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MONOCOT AND DICOT GENES ENCODING THE SMALL SUBUNIT OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE: STRUCTURAL ANALYSIS AND GENE EXPRESSION

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INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase (RUBISCO) is responsible for the assimilation of atmospheric CO_2 by photosynthetic organisms. The holoenzyme is composed of a large subunit (Mr = 55,000) and a small subunit (Mr = 12-15,000). The large subunit (L) is encoded by chloroplast DNA (COEN *et al.* 1977) and synthesized inside the organelle (BLAIR & ELLIS 1973). The small subunit (S) is coded for in the nucleus (KAWASHIMA & WILDMAN 1972) and synthesized as a larger precursor (pS) on free cytoplasmic ribosomes (DOBBERSTEIN *et al.* 1977; HIGHFIELD & ELLIS 1978; CHUA & SCHMIDT 1978; CASHMORE *et al.* 1978). The chloroplast transport and processing of pS have been shown to be post-translational events (HIGHFIELD & ELLIS 1978; CHUA & SCHMIDT 1978).

Although the large subunit gene has been cloned and its nucleotide sequence determined (McINTOSH *et al.* 1980; ZURAWSKI *et al.* 1981; SHINOZAKI & SUGIURA 1982), little is known concerning the structure, organization and expression of the nuclear genes encoding the small subunit. To study this question, we have isolated cDNA clones encoding this polypeptide from peas, *Pisum sativum* and from hexaploid wheat, *Triticum aestivum* (BROGLIE *et al.* 1981; BROGLIE *et al.* 1983). By Southern blot analyses of nuclear DNA we found that, in both plants, S is encoded by a small multigene family. Nucleotide sequence analysis of small subunit cDNA and genomic clones has allowed us to deduce the complete amino acid sequence of the mature small subunit polypeptides. The amino acid sequence for the wheat S is the first reported for any monocot.

In addition, our studies have provided amino acid sequence information for the precursor transit peptides, which are thought to be important for the transport of the precursor polypeptides across the chloroplast envelope (CHUA & SCHMIDT 1979). The results of these studies are summarized here.

DIFFERENTIAL EXPRESSION OF THE RUBISCO SMALL SUBUNIT POLYPEPTIDE

Although RUBISCO is the most abundant protein in leaf mesophyll cells, little is known concerning its distribution in other plant tissues. To address this question we first examined the levels of RUBISCO in leaf and root tissues from light and dark grown pea seedlings by native-SDS two dimensional polyacrylamide gel electrophoresis (SACHS *et al.* 1980). Figure 1 shows that the soluble polypeptide composition of leaves differs greatly from that of roots. The major soluble polypeptides in leaves from light grown plants correspond to L and S of RUBISCO

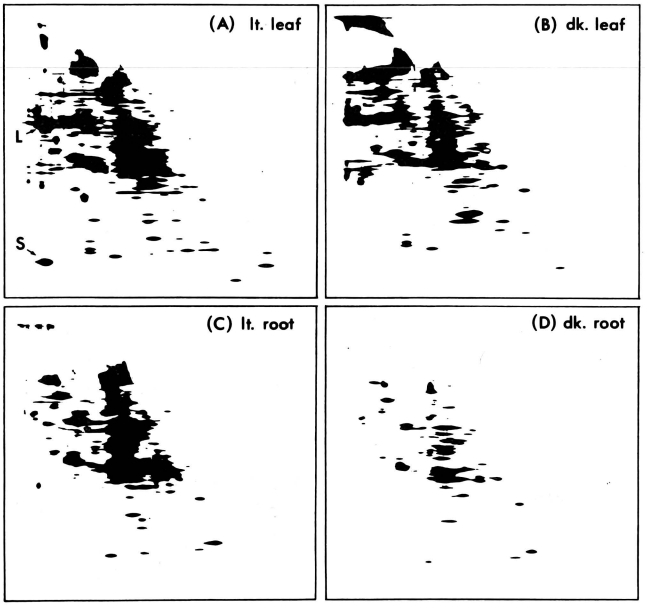

Figure 1. Two dimensional polyacrylamide gel electrophoresis of soluble proteins from (A) light leaves, (B) dark leaves, (C) light roots, and (D) dark roots. In these experiments, pea seedlings were grown for 7 days either in the light or in complete darkness. Soluble proteins obtained from leaves or roots were separated in the first dimension by electrophoresis in 4-15% native polyacrylamide gels (SACHS *et al.* 1980) and in the second dimension using 7.5-15% SDS-polyacrylamide gels (LAEMMELI 1970). Proteins were visualized by silver staining (MORRISSEY 1981).

(Figure 1A). In contrast, extracts from etiolated leaves or from roots show little or no detectable RUBISCO polypeptide subunits (Figure 1 B-D). Using monospecific antibodies raised against purified S in an ELISA assay, the levels of S in roots and etiolated leaves were shown to be less than 0.5% and 6%, respectively, of that found in leaves from light grown plants (EDWARDS & CHUA, unpublished results).

To determine whether the different levels of RUBISCO in these tissues reflect different steady-state levels of small subunit mRNAs, a Northern blot analysis (THOMAS 1980) of total RNA was performed. For this experiment a pea small subunit cDNA (pSS15) which shares homology with all members of the gene family (BROGLIE *et al.* 1981; CORUZZI *et al.* 1983) was used as a hybridization probe. RNA from both root and leaf tissues was denatured and resolved by electrophoresis in agarose gels. After transfer to nitrocellulose filters, the RNA was hybridized to ^{32}P-labeled pSS15 and the presence of small subunit transcripts was detected by autoradiography. Figure 2A shows that mRNA for the small subunit is present in both leaves and roots from light grown plants and in etiolated leaves; however, the level of this mRNA in leaves of light grown plants is at least

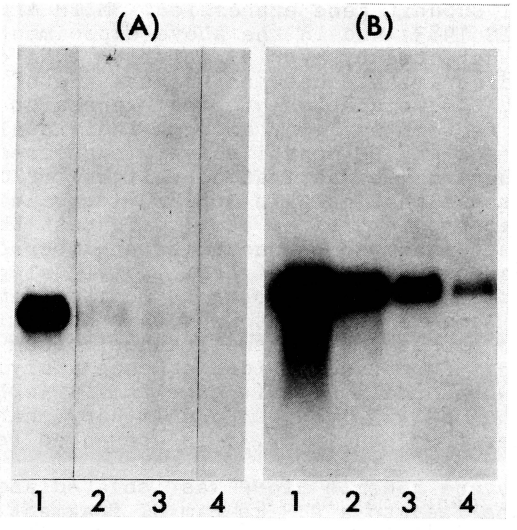

FIGURE 2. Northern blot analysis of total RNA isolated from light leaves (Lane 1), etiolated leaves (Lane 2), light roots (Lane 3) and dark roots (Lane 4). RNA (10 ug) was denatured with glyoxal and fractionated on 1% agarose gels. Panel A, probed with pSS15 (pea small subunit) and Panel B, probed with pAB96 (pea chlorophyll a/b binding polypeptide 15).

100-fold higher than in roots or etiolated leaves. Thus, the genes coding for the small subunit in peas can be regulated in both a light-dependent and a tissue-specific manner. Similar results demonstrating the light induction of the small subunit mRNA in pea leaves have been obtained previously (SMITH & ELLIS 1981). These studies, together with transcriptional studies using isolated pea nuclei (GALLAGHER & ELLIS 1983) provide evidence that the light-dependent expression of the small subunit is regulated at the level of mRNA synthesis. Figure 2B shows that when cDNA encoding the major chlorophyll a/b binding protein (BROGLIE *et al.* 1981), pAB96, is used to probe the same RNA samples, high levels of complementary mRNA can be detected in tissues lacking this polypeptide (CHUA unpublished results). These results suggest that the expression of chlorophyll a/b protein genes is less stringently regulated than for those genes encoding the small subunit. In this regard, BENNETT (1981) has shown that when light-grown pea seedlings are transferred to darkness, the chlorophyll a/b binding apoprotein continues to be synthesized but is rapidly degraded.

STRUCTURAL ANALYSIS OF GENOMIC CLONES CODING FOR pS FROM PEA AND WHEAT

As we have shown elsewhere, the RUBISCO small subunit is encoded by a small multigene family in both peas and wheat (CORUZZI *et al.* 1983; BROGLIE *et al.* 1983). In previous studies of RUBISCO small subunit gene expression (SMITH & ELLIS 1981; GALLAGHER & ELLIS 1983) and in the above experiments, small subunit mRNAs were detected by hybridization to cDNA clones which were unable to discriminate between transcripts of different genes. However, the regulation of gene expression by light or tissue-specific factors may vary amongst individual genes. For example, some genes may be constitutively expressed in leaf cells, while others may be activated by light, or differentially expressed in tissues that contain RUBISCO (e.g. leaf mesophyll cells, seed or embryonic tissue, etc.). To test these possibilities it is necessary to characterize a number of these genes to identify unique regions of DNA from individual genes which can be used as discriminating probes to monitor gene-specific transcription. As a first step toward this objective, we have isolated genomic clones for the small subunit from peas and wheat. A summary of our nucleotide sequence analysis for two members of the wheat small subunit gene family (cDNA clone, pW9 and genomic clone, pWS4.3) and our preliminary characterization of genomic clones from pea (pPS2.3) is presented below.

From wheat, one genomic clone was isolated and the small subunit gene localized to a 4.3 kb Bam HI fragment by Southern blot analysis (BROGLIE *et al.* 1983). This Bam HI fragment was subcloned into pBR322 (pWS4.3). From pea, we have isolated six different genomic clones. The small subunit gene from one of these clones has been localized to a 2.3 kb Eco RI-Cla I fragment which was subcloned into pBR325 (pPS2.3) (CORUZZI *et al.* in preparation). A schematic diagram summarizing the organization of these two genes is shown in Figure 3. Both genes contain an intron which separates the transit sequence from the main body of the mature protein. An intron has also been found at an

identical position in a soybean, *Glycine max*, small subunit gene (BERRY-LOWE et al. 1982). In addition, both the pean and soybean subunit genes contain a second intron separating the 47th and 48th amino acid residues of the mature protein pWS4.3.

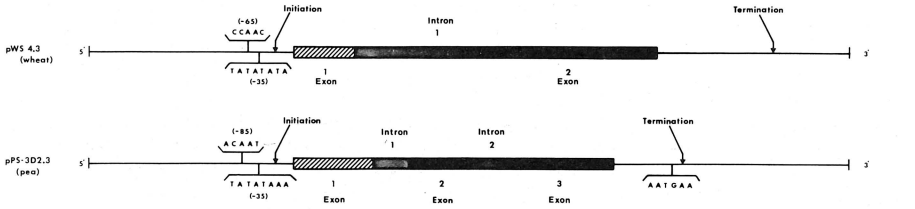

Figure 3. Structure of pea and wheat RUBISCO small subunit genomic clones. Regions of DNA coding for introns are shown in gray. Regions of DNA coding for the pS transit peptides and the mature S polypeptides are denoted by the cross-hatched and black areas, respectively. The 5' and 3' termini of the mRNA have been localized by S1 nuclease mapping.

We have localized the 5' and 3' boundaries of both small subunit genes by S1 nuclease mapping (BERK & SHARP 1977; WEAVER & WEISSMAN 1979). In each case, transcription is initiated at an A residue located within the sequence CAA. In wheat, this sequence is situated 42 nucleotides upstream of the initiator ATG, whereas in peas, this site occurs 32 nucleotides upstream of the ATG. Approximately thirty-five nucleotides upstream from the transcription initiation site in both genes is the sequence 5'-TATATA-3'. This sequence motif is similar to the "TATA" or Goldberg-Hogness box which has been shown to be required for the correct initiation of eucaryotic gene transcription by RNA polymerase II of animal cells (BREATHNACH & CHAMBON 1981). In addition, both genes contain sequences approximately 65 to 85 nucleotides upstream of their respective transcription start sites, which resemble the "CAT" sequences thought to be involved in the modulation of gene expression in animals (BREATHNACH & CHAMBON 1981).

The 3' boundary of the wheat pS gene has been mapped to a position 289 nucleotides downstream of the ochre termination codon and the 3' terminus of the pea pS gene to a position 150 nucleotides downstream of the opal terminator. In many eucaryotic mRNAs, the sequence "AAUAAA" is found 20 nucleotides upstream from the poly A tail. This sequence has been proposed to function as a signal for poly A addition (FITZGERALD & SHENK 1981). In the pea gene, a similar hexanucleotide sequence, AATGAA, is found 20 nucleotides upstream from the poly A tail. However, this sequence is not present at the 3' ends of pW9 and

Thus, at present, the significance of such sequences in the maturation of plant mRNAs is not known.

A comparison of the wheat small subunit gene encoded by pWS4.3 and the cDNA clone, pW9, revealed a marked nucleotide sequence divergence in both the 5' and 3' non-translated regions, which showed that the mRNA template for pW9 was not transcribed from the gene cloned in pWS4.3 (BROGLIE *et al.* 1983.). Such nucleotide sequence divergence in the 3' ends of different gene members identifies a DNA sequence unique to individual genes which can be used as a hybridization probe to monitor gene-specific transcription. For this purpose, we have subcloned a 302 bp Mbo I-Eco RI restriction fragment derived from the 3' non-translated region of pWS4.3. When this probe is used in a Southern blot analysis of wheat nuclear DNA, we observe hybridization to a single 4.3 kb Bam HI fragment, identical in size to pWS4.3. In addition, we have used the probe in a Northern blot analysis of wheat RNA to show that the gene cloned in pWS4.3 is transcriptionally active in vivo (BROGLIE *et al.* 1983).

In a similar experiment, a 3' gene specific probe from pea cDNA clone pSS15 was used to identify the gene from which the mRNA complementary to pSS15 was transcribed. This probe was hybridized to DNA dot blots of six genomic clones encoding the small subunit of RUBISCO. After stringent washing, hybridization to only a single genomic clone was detected. DNA sequence analysis of this clone (pPS2.3) confirmed that pSS15 is derived from this gene (CORUZZI *et al.* in preparation) and that this gene is expressed in pea leaves.

AMINO ACID SEQUENCE COMPARISON OF SMALL SUBUNIT PRECURSORS

The complete nucleotide sequence of pS encoded by pW9 and pWS4.3 (wheat) and pPS2.3 (pea) have provided information on the amino acid sequences and properties of their transit peptides which have been proposed to function in the post-translational transport of these proteins into chloroplasts (CHUA & SCHMIDT 1979). Figure 4 shows that both the wheat and pea transit peptides are characterized by a preponderance of basic amino acids and the absence of E (Glu), H (His), W (Trp), and Y (Tyr). We have suggested previously that the basic property of the transit peptide may be important in its interaction with the chloroplast envelope (CORUZZI *et al.* 1983; BROGLIE *et al.* 1983). A comparison of the amino acid sequence of the pS transit peptides from wheat, pea and soybean (BERRY-LOWE *et al.* 1982) shows that there is much less homology (30%) in this part of the precursor (Figure 4) as compared to the mature protein (80% homology) (Figure 6). This suggests that this part of the molecule is evolving at a faster rate than the mature polypeptide.

In spite of their weak amino acid homology, a number of structural features are conserved among the transit peptides of pS. For example, the number and positions of the positively charged amino acids and proline residues are strongly conserved. These factors contribute to an overall conservation in the peptide secondary structure as predicted by the rules of

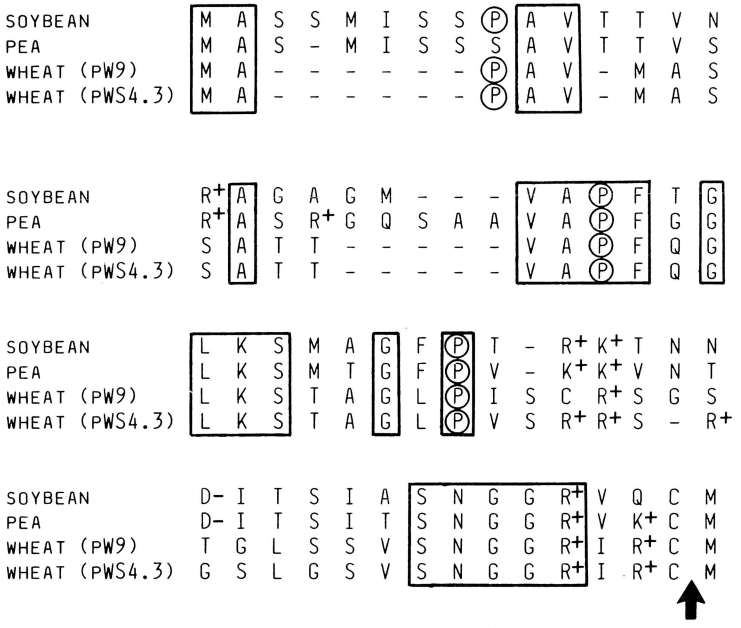

Figure 4. Amino acid sequence comparison of transit peptides for wheat pS, soybean pS (BERRY-LOWE *et al.* 1982) and pea pS. The amino acid sequences were deduced from the nucleotide sequence. The sequences were aligned to maximize regions of homology (boxed sequences). The arrow points to the cleavage site.

CHOU and FASMAN (1978). Figure 5 shows the predicted secondary structures for the wheat, pea and soybean transit peptides. The structures are largely composed of regions of beta sheet connected by a number of beta turns with alpha-helices at their NH_2-termini. The processing site is invariably composed of a beta sheet containing at least one positively charged residue, followed by a conserved beta turn. Such a structure may be important in the interaction of the precursor polypeptide with its appropriate processing enzyme. However, further support for this hypothesis requires a knowledge of the tertiary structures of these peptides and, also of the precursor polypeptides. The importance of these common structural features in the transport process is emphasized by the fact that pS from either peas or wheat (dicot and monocot, respectively) is imported into both homologous and heterologous chloroplasts (CORUZZI *et al.* 1983). Moreover, there is a striking amino acid homology at positions -4 to -8 and -1 to +5 surrounding the cleavage site in pS of these three plants. Therefore, it is not surprising that newly imported pS of pea and wheat are processed correctly by either chloroplast to yield a mature polypeptide of authentic size (CORUZZI *et al.* 1983).

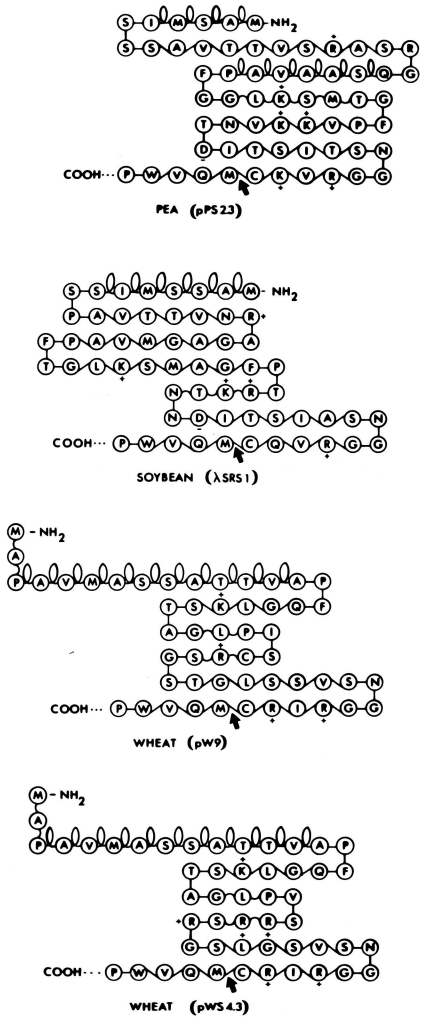

Figure 5. Proposed secondary structure for the transit peptides of wheat, soybean (BERRY-LOWE *et al.* 1982) and pea pS. The parameters proposed by CHOU & FASMAN (1978) were used to predict the structural features. Amino acids forming alpha-helices, beta-turns or random coils are interconnected with loops, lines and arcs, respectively. The arrow points to the cleavage site.

The complete amino acid sequence of wheat S deduced from our nucleotide sequence analysis of pW9 and pWS4.3 is the first reported for a monocot. A partial amino acid sequence for S of barley has been obtained by POULSEN *et al.* (1976). The first

twenty-five amino acid residues of wheat S are identical to barley S, although differences exist in other regions. Figure 6 shows a comparison of the amino acid sequences of mature S from wheat, pea, soybean (BERRY-LOWE *et al.* 1982), spinach, *Spinacia oleracea* (MARTIN 1979), and tobacco, *Nicotiana tabacum* (MULLER et al. 1983). The overall sequence homology among these

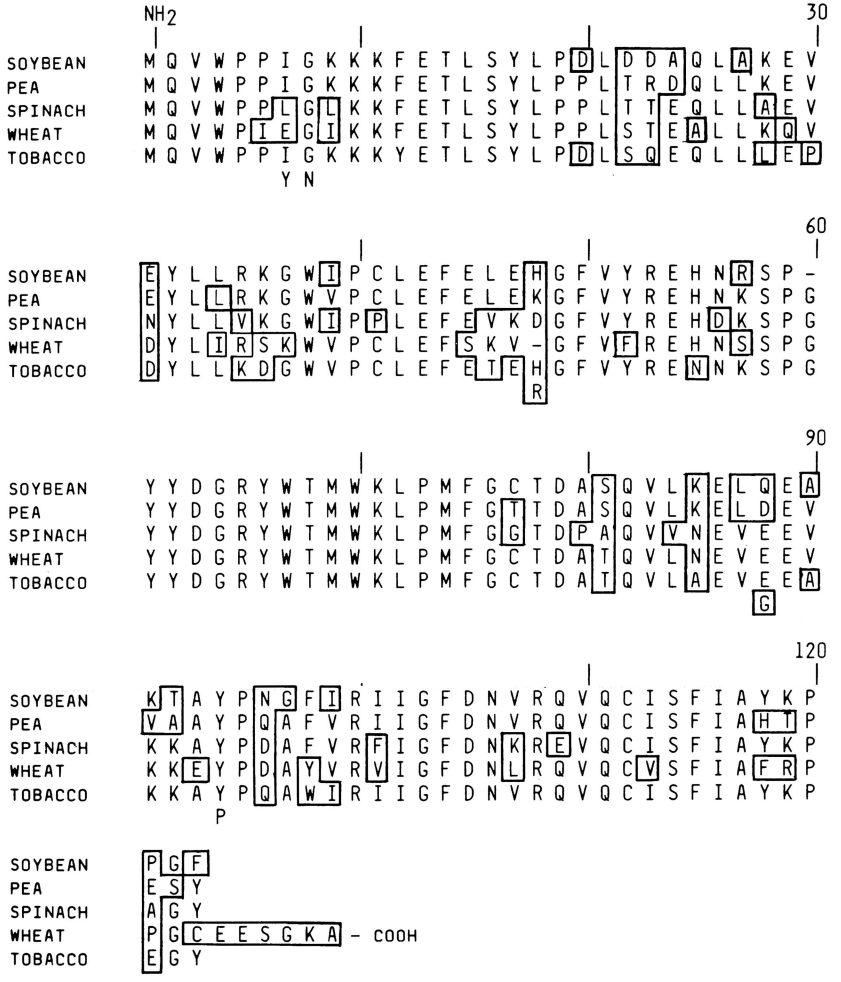

Figure 6. Amino acid sequence comparison of the mature small subunit polypeptide of wheat, pea, spinach (MARTIN 1979), soybean (BERRY-LOWE *et al.* 1982) and tobacco (MULLER *et al.* 1983). The amino acid sequences for wheat, pea and soybean were deduced from the nucleotide sequences. To maximize sequence homology a single amino acid deletion was placed at residue 60 and residue 48 of soybean and wheat, respectively. The boxed amino acids indicate differences in amino acid residues.

polypeptides is approximately 75-80%. However, four regions show an absolute conservation of amino acid sequences. These are located at the amino terminus of S (residues 1-5), at residues 10-19, at residues 61-76 and at residues 102-106. In view of recent experiments with the cyanobacterium *Synechococcus*, which show that S is required for the catalytic activity of RUBISCO *in vitro* (ANDREWS & BALLMENT 1983), we propose that these highly conserved regions of S may be important in its interaction with the large subunit. In this regard we have shown previously that newly-imported S of peas and wheat can assemble with homoenzymes, despite the fact that one is a dicot and the other a monocot (CORUZZI et al. 1983).

CONCLUSION

In this article we have summarized the similarities and differences in the structure and organization of nuclear genes encoding pS in wheat and peas. The wheat (pWS4.3) and the pea gene (pPS2.3) code for transit peptides and mature small subunits of different sizes; nevertheless, both genes contain an intron at an identical position in the coding sequence of S. In both plants, the first exon encodes the entire transit peptide plus the first two amino acids (Met-Gln) of S, which together play a role in the post-translational uptake and processing of pS (CHUA & SCHMIDT 1978). This structural feature of the RUBISCO small subunit gene supports the hypothesis that exons code for protein domains of distinct functions (GILBERT et al. 1979).

In contrast to the wheat gene, the pea gene contains an additional intron which interrupts the mature protein at approximately one third the length from the NH_2-terminus between amino acid residues 47 and 48. At present, we do not know whether the NH_2-terminal portion (residues 2-47) and the COOH terminal portion (residues 48-123) of the mature small subunit perform different functions either in the assembly of the RUBISCO holoenzyme or its catalytic properties. Similarly, we do not know whether the presence of a second intron in the pea gene reflects a fundamental difference between the structure of RUBISCO small subunit genes in monocots and dicots. Isolation and characterization of more small subunit genes from other plants are needed to establish this point.

The RUBISCO small subunit in both wheat and pea is encoded by a multigene family of at least 10 members (CORUZZI et al. 1983; BROGLIE et al. 1983). While the significance of such a small multigene family remains to be elucidated, RNA and protein analysis has already revealed that the small subunit genes are regulated in both a light-dependent and tissue-specific manner. The latter finding raised the possibility that individual gene members may be differentially expressed in different plant tissues or may respond differently to environmental factors. A thorough investigation of these questions requires the availability of hybridization probes that are specific to individual gene members. We have found that two wheat small subunit genes

contain divergent 5' and 3' non-coding sequences. We have exploited this finding by using a 3' non-coding region of pWS4.3 as a specific hybridization probe to demonstrate the expression of this gene in wheat leaves. A similar method was successfully employed to indentify from amongst six pea small subunit genomic clones the spinach gene which encodes the cDNA clone, pSS15. We expect this strategy to be useful in following the expression of individual members of the small subunit gene family during plant development in different plant tissues, and in response to environmental stimuli. Results obtained from these experiments may shed light on why higher plants have evolved a small multigene family to code for the small subunit of RUBISCO.

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