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5′ Analysis of the soybean leghaemoglobin lbc_3 gene: regulatory elements required for promoter activity and organ specificity

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The soybean leghaemoglobin lbc_3 gene promoter was analysed in transgenic *Lotus corniculatus* plants. Hybrid-promoter constructions and 5′ deletions were studied using chimeric genes composed of the various promoters, the chloramphenicol acetyltransferase (CAT) coding sequence and the lbc_3 3′ flanking region. A 5′ *Bal*31 deletion series mapped a strong positive regulatory element between −1100 and −950. A weaker element located between −230 and −170 defined the minimum 5′ region required for detectable promoter activity. Reactivation of inactive promoters with deletion endpoints between −230 and the transcription initiation site was obtained employing the constitutive cauliflower mosaic virus (CaMV) 35S enhancer. The position of *cis* regulatory element(s) required for nodule-specific expression was defined to 37 bp between −139 and −102. This region contains sequences conserved in other leghaemoglobin and nodulin genes. No indispensable control elements were found on the lbc_3 3′ flanking region.

Key words: promoter elements/*Bal*31 deletions/hybrid promoters/root nodule expression/transgenic legumes

Introduction

Few cases of organ development from differentiated somatic cells are known in higher eukaryotes. One such example is root nodule development from root cortex cells of legumes. After infection with soil bacteria of the genera *Rhizobium* or *Bradyrhizobium*, redifferentiation of root cortex cells into meristematic cells initiates nodule development, which is first visible as anticlinal cell division. At this stage the bacteria are still contained within the infection thread (Newcomb *et al.*, 1979). Differentiation of meristematic cells into the organized tissues ultimately leads to a functional nodule, where bacteroids enclosed within the peribacteroid membrane supply the plant with assimilated nitrogen. Induction of the bacterial *nod* genes required for invasion of the plant roots by rhizobia is mediated by flavones excreted by the plant (Peters *et al.*, 1986). This is probably one of the first signals in a complex process of signal exchange and differential gene expression leading to the mature symbiotic association. Several plant and bacterial genes are specifically expressed during different stages of root nodule formation (Marcker *et al.*, 1984; Govers *et al.*, 1986). The symbiotic associations can therefore be used to study both prokaryotic and eukaryotic gene expression during a process of signal transduction and cell differentiation.

Approximately 30 plant polypeptides are specifically synthesized in soybean root nodules (Legocki and Verma, 1980; Gloudemans *et al.*, 1987). Among the predominant 'nodulins' are the leghaemoglobin Lba, Lbc_1, Lbc_2, Lbc_3, polypeptides, which regulate the oxygen concentration within the nodule (Fuchsmann and Appleby, 1979). The corresponding *lb* genes have been characterized (Hyldig-Nielsen *et al.*, 1982; Wiborg *et al.*, 1982), and recently the lbc_3 gene was transferred into another legume *Lotus corniculatus* (Stougaard *et al.*, 1986, 1987b). Both the gene itself and a chimeric CAT-gene under control of the lbc_3 5′ and 3′ flanking regions were nodule-specifically expressed, indicating a conserved regulatory mechanism for *lb* gene expression among

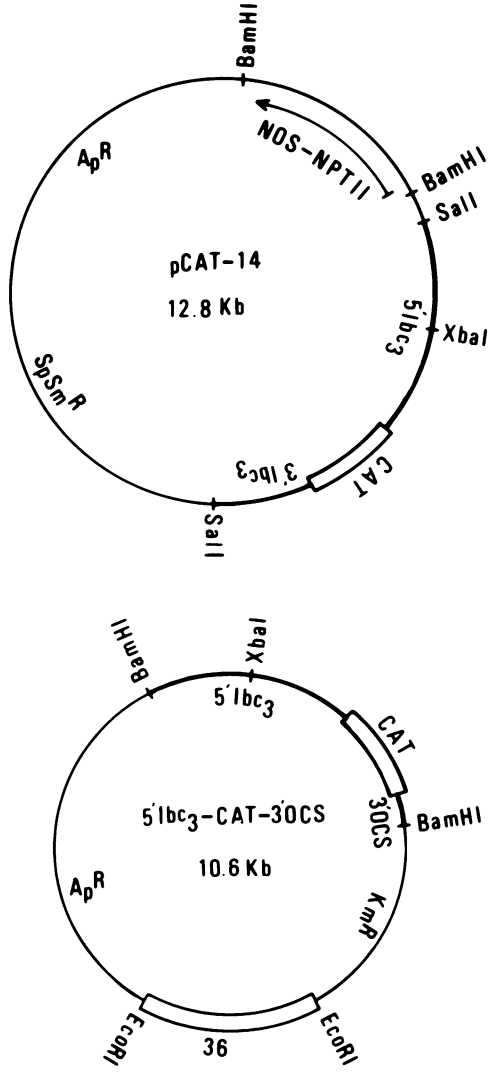

Fig. 1. Plasmids constructed for the 5′ deletion and 3′ replacement analysis of the soybean lbc_3 gene. The pIV2 vector carrying the *NOS–NPTII* reference gene was the basic vector used for the 5′ analysis. Deletions were inserted into the *Sal*I 275 bp from the *NOS* reference promoter in the same orientation as the original lbc_3 *5′3′-CAT* gene in pCAT-14. The lbc_3 *5′ -CAT-3′OCS* gene was constructed in the 'intermediate integration vector pAR6 Stougaard *et al.* (1987a) by replacing the 3′lbc_3 flanking region of the lbc_3 *5′3′-CAT* gene.

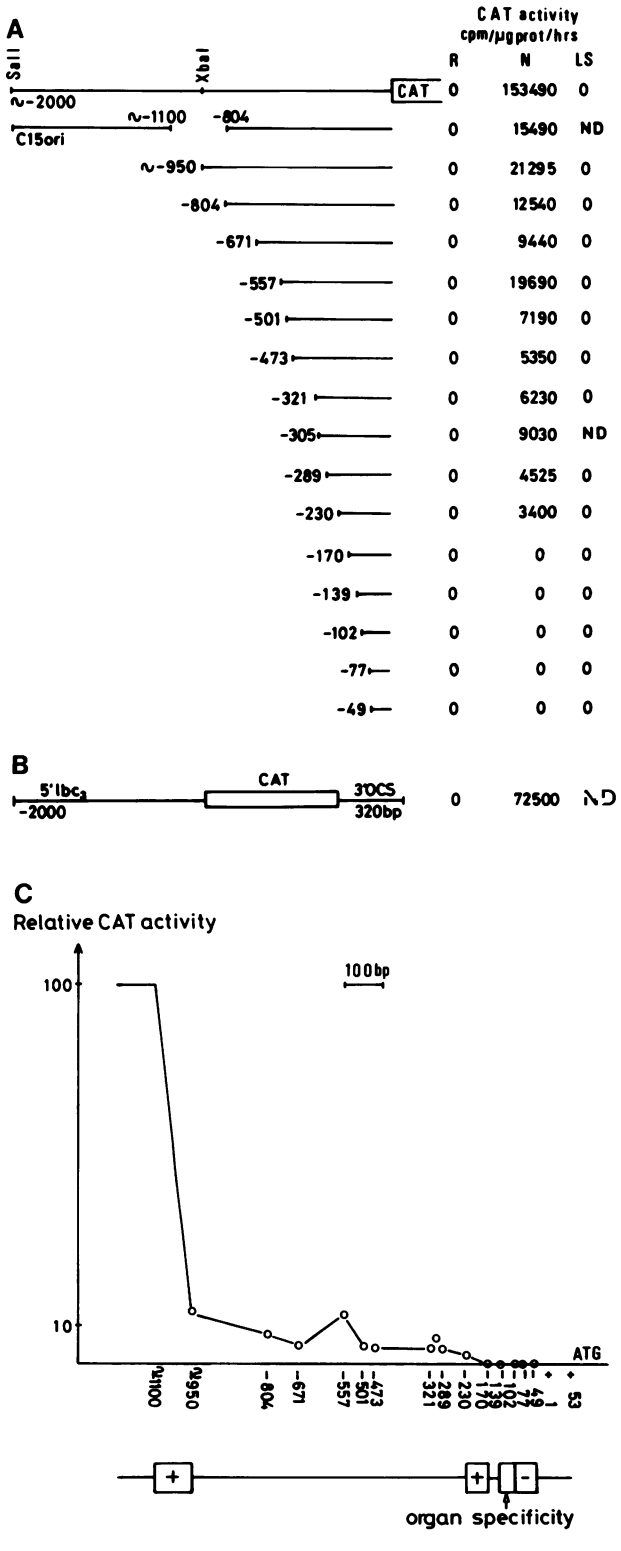

Fig. 2. (A) Deletion analysis of the 2 kb 5′ flanking region of the chimeric *lbc*$_3$ *5′3′-CAT* gene. The diagram presents the original *lbc*$_3$ *5′3′-CAT* construction together with the 5′ deletion series transferred into *L. corniculatus* plants. Deletion endpoints are given as distance in nucleotides from the cap site (Stougaard *et al.*, 1986). Putative regulatory DNA sequences are indicated in Figure 5. The internal deletion C15ori used to delimit the distal regulatory sequence is also shown. The CAT activity measured in roots (**R**), nodules (**N**) and leaves + stem (**LS**) is shown in the column. The lowest level of CAT activity detectable is approximately 0.1% of the full level. (**B**) CAT activity expressed from the chimeric *lbc*$_3$ *5′-CAT-3′OCS* gene. In (C) the derived CAT activity profile of the *lbc*$_3$ promoter region is shown relative to the activity of the 2 kb 5′ region.

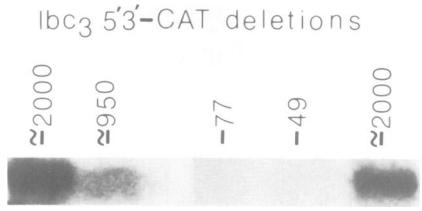

Fig. 3. Transcriptional activity from the deleted *lbc*$_3$ promoters. Total RNA was extracted from transgenic nodules and analysed by Northern hybridization. The complete *CAT* coding sequence was used to probe the transcripts from the *lbc*$_3$ *5′3′-CAT* 5′ deletions.

the various legume–*Rhizobium* associations. In this paper the 5′ flanking region of the soybean *lbc*$_3$ gene is analysed in order to define sequences required for both high level and organ-specific expression.

Results

A 6-fold variation in CAT activity among individual transformed *L. corniculatus* plants carrying the *lbc*$_3$ *5′3′-CAT* gene was previously found (Stougaard *et al.*, 1987a). To monitor this position and copy-number effect on the mutant promoter activities the *NOS–NPTII* gene expressing neomycin phosphotransferase activity under control of the T-DNA-derived constitutive promoter from the nopaline synthase gene was included on the pCAT-14 plasmid used for this analysis (Figure 1). Exonuclease *Bal*31 was subsequently used to generate a deletion series from the unique *Xba*I site located at position −950 in the *lbc*$_3$ 5′ flanking region. Religation of exonuclease-treated plasmid with *Sal*I linkers created a series of internal 5′ deletions. A subset of deletions with appropriate endpoints in the promoter region downstream of the −950 position was then reinserted into the unique *Sal*I site to form the series shown in Figure 2.

Expression of CAT activity from the 5′ deletions

The *Agrobacterium rhizogenes* AR1193 strain (Stougaard *et al.*, 1987a) was used to transfer the 5′ deletions into *L. corniculatus*. CAT activity expressed from the −950 to −49 promoter deletion series was determined in leaves and stem tissue, in roots and in nodules of 3–10 fully regenerated plants (Petit *et al.*, 1987). The transgenic tissues of the −305 deletion were generated by the short-cut transformation procedure only (see Materials and methods). No CAT activity was detected in root or in leaf and stem tissue of plants transformed with any of the 5′ *lbc*$_3$ promoter deletions (Figure 2). We conclude that the sequence element(s) conferring nodule specificity are linked closely to, or are an integral part of, the promoter structure. The shortest 5′ deletion with detectable CAT activity terminates in the −230 position.

Extension of the 5′ region from the −230 to the −950 position (*Xba*I site) yields a gradual increase in CAT activity. It appears that one or more positive elements are located in this region, most likely between positions −501 and −950. Only about 10% of the CAT activity observed with the 2 kb 5′ region is, however, attained with these deletions. Consequently a strong positive element must be located in the −2000 to −950 region. The internal deletion C15ori, devoid of the −1100 to −804 sequences, expresses the same level of CAT activity as the −950 or −804 deletions respectively. The positive element is therefore most likely located between −1100 and −950.

Transcriptional control of lbc$_3$ *gene expression*

The effect of deleting sequences from the *lbc*$_3$ promoter was also determined at the level of RNA. Total RNA was extracted

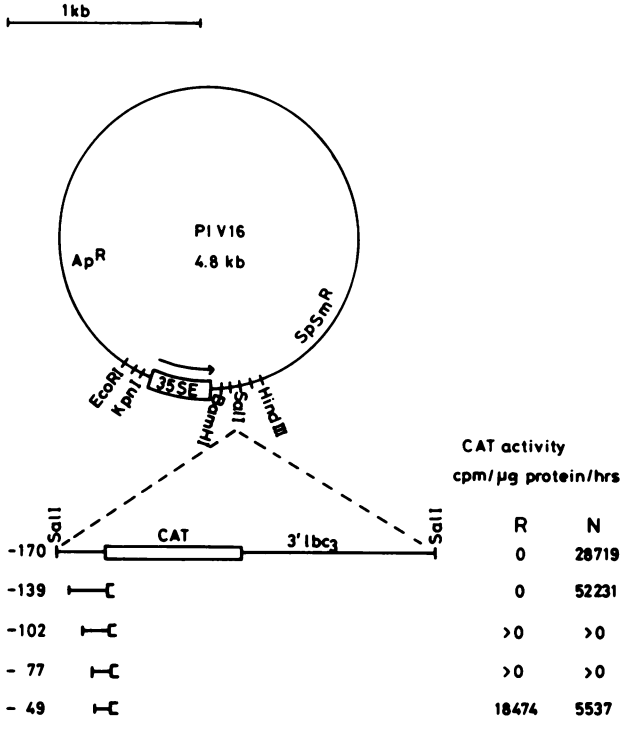

Fig. 4. Expression of the lbc_3 promoter deletions activated by the CaMV 35S enhancer. The inactive 5′ deletions −170, −139, −102, −77, −49 were linked to the (−418 to −90) CaMV 5′ enhancer region as shown in the figure. The CAT activities in root (**R**) and nodules (**N**) show the effect of the residual lbc_3 promoter elements present on the deletions.

from root nodules of at least three independent transgenic plants carrying the same construct and analysed by the Northern blotting procedure. Figure 3 presents the result using a CAT-specific probe. As expected from the corresponding CAT activities there is a drastically (20-fold) reduced level of steady-state CAT transcripts from the −950 deletion compared with the −2000 control. CAT transcripts were not detected from the −77 and −49 deletions. Attempts to use the neomycin phosphotransferase activity from the reference gene as control for the CAT activities were hampered by the high level of phosphotransferase activity in the *Rhizobium loti* strain used as inoculum. Control hybridizations with both the *NPTII* coding sequence from the *NOS–NPTII* gene and a *lb* cDNA probe showed, however, only small internal variation between the RNA preparations (data not shown). The RNA hybridizations therefore support the results obtained by measuring CAT activity and demonstrate that the effect of the 5′ deletions is on the level of transcription. This indicates that the organ-specific expression of the lbc_3 *5′3′-CAT* gene is regulated by a transcriptional control mechanism.

Enhancer activation of silent 5′ deletions

The low level of root-nodule-specific CAT activity expressed from the −230 deletion suggests that sequences involved in organ-specific regulation of the lbc_3 gene may be located between −230 and the +53 5′ lbc_3 CAT fusion point. In an attempt to localize these sequences further the cauliflower mosaic virus (CaMV) 35S gene enhancer was linked to the inactive −170, −139, −102, −77 and −49 deletions. The pIV16 vector (Figure 4) was used for these constructions, and the 5′ deletions were all cloned into the *SalI* site immediately downstream of the 35S enhancer. CAT activity was measured in transgenic *L. corniculatus* transformed by the short-cut procedure, with the results presented in Figure 4. The nodule-specific expression from hybrid promoters constructed from the −170 and −139 lbc_3 *5′3′-CAT5′* deletions demonstrates that the regulatory mechanism responsible for organ specificity can operate with the constitutive CaMV 35S enhancer. The very low level of CAT activity from the CaMV 35S −102 lbc_3 hybrid promoter indicates the presence of one or more elements involved in organ-specific control within the −139 to −102 sequence. DNA sequences in this region are apparently also required for expression of the lbc_3 promoter, since the residual −102 promoter contains both the concensus TATA and CAAT boxes in addition to the CACCC sequence necessary for mammalian β-globin gene expression (Dierks *et al.*, 1983). The −77 and −49 promoters do not contain the CAAT box (−81) and the CACCC box (−96) and may therefore not constitute fully functional promoters. Constitutive expression of the 35S enhancer-activated −49 promoter in transgenic roots and nodules, however, demonstrates that the 35S enhancer and the TATA box are sufficient for a low level of expression. We suggest that negative control elements located between positions −102 and −49 repress expression in roots and nodules of the −102 and −77 lbc_3 *5′3′-CAT* promoters linked to the 35S enhancer.

The 3′ region

The possible influence of the lbc_3 3′ flanking region on the expression of lbc_3 *5′3′-CAT* was investigated using the lbc_3 *5′-CAT-3′OCS* chimeric gene. A 320 bp 3′ flanking region carrying the polyadenylation site of the octopine synthase gene (*OCS*) was cloned into the lbc_3 *5′3′-CAT* gene resulting in the lbc_3 *5′-CAT-3′OCS* gene. CAT activity resulting from expression of this gene is shown in Figure 2. No major changes result from exchanging the lbc_3 3′ end, demonstrating that no indispensable regulatory sequences reside on the 3′ flanking region of the lbc_3 gene.

Discussion

Sequence analysis of the four active soybean *lb* genes shows that the 5′ regions of these genes are very conserved. Figure 5 shows the aligned 5′ flanking sequences of the active genes lbc_1, lbc_2, lbc_3, *lba*, together with the 5′ sequences of the pseudogene *Ps1* and of a truncated gene *Ps2*. Comparison of the 5′ regions of other nodulin genes from soybean and other legumes suggests two nodulin gene consensus sequences, AAAGAT and CTCTT (Sandal *et al.*, 1987). All four active soybean *lb* genes contain the AAAGAT sequence in a region homologous to the region required for organ-specific expression of the lbc_3 gene. The lbc_1, lbc_2 and lbc_3 genes do also contain the CTCTT sequence in this region; but the CTCTT motif is also present elsewhere in their 5′ regions. The deletion analysis of the soybean lbc_3 gene promoter delimited several 5′ sequences required for root nodule expression. One element responsible for a 20-fold increase in transcription was delimited to the −1100 to −950 upstream region. Another weaker element resulting in expression of about 5% of the CAT level obtained with the 2 kb 5′ lbc_3 region, was mapped to the −230 to −170 region. The 5′ deletion profile of CAT activities (Figure 2) indicates an additional weak positive element(s) in the −501 to −950 region. The internal variation between CAT activities on parallel plant lines makes the definition of positive elements between −230 and −950 difficult. This interpretation ignores possible cooperative effects and the possibility that regulatory elements overlap deletion endpoints. The identified positive elements are not absolutely required for the root-nodule-specific expression of the chimeric lbc_3 gene, since they can be replaced by the constitutive CaMV 35S

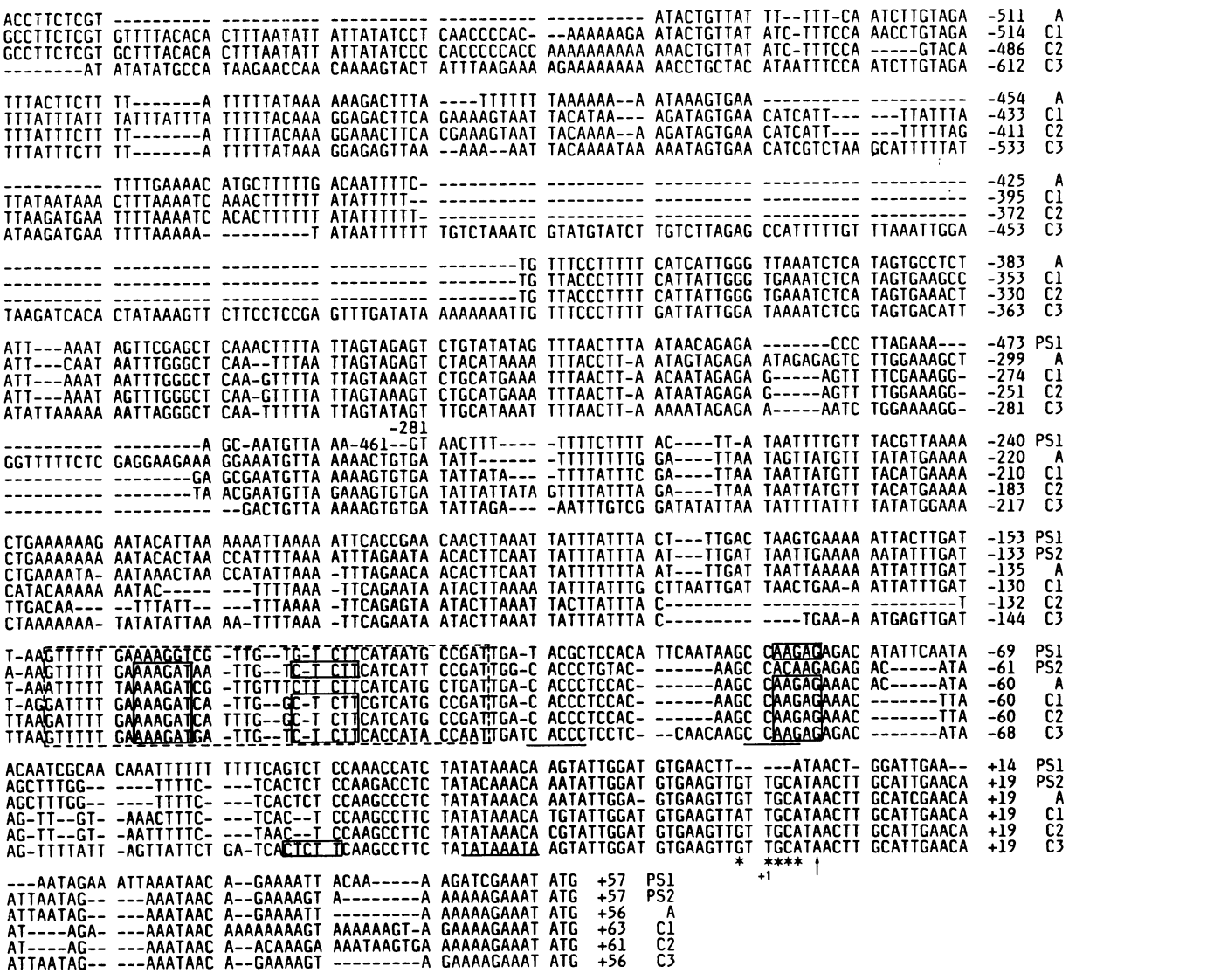

Fig. 5. Sequence alignment of the 5′ regions from the four active soybean leghaemoglobin genes *lba*, *lbc*$_1$, *lbc*$_2$, *lbc*$_3$, the pseudogene *PS1* and the truncated *PS2* gene. The sequences are aligned to maximize homology; vacant positions are indicated (−). Consensus promoter elements like the TATA box, the CAAT box and the CACCC sequence are underlined. The common nodulin sequences AAAGAT and CTCTT are framed with **solid lines**, while the region corresponding to the *lbc*$_3$ sequence implicated in the control of organ specificity is framed with a **dashed line**. The transcription initiation site determined by S$_1$ mapping (Brisson and Verma, 1982) is indicated with an **arrow**, and those determined by primer extension (Stougaard *et al.*, 1986) are marked with **stars**. Numbers to the right give the position from the cap site marked +1. Nucleotides between position −281 and −461 are omitted from the *PS1* sequence.

enhancer. However, it is still possible that these elements are nodule-specific control sequences that will not function in other organs. Such separately located positive elements have also been found in the chalcone synthase *chs* promoter and the chlorophyll a/b binding protein gene *lhcp* promoter, where sequences upstream and downstream of −350 positions determined the promoter strength (Kaulen *et al.*, 1986; Simpson *et al.*, 1985).

The presence of detectable CAT activity in root nodules of *L.corniculatus*, transformed with the −230 *lbc*$_3$ *5′3′-CAT* gene, located DNA sequences controlling organ specificity close to the TATA and CAAT boxes. Nodule-specific expression of the −170 and −139 deletions driven by the constitutive 35S enhancer confirms this interpretation and localizes the element(s) sufficient for organ-specific expression downstream of position −139. The minimal 35S enhancer reactivation of the −102 deletion carrying the classical eukaryotic promoter elements indicates that an element(s) required for expression of the *lbc*$_3$ gene in root nodules is located within the 37 bp region between positions −139 and −102. This element(s) might either be required to make other promoter elements accessible for cooperative mechanisms or to counteract a negative element located within the promoter. The soybean *lbc*$_3$ promoter is thus under complex control not unlike the light-regulated ribulose 1,5-biphosphate carboxylase small subunit genes *rbc*S E9 and *rbc*S ss3.6. Photoregulated expression of these *rbc*S genes could be detected with 5′ regions deleted to the −35 and −92 positions respectively (Morelli *et al.*, 1985; Timko *et al.*, 1985). Close association of the basic promoter structure and specific control elements is also known from plant genes regulated by light (Timko *et al.*, 1985; Fluhr *et al.*, 1986; Simpson *et al.*, 1985; Kaulen *et al.*, 1986) or anaerobiosis (Ellis *et al.*, 1987) or from promoters activated specifically in seeds (Chen *et al.*, 1986).

Sequence analysis of a number of root-nodule-specific genes suggests that the conserved sequences 5′AAAGAT and 5′CTCTT present on all 5′ regions analysed (Sandal *et al.*, 1987) might be involved in, or required for, the organ-specific regulation of nodulin genes. As demonstrated in this study for the *lbc*$_3$ promoter and by Jørgensen *et al.* (1987) for the nodule *N23* pro-

moter, these sequences are not sufficient (together with the TATA and CAAT boxes) to express a nodule-specific gene. Positive control elements are also required to drive the expression. The two consensus motifs described above are, however, both present in the 37 bp (−139 to −102) box of the lbc_3 promoter required for expression in root nodules and strongly indicate their involvement. The CTCTT sequence is also present in the lbc_3 promoter at position −44, immediately upstream of the TATA box, and in the reverse orientation at the −79 position overlapping the CAAT box. This motif may be a candidate for a negative regulatory element suggested to control the promoter. Low level constitutive expression of the −49 lbc_3 promoter linked to the 35S enhancer and the minimal constitutive expression of the similar −102, −77 constructions indicates that negative element(s) are located between −102 and −49. The one CTCTT sequence present at position −44 might be responsible for the low level expression of the −49 promoter, although the lack of CAAT and CACCC sequences could also account for this effect. A CTCTT sequence alone seems not sufficient to determine organ specificity. A schematic representation of the tentative regulatory elements is shown in Figure 2c.

Materials and methods

Plasmid constructions

The pIV2 vector was constructed by subcloning the *NOS–NPTII* 3'OCS gene (Herrera-Estrella *et al.*, 1983) into the *Bam*HI site of the pIV1 plasmid (Stougaard *et al.*, 1987a). pCAT-14 was subsequently constructed by cloning the lbc_3 *5'3'-CAT* gene (Jensen *et al.*, 1986) into the *Sal*I site of pIV2. The 320 bp (*Pvu*II/*Aha*III) 3'OCS (Gielen *et al.*, 1984) region inserted into the *Sma*I site of the SP6 polylinker was linked to the CAT coding sequence by cloning the distal 500 bp *Eco*RI fragment of CAT from lbc_3 *5'3'-CAT* into the *Eco*RI polylinker site. The lbc_3 *5'-CAT-3'OCS* was subsequently constructed by exchanging the *Nco*I/*Sal*I fragment of lbc_3 *5'3'-CAT* in pCAT-15 (J.Stougaard *et al.*, unpublished) with the *Nco*I/*Sal*I carrying the 3'OCS. The final construct was *Bam*HI subcloned into pAR6 (Stougaard *et al.*, 1987a). The deletion series of pCAT-14 was generated with exonuclease *Bal*31 treatment. pCAT-14 was linearized in the unique *Xba*I site at position −950, phenol treated and ethanol precipitated; 9 μg of linearized plasmid was treated with 0.08 units *Bal*31 enzyme (Biolab) and aliquots removed every 2 min for 20 min. The phenol treated reaction mixture was endfilled with Klenow polymerase before ligation to *Sal*I linkers. Transformants were selected on Amp (40 μg/ml) plates and analysed by gel electrophoresis. Suitable deletions were subcloned into the pIV2 *Sal*I site. Reaction conditions and assay buffers were used according to the manufacturers instructions or Maniatis *et al.* (1982). Endpoints of deletions were determined by dideoxy sequencing according to Hattori and Sakaki (1986); positions −950 and −1100 were determined with an accuracy of 20 bp only. The IV16 expression vector was derived from pIV10 (J.Stougaard *et al.*, unpublished) by insertion of the CaMV *Hin*dII/*Eco*RV (−418, −90) enhancer elements (Franck *et al.*, 1980) into the *Sma*I site of the pIV20 (pUC 19) polylinker.

RNA techniques

Northern blot analysis was according to Marcker *et al.* (1984). RNA extractions were performed as in Stougaard *et al.* (1986).

CAT assays

The method outlined in Stougaard *et al.* (1986) was used to assay for CAT activity. To avoid inhibition effects in crude extracts, CAT activity was determined in 100- and 500-fold dilutions.

Plant transformation

L.corniculatus plants were transformed and regenerated using the *A.rhizogenes* system (Petit *et al.*, 1987; Stougaard *et al.*, 1987a). A short-cut procedure generating transformed roots and root nodules on an untransformed plant shoot was also used. Hairy roots generated after infection with *A.rhizogenes* were nodulated directly on the plant after removal of the normal root system. The roots and plants were however first washed in chlaforan 500 μg/ml for 6 days to remove *Agrobacterium*.

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