Expression dynamics of the pea *rbcS* multigene family and organ distribution of the transcripts

Robert Fluhr, Phyllis Moses¹, Giorgio Morelli²,
Gloria Coruzzi and Nam-Hai Chua

Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, ¹National Research Council, Board on Agriculture JH550, 2101 Constitution Avenue NW, Washington, DC 20418, USA, and ² Università degli Studi di Roma, Facoltà di Scienze MFN, Istituto di Fisiologia Generale, Roma 00100, Italy

Communicated by H. Koessel

We have determined the nucleotide sequence of two members (*rbcS*-3A and -3C) of the pea nuclear gene family encoding the small subunit (*rbcS*) of ribulose-1,5-bisphosphate carboxylase. Both *rbcS*-3A and -3C are interrupted by two introns located at the same positions as those of the other three pea *rbcS* genes. Compared with the other pea *rbcS* genes the *rbcS*-3C gene has the most divergent 5'- and 3'-flanking sequences while the *rbcS*-3A gene has a larger and highly divergent intron 1. All five pea *rbcS* genes are conserved in their coding regions but show considerable sequence differences in their 3'-untranslated portion. The 3' sequence divergence of the *rbcS* genes has allowed us to use S1 nuclease mapping procedures to compare their expression levels in different organs and during light induction. All the *rbcS* genes are differentially expressed in various organs of the pea plants; moreover, specific *rbcS* transcripts are under-represented in seeds and petals. In leaves there is a 10-fold difference between the highest and lowest specific *rbcS* transcript levels. By quantitating the distribution of *rbcS* transcripts during light, phytochrome and blue light induction of immature (etiolated), and mature (green), pea leaves, we show that the genes are differentially activated during leaf development.

Key words: multigene family/phytochrome/blue-light, organ specificity/S1 nuclease digestion

Introduction

The small subunit of ribulose-1,5-bisphosphate carboxylase is encoded by a small multigene family (*rbcS*) in dicots (Berry-Lowe *et al.*, 1982; Dunsmuir *et al.*, 1983; Coruzzi *et al.*, 1984) and monocots (Broglie *et al.*, 1983; Wimpee *et al.*, 1983). The gene family is composed of at least five members which are linked and segregate as a single Mendelian unit (Polans *et al.*, 1985). The *rbcS* mRNA is a major transcript of higher plant nuclear DNA (Gallagher and Ellis, 1982) and its protein product is transported into the chloroplast concomitant with post-translational processing (Chua and Schmidt, 1978; Highfield and Ellis, 1978). Within the organelle the *rbcS* polypeptide is assembled together with eight chloroplast-encoded large subunits to form the holoenzyme which catalyzes the initial step in the fixation of CO₂ (Miziorka and Lorimer, 1983).

Light has been shown to modulate the *rbcS* mRNA levels in etiolated pea leaves by a phytochrome-mediated response (Thompson *et al.*, 1983; Sasaki *et al.*, 1983; Tobin and Silverthorne, 1985; Fluhr and Chua, 1986). This increase is due largely

to a direct increase in the rate of transcription, as established by nuclear run-off experiments (Silverthorne and Tobin, 1984; Berry-Lowe and Meagher, 1985). Recently, we have examined light induction of *rbcS* genes in mature pea leaves and shown that a putative blue light receptor, in addition to phytochrome, is active in mediating light responsiveness (Fluhr and Chua, 1986). We have also transferred three different pea *rbcS* genes into the petunia and tobacco genomes and studied their expression in transgenic plants (Nagy *et al.*, 1985; Fluhr and Chua, 1986). While *rbcS*-3A and -3C are highly expressed in transgenic plants, the expression of *rbcS*-E9 is at least 5–10 times lower. The results of these experiments have led us to extend the quantification and organ distribution of *rbcS* transcripts carried out previously for *rbcS*-E9 (Coruzzi *et al.*, 1984) to *rbcS*-3A and -3C. We report here the nucleotide sequences of two additional pea *rbcS* genes and use the sequence information of all five published genes [*rbcS*-3A and -3C (this work), *rbcS*-E9 (Coruzzi *et al.*, 1984), *rbcS*-3.6 and -8.0 (Timko *et al.*, 1985)] to establish the criteria for global analysis of *rbcS* gene expression in pea plants.

Results

Nucleotide sequence of two *rbcS* genes

Pea genomic DNA cloned into λ phage 1059 (Karn *et al.*, 1980) was screened with the cDNA clone pSS15, which encodes pea *rbcS* (Coruzzi *et al.*, 1983). Six positively hybridizing genomic clones designated λ PS 3D, 3C, 3A, 2C, 5A and 8B were isolated. The clone λ PS 3D had been shown by previous work to contain the *rbcS*-E9 gene (Coruzzi *et al.*, 1984). Digestion by *Xho*II of λ PS clones 3A and 3C yielded 2.5-kb and 3.8-kb fragments, respectively, which contain the *rbcS* coding sequence. These fragments were subcloned into the pEMBL8⁺ vector (Dente *et al.*, 1983) and their nucleotide sequences were determined by the dideoxy termination method (Sanger *et al.*, 1977 as modified by Biggins *et al.*, 1983). A total of 2061 nucleotides of *rbcS*-3A and 1381 nucleotides of *rbcS*-3C are presented in Figures 1 and 2.

Among all the five pea *rbcS* genes published, the protein coding sequences are identical with the exception of an Arg-Gly to Thr-Val amino acid replacement in the center of the transit peptide of *rbcS*-3A (Figure 1). At this position *rbcS*-8.0 has one amino acid replacement Gly to Val. The second intron is also extremely well conserved with only six base substitutions within its 86-bp length. The first intron is more variable; while *rbcS*-3C and *rbcS*-3.6 have identical sequence, compared with these two genes, *rbcS*-E9 and *rbcS*-8.0 have a 4–8 bp deletion in the center of an otherwise conserved 83-bp length. However, *rbcS*-3A shares only the 32 bp at the 3' end of its intron 1 sequence with the other pea *rbcS* genes. The 5' portion of *rbcS*-3A intron 1 consists of a 437-bp sequence which is absent from intron 1 of the other *rbcS* genes (Figure 1).

The 5' non-coding regions of the *rbcS* gene members share five highly conserved sequences which are underlined in Figure 1. These include the region surrounding the 'TATA' box and

rbcS-3A

-410 GATCCAAAAGCTTGGACAGGAACAAATGTTACCCATACATAAAAGATATTTGTGAAGTAACAGTCACAAAATTCATGAGGCCAATACACTACAATTTTCATGGATACAAATCTTTA

-289 CAAAATAAAAAATATCGACATAACCCATCACACATTTTACTCTTCACATGAAAAGATAAGTACAGTGGAGTAATCCACATGGCACTGTCTATTGGTGGCTTATGATAAGGCTAGCA

-168 CACAAAATTTCAAAATCTGTGTGGTTAATATGGCTGCAAACTTTATCATTTTCACTATCTAACAGATTGGTACTAGGCAGTAGCTAAGTACCACAAATATTAAGACCATAATTTGGAAAT

-47 AGATAAATAAAAAATTATATATAGCAAGTTTGTAGCAGAGCTTTGCAATTCATACAGAAAGTGAGAAAA Met Ala Ser Met Ile Ser Ser Ser Ala Val Thr Thr
ATG GCT TCT ATG ATA TCC TCT TCA GCT GTG ACT ACA

59 Val Ser Arg Ala Ser Thr Val Gln Ser Ala Ala Val Ala Pro Phe Gly Gly Leu Lys Ser Met Thr Gly Phe Pro Val Lys Lys Val Asn
GTC AGC CGT GCT TCT ACG GTG CAA TCG GCC GCG GTG GCT CCA TTC GGC GGC CTC AAA TCC ATG ACT GGA TTC CCA GTT AAG AAG GTC AAC

149 Thr Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val Lys Cys Met Gln
ACT GAC ATT ACT TCC ATT ACA AGC AAT GGT GGA AGA GTA AAG TGC ATG CAG GTCAGAAAAATATATATCTCTTTGTATAAACTTTTGGTTCTGTTTGTATGG

253 TTTATTTTGGTGTATCTATTGGCATAAGCACTTATTAGACCGCTTGAGAGAGGTTAGAGAAACAACTTATGATAAGTCCATAAACGGTTTCAACTTATTTTGTATGTTCTTCAGGTTAGG

374 TTATGAAAATAACTTATAGCTTATATGAAAACAGTTTGTAGTCTATTTATCTTGTTTTAGAAATAGCTTATACATAAGGAACATAATTAAGTTTTTTTAAATCTAAATAGCGCTCTAGTA

495 TATAAAGTTTTCATTTCAAAAAGTTCAAAATAGCTTATAAAATGAGAGATTTTGGAAAAAGTTATGTTTCGAAATAGCTTACATATTTGAGAACTAGAAGGCTATTTGAATAGTATTAAAT

616 GATTCATTGGCAAAAATGCACATAAGTTTGTAAACCTTTTGTGTAATATTTAG Val Trp Pro Pro Ile Gly Lys Lys Lys Phe Glu Thr Leu Ser Tyr Leu
GTG TGG CCT CCA ATT GGA AAG AAG AAG TTT GAG ACT CTT TCC TAT TTG

718 Pro Pro Leu Thr Arg Asp Gln Leu Leu Lys Glu Val Glu Tyr Leu Leu Arg Lys Gly Trp Val Pro Cys Leu Glu Phe Glu Leu Glu
CCA CCA TTG ACC AGA GAT CAG TTG TTG AAA GAA GTT GAA TAC CTT CTC AGG AAG GGA TGG GTT CCT TGC TTG GAA TTT GAG TTG GAG GTTTC

810 ATATTCATTCCTTTTTCATGATTTCTATAAACTATTTGTTGAAACCTTAATGAGTTGATTTTGACAACCTTGGTTGCAG Lys Gly Phe Val Tyr Arg Glu His Asn
AAA GGA TTT GTG TAC CGT GAG CAC AAC

919 Lys Ser Pro Gly Tyr Tyr Asp Gly Arg Tyr Trp Thr Met Trp Lys Leu Pro Met Phe Gly Thr Thr Asp Ala Ser Gln Val Leu Lys Glu
AAG TCA CCA GGA TAC TAT GAT GGA AGA TAC TGG ACA ATG TGG AAG CTT CCT ATG TTT GGT ACC ACT GAT GCT TCT CAA GTC TTG AAG GAG

1009 Leu Asp Glu Val Val Ala Ala Tyr Pro Gln Ala Phe Val Arg Ile Ile Gly Phe Asp Asn Val Arg Gln Val Gln Cys Ile Ser Phe Ile
CTT GAT GAA GTT GGT GCC GCT TAC CCC CAA GCT TTC GTC CGT ATC ATC GGT TTC GAC AAC GTT CGT CAA GTT CAA TGC ATC AGT TTC ATT

1099 Ala His Thr Pro Glu Ser Tyr OC
GCC CAC ACA CCA GAA TCC TAC TAA

rbcS-3C

-319 CATCTATGATATAGATATTTATGGAGGAATTAATAGTGACAAAACCTCCATGAGGCCAACATGCATACAATATAAAATTTATTTCAAATGACTAGCATCACACATTTATGTTCTTCACACATG

-198 AAAAGATAAGGTCATCATCCACGTGGCACTCTCATAGTGATGATAATGTATGGCTAGTATACAATTTCTTTTCAAACCTCGTGTGGTTAATGGTTCCATTGATGATCATTTTTCACAAAT

-77 CCAAAACCATTTGGTATACCTTGATCAGAAACCAATTTCTGAAACATTATATACCTATAGAACTTTGCAACCTAATTATAACAACA Met Ala Ser Met Ile Ser Ser Ser
ATG GCT TCT ATG ATA TCC TCT TCC

34 Ala Val Thr Thr Val Ser Arg Ala Ser Arg Gly Gln Ser Ala Ala Val Ala Pro Phe Gly Gly Leu Lys Ser Met Thr Gly Phe Pro Val
GCT GTG ACA ACA GTC AGC CGT GCC TCT AGG GGG CAA TCC GCC GCA GTG GCT CCA TTC GGC GGC CTC AAA TCC ATG ACT GGA TTC CCA GTG

124 Lys Lys Val Asn Thr Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val Lys Cys Met Gln
AAG AAG GTC AAC ACT GAC ATT ACT TCC ATT ACA AGC AAT GGT GGA AGA GTA AAG TGC ATG CAG GTGACAGAAACATATACATATATATATAGTTGAAT

224 ATCAGTAATGATTCAAGTTTGTAAACCGTTTATGTTGAATATTTAG Val Trp Pro Pro Ile Gly Lys Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Pro
GTG TGG CCT CCA ATT GGA AAG AAG AAG TTT GAG ACT CTT TCC TAT TTG CCA CCA

324 Leu Thr Arg Asp Gln Leu Leu Lys Glu Val Glu Tyr Leu Leu Arg Lys Gly Trp Val Pro Cys Leu Glu Phe Glu Leu Glu
TTG ACG AGA GAT CAA TTG TTG AAA GAA GTT GAA TAC CTT CTG AGG AAG GGA TGG GTT CCA TGC TTG GAA TTT GAG TTG GAG GTTTCATATTCAT

418 TCCTTTTTCAATGATTATATAAACTTTTGTGTTGAAACCGTAATGAGTTGATTTTACTGTTTGGTTGCAG Lys Gly Phe Val Tyr Arg Glu His Asn Lys Ser
AAA GGA TTT GTG TAC CGT GAG CAC AAC AAG TCA

524 Pro Gly Tyr Tyr Asp Gly Arg Tyr Trp Thr Met Trp Lys Leu Pro Met Phe Gly Thr Thr Asp Ala Ser Gln Val Leu Lys Glu Leu Asp
CCA GGA TAC TAT GAT GGA AGA TAC TGG ACA ATG TGG AAG CTT CCT ATG TTT GGT ACC ACT GAT GCT TCT CAA GTC TTG AAG GAG CTT GAT

614 Glu Val Val Ala Ala Tyr Pro Gln Ala Phe Val Arg Ile Ile Gly Phe Asp Asn Val Arg Gln Val Gln Cys Ile Ser Phe Ile Ala His
GAA GTT GTT GCC GCT TAC CCT CAA GCT TTC GTT CGT ATC ATC GGT TTC GAC AAC GTT CGT CAA GTT CAA TGC ATC AGT TTC ATT GCA CAC

704 Thr Pro Glu Ser Tyr OC
ACA CCA GAA TCC TAC TAA

Fig. 1. Nucleotide sequence of pea *rbcS-3A* (top) and *rbcS-3C* (bottom) genes. The nucleotide sequence shown is that of the sense strand and includes the 5'-flanking and protein-coding regions. The nucleotide designated +1 is the first major putative transcription initiation site (vertical arrows). Areas underlined in the 5'-flanking region and the introns are conserved in all five pea *rbcS* sequences published to date. Dotted lines under nucleotides 74–80 of *rbcS-3A* indicate sites of amino acid variance between *rbcS-3A* and the other members of the *rbcS* gene family.

a sequence resembling the SV40 enhancer-core sequence (Weiher *et al.*, 1983). The mRNA leader sequence is 22 nucleotides for *rbcS-3A* and nine nucleotides for *rbcS-3C* (Figure 3A); by comparison, the *rbcS-E9* mRNA leader is 34 nucleotides long. We have previously used 5' S1 nuclease analysis to distinguish individual *rbcS* gene transcripts (Coruzzi *et al.*, 1984). However,

a comparison of nucleotide sequences in the 5'-transcribed region of the five genes show that *rbcS-E9* and -8.0 are indistinguishable by 5' S1 nuclease protection assays using a probe from *rbcS-E9*. In addition, the leader RNAs of *rbcS-3A* and -3.6 are short and highly homologous, likewise rendering their discrimination by 5' S1 analysis impossible.

Fig. 2. Alignment of nucleotide sequence of the 3' non-coding region of pea *rbcS* genes using *rbcS-3A* as a reference. The sequence shown begins at the termination codon and extends for 529 bp for 3A and 341 bp for 3C. Published sequences for *rbcS-E9*, -3.6 and -8.0 have been aligned at the putative polyadenylation site (vertical arrows). A star above the nucleotide indicates homology with the *rbcS-3A* gene. Numbers on the right side indicate nucleotide positions with respect to the *rbcS-3A* gene (Figure 1). Numbers on top of the sequence begin at the conserved *Hind*III site (e.g. nucleotide 1037 for *rbcS-3A* and 664 for *rbcS-3C*), and indicate the expected S1-resistant fragment sizes shown in Table I and Figure 3. Lines drawn above sequences 3A, 3C and E9 are 30-mer oligonucleotide probes used in the experiments shown in Table II.

Development of gene-specific probes

In all the five pea *rbcS* genes the transcribed 3' non-coding region extends ~150 nucleotides downstream from the termination codon (Figures 2 and 3B). Twenty nucleotides upstream from the 3' end is the sequence AATAAA (3A) or AATGAA (3C, E9, 3.6 and 8.0) which resembles the consensus sequence for polyadenylation (Montell *et al.*, 1983). Sequence homology in the proximal half of the 3' non-coding region among four members of the *rbcS* gene family, *rbcS-E9*, -3A, -8.0 and -3.6 is ~90% whereas *rbcS-3C* shows no homology in these sequences immediately 3' to the gene (Figure 2). Despite this overall conservation, short regions of sequence divergence extending over at least 2–3 nucleotides are present. These divergent spots can be targets for S1 nuclease digestion when non-homologous probes are used (Shenk *et al.*, 1975; Dodgson and Wells, 1977; Myers *et al.*, 1985). Accordingly, we have chosen to prepare 3' end S1 probes that begin in the conserved coding

region (*Hind*III site 83 nucleotides upstream from the termination codon) and extend through the more variable 3' non-coding sequences. The sequence alignment in Figure 2 enables us to predict the sizes of the protected fragments resulting from S1 nuclease digestion of hybrids between specific probes and non-cognate transcripts (Table I). The results in Figure 3B bear out predictions for the *rbcS-3A* and -3C probes hybridized to total leaf RNA. With the *rbcS-3C* probe, two classes of protected fragments at 89 and 230 nucleotides were obtained. The *rbcS-3A* probe gave four protected fragments, 89, 110, 160 and 230 nucleotides, of which three were expected (Table I). The additional cleavage site which generated the 112–122 nucleotide fragments (Figure 3B) could be due to transcripts from an as yet unidentified *rbcS* gene, the juxtaposition of a few single homology breaks in a small region as is evident around 118 nucleotides in Figure 2, or to spurious digestion by S1 nuclease in regions of high A+T content.

To assign unambiguously specific gene transcripts responsible

Fig. 3. S1 nuclease mapping of 5' and 3' termini. (A) 5' DNA fragments from the *rbcS-3A* and *rbcS-3C* genomic clones were end-labeled at the *Hinf*I site (nucleotide 127 in *rbcS-3A*, and nucleotide 113 in *rbcS-3C*) and hybridized to total pea leaf RNAs (lane 2). The sequence ladder displays the position of the nucleotide G (lane 1). Dotted nucleotides indicate positions of the major S1-resistant fragments. The methionine initiation codon, ATG, is underlined. (B) 3' DNA fragments from the *rbcS-3A* and *rbcS-3C* genomic clones were end-labeled at the *Hind*III site (see Figure 2) and hybridized to total pea leaf RNAs (lane 1). The sequence ladder shown alongside and the sizes of the protected fragments are indicated (lanes 2–5). The positions of major S1-resistant fragments are dotted. The TAA termination codon is underlined.

for each S1-resistant DNA fragment, we took advantage of transgenic petunia plants that contain either the pea *rbcS-3A*, *-3C* or *-E9* gene. We have shown previously that the endogenous petunia *rbcS* transcripts do not hybridize with the pea *rbcS* probes used (Fluhr and Chua, 1986). When the *rbcS-3A* 3' end probe is hybridized to RNA from transgenic petunia plants, major fragments of 225–230 nucleotides and minor fragments at 112 nucleotides are obtained with petunia *rbcS-3A* (Figure 4, lane 1), 85–90 nucleotides with petunia *-3C* (Figure 4, lane 2) and 157–160 nucleotides with petunia *-E9* (Figure 4, lane 3) as predicted in Table I. Moreover, an artificial mix of RNA from all the three transgenic plants yields signals of similar qualitative distribution to that obtained with RNA from pea leaves (Figure 4, compare lanes 4 and 5). Since no new fragments appear with RNA from pea leaves, the results strongly suggest that these three pea *rbcS* transcripts hybridized with the probe, as predicted by our sequence comparisons and tabulated in Table I. Therefore, the *3A* probe can be used to provide global information about *rbcS* RNA content.

The *rbcS-3C* gene contains the most divergent sequence at its 3' end and hence, the breakpoint of homology immediately follows the stop codon. With the 3' end probe from *rbcS-3C*, both transgenic petunia *-3A* and *-E9* yield fragments at 85–90 nucleotides (Figure 4, lanes 6 and 8), while the cognate reaction

Table I. Expected fragment sizes, in nucleotides, generated by S1 nuclease digestion of hybrids between pea leaf *rbcS* RNA transcripts and different 3' end probes

3'	<i>rbcS</i> transcript				
	<i>3A</i>	<i>3C</i>	<i>E9</i>	<i>3.6</i>	<i>8.0</i>
<i>3A</i>	230	89	160	160	161
<i>3C</i>	89	230	89	89	89
<i>E9</i>	160	89	230	230	160

All gene-specific DNA probes start at the *Hind*III site which in all genes is 83 nucleotides upstream from the termination codon. S1 nuclease digestion can be expected at sequences containing at least two consecutive, non-homologous nucleotides.

with transgenic petunia *-3C* yields fragments of 225–230 nucleotides (Figure 4, lane 7). No unexpected fragment sizes were uncovered in reconstruction experiments (Figure 4, compare lanes 9 and 10). The use of the *rbcS-E9* probe with transgenic petunia (Figure 4, lanes 11–15) gives results analogous to those obtained with the *rbcS-3A* probe. Although we have not carried out any experiments using probes from the other two *rbcS* genes (*3.6* and *8.0*) inspection of the sequences shown in Figure 2 allows us to assign to their transcripts, fragments generated by S1 nuclease digestion (Table I).

Fig. 4. 3' S1 nuclease analyses of *rbcS*-3A, -3C and -E9 transcripts in peas and transgenic petunia plants. Transgenic petunia-3A, 3 μ g total RNA (lanes 1, 6 and 11); transgenic petunia-3C, 3 μ g total RNA (lanes 2, 7 and 12); transgenic petunia-E9, 20 μ g total RNA (lanes 3, 8 and 13); a mixture of total RNAs from all three types of transgenic plants (lanes 4, 9 and 14); 3 μ g total RNA from pea leaves (lanes 5, 10 and 15). Probes used were 3' end genomic probes, of *rbcS*-3A (lanes 1–5), *rbcS*-3C (lanes 6–10) and *rbcS*-E9 (lanes 11–16). Mol. wt markers are *Msp*I digests of pBR322.

Distribution of *rbcS* pea transcripts in leaves

The 3' end S1 nuclease protection assays can be used to provide quantitative information of the *rbcS* transcripts in leaves. The results of densitometric scanning of appropriately exposed autoradiograms (e.g. Figure 4, lanes 5, 10 and 15; Figure 5A–C, lane 1) are summarized in Table IIA. Additionally 3' end oligonucleotide probes as delineated in Figure 2 were prepared and hybridized to slot blots of leaf RNA. Table IIA shows that *rbcS* transcripts -3A and -3C and -8.0 together represent >90% of the total *rbcS* gene activity while *rbcS*-E9 and -3.6 accumulate to much lower levels.

We previously showed by using 5' end probes that the transcripts from the *rbcS*-E9 gene constituted ~30% of the total *rbcS* RNA level (Coruzzi *et al.*, 1984). Based on recently published sequence data, we now know that the *rbcS*-E9 5' probe could not discriminate between *rbcS*-E9 and *rbcS*-8.0 transcripts (see Figure 3 in Timko *et al.*, 1985). Transcripts from these two genes together represent 23–28% of the total *rbcS* RNA (Table IIA), which is very close to what we ascribed earlier to *rbcS*-E9 only (Coruzzi *et al.*, 1984).

Organ-specific distribution of *rbcS* pea transcripts

When equal amounts of total RNA are used from different organs

of the pea plant, a quantitative comparison of the *rbcS* transcript distribution can be made (Figure 5). The results of densitometric scanning of Figure 5 (A–C) are summarized in Table IIB, and were confirmed with 3' end oligonucleotide probes. Thus, *rbcS* transcripts accumulate in leaves and pericarps, to a greater extent than in stems, petals and seeds, and is barely detectable in roots.

We have previously shown using 5' S1 analysis that the *rbcS*-E9 transcript was underrepresented in pericarps, petals and seeds relative to leaves (Coruzzi *et al.*, 1984). Here we have used 3' S1 analysis to further discriminate among other members of the *rbcS* gene family (Figure 5). Amounts of RNA from various organs of the pea plants were adjusted to obtain approximately equal signals with respect to *rbcS*-3A transcript found in leaf RNA. When these samples were hybridized with the *rbcS*-3A 3' probe (Figure 5, lanes 8–13), it became clear that the signal strength of the 160-nucleotide fragment in petals and seeds is proportionally much lower than that found in leaves, stems and pericarps (Figure 5, compare lanes 11, 13 with 8, 9 and 12; Table IIB). Fragments of 160 nucleotides obtained with the *rbcS*-3A probe are ascribed to *rbcS*-E9, -3.6 and -8.0 (Table I), among which *rbcS*-8.0 is the most highly expressed gene. Thus, these genes are more poorly expressed in petals and seeds relative to their contributions in leaves and pericarps. Results obtained with a 3' probe which discriminates *rbcS*-E9 and -3.6 from *rbcS*-8.0 (Figure 5, panel C) also corroborate these results. A possible explanation of the discrepancy in *rbcS* transcript distribution in pericarps reported here and that observed previously may be the age of the materials used in that study (Coruzzi *et al.*, 1984).

Light induction of the *rbcS* gene family in etiolated and mature pea leaves

We next examined the expression behavior of individual *rbcS* genes in the developing pea plant starting from etiolated seedlings. To this end RNAs isolated at different times during greening were analyzed with the gene-specific 3' probes (Figure 6). Both the *rbcS*-3A and -3C genes have low levels of transcripts in etiolated leaves (Figure 6, 0 h lane). After 24 h of continuous illumination with white light, these levels increase to near the steady-state levels detected in mature green leaves. In contrast, the *rbcS*-E9 transcript (Figure 6, panel E9) is barely detectable after 5 and 24 h of continuous illumination, similarly at these times the 160-nucleotide fragment ascribed mainly to the *rbcS*-8.0 transcript is underrepresented (Figure 6, panel 3A). Thus, transcripts of these two genes begin to accumulate only at a later developmental stage. This may explain the reduced amounts of *rbcS*-E9 and *rbcS*-8.0 transcripts in petals and seeds, organs in which chloroplast development is arrested and diverted into different developmental strategies (Kirk and Tilney-Basset, 1978). To test this hypothesis, we examined the time course of light induction of *rbcS* transcript accumulation in mature leaves which contain fully developed plastids. We found that after 4 days in the dark the *rbcS* transcript levels decrease to <5% of the light levels (Figure 7, lane 1–4). Upon re-illumination with white light, the *rbcS*-3A transcript rapidly accumulates reaching the steady-state within 5 h (Figure 7, compared lanes 1 and 7). The fragment at 160 nucleotides (mainly the *rbcS*-8.0 transcript) shows no lag in its induction in mature green leaves and the ratio between it and the 230-nucleotide fragment (*rbcS*-3A transcript) and 89-nucleotide fragment (*rbcS*-3C transcript) remains constant throughout the re-induction (Figure 7, lanes 1, 6–7). These results are in contrast to those obtained during greening of etiolated leaves (Figure 6). Thus, the *rbcS*-8.0, -E9 and probably -3.6 are expressed later in development and their under-

Fig. 5. Quantitative 3' S1 analysis of *rbcS* transcripts from various organs of pea plants. Total organ RNA was analyzed by the *rbcS-3A* probe (panel A); the *rbcS-3C* probe (panel B); and the *rbcS-E9* probe (panel C). 1 μ g of total RNA was used for the following organs: leaves (lane 1); stems (lane 2); roots (lane 3); petals (lane 4); pericarps (lane 5); and seeds (lane 6). Varying amounts of total RNA from the various pea organs was hybridized with the *rbcS-3A* probe: leaves, 1 μ g (lane 8); stems, 16 μ g (lane 9); roots, 30 μ g (lane 10); petals, 6 μ g (lane 11); pericarps, 3 μ g (lane 12); and seeds, 7 μ g (lane 13). Mol. wt markers are the same as in Figure 4.

representation in organs which contain specialized plastids may be a reflection of this.

Phytochrome and blue light control of rbcS gene expression in etiolated and mature pea leaves

A red flash and continuous blue light are the effective wavelengths for the induction of *rbcS-3A* and *-3C* in immature and mature leaves, respectively (Fluhr and Chua, 1986). To extend these experiments to other gene members we irradiated etiolated pea leaves with a brief flash of red light, placed the plants in the dark for 24 h, and then analyzed the RNAs with the *rbcS-E9* 3' probe. A 230-nucleotide signal, associated with *rbcS-E9* and *-3.6* is induced compared with the dark levels (Figure 8A, lanes 1 and 2) and the induction is abrogated when the red flash is followed by a far red flash (Figure 8A, lane 3). These results demonstrate that the expression of *rbcS-E9* and *-3.6* is also controlled by phytochrome. The absolute transcript level of *rbcS-E9* and *-3.6* is very low when compared with that of *rbcS-3A* and *-3C* (160 and 89 nucleotides, respectively) and also when compared with the steady-state transcript levels of *rbcS-E9* and *-3.6* in mature green leaves (Figure 6).

We next examined the effects of light quality on the expression of *rbcS* genes in mature pea leaves using the *rbcS-3A* 3' probe to highlight the expression of *rbcS-E9* and *-8.0*. Figure 8B shows that blue light is much more effective than red light

in the induction of *rbcS* transcripts. Supplementation of blue light with red light does not increase the response (Figure 8B, lane 5). However, the induction of *rbcS* transcript by blue and red light can be partially reversed by a background of far red light (Figure 8B, lane 4). These results indicate that the full induction of *rbcS* genes in mature leaves requires the co-action of phytochrome and a blue light photoreceptor. Continuous blue light alone is sufficient to activate both photoreceptors and additional red light is not necessary. The spectral sensitivity of transcript induction is developmentally specific and qualitatively the same for all *rbcS* transcripts. However, in contrast to *rbcS-3A* and *-3C*, transcripts ascribed to *rbcS-8.0*, *-3.6* and *-E9* (Figure 8B, signal at 160 nucleotides), obtain their full response potential only in mature leaves. These results corroborate and extend the results of white light induction shown in the previous section.

Discussion

Analysis of pea genomic DNA with restriction endonucleases yields five or fewer fragments containing complete *rbcS* sequences. We and others have tentatively assigned all the isolated genes to individual fragments (Coruzzi *et al.*, 1984; Cashmore, 1983). Thus, the number of individual *rbcS* genes cannot be much greater than the five presented here. For comparison, the number

Table II. A. Relative abundance of *rbcS* transcripts in leaves as percent of total *rbcS* gene expression

Organ RNA	<i>rbcS</i> transcript			
	3A	3C	E9 + 3.6	8.0
Leaves ^a	41	36	7	16
Leaves ^b	40	32	7	21

B. Distribution of specific *rbcS* transcripts in pea organs as percent of expression in leaves

Organ RNA	<i>rbcS</i> transcript			
	3A ^c	3A ^d	3C ^c	Others ^e
Leaves	100	100	100	100
Stems	5	6	4	5
Roots	nd	3	nd	nd
Petals	10	10	13	1
Pericarps	40	33	48	40
Seeds	10	10	4	1

The distribution and relative abundance of specific RNA transcripts were estimated by densitometric tracing of appropriately exposed autoradiographs. Numbers are averages of three to five experiments.

^aSlot-blot hybridizations were carried out using oligonucleotides delineated in Figure 2. Total *rbcS* transcript was estimated by hybridization with an oligonucleotide (30-mer) specific for a sequence within the coding region conserved in all pea *rbcS* genes characterized (nucleotide 689–719 in *rbcS*-3A) (Figure 1). Hybridization of all labeled oligonucleotides to slot blots of transgenic petunia RNA was used to detect any minor differences in specific activity between probes. Abundance of transcript 8.0 is deduced by subtracting the sum of all other transcripts from 100%.

^bS1 signals were obtained with the 3' end-specific probes *rbcS*-3A, -3C and -E9 as described in the text (Figure 5). Transcript identity was ascribed as in Table I. The sum of all S1 signals in a particular lane gives total *rbcS* expression.

^cResults were obtained using the gene-specific oligonucleotide probes delineated in Figure 2. 5 µg of total RNA from each organ was used in all slots.

^dData extracted from Figure 5, panel A, lanes 8–13. The distribution was obtained by adjusting the varying amounts of RNA needed to obtain a signal of equal strength from each organ, and then compensating for remaining small differences in the intensities of the 230-nucleotide band.

^e'Other' includes transcripts ascribed to *rbcS*-E9, -3.6 and -8.0 which give S1 signals at 160 nucleotides with the *rbcS*-3A 3' end probe and at 230 nucleotides and 160 nucleotides with the *rbcS*-E9 probe.

of gene members in other *rbcS* gene families are: *N. plumbaginifolia*-2 (C. Poulsen, in preparation) and *Petunia*-8 (Dean *et al.*, 1985a).

The *rbcS* multigene family is clustered on pea chromosome 5 within <4 map units (Polans *et al.*, 1985) and at least two of the genes are linked in one genomic clone (Cashmore, 1983). The clustered configuration could have been derived from a rare duplication followed by unequal crossing over events. Analyses of the *rbcS* gene family reveal that this may be the case. The 5'-flanking sequence of *rbcS*-E9 is nearly identical to that of *rbcS*-8.0 while its 3'-flanking sequence resembles most closely that of *rbcS*-3.6. The *rbcS*-3A 5' non-coding sequence is identical to *rbcS*-3.6 for the first 101 nucleotides upstream of the initiator methionine but *rbcS*-3A differs from the latter gene in other regions. Most strikingly, *rbcS*-3A differs from the four other gene family members in having two amino acid replacements in its transit peptide and an exceptionally large intron 1. The 5'- and 3'-flanking sequences of *rbcS*-3C are the most divergent of the entire gene family.

Due to a high degree of sequence homology, analysis of even small gene families necessitates prudent use of sequence data and judicious selection of specific gene probes. In this case, the use

Fig. 6. Light induction of *rbcS* transcripts in etiolated pea leaves. Seedlings were grown in the dark for 8 days and exposed to a white-light source (1200 µE/M²/s) for the times indicated. Total RNA (2 µg) from leaves was hybridized with the 3' and S1 probes from *rbcS*-3A (left panel), *rbcS*-3C (center panel) and *rbcS*-E9 (right panel). Sizes of fragments protected from S1 digestion are given in numbers on the left.

Fig. 7. Decrease of *rbcS* transcripts in mature, green pea leaves in the dark and their re-induction in the light. Pea seedlings were grown in a 16 h light/8 h dark cycle for 3 weeks and transferred to the dark for 4 days. On the fourth day the plants were re-exposed to white light (1200 µE/M²/s) for 12 h. Leaves were harvested in the middle of the light phase of the light/dark cycle (lane 1); 1 day dark (lane 2); 2 days dark (lane 3); 3 days dark (lane 4); 4 days dark (lane 5); 4 days dark + 1 h light (lane 6); 4 days dark + 5 h light (lane 7). Total RNA (3 µg) was hybridized with the 3' *rbcS*-3A probe.

Fig. 8. Red and blue light induction of *rbcS* transcripts in immature and mature pea leaves. (A) Seedlings were grown in the dark for 8 days and exposed to red light (red fluorescent lamps, General Electric F20T12.R) at a fluence rate of $40 \mu\text{E}/\text{M}^2/\text{s}$ for 3 min or were given a 3-min red flash followed by 12 min of far-red light (Westlane FRF700 filter) at the same fluence rate. After light treatments plants were returned to the dark or 24 h before RNA extraction. Transcript levels were assayed quantitatively by 3' *rbcS-E9* probe: no light treatment (lane 1); red flash (lane 2); red flash followed by far-red flash (lane 3). (B) Mature pea leaves were grown as described in Figure 7 and transferred to the dark for 4 days. Plants were then irradiated continuously for 9 h with white light ($500 \mu\text{E}/\text{M}^2/\text{s}$); red light and/or blue light (blue fluorescent lamps, GTE Products, Type 246; peak 450 nm, 1/2 band width = 50 nm) both at $15 \mu\text{E}/\text{M}^2/\text{s}$. In some experiments, the continuous light was supplemented by 10 min pulses of far-red illumination every 20 min ($40 \mu\text{E}/\text{M}^2/\text{s}$). After light treatments, plants were harvested and RNA extracted. Transcript levels were assayed quantitatively by a 3' *rbcS-3A* probe: no light treatment (lane 1); white light (lane 2); blue light (lane 3); blue, red and far-red light (lane 4); blue and red light (lane 5); red light (lane 6).

of a 3' probe starting from within the conserved coding region and extending through non-coding diverged regions provided both an internal control and maximized our ability to detect gene-specific divergence in the non-coding regions. A similar modification of the Berk and Sharp (1977) S1 nuclease mapping procedure was first applied to the analysis of the 16-member actin gene family of *Dictyostelium* (McKeown and Firtel, 1981). The results here show that the more active genes (*rbcS-3A*, and *-3C* and *-8.0*) have transcript levels that are 10 times higher than the less active genes (*rbcS-E9* and *-3.6*). Similar variance in *rbcS* gene activities has been described in petunia (Dean *et al.*, 1985b) and *Lemna* (Tobin *et al.*, 1985).

Although there are large quantitative differences between *rbcS* gene transcripts with respect to their accumulation, the spectral sensitivity for gene activation is qualitatively the same. Phytochrome regulation of *rbcS* gene activity is present in immature and mature pea leaves, but in the latter, blue light alone and not red light alone, is effective in inducing *rbcS* accumulation. However, pulses of far-red light can reverse the blue light activation. The accumulation of *rbcS* transcripts in mature pea leaves is similar to the co-action of blue/u.v. light and phytochrome reported in light-mediated anthocyanin formation

in the *Sorghum vulgare* seedling (Oelmüller and Mohr, 1985). We have also documented blue light/phytochrome activation of *rbcS* pea genes in mature leaves of transgenic petunia (Fluhr and Chua, 1986) and tobacco (R. Fluhr, unpublished), hence for these species the regulatory mechanisms are conserved. It is intriguing to us that the photoregulation of these genes exhibit distinct developmental dependence.

Some transcripts, particularly those of *rbcS-8.0* and *-E9* are under-represented in petals and seeds and at early developmental stages in the leaves. These genes reach their full expression potential only in cells that contain mature chloroplasts. Recently, studies on the expression of members of the petunia *rbcS* family were reported, but no differential expression between organs was detected (Dean *et al.*, 1985b). The techniques used in those studies involved quantification of dot blots hybridized to gene-specific probes and statistical analysis of the distribution of cDNA clones.

We do not know whether the variation in transcript levels is a result of differential rates of transcription or RNA stability. The data in Figure 7 suggest that the *rbcS* transcripts turn over in the dark at similar rates. We have transferred into transgenic plants chimeric constructs composed of *rbcS* 5'-flanking fragments (*rbcS-3A* or *-E9*) plus heterologous (bacterial) coding sequences (Fluhr *et al.*, 1986). The light inducibility and organ distribution of the chimeric transcripts parallel those of *rbcS-3A* and *rbcS-E9* in peas. These results suggest that transcript stability does not play a key role.

The 5' upstream sequences of all *rbcS* genes contain conserved sequence motifs. Light inducibility has been localized to the TATA box region (Morelli *et al.*, 1985) and to an enhancer-like sequence further upstream (Timko *et al.*, 1985; Fluhr *et al.*, 1986). But inspection of these sequences shows no obvious reasons why some genes are expressed 3- to 10-fold less than others or other genes are developmentally tardy. We consider it most likely that subtle differences in the upstream sequences of these genes or in their local chromosomal environment are responsible for their differential expression patterns.

Materials and methods

Isolation of pea *rbcS* genomic clones and DNA sequencing

Recombinant lambda phage 1059 was generated from genomic DNA of *Pisum sativum* (var. Progress No. 9) (Coruzzi *et al.*, 1984). The *rbcS-3A* and *-3C* clones were subcloned as 2.5-kb and 3.8-kb subfragments, respectively, in two orientations in a modified pEMBL8⁺ vector (Dente *et al.*, 1983). To facilitate sequence determination, regions of the insert were specifically deleted between the sequencing primer and known single restriction sites within the *rbcS* region. Single strands from clones were sequenced by the dideoxy method (Sanger *et al.*, 1977 as modified by Biggins *et al.*, 1983).

Growth of pea plants 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 at 22°C for 8 days prior to harvesting of leaves, stems and roots. The first internodes were used as stem tissues. For dark-grown plants, seeds were imbibed and germinated in black lucite boxes contained within a dark environmental chambers at 22°C for 8 days. Petals were obtained from 3–4 week old flowering pea plants. Pericarps and seeds ($d = 2-4$ mm) were obtained from pea plants 5–6 weeks after sowing. RNA isolation was as described previously (Fluhr and Chua, 1986).

Slot blot hybridization

Total plant RNA ($5 \mu\text{g}$) was denatured in 4.6 M formaldehyde and $7.5 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}/0.015 \text{ M Na citrate}$) incubated at 65°C for 15 min and loaded on a minifold II Slot Blot System (Schleicher and Schuell). Oven-dried filters were pre-hybridized and hybridized in $6 \times \text{SSC}$, $5 \times$ Denhardt's solution ($1 \times$ Denhardt's solution contains 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone). Hybridization was at 42°C with DNA oligonucleotides labeled at their 5' ends. Filters were washed at 42°C in $2 \times \text{SSC}$ to reduce background before autoradiography.

5' and 3' S1 nuclease mapping

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 total RNA in a 10 μl solution containing 50% formamide, 0.4 M NaCl, 2 mM EDTA, and 20 mM Pipes (pH 6.8) for 6–12 h at 37°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 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 300 units/ml S1 nuclease and incubated for 1.5 h at 21°C. Products were sized on 6% acrylamide, 7 M urea gels.

Acknowledgements

We thank Jacqueline Carter and Peter Silverstein for skilled technical assistance and Wendy Roine for typing the manuscript. We thank Dr Chris Kuhlemeier for critical reading of this manuscript. Robert Fluhr holds a Chaim Weizmann Postdoctoral Fellowship. This work was supported by a grant from Monsanto Company.

References

- Berk, A.J. and Sharp, P.A. (1977) *Cell*, **12**, 721–732.
- Berry-Lowe, S.L. and Meagher, R.B. (1985) *Mol. Cell Biol.*, **5**, 1910–1917.
- Berry-Lowe, S.L., McKnight, T.D., Shah, D.M. and Meagher, R.B. (1982) *J. Mol. Appl. Genet.*, **1**, 483–498.
- Biggins, M.D., Gibson, T.J. and Hong, G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.
- Broglie, R., Coruzzi, G., Lampka, G., Keith, B. and Chua, N.-H. (1983) *Biotechnology*, **1**, 55–61.
- Cashmore, A.R. (1983) In Kosuge, T., Meredith, C.P. and Hollaender, A. (eds), *Genetic Engineering of Plants*. Plenum Press, NY, pp. 29–38.
- Chua, N.-H., Schmidt, G. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 6110–6114.
- Coruzzi, G., Broglie, R., Cashmore, A.R. and Chua, N.-H. (1983) *J. Biol. Chem.*, **258**, 1389–1402.
- Coruzzi, G., Broglie, R., Edwards, C. and Chua, N.-H. (1984) *EMBO J.*, **3**, 1671–1679.
- Dean, C., Van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedbrook, J.R. (1985a) *Proc. Natl. Acad. Sci. USA*, **82**, 4964–4968.
- Dean, C., Van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedbrook, J. (1985b) *EMBO J.*, **4**, 3055–3061.
- Dente, L., Cesareni, G. and Cortese, R. (1985) *Nucleic Acids Res.*, **11**, 1645–1655.
- Dodgson, J.B. and Wells, R.D. (1977) *Biochemistry*, **16**, 2374–2379.
- Dunsmuir, P., Smith, S. and Bedbrook, J. (1983) *Nucleic Acids Res.*, **11**, 4177–4183.
- Fluhr, R. and Chua, N.-H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2358–2362.
- Fluhr, R., Kuhlemeier, C., Nagy, F. and Chua, N.-H. (1986) *Science*, **232**, 1106–1112.
- Gallagher, T.F. and Ellis, R.J. (1982) *EMBO J.*, **1**, 1493–1498.
- Highfield, P.E. and Ellis, R.J. (1978) *Nature*, **271**, 420–424.
- Karn, J., Brenner, S., Barnett, L. and Cesareni, G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5172–5176.
- Kirk, J.T.O. and Tilney-Basset, R.A.E. (1978) *The Plastids*. Amsterdam National Holland Press.
- McKeown, M. and Firtel, R.A. (1981) *Cell*, **24**, 799–807.
- Miziorka, H.M. and Lorimer, G.H. (1983) *Annu. Rev. Biochem.*, **52**, 507–535.
- Montell, C., Fisher, E.F., Caruthers, M.H. and Berk, A.J. (1983) *Nature*, **305**, 600–605.
- Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N.-H. (1985) *Nature*, **315**, 1–5.
- Myers, R.M., Larin, Z. and Maniatis, T. (1985) *Science*, **230**, 1242–1246.
- Nagy, F., Morelli, G., Fraley, R.T., Rogers, S.G. and Chua, N.-H. (1985) *EMBO J.*, **4**, 3063–3068.
- Oelmüller, R. and Mohr, H. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6124–6128.
- Polans, N.O., Weeden, N.F. and Thompson, W.F. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5083–5087.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Sasaki, Y., Sakihama, T., Kamikubo, T. and Shinozaki, K. (1983) *Eur. J. Biochem.*, **133**, 617–620.
- Shenk, T.E., Rhodes, C., Rigby, P.W.J. and Berg, P. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 989–993.
- Silverthorne, J. and Tobin, E.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1112–1116.
- Thompson, W.F., Everett, M., Polans, N.O., Jorgensen, R.A. and Palmer, J.D. (1983) *Planta*, **158**, 487–500.
- Timko, M.P., Kausch, A.P., Hand, J.M., Cashmore, A.R., Herrera-Estrella, L., Vande den Broeck, G. and Van Montagu, M. (1985) In Steinbeck, K.E., Bonitz, S., Arntzen, C.J. and Bogorad, L. (eds), *Molecular Biology of the Photosynthetic Apparatus*. Cold Spring Harbor Laboratory Press, NY, pp. 381–396.
- Tobin, E.M. and Silverthorne, J. (1985) *Annu. Rev. Plant Physiol.*, **36**, 569–593.
- Tobin, E.M., Wimpee, C.F., Karlin-Neumann, G.A., Silverthorne, J. and Kohorn, B.D. (1985) In Steinbeck, K.E., Bonitz, S., Arntzen, C.J. and Bogorad, L. (eds), *Molecular Biology of the Photosynthetic Apparatus*. Cold Spring Harbor Laboratory Press, NY, pp. 378–380.
- Weiher, H., Konig, M. and Gruss, P. (1983) *Science*, **219**, 626–631.
- Wimpee, C.F., Stiekema, W.J. and Tobin, E.M. (1983) In Goldberg, R.B. (ed.), *Plant Molecular Biology. UCLA Symposium on Molecular and Cellular Biology*. Vol. **12**, pp. 391–401.

Received on 13 May 1986