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Photoregulated expression of a pea *rbc*S gene in leaves of transgenic plants

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A 2.4-kb pea genomic fragment, containing a member (*rbc*S-E9) of the multigene family encoding the small subunit (rbcS) of ribulose-1,5-bisphosphate carboxylase, was inserted into a non-oncogenic, Ti-plasmid vector and introduced into the genomes of *Petunia hybrida* (Mitchell) and *Nicotiana tabacum* (SR1) plants by *in vitro* transformation. Petunia and tobacco plants containing the introduced pea *rbc*S-E9 gene were regenerated from protoplasts. In these transgenic plants the *rbc*S-E9 gene is transcribed accurately using its own promoter and its expression is light-induced and organ-specific. A deletion mutant with 352 bp of 5′-upstream sequence still retains photoinducibility and leaf-specific expression. Clonal analysis of independent transgenic petunia plants revealed that chromosomal positions in the recipient plant genome affect the quantitative but not qualitative aspects of *rbc*S-E9 expression.

Key words: ribulose-1,5-bisphosphate carboxylase/light-inducibility/organ-specific expression/position effect/5′ deletion mutant

Introduction

Ribulose-1,5-bisphosphate carboxylase is the most abundant protein in chloroplasts where it accounts for up to 50% of the total organelle protein (Ellis, 1979). The carboxylase holoenzyme is made up of eight copies each of a large (M_r = 55 000) and a small (m_r = 16 000) subunit. The large subunit (rbcL) is encoded in the chloroplasts (Coen *et al.*, 1977) whereas the small subunit (rbcS) is encoded in the nuclear genome by a multigene family (Berry-Lowe *et al.*, 1982; Coruzzi *et al.*, 1983; Broglie *et al.* 1983; Dunsmuir *et al.*, 1983; Wimpee *et al.*, 1983). Expression of *rbc*S genes is induced by light (cf. Tobin and Silverthorne, 1985) and there is evidence that the regulation is exercised primarily at the transcriptional level (Gallagher and Ellis, 1982; Tobin and Silverthorne, 1985). In peas, the *rbc*S transcripts are distributed in an organ-specific manner, being most abundant in photosynthetic organs, such as leaves and pericarps (Coruzzi *et al.*, 1984).

The light-inducibility and organ-specificity of the pea *rbc*S gene expression render this gene family an attractive system for studying *cis*-regulatory elements required for gene regulation during plant development. As a first step, we have transferred one member (E9) of the pea *rbc*S gene family into petunia and showed that the expression of the *rbc*S-E9 gene in transformed calli continues to be light-regulated (Broglie *et al.*, 1984; Morelli *et al.*, 1985). Similar light-induced expression has been reported for another pea *rbc*S gene (SS3.6) (Herrera-Estrella *et al.*, 1984) and of a soybean *rbc*S gene (Facciotti *et al.*, 1985) in green tobacco calli. Although all three *rbc*S genes show light-induced expression in transformed petunia or tobacco calli, we did not know to what extent this regulated expression reflects the biochemical reactions in leaves. To address this issue and also to explore the question of whether the *rbc*S genes would be expressed in the appropriate organs of transgenic plants, we have used a 'disarmed' Ti-vector (Fraley *et al.*, 1985) to introduce the pea *rbc*S-E9 gene into petunia and tobacco genomes. Here we show that the pea *rbc*S-E9 gene is expressed in a light-regulated and leaf-specific manner in transgenic plants and that this expression pattern is preserved in a 5′ deletion mutant which has only one 352 bp sequence upstream from the S1 start site.

Results

Expression of the pea rbcS-E9 gene in transgenic plants is light-induced

In peas (*Pisum sativum*) the rbcS polypeptide is encoded by a small multigene family of five to ten members (Coruzzi *et al.*, 1983, 1984). We have determined previously the complete nucleotide sequence of one member (E9) of the *rbc*S gene family and showed that the expression of this gene in pea leaves is at least 10 and 100 times higher than in stems and roots, respectively. Moreover, the leaf-specific expression of the *rbc*S-E9 gene is induced by light (Coruzzi *et al.*, 1984). To see whether the light-induced and organ-specific expression of the *rbc*S-E9 gene would be retained in a heterologous nuclear background, we have transferred this gene into petunia and tobacco using the split end

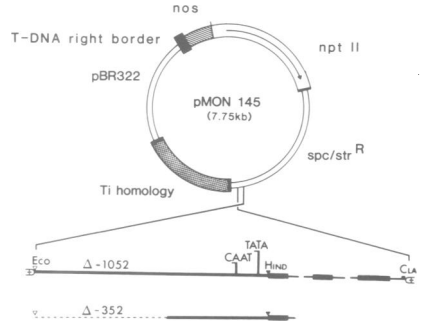

Fig. 1. A schematic diagram of pMON145 with the inserted pea *rbc*S-E9 gene or its 5′ deletion mutant. The intermediate cloning vector pMON145 has been described in detail (Broglie *et al.*, 1984). It contains a segment homologous to a T-DNA sequence found in the pTiT37 plasmid, a portion of pBR322 including the origin of replication, the nopaline synthase right border, a chimeric gene consisting of the nopaline synthase promoter (NOS) fused to the coding sequence of neomycin phosphotransferase II (NptII), and a segment from Tn7 which confers resistance to spectinomycin and streptomycin. The pea *rbc*S-E9 gene was isolated as a 2.4-kb *Eco*RI-*Cla*I fragment which contains 1052 bp of upstream sequence (*EcoRI-Hind*III) and ~1.4 kb of coding and 3′ sequence (*Hind*III-*Cla*I). The coding sequence of *rbc*S-E9 contains three exons which are represented as dark boxes in the figure. The genomic fragment was inserted between the *Eco*RI and *Cla*I sites of pMON145 as indicated. The 5′ deletion mutant, Δ-352, was constructed between identical restriction sites of the intermediate vector.

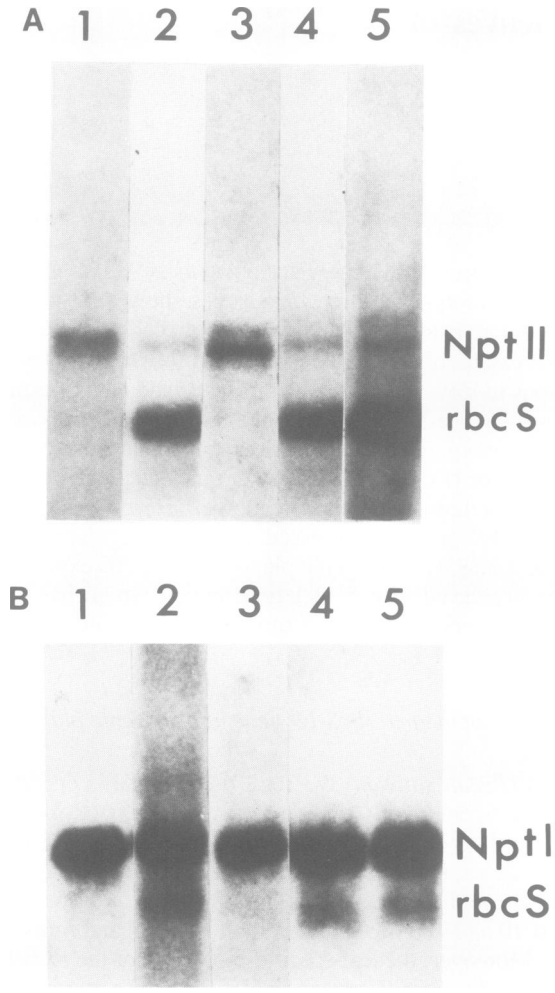

Fig. 2. Light-regulated expression of the pea *rbc*S-E9 gene in transgenic petunia and tobacco plants. Filters were hybridized to a probe containing the coding sequence of the *npt*II gene joined to a 3′ fragment (690 bp) of *rbc*S-E9 (Morelli *et al.*, 1985). **Panel A**: leaves were pooled from eight independent transgenic petunia clones and polyadenylated RNAs were purified (Broglie *et al.*, 1983). (**1**) pMON145 transgenic clones, 4 μg; (**2**) *rbc*S transgenic clones grown under 16 h light, 8 h dark photoperiodic cycle, 2 μg; (**3**) *rbc*S-E9 transgenic clones kept in darkness for 4 days, 4 μg; (**4**) *rbc*S-E9 transgenic clones dark-adapted for 4 days and then re-exposed to continuous light for 24 h, 2 μg; (**5**) *rbc*S-E9 transformants dark-adapted for 4 days and then re-exposed to continuous light for 48 h, 2 μg. **Panel B**: leaves were pooled from seven independent transgenic tobacco clones. **Lanes 1–5** same as **A** except that each lane contains 2 μg polyadenylated RNA.

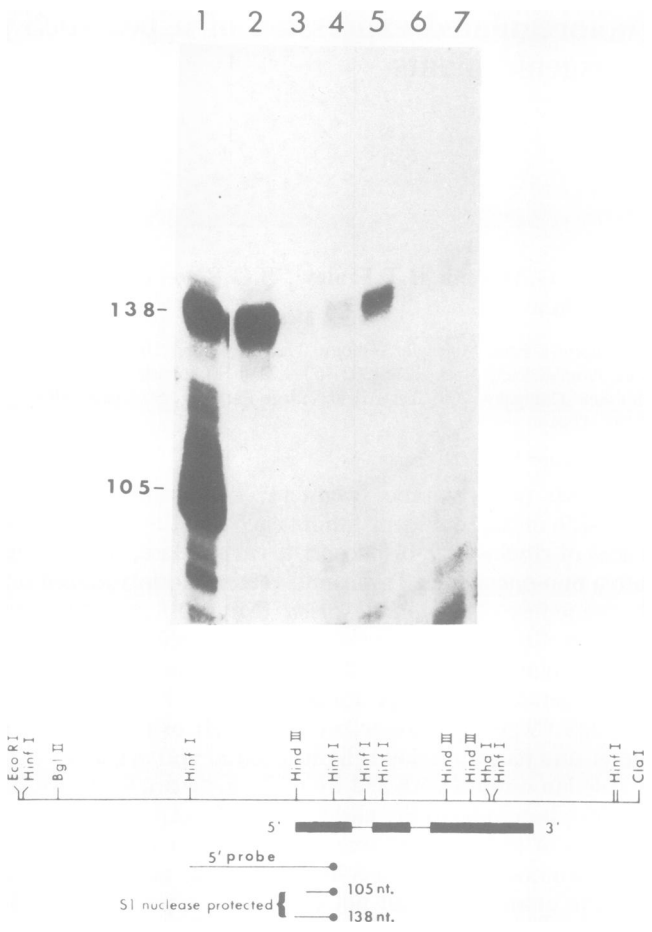

Fig. 3. 5′ mapping of the *rbc*S-E9 transcript in transgenic petunia plants by S1 nuclease protection assay. (**1**) Pea-leaf polyadenylated RNA, 60 ng; (**2**), (**3**) and (**4**) polyadenylated RNA (2 μg) from leaves, stems, and roots, respectively, pooled from eight independent transgenic petunia clones containing the *rbc*S-E9 gene with 1052 bp of 5′ upstream sequence; (**5**), (**6**) and (**7**) polyadenylated RNA (1 μg) from leaves, stems and roots, respectively, pooled from three independent transgenic petunia clones containing the mutant derivative 5′ Δ-352 *rbc*S-E9. Numbers on the extreme left indicate the size (nucleotide) of the protected fragment. Lower panel shows a schematic diagram of the *rbc*S-E9 gene with relevant restriction sites. The 5′ probe, as well as the expected lengths of the S1 nuclease protected fragments, are also indicated.

vector system of Fraley *et al.* (1985). Figure 1 shows a schematic diagram of the intermediate vector pMON145 and the position and orientation of the inserted pea *rbc*S-E9 gene. Eight independent transgenic petunia clones and seven independent transgenic tobacco clones were selected for analysis.

In the first series of experiments, the plants were grown under normal photo-periodic cycle (16 h light, 8 h dark), transferred to the dark for 4 days and then re-exposed to light for 24 h or 48 h. Leaves from individual plants were pooled before RNA extraction and the levels of the *npt*II mRNA and the *rbc*S-E9 mRNA were analyzed by Northern blots. A fragment isolated from a subclone containing the entire coding sequence (1200 bp) of the *npt*II gene joined to 690 bp from the 3′ non-coding region of the *rbc*S-E9 gene was labeled and used as a hybridization probe. Under the conditions used, this probe does not cross-hybridize with RNA from non-transformed plants (data not shown), whereas RNA from plants transformed with the pMON145 vector alone gives only one band corresponding in size to the NOS-*npt*II mRNA (Figure 2A, lane 1). To minimize any possible variations in the physiological state of the plants and/or the purity of the polyadenylated RNA preparations we have used the Nos-*npt*II mRNA level as an internal control. The expression of the pea *rbc*S-E9 gene in transgenic plants was evaluated as relative transcript level (RTL), which is the ratio of the *rbc*S-E9 mRNA to the Nos-*npt*II mRNA. To calculate RTL, bands corresponding to the *npt*II and *rbc*S-E9 mRNA were excised and their intensities quantified (Suissa, 1983). The 3′ fragment of *rbc*S-E9 hybridizes to only 220 nucleotides at the 3′ end of the *rbc*S-E9 mRNA, whereas the *npt*II coding sequence hybridizes to 1200 nucleotides of the *npt*II mRNA. To adjust for this difference, the value for *rbc*S-E9 was multipled by 5.45 before dividing by the value for *npt*II to give the RTL. Figure 2A, lane 2 shows that the light-grown transgenic petunia plants express the *rbc*S-E9 mRNA in addition to the NOS-*npt*II mRNA with an RTL

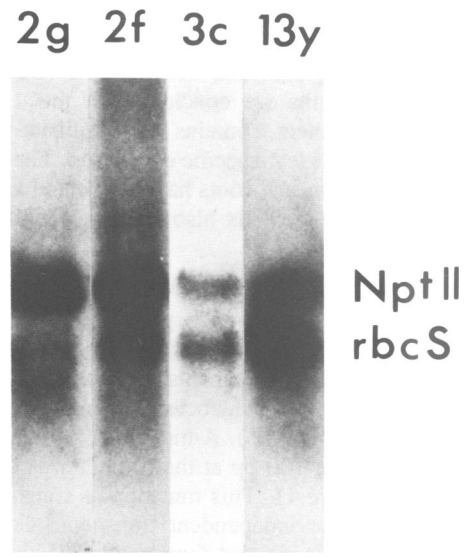

Fig. 4. Expression of the *rbc*S-E9 gene in four (clones 2g, 2f, 3c, 13y) independent transgenic petunia plants. Leaf polyadenylated RNAs (2 μg) were analyzed by Northern blot hybridization as described in Figure 2.

of ~15. The *rbc*S-E9 level is reduced by at least 15-fold in plants kept in dark for 4 days (lane 3); this low mRNA level has precluded a reliable estimation of the RTL in dark-adapted plants. The effect of darkness on the *rbc*S-E9 gene expression is reversible since the light RTL is nearly re-established after re-exposure to light for 24 h (Figure 2A, lanes 4–5). Figure 2A also shows that *npt*II mRNA transcribed from the NOS promoter remains at approximately the same level under these experimental conditions. This result is consistent with our previous observation that the Nos-*npt*II gene is insensitive to light (Odell *et al.*, 1985). The pea *rbc*S-E9 gene is also regulated by light in transgenic tobacco plants. However, its transcript level is lower than that in transgenic petunia (Figure 2B). The RTL is 15 in petunia leaves, but this value decreases to ~1 in tobacco leaves. These results demonstrate clearly that the *rbc*S-E9 gene retains photoregulated expression in leaves of transgenic petunia and tobacco plants.

The rbcS-E9 gene is transcribed accurately in transgenic plants

To determine the exact 5′ terminus of the *rbc*S-E9 mRNA in transgenic petunia, leaf RNAs were analyzed by S1 nuclease protection assays (Berk and Sharp, 1977). Figure 3, lane 1 shows that pea leaf polyadenylated RNA gives two major S1-protected

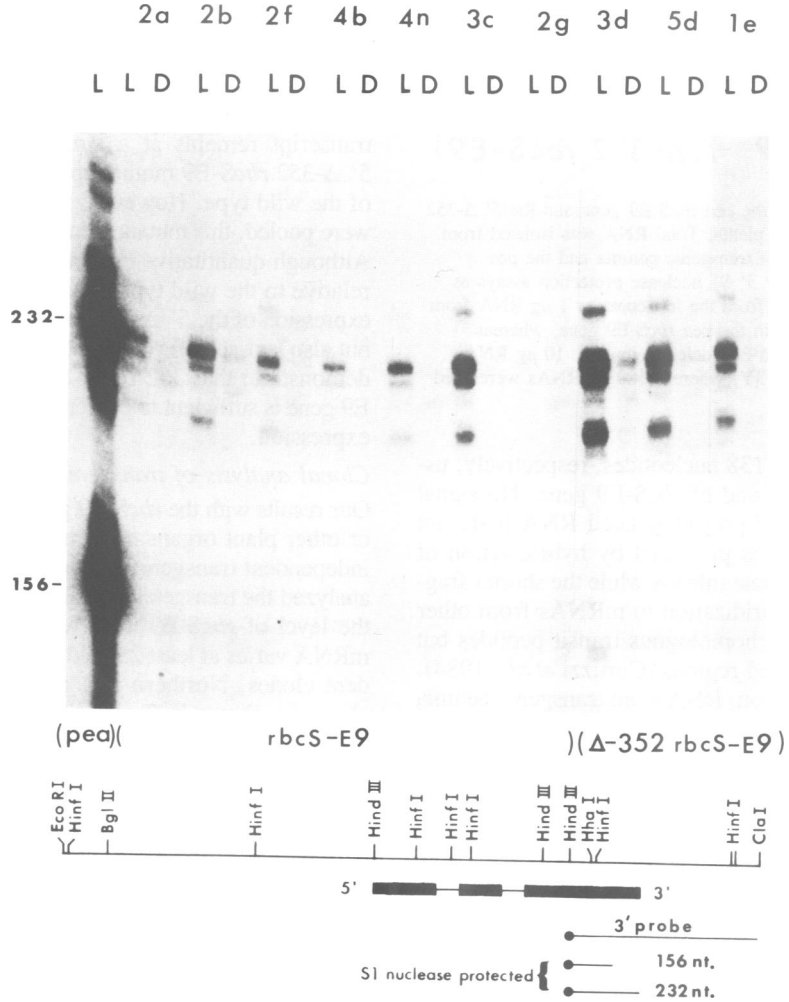

Fig. 5. Light-regulated expression of the pea *rbc*S-E9 gene and the 5′ Δ-352 mutant in independent transgenic petunia plants. Eight independent transgenic petunia clones (2a, 2b, 2f, 4b, 4h, 3c and 2g) containing the pea *rbc*S-E9 gene and three independent transgenic petunia clones (3d, 5d and 1e) containing the deletion mutant, 5′ Δ-352 *rbc*S-E9, were analyzed. Levels of *rbc*S-E9 transcript in 5 ug RNA from pea and 50 μg from transgenic petunia were quantitated by 3′ S1-nuclease protection assays (Morelli *et al.*, 1985). Lower panel shows a schematic diagram of the *rbc*S-E9 gene with relevant restriction sites. The 3′ probe and the expected lengths of the S1-nuclease protected fragments are also indicated.

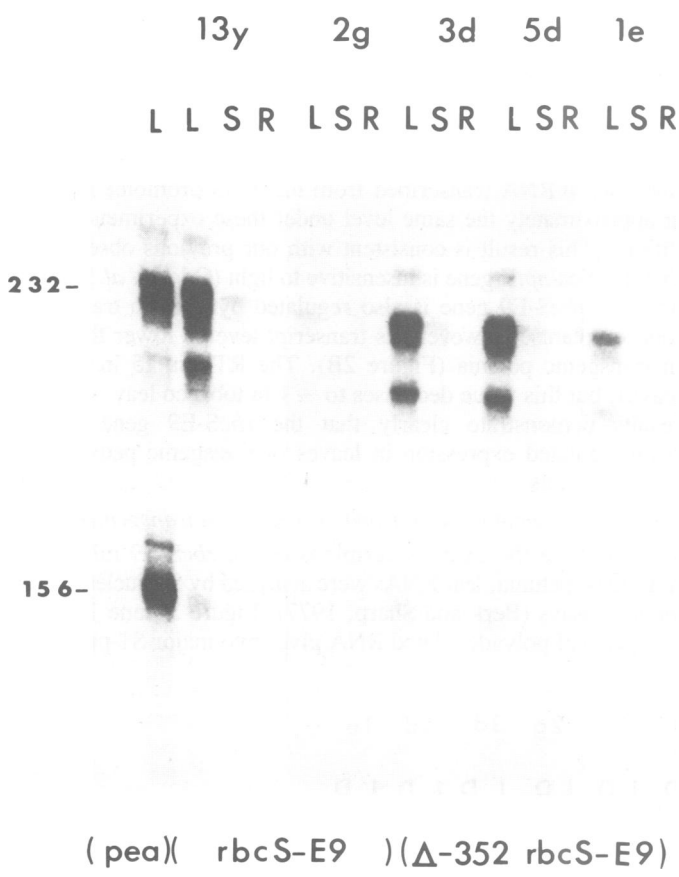

Fig. 6. Organ-specific expression of the pea *rbc*S-E9 gene and the 5′ Δ-352 deletion mutant in transgenic petunia plants. Total RNA was isolated from leaves (L), stems (S) and roots (R) of transgenic petunia and the pea *rbc*S-E9 transcript was quantitated by 3′ S1 nuclease protection assays as described in Figure 5. The first lane from the left contains 1 μg RNA from pea leaves. Clones 13y and 2g contain the pea *rbc*S-E9 gene, whereas clones 3d, 5d and 1e contain the 5′ Δ-352 deletion mutant. 10 μg RNAs were used for the anlaysis of clone 13Y, whereas 100 μg RNAs were used for the other clones.

fragments, 105 nucleotide and 138 nucleotides, respectively, using a probe that covers the 5′ end of *rbc*S-E9 gene. No signal was obtained with petunia leaf polyadenylated RNA (data not shown). The longer fragment is produced by hybridization of the *rbc*S-E9 probe with its cognate mRNA while the shorter fragment is the result of cross-hybridization to mRNAs from other pea *rbc*S genes which encode homologous transit peptides but have divergent 5′ non-translated regions (Coruzzi *et al.*, 1984). Consistent with this interpretation, RNA from transgenic petunia leaves gives only the 138-nucleotide protected fragment (Figure 3, lane 2). This result confirms that transcription initiation of the pea *rbc*S-E9 gene occurs accurately in transgenic petunia leaves, as is the case in transformed petunia calli (Broglie *et al.*, 1984).

Organ-specific expression of rbcS-E9

In peas, the level of the *rbc*S-E9 transcript is high in leaves, low in stems and not detectable in roots (Coruzzi *et al.*, 1984). To determine whether this expression pattern of the *rbc*S-E9 gene could be recapitulated after its insertion into the petunia genome, RNAs from leaves, stems and roots of transgenic petunia were analyzed. We found that the expression of the *rbc*S-E9 gene is indeed leaf-specific by 5′ S1 nuclear protection assays (Figure 3, lanes 2−4) and by Northern blot hybridizations (data not shown). In contrast, the co-transferred and linked NOS-*npt*II gene is expressed constitutively in all the plant organs examined (data not shown), confirming previous observations (Odell *et al.*, 1985). From these results we conclude that the 2.4-kb pea *rbc*S-E9 genomic fragment contains the requisite *cis*-acting regulatory elements for its leaf-specific expression. The low *rbc*S-E9 mRNA levels in stems and roots have prevented us from investigating whether the gene is also light-regulated in these organs.

Expression pattern of a 5′ deletion mutant

The photoinduced expression of the pea *rbc*S-E9 gene in appropriate organs of transgenic plants, allows the opportunity to define *cis*-acting regulatory elements. We constructed a series of 5′ deletion mutants by *Bal*31 nuclease digestion as detailed elsewhere (Morelli *et al.*, 1985). A mutant, 5′ Δ-352 *rbc*S-E9, that suffers a deletion of 700 bp at the 5′ end, was chosen for the present study (Figure 1). This mutant was transferred into petunia plants and three independent transgenic clones were recovered. RNAs were extracted from pooled leaves of these petunia plants and analyzed by S1 nuclease protection assays. Figure 3, lane 5 shows that transcription initiation of the 5′ Δ-352 *rbc*S-E9 mutant gene in leaves occurs at the correct site. Thus, deletion of the 700 bp 5′ upstream sequence from −1052 to −352 has no effect on the S1 start site, confirming previous observations with transformed calli (Morrelli *et al.*, 1985). Northern blot analysis revealed that the level of mRNA transcribed from the 5′ Δ-352 *rbc*S-E9 gene is reduced in dark-grown plants compared with that grown in the light, while the NOS-*npt*II transcript remains at a similar level (data not shown). The 5′ Δ-352 *rbc*S-E9 mutant appears to have a RTL similar to that of the wild type. However, since only three transformed plants were pooled, this mutant value may not be quantitatively reliable. Although quantitative estimation of the mutant expression level relative to the wild type is premature at this stage, we note that expression of the 5′ Δ-352 *rbc*S-E9 gene is not only light-induced but also leaf-specific (Figure 3, lanes 5−7). These results clearly demonstrate that 352 bp of 5′ upstream sequence of the *rbc*S-E9 gene is sufficient to confer light-inducibility and organ-specific expression.

Clonal analysis of transgenic plants

Our results with the *rbc*S-E9 gene have been obtained with leaves or other plant organs collected and pooled from seven or eight independent transgenic petunia or tobacco plants. We have also analyzed the transgenic petunia plants individually and found that the level of *rbc*S-E9 mRNA relative to that of the NOS-*npt*II mRNA varies at least 25-fold among leaves of the eight independent clones. Northern blot analyses of four of the transgenic clones are shown in Figure 4. Clones 13y and 2g have the highest and lowest RTL, respectively, whereas intermediate values are obtained with clones 2f and 3c. Since the *rbc*S-E9 and the NOS-*npt*II genes are linked and, therefore, presumably present in equal copy numbers, this variation is probably due to a position effect, resulting from insertion into different chromosomal sites among the eight clones (Feinstein *et al.*, 1982).

We have also compared the *rbc*S-E9 transcript level in the transgenic petunia plants relative to that in pea seedlings. DNA was prepared from individual transgenic petunia clones and the copy numbers of the inserted *rbc*S-E9 and NOS-*npt*II genes were determined by slot blot hybridizations (Tlsty *et al.*, 1982) using labeled probes specific to the *npt*II and *rbc*S-E9 genes. We found that the NOS-*npt*II and *rbc*S-E9 genes are present at equally low copy number (1−2 copies per diploid petunia genome) in all

the transgenic plants examined. To confirm these results S1 progeny from all the transgenic plants were tested for kanamycin resistance (Horsch *et al.*, 1984). Consistent with the low (1−2) copy numbers, the progeny in every case showed a normal 3:1 Mendelian inheritance with respect to the kanamycin-resistance marker. Since there are two copies of *rbc*S-E9 in the diploid pea genome (R.Fluhr, unpublished results), based on total RNA, we estimated that in the transgenic petunia plants the *rbc*S-E9 gene is expressed at ~0.2% (clone 2g) to 10% (clone 13y) relative to the level in peas (Figures 5 and 6). This low-level expression may be due to less efficient transcription of the *rbc*S-E9 gene and/or reduced stability of this transcript in petunia as compared with pea. Notwithstanding the quantitative variations, all of the transgenic petunia plants transformed with the *rbc*S-E9 (eight clones) and the 5′ Δ-352 *rbc*S-E9 gene (three clones) continue to show light-regulated expression (Figure 5). Moreover, their *rbc*S-E9 transcript level is 15−20 times higher in leaves than in stems and no transcript is detectable in roots of transgenic plants (Figure 6). These results suggest that the quantitative level of the *rbc*S-E9 gene expression is more sensitive to chromosomal positions in the recipient plant genome than its leaf-specific and photoregulated expression.

Discussion

So far, two pea *rbc*S genes (Broglie *et al.*, 1984; Morelli *et al.*, 1985; Herrera-Estrella *et al.*, 1984) and one soybean *rbc*S gene (Facciotti *et al.*, 1985) have been introduced into petunia and tobacco genomes. In these experiments, oncogenic Ti-plasmids were employed as vectors resulting in transformed calli that could proliferate in the absence of phytohormones. Although the transferred *rbc*S gene was expressed in a light-regulated manner in all three cases, it is not known to what extent the expression is influenced by abnormal levels of auxins and cytokinins within the transformed cells. Furthermore, since the transformed calli are non-morphogenic, the expression of the *rbc*S genes in intact plants under normal physiological conditions cannot be assessed. Here we have used the non-oncogenic Ti-vector system of Fraley *et al.* (1985) to transfer the pea *rbc*S-E9 gene into petunia and tobacco genomes and showed that the inserted gene retains its light-regulated expression in regenerated, transgenic plants. The *rbc*S-E9 transcripts possess the same 5′ and 3′ termini in the transgenic petunia plants as in pea seedlings. More importantly, the transcripts are distributed in an organ-specific manner being most abundant in leaves and not detectable in roots. Therefore the 2.4-kb pea genomic fragment must contain sequences that are recognized by the relevant transcriptional factors involved in light-regulated and organ-specific expression of petunia and tobacco genes. Light-regulated and organ-specific expression in transgenic plants have also been obtained with two additional members, 3A and 3C, of the pea *rbc*S gene family (R.Fluhr, unpublished results) and with a wheat nuclear gene encoding the chlorphyll a/b-binding protein (Lamppa *et al.*, 1985). In addition, Sengupta-Gopalan *et al.* (1985) have reported the seed-specific and developmentally regulated expression of a bean gene encoding the β phaseolin storage protein in transgenic tobacco plants. Taken together, these results strongly suggest that regulatory DNA sequences for organ-specific expression of these genes are conserved across evolutionarily distant plant species.

That the *rbc*S-E9 gene is expressed correctly in the appropriate organs of the transgenic plants provided an opportunity to define regulatory elements within the introduced pea genomic fragment. As a first step we have demonstrated that a 700-bp deletion (from −1052 to −352) from the 5′ upstream region of the *rbc*S-E9 gene has no noticeable affect on transcription initiation, light-regulation or leaf-specific expression. These results are comparable with those of Ornitz *et al.* (1985) who reported that the 213-bp sequence upstream of the elastase gene promoter is sufficient to confer tissue specificity in transgenic mice. In transformed petunia calli, a 5′ deletion mutant of *rbc*S-E9 that contains only 35 bp of 5′ upstream sequence still shows light-regulated expression (Morelli *et al.*, 1985). The expression pattern of this, as well as other mutants of *rbc*S-E9 in transgenic petunia clones, are being evaluated.

The expression of foreign genes in transgenic mice (cf. Palmiter and Brinster, 1985) and in *Drosophila* (Bourouis and Richards, 1985) is influenced greatly by their chromosomal insertion sites. Such position effects may be manifested in several ways. For example, there is generally no correlation between expression level of an introduced gene and its copy number in transgenic mice. In at least one case, the introduced gene is expressed in inappropriate tissues (Lacy *et al.*, 1983). Whether chromosomal positions similarly affect the expression of foreign genes in transgenic plants is not known. To address this question we have analyzed individual transgenic petunia plants carrying one or two copies of the *rbc*S-E9 gene (eight clones) or its deletion derivative (three clones). By Northern blot analysis of polyadenylated RNAs we found that the ratio of NOS-*npt*II transcript and *rbc*S-E9 transcript varied from 25- to 50-fold. A similar range of variation in *rbc*S-E9 transcript was also observed when total RNAs from the transgenic clones were assayed directly by 3′ S1 nuclease protection. None of the clones examined showed deregulated expression of the *rbc*S-E9 or expression in inappropriate tissues, as had been reported for a rabbit β-globin gene in transgenic mice (Lacy *et al.*, 1983). Therefore we conclude that the chromosomal positions of the *rbc*S-E9 gene in the petunia genome affect the quantitative but not the qualitative aspect of its expression.

We consistently observe a lower level of *rbc*S-E9 mRNA in tobacco as compared with petunia. This difference could be due to either differential transcription rate of the *rbc*S-E9 gene or differential transcript stability in the two recipient plants. The *rbc*S-E9 gene in transgenic petunia is expressed at only 0.2-10% of the level in pea seedlings. In our experiments, a chimeric gene consisting of the nopaline synthase (NOS) promoter fused to the coding sequence of neomycin phosphotransferase II (*npt*II) was used as a selectable marker (Fraley *et al.*, 1983). Since the transgenic plants were selected for their resistance to kanamycin (100 μg/ml) it is possible that the selection protocol might lead to a preferential recovery of clones that contain the NOS-*npt*II gene in favorable chromosomal sites and such sites may be suboptimal for the expression of the linked *rbc*S-E9 gene. Experiments are in progress to use a *rbc*S-E9-*npt*II chimeric construct as a selectable marker in an attempt to recover transgenic clones that might express the *rbc*S-E9 gene at a higher levels.

Materials and methods

Transfer of the pea rbcS-E9 gene into petunia and tobacco

The *rbc*S-E9 gene was isolated as a 2.4-kb *Eco*RI-*Cla*I genomic fragment which includes 1052 bp upstream of the transcription initiation site and 690 bp beyond the rbcS polypeptide termination codon (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985). The genomic fragment was inserted into the intermediate vector, pMON145, which contains the nopaline synthase (NOS) promoter fused to the Tn5 neomycin phosphotransferase II (nptII) coding sequence followed by the nopaline synthase polyadenylation site (Fraley *et al.*, 1983). The NOS-*npt*II chimeric gene confers kanamycin resistance to transformed plant cells. The pMON145 plasmid containing the pea *rbc*S-E9 gene was transferred by triparental crosses into a 'disarmed' *A. tumefaciens* and recombined into the modified pTiB6S3SE plasmid, in which

all phytohormone biosynthetic genes and the TL-DNA right border have been deleted (Fraley *et al.*, 1985). These *A. tumefaciens* cells were co-cultured with protoplasts of *Petunia hybrida* (Mitchell) or *Nicotiana tabacum* SR1, and transformed cells were selected by their resistance to kanamycin at 100 μg/ml (Fraley *et al.*, 1983). Shoots were regenerated from transformed petunia and tobacco calli (Marton *et al.*, 1979), and after root formation, young plantlets were transferred to soil and used for further analyses.

Preparation of total RNA and polyadenylated mRNA

Leaves, stems and roots were collected from transgenic plants grown in a greenhouse. The entire stem was used for RNA extraction. In some cases roots were also collected from transgenic plants grown in the light in MS medium. RNA was extracted from leaves, stems and roots using guanidium thiocyanate as a protein denaturant (Chirgwin *et al.*, 1979) and further purified by centrifugation through CsCl cushion (Gilsen *et al.*, 1974). Polyadenylated RNA was isolated by chromatography on poly(U)-Sepharose (Beroglie *et al.*, 1981).

Northern analysis

Aliquots of RNA were denatured in glyoxyl at 50°C (Carmichael and McMaster, 1980), electrophoresed on 1% agarose gels, and transferred onto nitrocellulose filters (Thomas, 1980). Conditions of hybridizations and the probes used are described under figure legends.

S1 nuclease analysis

End-labeled restriction fragments from the pea *rbc*S-E9 gene were used to detect the 5′ and 3′ ends, respectively, of the *rbc*S-E9 transcripts by S1 nuclease mapping (Berk and Sharp, 1977; Weaver and Weissman, 1979). For 5′ mapping, a *HinfI-HinfI* fragment (541 nucleotides) from *rbc*S-E9 (Broglie *et al.*, 1984) was labeled at the 5′ end and denatured and the dissociated strands separated by polyacrylamide gel electrophoresis. The isolated strand was hybridized with polyadenylated RNA in a solution (10 μl) containing 50% formamide, 0.4 M NaCl, 2 mM EDTA and 20 mM PIPES (pH 6.8) for 12 h at 42°C. After hybridization, the reaction mixture was diluted to 300 μl with a solution containing 0.5 M NaCl, 30 mM NaOAc (pH 4.6), 1 mM $ZnSO_4$, 20 μg/ml denatured salmon sperm DNA, 300 U/ml S1 nuclease and incubated at 37°C for 45 min. For 3′ mapping, a *Hind*III-*Cla*I fragment (690 bp) from the *rbc*S-E9 gene (Morelli *et al.*, 1985) was end-labeled by polymerase I Klenow large fragment at the *Hind*III site. Single-stranded probes were hybridized with either 50 μg RNA from transgenic petunia or 5 μg RNA from pea under conditions described previously (Morelli *et al.*, 1985). DNA fragments protected from S1 nuclease digestion were sized at 6% sequencing gels and visualized by autoradiography.

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Note added in proof

We have found recently that in transgenic plants, young stems (the upmost two internodes) contain *rbc*S-E9 mRNA at a level 25−50% of that in leaves and the expression is light-regulated. (October 24, 1985).