlings of six, five, and two independent transgenic plants were analyzed for the −166 construct, pseudowild type, and internal deletion, respectively.

binding sites for the nuclear protein GT-1 (8). These sequences are highly similar to the enhancer motifs of SV40 and adenovirus (7). Detailed *in vivo* analysis of boxes II and III has shown that, in plants, they can function as negative regulatory elements when placed between the promoter and enhancer of a constitutively expressed reporter gene (7). In this paper we demonstrate that both box II and box III also play a role as mediators for positive enhancement of transcription. Thus, the negative and positive cis-acting elements overlap or are perhaps identical. If positive and negative regulatory factors have different sequence requirements, it may be possible to obtain constitutive "dark-up" mutations with a fine mutagenesis approach similar to that carried out with the human β-interferon gene (18–20). Certainly, the number of elements required for negative and positive regulation is not identical. For instance, the −136 to −50 fragment, which contains box III but not box II, is a potent negative LRE, but the 5′ −136 deletion has very little enhancing activity (7). In addition, it is clear from Fig. 2 that both box II and box III are required for expression. Thus, it seems that the requirements for enhancer activity are more stringent than those for negative regulation.

The fact that positive and negative elements physically overlap raises interesting questions concerning the regulation of *rbcS-3A*. To understand how positive transcription factors compete with repressors for the same DNA sequences, it will be necessary to characterize the proteins that bind to boxes II and III. The *in vitro* protein-binding experiments (8) on the −170 to −50 fragment showed that mutations in either box II or box III, which abolish expression *in vivo* (Fig. 3), also impair GT-1 binding. This may be taken as correlative evidence for a role of GT-1 as a positive transcription factor. Direct proof, however, will require an *in vitro* transcription system for functional assay of GT-1.

Within the *rbcS-3A* gene there is a redundancy of regulatory elements (Fig. 3; see also ref. 7). But the data presented in Figs. 4 and 5 indicate that redundancy is not an absolute property but is influenced by the stage of development. The −166 deletion was fully active in mature green leaves, and under these assay conditions the distal upstream region is redundant. However, in very young leaves and in seedlings the transcript level was markedly lower than that of the intact *rbcS-3A* reference gene. An explanation for this phenomenon could be that there is a limiting concentration of (positive-acting) trans factors in etiolated and young tissues. Only under such conditions would cooperativity with additional cis elements offer an advantage in the competition for scarce trans factors. Alternatively, the distal and proximal elements could have distinct developmental specificities, with the −410 to −171 region being a general enhancer and the proximal region an adult enhancer. There is an interesting parallel case in the human α-fetoprotein gene. In that case, transfection studies have identified three redundant enhancer elements, but experiments in transgenic mice yielded a more diverse picture in which the elements are not functionally equivalent (21). In preliminary experiments designed to test whether the distal region is indeed functionally dissimilar to the proximal element, we have obtained data suggesting an internal redundancy within the distal element, making it more likely that it is a cooperation between functionally equivalent elements that leads to high expression in young leaves and seedlings.

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