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Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase

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We have examined the expression of a member of the multigene family encoding the small subunit (rbcS) of ribulose-1,5-bisphosphate carboxylase in various tissues of pea. The rbcS gene, *pPS-2.4*, was characterized by DNA sequence analysis and 5′ and 3′ end mapping of its mRNA transcript. rbcS polypeptides were shown to be differentially present in various tissues of light- and dark-grown plants. Northern analysis shows that compared with green leaves, the level of rbcS mRNA is reduced to ~50% in pericarps, 8% in petals and seeds, and 1 – 3% in etiolated leaves, stems, and roots. 5′ S1 nuclease mapping of total rbcS mRNA was used to quantitate the relative amount of *pPS-2.4* gene-specific transcripts in each tissue. *pPS-2.4* mRNA accounts for approximately 30 – 35% of total rbcS transcripts in green leaves, but only 5 – 10% in pericarps, 15 – 20% in seeds, and is below detection in petals and etiolated leaves. We conclude that the *pPS-2.4* gene is expressed in a tissue-specific, light-regulated fashion and that transcriptional controls of individual rbcS genes vary.

Key words: light-regulation/multigene family/*Pisum sativum*/ribulose-1,5-bisphosphate carboxylase/tissue-specific expression

Introduction

The small subunit (rbcS) of ribulose-1,5-bisphosphate carboxylase (RUBISCO) (EC 4.1.1.39) is encoded by a small multigene family in *Pisum sativum* (Coruzzi *et al.*, 1983; Bedbrook *et al.*, 1980), as well as in other higher plants (Berry-Lowe *et al.*, 1982; Broglie *et al.*, 1983; Dunsmuir *et al.*, 1983). The rbcS mRNA is a major transcript of higher plant nuclear DNA (Gallagher and Ellis, 1982) and is translated in the cytoplasm as a larger precursor containing an amino-terminal transit peptide which is cleaved during or shortly after its post-translational transport into chloroplasts (Chua and Schmidt, 1978). Inside the organelle, eight rbcS polypeptides assemble with an equal number of chloroplast-encoded large subunits (rbcL) to form the holoenzyme which catalyzes the initial fixation of CO_2 (Miziorko and Lorimer, 1983). Each plant cell contains multiple copies of chloroplast DNA ($0.5 - 1.0 \times 10^4$/cell) (Lamppa and Bendich, 1979; Scott and Possingham, 1980) and therefore multiple copies of the gene encoding rbcL. The nuclear genome contains 6 – 12 copies of the rbcS gene, depending on the species. Although some of these genes may be pseudogenes, there is evidence in pea (Coruzzi *et al.*, 1983; Bedbrook *et al.*, 1980), wheat (Broglie *et al.*, 1983), and petunia (Dunsmuir *et al.*, 1983), that more than one rbcS gene is expressed *in vivo*.

Light has been shown to modulate the steady-state levels of rbcS mRNA in pea leaves in a phytochrome-mediated response (Thompson *et al.*, 1983). Transcription experiments with isolated pea leaf nuclei provide evidence that light affects an increase in transcription of rbcS genes (Gallagher and Ellis, 1982). However, since the hybridization probes used in these experiments are homologous to all rbcS transcripts, the qualitative and quantitative responses of individual rbcS genes to light, could not be assessed. As shown for other multigene families of *Dictyostelium* (Devine *et al.*, 1982; McKeown and Firtel, 1981a), *Drosophila* (Fyrberg *et al.*, 1983), mouse (Shaw *et al.*, 1983) and human (Kim *et al.*, 1983), individual genes may be selectively expressed during different developmental stages or in different tissues. A similar situation may exist for the rbcS multigene family of higher plants. For example, the transcription of some rbcS genes may be enhanced by light or tissue-specific factors, while others may be expressed constitutively, and yet others may be inactive (pseudogenes). As a first step towards examining this issue we determined the level of rbcS polypeptide and mRNA in various tissues of light- and dark-grown pea plants. We isolated and characterized an rbcS gene (*pPS-2.4*) which is actively transcribed in green leaves, and followed its specific transcription products in various tissues of light- and dark-grown pea plants. Our results show that the rbcS gene, *pPS-2.4*, is expressed in a tissue-specific, light-regulated fashion and that the transcriptional controls of individual rbcS genes vary.

Results

Isolation of an rbcS gene which is expressed in pea leaves

Pea genomic DNA cloned into λ phage 1059 (Karn *et al.*, 1980) was screened with radioactive insert of cDNA clone, pSS15, which encodes pea rbcS (Coruzzi *et al.*, 1983). Six positively hybridizing genomic clones were isolated and designated λPS-3D, 3C, 3A, 2C, 5A, 8B. Southern blot and DNA restriction analysis revealed that four to five members of the rbcS gene family are present in this collection of genomic clones (data not shown). To isolate and characterize a gene which is transcriptionally active in pea leaves, we identified from the set of genomic clones, the one which encodes pSS15 (synthesized from pea leaf mRNA). A comparison of the DNA sequences of two independently isolated pea rbcS cDNA clones, pSS15 (Coruzzi *et al.*, 1983) and pSSU1 (Bedbrook *et al.*, 1980), showed that while the amino acid coding sequences are highly conserved, the 3′ non-coding regions are divergent. This is consistent with observations made for the rbcS gene families of other species (Broglie *et al.*, 1983; Dunsmuir *et al.*, 1983), as well as other multigene families (McKeown and Firtel, 1981b). Since the 3′ non-coding region contains a sequence specific to each gene, a DNA fragment from this region can be used as a hybridization probe to discriminate among members of the rbcS gene family. To this

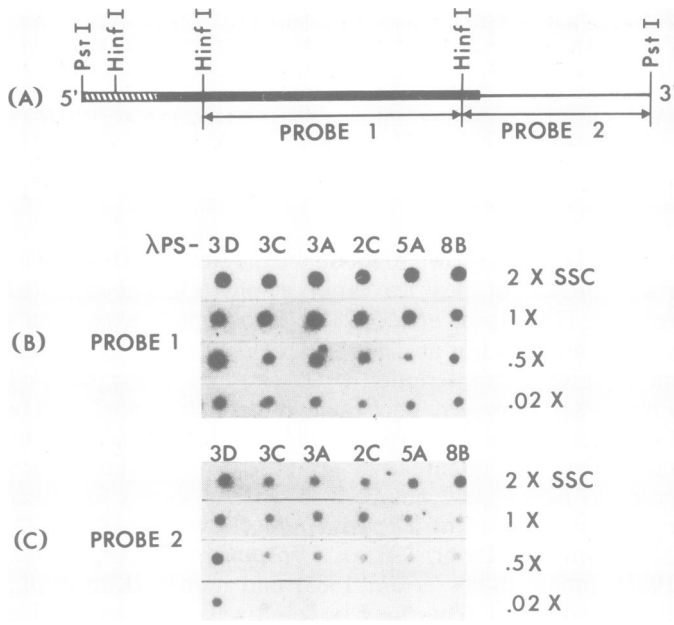

Fig. 1. DNA dot-blots of rbcS genomic clones. Nitrocellulose strips dotted with 0.5 μg of DNA from pea rbcS genomic clones λPS-3D, 3C, 3A, 2C, 5A, 8B, were hybridized to radioactively labeled probe 1 (**Panel B**) derived from the protein coding region of pea rbcS cDNA clone pSS15, or to probe 2 (**Panel C**) derived from the 3′ non-coding region of pSS15. Strips were washed with decreasing concentrations of SSC (2 x to 0.02 x SSC) (see Materials and methods). Nitrocellulose strips were exposed to X-ray film for autoradiography.

end, two *Hinf*I fragments were isolated from pSS15 (Figure 1A). The larger *Hinf*I fragment (probe 1) contained only amino acid coding sequences, while the smaller *Hinf*I fragment (probe 2) contained the 3′ non-coding sequences (Figure 1A). DNA dot-blots of phage DNA from the six rbcS genomic clones were hybridized with either ^{32}P-labeled probe 1 or 2, and replicate filters were washed under conditions of increasing hybridization stringency (2.0 x SSC to 0.02 x SSC) as described previously (Wahli and Dawid, 1980). Figure 1C shows that the 3′ non-coding fragment (probe 2) hybridizes most strongly to the genomic clone, designated λ PS-3D. The amino acid coding fragment (probe 1) hybridizes equally well to all clones following washes with 2.0 x and 1.0 x SSC, but shows preferential hybridization to λ PS-3D following the more stringent washes (0.5 x and 0.02 x SSC) (Figure 1B). These data indicate that of the six genomic clones examined, λ PS-3D is most closely related to pSS15, in both the coding and 3′ non-coding regions.

pPS-2.4 is the rbcS gene which encodes cDNA clone pSS15

Fragments of genomic clone λ PS-3D containing the entire rbcS gene were subcloned into pBR325 for subsequent analysis. Subclone pPS-4.0 contains a 4-kb *Eco*RI fragment, and subclone pPS-2.4 contains a 2.4-kb *Eco*RI-*Cla*I subfragment of pPS-4.0 (Figure 4A). The nucleotide sequence of the pea rbcS gene, *pPS-2.4*, is shown in Figure 2. In addition to the structural gene, this DNA fragment contains 1085 nucleotides of 5′ upstream sequences and 550 nucleotides of 3′ non-coding sequences. The pea rbcS precursor polypeptide is encoded by three exons. Exon 1 encodes the entire 57 amino acids of the precursor transit peptide plus the first two amino acids of the mature protein. Exons 2 and 3 encode 45 and 76 amino acids of the mature protein, respectively. Introns 1 and 2 are 79 and 86 nucleotides long, respectively. The placement of the intron-exon junctions is based on nucleotide sequence identity with pSS15. However, the presence of a direct repeat of five nucleotides at the boundaries of intron 1 allows the splice junction to be placed at any one of six possible positions to produce the nucleotide sequence of pSS15. Similarly, three splice junctions are possible for intron 2, due to the presence of a direct repeat of two nucleotides at the intron boundaries. The splice junctions shown in Figure 2 were the only ones homologous to the consensus sequence as determined for other eukaryotic mRNA splice junctions (Mount, 1982). The DNA sequence of genomic subclone pPS-2.4 is identical to the cDNA clone pSS15 throughout the amino acid coding region and the 3′ non-coding region. The nucleotide differences which occur within amino acid codons −33, +35 and +61 represent corrections of the previously published sequence of pSS15 (Coruzzi *et al.*, 1983).

As shown previously, a DNA probe encoding the rbcS polypeptide hybridizes to 5−6 pea genomic bands in a Southern blot experiment (Figure 3, lanes 1, 3, 6). Since the 3′ non-coding regions of individual rbcS genes are divergent (Coruzzi *et al.*, 1983; Bedbrook *et al.*, 1980; Broglie *et al.*, 1983; Dunsmuir *et al.*, 1983), we probed identical Southern blots with a DNA fragment from the 3′ non-coding region of *pPS-2.4* to determine which genomic fragment(s) contain this rbcS gene. In each case the *pPS-2.4* rbcS gene is contained within a single pea genomic fragment; 7.4-kb *Bam*HI, 6.0-kb *Eco*RI, 2.8-kb *Bgl*II (Figure 3, lanes 2, 5, 7). A restriction map of genomic subclone pPS-4.0 (Figure 4A) shows that the 2.8-kb *Bgl*II genomic fragment is contained within this subclone. However, the 4-kb *Eco*RI fragment of pPS-4.0 does not correspond in size to the genomic *Eco*RI fragment shown to contain the *pPS-2.4* gene. This suggests that the DNA on one or both sides of the 2.8-kb *Bgl*II fragment in pPS-4.0 is most likely from other pea genomic *Bgl*II fragments which were randomly ligated during the cloning process (see Materials and methods). Under less stringent hybridization conditions (see legend, Figure 3), the 3′ non-coding probe from *pPS-2.4* hybridizes to two additional *Eco*RI fragments of pea nuclear DNA (~3.6 and 3.4 kb) (Figure 3, lane 4). These genomic fragments most likely contain other rbcS genes which share homology with *pPS-2.4* in their 3′ non-coding regions. There is evidence in petunia for subsets of related rbcS genes (Dunsmuir *et al.*, 1983). The DNA sequence of the pea rbcS gene (*SS3.6*) encoded in the 3.6-kb *Eco*RI fragment has recently been reported (Cashmore, 1983). A comparison of the nucleotide sequences of the two pea rbcS genes shows that *SS3.6* is highly homologous to *pPS-2.4* in the coding and 3′ non-coding regions, while the 5′ non-coding regions of the two genes are more diverse.

Characterization of the pPS-2.4 mRNA transcript

The precise nucleotide location of the 5′ and 3′ ends of the mRNA transcript encoded by *pPS-2.4* were determined by S1 nuclease mapping (Berk and Sharp, 1977). DNA fragments spanning either the 5′ or 3′ boundary of the gene were isolated (Figure 4A), labeled at either their 5′ or 3′ ends, respectively, and the complementary strands separated by gel electrophoresis. The 5′ DNA probe is a 545 nucleotide *Hinf*I fragment, while the 3′ probe is a 550 nucleotide *Dde*I-*Cla*I fragment (Figure 4A). The end-labeled single strands were

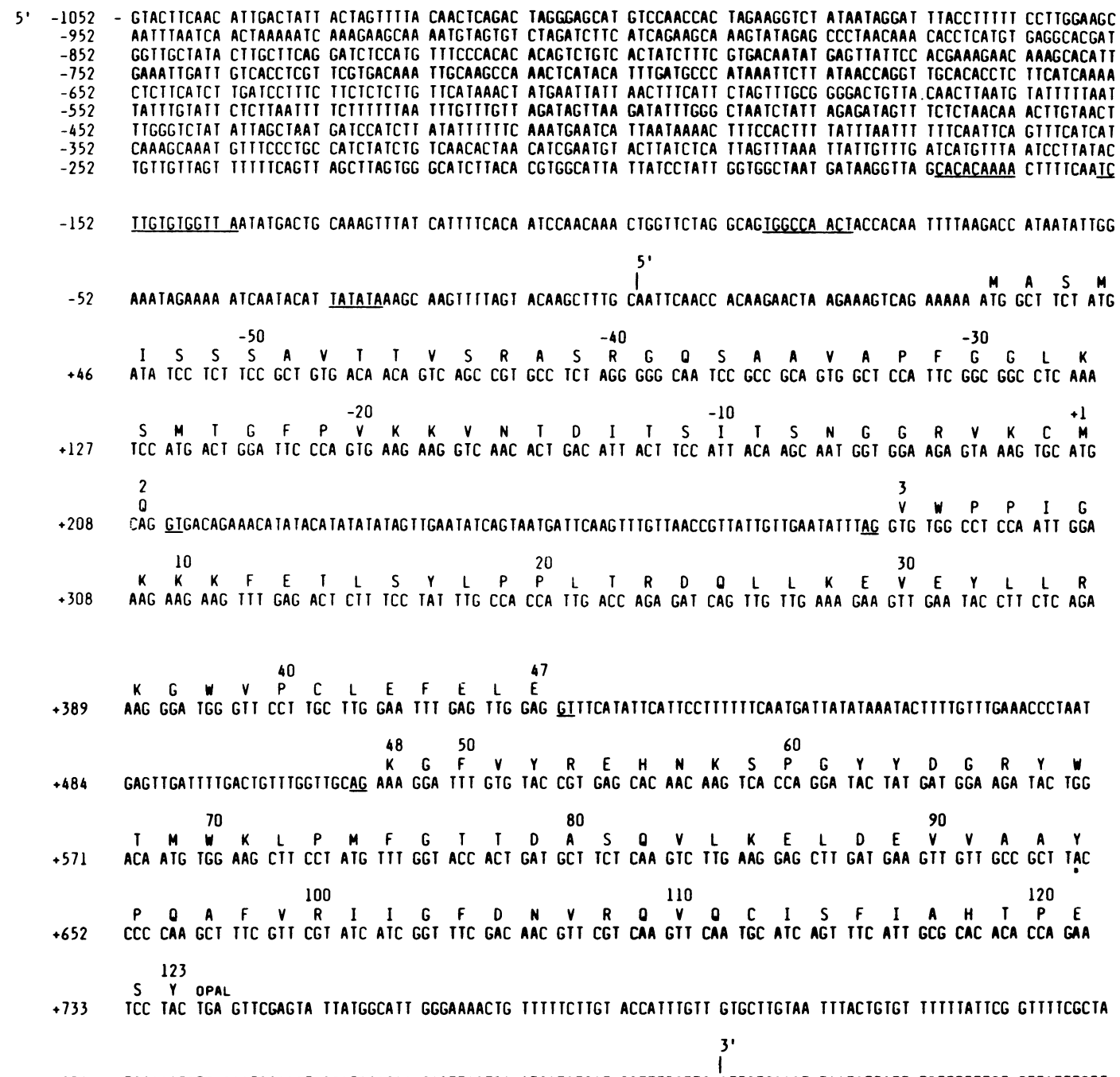

Fig. 2. Nucleotide sequence of pea genomic subclone *pPS-2.4* encoding rbcS. The nucleotide sequence shown is that of the non-coding strand (mRNA sense). The deduced amino acid residues are denoted above the nucleotide triplets in the standard one letter code. The amino acid designated +1 is the amino-terminal residue of the mature protein. The nucleotide designated +1 in numbering the DNA sequence is the putative transcription initiation site of *pPS-2.4*. The symbol at nucleotide position +891 denotes the 3′ terminus of the mRNA encoded by *pPS-2.4*. DNA sequences 5′ to the mRNA start site which resemble signals for transcriptional regulation of other eukaryotic genes are underlined.

hybridized to pea poly(A)$^+$ mRNA and digested with S1 nuclease. The DNA fragments protected from S1 nuclease digestion were electrophoresed alongside the end-labeled coding strand that had been chemically cleaved for sequence determination (Figure 4B, 4C). The 5′ end of the pPS-2.4 mRNA transcript falls within the sequence 'ATTCA' (Figure 4B, fragment *b*) 33 nucleotides upstream from the initiator ATG. Thirty-two nucleotides upstream from this putative initiation site is the sequence 'TATATA' which resembles the sequence motif of the Goldberg-Hogness 'TATA' box, shown to be required for the correct transcriptional initiation of animal genes by RNA polymerase II (Breathnach and Chambon, 1981). Further upstream of the transcription initiation site at position −85, is the sequence 'GGCCAACT'.

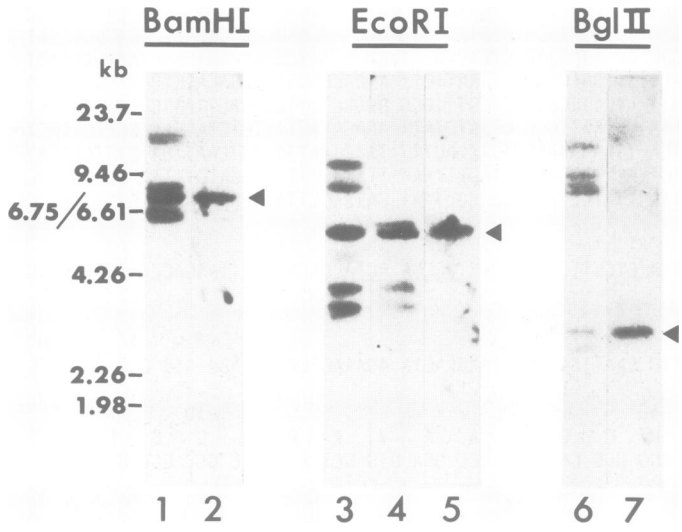

Fig. 3. Southern blot of pea genomic DNA probed with rbcS structural gene or *pPS-2.4* gene-specific DNA probes. 10 μg pea genomic DNA digested to completion with *Bam*HI (**lanes 1** and **2**), *Eco*RI (**lanes 3–5**), or *Bgl*II (**lanes 6** and **7**) was run on 0.8% agarose gel and transferred to nitrocellulose paper. **Lanes 1, 3** and **6** were hybridized with ^{32}P-labeled pSS15 at 42°C in: 50% formamide, 5 x SSC, 1 x Denhardt's solution, 100 g/ml denatured salmon sperm DNA. **Lanes 2, 5** and **7** were hybridized with ^{32}P-labeled *Dde*I-*Cla*I 3′ non-coding fragment of *pPS-2.4* (see Figure 4A) at 50°C in: 50% formamide, 2 x SSC, 1 x Denhardt's solution, 100 μg/ml denatured salmon sperm DNA. **Lane 4** was probed with the same *Dde*I-*Cla*I 3′ non-coding fragment under hybridization conditions described for **lanes 1, 3** and **6**. Lambda DNA fragments produced by *Hind*III digest were used as size markers. Arrows mark the genomic fragment in each digestion which contains the *pPS-2.4* gene.

This sequence motif resembles the 'GG$^{C}_{T}$CAATCT' or 'CAAT' box, located −70 to −80 nucleotides upstream of other eukaryotic genes, which is thought to be involved in the regulation of gene expression (Breathnach and Chambon, 1981). Also upstream of the *pPS-2.4* transcription initiation site is a sequence (−140 to −172) which contains features homologous to viral and eukaryotic enhancer elements (Weiher *et al.* 1983; Gillies *et al.*, 1983). These features, as outlined in Table I include homology to the 'core' nucleotides of enhancer elements 'GTGG$^{TTT}_{AAA}$G' (Weiher *et al.*, 1983) and the presence of an inverted repeat which includes the 'core' element (Gillies *et al.*, 1983). Although enhancer sequences have been shown to increase transcription of viral and eukaryotic genes (Weiher *et al.*, 1983; Gillies *et al.*, 1983), identification of homologous sequences as regulatory elements in plants awaits further investigation.

In Figure 4B the shorter S1 nuclease protected DNA fragment *a* corresponds to nucleotide +33 of the *pPS-2.4* gene (the ATG initiator of the rbcS precursor polypeptide). Fragment *a* results from S1 nuclease digestion of hybrids formed between the *pPS-2.4* DNA probe and RNAs transcribed from other rbcS genes homologous only within the rbcS coding region. Similar S1 nuclease experiments performed on RNA from petunia calli transformed with the *pPS-2.4* gene show only the larger S1 protected fragment *b* (Broglie *et al.* 1984), ruling out the possibility that the smaller S1 protected fragment *a* is the result of breathing of the hybrid around the ATG initiator.

The S1 nuclease mapping of the 3′ end of the *pPS-2.4* mRNA (Figure 4C) reveals an S1 protected DNA fragment *c* at a position 150 nucleotides downstream from the TGA terminator. This corresponds to the polyadenylation site in the

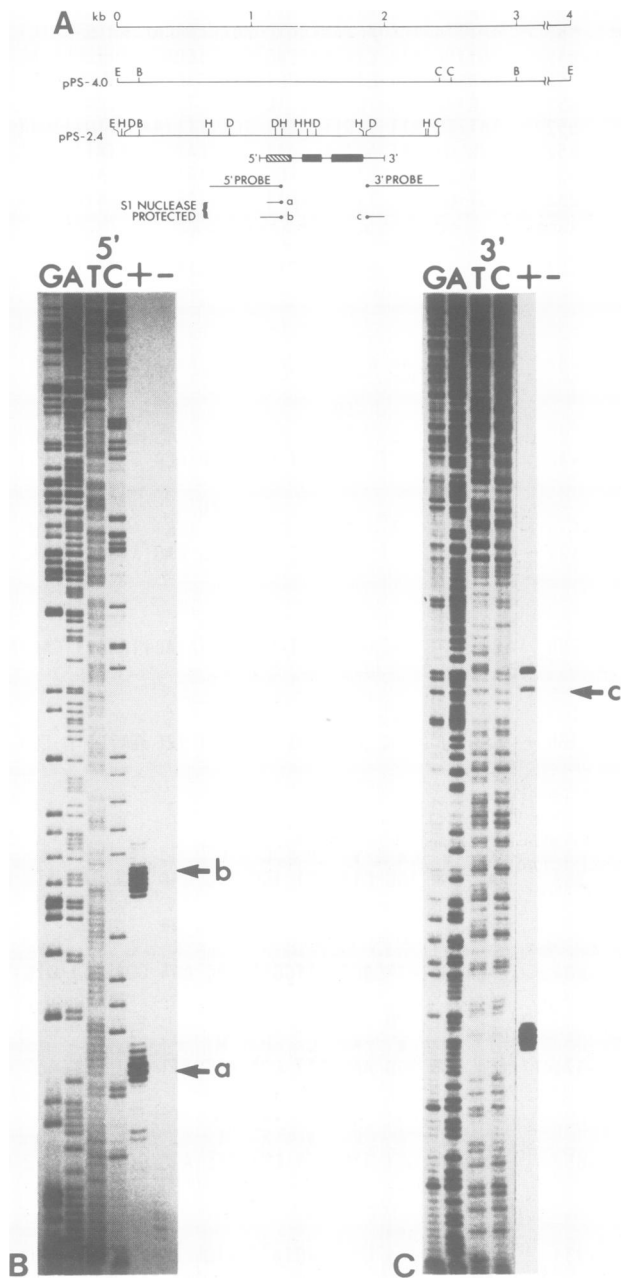

Fig. 4. Restriction map of rbcS genomic subclones pPS-4.0 and pPS-2.4 and S1 mapping of 5′ and 3′ ends of rbcS mRNA transcript. **(A)** Restriction endonuclease map of subclones of rbcS genomic clone λPS-3D: pPS-4.0 and pPS-2.4. B = *Bgl*II, C = *Cla*I, D = *Dde*I, E = *Eco*RI, H = *Hinf*I. Below the map is a schematic diagram of the gene coding region, consisting of three exons (boxed) and two introns. The first exon (hatched) includes the entire transit sequence. 5′ and 3′ non-coding region of *pPS-2.4* mRNA are marked as lines. The restriction fragments used for S1 mapping of the 5′ and 3′ boundaries of the mature rbcS mRNA are shown below the diagram of the rbcS gene. **(B)** S1 nuclease mapping of 5′ termini of pPS-2.4 mRNA. A 5′ DNA probe **(A)** was labeled at its 5′ end, hybridized to 6 μg pea leaf poly(A)$^+$ mRNA, and treated with S1 nuclease (see Materials and methods). DNA fragments protected from S1 nuclease digestion, in the presence (+) or absence (−) of pea RNA, are displayed alongside the DNA sequence ladder of the *Hinf*I strand complementary to the mRNA on 6% acrylamide/7 M urea gels. Fragment *a* = 105 nucleotides, Fragment *b* = 138 nucleotides. **(C)** S1 nuclease mapping of 3′ terminus of pPS-2.4 mRNA. A 3′ DNA probe **(A)** was labeled at its 3′ terminus, hybridized to 6 μg pea leaf poly(A)$^+$ mRNA, and treated with S1 nuclease (see Materials and methods). DNA fragments protected from S1 nuclease digestion in the presence (+) or absence (−) of pea RNA are displayed alongside the DNA sequence ladder of the *Dde*I-*Cla*I strand complementary to the mRNA on 6% acrylamide/7 M urea gels. Fragment *c* = 149 nucleotides.

Table I. rbcS sequences homologous to enhancer sequence elements

'GTGG$^{TTT}_{AAA}$G'	Enhancer 'core'[a]
+430 +457 AATAAAACCACTA—TGTGGTTTGAAGAAGTGGTTTTGAAAC	IgC intron[b]
−171 −152 +1 AGCACACAAAA—TCTTGTGTGGTTAA—ATTC—	Pea rbcS[c]
−197 −151 +1 GGTTGTGTGGCAGC——CACACACAATG—AACT—	Wheat rbcS[d]
−149 +1 +467 TCGTGTGGCCTA—ATCT—TCCACACATATATACACACAA (Intron 1)	Soybean rbcS[e]

[a]'Core' sequence of enhancer element (Weiher *et al.*, 1983).
[b]Gillies *et al.*, 1983.
[c]*pPS-2.4*, pea rbcS gene.
[d]*pWS-4.3*, wheat rbcS gene (Broglie *et al.*, 1983).
[e]*SRS1*, soybean rbcS gene (Berry-Lowe *et al.*, 1982).
Asterisks denote homology to enhancer 'core' element. Arrows mark inverted repeats.

rbcS cDNA clone pSS15 (Coruzzi *et al.*, 1983). Twenty nucleotides upstream from this site is the sequence 'AATGAA' which resembles the consensus sequence shown to be required for the cleavage step preceding mRNA polyadenylation (Montell *et al.*, 1983). We routinely observe an S1 protected fragment six nucleotides longer than fragment *c*, indicating that there may be two possible 3′ termination or processing sites for this transcript. The smaller fragments seen in Figure 4C may result from S1 nuclease digestion of the hybrid at this AT-rich region, or, alternatively, these fragments may correspond to S1 cleavage products of hybrids formed with mRNAs transcribed from other rbcS genes.

rbcS polypeptide is differentially present in pea tissues

While RUBISCO is the most abundant protein in leaf mesophyll cells, little is known concerning its distribution in other plant tissues. To determine if RUBISCO is present in tissues other than leaves, we compared the soluble polypeptide profile of the latter to that of stems, roots, pericarps, petals, and seeds by native-SDS, two-dimensional gel electrophoresis (Sachs *et al.*, 1980) (Figure 5). In addition, the soluble protein profiles of tissues from etiolated plants were also examined. The soluble polypeptide profile of roots from light-grown plants (data not shown) is identical to that of roots from etiolated plants (Figure 5C) and the same situation also applies in the case of stems (Figure 5D). In green leaves (Figure 5A), the major soluble proteins are the polypeptide subunits (rbcL and rbcS) of RUBISCO. In contrast, extracts of etiolated leaves (Figure 5B), roots (Figure 5C), and stems (data not shown but similar to 5D), show little or no detectable RUBISCO polypeptide subunits. Pericarps contain rbcL and rbcS at levels approaching those found in green

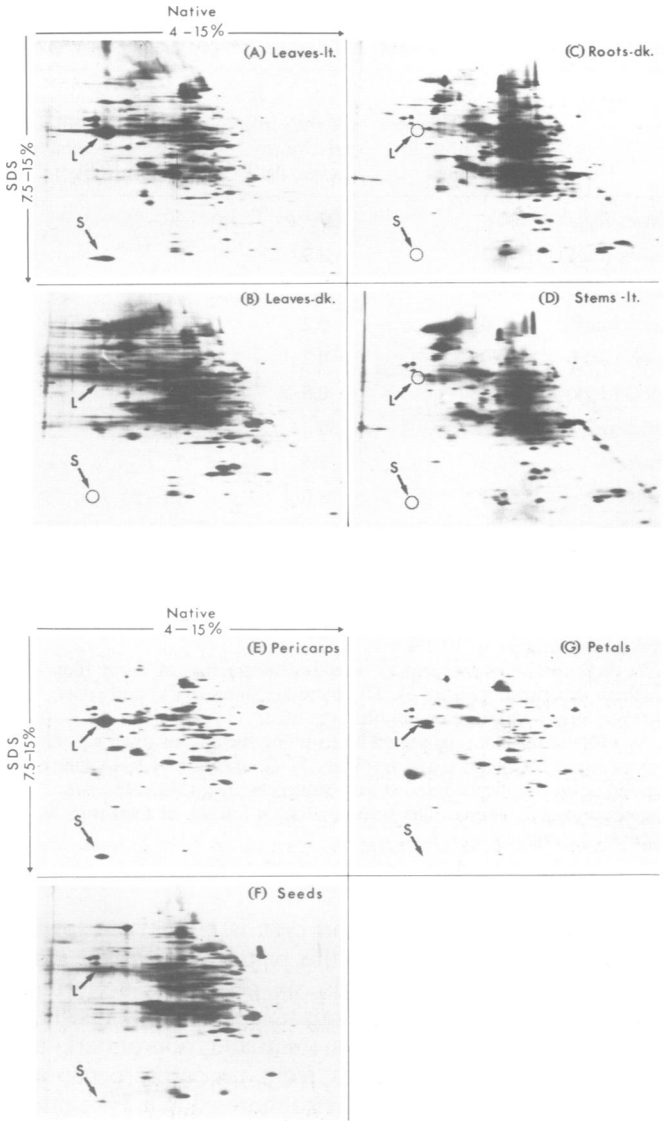

Fig. 5. Two-dimensional analysis of soluble polypeptides isolated from various tissues. Soluble protein profiles of various tissues isolated from light- and dark-grown pea plant proteins were analyzed by two-dimensional gel electrophoresis as described in Materials and methods. **(A)** Leaves from light-grown plants, 58 μg; **(B)** leaves from etiolated plants, 56 μg; **(C)** roots from etiolated plants, 46 μg; **(D)** stems from light-grown plants, 48 μg; **(E)** pericarps from light-grown plants, 14 μg; **(F)** seeds from light-grown plants, 78 μg, and **(G)** petals from light-grown plants, 40 μg. Lt, light; dk, dark; L and S refer to the (rbcL) and (rbcS), respectively. Amounts of rbcS in each tissue were quantitated by ELISA (see Materials and methods) and presented in Table II, column A.

leaves (Figure 5E), whereas seeds (Figure 5F), and petals (Figure 5G), have lower but detectable levels of the RUBISCO subunit polypeptides.

The amounts of rbcS polypeptide in the various tissues were measured quantitatively by ELISA (Voller *et al.*, 1976) and the results are presented in Table II, column A. In etiolated leaves, the amount of rbcS polypeptide is ~0.7% of that in green leaves. Compared with the latter, pericarps contain the same level of rbcS polypeptide, whereas petals and seeds contain ~50%. Other tissues examined (stems and roots) contain only trace amounts of this polypeptide.

rbcS mRNA levels vary in pea tissues

Results described in the previous section showed that the ex-

Table II. Distribution of rbcS polypeptide, rbcS mRNA and *pPS-2.4* rbcS mRNA in different tisues of light- and dark-grown pea plants

	A % rbcS polypeptide relative to leaves, light	B % rbcS mRNA relative to leaves, light	C % *pPS2.4* mRNA relative to total rbcS mRNA
Leaves (light)	100	100	30–35
Leaves (dark)	0.7	2.9	*
Stems (light)	<0.05	2.7	–
Stems (dark)	<0.05	0.2	–
Roots (light)	<0.05	0.7	–
Roots (dark)	<0.05	0.5	–
Pericarps	100	50	5–10
Petals	42.3	8.5	*
Seeds	58.3	8.0	15–20

*below detectable limit.
A, % rbcS polypeptide was quantitated by ELISA (Voller *et al.*, 1976) using monospecific antibody against rbcS. rbcS polypeptide is assumed to account for 20% of RUBISCO holoenzyme; for leaves light, the % rbcS of total soluble protein = 10.2%.
B, % rbcS mRNA quantitated by densitometer tracings of X-ray films of Northern experiment (Figure 6). To quantitate stems (dark) and roots (dark), a longer exposure of the film was used.
C, % *pPS-2.4* mRNA quantitated by counting sections of dried gel corresponding to fragments *a* and *b* (Figure 7). Counts in (–) RNA lane corresponding to fragments *a* and *b* were subtracted from the respective fragments in each sample. The percent *pPS-2.4* mRNA of total rbcS = fragment *b*/fragment *a* + *b*.

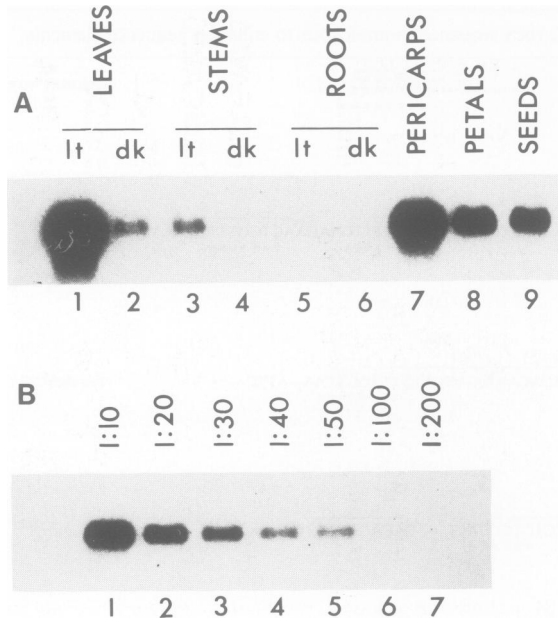

Fig. 6. Northern analysis of rbcS mRNA in various tissues of light- and dark-grown plants. 10 μg of total RNA isolated from leaves, stems and roots of light-grown plants (**A, lanes 1, 3, 5**), dark-grown plants (**A, lanes 2, 4, 6**) or from pericarps, petals and seeds (**A, lanes 7, 8, 9**) was denatured and displayed on 1% agarose gels and transferred to nitrocellulose paper (Thomas, 1980). Serial dilutions of leaf RNA (**B, lanes 1–7**) were included for quantitation. Northern blot filters were hybridized to ^{32}P-labeled insert of pSS15 and exposed to X-ray film. Lt = light, dk = dark. Signals on X-ray film were quantitated and presented in Table II, column B.

pression of rbcS polypeptide in peas is both tissue-specific and light-regulated. To see if this pattern is reflected in the steady-state rbcS mRNA levels, we performed a Northern analysis of rbcS mRNA in various tissues of pea plants. Total RNA was extracted from leaves, stems and roots of dark- and light-grown plants, as well as from pericarps, petals and seeds. Denatured RNA was electrophoresed in a 1% agarose gel, transferred to nitrocellulose paper (Thomas, 1980), and probed with the rbcS cDNA clone pSS15 (Figure 6). rbcS mRNA is most abundant in green leaf tissues (Figure 6A, lane 1), and is ~850 nucleotides in length. Serial dilutions of RNA from green leaves (Figure 6B, lanes 1–7) were used to quantitate the amount of rbcS mRNA in the various tissues relative to green leaves. Moderate amounts of rbcS mRNA occur in pericarps, petals, and seeds (Figure 6A, lanes 7–9), while low amounts of rbcS mRNA are found in etiolated leaves, stems and roots (Figure 6A, lanes 2–6). These amounts have been quantitated by densitometry and expressed as the percent of rbcS mRNA in each tissue relative to green leaves (Table II, column B). These results show that rbcS mRNA is expressed differentially in pea tissues. Furthermore, these data show that while light stimulates rbcS expression in leaves 35-fold, it also stimulates rbcS expression in tissues such as roots 2- to 5-fold, and stems, 12-fold. For roots, this value may be an underestimate since the roots receive indirect light. These results provide evidence that the light-regulation of rbcS gene expression is independent of the tissue-type. This is consistent with the finding that in petunia callus tissue transformed with the pea rbcS gene (*pPS-2.4*), light modulates levels of pPS-2.4 mRNA (Broglie *et al.*, 1984).

pPS-2.4 is expressed in a tissue-specific, light-regulated fashion

The hybridization probe (pSS15) used in the above experiments contains rbcS protein coding sequences and most likely detects transcripts of all rbcS genes. However, since it is possible that individual rbcS genes may be under different transcriptional controls (i.e., some may be constitutively expressed while the expression of others may be enhanced by light or tissue-specific factors), it was necessary to examine the transcriptional regulation of an individual rbcS gene. To this end we determined the level of *pPS-2.4* gene-specific transcripts in different tissues of light- or dark-grown pea plants using a modification of the S1 nuclease mapping procedure. This procedure was used previously to quantify the amount of gene specific actin mRNA in developmental stages of *Dictyostelium* (McKeown and Firtel, 1981a). For this experiment, a *Hinf*I fragment from the 5′ end of the *pPS-2.4* (Figure 4A) was hybridized to total RNA from green leaves, etiolated leaves, pericarps, petals and seeds. Since the DNA probe contains protein coding sequences, hybrids are formed with both the *pPS-2.4* transcripts as well as with the transcripts of other rbcS genes. In each tissue, DNA fragments of two sizes are protected from S1 nuclease digestion (Figure 7, lanes 1–8), corresponding to the digestion products of the two classes of DNA:RNA hybrids. S1 protected fragments from the *pPS-2.4* hybrid will be as long as the rbcS coding sequence plus the length of the 5′ leader (138 nucleotides = fragment *b*), whereas the fragments protected by other rbcS genes non-homologous in the 5′ non-coding region will be only as long as the rbcS protein coding se-

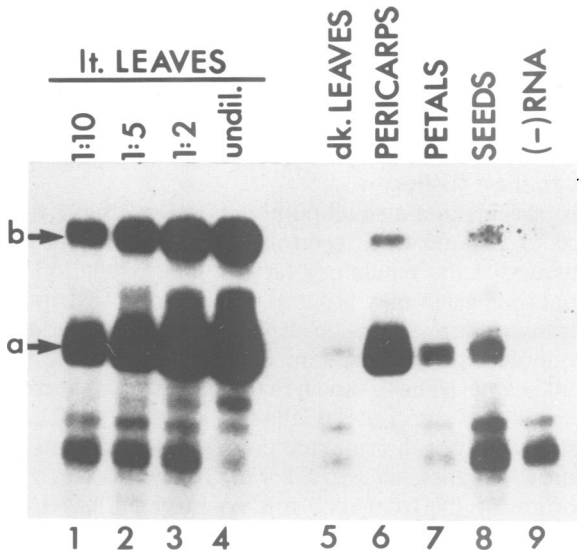

Fig. 7. S1 nuclease mapping of 5′ termini of *pPS-2.4* mRNA in green leaves, etiolated leaves, pericarps, petals and seeds. 20 μg of RNA from green leaves (**lane 4**), etiolated leaves (**lane 5**), pericarps (**lane 6**), petals (**lane 7**) and seeds (**lane 8**), was hybridized to a *HinfI* DNA fragment from the 5′ end of *pPS-2.4* (Figure 3A). The DNA fragments (*a* and *b*) protected from S1 nuclease digestion were sized on a 6% acrylamide/7 M urea gel. Control lanes include dilutions of green leaf RNA: 2, 4, 10 μg (**lanes 1–3**) and a minus RNA sample (**lane 9**). For these controls, *E. coli* tRNA was added to bring the final RNA concentration to 20 μg. *b* = 5′ end of *pPS-2.4* mRNA, *a* = other rbcS mRNAs. *a* and *b* were cut out of the dried gel and counted by liquid scintillation (Table II, column C).

quence (105 nucleotides = fragment *a*). Therefore, the longer fragment *b*, corresponds to the 5′ end of *pPS-2.4* mRNA, while the shorter fragment *a* corresponds to the transcripts of other rbcS genes. The ratio of fragment *b* to fragment *a* + *b* is the fraction of *pPS-2.4*-specific mRNA in the total rbcS mRNA population. These results show that the fraction of *pPS-2.4* mRNA varies in each tissue examined (Figure 7, lanes 4–8). The results are particularly striking when one compares the S1 nuclease digestion products of RNA from green leaves (Figure 7, lane 4) with those of pericarps (Figure 7, lane 6). Since Northern experiments (Figure 6) showed that pericarps contain 1:5 to 1:2 the amount of rbcS mRNA compared with green leaves, we compared the S1 nuclease digestion products of RNA from green leaves which was diluted 1:5 and 1:2 (Figure 7, lanes 2 and 3) with those of pericarps (Figure 7, lane 6). The dilutions of RNA from green leaves served as a control to ensure that the change in ratio of fragment *a* to *b* in each tissue is not a result of dilution of rbcS mRNA. In addition, the dilutions show that the S1 nuclease digestions were performed under conditions of DNA probe excess so that all rbcS transcripts are represented in the hybrids. To quantitate these results, the radioactivity in fragments *a* and *b* was determined by liquid scintillation counting. The percentage of *pPS-2.4* mRNA relative to total rbcS mRNA in each tissue is listed in Table II, column C.

In green leaves, 30–35% of the rbcS transcripts are *pPS-2.4* mRNA while this level is reduced to 5–10% in pericarps and 15–20% in seeds. Levels in etiolated leaves and petals were equal to background. From these experiments we conclude that the *pPS-2.4* gene is expressed primarily in leaves and that its expression is light-regulated. Furthermore, if all rbcS genes were coordinately regulated, the fraction of gene-specific RNA in each tissue would remain the same. Since the fraction of *pPS-2.4*-specific RNA varies in each tissue, these results also indicate that the transcriptional controls of individual members of the rbcS gene family vary.

Discussion

pPS-2.4 is the pea rbcS gene which encodes cDNA clone pSS15

In higher plants, rbcS is encoded by several nuclear genes (6–12), more than one of which is expressed *in vivo* (Coruzzi *et al.*, 1983; Bedbrook *et al.*, 1980; Broglie *et al.*, 1983; Dunsmuir *et al.*, 1983). To determine whether individual rbcS genes are expressed in a tissue- or developmental-specific fashion, we isolated a gene which is actively transcribed in green leaves and followed its specific expression in various tissues of light- and dark-grown plants. The rbcS gene studied (*pPS-2.4*) was selected from a set of six rbcS genomic clones on the basis of its hybridization to a DNA probe from the 3′ non-coding region of cDNA clone pSS15. Since pSS15 was synthesized from pea leaf mRNA, its corresponding genomic sequence is an rbcS gene which is transcribed in leaves. Nucleotide sequence analysis of rbcS gene *pPS-2.4* confirmed its identity with pSS15.

The organization of the *pPS-2.4* structural gene is similar to rbcS genes of wheat (Broglie *et al.*, 1983) and soybean (Berry-Lowe *et al.*, 1982). The first exon encodes the transit peptide plus the first two amino acids of the mature protein, which adjoin the processing site. This is consistent with the hypothesis that exons encode separate functional domains of polypeptides (Gilbert, 1979). The division of the remainder of the mature protein into two exons is similar to that found for other dicots (Berry-Lowe *et al.*, 1982; Cashmore, 1983) whereas in wheat, a monocot, these residues are encoded by a single exon (Broglie *et al.*, 1983). Although the sequence of the mRNA splice junctions of *pPS-2.4* can be predicted from the nucleotide sequence of its corresponding cDNA clone pSS15, the exact placement of the introns is obscured by the presence of direct repeats of five and two nucleotides, respectively, at the boundaries of the first and second introns. The mRNA splice junctions shown in Figure 2 were assigned by homology to other eukaryotic splice junctions which in many cases also contain terminal direct repeats (Mount, 1982). These repeats may serve as a proofreading mechanism to ensure accurate nucleotide splicing. The terminal repeats may be indicative of the mechanism by which these introns have evolved, since the repeats are highly reminiscent of the short duplications (5–9 bp) which occur at the boundaries of prokaryotic and eukaryotic transposable elements (Calos and Miller, 1980).

The non-coding regions of the *pPS-2.4* gene contain features homologous to other eukaryotic genes. Upstream from the mRNA start site are the 'TATA' and 'CAAT' box sequences shown to be involved in the transcription initiation of other eukaryotic genes (Breathnach and Chambon, 1981). Interestingly, we also observe in this region a short inverted repeat which shares homology to eukaryotic and viral enhancer elements (Weiher *et al.*, 1983; Gillies *et al.*, 1983) (see Table I). Similar sequences are also found in both wheat (Broglie *et al.*, 1983) and soybean (Berry-Lowe *et al.*, 1982) rbcS genes (Table I). In addition, these inverted repeats contain alternating purine and pyrimidine residues $(GT)_n$, $(CA)_n$, which have been postulated to regulate gene expression through the formation of Z-DNA (Rich, 1982). However, the genetic manipulation of these sequences in conjunction with

plant transformation experiments will be necessary to determine their role as potential regulators of rbcS gene expression. The 3′ non-coding region of *pPS-2.4* contains a sequence motif which resembles the sequence 'AATAAA' required for the cleavage step preceding mRNA polyadenylation (Montell *et al.*, 1983). The 3′ terminus of the mRNA occurs 20 nucleotides downstream from this sequence and corresponds to the site of the poly(A) tail of pSS15.

Southern blot experiments, using a hybridization probe from the 3′ non-coding region of *pPS-2.4*, showed that this gene was related to two other members of the pea rbcS gene family. A comparison of the DNA sequence of *pPS-2.4* with one of these related rbcS genes (*SS3.6*) (Cashmore, 1983), reveals that, although distinct, the genes are highly homologous within the coding as well as the 3′ non-coding region and the two introns. The highest nucleotide diversity is found between the 5′ leader of the *pPS-2.4* mRNA and the sequences 5′ to the *SS3.6* gene. It is not known however if the *SS3.6* gene is also transcribed.

rbcS polypeptide and mRNA are differentially present in various tissues of pea

To study the expression of individual rbcS genes, we first examined at the polypeptide and mRNA levels whether rbcS is expressed differentially in various tissues of light- and dark-grown plants. These results showed that both rbcS polypeptide and mRNA levels are high in green leaves, moderate in pericarps, petals and seeds, and low or undetectable in etiolated leaves, stems and roots. Although rbcS mRNA levels are greatly reduced in stems and roots, these levels are still enhanced by light, indicating that light stimulation of rbcS expression is independent of tissue-type. However, since the fold stimulation by light is greater in leaves than in stems or roots, it is possible that these two factors (light and tissue-type) exhibit a synergistic effect on rbcS gene expression.

Gene pPS-2.4 is expressed in a tissue-specific, light-regulated fashion

Although Northern analysis revealed that the level of rbcS mRNA varied among the tissues examined, the hybridization probe (pSS15) used in that experiment contained protein coding sequences and therefore detected all rbcS transcripts. Since transcriptional controls of individual rbcS genes may vary in response to light or tissue-specific factors, we examined further the levels of the *pPS-2.4* gene-specific transcripts in the various tissues of pea.

pPS-2.4-specific mRNA was monitored in various tissues from light- and dark-grown plants using a modification of the 5′ S1 nuclease mapping technique previously used for actin genes of *Dictyostelium* (McKeown and Firtel, 1981a). In our experiments S1 nuclease digestion of hybrids formed with RNA from various tissues produces two types of DNA fragments (Figure 7). Fragment *b* corresponds to the 5′ end of *pPS-2.4* RNA, while fragment *a* corresponds to other rbcS transcripts. The ratio of these two fragments quantitates the fraction of *pPS-2.4* RNA relative to total rbcS mRNA in each sample. *pPS-2.4* mRNA accounts for ~30–35% of the rbcS transcripts of green leaves, while it constitutes only 5–10% of the rbcS transcripts in pericarps, 15–20% in seeds, and is below detection in the RNA samples of etiolated leaves and petals (Table II, column C). Thus, the *pPS-2.4* gene is a major contributor to the rbcS mRNA population in green leaves, but is a minor contributor in pericarps, petals, seeds and etiolated leaves. These results also provide information about the expression of the rbcS gene family as a whole. If all rbcS genes were coordinately regulated, the fraction of gene-specific mRNA would remain the same in each tissue. Since the *pPS-2.4* transcript makes up a different proportion of the rbcS mRNA in each tissue examined, this indicates that the expression of individual members of the rbcS gene family varies in these tissues.

It is possible that a small number (5–6) of rbcS genes are needed to respond to a repertoire of environmental stimuli and tissue-specific regulatory factors. The regulation of individual rbcS genes may occur at the level of transcription or post-transcription. Although we have not directly addressed this question in our experiments, previous studies using isolated pea nuclei have shown that light affects an increase in transcription of rbcS genes (Gallagher and Ellis, 1982). While that study did not discriminate among the transcripts of individual rbcS genes, since *pPS-2.4* mRNA constitutes a major proportion of the total rbcS mRNA in green leaves and is undetectable in etiolated leaves, it is most likely that light is acting at the transcriptional rather than post-transcriptional level on the *pPS-2.4* gene. However, this does not exclude the possibility that light may, in addition, affect the stability of rbcS mRNA.

The features of an rbcS gene which is expressed primarily in leaves and regulated by light, hallmark the *pPS-2.4* gene for use in plant transformation experiments. As reported elsewhere (Broglie *et al.*, 1984), we have transferred this pea rbcS gene into petunia calli and have shown that it is expressed both at the level of mRNA and protein in the transformed calli, and furthermore, that its expression is light-regulated. Sequence alterations of the promoter region by deletion and site specific mutagenesis will enable us to define the DNA sequence elements required for transcription and light-regulation. Once defined, these sequences may be used to promote high level expression of foreign genes in plants.

Materials and methods

Isolation of pea rbcS genomic clones

Genomic DNA from *P. sativum* (var. Progress No. 9) was digested to completion with *Bgl*II. The unphosphatased pea DNA was ligated to *Bam*HI-digested 1059 DNA (Karn *et al.*, 1980). Recombinant phages were screened with ^{32}P-labeled insert of the cDNA clone pSS15 (Coruzzi *et al.*, 1983). Six recombinant phages encoding rbcS were isolated and designated λ PS-3D, 3C, 3A, 2C, 5A, 8B. A 4.0-kb *Eco*RI fragment of genomic clone λPS-3D, containing the entire rbcS gene was subcloned into pBR325 and designated pPS-4.0. A 2.4-kb *Eco*RI-*Cla*I fragment of pPS-4.0 determined to contain the entire rbcS gene was further subcloned into pBR325 and designated pPS-2.4.

DNA dot-blots of rbcS genomic clones

DNA (0.5 μg) from the rbcS genomic clones (λ PS-3D, 3C, 3A, 2C, 5A, 8B) was denatured in 0.3 M NaOH, neutralized in 0.6 M Tris-Cl pH 3.0 and applied to a nitrocellulose filter. Filters were pre-hybridized and hybridized in: 50% formamide, 0.6 M NaCl, 50 mM $NaPO_4$ pH 7.0, 5 x Denhardt's solution (1 x Denhardt's solution = 0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 100 μg/ml denatured salmon sperm DNA, at 37°C. DNA probes were labeled at their 5′ ends with ^{32}P and included in the hybridization. Identical filters were washed at 37°C in: 50% formamide, 0.5% SDS in the presence of either; 2 x SSC, 1 x SSC, 0.5 x SSC or 0.02 x SSC (1 x SSC = 0.15 M NaCl/0.015 M Na-citrate). Filters were exposed to X-ray films for autoradiography.

DNA sequencing

Restriction of fragments of DNA were labeled at their 5′ ends with [γ-^{32}P]-ATP in the presence of T4 polynucleotide kinase. End-labeled fragments were denatured and complementary strands separated by electrophoresis on 5%, 6% or 8% polyacrylamide gels. DNA sequence analysis of the single-stranded DNA fragments was performed according to Maxam and Gilbert (1977).

5' and 3' S1 nuclease mapping of the pPS-2.4 transcript

DNA fragments used as hybridization probes for 5' and 3' S1 nuclease mapping of rbcS mRNA transcripts were isolated and labeled with ^{32}P at either their 5' or 3' ends and the complementary strands separated by polyacrylamide gel electrophoresis. The separated strands were hybridized with pea poly(A)$^+$ mRNA (6 μg) or total RNA (20 μg), in a solution 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 was diluted to 300 μl with a solution containing; 0.3 M NaCl, 30 mM NaOAc (pH 4.6), 1 mM $ZnSO_4$, 20 μg/ml denatured salmon sperm DNA, 300 units/ml S1 nuclease, and incubated at 37°C for 30 min. DNA fragments protected from S1 nuclease digestion were sized on 6% acrylamide, 7 M urea gels.

Growth of pea plants for protein and RNA isolation

Pea seeds (var. Progress No. 9) were imbibed and germinated in a Conviron environmental chamber with a 16 h light, 8 h dark photoperiod (4000 lux) at 22°C for 7 days prior to harvesting of leaves, roots and stems. Stem tissue collected corresponds to the portion of the stem between the cotyledon and the first node. For dark-grown plants, seeds were imbibed and germinated in black lucite boxes contained within a dark environmental chamber at 22°C for 7 days. Petals were obtained from 2–3 weeks old flowering pea plants. Pericarps and seeds (d = 2–4 mm) were obtained from pea plants ~5 weeks after sowing.

Protein isolation and two-dimensional gel electrophoresis

1 or 2 g of fresh weight of various plant tissues were ground vigorously in 10 ml of medium A (50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine and 1 mM ε-amino-n-caproic acid) with a pestle and mortar at 4°C. The homogenate was passed through four layers of cheese cloth and the filtrate centrifuged at 50 000 g_{max} for 10 min. After centrifugation, solid powder of $(NH_4)_2SO_4$ was added to the supernatant to 75% saturation and the suspension was kept on ice for at least 30 min for complete protein precipitation. Following centrifugation, the supernatant was completely removed by aspiration and the protein pellet dissolved slowly in 0.5–0.7 ml of medium A. Residual $(NH_4)_2SO_4$ in the suspension was removed by centrifugation through a column of Sephadex G-50 equilibrated in 0.1 M sodium phosphate (pH 7.0) (Helmerhorst and Stokes, 1980). Protein concentrations were determined using defatted crystalline bovine serum albumin as standard (BioRad Laboratories, 1977) and 5–10 μl of the extract containing 20–50 μg protein were used in two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was performed according to Sachs *et al.* (1980). The first dimension consisted of a native 4–15% gradient gel and the second dimension a 7.5–15% SDS gradient gel. Both gel systems employed the running and reservoir buffers of Laemmli (1970). Protein spots were visualized by silver-staining (Morrissey, 1981).

RUBISCO was purified from pea chloroplast stroma by two consecutive centrifugation steps using 10–30% sucrose gradients (Chua and Schmidt, 1978). The level of RUBISCO in various tissue fractions were quantitated by an indirect enzyme linked immunosorbent assay (ELISA) (Voller *et al.*, 1976), using purified pea RUBISCO as a standard. Mono-specific antibody to pea rbcS and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins were used with phenylenediamine as indicator to develop the assay. The absorbance at 488 nm was determined in an Artek V Beam Reader.

Northern analysis of rbcS mRNA

RNA was extracted from pea tissues using guanidinium thiocyanate as a protein denaturant (Chirgwin *et al.*, 1979). Aliquots of RNA (10 μg) were denatured in glyoxal at 50°C (Carmichael and McMaster, 1980), electrophoresed on 1% agarose gels, and transferred to nitrocellulose filters (Thomas, 1980). Filters were hybridized with DNA probes made radioactive by nick-translation (sp. act. 1–5 x 10^8/μg) at 42°C in the presence of: 50% formamide, 5 x SSC, 1 x Denhardt's solution, 20 mM $NaPO_4$, pH 6.5, 100 μg/ml denatured salmon sperm DNA.

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