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Light-inducible and tissue-specific expression of a chimaeric gene under control of the 5′-flanking sequence of a pea chlorophyll *a/b*-binding protein gene

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We have investigated the regulatory functions of the 5′-flanking sequences of a chlorophyll *a/b*-binding protein gene from *Pisum sativum*, using the neomycin phosphotransferase (II) activity from Tn*5* as an enzymatic reporter. We show that 0.4 kb of the upstream flanking sequences of this gene are sufficient for both organ-specific and light-regulated expression of our chimaeric constructs in transformed tobacco plants. In addition, we show that sequences farther upstream have a significant influence on the level of transcription of these constructions.

Key words: plant cell transformation/chimaeric gene/tissue-specific expression/light-inducible promoter

Introduction

Plants are complex organisms responding to many external stimuli, such as light, heat and soil conditions. We are interested in examining how these stimuli affect gene expression at the molecular level and in determining which DNA sequences may be involved in regulating the responses to these stimuli, not just over the whole plant, but within specific plant organs.

A variety of plant nuclear genes have now been isolated, cloned and sequenced, however, little is known about the promoter sequences and regulatory elements associated with these genes which, although constitutively expressed in plants and transcribed by plant RNA polymerase II, cannot strictly be classified as plant genes, namely the nopaline and octopine synthase genes from the *Agrobacterium* Ti plasmids (Koncz *et al.*, 1983; Shaw *et al.*, 1984) and the inclusion body protein (IBP) and 35S genes of the cauliflower mosaic virus (CMV) (Koziel *et al.*, 1984; Odell *et al.*, 1985). These studies showed that the 5′-upstream flanking sequences of these genes are necessary for transcription, and Odell *et al.* (1985) identified several sequences which may be important for transcription regulation.

The most extensively studied promoter region of an endogenous plant gene is that of the small subunit of the ribulose-1,5-bisphosphate carboxylase (Herrera-Estrella *et al.*, 1984; Morelli *et al.*, 1985; Timko *et al.*, 1985). These studies have concentrated on determining sequences involved in the light-inducible expression of the genes encoding the ribulose-1,5-bisphosphate carboxylase.

Light-harvesting chlorophyll *a/b* proteins (LHCPs) are among the more abundant proteins found in the leaves of all green plants. They are involved in the most important and characteristic reaction occurring in plants, i.e., photosynthesis. The majority of chlorophyll *a* and *b* found in plant cells is associated with the LHCPs to form the light-harvesting complex (LHC). This complex becomes integrated in the thylakoid membranes of the chloroplast where it absorbs energy from light and transfers the resulting excitation energy to photosystems 1 and 2 (Arntzen, 1978; Boardman *et al.*, 1978). The LHCPs are synthesized by free cytoplasmic ribosomes as precursor proteins, which contain an amino acid terminal extension or transit peptide (Apel and Kloppstech, 1978; Schmidt *et al.*, 1981). This transit peptide is cleaved either during or immediately after translocation through at least one membrane system, to produce the mature polypeptide (Schmidt *et al.*, 1981).

The LHCPs are encoded by small gene families as part of the nuclear genome in several plant species (Coruzzi *et al.*, 1983; Dunsmuir *et al.*, 1983; Dunsmuir, 1985; Timko and Cashmore, 1983). These genes code for at least two differently sized polypeptides and some genes seem to be expressed differentially during the various stages of leaf growth (Dunsmuir, 1985).

We chose to study a gene encoding a chlorophyll *a/b*-binding polypeptide, AB80 isolated from *Pisum sativum* (pea), since it has an interesting pattern of regulation. The expression of *LHCP* genes, is regulated by light (Tobin, 1981a; Cuming and Bennett, 1981) and is specific for chloroplast-containing tissues (Müller *et al.*, 1980). In addition, phytochrome plays a role in *LCHP* gene regulation (Stiekema *et al.*, 1983; Thompson *et al.*, 1983; Kaufman *et al.*, 1984), RNA accumulation is stimulated by red light and repressed by far-red light, although expression of *LHCP* genes occurs even under far-red light conditions.

Run-off transcription experiments have shown that light-regulated gene expression is at least partly mediated at the transcriptional level (Gallagher and Ellis, 1982; Silverthorne and Tobin, 1984) and transformation studies involving the ribulose-1,5-bisphosphate carboxylase small subunit genes have shown that 5′-flanking sequences are responsible for regulating the induction of transcription by light (Herrera-Estrella *et al.*, 1984; Morelli *et al.*, 1985; Timko *et al.*, 1985). Therefore, we decided to examine the 5′-flanking sequences of the *AB*80 gene by fusing these sequences to the neomycin phosphotransferase (II) [NPT(II)] gene from Tn*5* and utilising NPT(II) activity as an enzymatic reporter.

Results

Sequence of 1 kb of 5′-flanking region

To identify putative *cis*-acting DNA sequences involved in the regulation of the expression of the *LHCP* genes, it was first necessary to determine the sequence of at least part of the upstream 5′-flanking region of the *AB*80 gene. Therefore, a 1-kb sequence was determined using the Sanger sequencing method from a *Pst*I site (941 bp) upstream from the 5′ end of the corresponding AB80 transcript (Cashmore, 1984) (Figure 1). The sequence uncovered several interesting features within the 5′-flanking region of the gene.

The cap site of this gene and the presence of two putative 'TATA' boxes has been previously described (Cashmore, 1984). Only one of these TATA signals is in the appropriate functional

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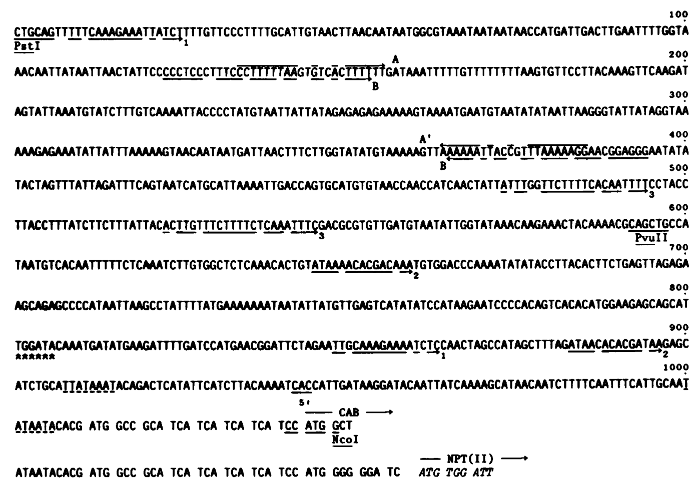

Fig. 1. Sequence of the upstream flanking region of the *AB*80 gene. The nucleotide sequence of 940 bp of the *AB*80 5′-flanking region was determined by the Sanger sequencing method. Arrows indicate direct and inverted repeats, the Goldberg-Hogness TATA boxes are underlined (- - -), and the homology to the animal enhancer sequence (Gruss, 1984) is indicated by asterisks (***). Also shown are the starting points of transcription (5′) and the initiation codon of the *AB*80 gene (—CAB→). The last lane shows the DNA sequence of the AB-NPT(II) chimaeric genes around the fusion point between the *AB*80 promoter and the NPT(II) coding sequence as determined by the Maxam and Gilbert method. The initiation codon of the chimaeric genes is also shown [—NPT(II)→].

position, 31 nucleotides upstream from the major cap site, the second occurring 91 nucleotides 3′ from this sequence. It is unknown whether these two TATA boxes allow differential transcription as occurs in similar cases for some *Drosophila* and mouse genes (Benyajati *et al.*, 1983; Schibler *et al.*, 1983).

The sequence also shows the presence of several direct and inverted repeats throughout the 942-bp 5′-flanking sequence (Figure 1), some of which may be involved in determining the level or specificity of the regulation of this gene.

Construction of vectors

To examine whether the *cis*-acting DNA sequences regulating the expression of genes encoding LHCPs reside in the 5′-flanking sequences of the gene, chimaeric gene constructions were made fusing 5′ fragments of the *AB*80 gene to the NPT(II) gene from Tn*5*. To this end, a promoter probe vector consisting of the NPT(II) coding sequence, the polyadenylation and termination signals of the octopine synthase gene and a polylinker 5′ to the initiation codon of the NPT(II) gene was constructed.

Figure 2 illustrates the construction of these vectors. The *Eco*RI-*Bam*HI fragment from the multilinker of pUC18 was used to replace an *Eco*RI-*Bcl*I fragment containing the nopaline synthase (*nos*) promoter present in pLGV1103neo. The resulting plasmid, pJ21LΔ, contains *Eco*RI, *Kpn*I, *Sst*I and *Sma*I restriction sites upstream of the NPT(II) gene and allows fragments with the corresponding ends or any blunt-ended fragment to be cloned upstream of the NPT(II) coding sequence.

Two fragments containing either 2.5 or 0.4 kb of 5′-flanking sequences of the *AB*80 gene were obtained by digestion with *Nco*I and *Bgl*II, or *Nco*I and *Pvu*II, and isolated from 1% agarose gels. After filling-in the recessed ends using DNA polymerase I, these fragments were cloned into the *Sma*I site of pJ21LΔ to produce pJ21LΔB and pJ21LΔP, respectively. These fragments contained 27 nucleotides from the coding region of the *AB*80 gene, and consequently two ATG codons were placed out of frame, upstream of the coding sequence.

A *Sal*I fragment containing the kanamycin resistance gene from Tn*903* was cloned into the *Sal*I site of both of these resulting plasmid vectors carrying the AB-NTP(II) fusions, in order to facilitate the selection of *Agrobacterium* exconjugants harbouring co-integrates between these plasmids and an acceptor Ti plasmid. This gave the final plasmids, pBC and pPC carrying 2.5 kb and 0.4 kb of AB 5′-flanking sequences, respectively.

Escherichia coli strains harbouring pBC and pPC were then conjugated (Van Haute *et al.*, 1983) to an *Agrobacterium* strain which harbours the non-oncogenic Ti plasmid derivative pGV3850, which carries a T-DNA containing homology to pBR322, and thus allows the easy integration of pBR322 derivatives containing the desired constructs in between T-DNA borders for subsequent transfer to plant cells (Zambryski *et al.*, 1983).

The presence of co-integrates with the correct structure in *Agrobacterium* exconjugates containing either pBC(pGV3850::pBC) or pPC(pGV3850::pPC) in between T-DNA borders was confirmed by Southern hybridization analysis using total bacterial DNA and specific probes, as described previously (Dhaese *et al.*, 1979) (data not shown). An *Agrobacterium* strain (pGV3850::1103neo) harbouring a chimaeric gene, where the NPT(II) coding sequence is under the control of the nopaline synthase promoter (*nos*-NPT(II)) was used as control to compare its expression with that of the AB-NPT(II) constructs, since the *nos* promoter is known to be constitutively expressed in all tissues of transformed plants.

Transfer of chimaeric genes to plant cells

The AB-NPT(II) and the [*nos*-NPT(II)] chimaeric gene constructions were introduced into the genome of tobacco cells by incubating leaf discs with the resulting *Agrobacterium* strains for 2 days using a modification of the method described by Horsch *et al.* (1985). The discs were then transferred to medium containing 75 μg/ml kanamycin in order to select for transformed shoots (see Materials and methods). After subsequent transfer to kanamycin-containing medium, green plantlets were tested for the presence of nopaline synthase, the gene product of a T-DNA-encoded gene present in the vector pGV3850, to confirm their transformed nature. A correlation >90% was observed between the kanamycin resistance and the presence of the nopaline synthase gene marker.

It is worth mentioning that the leaf disc infection method allows direct selection of transformed cells expressing selectable marker genes linked to shoot- or root-specific promoters since these organs can be directly obtained from leaf discs, whereas in the co-cultivation method (Márton *et al.*, 1979; Wullems *et al.*, 1981) the selection must be applied at the level of callus tissue, and therefore only allows the selection for markers under the control of genes which are expressed in undifferentiated tissue. Plants obtained from pGV3850::pBC- or pGV3850::pPC-transformed shoots, although capable of growing on kanamycin-containing media, failed to form roots on that medium, whereas similar shoots transformed using the *nos*-NPT(II) construction did form roots on kanamycin-containing media. This gave an early indication that the 5′-flanking regions of the *AB*80 gene used in these constructions were not sufficiently expressed in roots to protect these cells from inhibition by kanamycin.

Southern analysis

DNA was prepared from plants containing in their genome the pBC or pPC constructions (regenerated plants, rBC1, rPC2) and also from plants transformed using pGV3850::1103neo (r11031). This DNA was digested by *Eco*RI and *Nco*I restriction enzymes,

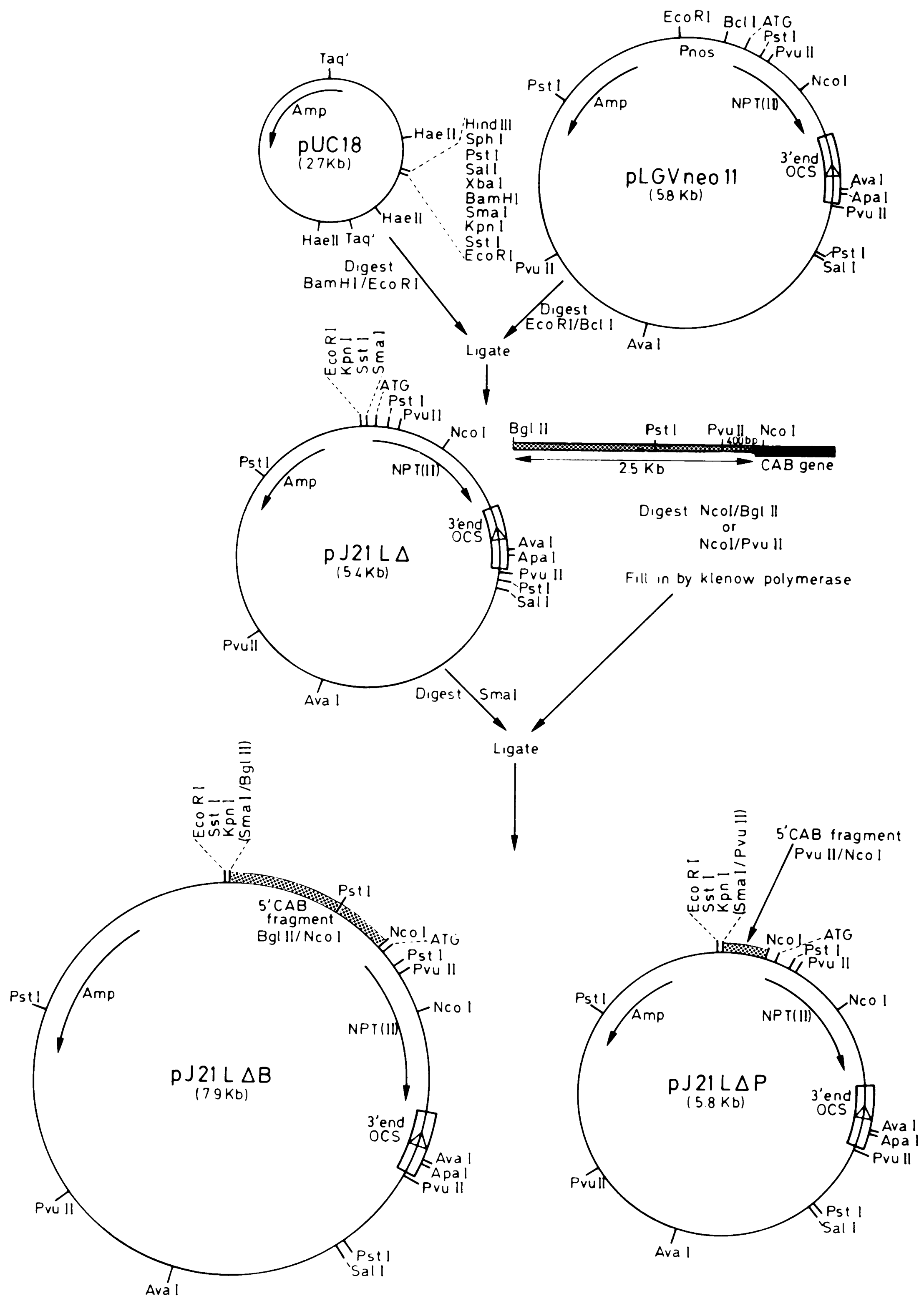

Fig. 2. Diagram of construction of chimaeric genes. The *Eco*RI-*Bcl*I nopaline synthase promoter (pnos) fragment from pLGVneo11 was replaced by the *Eco*RI-*Bam*HI fragment from the linker region of pUC18, to produce the promoter probe vector, pJ21LΔ. The 2.5-kb *Bgl*II-*Nco*I or the 0.4-kb *Pvu*II-*Nco*I fragments containing the 5'-flanking sequences of the *AB*80 gene were subsequently cloned into the *Sma*I site within the linker region to produce the 2.5-kb AB-NPT(II) and 0.4-kb AB-NPT(II) chimaeric genes. The single line → represents the vector, [▬] the AB80 coding sequence, [— — —] the octopine synthase 3' region, and [▒] the 5'-flanking region of the *AB*80 gene.

resolved on a 1% agarose gel and blotted onto nylon filters for Southern hybridisation. The vector pBC was digested also by *Eco*RI and *Nco*I, labelled with [α-^{32}P]dCTP and used as a probe. The results of this hybridisation are shown in Figure 3.

For the pBC plant, a band of 2.5 kb, corresponding to the *Eco*RI-*Nco*I fragment containing the upstream *AB*80 sequence, and a 490-bp band, corresponding to a fragment from the AB-NTP(II) fusion point to an *Nco*I site internal to the NPT(II) gene,

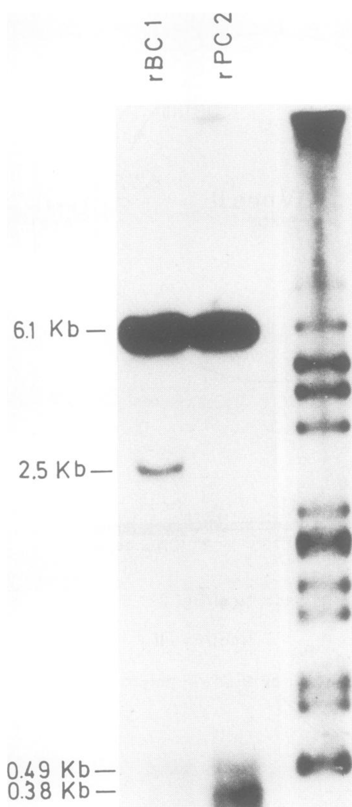

Fig. 3. Southern blot hybridisation analysis of DNA prepared from the rBC1 plant transformed by pLGV3850::pBC (containing 2.5 kb of the *AB*80 flanking region) and rPC2, a pLGV3850::pPC-transformed plant (containing 0.4 kb of the AB flanking region). Total plant DNA was digested by *Nco*I and *Eco*RI restriction enzymes, resolved on a 1% agarose gel and blotted onto nylon membranes. The filter was then hybridized against pBC ^{32}P-labelled by nick translation to give 10^7 c.p.m./μg. The figure shows the expected bands of 6.1 kb, 2.5 kb and 0.49 kb for rBC1, and 6.1 kb, 0.49 kb and 0.38 kb for rPC2. The third track is a mixture of phage λ DNA digested by *Hind*III alone and by *Eco*RI/*Hind*III used as a mol. wt. marker.

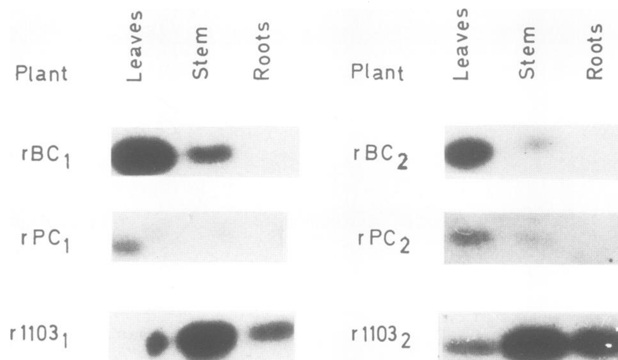

Fig. 4. Comparison of NPT(II) activity in individual organs of transformed plants. Total extracts from leaves, stem or roots were obtained from plants transformed by pLGV3850::pBC, pGV3850::pPC and pGV3850::1103, i.e., regenerants rBC_{1-2}, rPC_{1-2} and $r1103_{1-2}$, respectively, and the NPT(II) activity present in equal amounts of protein from each extract was determined by the *in situ* detection method on non-denaturing polyacrylamide gels as described by Reiss *et al.* (1984). The left-hand column shows the results of an autoradiogram indicating NPT(II) activity from plants grown under non-sterile conditions in vermiculite supplemented by commercial plant food (Substral). The right-hand column shows results from plants grown under sterile conditions in medium containing Murashige and Skoog salts plus 1% sucrose.

Table I. Comparison of NPT(II) activity in different organs of transformed plants

Plant type	C.p.m./μg of total protein		
	Leaves	Stem	Roots
r1103	9.6	12.91	10.38
rBC	24.6	7.5	B.D.
rPC	3.12	2.4	B.D.

B.D.: below level of detection.

are expected. A third fragment of 6.1 kb corresponding to the rest of the vector is also expected.

For the pPC-containing plant, the 6.1-kb and 490-bp band as already described are expected and also a 380-bp band corresponding to the *Eco*RI-*Nco*I fragment containing the shorter *AB*80 upstream sequence.

For pGV3850::1103neo-transformed plants (Herrera-Estrella *et al.*, in preparation), a band of 1.07 kb, corresponding to the *Eco*RI-*Nco*I fragment containing the *nos* promoter and part of the NPT(II) gene, and a fragment of 6.0 kb, corresponding to the rest of the vector, were observed (data not shown).

Tissue-specific expression of AB-NPT(II) genes

To determine whether the 5′-flanking region of the *AB*80 gene present in the chimaeric constructions is sufficient to direct tissue-specific expression in transformed plants, the NPT(II) activity present in extracts taken from leaves, stem and roots of plants transformed with pGV3850::pBC or pGV3850::pPC were compared with the activity present in corresponding extracts of pGV3850::pLGV1103neo-transformed plants. In the latter plants the NPT(II)-coding sequence is under control of the nopaline synthase promoter which has been shown to be transcribed at a similar level in all the tissues of transformed plants (De Block *et al.*, 1984). The relative amounts of NPT(II) activity in each tissue were assessed by separating similar amounts of protein in non-denaturing polyacrylamide gels, as described previously (Reiss *et al.*, 1984; Van den Broeck *et al.*, 1985). Figure 4 and Table I show that, whereas pLGV1103neo-containing plants with the NPT(II) gene under control of the *nos* promoter show similar levels of enzymatic activity in all plant tissues, both pBC and pPC show high levels of activity in leaves, weaker activity in stem and no detectable activity in roots (Figure 4, left panel). Moreover, even when plants were grown in sucrose-containing medium, where pLGV1103-transformed plants showed higher NPT(II) activity in the roots than in leaves, no activity was detected in roots of transformed plants containing pPC or pBC in their genome (Figure 4, right panel). Since the roots of plants grown on sucrose-containing medium were exposed to light for several weeks, this suggests that although light regulates the expression of the AB-NPT(II) constructions, it is not sufficient to induce their expression in plant tissues which do not contain chloroplasts.

The level of NPT(II) activity present in extracts containing equal amounts of total protein from the leaves, stem and roots of plants transformed with the different chimaeric gene constructions, was quantitated by measuring the Km $^{32}PO_4$ bound to the phosphocellulose paper in a liquid scintillation counter (Table I). This data also shows that there is an 8-fold higher level of NPT(II) activity in the leaves of rBC plants than in the leaves of rPC plants.

Since the pPC construction contains only 400 bp of the

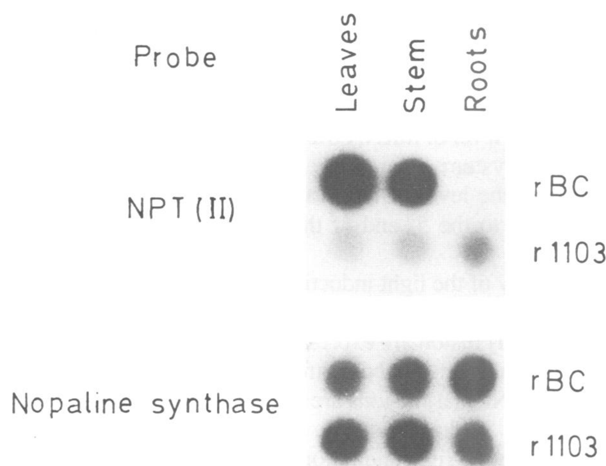

Fig. 5. Detection of transcripts homologous to NPT(II) by dot blot hybridisation analysis. Total RNA was prepared from leaves, stems or roots of plants transformed by either pGV3850::pBC (rBC) or pGV3850::1103 (r1003). 10 μg of RNA were spotted onto nylon filters using the minifold device and following the quick blot protocol (Bresser *et al.*, 1983) to selectively bind mRNA to the membrane. The RNAs were then hybridized to a DNA fragment containing the NPT(II) coding region ^{32}P-labelled by nick translation. **Upper panel**: top line shows autoradiogram of hybridisation of the labelled NPT(II) fragment to the RNA from the rBC plant; lower line shows hybridisation of the same probe to an r1103 plant. **Lower panel**: hybridisation of the ^{32}P-labelled *Hind*III-23 fragment from pTiC58 which contains the nopaline synthase gene to the RNA extracted from leaves, stem and roots of the rBC plant (upper line) or to the r1103 plant (lower line).

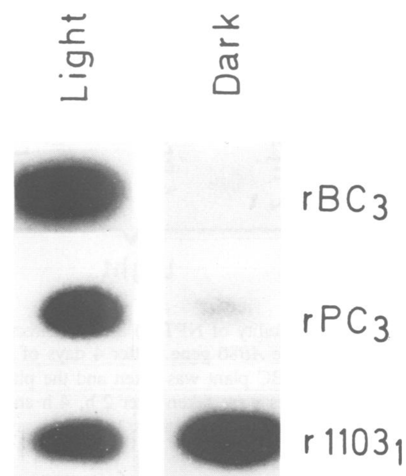

Fig. 6. Effect of light on the level of NPT(II) activity directed by either AB80 5′-flanking sequences or the nopaline synthase promoter. Samples were taken from plants grown in light conditions and analysed for NPT(II) activity (Reiss *et al.*, 1984). The plants were placed in darkness for 4 days when samples were again taken and assayed for NPT(II) activity. Equivalent amounts of protein were used in each assay. The left-hand column shows results of autoradiograms from light-grown samples, the right-hand column shows results from the same plants after a period of 4 days darkness.

5′-flanking *AB*80 sequence, this suggests that this fragment contains the necessary signals to control the expression of the *AB*80 gene in different organs of a plant in a similar fashion to that found for the *LHCP* genes normally present in the genome of plants, and that sequences upstream to 400 bp play an important role in determining the final level of transcription.

RNA analysis

To assess whether the level of NPT(II) activity present in the different organs of transformed plants is representative of the levels of the NPT(II) mRNA present in the corresponding organs, total RNA was prepared separately from leaves, stem and roots of individual AB-NPT(II)-containing plants, and a *nos*-NPT(II)-containing plant r11031. 10 μg of these RNAs were bound to a nylon membrane using a minifold system and the 'quick blot' protocol which selectively immobilises mRNA in the membrane (Bresser *et al.*, 1983). The filters were then hybridised against either a ^{32}P-labelled NPT(II) DNA fragment or a similarly labelled nopaline synthase fragment. Figure 5 shows clearly that NPT(II) mRNA is present at high levels in the leaves and lower levels in the stem of the rBC1 plant with the NPT(II) under control of the *AB*80 promoter region, but is undetectable in root tissue, in contrast in the r1103 plant with the NPT(II) under control of the nopaline synthase promoter, there is a lower overall level of mRNA, but it is present at similar levels in all the plant organs.

Figure 5 also shows the results obtained using the same RNA preparations hybridized to detect mRNA homologous to the nopaline synthase gene, and indicates that mRNA from this gene was present in similar amounts in all plant tissues of both rBC1 and r11031 plants.

Light inducibility

To test whether the expression directed by the 5′-flanking sequences of the *AB*80 gene is not only tissue-specific but also light-inducible, extracts from the leaves of plants containing pBC, pPC and pLGV1103 were taken as before and tested for NPT(II) activity. These plants were then placed in darkness for 4 days after which time they were again assayed for NPT(II) activity (Figure 6). Alternatively, a plant of each type was divided in two eventually forming two plants, one of which was kept in the light, while the other was put into darkness for 4 days. After this time extracts containing equivalent amounts of protein were assayed for NPT(II) activity. Similar results were obtained from both types of experiments.

As shown in Figure 6, there is clearly a higher level of NPT(II) activity when the rBC and rPC plants are grown in light conditions as compared with growth in darkness, whereas the level of NPT(II) activity in r1103-transformed plants remains similar in both growth conditions. The fact that the level of expression of chimaeric genes under control of the *nos* promoter is not significantly altered by the light conditions under which plant cells are grown has been shown previously (Herrera-Estrella *et al.*, 1984).

To quantitate the NPT(II) activity present in the different extracts, the radioactivity bound to the phosphocellulose paper was measured in a scintillation counter and the c.p.m./μg of protein for each extract was calculated. These results indicate 8- to 50-fold higher activity in both rBC and rPC plants grown in light conditions when compared with dark-grown plants, but only a maximum 2-fold higher activity in light-grown pLGV1103 plants in comparison with those kept in darkness. This latter variation was not correlated with the light conditions since many times the dark-grown plants showed higher activity than those grown under light conditions. Since not only pBC but also pPC plants show a similar level of induction by light, this indicates that the signals necessary for the light inducibility of the *AB*80 gene also reside within the 400-bp sequence upstream of the start point of transcription. These results are in agreement with those obtained by Tobin (1981b) and Silverthorne and Tobin (1984), which showed that not only is the LHPC absent in dark-grown plants, but also that there is a dramatic drop in the transcription rate

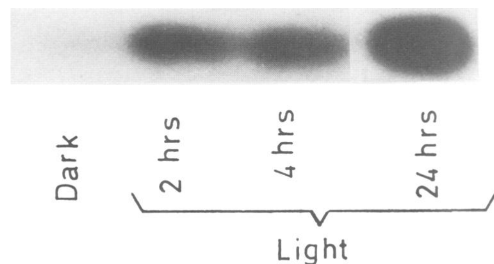

Fig. 7. Kinetics of light inducibility of NPT(II) activity directed by the 2.5-kb flanking sequence of the *AB*80 gene. After 4 days of growth in darkness a leaf piece from a rBC plant was taken and the plant brought into the light. Subsequently, samples were taken after 2 h, 4 h and 24 h; equal amounts of total protein from each sample were used to assay for NPT(II) activity.

of these genes in the dark. In contrast to the LHCPs which are absent in dark-grown plants, it was observed that rBC or rPC plants grown in the dark for extended periods of time, always contained a low but readily detectable level of NPT(II) activity. This can be explained by the fact that there is a low but significant level of transcription of the *LHCP* genes in dark-grown plants (Tobin, 1981b). Moreover, this also suggests that the NPT(II) protein is not subjected to the same specific degradation as the LHCPs in dark-grown plants.

To estimate the time of induction by light of an increase in NPT(II) activity in pBC-transformed plants, samples were taken at various time periods after plants were removed from darkness. It is apparent that induction of the gene by light occurs relatively quickly, as judged by the level of NPT(II) activity, i.e., within 2 h, and reaches a maximum by 24 h (Figure 7).

Discussion

Here we have examined the 5′-flanking sequences of a chlorophyll *a/b*-binding polypeptide, AB80 from pea, using NPT(II) activity as an enzymatic reporter. Our results show that the 5′-flanking region is involved in both organ-specific and light-regulated expression of the gene.

In comparison with chimaeric constructs involving *nos*-NPT(II) fusions, which show activity in all organs of regenerated plants, the AB-NPT(II) fusions show strong NPT(II) activity in leaves, weaker activity in stems and no detectable activity in roots. Fusions involving either the 2.5-kb AB sequence or the 0.4-kb sequence, both show this organ-specific regulation, suggesting that the 0.4-kb sequence contains most of the information necessary to control this regulation.

The enzyme activity mirrors the presence of mRNA transcripts of the gene. High levels of NPT(II) mRNA are found in leaves, lower levels in stems and no detectable NPT(II) mRNA is found in roots of plants containing the AB-NPT(II) fusion. In comparison, NPT(II) mRNA is found in all organs of a plant containing the *nos*-NPT(II) fusion. Since most of the constitutively expressed *nos*-NPT(II) gene transcript is identical to the AB-NPT(II) transcript, it is unlikely that they are subjected to different post-translational regulation, and therefore, it is probable that the light-inducible and organ-specific regulation mediated by the *AB*80 5′-flanking region is regulated at the level of transcription initiation. However, we cannot exclude the possibility that the small segment of the *AB*80 sequence, present in the AB-NPT(III) mRNA, might also play a role in determining the stability of messenger in the different organs or light conditions.

The mRNA analysis also indicates that the 2.5-kb AB-NPT(II) fusion is expressed at much higher levels than the *nos*-NPT(II) fusion. This observation is not reflected in the enzyme assays, and can be explained by the presence of two extra out of frame ATGs in the AB-NPT(II) fusions, which would have no effect on the overall level of mRNA transcribed but, according to studies with animal systems (Liu *et al.*, 1984), would have a pronounced effect on the level of functional protein produced, since the closest AUG to the 5′ end of the messenger is translated most efficiently.

In our study of the light induction of the chimaeric AB-NPT(II) genes, we report that the 2.5-kb AB-NPT(II) fusion and the 0.4-kb NPT(II) fusion are expressed up to 50 times more strongly in light-grown plants as compared with those kept in darkness, whereas *nos*-NPT(II) fusions are expressed only twice as strongly as those kept in darkness.

The kinetics of light induction indicates that the induction of the level of enzymatic activity occurs relatively quickly, i.e., within 2 h, reaching a maximum after 24 h. It may be that transcription is induced almost immediately, but that the maximum of enzymatic activity found at 24 h can only be reached when the plant regains its normal physiological state.

Müller *et al.* (1980) showed that LHCP mRNA is only present in green chloroplast-containing tissue, and Herrera-Estrella *et al.* (1984) reported that the promoter region of a ribulose-1,5-bisphosphate carboxylase gene was active in green callus tissue, but not in white undifferentiated tissue. We have observed low levels of NPT(II) activity in roots which have been induced by addition of cytokinins to the growth medium to become green, thus suggesting that the tissue-specific expression of the LHCP and ribulose-1,5-bisphosphate carboxylase genes might be determined by a factor produced by, or associated with, the presence of chloroplasts or other developed plastids, such as etioplasts. This is supported by the fact that etiolated plants are able to rapidly express these genes simultaneously with the development of etioplasts into chloroplasts.

Since in all of the cases we have examined, the pBC construct is expressed at a significantly higher level than the pPC construct, this suggests that, although 400 bp of 5′-flanking sequences are sufficient to direct light-inducible and tissue-specific expression, there are sequences up to 400 bp upstream from the start point of transcription of the *AB*80 gene which play an important role in determining the high overall level of expression. This is in contrast to the organisation of the promoters for constitutively expressed octopine and nopaline synthase genes which contain all essential sequences within 100 bp of the start of transcription (Koncz *et al.*, 1983; Shaw *et al.*, 1984) for normal expression, and similar to that found for the small subunit of ribulose-1,5-bisphosphate carboxylase (Morelli *et al.*, 1985; Timko *et al.*, 1985).

Recently, Morelli *et al.* (1985) have suggested that repeats close to the TATA box of the *rbc*S-E9 gene may play an important role in the light regulation of this gene. We have been unable, however, to find any closely related sequences within our 1-kb flanking region. On the other hand, Timko *et al.* (1985) have shown that an 'enhancer-like' sequence is involved in the light inducibility of a ribulose-1,5-bisphosphate carboxylase gene, whether the 5′-flanking region of the *AB*80 gene also behaves as an enhancer sequence is under investigation.

Although we cannot pinpoint precisely which sequences determine the light-inducible and organ-specific expression of the chimaeric constructions, we have shown that a *cis*-acting 400-bp sequence upstream to the transcription startpoint is capable of mediating this regulation. We also suggest that sequences further upstream are required for regulation of the levels of expres-

sion in a fashion similar to that observed for the small subunit of ribulose-1,5-bisphosphate carboxylase (Morelli *et al.*, 1985; Timko *et al.*, 1985). These sequences may now potentially be used to regulate and target the expression of foreign genes to specific plant organs.

Materials and methods

Chimaeric gene constructs were made using standard DNA techniques as described by Maniatis *et al.* (1982).

Confirmation of co-integrates with the correct structure in *Agrobacterium* exconjugants was determined by the method of Dhaese *et al.* (1979) and of plant transformation by Southern hybridization (Southern, 1975).

Leaves of SR1 tobacco plants were cut into pieces of 1 cm^2 and floated upside down in K3 medium containing 0.2 mg/l BAP, 0.1 mg/l NAA. 50 μl of an undiluted *Agrobacterium* culture grown overnight in minimal A medium, were then added. The plates were sealed and left in the light in a plant growth chamber for 3 days. The leaf pieces were then washed in K3 containing 500 μg/ml Claforan®, (Hoechst) and transferred to solid Linsmaier and Skoog medium containing 75 μg/ml kanamycin sulfate (Sigma), 500 μg/ml Claforan®, and hormones to stimulate callus growth, i.e., 1 mg/l NAA, 0.2 mg/l BAP. After ~10 days they were transformed to similar media, but without NAA and with 1 mg/ml BAP to stimulate shoot formation.

When shoots reached ~0.5 cm they were transferred to media without hormones but containing kanamycin and Claforan® as before. Shoots remaining green after 2–3 weeks were tested for nopaline synthase activity (Otten and Schilperoort, 1978; Aerts *et al.*, 1979). Positive shoots were then transferred to non-selective media to allow root formation.

Neomycin phosphotransferase [NPT(II)] activity was determined from extracts of various plant tissues of transformed plants by the modification for plant tissues (Van den Broeck *et al.*, 1985) of the *in situ* detection of NPT(II) activity in non-denaturing polyacrylamide gels (Reiss *et al.*, 1984).

In the experiments described here, we used 100 mg of plant tissue ground in 50 μl of extraction buffer. The residue was pelleted in an Eppendorf centrifuge and an aliquot of the supernatant was taken to measure protein content using the Bio-Rad assay system (Bio-Rad product profile). Comparable amounts of each extract were then loaded on a 10% non-denatured polyacrylamide gel. After 4–5 h, the gel was washed in distilled water, then equilibrated by shaking in 2 × reaction buffer for 30 min. The polyacrylamide gel was then overlaid with 1% agarose gel containing 30 μg/ml kanamycin sulfate and 200 μCi [γ-^{32}P]ATP. After 30 min the gel sandwich was covered by Whatman P81 paper and blotted in the manner of Southern for 2–3 h after which time the filter was washed several times, then autoradiographed overnight.

RNA dot blot analysis

Total RNA was prepared separately from the leaves, stem and roots of rBC1 and r1103 plants by a modification of the technique described by Hepburn *et al.* (1983).

10 μg of total RNA from each organ of both plants were spotted onto nylon filters using the minifold device (Bresser *et al.*, 1983).

DNA fragments containing the NPT(II) gene and the nopaline synthase gene were labelled by nick translation with [^{32}P]dCTP and used as a hybridisation probe against the RNA spots.

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References

Aerts,M., Jacobs,M., Hernalsteens,J.-P., Van Montagu,M. and Schell,J. (1979) *Plant Sci. Lett.*, **17**, 43-50.
Apel,K. and Kloppstech,K. (1978) *Eur. J. Biochem.*, **85**, 581-588.
Arntzen,C.J. (1978) *Curr. Top. Bioenerget.*, **8**, 111-160.
Benyajati,C., Spoerel,N., Haymerle,H. and Ashburner,M. (1983) *Cell*, **33**, 125-133.
Boardman,N.K., Anderson,J.M. and Goodchild,D.J. (1978) *Curr. Top. Bioenerget.*, **8**, 36-109.
Bresser,J., Doering,J. and Gillespie,D. (1983) *DNA*, **2**, 243-254.
Cashmore,A.R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2960-2964.
Coruzzi,G., Broglie,R., Cashmore,A. and Chua,N.-H. (1983) *J. Biol. Chem.*, **258**, 1399-1402.
Cuming,A.C. and Bennett,J. (1981) *Eur. J. Biochem.*, **118**, 71-80.
De Block,M., Herrera-Estrella,L., Van Montagu,M., Schell,J. and Zambryski,P. (1984) *EMBO J.*, **3**, 1681-1689.
Dhaese,P., De Greve,H., Decraemer,H., Schell,J. and Van Montagu,M. (1979) *Nucleic Acids Res.*, **7**, 1837-1849.
Dunsmuir,P. (1985) *Nucleic Acids Res.*, **13**, 2503-2518.
Dunsmuir,P., Smith,S.M. and Bedbrook,J. (1983) *J. Mol. Appl. Genet.*, **2**, 285-300.
Gallagher,T.F. and Ellis,R.J. (1982) *EMBO J.*, **1**, 1493-1498.
Gruss,P. (1984) *DNA*, **3**, 1-5.
Hepburn,A.G., Clarke,L.E., Pearson,L. and White,J. (1983) *J. Mol. Appl. Genet.*, **2**, 315-329.
Herrera-Estrella,L., Van den Broeck,G., Maenhaut,R., Van Montagu,M., Schell,J., Timko,M. and Cashmore,A. (1984) *Nature*, **310**, 115-120.
Horsch,R.B., Fry,J.E., Hoffmann,N.L., Eichholtz,D., Rogers,S.G. and Fraley,R.T. (1985) *Science (Wash.)*, **227**, 1229-1231.
Kaufman,L.S., Thompson,N.F. and Briggs,W.R. (1984) *Science (Wash.)*, **226**, 1447-1448.
Koncz,C., De Greve,H., André,D., Deboeck,F., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 1597-1603.
Koziel,M.G., Adams,T.L., Hazlet,M.A., Damm,D., Miller,J., Dahlbeck,D., Jayne,S. and Staskawicz,B.J. (1984) *J. Mol. Appl. Genet.*, **2**, 549-562.
Liu,C.-C., Simonsen,C.C. and Levinson,A.D. (1984) *Nature*, **309**, 82-85.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Márton,L., Wullems,G.J., Molendijk,L. and Schilperoort,R.A. (1979) *Nature*, **277**, 29-130.
Morelli,G., Nagy,F., Fraley,R.T., Rogers,S. and Chua,N.-H. (1985) *Nature*, **315**, 200-204.
Müller,M., Viro,M., Balke,C. and Kloppstech,K. (1980) *Planta*, **148**, 444-447.
Odell,J.T., Nagy,F. and Chua,N.-H. (1985) *Nature*, **313**, 810-812.
Otten,L.A. and Schilperoort,R.A. (1978) *Biochim. Biophys. Acta*, **527**, 497-500.
Reiss,B., Sprengel,R., Will,H. and Schaller,H. (1984) *Gene*, **30**, 211-218.
Schibler,U., Hagenbüchle,O., Wellauer,P.K. and Pittet,A.C. (1983) *Cell*, **33**, 501-508.
Schmidt,G.W., Bartlett,S.G., Grossman,A.R., Cashmore,A.R. and Chua,N.-H. (1981) *J. Cell Biol.*, **91**, 468-478.
Silverthorne,J. and Tobin,E.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1112-1116.
Shaw,C.H., Carter,G.H., Watson,M.D. and Shaw,C.H. (1984) *Nucleic Acids Res.*, **12**, 7831-7846.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-518.
Stiekema,W.J., Wimpee,C.F., Silverthorne,J. and Tobin,E.M. (1983) *Plant Physiol.*, **72**, 717-724.
Thompson,W.F., Everett,M., Polan,N.O., Jorgensen,R.A. and Palmer,J.D. (1983) *Planta*, **158**, 487-500.
Timko,M.P. and Cashmore,A.R. (1983) in Goldberg,R.B. (ed.), *Plant Molecular Biology UCLA Symposia on Molecular and Cellular Biology, New Series*, Vol. **12**, Alan R.Liss, NY, pp. 403-412.
Timko,M.P., Kausch,A.P., Castresana,C., Fassler,J., Herrera-Estrella,L., Van den Broeck,G., Van Montagu,M. and Cashmore,A.R. (1985) *Nature*, in press.
Tobin,E.M. (1981a) *Plant Mol. Biol.*, **1**, 35-51.
Tobin,E.M. (1981b) *Plant Physiol.*, **67**, 1078-1083.
Van den Broeck,G., Timko,M.P., Kausch,A.P., Cashmore,A.R., Van Montagu,M. and Herrera-Estrella,L. (1985) *Nature*, **313**, 358-363.
Van Haute,E., Joos,H., Maes,M., Warren,G., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 411-418.
Wullems,G.J., Molendijk,L., Ooms,G. and Schilperoort,R.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4344-4348.
Zambryski,P., Joos,H., Genetello,C., Leemans,J., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 2143-2150.

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