The structure and expression of genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase in *Phaseolus vulgaris* L.

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Pour Maman et Papa,

toujours votre Petit Sanglier.

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SUMMARY

The multigene family encoding the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase [EC 4.1.1.39] in Phaseolus vulgaris L. contains at least three members. cDNA clones representing 3 members of the family (rbcS genes) have been characterised in terms of structure and expression. These rbcS genes show very high nucleotide sequence homologies in the coding regions for both the transit peptide and the mature polypeptide. The nucleotide sequences for the 5′- and 3′-untranslated regions of these genes which were present in the cDNA clones showed far more divergence. One of the rbcS cDNA clones contained two regions of intronic sequences, the first lying between the codons for the 2nd and 3rd amino acids of the mature polypeptide and the second lying between the codons for the 47th and 48th amino acids of the mature polypeptide.

The steady-state levels of total rbcS transcripts were measured by RNA dot-blot hybridisation analysis. Readily detectable levels of rbcS transcripts were present in dark-grown primary leaves and also in non-leaf tissue. Exposure to white light for 48h was found to increase levels of rbcS transcripts by 5 to 10-fold in the primary leaves of etiolated seedlings. A brief illumination with red light produced a comparable increase after 24h, this increase being partially reversible by a subsequent brief illumination with far-red light, implying the involvement of the photoreceptor phytochrome.

Accumulation of the transcripts of different rbcS genes was studied by S1 nuclease analysis of total RNA. This study indicated that rbcS genes in the primary leaves of P. vulgaris were responding differentially to white light when etiolated seedlings were illuminated. In order to study the accumulation of different rbcS transcripts more specifically, oligonucleotide probes were made to unique

sequences from the 3'-untranslated regions of the three rbcS genes identified. RNA dot-blot analysis using these probes showed there to be differences in the accumulation of the different rbcS transcripts in response to white light, red light and developmental status.

INTRODUCTION

1.1 Rubisco

1.1.1 Structure and activity

Ribulose 1,5-bisphosphate carboxylase/oxygenase [EC 4.1.1.39] (Rubisco) is a chloroplastic enzyme with activities involved in the Calvin cycle of photosynthesis (carboxylase) and in the process known as photorespiration (oxygenase) (Ogren and Bowes, 1971). This enyzyme is particularly abundant and can comprise up to 50% of total leaf soluble protein (Kung, 1976). Two types of polypeptide subunits are present in the functional holoenzyme, known as the small- and large-subunits of Rubisco. In higher plants the holoenzyme consists of 8 small subunits and 8 large subunits (Baker et al., 1975). The large subunit carries the active site of the enzyme which is believed to be identical for both the carboxylase and oxygenase activities (Lorimer and Mizioko, 1980; Lorimer, 1981). The small subunit does not appear to have catalytic activity, but is thought to be necessary for full activity of the enzyme and probably performs a regulatory role (Jensen and Bahr, 1977) The genes encoding the small and large subunits of Rubisco are known as rbcS and rbcL respectively. In higher plant cells, rbcL genes are located in the plastid genome whereas rbcS genes are located in the nuclear genome (Chan and Wildman, 1972; Kawashima and Wildman, 1972).

1.1.2 Biogenesis

Synthesis of the precursor to the small subunit of Rubisco in higher plants involves the transcription of rbcS genes, processing of the transcripts and translation of the transcripts on the free cytoplasmic 80s ribosomes (Ellis

et al., 1984). Small subunit precursors have an N-terminal transit peptide which is between 46-57 amino acid residues in length depending upon the species (Dean et al., 1989). The interaction of the transit peptide of a pea rbcS polypeptide precursor with import receptors located at the contact zones of the envelopes of isolated pea chloroplasts has been demonstrated (Pain et al., 1988). The transit peptide itself contains sufficient protein sequence information to allow the transport of the precursor into chloroplasts. This feature has been demonstrated by the successful targetting of bacterial proteins to chloroplasts by the fusion of rbcS transit peptide coding sequences to bacterial coding regions (Van den Broek et al., 1985). Once within the stroma the precursor is processed to the mature size by a metal ion-dependent protease. (Robinson and Ellis, 1984a). This process involves two separate cleavage events, the second of which occurs at the Cys-Met junction between the transit and mature polypeptide sequences (Robinson and Ellis, 1984b). The assembly of the large and small subunits of Rubisco into functional Rubisco holoenzyme occurs within the chloroplast, and involves another nuclear-encoded protein, the Rubisco subunit binding protein (Barraclough and Ellis, 1980). It has been shown that this protein does not form part of the final holoenzyme but acts as a molecular 'chaperone' (Hemmingsen et al., 1988) assisting the assembly process.

1.2 rbcS gene structure and organisation

Interest in rbcS genes stemmed from the fact that they encoded the subunits of an important enzyme in plant metabolism, an enzyme whose biogenesis was found to involve the interaction of both plastid and nuclear genetic systems and whose abundance and activity were evidently regulated by light. Cloning the genes encoding

the subunits of Rubisco, therefore, became a focus of much research. Purification was facilitated by the high levels of this enzyme present in higher plants enabling the production of antibodies to Rubisco. Furthermore, the strong light-regulation of rbcS transcript accumulation facilitated cloning by differential screening, and thus allowed a rbcS gene to become the first cloned plant gene (Bedbrook et al., 1980). This secured a place for rbcS genes as 'model' plant genes for study. Studies of the regulation of their expression have provided insights into the mechanisms involved in the regulation of plant genes by light (section 1.3.2), tissue-specific factors and development (section 1.3.1).

1.2.1 Organisation of rbcS multigene families

The rbcS genes of higher plants are encoded in the nuclear genome. These genes are present as small multigene families in most of the higher plant species studied. Petunia hybrida (Mitchell) has 8 rbcS genes (Tumer et al., 1986; Dean et al., 1987a), tomato has 5 (Pichersky et al., 1986; Vallejos et al., 1986), Arabidopsis has 4 (Krebbers et al., 1988), potato has at least 4 (Wolter et al., 1988), pea has at least 5 (Polans et al., 1985), soybean has between 6-10 (Berry-Lowe et al., 1982; Grandbastien et al., 1986), Nicotiana tabacum has at least 5 (Mazur et al., 1985; O'Neal et al., 1987), Nicotiana sylvestris has at least 7 (Pinck et al., 1986), Lemna gibba has around 13 (Wimpee et al., 1983), wheat has more than 10 (Smith et al., 1983) and maize has at least 4 rbcS genes (Sheen and Bogorad, 1986; Lebrun et al., 1987). In some species, these genes have been shown to be linked within the nuclear genome. Petunia has 5 rbcS genes from a 6 gene subfamily closely linked in such a way that all 5 genes are contained within a 25kbp stretch of the genome (Dean et al., 1985a). Similarly both tomato (Sugita et al.,

1987) and potato (Wolter et al., 1988) have 3 rbcS genes within a 10kbp stretch of genomic DNA. In the case of potato these three genes are within 12 kbp of a fourth rbcS gene. All 5 rbcS genes so far characterised in pea are found on the same chromosome and map to within 4 map units (Polans et al., 1985).
Other species from which rbcS cDNA clones have been isolated, but not genomic clones, include spinach (Tittgen et al., 1986), Silene pratensis (Smeekens et al., 1986), Amaranthus hypochondriacus (Berry et al., 1985), Flaveria trinervia (Adams et al., 1987), Helianthus annuus (Waksman and Freyssinet, 1987), Sinapis alba (Oelmuller et al., 1986) and cucumber (Greenland et al., 1987). This last species, cucumber, is notable in that it appears to have only one rbcS gene.

1.2.2 rbcS gene structure

A feature of the rbcS polypeptides of higher plants is the high degree of amino acid sequence homology. This homology is much greater in the sequences of the mature polypeptide than in those of the transit peptide. For instance the mature polypeptide of Lemna is 70-75% homologous to those of soybean, pea and wheat whereas the transit peptide is only 50, 40 and 25% homologous respectively (Stiekema et al., 1983).
The greatest homology between the transit peptides of different species occurs at the N-terminus and around the processing site, and charged residues tend also to be conserved (Stiekema et al., 1983). The N-terminal conserved region of rbcS transit peptides may be involved in the recognition of the rbcS precursor by chloroplasts, and those around the processing site may be required for correct cleavage.
As well as transit peptide sequence divergence, the length of the transit peptide varies between species. In petunia

(Dean et al., 1987a), pea (Fluhr et al., 1986b), tomato (Pichersky et al., 1986; Sugita et al., 1987), tobacco (Mazur et al., 1985; O'Neal et al., 1987), potato (Wolter et al., 1988) and Lemna (Wimpee et al., 1983) most rbcS genes encode precursors which have transit peptides 57 amino acids in length, although one gene from each of the Solanaceous species (petunia, tomato and potato) has one extra amino acid. Soybean rbcS genes encode precursors which have transit peptides 55 amino acids in length (Grandbastien et al., 1987) and those of wheat (Broglie et al., 1983) and maize (Lebrun et al., 1987) 46 or 47 amino acids in length.

Both the nucleic acid sequences and the protein sequences of the mature polypeptide are highly conserved. Conservation of protein sequence between different rbcS polypeptides within the same cell is especially apparent, and this conservation is greater than between rbcS polypeptides of different species. For instance Lemna gibba has 6 identical rbcS polypeptides (Wimpee et al., 1983), whereas the mature rbcS polypeptides of tomato and petunia (both Solanaceous species) diverge by as much as 18% (Dean et al., 1989). Manzara and Gruissem (1988) describe a large number of changes in the nucleotide sequences of tomato rbcS genes which are silent at the amino acid sequence level. This suggests that the need for protein sequence homogeneity within the cell is responsible for the concerted evolution of rbcS genes in the same multigene family. However in tomato (Sugita et al., 1987) 2 rbcS genes have identical nucleotide sequence for all three exons and 2 introns and only have a few nucleotide changes with respect to a third rbcS gene. This suggests, in this case at least, the involvement of other mechanisms such as gene conversion, explaining the high degree of nucleotide sequence homology between rbcS genes in tomato (Sugita et al., 1987).

Nucleotide sequence homologies of the mature polypeptide

coding regions of rbcS genes which are linked tend to be higher than those that are not. For instance in petunia there is a 10.2% nucleotide sequence divergence between the members of different subfamilies, whereas members from the same, linked subfamily show a nucleotide sequence divergence of only between 0-3% (Dean et al., 1989). Similarly in tomato (Pichersky et al., 1986) the nucleotide sequence divergence of the coding regions of non-linked rbcS genes ranges from 10-14% whereas between linked genes it is only 1.6%.

The nucleotide sequences of the 3' and 5' flanking sequences of rbcS genes tend to be more divergent than the coding regions. In the 3' flanking regions of pea (Fluhr et al., 1986b) and petunia (Dean et al., 1989) the most homology between genes from the same multigene family is found immediately preceding the polyadenylation sites of the different rbcS genes. In petunia, (Dean et al., 1989) all the petunia genes share a 60 bp region of nucleotide sequence homology in the region preceding the polyadenylation sites suggesting that these sequences may be involved in mRNA processing and polyadenylation. Petunia rbcS transcripts have been shown to be polyadenylated at several different sites (Dean et al., 1987b).

Although conservation of rbcS protein sequence between species is lower than within species, certain regions are highly conserved. One region which is absolutely conserved in all species which have been studied to date is the hexadecapeptide YYDGRYWTMWKLPMFG located between amino acid residues 61 to 76 (Dean et al., 1989). The 16 amino acids between positions 102 to 117 are also relatively well conserved. Dean et al. (1989) suggest that the conservation of the hexadecapeptide sequence in the context of the relatively high level of interspecific protein sequence divergence favours the theory that this region is important in the structure or the function of

the small subunit. They point out that 6 of the 16 residues are aromatic, suggesting that this part of the subunit would not be exposed at the surface of the Rubisco holoenzyme. Thus, it is possible that this region may be involved in the binding of subunits at the core of the holoenzyme.

All dicotyledonous plants so far studied have at least two introns in their rbcS genes, at conserved positions between amino acids 2 and 3 and amino acids 47 and 48 of the mature polypeptide (Dean et al, 1989).

In monocots one intron may be absent. For instance in Lemna the first intron is absent (Wimpee et al., 1983) and in wheat (Broglie et al., 1983) and maize (Lebrun et al., 1987) the second intron is absent.

In addition to introns at these two conserved sites, some members of the Solanaceae, petunia (Dean et al., 1985a), tomato (Sugita et al., 1987) and potato (Wolter et al., 1988) have a third intron positioned within the codon for the 61st amino acid of the mature polypeptide.

1.3 rbcS gene expression

Expression of rbcS genes has been monitored by the direct measurement of the rate of transcription of these genes as well as by measuring the steady-state levels of rbcS transcripts.

Rates of rbcS gene transcription can be measured directly by run-off transcription assays performed on isolated nuclei. Assays of this kind on rbcS genes were first described by Gallagher and Ellis (1982) using nuclei isolated from pea seedlings, and were subsequently performed on nuclei from Lemna gibba (Silverthorne and Tobin, 1984), soybean (Berry-Lowe and Meagher, 1985), rye (Ernst et al., 1987) and petunia (Dean et al., 1989). Such experiments provide a measurement of the relative rate of rbcS transcription already initiated in vivo.

The steady-state levels of rbcS transcripts can be measured by northern blot analysis (eg. Bedbrook et al., 1980) and dot or slot blot analysis of RNA (eg. Barnett et al., 1987) using a probe to rbcS. These techniques can be extended to measure the steady-state levels of transcripts from different rbcS genes using gene-specific oligonucleotide probes. Gene-specific oligonucleotide probes have been used in tomato (Sugita and Gruissem, 1987), petunia (Tumer et al., 1986) and in pea (Fluhr et al., 1986a) and Lemna (Tobin et al., 1985). Other techniques which have been used to measure the levels of different rbcS transcripts from the same tissue include S1 nuclease or RNase protection analysis (Poulsen et al., 1986; Coruzzi et al., 1984; Fluhr et al., 1986a), primer extension analysis (Dean et al., 1987b) and the measurement of frequency of cloning in a cDNA library (Dean et al., 1985b). Dean et al. (1989) argue that primer extension analysis and S1 nuclease or RNase protection analysis provide the most accurate results as hybridisation reactions are carried out in solution and a large excess of probe can be added to drive the hybridisation reaction to completion. Several techniques have been used in this thesis to study the steady-state levels of different rbcS transcripts. The relative merits of these techniques specifically relevant to this particular study are discussed in section 4.3.

In all, these experimental studies demonstrate that expression of the rbcS genes of higher plants is controlled in several ways. Firstly it is controlled spatially in that different levels of expression are found in different tissues and cell-types (section 1.3.1.1). Secondly it is developmentally regulated and changes are seen during ontogenesis eg. in leaves and fruits (section 1.3.1.2) and thirdly it is photoregulated (section 1.3.2).

1.3.1 Tissue-specific and developmental regulation

1.3.1.1 Tissue-specific expression

Expression of rbcS genes to maximal levels is only observed in tissue containing plastids that have developed beyond a certain stage towards becoming chloroplasts (Eckes et al., 1985; Tobin and Silverthorne, 1985; Oelmuller et al., 1986). The variation in the levels of rbcS transcripts in different tissues is quite marked. For instance in petunia (Dean et al., 1985b) the levels of rbcS mRNA in sepals, petals, stems, stigmas/anthers and roots are 11.2, 3.0, 2.3, 0.25 and 0.2% of those found in light-grown leaf tissue. In tomato (Sugita and Gruissem, 1987) rbcS mRNA is undetectable in roots and ripe fruit but is present in stem, immature fruit and etiolated seedlings at levels of 3.2, 6.5 and 4.0% respectively of those found in light-grown leaf tissue.

The levels of transcripts of different rbcS genes from the same multigene family have been measured in petunia (Dean et al., 1985b), pea (Fluhr et al., 1986b) and tomato (Sugita and Gruissem, 1987). In both petunia and pea, different rbcS genes showed variations in their relative expression patterns in different organs but, in these two species at least, the relative ranking of the different rbcS transcripts was the same in all the tissues studied. In tomato, on the other hand, different tissues show great variation in the relative ranking of the abundance of different rbcS transcripts. For instance in immature fruit, mRNA from the tomato rbcS-2 gene accounts for 60% of the total rbcS mRNA in this tissue, whereas rbcS-3B mRNA is not detected. In roots the relative levels of rbcS-2 and rbcS-3B are 18% and 45%, demonstrating dramatic changes in the relative abundances between different rbcS mRNAs in tomato.

The differences between the most expressed and least

expressed rbcS genes in different tissues are 25, 10 and 4-fold for petunia, pea and tomato respectively (Dean et al., 1989). Thus it seems that variable expression of the rbcS genes among plant organs is typical in higher plants, but the pattern of differential tissue-specific expression can vary dramatically between species (Manzara and Gruissem, 1988).

The kinetics of rbcS gene expression have also been shown to vary between plant cell-types. For instance in maize (Sheen and Bogorad, 1986) the transcripts of three rbcS genes are present in both bundle-sheath cells and mesophyll cells of etiolated maize leaves. The levels of each transcript differ in etiolated mesophyll cells. Upon illumination, the levels of all three transcripts drop in mesophyll cells, but follow a pattern of transitory rise and fall in bundle-sheath cells.

In plant transformation experiments using rbcS gene promoter and enhancer elements (section 1.4), tissue-specific control of expression is apparent in transformed callus tissue as well as whole transgenic plants (Herrera-Estrella et al., 1984; Simpson et al., 1986), with maximal levels of expression being found only in chloroplast-containing tissue.

The correlation of higher levels of rbcS gene expression in tissues containing chloroplasts seems to indicate that some factor is present in these tissues which in some way exerts control over rbcS gene expression, or at least modulates it in some way. The molecular basis for the tissue-specific control of rbcS expression has not been fully defined, however there is strong evidence that the export of a plastid factor is involved (Simpson et al., 1986; Taylor, 1989; section 1.3.1.3).

1.3.1.2 Developmental regulation

rbcS genes show distinct developmental regulation. During tomato leaf development, the level of rbcS gene expression changes such that although the rbcS-2 and rbcS-3B genes are the most highly expressed in immature leaves, the most highly expressed rbcS genes in mature tissue are rbcS-3B and rbcS-3C (Manzara and Gruissem, 1988). Developmental regulation of rbcS genes is also seen in tomato fruit development and ripening (Piechulla et al., 1986). Following pollination, the levels of rbcS mRNA in tomato fruit pericarp increases for 15 days, and then decreases, so that in red, ripe fruit rbcS mRNA is undetectable. This decrease is not thought to be an overall response to senescence as ripening-specific genes are activated at this time and the constitutive expression of other genes is maintained. In etiolated seedlings of amaranth (Berry et al., 1985) and maize (Nelson et al., 1984) the steady-state levels of rbcS mRNA increase independently of light during the development of the seedlings. In tomato (Manzara and Gruissem, 1988), a subset of the rbcS multigene family consisting of rbcS-1, -2 and -3A is responsible for expression of rbcS in etiolated seedlings, and rbcS1 in particular is thought to be developmentally regulated. In pea, the kinetics of the changes in the transcription of rbcS genes in response to light are substantially different in mature, green tissue to those observed in etiolated pea seedlings (Gallagher et al., 1985) This suggests that developmental factors may modulate the response of rbcS genes to other factors such as light.

Although transcriptional control seems to be the most important factor in controlling the levels of rbcS polypeptide, translational control of rbcS polypeptide levels has also been described. These levels seem to be developmentally controlled. For instance, in amaranth

cotyledons, levels of rbcS mRNA show little correlation to the amount of rbcS polypeptide (Berry et al., 1985). In this species a 3 day burst of rbcS polypeptide synthesis is observed a few days after germination whereas the levels of functional rbcS mRNA remain similar over the same timescale. This suggests, in this species at least, the involvement of developmental translational control in rbcS gene expression.

1.3.1.3 Role of plastid factors

As described in section 1.3.1.1, high levels of expression of rbcS genes are correlated to the presence of chloroplasts in the plant cell in which they are expressed. When mutations or herbicides (eg. norflurazon) which inhibit the accumulation of carotenoid pigments in higher plants grown in the light, chloroplast development is arrested at a very early stage due to the damage by photooxidation (Mayfield and Taylor, 1987). The extent of the damage depends upon the light intensity, and when carotenoid-deficient seedlings are grown in very dim light normal chloroplast development occurs. The conditional nature of carotenoid deficiency has been a useful experimental tool to gauge the extent of the involvement of chloroplast development in rbcS gene expression. Light conditions permissive for chloroplast development in carotenoid-deficient maize (Mayfield and Taylor, 1987), mustard (Oelmuller et al., 1986) and barley (Batschauer et al., 1986) promoted the normal accumulation of rbcS mRNA. When carotenoid-deficient maize or mustard plants are transferred to higher light intensities, rbcS mRNA drops to very low levels.

The involvement of a plastid factor in the regulation of rbcS gene expression is further supported by work on mustard seedlings (Oelmuller et al., 1986). Chloroplast ultrastructure develops in mustard cotyledons between 36

to 120h after germination. rbcS mRNA accumulates over the same period. The addition of chloramphenicol (to specifically inhibit plastid protein synthesis) at 36h after germination blocks both chloroplast development and rbcS mRNA accumulation. Addition of chloramphenicol 48h after germination has little effect on rbcS mRNA accumulation. These data suggest, therefore, that the plastid factor is synthesised at an early stage of development.

Photooxidative damage to the chloroplasts affects rbcS gene expression at a trancriptional level. This has been demonstrated by Simpson et al. (1986) who showed that the expression of chimeric gene constructs with rbcS promoters was blocked by treatment with norflurazon in normal light conditions, but chimeric constructs with nopaline synthase (nos) promoters were unaffected. Trancription assays have shown that rbcS transcription is blocked by photooxidation in tomato (Giuliano and Scolnik, 1988). On the other hand, rbcS transcription in barley is neither affected by norflurazon treatment nor in carotenoid-deficient mutants. It seems, therefore, that a plastid factor is involved in the modulation of the expression of rbcS genes, and this factor is either destroyed, or more likely its synthesis is inhibited, as a result of photooxidation (Taylor, 1988). The extent to which this factor affects rbcS gene expression varies considerably between species.

Chory et al. (1989) have characterised a mutant of Arabidopsis (det mutant) which exhibits the phenotype of a light-grown plant even when grown in darkness. These det mutants show normal leaf and chloroplast development even in darkness. Their chloroplasts are deficient of chlorophyll as light is a factor required for its biosynthesis. Wild-type Arabidopsis seedlings grown in darkness contain small amounts of rbcS mRNA but levels accumulate upon exposure to white light. The Arabidopsis det mutants contain levels of rbcS mRNA as high as

light-grown wild-type Arabidopsis irrespective of whether they are illuminated or not. Although it is possible that the det locus encodes a factor which is responsible for directly repressing rbcS gene expression in the dark, it seems more likely that the effect on rbcS gene expression is mediated by a plastid factor produced from the developing chloroplasts or a developmental factor produced as a result of leaf development.

1.3.2 Photoregulation

1.3.2.1 Effects of light

Light has a marked effect on plant gene expression. Lamb and Lawton (1983) describe a large number of gene products whose levels or activities are modified by light. This effect can also be seen to influence mRNA levels. DeVries et al. (1982) showed differences in 30 different translatable mRNA species in response to light. The levels of rbcS mRNA have been shown to be dramatically stimulated by light in several species (Tobin and Siverthorne, 1985). However there are great differences in both the extent and nature of this response between different higher plant species. For instance the level of rbcS transcripts in barley is hardly affected by light (Batschauer et al., 1986). This contrasts the situation in pea where illumination of etiolated seedlings with white light brings about a 100-fold increase in rbcS transcript levels (Ellis et al., 1984).
In pea, rbcS gene transcription shows three distinct kinetic responses to light (Gallagher et al., 1985). The first is a short, transient increase observed when dark-grown seedlings are first exposed to white light. The second response is a slow, steady increase in transcription to a maximum rate after about 36h. The third type of response is a 'rapid switch' in

transcription observed when plants transcribing rbcS genes at maximal rates are transferred to darkness and subsequently returned to white light. When pea plants are transferred to darkness for 5h, transcription of rbcS genes decreases by approximately 75%. Maximal rates of transcription are restored by re-illumination for 20 minutes. These data suggest that these plants are rendered 'competent' to transcribe maximally during the slow increase in transcription during greening. The drop in the rate of transcription after transfer to darkness is much lower in pea seedlings exposed to red light as opposed to white light (Barnett et al., 1987). Soybean, in contrast to pea, shows no transient increase and the increase to maximal rates is more rapid (Berry-Lowe and Meagher, 1985). The initial transient increase in rbcS transcription has been observed at the transcript level (Ellis et al., 1984; Scharnhorst et al., 1985; Kaufman et al., 1986).

As well as changes in transcription, the effect of transcript stability on the levels of rbcS transcripts must be taken into account. Differences in rbcS transcript stability under varying conditions have been observed by Barnett et al. (1987). Ernst et al. (1987) showed that light increases the transcription of rbcS genes 3-fold less than the increase in rbcS transcript levels, indicating some form of post-transcriptional control, most probably at the level of RNA stability.

1.3.2.2 Photoreceptors

The use of the red/far-red photoreversibilty test has shown that the light-induced changes in rbcS gene expression when dark-grown plants are first illuminated are mediated by phytochrome (Tobin and Silverthorne, 1985; Thompson, 1985; Kuhlemeier et al., 1987a; Jenkins, 1988). In pea, phytochrome mediates two effects on rbcS

transcription when dark-grown plants are first illuminated, an initial increase and induction of the capacity to subsequently transcribe rbcS genes maximally in white light (Barnett et al., 1987). Kaufman et al. (1984; 1985) investigated the fluence response relationships of rbcS transcript accumulation mediated by phytochrome in pea. They found that illumination at very low fluence (VLF; threshold 10^{-4} µmol m^{-2}) although capable of inducing cab expression was not able to induce rbcS expression. On the other hand low fluence (LF; threshold 1µmol m^{-2}) was capable of inducing rbcS gene expression.

The role of phytochrome in the control of rbcS gene expression seems to decrease with the maturity of the plant (Jenkins and Smith, 1985) as a wide variation of the red:far-red ratio in a white light background has a negligible effect on the abundance of rbcS transcripts.

Differences in the relative amounts of photoreceptors between different tissues may account for developmental changes in light-mediated rbcS gene expression (section 1.3.1.2) For instance, the ratio between the two immunologically distinct forms of phytochrome (phytochromes I and II) varies substantially between dark-grown leaves and green leaves (Abe et al., 1985; Shimazaki and Pratt, 1985; Tokuhisa et al., 1985; Jordan et al., 1986). This may be correlated with the observation that large changes in the phytochrome equilibrium have no significant effect on the level of rbcS transcripts in light-grown pea plants (Jenkins and Smith, 1985), whereas rbcS trancription is markedly stimulated by red light in etiolated pea seedlings (Barnett et al., 1987). In the mature, green leaves of P. vulgaris increases in light quantity substantially increase the levels of rbcS mRNA (Jenkins, 1986). This suggests that, in this species at least, a photoreceptor capable of detecting light quantity rather than light

quality plays a dominant role in the control of rbcS genes in green tissue. The identity of the photoreceptor that mediates this fluence-dependent control in green tissue is not yet known, however a blue-light photoreceptor is implicated (Jenkins, 1988). In pea, Fluhr and Chua (1986) and Fluhr et al. (1986b) have shown that a blue-light photoreceptor acting in conjunction with phytochrome mediates rbcS transcript accumulation in greened leaf tissue. However, in these experiments, the greened plants were dark-adapted for 4 days prior to illumination so that rbcS transcript levels could be measured, and therefore these results may not reflect the true situation in greened, light-grown plants.

1.3.2.3 Differential expression within multigene families

As well as variations in rbcS gene expression in response to light between different species, variations arise between different members of the same rbcS multigene family. This has been described in Lemna gibba (Tobin et al., 1985), Petunia (Dean et al., 1985b; Tumer et al., 1986), pea (Fluhr et al., 1986b) and tomato (Sugita and Gruissem, 1987). In pea, two of the five rbcS genes (rbcS-3A and rbcS-3C) expressed account together for approximately 75% of the accumulated rbcS mRNA in light-grown leaves. In petunia, expression of all 8 rbcS genes is detected in light-grown leaf tissue, and the relative abundance of the different rbcS transcripts varies between 47.3% (SSU301) and less than 0.5% (SSU991) of the total level of rbcS transcripts (Dean et al, 1985b). Similarly, in pea (Fluhr et al., 1986b), the levels of expression of 5 rbcS genes in light-grown leaf tissue vary between 41% and 7%. In tomato (Sugita and Gruissem, 1987), the levels of the mRNAs of the 5 rbcS genes are quite similar in light-grown leaf tissue with levels of 10, 15, 11, 29 and 35% of total rbcS mRNA for rbcS-1, -2,

-3A, -3B and 3C respectively. Clearly then, light affects the expression of the different members of rbcS multigene families to different extents.

Qualitative differences in expression are also observed in pea (Fluhr et al., 1986b). Two pea rbcS genes are expressed earlier during the greening of pea seedlings than the other three genes in the same multigene family. Similar qualitative differences are reported for tomato (Sugita and Gruissem, 1987). Two of the tomato rbcS genes (rbcS-3B and rbcS-3C) produce transcripts which are highly abundant in light-grown leaf tissue but undetectable in dark-grown leaf tissue. In contrast, the transcripts from two other tomato rbcS genes (rbcS-1 and rbcS-3A) are easily detected in dark-grown leaf tissue but show only slight increases in abundance in response to illumination.

1.4 Regulatory elements of rbcS genes

Much progress has been made in recent years in identifying and characterising the regulatory elements involved in the control of rbcS gene expression. This success has stemmed from the development of plant transformation vectors based on the natural functions of the Ti (tumour-inducing) plasmid of Agrobacterium tumefaciens. These vectors have allowed the stable introduction of chimeric plant genes into the genomic DNA of plant cells. These chimeric constructs produced by the fusion of putative photoregulatory promoter and enhancer sequences to easily assayable 'reporter' coding sequences allow the expression of the genes to be monitored in both callus cells and regenerated transformed plants. In this way, various constructs have been made to test the activity of possible regulatory sequences from rbcS genes.

1.4.1 5' flanking regions

Broglie et al. (1984) first transferred a pea rbcS gene (rbcS-E9) into Petunia cells and monitored expression in callus cell cultures of the transformed cells. They observed that this gene was faithfully transcribed in these calli in a light-dependent manner similar quantitatively to that observed in pea. Herrera-Estrella et al. (1984) showed that 970bp of 5' flanking sequences of the pea rbcS-ss3.6 gene were sufficient to drive the light-regulated transcription of a bacterial chloramphenicol acetyltransferase (CAT) coding sequence fused to it. Similarly the 5' flanking sequences of a soybean rbcS gene were demonstrated to confer photoregulation of the transcription of a kanamycin resistance coding region in soybean callus cells (Facciotti et al., 1985). The 5' flanking sequences of the pea rbcS-E9 gene were further dissected by monitoring the expression of 5' flanking region deletion mutants. This was performed in an attempt to more clearly define the functional elements contained within the 5' flanking sequences of rbcS-E9 (Morelli et al., 1985). These sequences were shown to contain two regions involved in controlling expression. The first was located between -35 and -2 and contained the TATA box for the rbcS-E9 gene. This region conferred the two levels of regulated transcription of the rbcS-E9 gene which had been previously described. The second region was further upstream and contained an element which was necessary for maximal expression of the rbcS-E9 gene.

Subsequent studies of photoregulatory DNA sequence elements employed 'disarmed' non-tumourigenic Ti-plasmid-based plant transformation vectors. This allowed studies of expression to be made in whole, regenerated transgenic plants as opposed to callus tissue, and so allowed the monitoring of the expression of

chimeric genes in a more developmental context.

The 5' flanking regions of several other rbcS genes were subsequently shown to drive the photoregulated transcription of reporter coding sequences in regenerated transformed plants (Schreier et al., 1985; Fluhr et al, 1986a; Simpson et al., 1986; Poulsen et al., 1986; Kuhlemeier et al., 1987b; Shirley et al., 1987). In most cases expression of these transferred gene-constructs showed the correct pattern of tissue-specific expression as well as photoregulation. Two regulatory elements were characterised in the pea rbcS-ss3.6 gene (Timko et al., 1985). In this case, the upstream sequences between -90 and -973 were found to function in either orientation in conferring light regulated expression of a fused reporter coding region in transformed callus cells. This feature is a characteristic of eukaryotic enhancer elements (Serfling et al., 1985), and so elements present in rbcS genes with this property are termed 'enhancer-like elements'. Such an element from the pea rbcS-3A gene (-347 to -48) was fused to a truncated cauliflower mosaic virus (CaMV) 35S promoter containing a TATA box, and together these sequences were used to drive the transcription of the coding region of a CAT gene (Fluhr et al., 1986a). This element, in either orientation, was sufficient to confer light-regulated expression of the reporter sequences. An 'enhancer-like element' from the pea rbcS-E9 gene from -317 to -82 functioned similarly, and was shown to contain the sequences necessary for the phytochrome-mediated regulation of rbcS gene expression in dark-grown transgenic tobacco seedlings.

The 'enhancer-like element' from the pea rbcS-3A gene was dissected even further (Kuhlemeier et al., 1987b). This element was found to contain both positive and negative photoregulatory elements. The region from -169 to -112 contains three sequence elements termed 'boxes' I, II and III. These three elements are conserved in all pea rbcS

genes (Fluhr et al., 1986a; Kuhlemeier et al., 1987b). Boxes II and III show certain homology to viral and mammalian enhancer elements. A 58bp sequence containing all three boxes fused to a CaMV 35S promoter was found to be unable to drive the transcription of a fused CAT coding region. One or several copies of box II alone also failed to drive transcription. The inclusion of sequences from further upstream in the chimaeric construct allowed expression of the reporter gene so it was concluded that the three elements alone were not sufficient. When the 58bp sequence containing the three boxes was placed between the constitutive CaMV enhancer and the CaMV 35S promoter, expression of the reporter gene was suppressed in darkness. Boxes II and III were shown to be specifically involved in this phenomenon, thus effectively conferring photoregulation of transcription.

Deletions of the rbcS-3A flanking sequences were made and expression relative to an intact rbcS-3A gene introduced on the same vector was compared in an attempt to identify positive regulatory elements. A -166 deletion mutant showed photoregulated expression whereas a -149 deletion mutant did not. The region between -166 and -149 contains box I (whose function has not been determined) and part of box II. Elements with homology to boxes II and III (termed boxes II* and III*) are found further upstream (between -250 and -210). When boxes I, II and III are deleted boxes II* and III* are able to confer photoregulation of gene expression.

The promoter regions of the two most abundantly expressed petunia rbcS genes SSU301 and SSU611 show sequence homology, in their 5' flanking sequences, which is not present in other petunia genes (Dean et al., 1988). Furthermore, this homology is also present in two of the tomato rbcS genes, rbcS-2 and rbcS-3a, the Nicotiana tabacum NtSS23 gene and the Nicotiana plumbaginifolia gene rbcS-8B (Dean et al., 1989). Specific sequences from the

5' flanking sequences of the petunia SSU301 gene (the most strongly expressed) were fused to equivalent positions in the SSU911 (the most weakly expressed gene) promoter and expression of these constructs was analysed in transgenic tobacco plants (Dean et al., 1989). An 80 bp fragment was identified which acted as an 'enhancer-like' element. This fragment contained the promoter sequences also present in the petunia SSU611 gene and the tomato and tobacco genes with homologies to SSU301 described above.
Poulsen et al. (1986; 1988) carried out deletion analysis on the 5' flanking sequences, from -1038 to +32, of the rbcS-8B gene of N. plumbaginifolia . They found that this fragment contained two 'enhancer-like' elements. A proximal element, located between -312 and -102, confers organ-specific and light-inducible expression. A distal element, located between -1038 and -589, has enhancer-like characteristics if a basic promoter (eg. 35S CaMV promoter) is present in the construct. This element also confers organ-specificity on the heterologous promoter but not light-inducibility.
5' flanking region elements have also been shown to confer light inducibility of the soybean SRS1 gene (Facciotti et al., 1985) and the light-regulated and tissue-specific expression of the Ats1-A gene of Arabidopsis (Dean et al, 1989).

1.4.2 3' flanking regions and introns

In petunia, sequences both 5' and 3' to the translation initiation codon contribute to the quantitative differences in the expression of the genes (Dean et al., 1988a; 1988b). These workers investigated which sequences 3' to the translation initiation codon were causing these effects by constructing a series of fusions that exchanged the sequences of SSU301 (a three intron rbcS gene) and SSU911 (which has two introns) within each intron and

immediately following the end of the coding region. Another construct of SSU301 was made without introns. The expression of these constructs was assayed in transgenic tobacco plants. The resultant data suggest that sequences downstream of the coding region and sequences within the introns themselves were contributing to the differential expression of the rbcS genes of petunia (Dean et al., 1989). Further work measuring rbcS transcription directly in petunia has indicated that the 3' untranslated sequences of the petunia rbcS genes affect trancriptional rates (Dean et al., 1989).

1.4.3 DNA-binding factors

DNase 1 footprinting and gel-retardation assays have been used to detect the binding of a nuclear factor (GT-1) to the rbcS-3A enhancer-like element (Green et al., 1987). GT-1 has multiple binding sites in the enhancer-like element of rbcS-3A, binding to boxes II, III, II* and III*. It showed little affinity for box I. This factor bound with different affinities to the different types of box II and box III element, as determined by competition experiments. By using methylation interference, it was shown that specific G residues within the boxes were essential for binding to GT-1 (Green et al., 1988). GT-1 binding activity was shown to be present in both light and dark, although it was lower in the dark than in the light. This seems to suggest that light mediates the expression of the rbcS-3A gene by altering the activity of the GT-1 protein rather than by regulating the amounts that are present. Complexes of this factor with DNA from both light and dark-adapted plants show slightly different gel-mobilities which might support the view that the factor itself is in some way modified. As the different boxes of the enhancer-like element of the rbcS-3A gene overlap (Kuhlemeier et al., 1987) it has not been possible

to determine whether GT-1 acts as a positive or negative regulatory factor. GT-1 may act as a general factor for different light-regulated plant genes and 7 distinct GT-1 binding sites have been found in the Cab-E gene of Nicotiana plumbaginifolia (Schindler and Cashmore, 1989). In this case competition studies have shown that these DNA binding sites bind GT-1 with different affinities and at least two such sites need to be present to allow binding. Other 'boxes' within rbcS gene promoters to which factor-binding has been demonstrated are 'G-boxes' (Giuliano et al., 1988; Schulze-Lefert et al., 1989) and the 'AT-1 boxes' (Datta and Cashmore, 1989). The protein which binds to 'G-boxes' is known as GBF and it binds to the 'G-box' of most rbcS, Adh and Chs promoters (Giuliano et al., 1988; Schulze-Lefert et al., 1989) and to the Cab-E promoter of N. plumbaginifolia (Schindler and Cashmore, 1989). Trancription factors isolated from yeast and mammalian nuclei have been shown to bind to similar sequence motifs (Beckmann and Kadesch, 1989) and Schindler and Cashmore (1989) report that the methylation of identical G-residues interferes with the binding of the yeast and plant factors. AT-1 is a DNA-binding protein which binds to specific AT-rich elements (the AT-1 box) within the promoters of the pea rbcS 3.6, tomato rbcS 3A and tobacco cab E genes (Datta and Cashmore, 1989). The binding activity of AT-1 is reversibly modulated by phosphorylation, DNA-binding activity being lost as a result of phosphorylation. Datta and Cashmore (1989) report that the kinase involved in this process has the characteristics of a NII type casein kinase. Analysis of the promoter of the tomato rbcS 3A gene suggests that AT-1 might be a positive regulatory element (Ueda et al., 1989)

1.5 Summary

In the majority of higher plant species studied, rbcS genes are present as small multigene families. Individual members of these families tend to be linked in the plant genome. rbcS polypeptides show a high degree of protein sequence homology, and this homology is very high between rbcS polypeptides produced in the same plant cell, implying the concerted evolution of the different members of rbcS multigene families.

rbcS gene expression is highest in chloroplast-containing tissue and the involvement of a plastid factor in the regulation of rbcS gene expression is implicated.

In many species light has a marked effect on rbcS gene expression. In species in which the effect of different light qualities on rbcS gene expression has been studied, it has been shown that rbcS genes are regulated by phytochrome at the seedling stage, but that after the plants have matured, some if not all rbcS genes are regulated via a blue-light receptor.

The control of rbcS polypeptide levels is thought to be mainly at the level of transcription. 5' and 3' flanking sequences as well as intronic sequences of rbcS genes have been implicated in this control of tissue-specific and photoregulated expression .

Discrete elements within the 5' flanking sequences which control rbcS gene expression have been identified, as have some of the protein factors which bind to them. Current research is involved in characterising these trans-acting factors further, and in investigating the levels at which their own activities are controlled.

1.6 Aim of project

Preliminary studies of the effect of light on the accumulation of rbcS transcripts in the primary leaves of P. vulgaris indicate that the stringency of photoregulation is not as strict as in many other species such as pea (G.I. Jenkins, unpublished work). Quite a substantial amount of rbcS transcripts is present in the primary leaves of dark-grown P. vulgaris seedlings, and this is readily detectable in northern blot analysis. In pea, rbcS transcripts are barely detectable in comparable tissue using the same analysis. Furthermore the light-stimulated accumulation of rbcS transcripts is much less marked than in pea. This relatively high level of expression in the dark raises the question of whether all the rbcS genes in P. vulgaris are equally and loosely regulated by light or whether they respond differentially in light- and dark-grown tissue. The primary leaves of P. vulgaris were chosen for this study as they provided a good developmental system for more longer term studies of rbcS gene expression. The primary leaves are distinct in morphology from the subsequent trifoliate secondary leaves, so can be readily identified throughout the development of the plant. The primary leaves are exposed throughout their development following germination, allowing illumination experiments to be performed throughout the lifespan of the leaf (about 40 days from germination to senescence). Furthermore, cell division ceases early in the development of the primary leaves, most leaf growth being due to cell expansion (Volkenburg and Cleland, 1979; 1980; 1981), thus providing a simpler system for study. The aim of the project described in this thesis was to investigate the structure and possible differential expression of the rbcS genes of P. vulgaris. Analysis of gene structure was a prerequisite to enable the production of probes suitable for use in studying rbcS

transcript levels. Measurements of total rbcS transcripts were to be made as well as measurements of the levels of individual rbcS transcripts from the different rbcS genes of P. vulgaris. The long term aim of this work (beyond this project) was to elucidate the reasons for the differences in the expression of the rbcS genes of P. vulgaris with respect to the rbcS genes of other species, as well to elucidate the reasons for any differences observed in this study between the members of the rbcS multigene family of P. vulgaris. This continuing work would attempt to correlate such differences in the expression of the rbcS genes of P. vulgaris to differences in the regulatory sequences of the genes involved.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1. Chemicals

Analar (BDH Ltd., Poole, Dorset) and Formachem (Formachem Research International, Strathaven, Strathclyde) chemicals were used throughout unless otherwise specified.

2.1.2. DNA- and RNA-modifying enzymes

All restriction endonucleases (isolated from various sources) and T4 DNA ligase (isolated from *E. coli* lysogen NM989) were supplied with their reaction buffers by Gibco-BRL, Paisley, Strathclyde. Polynucleotide kinase (isolated from T4 phage-infected *E. coli*, >40 kU/mg) was supplied by BCL, Lewes, E.Sussex. S1 nuclease was supplied either by BCL or Gibco-BRL (both isolated from *Aspergillus oryzae*). Klenow polymerase was either supplied by BCL (sequencing grade isolated from *E. coli* lysogen NM964 at 1U/µl) or Amersham International, Amersham, Bucks (from cloned Klenow fragment; at 1U/µl and 5U/µl). DNase 1 was either supplied by Cooper Biomedical, Worthington Products, New Jersey, USA (specially purified from bovine pancreas by chromatography to reduce RNase and protease levels, 1kU/mg) or Amersham International (standard grade). RNase A (purified from bovine pancreas type XIIA, 118kU/mg) was supplied by Sigma, Poole, Dorset. DNA polymerase 1 (isolated from *E. coli*) was supplied by Amersham International. Sequenase Version 2.0 (modified T7 DNA polymerase) was supplied by UBB, Ohio, USA.

2.1.3 Nucleotides and radio-labelled nucleotides

[α-^{32}P]dCTP (3000Ci/mmol), [α-^{32}P]dATP (3000Ci/mmol), [α-^{32}P]$_{\wedge}^{dATP}$ (>400Ci/mmol) and [γ-^{32}P]ATP (>5000Ci/mmol) were all supplied in aqueous solution with 2-mercaptoethanol (5mM) at 10mCi/ml by Amersham International.
[α-^{35}S]dATP (>600Ci/mmol) was supplied in aqueous solution with 20mM DTT at 10mCi/ml by Amersham International.
2'-deoxy-adenosine-5'-triphosphate (dATP),
2'-deoxy-cytidine-5'-triphosphate (dCTP),
2'-deoxy-guanosine-5'-triphosphate (dGTP),
2'-deoxy-thymidine-5'-triphosphate (dTTP) and
adenosine-5'-triphosphate (ATP) were all supplied as crystaline disodium or dilithium salts by BCL.

2.2 Plant material

Phaseolus vulgaris cv. 'Tendergreen' seeds were supplied by Booker Seeds, Sleaford, Lincs. Germination was acheived by planting seeds at a depth of approx. 2cm in John Innes no.2 compost. All plants were germinated and grown at 20°C.

2.3 Bacterial strains

E. coli TG1 (derivative of JM101 (lacpro, thi, supE, F' traD36, proAB, lacI^{q}ZΔM15; Messing et al., 1981) modified by T. Gibson (unpublished) to give additionally hsd 5(EcoK r$^-$ m$^-$)) was used for transformation with M13 RF DNA ligations prior to DNA sequencing (section 2.20), as well as a general recipient for plasmid DNA to provide cells for plasmid DNA preparations.
The P. vulgaris var. 'Tendergreen' cDNA plasmid clones (provided by Dr J. Cullimore, Warwick University; section 2.4) were contained in an E. coli JM83 host (Lightfoot et al., 1988), from which plasmid DNA was regularly prepared.

2.4 Plasmids and phage DNA

Table 1 describes the plasmids used during the course of this project. Strains containing these plasmids were stored as slopes or stabs (section 2.6.2) for several months. Plasmid DNA was prepared as in section 2.9.2.1 and stored at -20°C ready for bacterial cell transformation (section 2.20.5) to provide longer term storage.
M13mp18 and M13mp19 (Yannisch-Perron et al., 1985) RF DNA and phage DNA was supplied by Amersham International.

2.5 Synthesis of oligonucleotides

Single-stranded DNA oligonucleotides were synthesised by Dr. V. Math of the Department of Biochemistry, University of Glasgow, using an Applied Biosystems oligonucleotide synthesiser.

2.6 Growth media

2.6.1 Liquid media

L-broth (1% (w/v) bacto-tryptone (supplied by Difco Laboratories, Michigan, USA), 1% (w/v) NaCl, 0.5% (w/v) yeast extract (supplied by Difco), pH 7.3) was used with appropriate antibiotic for the culture of E. coli for plasmid DNA preparations (section 2.9.2).

2xTY medium (1.6% (w/v) bacto-tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) was used for all cultures of E. coli TG1 (section 2.20).

Both these liquid media were sterilised by autoclaving at 15psi for 20 min as in section 2.7.2.1.

TABLE 1

PLASMID	DESCRIPTION	source
pPvSS191 through to pPvSS2415	cDNA clones in pUC8 from a library made to polyA$^+$ RNA from light-grown primary leaves of *P. vulgaris*. The clones selected hybridise to pea *rbcS* sequences (pSSU60, pSSU160; figure 1)	Gifted by Dr J.Cullimore (University of Warwick)
pSSU61 & pSSU161	Subclones of the pea *rbcS* cDNA clone pSSU1 (figure 1)	L.K.Barnett & G.I. Jenkins (Unpublished)
pUC8	Vector with multi-restriction sites used for construction of pBS1 and to produce DNA size markers.	Vieira et al. (1982)
pAT153	Used for the production of DNA size markers	Twigg & Sherratt (1980)
pBS1	Subclone of the *P. vulgaris rbcS* cDNA clone pPvSS1672 in pUC8 (figure 15)	M.R. Knight, A-M. Clark& G.I.Jenkins (Unpublished)

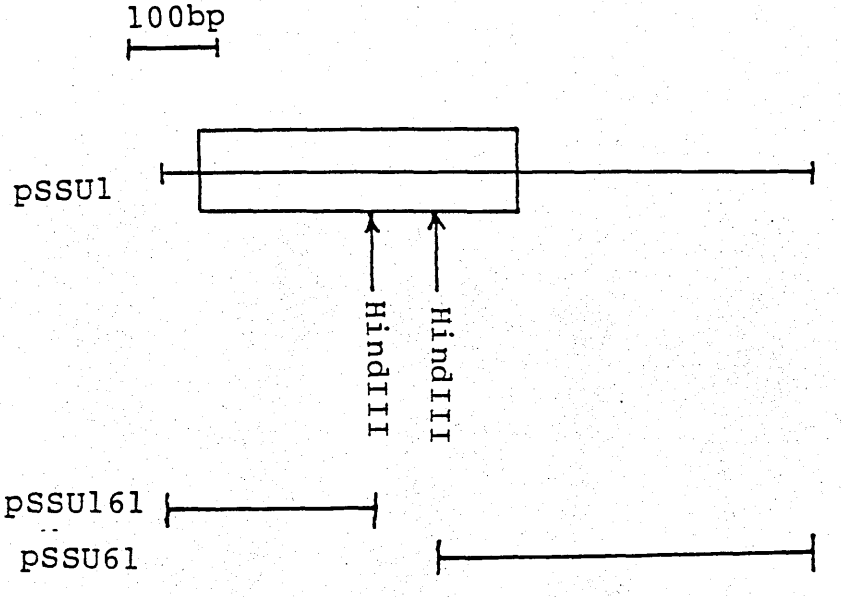


Figure 1 - subclones of pea rbcS cDNA clone used as probes

Physical map of the pea rbcS cDNA clone pSSU1 (Bedbrook et al., 1980) and subclones pSSU161 and pSSU61. The boxed region represents the coding region for the mature polypeptide. pSSU1 is orientated 5'---->3' (left to right).

2.6.2 Solid media

L-agar was made by adding bacto-agar to L-broth (section 2.6.1) to various concentrations prior to autoclaving at 15psi for 20 min (section 2.7.2.1). Agar was added to 1.5% (w/v) for plates which were used to plate out *E. coli* to provide fresh cells to inoculate a liquid culture; 0.8% (w/v) for 'stabs' and 2.5% (w/v) for 'slopes' in bijoux bottles which were used to preserve bacterial strains for a few months.

H-plate agar (1% (w/v) bacto-tryptone, 0.8% (w/v) NaCl, 1.2% (w/v) bacto-agar) and H-top agar (1% (w/v) bacto-tryptone, 0.8% (w/v) NaCl, 0.8% (w/v) bacto-agar) were sterilised as above and used for the plating out of M13 phage (section 2.20.6).

Glucose/minimal medium plates were used to plate out *E. coli* TG1 prior to inoculation of liquid medium (2xTY). The following reagents were autoclaved separately at 15psi for 20 min, with the exception of the glucose which was autoclaved at 5psi for 50 min:

(i) Oxoid agar in water (15g/900ml)
(ii) 1M $MgSO_4$
(iii) 0.1M $CaCl_2$
(iv) 1M thiamine HCl
(v) 20% glucose (w/v)
(vi) 10xM9 salts (6% Na_2HPO_4 (w/v), 3% KH_2PO_4 (w/v), 1% NH_4Cl (w/v), 0.5% NaCl (w/v))

The agar was allowed to cool to 50°C, and the other reagents to room temperature, and then they were mixed aseptically in the volume to volume ratio 900:100:1:1:1:10 (i:ii:iii:iv:v:vi).

2.6.3 Antibiotics

Both ampicillin and chloramphenicol were supplied by the Sigma Chemical Co.
Ampicillin at 25mg/ml in water, filter-sterilised by being passed through a Flowlabs filter (pore diameter 0.2μM), was added to L-broth and L-agar when required after allowing the media to cool to or below 50°C. The final concentration of ampicillin was 50μg/ml.
Chloramphenicol (1.75% (w/v) in ethanol), freshly prepared and filter-sterilised, was used to amplify plasmids before large-scale plasmid DNA isolations (section 2.9.3).

2.7 General Methods

2.7.1 Measurement of pH

Measurement of pH was carried out using Corning 220 and Radiometer 26 pH meters as per operating instructions.

2.7.2 Sterilisation of solutions and equipment

2.7.2.1 Autoclaving

Sterilisation of heat-stable solutions and plastic equipment such as pipette-tips and microcentrifuge tubes was carried out at 15psi for 20 min in a Lab Thermal Equipment 225E autoclave or in small batches in a Prestige Hi-Dome pressure cooker.

2.7.2.2 Filter-sterilisation

Heat-labile solutions were sterilised by passing them from a syringe through a Flowlabs filter (pore diameter, 0.2μm) into a sterile receptacle.

2.7.2.3 Glassware

Glassware was sterilised by baking in a dry-oven at 180°C for 12h.

2.8 Illumination of plants

Plants to be illuminated with white light were grown beneath banks of Osram 45W 'warm white' fluorescent tubes at a height which gave the desired irradiance; usually 100-200 µmol $m^{-2}s^{1}$ (400-700 nm).
Plants to be illuminated with red or far-red light were placed in a light-tight cabinet constructed with a hole in the top to allow the attachment of appropriate interference filters (Balzers Aktiengessellschaft, Liechtenstein). Light from Rank Aldis Tutor 2 335W projectors was then passed through these filters to acheive illumination. Red light was at 662nm (λmax; half-band width 15nm), 15 µmol $m^{-2}s^{-1}$ and far-red light was at 735nm (λmax; half-band width 15nm), 8 umol $m^{-2}s^{-1}$. Light intensity was measured using a Li-Cor LI-185B quantum sensor.
Dark-grown plants were grown within light-tight wooden cabinets which were in turn housed within a light-tight growth room.

2.9 DNA preparations

2.9.1 Plant genomic DNA preparation

The method used was a plant genomic DNA 'miniprep' as described by Dellaporta *et al.* (1983), with slight modifications.

P. vulgaris seeds were sown in trays and germinated in the

dark at 20°C. After six days, primary leaf tissue was harvested; for each preparation 2g of tissue was weighed, wrapped in aluminium foil and quick-frozen in liquid nitrogen. The tissue was then ground into a fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a 100ml Prepspin centrifuge tube with a screwcap using a spatula cooled in the liquid nitrogen. To the tissue was added 45ml of plant DNA extraction buffer (100mM Tris pH 8.0, 50mM Na_2EDTA pH 8.0, 500mM NaCl, 10mM 2-mercaptoethanol (supplied by Koch-Light)), followed by 3ml 20% SDS. The contents of the tube were mixed thoroughly by vigorous shaking and were then incubated at 65°C for 10min, after which time 15ml 5M potassium acetate was added. Once again the contents of the tube were vigorously mixed and placed on ice for 20min. The tube was then centrifuged at 16krpm (approx. 80 000x*g*) for 20min in a Prepspin 50 centrifuge using a 6x100ml rotor. The supernatant was poured through two layers of sterilized muslin into a fresh 100ml Prepspin tube containing 30ml isopropanol. The contents of this tube were mixed and incubated at -20°C for 30min. Nucleic acid was pelleted by centrifugation at 13krpm (approximately 65 000x*g*) for 15min in the same rotor as before. The supernatant was poured off and the pellet dried by draining the tube inverted over some paper towels. The pellet was resuspended in 2.1ml of 50mM Tris, 10mM $Na_2EDTA_{\wedge}^{pH\,8.0}$ by allowing it to dissolve at 65°C (this often took several hours, and was often left overnight). From this point on, any handling of the DNA solution involved the use of pipette tips with their ends cut off to give a reasonably wide bore, so as not to shear the genomic DNA. The DNA solution was transferred to a 15ml Corex tube and was centrifuged briefly in a MSE High Speed 18 centrifuge at 10krpm (15 000x*g* to pellet out debris. Once transferred to a fresh 15ml Corex tube, 225µl 3M NaOAc pH 6.0 and 1500ul isopropanol were added, and the

contents of the tube mixed very gently. The DNA was then pelleted by centrifugation once again at 10krpm for 10 min, the pellet washed in 80% EtOH and then dried by allowing the tube to drain off inverted over paper towels for about 1h. This pellet was allowed to rehydrate in 300ul 1xTE (10mM Tris, 1mM Na_2EDTA pH 8.0) for 1h at 4^oC, and then resuspended by very light vortexing. RNase A was included (6kU/µg DNA) in subsequent restriction digests of DNA isolated in this way to digest the RNA. No evidence of nuclease contamination of the DNA preparation was detected using this protocol. The size of the DNA obtained was in excess of 50kbp as shown by comparison to undigested DNA on a standard agarose gel (section 2.13.1).

2.9.2 Small-scale plasmid DNA preparations

2.9.2.1 Birnboim and Doly

This preparation is as described by Birnboim and Doly (1979) and modified by Maniatis (1982).
Overnight cultures of *E. coli* grown in universal bottles were dispensed as 1.5ml aliquots to 1.5ml microcentrifuge tubes. These were spun for 5 minutes in a microfuge. The supernatant was discarded and the pellets were resuspended in 95µl ice-cold GET (50mM glucose, 10mM Na_2EDTA, 25mM Tris-HCl pH 8.0) by vortexing gently and 5µl of lysozyme (isolated from chicken egg white, 53.3 kU/mg, supplied by Sigma) in GET (40mg/ml; made freshly) was added. The tubes were then left on ice for 5 min after which time 200µl 0.2M NaOH, 0.1% SDS was added. The tubes were then left on ice for a further 5 min after which time 150µl 3M NaOAc (pH 4.5) was added and the tubes were vortexed thoroughly. After 10 min on ice the tubes were centrifuged as before for 10 min. Supernatants were transferred into fresh tubes containing 200µl of

re-distilled phenol saturated with TE, and 200µl chloroform : iso-amyl alcohol (24:1 v/v). The contents were then mixed thoroughly and centrifuged once again for 5 min. Upper aqueous phases from each of the tubes were transferred into fresh tubes containing 600µl of chloroform : amyl alcohol. The contents were mixed thoroughly and then centrifuged as before for 5 min, after which time the upper aqueous phases were once again removed into fresh tubes containing 600µl of diethyl ether. After thorough mixing and centrifugation for 5 min as before, the upper ethereal layer was discarded and 5µl of RNaseA (5mg/ml) was added and the tubes were incubated at 37°C for 15 min. Ethanol was then added (2 volumes) and the DNA precipitated by leaving the tubes at -20°C overnight or on cardice for 10 min. The pellets were washed in 80% ethanol and dried *in vacuo* to remove excess ethanol. The pellets were then resuspended in either 1xTE or SDW.

2.9.2.2 'Zippy' preparation

The method used here was as described by Holmes and Quigley (1981) modified by Maniatis (1982).

Overnight cultures of *E. coli* grown in universal bottles were dispensed as 1.5ml aliquots to 1.5ml microcentrifuge tubes, and the bacterial cells pelleted by spinning in a microfuge for 2min. The bacterial pellet was resuspended in 350µl STET (8% sucrose, 5% Triton X-100, 50mM Na_2EDTA, 50mM Tris-HCl pH 8.0). To this was added 25µl 10mg/ml lysozyme (Sigma), and the samples were boiled for 45s. The tubes were spun in a microfuge for 5min, after which time the pellets were removed using sterile cocktail sticks. The plasmid DNA was precipitated by the addition of 350µl isopropanol and incubation at -20°C for 30min, and was spun down in a microfuge for 10min, washed in 80%

ethanol, dried and resuspended in TE_2 (10mM Tris, 2mM Na_2EDTA). Plasmid preparations were stored at -20°C, and RNase A was added to any subsequent restriction digest.

2.9.3 Large-scale plasmid DNA preparation

The method used was a scaled-up version of the method described by Birnboim and Doly (1979).

Overnight cultures (10ml) of *E. coli* were added to separate 2l conical flasks containing 750ml sterile L-broth with appropriate antibiotic. The contents were incubated, shaking, at 37°C, until the culture had an optical density of about 0.8 at 600nm against a blank of medium (after an incubation of approx. 8h). To each culture was added 7.5ml 1.75% chloramphenicol in ethanol (w/v, freshly prepared) and the cultures were incubated, shaking, at 37°C once again overnight to amplify the plasmids.

Each culture was poured into a sterile one-litre polypropylene centrifuge bottle with a screw cap and spun at about 3000rpm (approx. 5000xg) in a MSE 6LB centrifuge for 20min. The supernatants were discarded and the pellets well drained. The pellets were resuspended in 3.2ml GET and put on ice. The suspensions were transferred to separate NaOH-washed 50ml polypropylene centrifuge tubes and 200µl 40mg/ml lysozyme in GET (made freshly) was added to each. The contents of the tubes were then incubated on ice for 30min, and 6.6ml NaOH, 0.1% SDS was added and mixed by vortexing. The tubes were left on ice for a further 15min. To this mixture was added 5ml 3M NaOAc pH 4.5 followed by vortexing and an incubation on ice for 30 min. The tubes were then spun in an MSE High Speed 18 centrifuge at 10krpm (apprx. 15 000xg) at 4°C for 15 min to pellet the chromosomal DNA. The supernatants were removed to fresh 50ml tubes and 8.3ml isopropanol was

added to each. The contents were mixed and the tubes were left at room temperature for 10 min to precipitate the plasmid DNA. The tubes were centrifuged exactly as before and the supernatant containing RNA was discarded. The pellets were lightly dried and resuspended in 2ml 1xTE. The volume was made up to 7.1ml with 1xTE and was added to 6.72g of CsCl in an ultracentrifuge tube fitting a Beckman Ti70.1 rotor. The CsCl was dissolved by inverting the tube and 425ul EtBr (10mg/ml) was added and mixed in. The tubes were balanced in pairs to within 0.2g, and were spun at 49krpm for approx. 16h. The plasmid band was then removed into a 15ml Corex tube using a Hamilton syringe, which was rinsed in sterile water and ethanol between different plasmids preparations. Two volumes of butan-1-ol saturated with water was added to the plasmid DNA solution, and the two phases mixed by vortexing. The phases were separated by spinning at 1.5krpm (approximately 2 000xg) in a MSE High Speed 18 centrifuge for 3min at 20°C, and the butan-1-ol phase was removed. Two further extractions with equal volumes of butan-1-ol were carried out in the same way. The volume of the aqueous phase was made up to 4ml with 1xTE and 400ul 3M NaOAc pH 6.0 was added followed by 10ml of ethanol. The DNA was precipitated by incubating at -20°C for at least 1 hour, after which time it was pelleted by centrifugation at 10krpm (approximately 15 000xg). The pellet was washed in 80% ethanol and spun again, left to drain to dry, and then resuspended in 500µl 1xTE.

2.10 Total plant RNA preparation (Parish and Kirby, 1966)

Glassware immersed in 0.1% DEPC (v/v) overnight at room temperature subsequently baked at 180°C for at least 2h was used throughout this procedure. Solutions other than those containing Tris were rendered RNase-free by the

addition of 0.001 volume DEPC, incubation at room temperature overnight followed by autoclaving at 15psi for 20min. Tris stock solutions were made from new containers of Tris solid, by weighing the solid out directly into DEPC-treated glassware and the addition of DEPC-treated water. Sterile plastic pipettes were used for the dispensing of all solutions.

Plant samples were harvested into liquid nitrogen and stored at -80°C. Plant material from each individual treatment was then homogenised as in section 2.9.1 and transferred to separate 30ml Corex tubes each containing 10ml phenol reagent and 10ml freshly-prepared Kirby reagent (section 2.10.1). The contents of the tubes were mixed well and then spun at 3500rpm (approximately 2500x*g*) for 7 min at 10°C in a MSE Mistral 21 centrifuge, using a swing-out rotor, slowing with the brake off. The upper aqueous layers were transferred to separate 30ml Corex tubes containing 5ml phenol reagent and the contents of the tubes were mixed well. To this mixture 5ml of chloroform was added and the contents of the tubes were once again mixed well. The tubes were then centrifuged as before. The upper aqueous layers were collected, and 7ml of each were put into separate 30ml Corex tubes to which was added 16ml of ethanol at -20°C. The tubes were covered with Nescofilm and the contents mixed gently. The tubes were left at -20°C overnight. The tubes were centrifuged as before but with the brake on. The pellets were resuspended in 5ml cold 70% ethanol 50mM NaCl and transferred to separate 15ml Corex tubes. These tubes were centrifuged with the brake on as before and the supernatant was discarded. The tubes were inverted and allowed to drain at 4°C and the pellets were partially dried *in vacuo*. The pellets were resuspended in 2ml MES-Mg buffer (50mM MES, 2mM Mg acetate, pH adjusted to 7.0 with NaOH) and 1-20 units DNase 1 (RNase-free; Worthington Products) was added and the contents of the

tubes were then mixed and left on ice for 30 min. The amount of DNase added was the minimum required to digest all the genomic DNA in 30 min, so as to reduce the likelihood of RNase contamination. Phenol reagent (2ml) was added and the contents of the tubes were mixed well and centrifuged as before with the brake off. The upper aqueous layer was removed from each tube and transferred to separate 15ml Corex tubes containing 1ml phenol reagent. These tubes were mixed well and 1ml chloroform was added to each and the contents were mixed well again. The tubes were centrifuged again as before with the brake off. The upper aqueous layer was removed from each tube into fresh 15ml Corex tubes containing 0.2ml 3M NaOAc followed by the addition of 6ml ethanol at -20°C. The tubes were gently mixed and placed at -20°C overnight. Precipitates were spun down as before with the brake on. The pellets were resuspended in 5ml 70% ethanol 50mM NaCl and pelleted as before; this was then repeated. The pellets were then drained and partially dried as before, and then dissolved in 0.5-1.0ml sterile distilled water and tranferred to microcentrifuge tubes.
The concentration of RNA solutions was estimated by reading the absorbance at 260nm (section 2.12.1). Purity was ascertained by the ratio of absorbance at 260nm:280nm; a ratio of 2.0 being optimal. Any RNA preparation with a ratio lower than 1.8 was discarded.

2.10.1 Preparation of Kirby and phenol reagents

Phenol reagent:
500g phenol re-distilled in an air condenser
70ml m-cresol redistilled in a water condenser
0.58g 8-OH quinoline

Cresol was added to the warm phenol followed by the 8-OH quinoline. Whilst still warm this mixture was saturated

with 10ml Tris-HCl pH 7.6. Phenol reagent was stored at 4°C covered in aluminium foil.

Kirby reagent:
6% (w/v) 4-aminosalycilic acid (Na salt; Sigma Chem. Co.)
10mM Tris-HCl pH 7.6
50mM KCl
1% (w/v) TNS (tri-iso-propylnaphthalenesulphonic acid, Na salt; Eastman/Kodak Chemicals)
Approx. 8% phenol reagent

Kirby reagent was made freshly for each set of extractions. The 4-AS was dissolved in a small amount of sterile distilled water and then the KCl was added. The TNS was then added rendering the solution cloudy. Addition of the phenol reagent dispersed this cloudiness, and sterile distilled water was added to make up the full volume. When made up this reagent was kept on ice.

2.11 Extraction and precipitation of nucleic acids

2.11.1 Phenol extraction of nucleic acids in solution

To the solution of nucleic acid to be extracted in a microcentrifuge tube was added one-half volume re-distilled phenol saturated with 1xTE and one-half volume chloroform:iso-amyl alcohol (24:1 (v/v)). The two phases were mixed by thorough vortexing and separated out by centrifugation for 5 min. The upper, aqueous layer was transferred to a fresh microcentrifuge tube containing an equal volume of chloroform:iso-amyl alcohol (24:1 (v/v)). The phases were mixed and separated out as before and the upper aqueous layer was transferred to a fresh microcentrifuge tube in which the nucleic acid was ethanol precipitated (section 2.11.3).

2.11.2 Butanol extraction of nucleic acids in solution (reduction of volume of nucleic acid solutions)

An equal volume of butan-1-ol was added to the solution of nucleic acid to be concentrated, and the two phases were vortexed together and separated out by centrifugation for 3min. The top butan-1-ol layer was removed and the procedure repeated until the lower aqueous phase had reached the desired volume. When as much butan-1-ol was removed as possible the remaining aqueous phase was extracted with water-saturated diethyl ether to remove any final traces. As much ether was removed as possible, and the rest was allowed to evaporate off before the nucleic acid solution was used.

2.11.3 Ethanol precipitation of nucleic acids

2.11.3.1 Ethanol precipitation in large volumes (>200µl)

To the nucleic acid solution was added one-tenth volume 3M NaOAc pH 6.0 and either 2 volumes (for DNA) or 2.5 volumes (for RNA) ethanol (stored at -20°C).

2.11.3.2 Ethanol precipitaion in small volumes (<200µl)

To the nucleic acid solution was added one-half volume 7.5M NH_4Ac and 3 volumes ethanol (stored at 20°C).

In both cases the nucleic acids were precipitated by incubation at -20°C for 1-16h, -70°C for 1h or on cardice for 20min, after which time they were centrifuged for 10min. The supernatant was removed and the pellets were gently washed in 80% ethanol (stored at -20°C) by adding it gently and then rotating the tube to wash the pellet. Following washing, the pellet was spun for a further 5min,

and all the ethanol was removed. The pellet was either dried *in vacuo* or allowed to dry by inverting the tube on paper towels. SDW or 1xTE was added to the dried pellet and the nucleic acid was allowed to rehydrate for 5 min without mixing. Once rehydrated the DNA solution was vortexed.

For smaller quantities of nucleic acids (<500ng) carrier such as sheared salmon sperm DNA (sodium salt type III, supplied by Sigma), tRNA (from *E. coli*, RNase-free, supplied by BCL) or glycogen (molecular biology grade, isolated from mussels, supplied by BCL) was added before precipitation.

2.12 Quantification of nucleic acid solutions

2.12.1 Quantification by spectrophotometry

Reading of the absorbance at 260nm of nucleic acid solutions was used as an estimate of their concentration. Dilutions were made so that readings were in the range 0.000-0.500 and, if possible, several different dilutions were read. An A_{260} reading of 1.0 was taken as representing a concentration of 50µg/ml for double-stranded DNA, 40µg/ml for single-stranded DNA and RNA and 20µg/ml for oligonucleotides.

2.12.2 Quantification by agarose gel electrophoresis

Based on the principle that staining with EtBr on agarose gels is linear in the range 10-100ng, lamda DNA in different amounts within this range was loaded on gels in separate tracks alongside DNA to be quantified. The gels were run sufficiently for the EtBr to bind fully to the DNA and then an estimate was made.

2.13 Agarose gel electrophoresis

All agarose was ultra pure electrophoresis grade supplied by Life Technologies Inc., Gaithernburg, USA.

2.13.1 Agarose gel electrophoresis of DNA

Agarose gels (0.6-2.0% depending on the size of DNA fragments to be separated; Maniatis, 1982) of various volumes were made by adding the appropriate amount of agarose to 1xTBE (1.08% (w/v) Tris, 0.55% (w/v) boric acid, 0.093% (w/v) Na_2EDTA) or 1xTAE (0.485% (w/v) Tris, 0.114% (v/v) glacial acetic acid, 0.2% (v/v) 0.5M Na_2EDTA (pH 8.0) in a conical flask, covering the top with aluminium foil and heating to boiling on a heating stirrer. The gel solution was then cooled to hand-hot and 10mg/ml EtBr was added to a final concentration of 1µg/ml and the gel solution poured into the electrophoresis apparatus. Electrophoresis running buffer (1xTBE or 1xTAE) was added just sufficiently to cover the gel. To the DNA samples in solution to be loaded was added one-tenth volume sample buffer (125mM Na_2EDTA, 0.1% SDS, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and the samples were loaded into the wells with an automatic pipette. The gel was run at between 40-100mA constant current for however long was necessary (the bromphenol blue dye in the sample buffer serving as a size indicator).

2.13.2 Agarose gel electrophoresis of RNA

For the purpose of checking a total RNA preparation (section 2.10), a standard 1% agarose/TBE gel (section 2.13.1) was used but, for northern hybridization analysis an agarose/formaldehyde gel cast in MOPS buffer was used:

For 100ml of gel solution 1-1.5g of agarose was added to 10ml 10xMOPS buffer (0.2M MOPS, 0.05M NaOAc pH 7.0, 0.01M Na_2EDTA) and 73ml of water, and the solution heated to boiling. The gel solution was then cooled to hand-hot and 17ml formaldehyde (37% v/v; May and Baker, Dagenham, Essex) was added and the solution was mixed and poured into the electrophoresis apparatus. Running buffer (1xMOPS buffer) was added to just cover the gel. RNA (1-10µg) in a solution of 50% (v/v) de-ionised formamide (Fluka Biochemicals, BDR), 1xMOPS buffer, 5.92% formaldehyde (16% of a 37% v/v solution) was heated to 65°C for 5 min and then chilled on ice. One-tenth volume RNA sample buffer (50% glycerol (v/v), 0.1mg/ml bromophenol blue) was added and the samples loaded. The gel was run at 40mA until the bromophenol blue had travelled 15 cm, and was then stained by washing in 10ug/ml EtBr for 30min followed by destaining in water twice for 15min. The gel was then checked by illuminating with short-wave UV light before setting up a Northern blot (section 2.17.2).

2.14 Digestion of DNA with restriction endonucleases

Restriction digests were set up as follows:

DNA in solution
0.1x final volume appropriate x10 restriction buffer
0.1x final volume spermidine (10mg/ml)
1-20 U restriction endonuclease per ug DNA
SDW to make up final volume

The glycerol content was never allowed to go over 5% so as not to inhibit the enzyme. Once set up, the contents of the tube were mixed gently and incubated in a water bath at a temperature appropriate for the enzyme in question. Incubation times varied from 1-16h.

2.15 Purification of DNA fragments from agarose gels

Each of the following methods were used during the course of this work.

2.15.1 By electo-elution

DNA was run on a standard agarose gel and visualised by illumination with short-wave UV light. A slice of agarose containing the DNA fragment to be eluted was cut out of the gel, and placed into a piece of dialysis tubing (previously boiled in 2% sodium bicarbonate, 1mM Na_2EDTA for 10 min followed by SDW for 10 min) closed off at one end with a dialysis clip. To the tubing was added ample 0.5xTBE and the other end of the tubing was closed off with a dialysis clip in such a way that there was the minimum volume of buffer left and the gel slice was aligned with its longest side parallel to the tubing (perpendicular to the clips). The tubing was placed into a gel electrophoresis tank with the clips aligned with the direction of electrophoresis. Electrophoresis buffer (0.5xTBE) was added to the tank to cover the tubing, and voltage was applied at 100V until the DNA had eluted out of the gel slice (checked by illumination with short-wave UV light). The current was then reversed and run for 2-3 min to free DNA from the membrane. The eluate was then phenol extracted (section 2.11.1) and ethanol precipitated (section 2.11.3).

2.15.2 Isolation of DNA fragments onto filter paper

DNA was run on a standard agarose gel and visualised by illumination with short-wave UV light. A slot was cut into the gel about 5mm ahead of, and parallel to, the DNA band to be collected and about 20mm wider on either side.

A piece of Whatman's no.1 filter paper was cut to be 10mm high and 20mm wider than the DNA band, and was moistened with 1xTE. A piece of dialysis membrane (boiled freshly in 1xTE for 10 min) was placed on top of the filter paper and air bubbles were smoothed out. The membrane was then cut around the filter paper. This 'sandwich' was then inserted into the slot, in the gel, symmetrical with respect to the DNA band and so that it was inserted as far as possible to the bottom of the gel and so that the filter paper side was nearest the DNA band. The DNA was then run completely into the filter paper (approx. 15min at 40mA), and then the current was reversed for 1 min to dislodge any DNA bound to the membrane. The filter paper was then removed and rolled up and placed into a small microcentrifuge tube perforated at the base, which was in turn placed into a large (1.5ml) microcentrifuge tube. This tube was spun for 3 min to collect the DNA fraction. The small tube was placed in a fresh large tube, and 100µl of 1xTE added to the filter paper and a fraction collected in the same way. This was repeated once more. The combined fractions (approx. 300µl) were then phenol extracted (section 2.11.1) and ethanol precipitated (section 2.11.3).

2.15.3 Isolation of DNA fragments onto Whatman's DE81 paper

DNA was run on a standard agarose gel and the band of interest located by illumination with short-wave UV light. A slit was cut in the agarose perpendicular to the DNA band (ie. parallel to the direction of electrophoresis) as close as possible to the DNA band. A piece of Whatman's DE81 ion-exchange paper was cut (5x10mm) and was inserted into the slot so that one of the short sides was right down the bottom of the gel. The gel was then re-orientated in the gel apparatus so that the direction

of electrophoresis was perpendicular to the original direction (ie. so that the DNA band travelled towards the paper). The gel was run until all the DNA had fixed to the paper (checked by illumnation with short-wave UV light), and the paper was removed to a 1.5ml microcentrifuge tube containing 1ml 1xTE. The paper was washed by gently rotating the tube, removed allowing the excess to drain off, and transferred to a tube containing 0.4ml 1.5M NaCl, 1xTE. The paper was incubated for 2h at 37°C, and was then removed from the tube and illuminated with UV to check that all the DNA had eluted off. The eluate was then spun in a microfuge for 5min to pellet paper fragments, and was transferred to a fresh 1.5ml microcentrifuge tube. The eluate was then phenol extracted (section 2.11.1) and ethanol precipitated (section 2.11.3).

2.15.4 Isolation of DNA fragments from low melting-point agarose gels

DNA was run on a standard agarose gel made from low-melting point agarose (ultra pure electrophoresis grade Life Technolgies Inc.), run at 50mA constant current in the cold-room. The DNA fragment to be isolated was located by illumination with short-wave UV light and then cut out of the gel and transferred to a 1.5ml microcentrifuge tube. The volume of the agarose slice was estimated by weighing (1g = 1ml) in a microcentrifuge tube (as the difference between an empty microcentrifuge tube and the one containing the slice) and 1-5 volumes of T_2E (20mM Tris, 1mM Na_2EDTA) was added. The gel slice was melted by incubation in a 65°C waterbath for 15 min. To the melted agarose was added an equal volume of re-distilled phenol saturated with 1xTE, pre-heated to 37°C, and the contents of the tube were mixed thoroughly by vortexing. The tube was left to stand at room

temperature for 15 min, after which time it was spun for 5 min and the top aqueous phase was removed to a fresh 1.5ml microcentrifuge tube. To the phenol phase was added 200µl of T_2E, and the contents of this tube was mixed by vortexing and the phases separated by centrifugation for 5 min. Again the aqueous phase was removed and combined with the aqueous phase from the first extraction. To this was added one-half volume phenol saturated with TE and one-half volume chloroform:iso-amyl alcohol (24:1, v/v), and the contents of the tube were mixed thoroughly by vortexing. The tube was spun for 5min to separate the phases, and the top aqueous phase was removed to a fresh 1.5ml microcentrifuge tube and ethanol precipitated (section 2.11.3)

2.15.5 Isolation of DNA fragments using 'Geneclean' kit

The method enclosed with the kit (no. BIO 101) supplied by UBB, Ohio, USA was followed.

DNA was run on a standard agarose gel cast in 1xTAE containing 10ug/ml EtBr. The band required was located by illumination with short-wave UV light, punched-out and removed in a disk of agarose using the large end of a disposable pipette tip. The volume of the agarose disk was estimated by weighing (1g = 1ml) in a 1.5ml microcentrifuge tube (as the difference between an empty microcentrifuge tube and the one containing the disk) and 2.5 volumes of NaI stock solution (supplied with the kit) was added. The agarose disk was dissolved by incubation at 55°C for about 5min. 'Glassmilk' (supplied with the kit) was added (5µl for up to 5µg DNA and an additional 1ul for every 0.5µg above 5µg) and the contents of the tube were vortexed and allowed to stand at room temperature for 5min. The 'Glassmilk' pellet with the DNA bound was spun down in a microcentrifuge for 5s, and the

supernatant removed. The pellet was then resuspended in 500µl ice-cold 'NEW' (supplied with the kit) by vortexing and the tube centrifuged for 5s again to pellet the 'Glassmilk'. The supernatant was removed and the washing of the pellet was repeated twice more. Once every trace of 'NEW' was removed from the pellet it was resuspended by vortexing in 5-20µl SDW or 1xTE. The DNA was eluted by incubaton at 55°C for 3 min, after which time the 'Glassmilk' was pelleted by centrifugation for 2min. The DNA solution was removed to a fresh microcentrifuge tube, leaving the 'Glassmilk' pellet which was re-extracted once more with more SDW or 1xTE.

2.16 Gel-purification of oligonucleotides

Crude oligonucleotide preparations were quantified by measuring absorbance at 260nm (section 2.12.1), and 5 OD units of each preparation were resuspended in 20µl loading buffer (de-ionized formamide:TBE, 70:30 (v/v)). These samples were then incubated at 90-95°C for 3 min and loaded on a denaturing acrylamide gel (16% (w/v) acrylamide:bisacrylamide (38:2), 8M urea, 1xTBE) cast using a sequencing gel apparatus to give a gel 200x400mm, 0.3mm thick, using a comb which gave slots 30mm wide. In one of the spare wells was loaded 20µl of formamide dye mix (section 2.21.2). The gels were run at 37W constant power (starting voltage 1.5kV, starting current approx. 25mA), until the bromophenol blue in the formamide dye mix had reached the bottom of the gel. The gel plates were separated and clingfilm was placed on top of the gel. The gel was then placed with the cling film facing down, on top of a fluorescent TLC plate and was illuminated with short-wave UV light using a hand-held illuminator. The band with the highest molecular weight and strongest abundance was removed using a scalpel (the upper half of the band only was taken to avoid contamination from the

(n-1) band). Each gel slice was placed in a separate 1.5ml microcentrifuge tube, and 1.5ml of 1xTE added. Gel slices were then incubated at 37°C overnight with moderate agitation. Each oligonucleotide solution was then passed through a blue pipette tip plugged with siliconised glass wool (previously rinsed through with 1ml 1xTE) to remove small gel fragments. Purified oligonucleotides were ethanol precipitated (section 2.11.3), resuspended in 50µl sterile Analar water and their concentration was estimated by absorbance at 260nm (section 2.12.1). This procedure gave recoveries of between 15-20%.

2.17 Blotting of nucleic acids onto nitrocellulose

2.17.1 Southern blotting (Southern, 1975)

Genomic DNA was digested with restriction endonucleases and run on a 0.8% agarose/TBE gel until the bromophenol blue had run three-quarters the way down the gel. Regions of the gel not containing DNA were trimmed away as much as possible and the remaining gel was then measured. The gel was then washed twice in 1.5M NaCl, 0.5M NaOH for 15 min and then twice in 1.5M NaCl, 1M Tris-HCl pH 8.0 for 15 min. Whilst washing the gel a piece of nitrocellulose (supplied by Alderman & Co. Ltd., Kingston upon Thames, Surrey) or Hybond-N (supplied by Amersham International) filter was cut to be just a few millimetres bigger than the gel. This filter was pre-soaked in water for 5 min followed by 10xSSC (1xSSC=0.15M NaCl, 0.015M trisodium citrate) for 20 min. The gel was placed (well-side down) on a wick of 3 layers of Whatman's 3MM paper dipping into and thoroughly wetted with 10xSSC, and any air bubbles under the gel were removed. Cling-film was placed over all the exposed areas of the paper wick to a few millimetres from the gel. On top of the gel was placed the filter with the extra few millimetres overlapping the

edge of the gel. Once again air bubbles between the gel and filter were removed by rolling a glass pipette over the filter. Pieces of 3MM paper roughly the size of the filter were cut and 10-20 were piled on top of the filter. On top of this was placed a large quantity of paper towels and finally a glass plate with a weight eg. a bottle containing 500ml of solution. The blotting apparatus was left for at least 4h, after which it was dismantled. The gel was re-stained with EtBr to check that transfer of DNA had been successful, and the filter was air-dried and then baked *in vacuo* at 80°C for 2h.

2.17.2 Northern blotting

Agarose/formaldehyde/MOPS gels (section 2.13.2) were run with appropriate markers, and were trimmed to remove agarose gel below the tRNA bands and any spare gel to the side of the tracks. The gel was set up for blotting as in section 2.17.1.

2.17.3 DNA dot-blotting

Aliquots of DNA in solution were denatured at 95°C for 2 min and then chilled rapidly on ice. Once chilled an equal volume of ice-cold 20xSSC was added and the samples mixed.

A nitrocellulose or Hybond-N filter (120x80mm) was cut and immersed in distilled water for 5min, followed by 10xSSC for 20min. This filter was then set up in a BRL HYBRI-DOT 96-hole manifold apparatus (no.580-1050 MM) and suction sufficient to pull 1ml of water up a 2ml glass pipette applied. Each well was washed through with 200µl 10xSSC, then the samples were loaded in separate wells (100-200µl). Following loading of the DNA solutions, the wells were flushed with two applications of 200µl 10xSSC. The manifold apparatus was then dismantled and the filter

was first soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5min followed by 1min in neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001 Na_2EDTA). The filter was dried, and then baked *in vacuo* oven at 80°C for 2h.

2.17.4 RNA dot-blotting

To aliquots of RNA was added 3 volumes RNA denaturation buffer (65.6% formamide (v/v), 7.87% formaldehyde (v/v), 1.31xMOPS buffer). Samples were then incubated at 65°C for 5min and then chilled on ice, after which time an equal volume of ice-cold 20xSSC was added and the samples mixed.
The samples were loaded and fixed onto a nitrocellulose or Hybond-N filter as in section 2.17.3 without the denaturation and neutralisation stages.

2.18 Preparation of radio-labelled DNA probes

2.18.1 DNA probes used

2.18.1.1 rbcS cDNA probes

Inserts from pea *rbcS* cDNA clones pSSU61 and pSSU161 (figure 1) were used for Southern blot analysis (section 2.17.1) and for RNA dot blot analysis (section 2.17.4), as was the *P. vulgaris rbcS* cDNA clone pPvSS1672 (figure 15). In both cases inserts were purified as in section 2.15.

2.18.1.2 Oligonucleotide probes

Oligonucleotides (21^{mers}) were synthesised for use either as probes specific to the individual *P. vulgaris rbcS* genes (probes *rbcS1*, *rbcS2* and *rbcS3*) or as a general *P. vulgaris rbcS* probe (probe *rbcSG*).

The sequences for these oligonucleotides were:
5' AGCTTTCAACAGAAGATAATC 3' (rbcS1),
5' GAAGATGAAATCTCAAAATGC 3' (rbcS2),
5' ATAAATTTCCTTACGCAGAAG 3' (rbcS3) and
5' AATCCAATGATACGGATGAAA 3' (rbcSG).

The design of these probes is described in section 3.5.

2.18.2 Nick-translation labelling of DNA

2.18.2.1 Standard reaction

The following reaction mixture was prepared in a sterile microcentrifuge tube:

2ul 10x nick-translation buffer (0.5M Tris-HCl pH 7.2, 0.1M $MgSO_4$, 1mM DTT (BCL) 500µg/ml BSA (Cohn Fraction V, Sigma)
2.5µl 10µm dATP (BCL)
2.5µl 10µm dTTP (BCL)
2.5µl 10µm dGTP (BCL)
1µl DNase 1 (supplied by Amersham, diluted to 0.0005 units/µl)
2.5µl (25µCi) [∝-32P]dCTP (3000Ci/mmol)
6µl SDW containing 50ng DNA

The contents were incubated at 37°C for 15 min. DNA polymerase 1 (1µl at 1U/µl) was added and mixed-in by repeated pipetting of the contents of the tube and the mixture was incubated at 15°C for 2.5-3h.
The unincorporated nucleotides were removed (section 2.18.6)) and the labelled DNA resuspended in 100µl SDW. The specific activity of the labelled DNA was measured by

TCA-precipitation (section 2.18.7.1).

2.18.2.2 Using Amersham Nick Translation Kit no. N.5500

Reaction mixture:

DNA (50ng)	5µl
Nucleotide/buffer solution (solution 1)	10µl
[α-^{32}P]dCTP (50µCi, 3000Ci/mmol)	5µl
Sterile Analar water	25µl
Enzyme solution (solution 2)	5µl

This reaction mixture was set-up in this order in a 1.5ml microcentrifuge tube, and was mixed by pipetting the contents of the tube gently in and out of a disposable pipette tip several times. The reaction was incubated at 15°C for 3h, and was then terminated by the addition of 5µl 0.2M Na_2EDTA pH 8.0.
The unincorporated nucleotides were removed (section 2.18.6) and the labelled DNA resuspended in 100µl SDW. The specific activity of the labelled DNA was measured by TCA-precipitation (section 2.18.7.1).

2.18.3 Uniform labelling of single-stranded DNA with Klenow polymerase

The method described by Burke (1984) was used with slight modifications.

The DNA insert to be labelled was subcloned into M13 (mp18 or mp19 depending on which strand required labelling), and single-stranded templates were made as for DNA sequencing (section 2.20). M13 sequencing primer (30ng; 5'GTAAAACGACGGCCAGT3' supplied by BCL) was annealed to the single-stranded DNA (150ng) in a microcentrifuge tube in a volume of 48.2µl of prime-cut probe buffer (20mM Tris-HCl

pH 7.5, 2mM DTT and 12mM $MgCl_2$) by heating to 90°C and cooling to 65°C for 10 min. The solution was quenched on ice and dATP, dGTP and dTTP were added to 100µm (final volume 50µl). Klenow polymerase (supplied by Amersham),was added to the annealed template (1µl; 5 units) and the contents of the tube were mixed by gently pipetting the contents in and out, then 10µCi (1µl) of [α-^{32}P]dATP (>400Ci/mmol) or 50µCi (5µl) of [α-^{32}P]dCTP (~3000Ci/mmol) was added. The reaction mixture was incubated at 18°C for 20 min followed by the addition of 10mM dATP or dCTP (depending on the label used) to 1mM and a further incubation at 18°C for 20 min. The reaction was then terminated by heating at 65°C for 15 min. The reaction mixture was then cooled to 4°C and 500mM NaCl was added to 50mM. Restriction endonuclease (2ul @ 10U/µl) was added (HindIII for mp19 or EcoR1 for mp18) and the restriction mixture was then incubated at 37°C for 30 min. Following synthesis and digestion the probe was denatured and separated from the unlabelled template using one of four different methods:

i) The reaction mixture was ethanol precipitated on cardice (section 2.11.3) and resuspended in 10µl SDW. To this was added 2µl 1M NaOH followed by 10µl 1M Tris-HCl pH 4.6, and the sample loaded and run on a standard 1.2% agarose/TBE gel at 40mA (section 2.13.1) (Burke, 1984)

ii) The reaction mixture was ethanol precipitated on cardice (section 2.11.3) and resuspended in 0.1M NaOH, 1mM Na_2EDTA, 8% sucrose and 0.05% bromophenol blue. The sample was then loaded on a 1.2% agarose gel cast and run in 36mM Tris-HCl NaH_2PO_4, 1mM Na_2EDTA (Maniatis, 1982) and run at 60mA.

iii) The reaction mixture was ethanol precipitated on cardice (section 2.11.3) and resuspended in 5µl formamide dye mix and run on a standard denaturing polyacrylamide gel (section 2.21.2).

iv) To the reaction mixture was added 6µl of denaturation buffer (4M NaOH, 0.2M Na_2EDTA). After 2-3 minutes at room temperature 10µl of agarose gel sample buffer was added and the sample was loaded on a 1.2% agarose gel cast and run in 1xTAE (section 2.13.1). The gel was run initially at 100mA for 20 minutes to prevent renaturation of the probe after which it was run at 40mA (Calzone, 1987).

In each case after electrophoresis the gel was wrapped in plastic film and the probe band was located by autoradiography (at the workbench, behind a screen) with X-ray film double-wrapped in aluminium foil. The film was pressed firmly against the gel by placing a glass plate on top of it upon which a weight was applied. The exposure time was 5 min when [α-32P]dCTP was used and 15-30min when [α-32P]dATP was used. Once located the probe band by was excised from the gel and electo-eluted (section 2.15.1). The probe was then ethanol precipitated (section 2.11.3), washed twice in 80% ethanol, dried and resuspended in 50ul SDW.

2.18.4 Polynucleotide kinase labelling of oligonucleotide probes

The method described by Maxam and Gilbert (1980) was followed with slight modifications.

Reaction mixture:

50mM $MgCl_2$	10µl
1M Tris-HCl pH7.6	5µl
200mM 2-mercaptoethanol (Koch-Light)	5µl
Sterile Analar water containing	
200ng oligonucleotide	19µl
[γ-^{32}P]dATP (100µCi, ~5000Ci/mmol)	10µl
T4 polynucleotide kinase (10U)	1µl

This reaction mixture was set up in the above order in a 1.5ml microcentrifuge tube, and mixed together by repeated pipetting in and out of a disposable pipette tip. The reaction was incubated at 37°C for 1h and the reaction was terminated by the addition of 5ul 0.5M Na_2EDTA. Incorporation of label was estimated by binding to DE81 paper (section 2.18.7.2).

2.18.5 End-labelling of DNA with Klenow polymerase

Labelling was either carried out directly after restriction digestion of the DNA (section 2.14) in the buffer used for the digest, or with DNA purified as in section 2.15, in which case 10x Klenow buffer (section 2.21.1.1) was added to 1x. Unlabelled dNTPs (if needed) were added to 0.5uM, with a final volume of 25µl.

DNA (<=1ug) in buffer solution	25.0µl
[α-^{32}P]dNTP (5µCi, ~3000Ci/mmol)	0.5µl
Klenow polymerase (1U/µl)	1.0µl

The labelling mixture was set-up in the order above in a 1.5ml microcentrifuge tube, and mixed by repeated pipetting in and out of a disposable pipette tip. The labelling reaction was incubated at room temperature for 10 min, after which time the enzyme was inactivated by incubation at 65°C for 10 min. The unincorporated nucleotides were removed as in section 2.18.6.

2.18.6 Removal of unincorporated radio-nucleotides

To the probe/nucleotide mixture was added 2µl 5mg/ml sheared salmon sperm DNA (supplied by Sigma) as carrier, one-half volume 7.5M NH_4Ac and 3 volumes ethanol (stored at -20°C). The contents of the tube were mixed and left

to incubate on cardice for 20 min. The precipitated probe was pelleted by centrifugation in a microcentrifuge for 10 min and the supernatant was discarded. The pellet was then washed in 1ml 300mM NaOAc, 80% ethanol (stored at -20°C), and spun for a further 7min. The supernatant was removed and the pellet dried in vacuo, and resuspended in 100µl SDW. The incorporation of radio-nucleotides into the probe was then measured (section 2.18.7). Using this method after nick-translation of DNA (section 2.182) the radioactive component attributable to unincorporated nucleotides was always <5%. Levels lower than this were acheived by carrying out several such ethanol precipitations in succession.

2.18.7 Estimation of incorporation of radio-nucleotide into probes

Both methods are as described by Maniatis (1982).

2.18.7.1 By TCA-precipitation

Squares (10x10mm) of Whatman's no.1 filter paper were cut and soaked in 5% TCA and allowed to dry. For each probe preparation a small volume (1-2µl) of probe solution was applied to two separate squares. These were allowed to dry completely in a fume-hood after which one was washed well in 5% TCA, followed by diethyl ether:ethanol (1:1 v/v) followed by diethyl ether, and was then allowed to dry completely again. The two squares were placed into scintillation vials and 4ml Ecoscint scintillation fluid was added. The c.p.m obtained from the washed square were taken as c.p.m. incorporated into the probe and the difference between the two squares was taken as c.p.m. attributable to unincorporated nucleotides present in the probe solution.

2.18.7.2 By binding to Whatman's DE81 paper

Up to 5µl of the labelled DNA solution was spotted on to two 2.4 cm disks of Whatman's DE-81 ion-exchange paper. One of the disks was washed six times for 5 min in 0.5M Na_2HPO_4, twice for 1 min in water and twice for 1 min in 95% ethanol, then both were allowed to dry in a fume hood. Both disks were placed in separate scintillation vials and 4ml of Ecoscint scintillation fluid added. The c.p.m. obtained from the washed disk was taken as c.p.m. incorporated into the probe and the difference between the two disks was taken as c.p.m. attributable to unincorporated nucleotides present in the probe solution.

2.19 Hybridisation analysis of nucleic acids

2.19.1 Hybridisation of nucleic acids bound to nitrocellulose

2.19.1.1 Using nick-translated probes:

Filters prepared as in section 2.17 were pre-hybridised by incubating them in pre-hybridising solution (5xSSC, 50% de-ionised formamide pH 7.6 (v/v), 100-200µg/ml denatured sheared salmon sperm DNA, 5x Denhardt's solution (50x Denhardt's solution = 1% (w/v) Ficoll 400 (Sigma), 1% (w/v) PVP 360 (Sigma), 1% (w/v) BSA (Cohn fraction V; Sigma)), 50mM HEPES pH 7.0) in a sealed bag for 4h at the temperature chosen for hybridisation. After pre-hybridisation the filter was removed to a new sealed bag and hybridisation solution (5xSSC, 50% deionised formamide pH 7.6 (v/v), 100-200µg/ml denatured sheared salmon sperm DNA, 1x Denhardt's solution, 50mM HEPES pH 7.0) was added. The DNA probe was denatured by the addition of one-tenth volume 1M NaOH and incubation at

65°C for 10min, cooling on ice, followed by neutralisation with an equal volume of 1M HCl. The probe was then added to the hybridisation solution and the bag sealed avoiding the inclusion of air bubbles. Incubation was carried out at the chosen hybridisation temperature for 16h, following which the filters were washed in solutions at salt concentrations and temperatures chosen to be appropriate. The filters were then re-sealed in bags without drying (to allow further washing if necessary) and autoradiographed.

2.19.1.2 Using oligonucleotide probes

The method described by Sugita and Gruissem (1987) was used.

Filters prepared as in section 2.17 were pre-hybridised by incubating them in (0.9M NaCl, 50mM NaH_2PO_4 pH 7.4, 5mM Na_2EDTA, 0.1% SDS, 100µg/ml denatured sheared salmon sperm DNA, 5x Denhardt's solution) for 5h at 46°C. After pre-hybridisation end-labelled oligonucleotide (section 2.18.4) was added (to approx. 0.35pmol/ml hybridisation solution) and the bag sealed. Hybridisation was carried out again at 46°C for 20-48h, following which the filters were washed in solutions at salt concentrations and temperatures chosen to be appropriate. The filters were then sealed in bags without drying (to allow further washing if necessary) and autoradiographed.

2.19.2 S1 nuclease analysis of RNA

2.19.2.1 Using single-stranded DNA probes

2.19.2.1.1 Without formamide (Burke, 1984)

RNA (1, 5 or 10µg) and 5µl single-stranded probe (section 2.18.3) were co-precipitated (section 2.11.3) and

resuspended in 10µl hybridisation buffer (0.4M NaCl, 10mM PIPES pH 6.5). The mixture was heated to 95°C for 3 min and quickly transferred to a 65°C waterbath. After a 1h incubation 200µl S1 nuclease buffer (180mM NaCl, 30mM NaOAc pH 4.5, 4.5mM ZnAc, 1kU/ml S1 nuclease) was added and the digestion carried out at 37°C for 30 min. The digestion was stopped by the addition of Na_2EDTA to 10mM and tRNA to 5µg, and 3 volumes of ethanol were added. After precipitation the pellet was resuspended in 5µl formamide dye mix and this was loaded and run on a polyacrylamide sequencing gel (section 2.21.2). After drying, the protected fragments were visualised by exposing X-ray film to the gel at -80°C using an intensifying screen.

2.19.2.1.2 With formamide (Nagy et al., 1988)

To 1, 5 or 10µg of total RNA in a 1.5ml screw-top microcentrifuge tube was added 5ul uniformly labelled single-stranded probe (section 2.18.3) and the mixture was dried down completely in vacuo. To this mixture was added 8µl 62.5% formamide and the nucleic acid mixture was dissolved by vortexing. After the pellet was dissolved, 2µl 5X S1-hybridisation buffer was added (1x S1-hybridisation buffer=0.4M NaCl, 2mM Na_2EDTA (pH 7.0), 20mM PIPES (pH 6.8)). The sample was then incubated at 95°C for 2 minutes after which it was directly transferred to a 37°C waterbath and incubated overnight. After incubation the sample was diluted with 150µl S1 digestion mixture (0.28M NaCl, 0.05M NaOAc pH 4.6, 4.5mM ZnSO4, 20ug/ml sheared, denatured salmon sperm DNA, 4000 U/ml S1 nuclease) and incubated at room temperature for 90 min. After this time 30µl S1 termination solution (2.5M ammonium acetate, 0.05M Na_2EDTA, 300ug/ml tRNA) was added, followed by 400µl ethanol. The sample was mixed thoroughly and precipitated at -20°C for 30 min. The

sample was spun down at 15 000rpm for 10 min. The supernatant was discarded and the pellet washed twice in 80% ethanol. The pellet was then dried completely *in vacuo* and resuspended in 5µl formamide dye mix (section 2.21.2). Part of the sample (approx. 2µl) was then loaded and run at 1.5kV constant voltage for 2h on a sequencing gel (section 2.21.2). After drying, the protected fragments were visualised by exposing X-ray film to the gel at -80°C using an intensifying screen (usually for 4h).

2.19.2.2 Using double-stranded probes

RNA (1, 5 or 10µg) and 10ng of end-labelled double-stranded probe (section 2.18.5) were co-precipitated (section 2.11.3) in a screw-cap microcentrifuge tube and then resuspended in 20µl hybridisation solution(40mM PIPES pH 6.4, 400mM NaCl, 80% (v/v) deionised formamide). The tube was then placed in a 85°C waterbath for 10 min, after which time the waterbath was turned down to the chosen hybridisation temperature (52°C), and was allowed to equilibrate to this temperature over 30-60 min. After an incubation for 3-4h and keeping the tubes submerged, 300µl of chilled S1 digestion solution (280mM NaCl, 30mM NaOAc pH 4.4, 4.5mM ZnAc, 100µg/ml denatured sheared salmon sperm DNA, 1kU/ml S1 nuclease) was added and mixed-in by vortexing. Digestion was carried out at 37°C for 45 min, and was terminated by the addition of 75µl termination solution (2.5M NH_4Ac, 0.05M Na_2EDTA) and 10µg tRNA. After mixing by vortexing, 400µl isopropanol was added, and after a 5 min incubation at room temperature the tube was centrifuged for 10 min. The pellet was washed in 80% ethanol, dried and resuspended in 5µl formamide dye mix (section 2.21.2) and part of the sample (approx. 2µl) was then loaded and run on a sequencing gel (section 2.21.2). After drying the

protected fragments were visualised by exposing X-ray film to the gel at -80°C using an intensifying screen.

2.19.3 S1 nuclease analysis of DNA

Single-stranded templates (section 2.20.7) containing the probe and test sequences were added (10µl of each) to 10ug *E. coli* tRNA in a screw-cap microcentrifuge tube. The samples were dried *in vacuo* and resuspended in 8µl SDW. After the pellet was dissolved, 2µl 5X S1-hybridisation buffer was added (section 2.19.2.1.2). Hybridisation was carried out by heating 1000ml water to 100°C and then placing the samples in it and allowing the temperature to slowly come down to 40°C (usually approx. 1.5h). S1 nuclease buffer (150µl; section 2.19.2.1.2) was then added and the digestion carried out at 37°C for 30 min, after which 30µl S1 termination solution (section 2.19.2.1.2) was added, followed by 400µl ethanol. After precipitation (section 2.11.3) the pellets were resuspended in SDW and run on standard 2% agarose/TBE gels to visualise the protected fragments.

2.20 Subcloning into M13

2.20.1 Preparation of digested RF M13 vector DNA

For digestion with a single enzyme or two enzymes sharing the same salt-buffer requirements (e.g BamH1 and EcoR1) a standard restriction digestion was set up (section 2.14). Following this the DNA was phenol extracted (section 2.11.1), ethanol precipitated (section 2.11.3) and resuspended in a convenient volume of 1XTE.

For digestion with enzymes with different salt-buffer requirements (eg. EcoR1 and HindIII) or different optimal temperatures for activity (eg. EcoR1 and Sma1) digestion

was carried out first with one enzyme, the DNA then being phenol extracted, ethanol precipitated and resuspended. Successful linearisation of the DNA was then checked on a 1% TBE-agarose gel (section 2.13.1), then the digestion with the second enzyme was carried out. Following this the DNA was phenol extracted, ethanol precipitated and resuspended in a convenient volume of 1XTE.

In both cases the concentration of the digested M13 RF DNA was checked by quantification on an agarose gel (section 2.12.2) and made up to a concentration of 10ng/ul.

The cut vector was further checked the first time it was used in a ligation by setting up a ligation control reaction (section 2.20.3) to monitor the efficiency of linearisation.

2.20.2 Preparation of insert DNA

Insert DNA was prepared as in section 2.15. DNA was quantified on an agarose gel (section 2.12.2). This DNA was then made up to a concentration of 50ng/µl.

2.20.3 Ligations

Reaction mixture:		Ligation control:	
DNA insert (100ng)	2µl	RF DNA vector (20ng)	2µl
RF DNA vector (20ng)	2µl	5X T4 DNA ligase buffer	2µl
5X T4 DNA ligase buffer	2µl	ATP (10mM)	1µl
ATP (10mM)	1µl	DTT (50mM)	1µl
DTT (50mM)	1µl	T4 DNA ligase (1U)	1µl
T4 DNA ligase (1U)	1µl	H_2O	3µl
H_2O	1µl		

Ligations involving a 'sticky-end' were incubated in a water bath set at 14-16^{o}C in the cold room, for 4-16h.

Ligations where only 'blunt-ends' were involved were incubated at 4°C for 12-24h.
Both types of reactions were terminated by heating to 65°C for 10 min.

2.20.4 Preparation of competent cells

A single colony of *E. coli* TG1 was picked from a minimal medium plate and grown up in 10ml 2X TY medium in a universal, shaking overnight at 37°C.
For every 6 ligations, 20ml of 2X TY medium in a universal bottle was inoculated with 1ml of the overnight culture. Additionally, a further 20 ml of 2X TY medium was inoculated with a single drop of the overnight culture to provide fresh cells for the plating-out stage. The '1ml' cultures were grown at 37°C for 2h and chilled on ice for 15 min. Cells were spun down in the universals in a Beckman Model TJ-6 bench-top centrifuge at 3000rpm (approx. 4 000xg) for 2min, then resuspended in 10ml ice-cold 50mM $CaCl_2$ and left on ice for 20min. Cells were then centrifuged as before and resuspended in 2ml ice-cold 50mM $CaCl_2$.

2.20.5 Transformation of competent cells with ligated DNA

Aliquots of competent cells (0.3ml) were transferred to labelled plastic bijoux bottles and 5µl of the ligation mixture (or control) added to each separate one. Controls containing cut vector DNA (10ng) and uncut vector (1ng) were also included here. The cell/DNA mixtures were then left on ice for 2-6h after which the cells were heat-shocked at 42°C for 3min before being returned to the ice for at least 5min.

2.20.6 Plating-out of transformants

For each 8 separate transformations the following plating-out mixture was made:

360µl 100mMβIPTG (BRL, 8.6mg in 360µl H_2O)
360µl 2%βX-GAL (BRL, 7.2mg in 360µl dimethylformamide)
1800µl fresh exponential culture of E. coli (1 drop culture from section 2.20.4).

The bijoux bottles containing the heat-shocked cells were removed from the ice and 270µl of plating-out mixture was added. To this 3ml of molten H-top agar at 42°C was added and the mixture was plated out on H-plates after a brief mix. The soft agar was allowed to set at room temperature after which the plates were incubated inverted at 37°C overnight.

2.20.7 Preparation of single-stranded templates

An overnight culture of E. coli TG1 was set up (5ml 2X TY medium in an universal bottle, held horizontally, shaking vigorously at 37°C. A 1:100 dilution of this culture was made (20ml for each 12 plaques to be processed). Aliquots of this diluted culture (1.5ml) were dispensed into bijoux bottles. Each bottle was inoculated with a colourless plaque from a ligation plate using a glass Pasteur pipette to punch-out and transfer the plaque. These inoculated cultures were then shaken at 37°C for 5h, after which time they were transferred to 1.5ml microcentifuge tubes. After spinning for 5 min, the supernatants were transferred to separate 1.5ml microcentrifuge tubes (supernatants were often stored at -20°C at this stage) and spun again for 5min to remove all the bacterial cells. Once again the supernatants were transferred to separate 1.5ml microcentrifuge tubes. To each supernatant was added 200µl 20% PEG 6000, 2.5M NaCl and the tubes were

mixed and left to stand at room temperature for 15 min, after which they were centrifuged for 5 min. The supernatant was removed with a pipette tip, and the tubes were spun for a further 2 min to collect the last remaining liquid. To the viral pellets was added 450µl of 1xTE buffer followed by 225µl of re-distilled phenol saturated with 1xTE buffer. The pellets were resuspended by vortexing for 20s and the tubes were left to stand at room temperature for 15min, after which time 225µl of chloroform:iso-amyl alcohol (24:1 (v/v)) was added. The tubes were vortexed for a further 15s, centrifuged for 5 min and 400µl of the upper aqueous phase was removed and transferred to a fresh 1.5ml microcentrifuge tube containing 40µl 3M NaOAc (pH 6.0). Ethanol (1ml) was added and the DNA precipitated at -20°C overnight or at -70°C for 1h, after which the tubes were spun for 10min. The DNA pellets were washed by adding 1ml cold ethanol which was then poured off. The pellets were allowed to drain to dry (about 15 min) to prevent over-drying, and resuspended in 30-50ul 1xTE buffer. Final DNA concentrations were on average around 200ng/µl. Templates prepared this way were stored at -20°C until needed.

2.21 Dideoxy DNA sequencing

2.21.1 Annealing primer to template and sequencing reactions

2.21.1.1 Using Klenow polymerase (Amersham kit no. N.4501)

Annealing reaction mixture:

ssDNA template (approx. 1µg)	5.0µl
oligonucleotide primer (1.2ng)	1.0µl
Sterile Analar water	2.5µl
Klenow reaction buffer (x10)	1.5µl

(1x=10mM Tris-HCl pH 8.0, 5mM $MgCl_2$)

This reaction mixture was set up in a 1.5ml microcentrifuge tube, mixed and spun to the bottom of the tube. Annealing was achieved by incubation in an oven set at approx. 57°C for 2h. After incubation the contents of the tube were spun down to the bottom. Annealed templates were stored at -20°C until required for the sequencing reaction:

Template/primer/enzyme mixture:

Annealed primer/template	11.0µl
[α-^{35}S]dATP (15µCi, >600Ci/mmol)	1.5µl
Klenow fragment (BCL or Amersham, 1U/µl)	1.0µl

This reaction mixture was set up in each of the tubes containing the primer/template and the contents mixed. For each clone to be sequenced 4 tubes labelled 'A', 'C', 'G' and 'T' were placed into Eppendorf 5413 microcentrifuge racks which held the tubes horizontally. To the bottom of each tube was added 2.5µl of the template/primer/enzyme reaction mixture. dNTP/ddNTP (see table 2) mixes were thawed and 2µl added to the corresponding tubes (ie. tubes marked 'A' received 2µl A°/ddATP, tubes marked 'C' received 2µl C°/ddCTP etc.) just beneath the rim, keeping the tubes horizontal. The tubes were then spun briefly to mix the contents of the tube and start the reaction which was carried out at room temperature for 20min, after which time 2µl of chase solution (10mM dATP, 10mM dCTP, 10mM dGTP and 10mM dTTP) was added to all the tubes and mixed the same way as the dNTP/ddNTP mixes previously. This chase reaction was carried out for a further 15min at room temperature, after which time 4µl of formamide dye mix (96% (v/v) deionised formamide, 20mM Na_2EDTA, 0.1% (w/v) xylene cyanol FF, 0.1%

TABLE 2

Deoxynucleotides	MIXTURES A°	C°	G°	T°
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dGTP	20µl	20µl	1µl	20µl
0.5mM dTTP	20µl	20µl	20µl	1µl
1xTE	20µl	20µl	20µl	20µl

ddNTP/N° mixes were made by making a 50:50 (v/v) solution of the above A°, C°, G° and T° mixes with 0.15mM ddATP , 0.02mM ddCTP , 0.05mM ddGTP and 0.5mM ddTTP respectively.

(w/v) bromophenol blue) was added to stop the reaction. Completed sequencing reaction mixtures were stored at -20°C for up to a week before electrophoresis.

2.21.1.2 Using modified T7 polymerase (USB 'Sequenase' version 2.0 kit no.70770)

Annealing mix:

Oligonucleotide primer (approx. 1.5ng)	1µl
Template DNA (approx. 1.5µg)	7µl
Sequenase buffer (x5)	2µl

(5x=200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl)

For each clone this annealing mix was set up in a capped 1.5ml microcentrifuge tube. This tube was incubated at 65°C for 2min in a 50ml glass beaker half filled with water on a stiring hotplate, and then allowed to cool to below 30°C on an ordinary stirrer, this taking about 30min. Annealed templates were stored at -20°C until needed for the labelling and termination reactions:

For each clone, 4 tubes labelled 'A', 'C', 'G' and 'T' were set up and 2.5µl of the relevant termination mix (ddATP, ddCTP, ddGTP and ddTTP respectively; see table 3) placed into the bottom of each. Normally the termination mixes labelled 'dGTP' were used unless sequencing a sequence where compressions due to a G-C rich region was known to be a problem, in which case the termination mixes labelled 'dITP' were used. These tubes were kept on ice in the racks for the Eppendorf 5413 microcentrifuge until the labelling reaction was started.

TABLE 3

'dG' TERMINATION MIXES

Components	ddA	ddC	ddG	ddT
dATP	80μM	80μM	80μM	80μM
dCTP	80μM	80μM	80μM	80μM
dGTP	80μM	80μM	80μM	80μM
dTTP	80μM	80μM	80μM	80μM
ddATP	8μM	-	-	-
ddCTP	-	8μM	-	-
ddGTP	-	-	8μM	-
ddTTP	-	-	-	8μM
NaCl	50mM	50mM	50mM	50mM

'dI' TERMINATION MIXES

Components	ddA	ddC	ddG	ddT
dATP	80μM	80μM	80μM	80μM
dCTP	80μM	80μM	80μM	80μM
dITP	160μM	160μM	160μM	1600μM
dTTP	80μM	80μM	80μM	80μM
ddATP	8μM	-	-	-
ddCTP	-	8μM	-	-
ddGTP	-	-	8μM	-
ddTTP	-	-	-	8μM
NaCl	50mM	50mM	50mM	50mM

Labelling reaction mix:

Template-primer	10.0µl
DDT (0.1M)	1.0µl
Diluted labelling mix	2.0µl
[α-^{35}S]dATP (5µCi, >600Ci/mmol)	0.5µl
Diluted Sequenase (Version 2.0)	2.0µl

The labelling mix was either diluted 5-fold for sequencing up to 400bp from the primer or used undiluted for sequencing beyond 400bp. Usually the labelling mix labelled 'dGTP' (7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP) was used unless sequencing sequences which were known to give compression problems due to G-C rich regions in which case the labelling mix labelled 'dITP' (15µM dITP, 7.5µM dCTP, 7.5µM dTTP) was used.
The Sequenase Version 2.0 enzyme was always diluted 1:8 in Sequenase dilution buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 5mg/ml BSA)

The labelling reaction was set up in a 1.5ml microcentrifuge tube and mixed by pipetting the contents of the tube in and out a few times. The reaction was incubated at room temperature for 4 min. During the labelling reaction, the tubes containing the termination mixes were put into a 37°C waterbath to pre-warm for at least 1 min. To these tubes (and well before the end of the labelling reaction) 3.5µl of the labelling reaction mix was added to each separate tube labelled 'A', 'C', 'G' and 'T', just inside the rim, so that the termination reaction could be started at 4 min after the beginning of the labelling reaction by a brief spin in an Eppendorf 5413 microcentrifuge. The termination reaction was

incubated at 37°C for a further 4 min, after which time it was stopped by the addition of 4µl of stop solution (95% formamide (v/v), 20mM Na_2EDTA, 0.05% bromophenol blue (w/v), 0.05% xylene cyanol FF (w/v)), added in the same way as the labelling reaction mix previously. Completed reactions were stored for up to a week at -20°C. Samples were denatured by heating to 75-80°C for 2 min and loaded and run on gels as in section 2.21.2.

2.21.2 Sequencing gel electrophoresis

For each 20x40cm, 0.4mm thick gel, 50ml of linear sequencing gel mix (6% acrylamide:bisacrylamide (38:2 (w:w), 42% urea (w/v), 1xTBE) was used. To this mix was added 300µl 10% (w/v) ammonium persulphate, and the acrylamide was de-gassed in a vacuum flask for about 5mins (when no more bubbles were produced). To the de-gassed mix, 50µl TEMED was added and the gel poured immediately and the comb put into place. Gels were sometimes stored overnight at this stage. Excess acrylamide was then trimmed from the comb area, and the gel was set up on the electrophoresis apparatus with two aluminium plates to give the gel an even distribution of temperature during electrophoresis. The heat-denatured sequencing reaction mixtures (sections 2.21.1.1 and 2.21.1.2) were loaded into individual wells (2µl in each), each clone occupying 4 adjacent wells for each tube marked A, C, G and T, with at least one space between separate clones. Gels were run at a constant power setting of 40W, with the initial voltage at about 1.5kV and initial current at about 27mA. For reading the maximum number of nucleotides each sample was run for 2h, 5h and 8h.
After electrophoresis the apparatus was dismantled and the gel plates separated, leaving the gel on top of one of them. The gel was then fixed in 10% acetic acid, 10% methanol (v/v) for at least 15min, after which it was

dried on a slab gel-drier at 80°C for 30min and autoradiographed overnight.

2.21.3 Selective screening by sequencing ('A-tracking')

This method is a modification of the full sequencing protocol, and generates only one track (the 'A'-track) on the final sequencing gel, but is very useful for the comparison of a large number of clones to be sequenced. The protocol is such that it allows large numbers of clones to be handled:

Priming mix:

Oligonucleotide primer (5ng)	4µl
Klenow reaction buffer (x10)	6µl
H_2O	12µl

This priming mix was set up for each ten clones to be screened. To separate, labelled, 1.5µl microcentrifuge tubes was added 2µl of priming mix and 2µl (approx. 400ng) of DNA template (section 2.20.2). The contents of these tubes were mixed and spun briefly to the bottom. Annealing of the primer to the template was carried out by incubation in an oven set at approx. 57°C for 2h. After allowing the contents of the tubes to cool down they were spun to the bottom of the tubes.

Sequencing reaction mix:

dATP/ddATP mix (table 2)	16µl
[α-^{35}S]dATP S (30µCi, >600mCi/mmol)	3µl
Klenow polymerase (1U/µl)	2µl

This reaction mix was set up and mixed. To each tube containing annealed template/primer (above) 2µl of this

reaction mix was added just inside the rim, keeping the tubes horizontal in the racks for the Eppendorf 5413 microcentrifuge. The tubes were then spun briefly to start the reaction which was carried out at room temperature for 20min, after which time 1µl of chase solution was added to all the tubes and mixed the same way as the sequencing reaction mix previously. This chase reaction was carried out for a further 15min at room temperature, after which time 2µl of formamide dye mix was added to stop the reaction. Completed reactions were stored at -20°C for up to a week before electrophoresis (section 2.21.2)

2.22 Computer programs used for DNA sequence data analysis

Two separate program packages were used to store, analyse and modify DNA sequences read from DNA sequencing gel autoradiograms (section 2.21.2). The first was a package devised by Staden (1978), and was run on a Digital PDP 11-34 computer with a multi-user facility in the Department of Biochemistry, University of Glasgow. The second package was the UWGCG (University of Wisconsin Genetics Computer Group) package (Devereux et al., 1984) which was accessed on the ERCC (Edinburgh Regional Computer Centre) Digital VAX 11/750 computer. As well as programs to analyse DNA sequences this package contained programs which allowed access to sequences in the GenBank and EMBL databases.

2.22.1 Staden programs used

SEQEDT: allowed the creation and editing of files of DNA sequences.
SEQLST: displayed a sequence file in blocks of 10 nucleotides or base pairs in the Staden format.

TRNTRP: translated nucleotide sequence into peptide sequence in any reading frame required.
CUTSIT: generated a restriction enzyme map of a particular DNA sequence.

2.22.2. UWGCG programs used

SEQED: allowed the creation and editing of files of DNA sequences.
BESTFIT: found the region of greatest similarity between two DNA sequences, inserting gaps where necessary to optimise the alignment.
GAP: produced optimal alignment between two sequences by inserting gaps in either one where necessary. The outputs from this program was used as input for the PRETTY program.
PRETTY: displayed the alignment of a number of related sequences. PRETTY did not align sequences on the basis of similarity but simply formatted the GAP program outputs.
STRINGS: searched the GenBank or EMBL databases for entries containing certain (chosen) sequences or text patterns.
FETCH: transferred a copy of a file from either the GenBank or EMBL databases to the user directory for use in the above programs.
MAP: Produced a restriction enzyme maps and deduced amino acid sequences from nucleotide sequence input files.

3. RESULTS

3.1 Investigation of the number of rbcS genes in P. vulgaris genomic DNA

To obtain an estimate of the number of rbcS sequences in the genome of P. vulgaris, Southern blot analysis was performed on the genomic DNA of P. vulgaris digested with various restriction endonucleases. This analysis used rbcS cDNA sequences of both pea and P. vulgaris as hybridisation probes (section 2.18.1.1), as well as the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG (section 2.18.1.2).

3.1.1 Using rbcS cDNA probes

A southern blot of P. vulgaris genomic DNA, digested to completion with BamH1, EcoR1 and HindIII was hybridised to two different rbcS cDNA probes in two separate experiments. Hybridisation **a** was performed as in section 2.19.1.1 using the EcoR1-EcoR1 insert of pPvSS1672 (figure 15) and hybridisation **b** was performed as in section 2.19.1.1 using the HindIII-HindIII inserts of pSSU61 and pSSU161 (figure 1). Both probes were labelled by nick-translation (section 2.18.2). Figure 2 shows the autoradiograms of the filter after the two separate hybridisations and washings. After hybridisation **a** the filter was washed at relatively more stringent conditions than after hybridisation **b**. Figure 3b shows a plot of distance migrated against log [size in kbp] of DNA fragments, produced by the digestion of λDNA by EcoR1 and HindIII, which were run on the same gel as the genomic DNA (figure 3a). This plot was linear in the range 0.5-5.0 kbp and was used to extrapolate approximate sizes for hybridising fragments in this range. The sizes of hybridising fragments greater than 5.0 kbp were

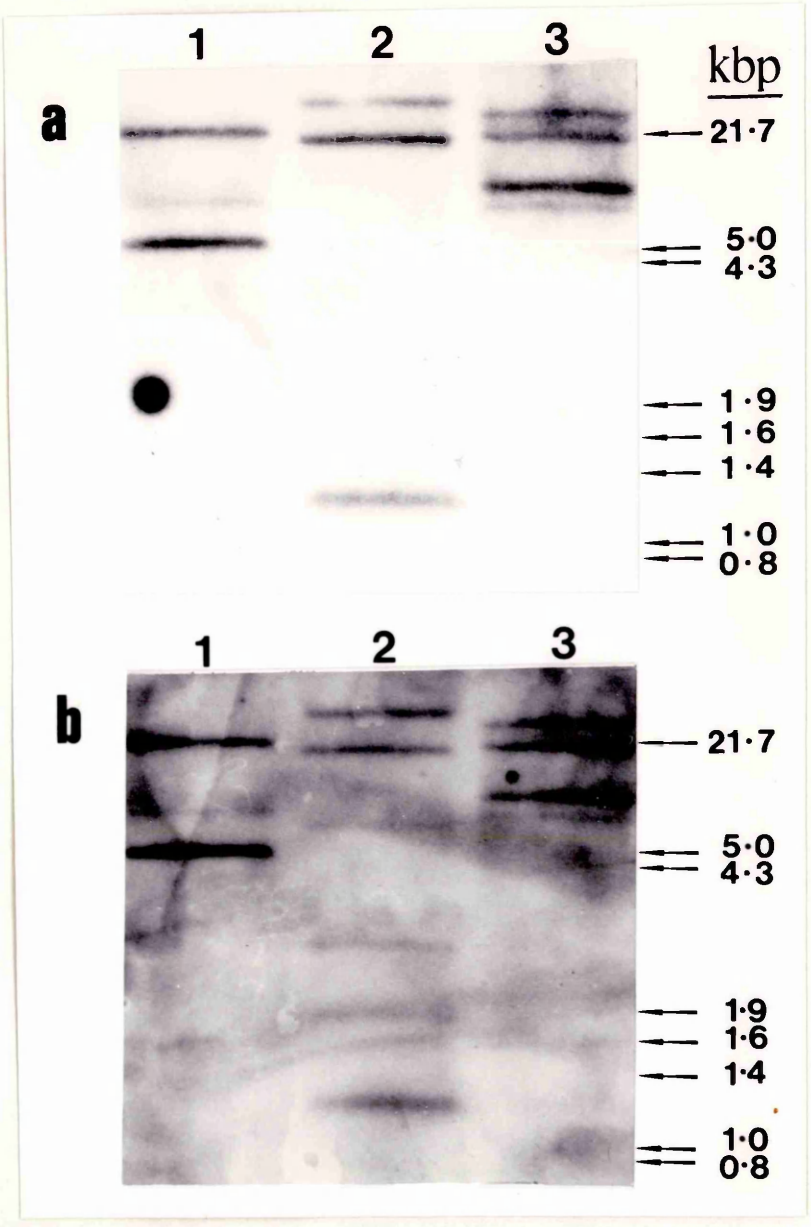
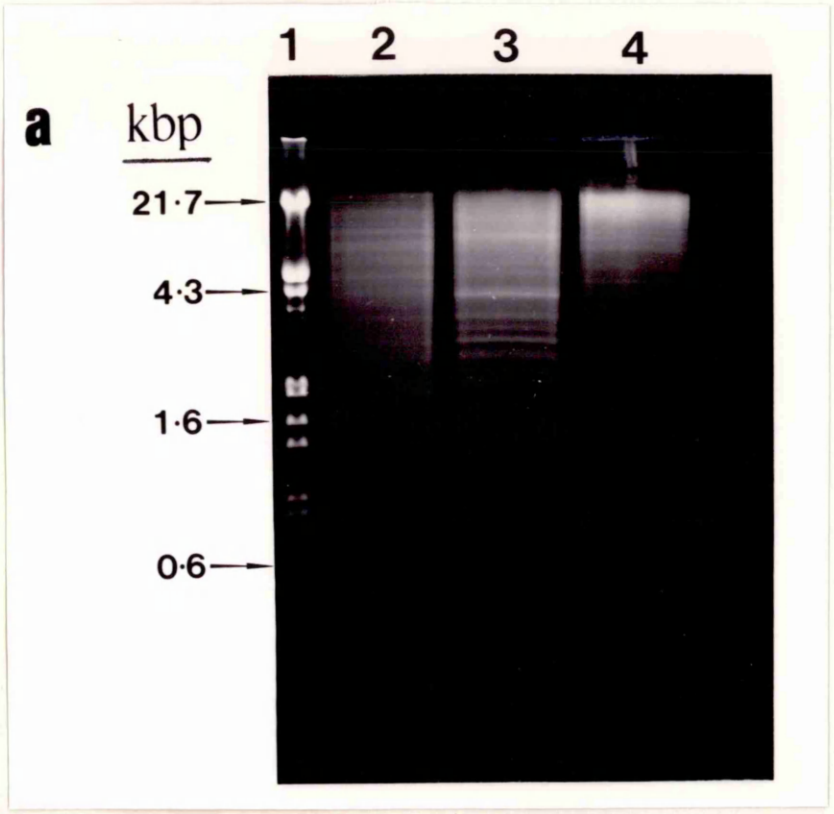


Figure 2 - Southern blot analysis of P. vulgaris genomic DNA

Autoradiograms of a Southern blot of P. vulgaris genomic DNA, digested to completion with BamH1, EcoR1 and HindIII (10ug of DNA per track), after hybridisation to two different rbcS cDNA probes. Hybridisation **a** was performed as in section 2.19.1.1 using the EcoR1-EcoR1 insert of pPvSS1672 (figure 15), which had been labelled by nick-translation (section 2.18.2) to approximately $1x10^9$cpm/ug, in hybridisation solution containing $2x10^6$cpm/ml. Hybridisation temperature was 45°C, and the critical washes of the filter were at 60°C in 0.5xSSC twice for 30 min. Hybridisation **b** was performed as in section 2.19.1.1 using the HindIII-HindIII inserts of pSSU61 and pSSU161 (figure 1) which had been labelled by nick-translation (section 2.18.2) to $7.5x10^8$cpm/ug in hybridisation solution containing $6x10^6$cpm/ml. Hybridisation temperature was 35°C, and the critical washes of the filter were at 50°C in 0.5xSSC twice for 30 min.

Tracks 1a and 1b contained HindIII-digested DNA, tracks 2a and 2b contained EcoR1-digested DNA and tracks 3a and 3b contained BamH1-digested DNA.



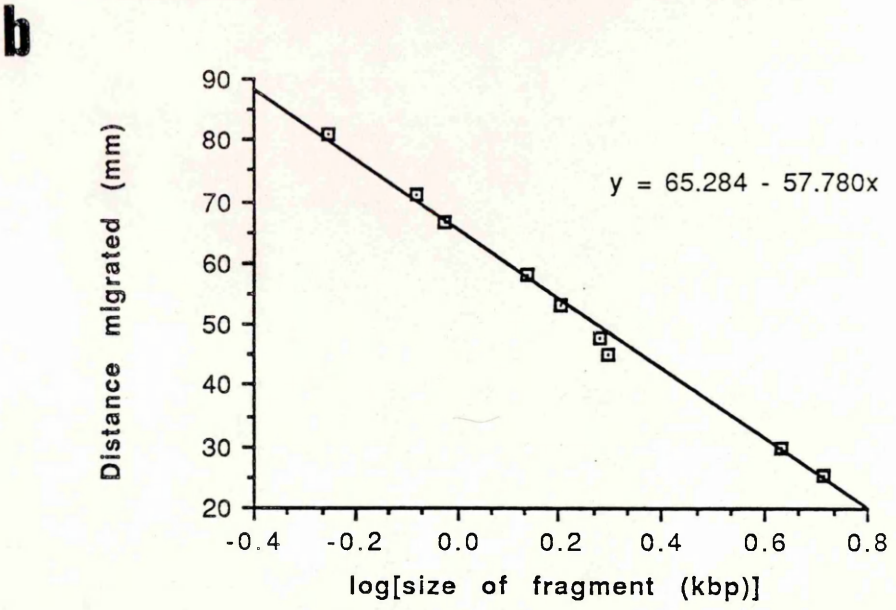


Figure 3 - Agarose gel electrophoresis of P. vulgaris genomic DNA

P. vulgaris DNA was digested with HindIII, EcoRl and BamHl and run on a 0.8% agarose/TBE gel (10ug/track). This gel was blotted as in section 2.17.1 to provide the blot for hybridisation (figure 2).

(a) Photograph of EtBr-stained agarose gel. Track 1 contained λDNA digested with EcoRl and HindIII and tracks 2, 3 and 4 contained P. vulgaris genomic DNA digested with HindIII, EcoRl and BamHl respectively.

(b) Plot of distance migrated against log[size(kbp)] of DNA fragments produced by the digestion of λDNA by EcoRl and HindIII and which were run alongside the P. vulgaris DNA digests on the same gel.

approximated by eye.

Table 4 shows the sizes of the hybridising fragments in both analyses. The pattern of sizes obtained was the same in both hybridisations **a** and **b** for DNA digested with BamH1 and HindIII. However, hybridisation **b** produced 6 hybridising fragments in DNA digested with EcoR1 whereas the corresponding track after hybridisation **a** produced only 3. Three hybridising fragments in EcoR1-digested genomic DNA were common to both analyses and after hybridisation **b** these 3 fragments hybridised more strongly than the other 3 fragments in the same track. The 3 extra hybridising fragments in blot B are shown in brackets in table 4. The result with the P. vulgaris rbcS cDNA probe was consistent when the experiment was repeated several times.

The relative degree of hybridisation to different hybridisation fragments also varied between the hybridisations to the pea rbcS cDNA probe and to the P. vulgaris rbcS cDNA probe. After hybridisation to the P. vulgaris cDNA probe (hybridisation **a**) one of the hybridising fragments (approximate size 20.0 kbp) in EcoR1-digested genomic DNA hybridised more strongly than the other two (approximate sizes 28.0 and 1.3 kbp) in the same track. Furthermore one of the hybridising fragments (approximate size 10.0 kbp) in BamH1-digested genomic DNA hybridised more strongly than those of approximate sizes 26.0 and 22.0 kbp in the same track. After hybridisation to the pea rbcS cDNA probe, on the other hand, the hybridising fragments in EcoR1-digested genomic DNA of approximate sizes 28.0, 20.0 and 1.3 kbp and those in BamH1-digested genomic DNA of approximate sizes 26.0, 22.0 and 10.0 showed very similar degrees of hybridisation. No differences in the relative hybridisation to fragments in HindIII-digested genomic DNA were observed between blots hybridised with the two different rbcS cDNA probes.

TABLE 4 - Distance migrated and approximate sizes of hybridising fragments in hybridisations (a) and (b) (figure 2)

Experimental conditions were as described in the legend to figure 2.

RESTRICTION ENDONUCLEASES

HindIII		EcoR1		BamH1	
Distance (mm)	Size (kbp)	Distance (mm)	Size (kbp)	Distance (mm)	Size (kbp)
9.5	24.0	5.5	28.0	7.5	26.0
19.0	9.0	10.5	20.0	10.5	22.0
24.5	5.0	(21.0)	(7.0)	17.0	10.0
		(37.5)	(3.8)	19.5	8.0
		(47.0)	(2.3)		
		59.0	1.3		

3.1.2 Using oligonucleotide probes

Four identical southern blots of P. vulgaris genomic DNA, digested to completion with BamH1, EcoR1 and HindIII were hybridised individually to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG as in section 2.19.1.2. Figure 4 shows the autoradiograms of these four blots after hybridisation and washing. Figure 5b shows a plot of distance migrated against log [size in kbp] of DNA fragments, produced by the digestion of λDNA by EcoR1 and HindIII, which were run on the same gel as the genomic DNA (figure 5a). This plot was linear in the range 0.5-5.0 kbp and was used to extrapolate approximate sizes for hybridising fragments in this range. The sizes of hybridising fragments greater than 5.0 kbp were approximated by eye.

Table 5 shows the sizes of the most strongly hybridising fragments observed in the blot hybridised to rbcSG. The patterns of hybridising fragments in BamH1- and HindIII-digested genomic DNA were very similar to those obtained in the corresponding tracks of the genomic blots hybridised to rbcS cDNA probes (section 3.1.1), although the hybridising fragment with an approximate size of 9.0 kbp in HindIII-digested genomic DNA was observed only very faintly in this blot. Five main hybridising fragments were observed in the EcoR1-digested DNA of this blot, three (approximate sizes 30.0, 22.0 and 1.1 kbp) were similar in size to those observed after hybridisations **a** and **b** (section 3.1.1). The other two hybridising fragments in the EcoR1-digested DNA (approximate sizes 14.0 and 5.8) were unique to this blot. The relative extents of hybridisation to the fragments described above were very similar.

The blot hybridised to rbcS1 had 1 main hybridising fragment in the EcoR1-digested genomic DNA, 2 main hybridising fragments in the BamH1-digested genomic DNA

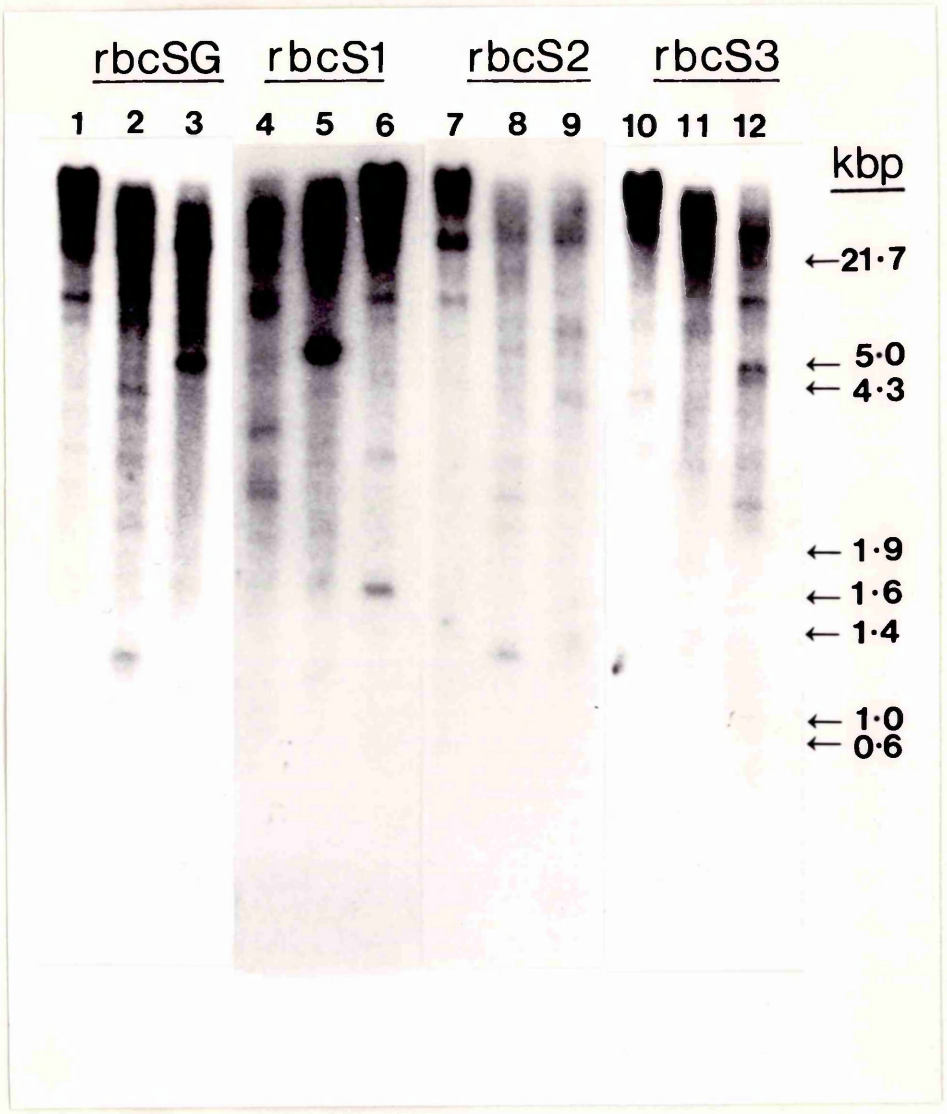
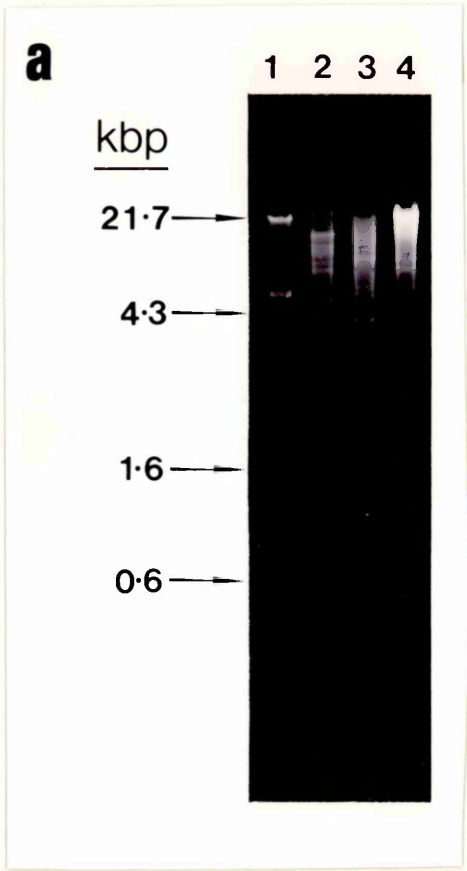


Figure 4 - Southern blot analysis of P. vulgaris genomic DNA using oligonucleotide probes

Autoradiograms of 4 identical southern blots of P. vulgaris genomic DNA, digested to completion with BamHl, EcoRl and HindIII (5ug of DNA per track), after having been hybridised to the oligonucleotide probes rbcSG, rbcSl, rbcS2 and rbcS3 (section 2.18.1.2). Hybridisation was carried out at 46°C as described in section 2.19.1.2 in hybridisation solution containing approximately $1x10^6$cpm/ml. The filters were then washed at room temperature three times in 6xSSC for 20 min and then autoradiographed. Tracks 1, 6, 7 and 10 contained BamHl-digested DNA, tracks 2, 5, 8 and 11 contained EcoRl-digested DNA and tracks 3, 4, 9 and 12 contained HindIII-digested DNA.



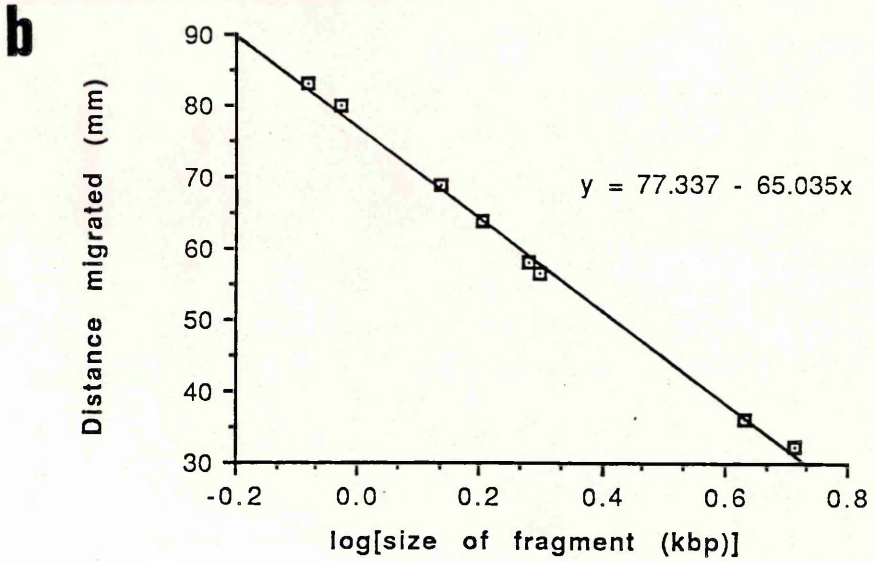


Figure 5 - Agarose gel electrophoresis of P. vulgaris genomic DNA

P. vulgaris DNA was digested with HindIII, EcoR1 and BamH1 and run on a 0.8% agarose/TBE gel (5ug/track). This gel was blotted as in section 2.17.1 to provide the blots for hybridisation (figure 4).

(a) Photograph of EtBr-stained agarose gel, showing 3 of the twelve tracks run to provide four replicates. Track 1 contained λDNA digested with EcoR1 and HindIII and tracks 2, 3 and 4 contained P. vulgaris genomic DNA digested with HindIII, EcoR1 and BamH1 respectively.

(b) Plot of distance migrated against log[size(kbp)] of DNA fragments produced by the digestion of λDNA by EcoR1 and HindIII which were run alongside the P. vulgaris DNA digests on the same gel.

TABLE 5 - Distance migrated and approximate sizes of hybridising fragments in the the Southern blot hybridised to the oligonucleotide probe rbsSG shown in figure 4.

Experimental conditions were as described in the legend to figure 4.

RESTRICTION ENDONUCLEASES

HindIII		EcoR1		BamH1	
Distance (mm)	Size (kbp)	Distance (mm)	Size (kbp)	Distance (mm)	Size (kbp)
16.0	25.0	11.0	>30.0	12.5	28.0
32.0	5.0	17.0	22.0	16.0	25.0
		22.5	14.0	23.5	12.0
		36.5	5.8		
		72.5	1.1		

and 6 main hybridising fragments in the HindIII-digested genomic DNA. The hybridising fragment with an approximate size of 12 kbp in the BamH1 track and the one with an approximate size of 25.0 kbp in the HindIII track were similar in size to fragments observed in the blot hybridised to rbcSG, but neither appeared to be the most strongly hybridising fragment in the track in which they were observed. The hybridising fragment in the EcoR1-digested DNA did not correspond in size to any observed in the blot hybridised to rbcSG.

The blot hybridised to rbcS2 had several hybridising fragments in the EcoR1-digested genomic DNA, 3 main hybridising fragments in the BamH1-digested genomic DNA and 3 main hybridising fragments in the HindIII-digested genomic DNA. The hybridising fragment with an approximate size of 25.0 kbp in the BamH1 track, the one with an approximate size of 25.0 kbp in the HindIII track and the one with an approximate size of 1.1 kbp in the EcoR1 track were similar in size to fragments observed in the blot hybridised to rbcSG and were, in each case, the most strongly hybridising fragment in the track in which they were observed.

The blot hybridised to rbcS3 had about 4 main hybridising fragments in the EcoR1-digested genomic DNA, 2 main hybridising fragments in the BamH1-digested genomic DNA and 5 main hybridising fragments in the HindIII-digested genomic DNA. The hybridising fragment with an approximate size of 28.0 kbp in the BamH1 track, the one with an approximate size of 5.0 kbp in the HindIII track and the one with an approximate size of 30.0 kbp in the EcoR1 track were similar in size to fragments observed in the blot hybridised to rbcSG and were, in the case of EcoR1- and BamH1-digested genomic DNA, the most strongly hybridising fragments in the tracks in which they were observed. The hybridising fragment with an approximate size of 5.0 in HindIII-digested DNA hybridised as strongly

as the other hybridising fragments in the same track.

3.2 S1 nuclease analysis of P. vulgaris total RNA

S1 nuclease analysis of total RNA was attempted to measure the expression of different P. vulgaris rbcS genes. This section describes the development of a S1 protection assay which would allow such a study, along with the results obtained using this method.

3.2.1 Development of a S1 protection assay for use in measuring expression of individual rbcS genes

During this study several protocols for the S1 nuclease analysis of RNA were used, with varying degrees of success. This section describes the attempts at using double-stranded probes labelled by two separate methods and single-stranded DNA probes labelled using one method but with labelled nucleotides of different specific activities.

3.2.1.1 Using double-stranded probes

This section describes attempts to optimise the conditions for using double-stranded DNA probes for the S1 nuclease analysis of total RNA. Theoretical optimal temperatures of hybridisation (temperatures at which re-annealing of the probe is minimal but hybridisation to RNA still occurs) were calculated empirically (section 3.2.1.1.1) and by the use of a formula (section 3.2.1.1.2).

3.2.1.1.1 Using end-labelled probe

The DNA probe used was the Hinf1-BamH1 fragment of pPvSS1672 (figure 15) which was end-labelled using Klenow polymerase and [α-32P]dATP (3000Ci/mmol) as in section

2.18.5. Hybridisation to total RNA and subsequent digestion with S1 nuclease was as described in section 2.19.2.2. The optimal hybridisation temperature was obtained empirically by testing for S1 nuclease sensitivity of the probe DNA in hybridisation solution at various temperatures. The temperature for sensitivity (Tm) was determined as 52°C. Under these conditions DNA and RNA hybridise optimally at Tm+4°C and RNA and RNA at Tm+10°C (Hopwood et al., 1985), thus the hybridisation temperature was chosen as 56°C.

Figure 6 shows that after the S1-digestion products were run on a sequencing gel most of the label was located in the undigested probe band. A band 10-15bp smaller was just visible. No other bands were detected.

The optimal hybridisation temperature estimated empirically was therefore unsuccessful and allowed the probe to re-anneal rather than hybridise to the RNA. Therefore another method involving the calculation of the optimal hybridisaing temperature using a mathematical formula was subsequently attempted (section 3.2.1.1.2).

3.2.1.1.2 Using probe synthesised by primer extension

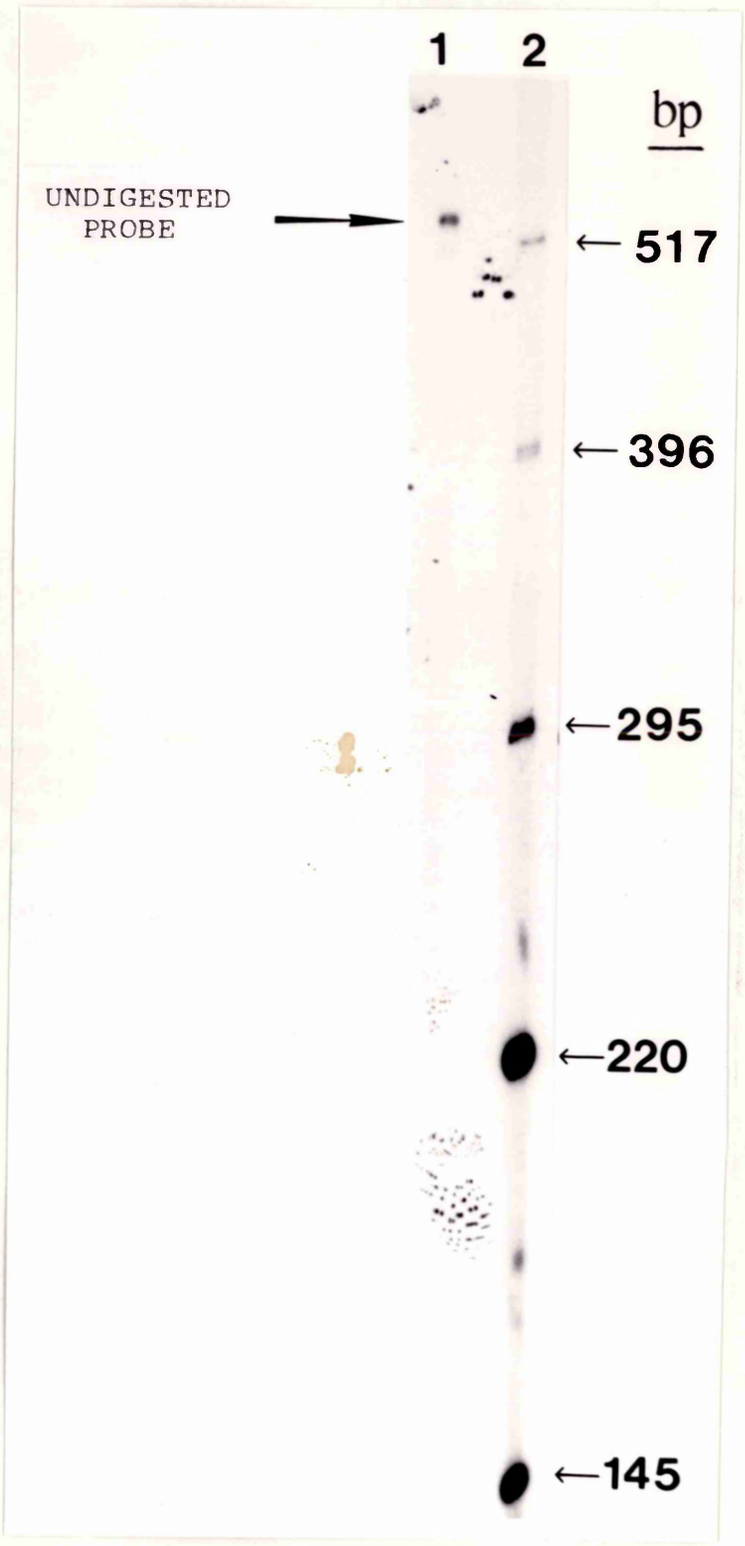
The DNA probe used was the EcoR1-BamH1 fragment of pBS1 (figure 15) inserted into M13mp19. The probe was labelled as for a single-stranded probe synthesis reaction (section 2.18.3), except that the labelled probe fragment was released by digestion with both BamH1 and EcoR1 before isolation on a standard 2% agarose/TBE gel, thus providing a double-stranded probe with one strand uniformly labelled. As the full sequence of this probe fragment was known, the hybridisation temperature was determined as 52°C using the formula:

$$[74.9 + 0.41(\%G+C) - 0.6465(\%formamide) - 500/L - 5]$$

(Calzone et al., 1987)

Figure 6 - S1 nuclease analysis of P. vulgaris total RNA using a double-stranded DNA probe.

The probe used was the Hinf1-BamH1 fragment (approx. 500bp) of pPvSS1672 (figure 15) which was end-labelled using Klenow polymerase and [α-^{32}P]dATP (3000Ci/mmol) as in section 2.18.5. Hybridisation was at 56^{o}C in 80% de-ionised formamide (v/v), 0.4mM NaCl, 40mM PIPES pH 6.4, and carried out as in section 2.19.2.2, as was digestion with S1 nuclease. Digestion products were then run on a 4% acrylamide sequencing gel (section 2.21.2) against pAT153 plasmid DNA digested with Hinf1 and end-labelled in the same way as the probe.
Track 1 contained total RNA incubated with probe and digested with S1 nuclease and track 2 contained pAT153/Hinf1 markers. The position of the undigested probe is indicated in track 1.


1
2
bp
UNDIGESTED
PROBE
517
396
295
220
145

where L=length of probe in base-pairs.

Hybridisation and digestion with S1 nuclease were as described in section 2.19.2.2.. Figure 7 shows the result of two separate experiments where the labelled nucleotide ([α-32P]dCTP) was at different specific activities (300Ci/mmol and 3000Ci/mmol). In both cases the strongest band was that representing undigested probe. The background:signal ratio was very much lower when the label was at 300Ci/mmol than at 3000Ci/mmol. When using the label with the higher specific activity, many other smaller additional bands were detected all the way down the gel, whereas when the label at the lower specific activity was used, only a few smaller faint additional bands were detected, the most noticeable of which was only slightly smaller than the probe band. It was apparent that in the experiment using the label at the lower specific activity, the intensity of the probe band varied between tracks even though equal amounts of probe were added to each hybridisation reaction.
The optimal hybridisation temperature calculated was therefore unsuccessful and allowed the probe to re-anneal rather than hybridise to the RNA (as in the method described in section 3.2.1.1.1). As re-annealing of the probe was such a recurrent problem when using double-stranded DNA probes, S1 nuclease analysis of total RNA using single-stranded DNA probes was subsequently performed (section 3.2.1.2).

3.2.1.2 Using single-stranded probes

The DNA probe used was the EcoR1-BamH1 insert of pBS1 (figure 15) inserted into M13mp19. Single-stranded templates were made as in section 2.20.7. Single-stranded probe was synthesised as in section 2.18.3.

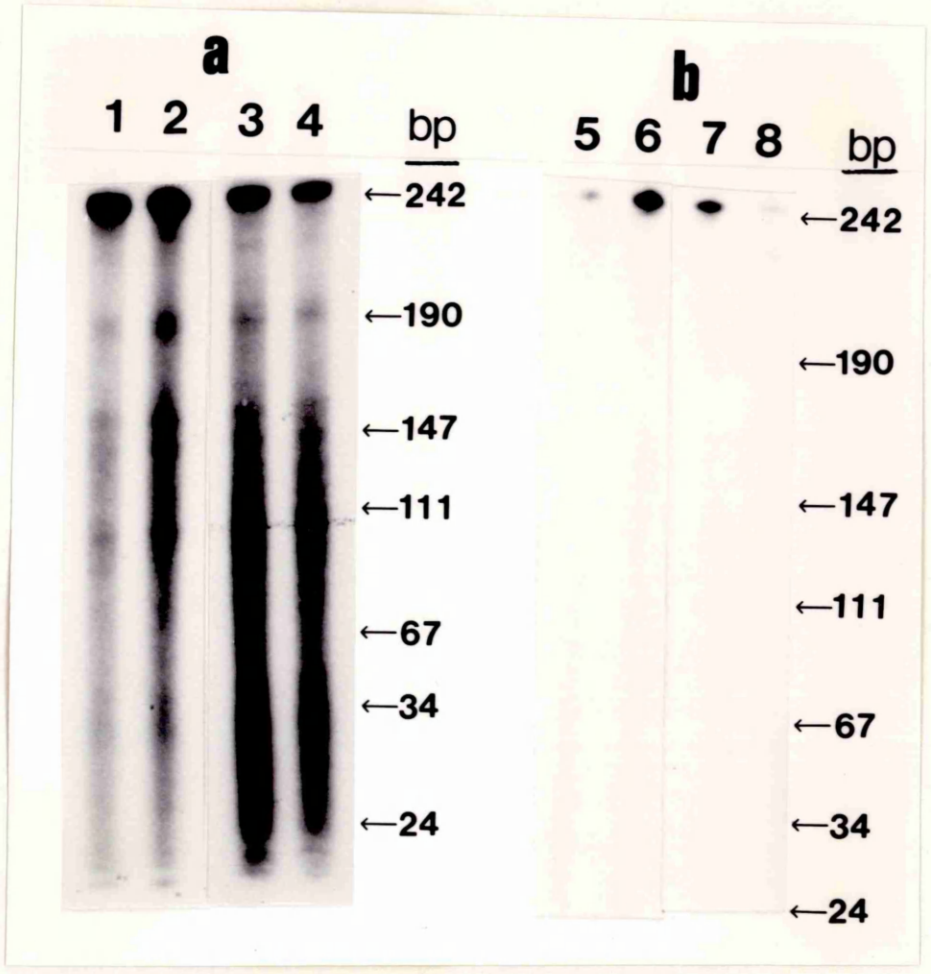


Figure 7 - S1 nuclease analysis of P. vulgaris total RNA using a double-stranded DNA probe

S1 nuclease analysis of total RNA (5 and 10ug) from dark-grown and light-grown primary leaves of P. vulgaris, using a double-stranded DNA probe (10ng). The probe used was the BamH1-EcoR1 fragment (approx.230bp) of pPvSS1672 (figure 15) in M13mp19 which was labelled by primer extension as in section 2.18.3 and isolated after digestion with BamH1 and EcoR1. Hybridisation was at 52°C in 80% de-ionised formamide (v/v), 0.4mM NaCl, 40mM PIPES pH 6.4, carried out as in section 2.19.2.2, as was digestion with S1 nuclease. Digestion products were then run on a 6% acrylamide sequencing gel (section 2.21.2) against pUC8 plasmid DNA digested with HpaII and end-labelled in the same way as in section 2.18.5. Tracks 1, 2, 5 and 6: RNA from dark-grown primary leaves; tracks 3, 4, 7 and 8: RNA from light-grown primary leaves. Tracks 1, 3, 5 and 7: 5ug RNA; tracks 2, 4, 6 and 8: 10ug RNA. Tracks 1-4: the probe was labelled with [α-^{32}P]dCTP at 3000Ci/mmol; tracks 5-8 the probe was labelled with [α-^{32}P]dCTP at 300Ci/mmol.

3.2.1.2.1 Probe isolation

Various gel-systems were used to isolate the single-stranded probe fragment (section 2.18.3); a standard TBE/agarose gel described by Burke (1984), a neutral agarose gel system as described by Maniatis et al. (1982), denaturing acrylamide sequencing gels (section 2.21.2) and latterly a TAE gel system as described by Calzone et al. (1987).

Using a TBE/agarose gel, a neutral agarose gel or a denaturing acrylamide sequencing gel, the majority of the label was always located in the well rather than in the probe band whereas the TAE/agarose gel system resulted in the majority of the label being located in the probe band (figure 8). The gel system described by Burke (1984) was also inconsistent with regards to the denaturation of the probe and template before separation on an agarose gel, as often most of the label which had not migrated out of the well was located in a high-molecular weight band corresponding to undenatured template (not shown). The method described by Calzone et al. (1987), therefore, was favoured to isolate single-stranded DNA probes for use in S1 nuclease analysis of total RNA.

3.2.1.2.2 Hybridisation and specific activity of [^{32}P]labelled nucleotide

Single-stranded probe was synthesised initially using [α-^{32}P]dCTP at 3000Ci/mmol, but latterly using [α-32P]dATP at >410Ci/mmol (minimum specified by Amersham International).

Two methods were used for the hybridisation of the single-stranded probes to the RNA. Initially the method described by Burke (1984; see section 2.19.2.1.1) was used but latterly the method described by Nagy et al. (1988; see section 2.19.2.1.2) was used.

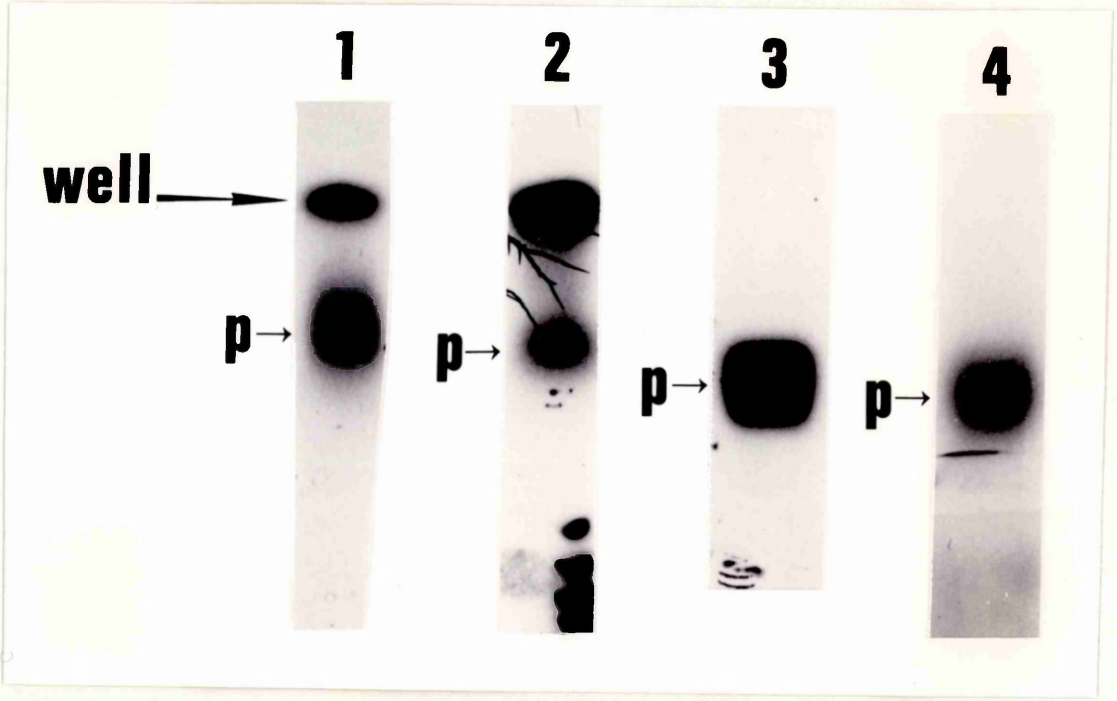


Figure 8 - Autoradiograms used to locate single-stranded probe in agarose gels after synthesis.

The positions of the probe band (P) and the loading well (WELL) of the gels are indicated. The different autoradiograms were used to locate probe bands from the different gel systems described in section 2.18.3.

1. Probe synthesised using [α-^{32}P]dCTP at ~3000Ci/mmol, denatured and run on a standard 1.2% agarose/TBE gel as described by Burke (1984).
2. Probe synthesised using [α-^{32}P]dCTP at ~3000Ci/mmol, denatured and run on a standard 1.2% neutral agarose gel as described by Maniatis (1982).
3. and 4. Probe denatured and isolated on a 1.2% agarose/TAE gel as described by Calzone (1987). Label used for probe synthesis was [α-^{32}]dCTP at ~3000Ci/mmol (3.) and [α-^{32}P]dATP at >400Ci/mmol (4.).

Using the method described by Burke (1984) with probe synthesised using [α-32P]dCTP at 3000Ci/mmol, protected fragments with sizes of approximately 230bp and 130bp were detected after hybridisation to RNA isolated from dark-grown and light-grown primary leaves of P. vulgaris (figure 9). The use of [α-32P]dCTP at 3000Ci/mmol always resulted in a relatively high background:signal ratio, thus preventing any estimation of the relative amounts of the different protected fragments.
Using the method described by Nagy et al.(1988) with probe synthesised using [α-32P]dATP at >410Ci/mmol, protected fragments with sizes of approximately 230bp, 220bp, 125bp and 115bp were detected after hybridisation to RNA isolated from dark-grown and light-grown primary leaves of P. vulgaris (figure 10). Using label at this specific activity considerably reduced the background:signal ratio, allowing a better estimation of the relative amounts of the different protected fragments. This ratio was relatively much lower than when the probe was synthesised from [α-32P]dCTP at 3000Ci/mmol (not shown).

3.2.2 S1 protection assay on RNA from dark- and light-grown primary leaves.

Figure 10 shows the result of an S1 protection assay (described in section 3.2.1.2.2) carried out on total RNA extracted from the dark-grown primary leaves of 6 day-old P. vulgaris plants, and on total RNA from such plants subsequently illuminated with continuous white light at 100 μmol $m^{-2}s^{-1}$ for 48h. Protected fragments with sizes of approximately 230bp, 220bp, 125bp and 115bp were detected as a result of hybridisation to both these types of RNA. Controls of probe hybridised to rat liver total RNA (provided by Dr. S. Griffifths of this department), probe alone with or without subsequent digestion with S1 nuclease showed no prominent protection fragments (not

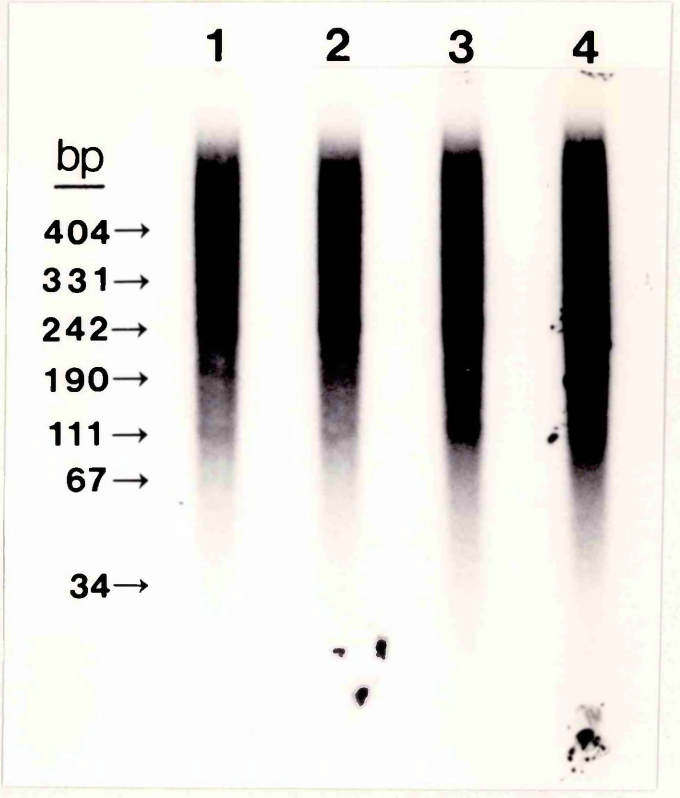


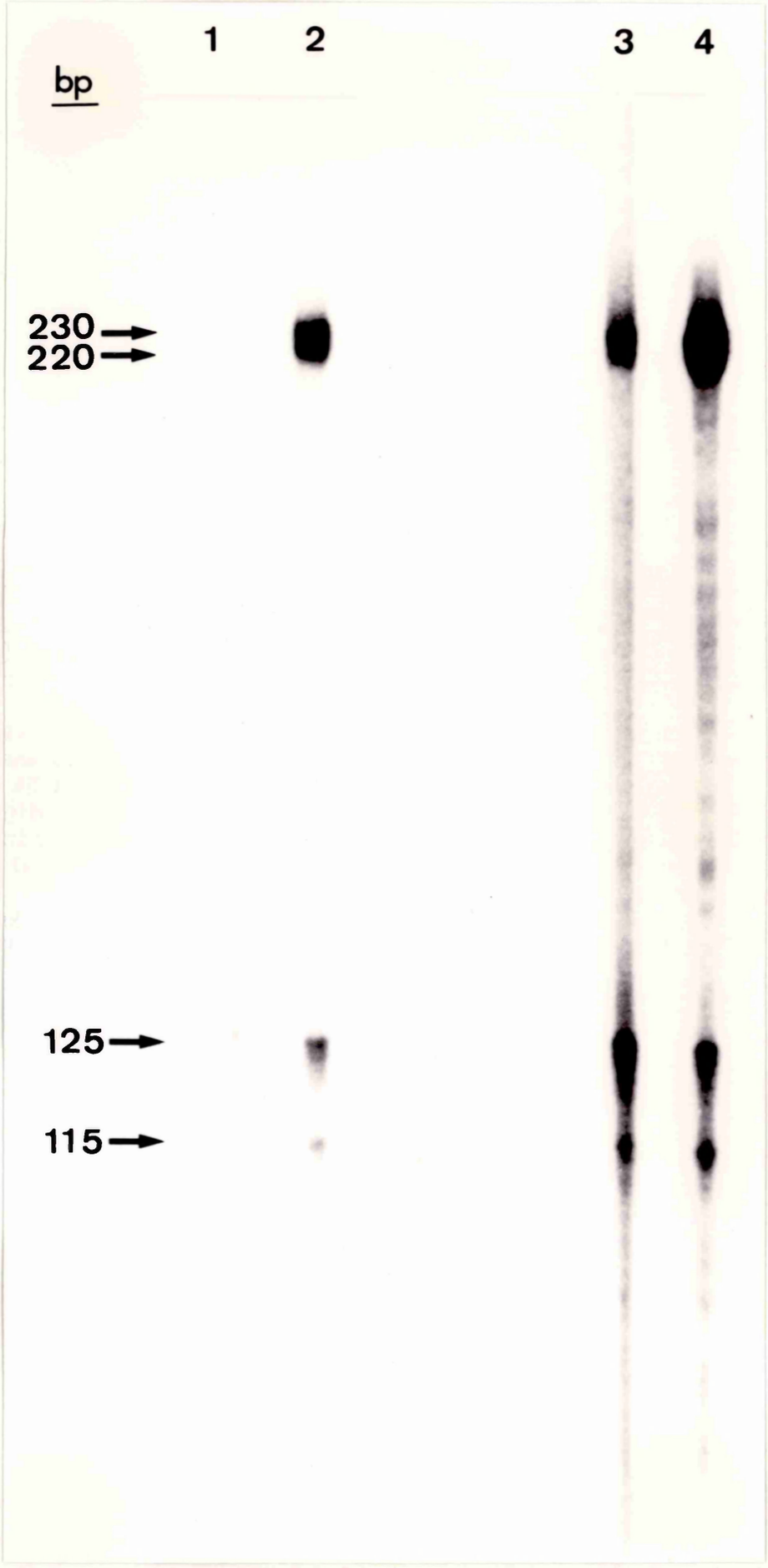
Figure 9 - S1 nuclease analysis of P. vulgaris total RNA using a single-stranded probe

The probe used was the BamH1-EcoR1 fragment (approx.230bp) of pPvSS1672 (figure 15) in M13mp19 which was labelled (using [α-^{32}P]dCTP at 3000Ci/mmol) by primer extension as in section 2.18.3 and isolated after digestion with EcoR1. Hybridisation was at 65^{o}C in 0.4mM NaCl, 10mM PIPES pH 6.5, carried out as in section 2.19.2.1.1, as was digestion with S1 nuclease. Digestion products were then run on a 6% polyacrylamide sequencing gel (section 2.21.2).
Tracks 1 and 3: RNA from dark-grown primary leaves; tracks 2 and 4: RNA from light-grown primary leaves. Tracks 1 and 2: 5ug RNA; tracks 3 and 4: 10ug RNA. pUC8 plasmid DNA digested with HpaII and end-labelled as in section 2.18.5 were used as size markers.

Figure 10 - S1 nuclease analysis of P. vulgaris total RNA using a single-stranded DNA probe

The probe used was the BamH1-EcoR1 fragment (approx.230bp) of pPvSS1672 (figure 15) in M13mp19 which was labelled (using [α-^{32}P]dATP at approximately 400Ci/mmol) by primer extension as in section 2.18.3 and isolated after digestion with EcoR1. Hybridisation was at 37°C in 50% de-ionised formamide (v/v), 0.4mM NaCl, 20mM PIPES pH 6.8, 2mM Na_2EDTA pH 7.0, carried out as in section 2.19.2.1.2, as was digestion with S1 nuclease. Digestion products were then run on a 6% acrylamide sequencing gel (section 2.21.2), with pUC8 plasmid DNA digested with HpaII and end-labelled as in section 2.18.5 to provide size markers. The sizes of the protected fragments were calculated from the migration of these labelled marker fragments.

Tracks 1 and 3: RNA from dark-grown primary leaves; tracks 2 and 4: RNA from light-grown primary leaves. Tracks 1 and 2: 5ug RNA; tracks 3 and 4: 10ug RNA.


1
2
3
4
bp
230
220
125
115

shown). The two protected fragments with sizes of approximately 230bp and 220bp showed very similar relative adundances to each other in both treatments. The protected fragment of size approximately 125bp was more abundant than that of size approximately 115bp in both treatments, the ratio of their abundances relative to each other remaining roughly similar between treatments. The ratio of the two largest fragments to the two smallest fragments was much higher in the light-grown RNA relative to the dark-grown RNA. Comparison of the two tracks where 5ug RNA was loaded showed that although all 4 protected fragments showed increases in abundance after hybridisation to light-grown RNA relative to dark-grown RNA, this increase was far more marked in the two largest fragments than the two smallest. Comparison of the two tracks where 10ug RNA was loaded shows an appreciable increase in abundance for the two largest fragments with little change in the abundance of the two smallest fragments.

3.3 Screening of P. vulgaris rbcS cDNA clones

Various methods were used in an attempt to divide the P. vulgaris rbcS cDNA clones into groups, so that the nucleotide sequences from representatives of each group could be determined. The methods used were restriction analysis, S1 nuclease analysis and 'A'-track sequencing analysis.

3.3.1 By restriction mapping

Restriction analysis was carried out to divide the rbcS cDNA clones on the basis of the pattern of fragments obtained after their digestion with restriction endonucleases.

The BamH1-HindIII inserts of 17 rbcS clones were subsequently digested with Alu1 and run on standard 1.5% agarose/TBE gels (figure 11). Table 6 shows the restriction fragment sizes obtained.
pPvSS328, pPvSS381, pPvSS386, pPvSS1451 and pPvSS2314 showed the most similarity, in their restriction fragment pattern, to pPvSS1672, all having fragments with approximate sizes of 220bp and 270-280bp. Digestions of pPvSS814, pPvSS1066, pPvSS1380, pPvSS1483, pPvSS2284 and pPvSS2285 all produced a restriction fragment of approximate size 500bp

3.3.2 By S1 nuclease analysis

S1 nuclease analysis was attempted on rbcS cDNA sequences to assign specific rbcS cDNA clones to specific rbcS mRNAs, by matching protection fragment sizes to those obtained in section 3.2.2

3.3.2.1 Using radio-labelled single-stranded DNA probe

The feasibilty of using this system was tested by performing S1 nuclease analysis exactly as described for figure 10 (section 3.2.1.2.2) but with the following samples:

(i) 5µg RNA from light-grown primary leaves (protection fragments resulting from hybridisation of this RNA to the probe serving as size markers).
(ii) 5ng linearised pPvSS1672 plasmid DNA (representative rbcS cDNA clone with a predictable protection fragment size, used as a test for assay).
(iii) 5ng linearised pUC8 plasmid DNA (control).

Sample (i) gave the same 4 protection fragments as described in section 3.2.2, but no protected fragments

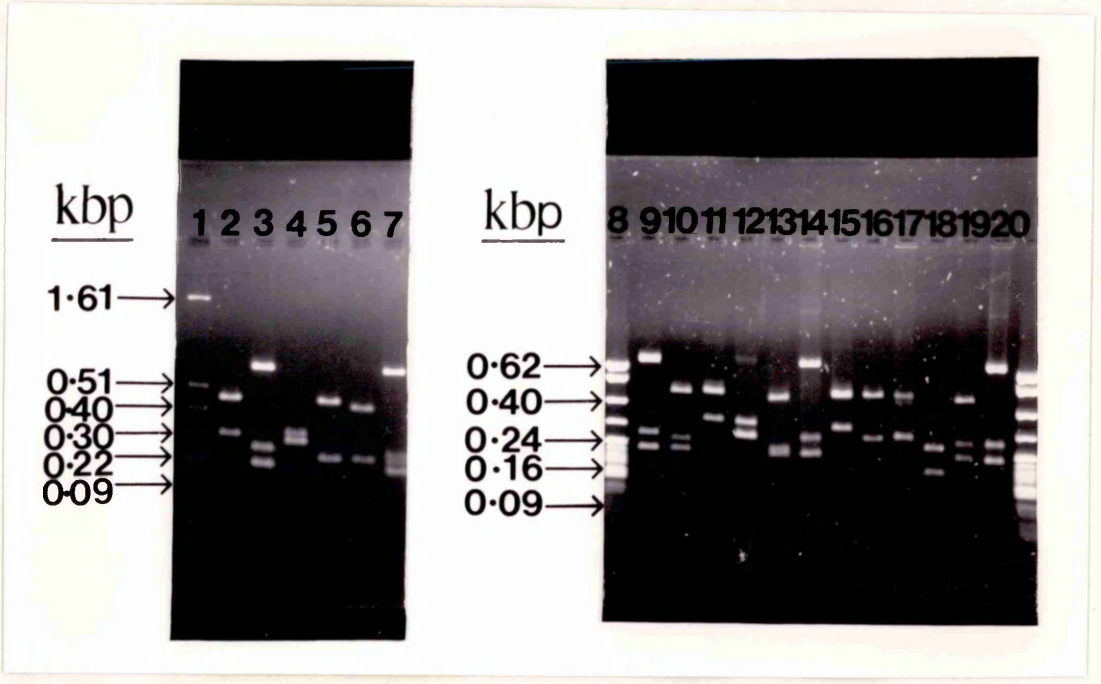


Figure 11 - Agarose gel electrophoresis of rbcS cDNA inserts after digestion with AluI.

The BamHI-HindIII inserts of 17 rbcS cDNA clones were digested with AluI and run on standard 1.5% agarose/TBE gels against pBR322 plasmid DNA digested with MspI (supplied by New England Biolabs, USA) or pAT153 plasmid DNA digested with HinfI as DNA size markers.
Track 1: pAT153/HinfI size markers; tracks 8 and 21: pBR322/MspI size markers; tracks 2 to 7: pPvSS2285, pPvSS1672, pPvSS1644, pPvSS1483, pPvSS636 and pPvSS328 respectively; tracks 9 to 20: pPvSS1672, pPvSS2314, pPvSS2284, pPvSS2227, pPvSS1674, pPvSS1451, pPvSS1380, pPvSS1066, pPvSS814, pPvSS444, pPvSS386 and pPvSS381 respectively.

TABLE 6 - Sizes of fragments obtained after digestion of the BamHI-HindIII inserts of selected P. vulgaris rbcS cDNA clones by AluI.

Experimental conditions were as described in the legend to figure 11.

	CLONE	Fragment sizes (bp)
1.	pPvSS328	700, 270, 250, 220
2.	pPvSS381	650, 280, 220
3.	pPvSS386	490, 280, 220
4.	pPvSS444	270, 200, 125
5.	pPvSS636	470, 250
6.	pPvSS814	500, 310
7.	pPvSS1066	500, 300
8.	pPvSS1380	500, 350
9.	pPvSS1451	700, 270, 220
10.	pPvSS1483	500, 250
11.	pPvSS1644	360, 300
12.	pPvSS1672	700, 270, 220
13.	pPvSS1674	500, 270, 250
14.	pPvSS2227	350, 280
15.	pPvSS2284	500, 350
16.	pPvSS2285	500, 340
17.	pPvSS2314	480, 250, 220

were detected with samples (ii) and (iii).

3.3.2.2 S1 nuclease analysis of the hybridisation products of single-stranded templates.

Single-stranded templates were prepared as in section 2.20.7 and S1 nuclease analysis carried out as in section 2.19.3.
Figure 12a shows the protection fragment generated when the assay was carried out on the BamH1-HindIII insert of pPvSS1672 in M13mp19, using the EcoR1-BamH1 insert of pBS1 in M13mp19 as a probe, against pAT153/Hinf1 markers. A protected fragment roughly co-migrating with the 220bp marker fragment was generated from pPvSS1672. When, in the same experiment, the assay was carried out with templates containing the EcoR1-BamH1 insert of pBS1 in opposite, complementary orientations (ie. in both M13mp19 and M13mp18) a protected fragment roughly co-migrating with the 298bp marker fragment was detected. Figure 12b shows the protection fragments generated when the assay was carried out on the BamH1-HindIII inserts of pPvSS636, pPvSS1483, pPvSS2227, pPvSS2284 and pPvSS1672 (all in M13mp19) using the EcoR1-BamH1 insert of pBS1 in M13mp19 as a probe, against pBR322/MspI markers. Protected fragments roughly co-migrating with the 217bp and 180bp marker fragments were generated from pPvSS1672, whereas the inserts from pPvSS636 and pPvSS1483 both gave protected fragments roughly co-migrating with the 123bp marker fragment. No protected fragments were detected for pPvSS2284 and pPvSS2227.

3.3.3 By 'A'-track sequencing

BamH1-HindIII inserts were prepared from the plasmid DNA of 42 of the P. vulgaris rbcS cDNA clones. These inserts were ligated into M13mp18 digested with the restriction

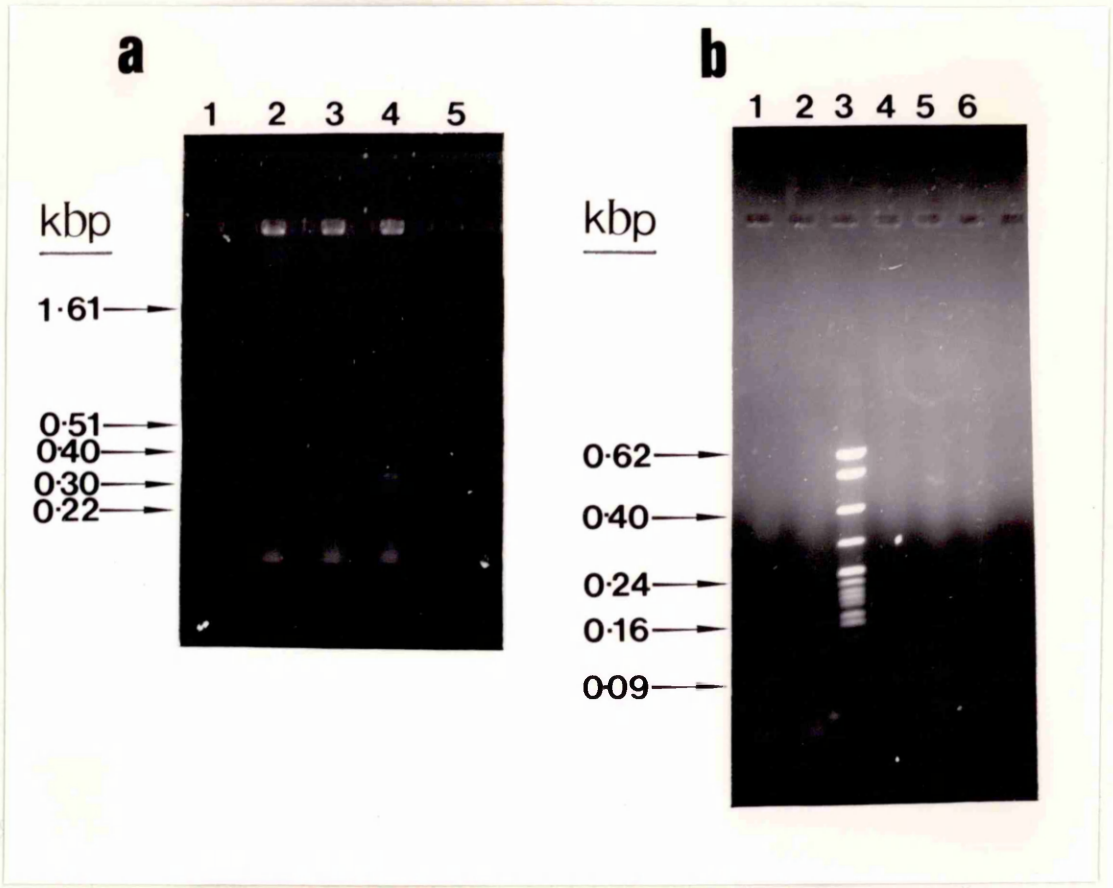


Figure 12 - S1 nuclease analysis of rbcS cDNA clones

(a) S1 nuclease analysis of the hybridisation products of the BamH1-HindIII insert of pPvSS1672 as a probe with the EcoR1-BamH1 insert of pBS1 in M13mp19. The probe insert was contained in M13mp18 and M13mp19 (tracks 2a and 3a respectively). Also shown are the hybridisation products of the EcoR1-BamH1 insert of pBS1 in opposite, complementary orientations (ie. in both M13mp19 and M13mp18) (track 4a).

(b) S1 nuclease analysis of the hybridisation products of the BamH1-HindIII inserts of pPvSS636, pPvSS1483, pPvSS2227, pPvSS2284 and pPvSS1672 (all in M13mp19; tracks 1b, 2b, 4b, 5b and 6b respectively) when hybridised to the EcoR1-BamH1 insert of pBS1 in M13mp19.

After hybridisation and digestion with S1 nuclease (section 2.19.3), protected fragments were run on 1.5% agarose/TBE gels and sized against either pAT153/HinfI (tracks 1a and 5a) or pBR322/MspI DNA markers (track 3b).

enzymes BamHI and HindIII, single-stranded templates were made (section 2.20) and 'A'-track analysis was carried out as in section 2.21.3, using the oligonucleotide primer no. 360 (section 3.4).

Table 7 shows that all the rbcS cDNA clones fell into one of three separate categories designated 'type 1' 'type 2' and 'type 3' (figure 15). Clones which represented rbcS mRNA of 'type 2' and 'type 3' showed evidence of two separate polyadenylation sites (discussed fully in section 4.1). Figure 13 shows that although pPvSS1676, pPvSS1750, pPvSS2122, pPvSS2019 and pPvSS1481 were all 'type 2' clones the latter two clones had polyA tails further downstream with respect to the other three clones. Figure 14 shows the autoradiogram of a sequencing gel run with two 'type 2' cDNA clones pPvSS1483 and pPvSS2285, sequenced using the same primer as for 'A'-track analysis. This shows that the polyA tail of pPvSS1483 starts about 30bp nearer the 'TAA' termination codon than did the polyA tail of pPvSS2285. Similarly the polyA tail of the 'type 3' rbcS cDNA clone pPvSS631 starts about 85bp nearer the 'TAA' termination codon than did the polyA tails of the other 'type 3' rbcS clones pPvSS191, pPvSS814 and pPvSS2235 (not shown). 'Type 2' and 'type 3' clones were classed as 'short' where the polyA tail started at the polyadenylation site nearest the termination codon, and 'long' where it started at the second site further downstream (table 7).

Out of 42 rbcS cDNA clones analysed 23 were 'type 1', 15 were 'type 2' and 4 were 'type 3'. As relative percentage 'type 1' accounted for 54.8%, 'type 2' 35.7% and 'type 3' 9.5% of rbcS cDNA clones.

3.4 DNA sequencing of P. vulgaris rbcS cDNA clones

Nucleotide sequencing of representatives of 'type 1', 'type 2' and 'type 3' rbcS clones (section 3.3.3) was

Table 7 - Types of rbcS mRNA represented by 42 selected P. vulgaris rbcS cDNA clone, as determined by 'A'-track sequencing.

Experimental conditions were as described in the legend to figure 13.

	CLONE	rbcS mRNA type
1	pPvSS191	3 (long)
2	pPvSS328	1
3	pPvSS381	1
4	pPvSS386	1
5	pPvSS444	2 (long)
6	pPvSS610	1
7	pPvSS631	3 (short)
8	pPvSS636	2 (long)
9	pPvSS783	1
10	pPvSS814	3 (long)
11	pPvSS965	2 (long)
12	pPvSS1066	2 (long)
13	pPvSS1106	1
14	pPvSS1191	1
15	pPvSS1240	1
16	pPvSS1256	1
17	pPvSS1269	1
18	pPvSS1292	1
19	pPvSS1363	1
20	pPvSS1380	2 (long)
21	pPvSS1446	2 (long)
22	pPvSS1451	1
23	pPvSS1481	2 (long)
24	pPvSS1483	2 (short)
25	pPvSS1530	1
26	pPvSS1644	2 (long)
27	pPvSS1651	1
28	pPvSS1672	1
29	pPvSS1674	2 (long)
30	pPvSS1676	2 (short)
31	pPvSS1750	2 (short)
32	pPvSS1793	1
33	pPvSS2019	2 (long)
34	pPvSS2122	2 (short)
35	pPvSS2169	1
36	pPvSS2227	1
37	pPvSS2235	3 (long)
38	pPvSS2284	1
39	pPvSS2285	2 (long)
40	pPvSS2314	1
41	pPvSS2352	1
42	pPvSS2415	1

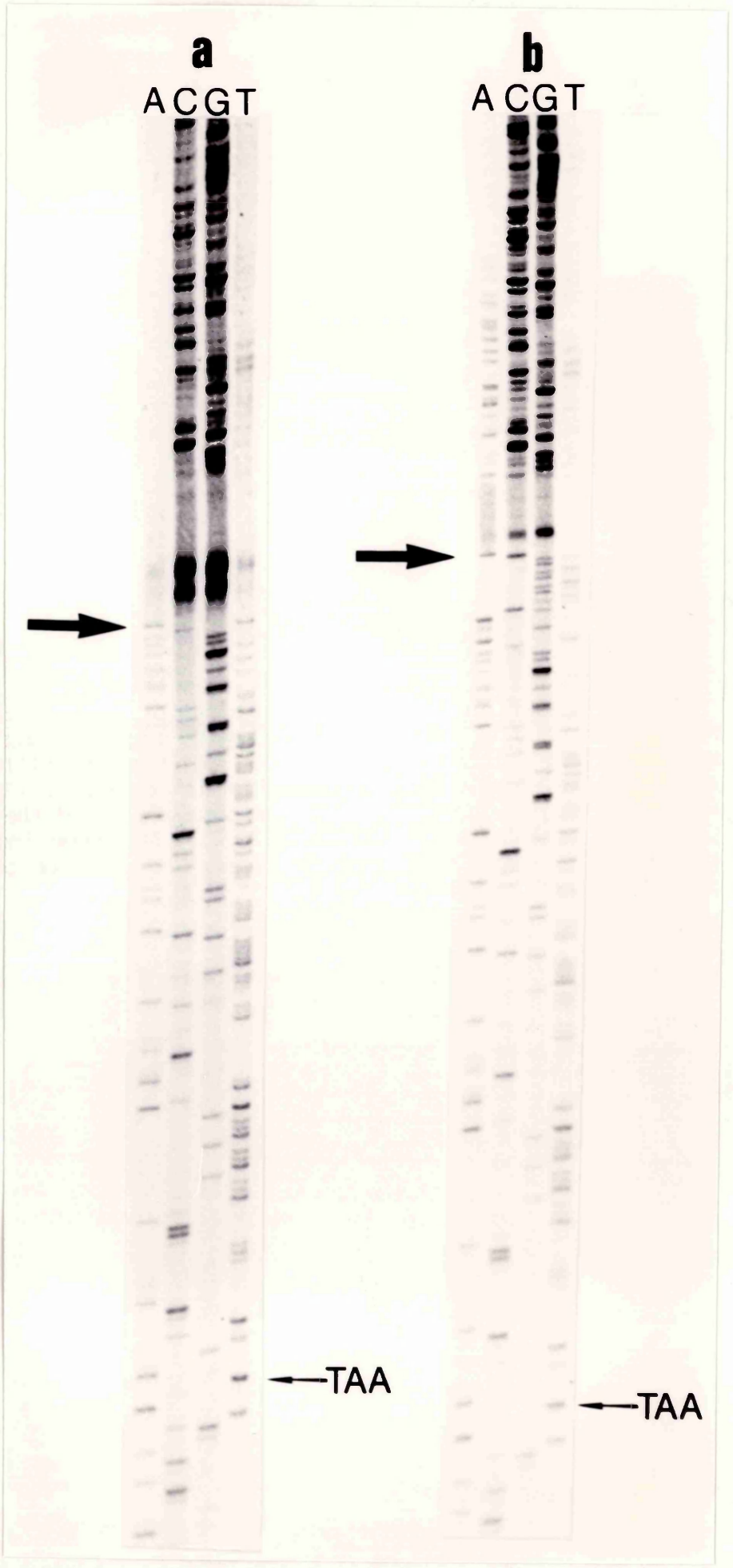
Figure 13 - 'A'-track sequencing analysis of rbcS cDNA clones

Analysis was carried out as in section 2.21.3 using the oligonucleotide primer no.360 (section 3.4). Clones were categorised as 'type 1', 'type 2' or 'type 3' depending upon the sequence obtained. The position of the 'TAA' termination codon is also indicated.

Tracks 1 to 7: pPvSS1676, pPvSS1750, pPvSS1793, pPvSS2019, pPvSS1481, pPvSS2235 and pPvSS2122 respectively.


1 2 3 4 5 6 7
TAA

Figure 14 - multiple polyadenylation sites of 'type 2' rbcS mRNA
Autoradiogram of a DNA sequencing gel, with sequence from rbcS cDNA clones pPvSS1483 (a) and pPvSS2285 (b). The BamH1-HindIII inserts of these clones in M13mp18 were sequenced as in section (2.21.1.1) using the oligonucleotide primer no. 360 (section 3.4), and the sequencing gel was run for 6h as in section 2.21.2. An arrow indicates the beginning of the polyA tail in each case. The position of the 'TAA' termination codod is also indicated.


a
A C G T
TAA
b
A C G T
TAA

performed to provide nucleotide sequence information for use in the design of rbcS gene-specific oligonucleotide probes.

The BamH1-HindIII inserts of three rbcS cDNA clones (pPvSS1672, pPvSS965 and pPvSS191) were subcloned either intact or after digestion with EcoR1 or Alu1 into M13mp18 and M13mp19 (section 2.20). Three oligonucleotide primers were used for DNA sequencing (section 2.21): a 17^{mer} M13-specific primer (5' GTAAAACGACGGCCAGT 3'; supplied by BCL) and two 18^{mer} oligonucleotide primers no. 360 (5' AAGCTGCCTATGTTTGGG 3') and no. 457 (5' AATCCAATGATACGG -ATGAAA 3') specific to the coding region of all three rbcS cDNA clones (section 2.5).

Figure 15 shows the stategy adopted for the DNA sequencing of the three representative rbcS cDNA clones. Figures 16, 17 and 18 show the sequences obtained from pPvSS1672, pPvSS965 and pPvSS191 respectively. Along with this sequence the déduced amino acid sequences of the coding regions of these three clones are shown, as well as the sites of restriction endonucleases relevant to this thesis (all from outputs of the 'MAP' computer program from the UWGCG sequence-handling package; section 2.22). The annealing sites of the oligonucleotide primers no. 360 and no. 457 are also shown in these figures with arrows indicating the direction of sequencing primed by them.

All coding region nucleotide sequences were 100% homologous between pPvSS1672, pPvSS965 and pPvSS191. This included the 171bp encoding the transit peptides of pPvSS1672 and pPvSS191. pPvSS965 contained only 14 amino acids of the transit peptide, but these are identical to the corresponding amino acids of pPvSS1672 and pPvSS965.

Two regions of intronic sequences were detected in pPvSS1672. The first region was located between the second and third amino acid of the mature polypeptide and was 98bp in length. Figure 19 shows sequencing gels for

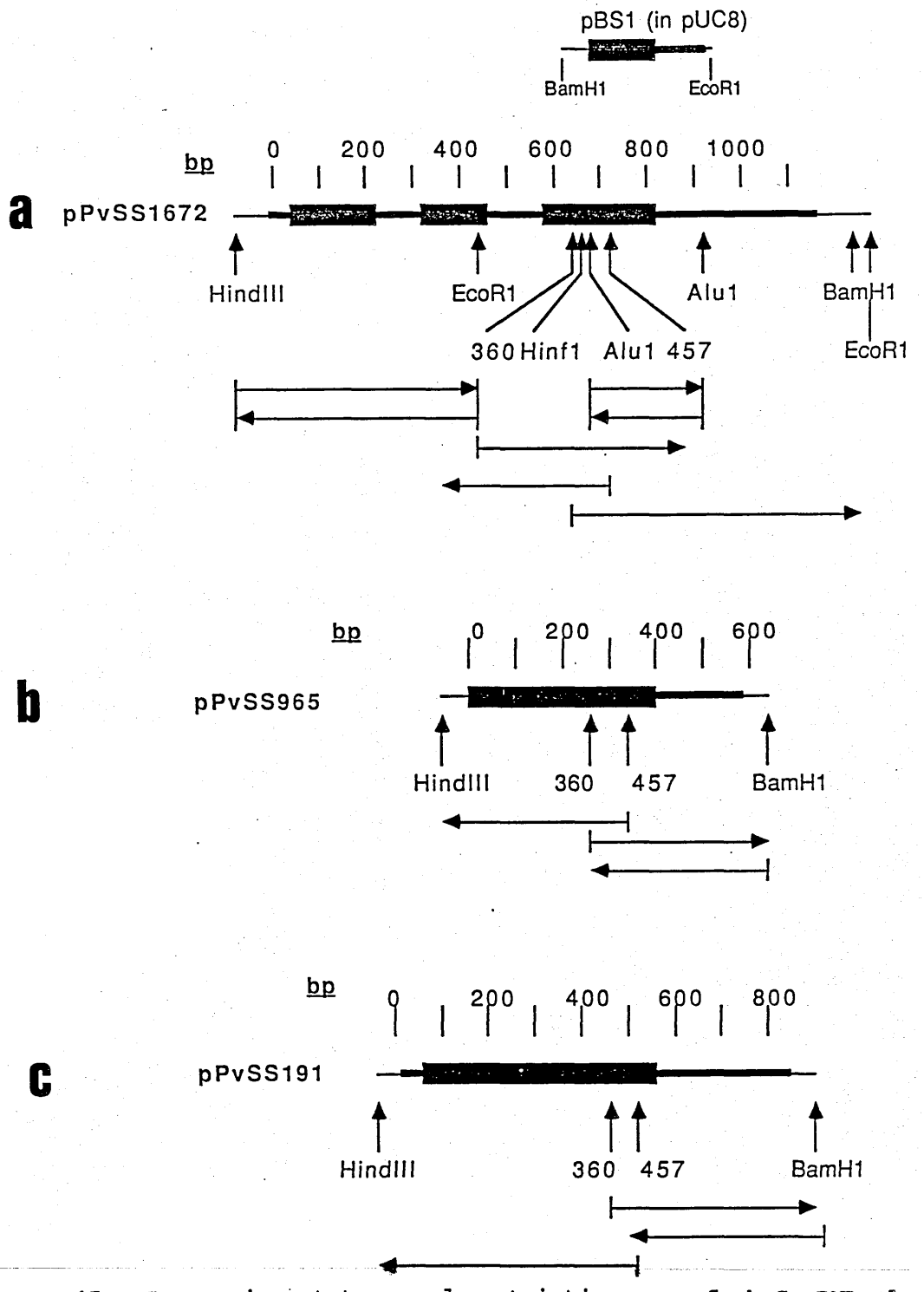


Figure 15 - Sequencing stategy and restriction map of rbcS cDNA clones
Figure showing the strategy adopted for sequencing the rbcS cDNA clones (a) pPvSS1672, (b) pPvSS965 and (c) pPvSS191. Positions of restriction endonucleases relevant to this thesis are shown as well as the positions from which the oligonucleotide primers nos.360 and 457 initiated the sequencing reactions in which they were involved (labelled '360' and '457'). Also shown is pBS1, a subclone of pPvSS1672, containing the 225 bp Alu1-Alu1 insert from nucleotides 687 to 912 (figure 16). This fragment was subcloned into the Sma1-site of M13mp19, from which it was removed as a BamH1-EcoR1 insert and inserted into EcoR1/BamH1-digested pUC8 vector to give pBS1.
In the map of each clone, the thick blocks (▬) correspond to coding sequence, the thick lines (▬) non-coding sequence and the thin lines (——) vector sequence. The horizontal lines with arrows (⟶) below the maps of the clones indicate the directions in which parts of the clones were sequenced.

Figure 16

The complete nucleotide sequence of the rbcS cDNA clone pPvSS1672 from P. vulgaris along with the deduced amino-acid sequence of the coding region. Also shown are the positions of the sites of restriction endonucleases relevant to this thesis.

```
        10        20        30        40                50
5'      .         .         .         .                 .
                                      Met Ala Ser Ser Met Ile
GAAATATAGCAGCAGAAAGAAGTAATTCAGAAGTTAGGAA ATG GCT TCT TCA ATG ATC

 60           70           80           90           100
 .            .            .            .            .
Ser Ser Pro Ala Val Thr Thr Val Asn Arg Ala Gly Ala Gly Ala Gly
TCC TCC CCC GCT GTG ACG ACC GTT AAC CGT GCC GGT GCC GGT GCC GGT

   110          120          130          140          150
    .            .            .            .            .
Met Val Ala Pro Phe Thr Gly Leu Lys Ser Leu Gly Gly Phe Pro Ser
ATG GTG GCT CCT TTC ACT GGG CTG AAG TCC CTG GGA GGG TTC CCA AGC

        160          170          180          190          200
         .            .            .            .            .
Arg Lys Met Asn Asn Asp Ile Thr Ser Val Ala Asn Asn Gly Gly Arg
AGG AAG ATG AAC AAT GAT ATT ACT TCC GTT GCG AAC AAC GGT GGA AGA

           210          220          230          240          250
            .            .            .            .            .
Val Gln Cys Ile Gln
GTG CAA TGC ATT CAG GTAAGAGAAGATATTTATGTGATAGGAAAATGTGAAGATTTGG

 260          270          280          290          300          310
  .            .            .            .            .            .
                                                                  Val Trp
AAGTTGGTTGGTTAGTTAGTTTGTTAACGAATTATGATGGAAATGAAATGAGTAG GTG TGG

320          330          340          350          360
 .            .            .            .            .
Pro Thr Val Gly Lys Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Pro
CCA ACA GTT GGG AAG AAG AAG TTC GAG ACT CTT TCG TAC CTG CCA CCC

   370          380          390          400          410
    .            .            .            .            .
Leu Thr Lys Gln Gln Leu Ala Lys Glu Val Asp Tyr Leu Leu Arg Lys
CTG ACA AAA CAA CAA TTG GCA AAG GAA GTA GAC TAC CTT CTT CGG AAA
```

```
      420           430           440           450       460
       .             .             .             .         .
Gly Trp Val Pro Cys Leu Glu Phe Glu Leu Glu
GGA TGG GTT CCG TGC TTG GAA TTC GAA TTG GAG GTGAATTTGTTGAATTGAA
                        EcoR1

  470       480       490       500       510       520
   .         .         .         .         .         .

TTGAAAGGGTTATTATGGTTAATTTATGAATGAATGAATGAATGAATGAATGATGTTGATGTT


530       540       560       570          580          590
 .         .         .         .            .            .
                               His Gly Phe Val Tyr Arg Glu His Asn
TGTGTTTGATGTTGATGTGTGTTGATAG CAT GGT TTC GTG TAC CGT GAA CAC AAC


    600          610          620          630          640
     .            .            .            .            .
Lys Ser Pro Gly Tyr Tyr Asp Gly Arg Tyr Trp Thr Met Trp Lys Leu
AAG TCA CCT GGA TAC TAT GAT GGA AGG TAC TGG ACG ATG TGG AAG CTG
                                                    5'AAG CTG
                                                      Alu1
    650          660          670          680          690
     .            .            .            .            .
Pro Met Phe Gly Cys Thr Asp Ser Ser Gln Val Leu Lys Glu Leu Tyr
CCT ATG TTT GGG TGC ACT GAT TCT TCT CAG GTG TTG AAG GAG CTT TAC
CCT ATG TTT GGG 3'-----> Hinf1                      Alu1
Primer no. 360
        700          710          720          730          740
         .            .            .            .            .
Glu Ala Gln Thr Ala HIs Pro Asp Gly Phe Ile Arg Ile Ile Gly Phe
GAG GCT CAG ACT GCT CAC CCC GAT GGT TTC ATC CGT ATC ATT GGA TTT
                       <----- 3'A AAG TAG GCA TAG TAA CCT AA 5'
                                   Primer no. 457
           750          760          770          780
            .            .            .            .
Asp Asn Val Arg Gln Val Gln Cys Ile Ser Phe Ile Ala Tyr Lys Pro
GAC AAC GTT CGT CAA GTG CAG TGC ATC AGT TTC ATT GCC TAC AAG CCA


790          800          810          820          830          840
 .            .            .            .            .            .
Pro Gly Tyr  *
CCA GGC TAC TAA GTCTCCAAAATTTCCAAAACAGTTCTAGCCACCCCACTTTGCTTGTT


  850          860          870          880          890          900
   .            .            .            .            .            .
TGTACTTAAACTAAACTTTCATTTCGTTTTTTCCCTTTTGTTGTCCGGATTATCTTCTGTTGA
```

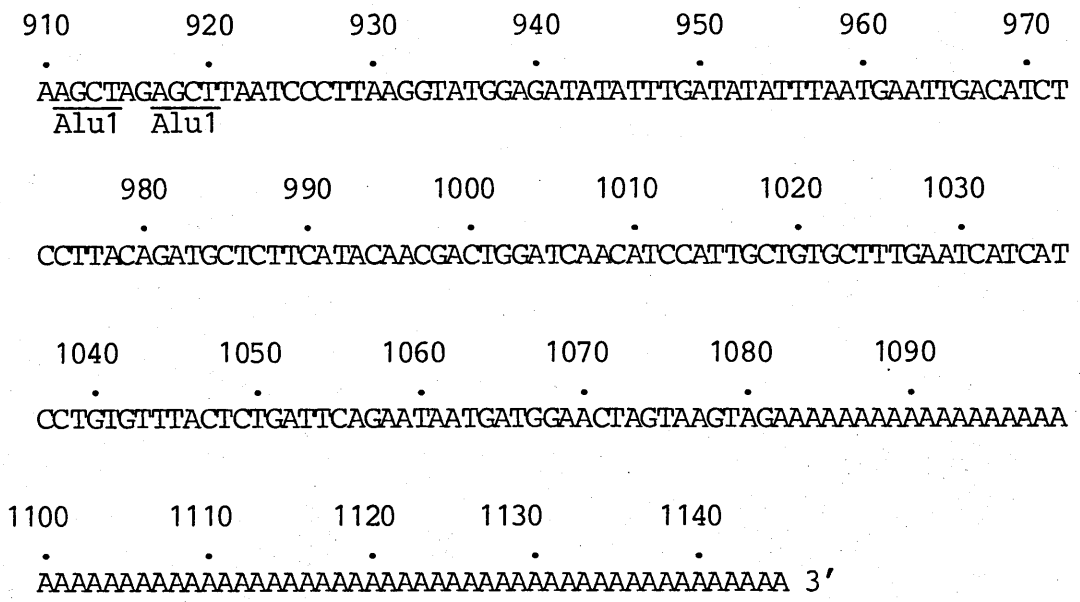

910 920 930 940 950 960 970
AAGCTAGAGCTTAATCCCTTAAGGTATGGAGATATATTTGATATATTTAATGAATTGACATCT
Alu1 Alu1
980 990 1000 1010 1020 1030
CCTTACAGATGCTCTTCATACAACGACTGGATCAACATCCATTGCTGTGCTTTGAATCATCAT
1040 1050 1060 1070 1080 1090
CCTGTGTTTACTCTGATTCAGAATAATGATGGAACTAGTAAGTAGAAAAAAAAAAAAAAAAA
1100 1110 1120 1130 1140
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3′

Figure 17

The complete nucleotide sequence of the rbcS cDNA clone pPvSS965 from P. vulgaris along with the deduced amino-acid sequence of the coding region. Also shown are the positions of the sites of restriction endonucleases relevant to this thesis.

```
                    10          20          30          40
                     .           .           .           .
          Thr Ser Val Ala Asn Asn Gly Gly Arg Val Gln Cys Ile
5' T ACT TCC GTT GCG AAC AAC GGT GGA AGA GTG CAA TGC ATT

          50          60          70          80
           .           .           .           .
Gln Val Trp Pro Thr Val Gly Lys Lys Lys Phe Glu Thr Leu Ser Tyr
CAG GTG TGG CCA ACA GTT GGG AAG AAG AAG TTC GAG ACT CTT TCG TAC

90          100         110         120         130
 .           .           .           .           .
Leu Pro Pro Leu Thr Lys Gln Gln Leu Ala Lys Glu Val Asp Tyr Leu
CTG CCA CCC CTG ACA AAA CAA CAA TTG GCA AAG GAA GTA GAC TAC CTT

   140         150         160         170         180
    .           .           .           .           .
Leu Arg Lys Gly Trp Val Pro Cys Leu Glu Phe Glu Leu Glu His Gly
CTT CGG AAA GGA TGG GTT CCG TGC TTG GAA TTC GAA TTG GAG CAT GGT
                                    EcoR1

     190         200         210         220         230
      .           .           .           .           .
Phe Val Tyr Arg Glu His Asn Lys Ser Pro Gly Tyr Tyr Asp Gly Arg
TTC GTG TAC CGT GAA CAC AAC AAG TCA CCT GGA TAC TAT GAT GGA AGG

        240         250         260         270         280
         .           .           .           .           .
Tyr Trp Thr Met Trp Lys Leu Pro Met Phe Gly Cys Thr Asp Ser Ser
TAC TGG ACG ATG TGG AAG CTG CCT ATG TTT GGG TGC ACT GAT TCT TCT
                 5'AAG CTG CCT ATG TTT GGG 3'-----> Hinf1
                   Alu1   Primer no. 360
           290         300         310         320
            .           .           .           .
Gln Val Leu Lys Glu Leu Tyr Glu Ala Gln Thr Ala His Pro Asp Gly
CAG GTG TTG AAG GAG CTT TAC GAG GCT CAG ACT GCT CAC CCC GAT GGT
                Alu1                                <-----3'A

   330         340         350         360         370
    .           .           .           .           .
Phe Ile Arg Ile Ile Gly Phe Asp Asn Val Arg Gln Val Gln Cys Ile
TTC ATC CGT ATC ATT GGA TTT GAC AAC GTT CGT CAA GTG CAG TGC ATC
AAG TAG GCA TAG TAA CCT AA 5'
     Primer no. 457
```

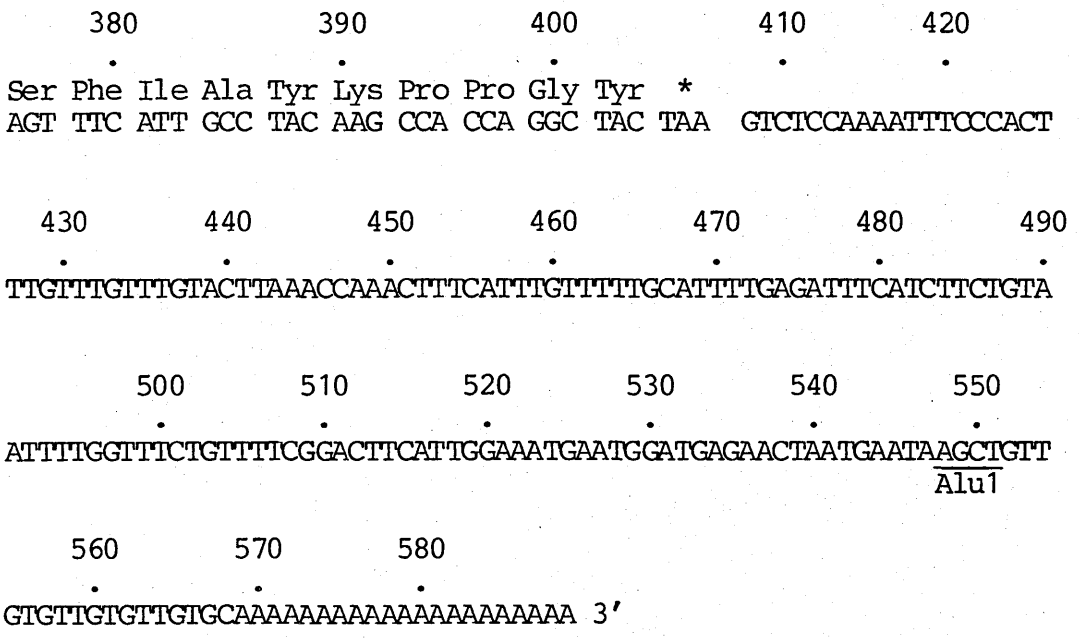

380 390 400 410 420
Ser Phe Ile Ala Tyr Lys Pro Pro Gly Tyr *
AGT TTC ATT GCC TAC AAG CCA CCA GGC TAC TAA GTCTCCAAAATTTCCCACT
430 440 450 460 470 480 490
TTGTTTGTTTGTACTTAAACCAAACTTTCATTTGTTTTTGCATTTTGAGATTTCATCTTCTGTA
500 510 520 530 540 550
ATTTTGGTTTCTGTTTTCGGACTTCATTGGAAATGAATGGATGAGAACTAATGAATAAGCTGTT
Alu1
560 570 580
GTGTTGTGTTGTGCAAAAAAAAAAAAAAAAAAAA 3'

Figure 18

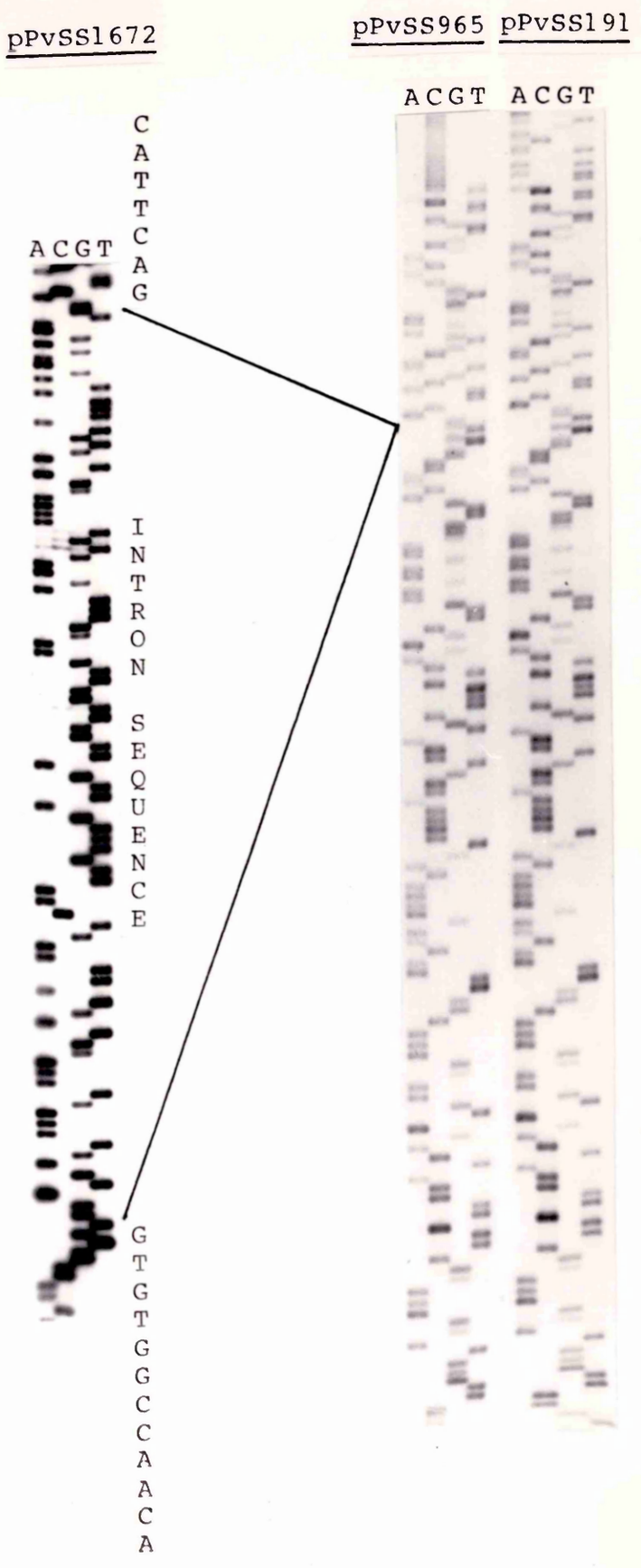
The complete nucleotide sequence of the rbcS cDNA clone pPvSS191 from P. vulgaris along with the deduced amino-acid sequence of the coding region. Also shown are the positions of the sites of restriction endonucleases relevant to this thesis.

```
                                                                  10
                                                                  .
                                                  5' GCAGCAGACAGCAGT

   20          30          40          50          60
   .           .           .           .           .
                           Met Ala Ser Ser Met Ile Ser Ser Pro Ala
ATTCAGAGTAGAGAGAGAGGGAA ATG GCT TCT TCA ATG ATC TCC TCC CCC GCT

70          80          90          100         110
.           .           .           .           .
Val Thr Thr Val Asn Arg Ala Gly Ala Gly Ala Gly Met Val Ala Pro
GTG ACG ACC GTT AAC CGT GCC GGT GCC GGT GCC GGT ATG GTG GCT CCT

   120         130         140         150         160
   .           .           .           .           .
Phe Thr Gly Leu Lys Ser Leu Gly Gly Phe Pro Ser Arg Lys Met Asn
TTC ACT GGG CTG AAG TCC CTG GGA GGG TTC CCA AGC AGG AAG ATG AAC

     170         180         190         200         210
     .           .           .           .           .
Asn Asp Ile Thr Ser Val Ala Asn Asn Gly Gly Arg Val Gln Cys Ile
AAT GAT ATT ACT TCC GTT GCG AAC AAC GGT GGA AGA GTG CAA TGC ATT

       220         230         240         250         260
       .           .           .           .           .
Gln Val Trp Pro Thr Val Gly Lys Lys Lys Phe Glu Thr Leu Ser Tyr
CAG GTG TGG CCA ACA GTT GGG AAG AAG AAG TTC GAG ACT CTT TCG TAC

           270         280         290         300
           .           .           .           .
Leu Pro Pro Leu Thr Lys Gln Gln Leu Ala Lys Glu Val Asp Tyr Leu
CTG CCA CCC CTG ACA AAA CAA CAA TTG GCA AAG GAA GTA GAC TAC CTT

310         320         330         340         350
.           .           .           .           .
Leu Arg Lys Gly Trp Val Pro Cys Leu Glu Phe Glu Leu Glu His Gly
CTT CGG AAA GGA TGG GTT CCG TGC TTG GAA TTC GAA TTG GAG CAT GGT
                                    EcoR1
```

```
   360          370          400          410          420
    .            .            .            .            .
Phe Val Tyr Arg Glu His Asn Lys Ser Pro Gly Tyr Tyr Asp Gly Arg
TTC GTG TAC CGT GAA CAC AAC AAG TCA CCT GGA TAC TAT GAT GGA AGG


     430          440          450          460          470
      .            .            .            .            .
Tyr Trp Thr Met Trp Lys Leu Pro Met Phe Gly Cys Thr Asp Ser Ser
TAC TGG ACG ATG TGG AAG CTG CCT ATG TTT GGG TGC ACT GAT TCT TCT
                5' AAG CTG CCT ATG TTT GGG 3'-----> Hinf1
                    Alu1    Primer no. 360
       480          490          500          510          520
        .            .            .            .            .
Gln Val Leu Lys Glu Leu Tyr Glu Ala Gln Thr Ala His Pro Asp Gly
CAG GTG TTG AAG GAG CTT TAC GAG GCT CAG ACT GCT CAC CCC GAT GGT
                Alu1                                  <-----3' A

           530          540          550          560
            .            .            .            .
Phe Ile Arg Ile Ile Gly Phe Asp Asn Val Arg Gln Val Gln Cys Ile
TTC ATC CGT ATC ATT GGA TTT GAC AAC GTT CGT CAA GTG CAG TGC ATC
AAG TAG GCA TAG TAA CCT AA 5'
     Primer no. 457
570          580          590          600          610          620
 .            .            .            .            .            .
Ser Phe Ile Ala Tyr Lys Pro Pro Gly Tyr  *
AGT TTC ATT GCC TAC AAG CCA CCA GGC TAC TAA GTCTCCAAAATTTCCCATT


       630          640          650          660          670          680
        .            .            .            .            .            .
TCATTTTGCTTTTTTGTTGCCCAGATTATCCTCATCCGAAACCTAGAGCCTAATCCTTCTGCG


     690          700          710          720          730          740
      .            .            .            .            .            .
TAAGGAAATTTATTTAATGAGATGACCTCTCTCAACATATGGTTTTATATATATTTATATATA


   750          760          770          780          790          800
    .            .            .            .            .            .
TATATGTATCTGGGATGGAACCAATTACCATTTGCTTTCTGATATTCACATTCTGTACTTTTG


 810          820          830          850          860          870
  .            .            .            .            .            .
GTTTCTGTTATCAGATATGAAAGGGTGAGAAATTAATGAATTAAAAAAAAAAAAAAAAAAA 3'
```

Figure 19 - Intronic sequences within an rbcS cDNA clone

Autoradiogram of sequencing gels for regions of the rbcS cDNA clones pPvSS1672, pPvSS965 and pPvSS191 around the site of the first intron. The position of intronic sequences within pPvSS1672 is indicated as well as the exonic sequence to either side.


pPvSS1672
pPvSS965
pPvSS191
ACGT
ACGT
ACGT
CATTCAG
INTRON SEQUENCE
GTGTGGCCAACA

this region along with the corresponding regions of pPvSS965 and pPvSS191 nucleotide sequence. The second region was located between the 47th and 48th amino acid of the mature polypeptide and was 111bp in length. Both these intronic regions had a very low frequency of cytidine residues (one C residue in the first region and none in the second). Comparison of the exon-intron junctions of pPvSS1672 with the eukaryotic consensus sequence and the sequences for the soybean rbcS gene λSRS1 (Berry-Lowe et al., 1982) showed them to have strong homology:

Sequence		Donor	-Intron-	Acceptor
pPvSS1672	1.	CAG GTAAGA		AAATGAAATGAGTAG G
	2.	GAG GTGAAT		GATGTGTGTTGATAG C
Soybean SRS1	1.	CAG GTAAGA		TATGCAAATTACTAG G
	2.	GAG GTCAAT		TGTGTTTGTATATAG C
Eukaryotic consensus		CAG GTAAGT		TTTTTTTTTTTNTAG G
		A G		CCCCCCCCCCC C

Figure 20 shows the comparison between the short lengths of 5'-untranslated sequence obtained from pPvSS1672 and pPvSS191. Homology in these regions was 44%. Figure 21 shows a comparison of the amino acid sequence of the transit peptide and mature polypeptide deduced from the nucleotide sequence of these three rbcS cDNA clones with the corresponding amino acid sequences from other species. The different extents of the homologies to other species are discussed in section 4.1.

```
pPvSS1672 5' GAAATATAGCAGCAGAAAGAAGTAATTCAGAAGTTAGGAAATG
                 *     ******  *       * *     **  ********
pPvSS191  5'  GCAGCAGACAGCAGTGATTCAG.AGTAGAGAGAGAGGAAATG
```

Figure 20 - 5' flanking sequences present within rbcS cDNA clones
Comparison of the 5'-untranslated sequences from the P. vulgaris rbcS cDNA clones pPvSS1672 and pPvSS191. The first ATG codon of the coding region of the transit peptide is underlined in each case. (An output from the 'GAP' computer program from the GCG sequence-handling package as described in section 2.22). Homology is indicated by asterisks (*).

(a)

```
                        10         20         30         40         50
P. vulgaris   IQVWPTVGKKKFETLSYLPPLTKQQLAKEVDYLLRKGWVPCLEFELEHGFVYREHNK
Lemna         MQVWPPEGLKKFETLSYFPLSSVEDLAKEVDYLLRNDWVPCIEFSKE-GFVYRENHA
Soybean       MQVWPPIGKKKFETLSYLPDLDDAQLAKEVEYLLRKGWIPCLEFELEHGFVYREHNR
Pea           MQVWPPIGKKKFETLSYLPPLTRDQLLKEVEYLSRKGWVPCLEFELEKGFVYREHNK
Wheat         MQVWPIEGIKKFETLSYLPPLSTEALLKQVDYLIRSKWVPCLEFSKV-GFVFREHNS
Spinach       MQVWPPLGLKKFETLSYLPPLTTEQLLAEVNYLLVKGWIPPLEFEVKDGFVYREHDK
Petunia       MQVWPPIGKKKYETLSYLPDLTGEQLLKEVEYLLDKGWVPCLEFELKHKFIYREYHA
Tobacco       MQVWPPIGKKKYEDLSYLPDLSQEQLLSEVDYLLKNGWVPCLEFETEHGFVYRENNK

  60         70         80         90         100        110        120
SPGYYDGRYWIMWKLPMFGCTDSSQVLKELYEAQTAHPDGFIRIIGFDNVRQVQCISFIAYKPPGY
SPGYYDGRYWIMWKLPMFGCTDASQVIAEVEEAKKAYPEYFVRIIGFDNKRQVQCISFIAYKPT
SP-YYDGRYWIMWKLPMFGCTDASQVLKELQEAKTAYPNGFIRIIGFDNVRQVQCISFIAYKPPGF
SPRYYDGRYWIMWKLPMFGITDASQVLKELDEVVAAYPQAFVRIIGFDNVRQVQCISFIAHTPESY
SPGYYDGRYWIMWKLPMFGCTDATQVLNEVEEVKKEYPDAYVRVIGFDNLRQVQCVSFIAFRPPGCEESGKA
SPGYYDGRYW---KLPMFGGIDPAQVVNEVEEVKKAYPDAFVRFIGFDNKREVQCISFIAYKPAGY
SPGYYDGRYWIMWKLPMFGCTDATQVLGELQEAKKAYPNAGSGIIGFDNVRQVQCISFIAYKPPGY
SPGYYDGRYWIMWKLPMFGCTDATQVLAEVEEAKKAYPQAWIRIIGFDNVRQVQCISFIAYKPEGY
```

Species	Percent homology to P. vulgaris
Lemna	71
Soybean	86
Pea	81
Wheat	69
Spinach	70
Petunia	77
Tobacco	78

(b)

```
P.vulgaris MASSMISSPAVTTVNRAGA-GAGMVAPFTGLKSLGGFP-SRKMNNDITSVANNGGRVQC
Lemna      MASSMMVSTAAVARVRPAQ-T-NMVGAFNGCRSSVAFPATRKANNDLSTLPSSGGRVSC
Soybean    MASSMISSPAVTTVNRAGA-G--MVAPFTGLKSMAGFP-TRKTNNDITSIASNGGRVQC
Wheat            MAPAVMASSA--T-T---VAPFQGLKSTAGLPISCRSGSTGLSSVSNGGRIRC
Pea        MAS-MISSSAVTTVSRASRGQSAAVAPFGGLKSMTGFPV-KKVNTDITSITSNGGRVKC
```

Species	Percent homology to P. vulgaris
Lemna	39
Soybean	89
Wheat	43
Pea	65

Figure 21 - Transit and mature rbcS polypeptide sequence comparison
(a) comparison of the deduced rbcS mature polypeptide amino acid sequence for P. vulgaris to 7 other plant species with percent homologies shown.
(b) comparison of the deduced rbcS transit peptide amino acid sequence for P. vulgaris to 4 other plant species with percent homologies shown.

3.5 Comparison of the 3'-untranslated sequences of three rbcS cDNA clones and the design of oligonucleotide probes.

Figure 22 shows the comparison between the 3'-untranslated sequences of the rbcS cDNA clones pPvSS1672, pPvSS965 and pPvSS191 as represented by the 'GAP' computer program from the UWGCG DNA sequence-handling package (section 2.22). The sequences complementary to the oligonucleotide probes rbcS1, rbcS2 and rbcS3 (section 2.18.1.2) are also shown. These probes were designed from sequences unique to the 3' untranslated nucleotide sequence of each rbcS clone so that they could be used as probes specific to these sequences. Additionally, all three probes were designed with identical G-C contents.

The homologies between the first 120bp downstream of the 'TAA' termination codons of pPvSS965 and pPvSS191 to the corresponding sequence of pPvSS1672 were 68% and 47% respectively. The homology between the first 120bp downstream of the 'TAA' codon of pPvSS965 and the corresponding sequence of pPvSS191 was 41%.

3.6 Studies of rbcS gene expression by dot blot analysis of P. vulgaris total RNA

Dot blot analysis of P. vulgaris total RNA was performed to obtain measurements of the steady-state levels of total rbcS transcripts and of individual rbcS transcripts, under various conditions. Previous experiments (Jenkins, 1986; unpublished) in which northern blots of total P. vulgaris RNA were hybridised to pea rbcS cDNA probes had shown that one major hybridising band was observed and that the amount of rbcS transcripts increased following the exposure of dark-grown plants to light. However, no quantitative data were obtained and no identification of the photoreceptors involved in controlling rbcS expression

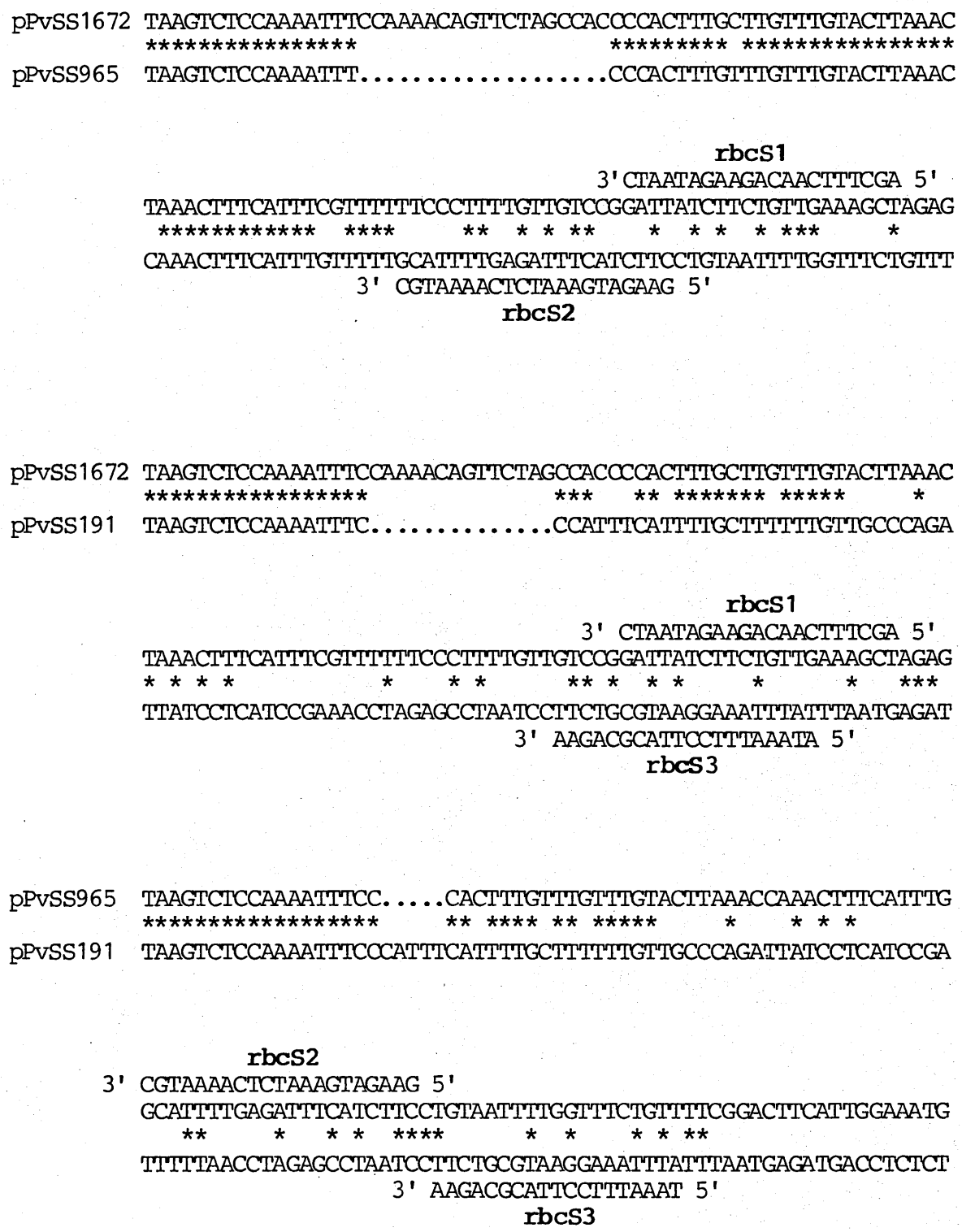


Figure 22

Comparison between the 3'-untranslated sequences (from the TAA termination codon to about 120bp downstream) of the rbcS cDNA clones pPvSS1672, pPvSS965 and pPvSS191 as represented by the 'GAP' computer program from the GCG sequence-handling package (2.22). Homology is indicated by asterisks (*). The sequences complementary to the oligonucleotide probes rbcS1, rbcS2 and rbcS3 are also shown.

was carried out. This section describes attempts to quantify the increase in rbcS transcript abundance during light-induced greening, the levels of rbcS transcripts in dark-grown plants and in various non-leaf tissues and phytochrome-mediated effects on rbcS transcript abundance.

3.6.1 Using rbcS cDNA probes

Dot blots of total RNA were hybridised to rbcS cDNA sequences from pea and P. vulgaris as probes, to obtain measurements of the steady-state levels of total rbcS mRNA.

3.6.1.1 Probed with pSSU61/pSSU161

RNA dot blots (1µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from at least 3 different plants. Pre-hybridisation and hybridisation was performed as in section 2.19.1.1. The DNA probe used was an equi-molar mixture of the HindIII-HindIII inserts of pSSU61 and pSSU161 (subclones of the pea rbcS cDNA clone pSSU1; figure 1) labelled by nick-translation (section 2.18.2). After the washing and autoradiography of filters, individual dots were cut from the filter and hybridisation estimated by scintillation counting. Results are given as average cpm per dot and average cpm per dot after background is subtracted. The cpm for background represents the average cpm hybridised to 1µg E. coli tRNA to take into account non-specific hybridisation as well as natural background.

3.6.1.1.1 Greening time course

Table 8 shows the results from dark-grown P. vulgaris seedlings (6 days old) which were illuminated with continuous white light at 100µmol $m^{-2}s^{-1}$ as in section 2.8

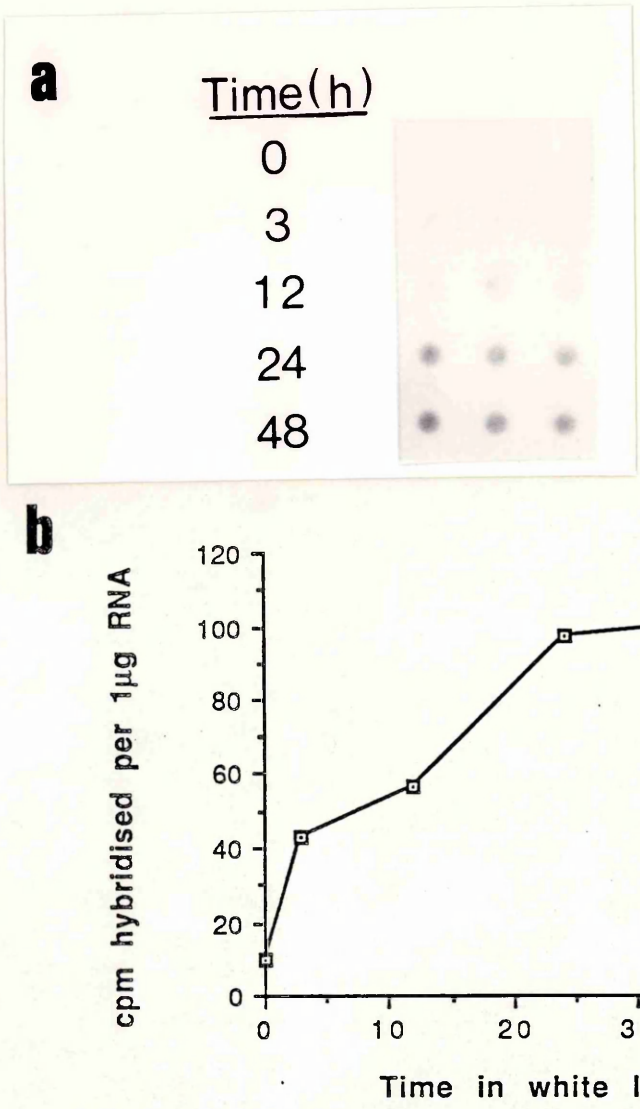


Figure 23 - Hybridisation of a pea rbcS cDNA probe to dots of RNA isolated from P. vulgaris primary leaf tissue harvested during a 48h time course in continuous white light

RNA was isolated from dark-grown P. vulgaris plants transferred to continuous white light for the number of hours indicated. RNA dot blots (1µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from at least 3 different plants. Pre-hybridisation and hybridisation was as in section 2.19.1.1. The DNA probe used was an equi-molar mixture of the HindIII-HindIII inserts of pSSU61 and pSSU161 (subclones of the pea rbcS cDNA clone pSSU1; figure 1), labelled by nick-translation (section 2.18.2) to approximately 4.5×10^8 cpm/ug, and was added to give 1.6×10^6 cpm/ml hybridisation solution. After hybridisation, filters were washed twice for 20 min in 2xSSC, 0.2% SDS (w/v) at 35°C, once for 20 min in 0.5xSSC, 0.2% SDS (w/v) at 35°C, once for 20 min in 0.5xSSC at 35°C, twice for 40 min in 0.5xSSC at 45°C, twice for 15 min in 0.25xSSC at 45°C and once for 15 min in 0.25xSSC at 50°C and were then autoradiographed.

(a) Autoradiogram of dots after hybridisation to the pea rbcS cDNA probe.

(b) Plot of cpm above background against time in white light (table 8).

TABLE 8 - Hybridisation of pea rbcS probe to RNA dots isolated from tissue collected during a 48h time course in continuous white light.

Experimental conditions were as described in the legend to figure 23

TIME	Average cpm per dot	Average cpm -background
0h	79	10
3h	112	43
12h	125	56
24h	166	97
48h	174	105
BACKGROUND	69	0

TABLE 9 - Hybridisation of pea rbcS probe to RNA dots isolated from tissue collected after various light treatments .

Experimental conditions were as described in the legend to figure 24.

TREATMENT	Average cpm per dot	Average cpm -background
R	249	180
FR	98	29
R/FR	100	31
24L	166	97
24D	79	10
BACKGROUND	69	0

and from which samples of primary leaves were taken after 0, 3, 12, 24 and 48h. Figure 23a shows the autoradiogram of the dots after hybridisation. Figure 23b shows a plot of cpm above background against time in white light. This shows that rbcS transcripts were detected and accumulated during the greening timecourse. rbcS transcripts were detected at 0h (dark-grown tissue).

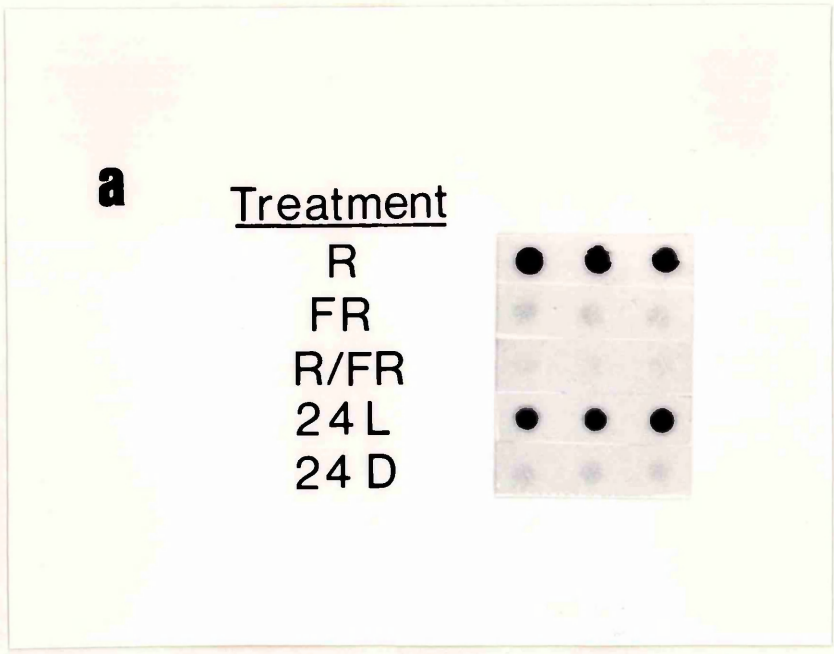
3.6.1.1.2 Phytochrome experiment

Table 9 shows the results from an experiment with dark-grown P. vulgaris seedlings (6 days old) which were given light treatments as in section 2.8 and returned to darkness for 24h before the primary leaves were harvested.

The treatments were red light for 3 min (treatment R), far-red light for 3 min (treatment FR) and red light for 3 min followed immediately by 3 min far-red light (treatment R/FR). Treatment '24D' represents a control which was not illuminated prior to the 24h in the dark, and '24L' represents the sample taken at 24h in the greening experiment from the same batch of plants (section 3.6.1.1.1). Figure 24a shows the autoradiogram of the dots after hybridisation. Figure 24b shows a plot of cpm over background for each treatment expressed as a percentage relative to the '24L' treatment. This shows that red light induces a large increase in the level of rbcS transcripts. This red-light mediated effect was substantially reversed by the subsequent illumination with far-red light.

3.6.1.2 Probed with pPvSS1672

RNA dot blots (1µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from at least 5 different plants. Pre-hybridisation and hybridisation was as in section 2.19.1.1. The DNA probe



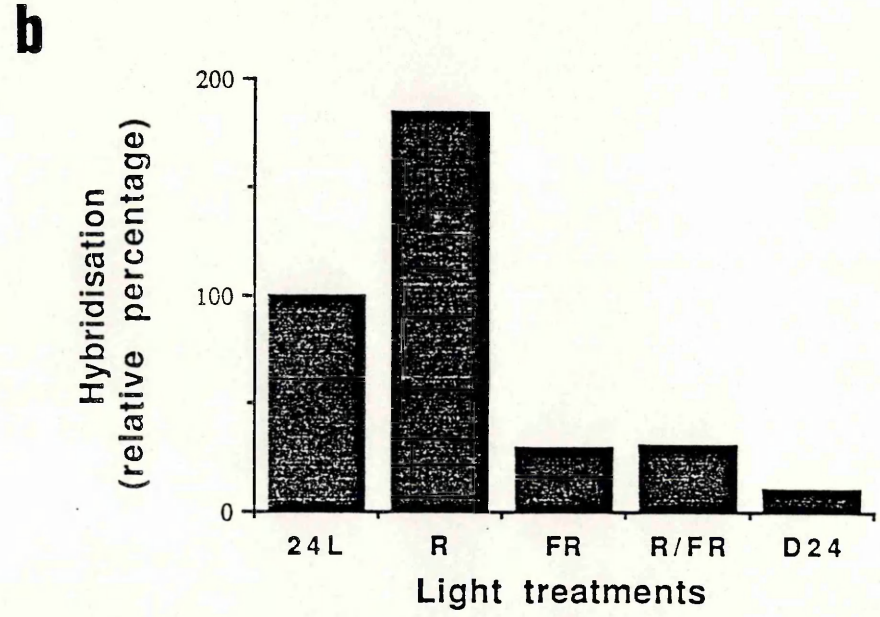


Figure 24 - Hybridisation of a pea rbcS cDNA probe to dots of RNA isolated from P. vulgaris primary leaf tissue harvested after various light treatments - phytochrome experiment

Dark-grown plants were exposed either to 3 min red-light (R), 3 min far-red light (FR), 3 min red light followed by 3 min far-red light (R/FR). Following these illuminations plants were grown for a further 24h in darkness. Controls used for comparison were plants not illuminated prior to 24h in the dark (24D) and dark-grown plants transferred to continuous white light for 24h. Dots blots were made, hybridised and washed exactly as described for figure 23.

(a) Autoradiogram of dots after hybridisation to the pea rbcS cDNA probe.

(b) Plot of cpm over background for each treatment expressed as a percentage relative to cpm obtained from RNA from leaves grown in continuous white light for 24h (table 9).

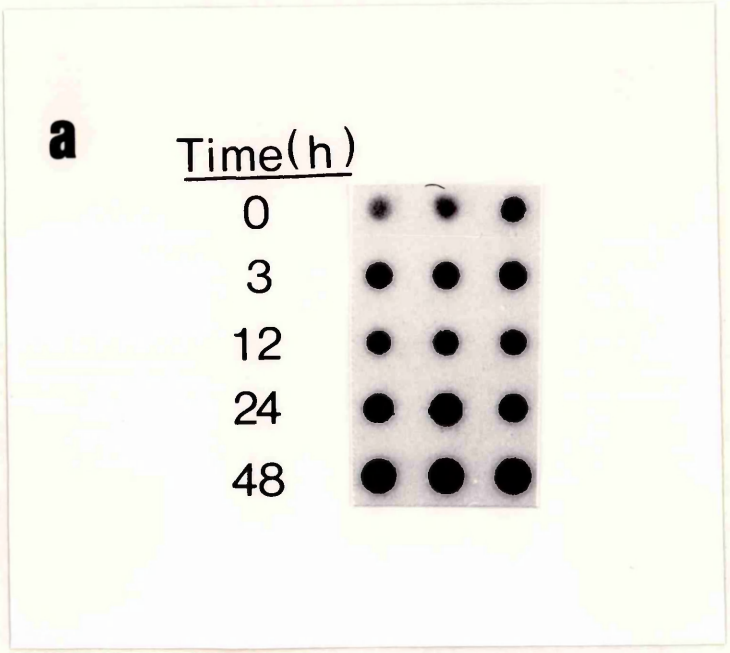
used was the EcoR1-EcoR1 insert of pPvSS1672 (figure 15), labelled by nick-translation (section 2.18.2). After washing and autoradiography of the filters individual dots were cut from the filter and hybridisation estimated by scintillation counting. Results are given as average cpm per dot and average cpm per dot after background is subtracted. The cpm for background represents the counts hybridised to 1µg *E. coli* tRNA to take into account non-specific hybridisation as well as natural background. Experiments are labelled as 'experiment A' or 'experiment B' both of which had RNA concentration series included with them (section 3.6.1.2.4)

3.6.1.2.1 Greening timecourse (experiment A)

Table 10 shows the results from dark-grown *P. vulgaris* seedlings (6 days old) which were illuminated with continuous white light at 100µmol $m^{-2}s^{-1}$ as in section 2.8 and from which samples of primary leaves were taken after 0, 3, 12, 24 and 48h. Figure 25a shows the autoradiogram of the dots after hybridisation. Figure 25b shows a plot of cpm above background against time in white light. This shows that *rbc*S transcripts were detected and accumulated during the greening timecourse. A transient increase in the level of *rbc*S transcripts was observed in the first few hours of the timecourse. *rbc*S transcripts were detected at 0h (dark-grown tissue).

3.6.1.2.2 Phytochrome experiment (experiment B)

Table 11 shows the results from an experiment with dark-grown *P. vulgaris* seedlings (5 days old) which were given light treatments as in section 2.8 and replaced in the dark for 24h before the primary leaves were harvested. The treatments were red light for 3 min (treatment R), far-red light for 3 min (treatment FR) and red light for 3



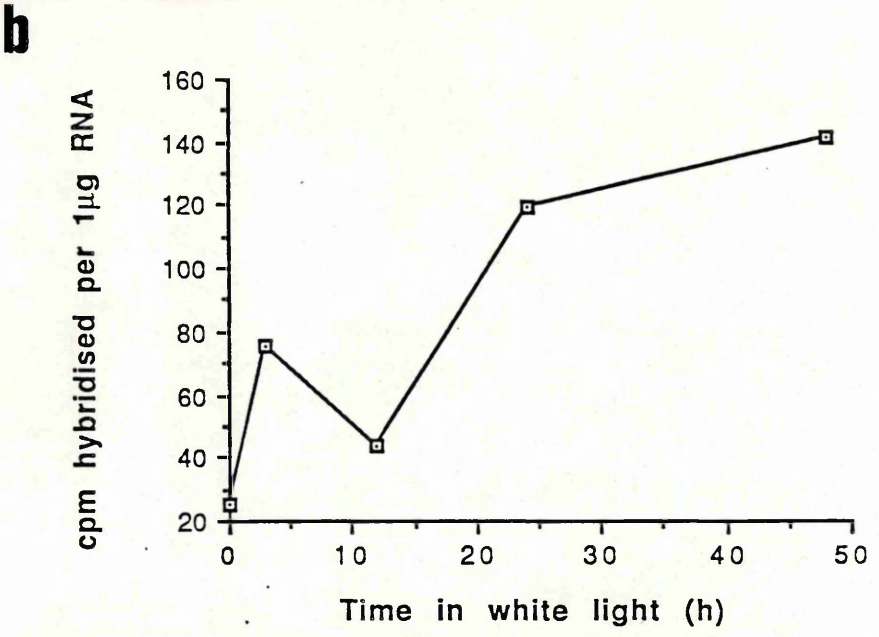


Figure 25 - Hybridisation of a P. vulgaris rbcS cDNA probe to dots of RNA isolated from P. vulgaris primary leaf tissue harvested during a 48h time course in continuous white light

RNA dot blots (1µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from at least 5 different plants. Pre-hybridisation and hybridisation was as in section 2.19.1.1. The DNA probe used was the EcoR1-EcoR1 insert of pPvSS1672 (figure 15), labelled by nick-translation (section 2.18.2) to $\sim 8 \times 10^8$ cpm/ug. Probe was added to give 2.5×10^6 cpm/ml hybridisation solution. After hybridisation filters were washed twice for 20 min in 0.5xSSC at 35°C, three times for 20 min in 0.1xSSC at 35°C and twice for 30 min in 0.1xSSC at 45°C and then autoradiographed.

(a) Autoradiogram of dots after hybridisation to the P. vulgaris rbcS cDNA probe.

(b) Plot of cpm above background against time in white light (table 10)

TABLE 10 - Hybridisation of P. vulgaris rbcS cDNA probe to RNA dots isolated from tissue harvested during a 48h time course in continuous white light.

Experimental conditions were as described in the legend to figure 25.

TIME	Average cpm per dot	Average cpm -background
0h	65	25
3h	116	76
12h	84	44
24h	160	120
48h	181	141
BACKGROUND	40	0

TABLE 11 - Hybridisation of P. vulgaris rbcS cDNA probe to RNA dots isolated from tissue harvested after various light treatments .

Experimental conditions were as described in the legend to figure 26.

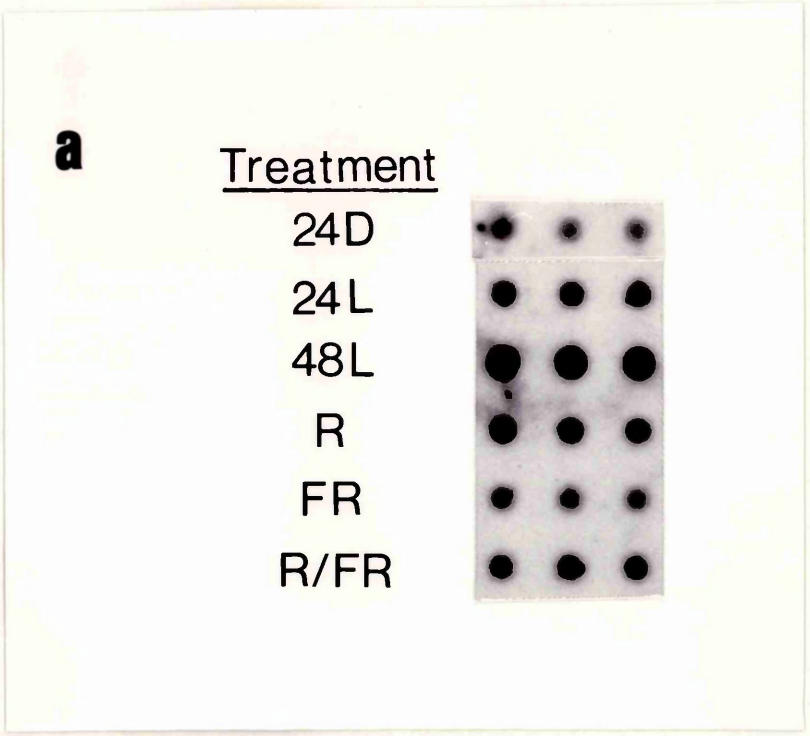
TREATMENT	Average cpm per dot	Average cpm -background
R	206	132
FR	122	47
R/FR	149	75
24L	166	92
24D	109	35
48L	307	233
BACKGROUND	74	0

min followed immediately by 3 min far-red light (treatment R/FR). Treatment '24D' represents a control which was not illuminated prior to the 24h incubation in the dark and '24L' and '48L' represent the samples taken at the 24h and 48h time-points in a greening experiment from the same batch of plants (section 3.6.1.2.1). Figure 26a shows the autoradiogram of the dots after hybridisation. Figure 26b shows a plot of cpm over background for each treatment expressed as a percentage relative to the '24L' treatment. This shows that red light induced a large increase in the level of rbcS transcripts. This red-light mediated effect was reversible by the subsequent illumination with far-red light.

3.6.1.2.3 Tissue-specific

Experiment A.
Various types of P. vulgaris tissue were extracted at various stages of development: dark-grown leaves and dark-grown cotyledons were harvested at the 0h time-point of a greening timecourse from the same batch of plants (section 3.6.1.2.1); light-grown leaves, light-grown cotyledons, stems and roots were harvested at the 48h time-point of the same timecourse. Immature pods were harvested from mature plants grown in white light with a 16h photoperiod. Hybridisation is represented as a relative proportion of light-grown leaves (table 12). The levels of rbcS transcripts was low in tissues other than light-grown primary leaves. Apart from dark-grown primary leaves (18% of the levels found in light-grown primary leaves), these tissues all contained around 5-10% the level of rbcS transcripts found in light-grown primary leaves.

Experiment B.
Various types of P. vulgaris tissue were extracted at



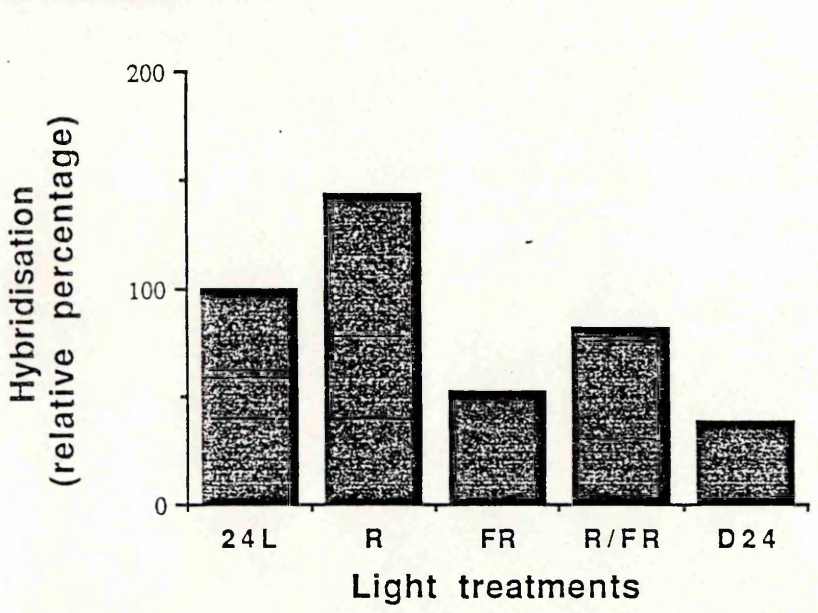


Figure 26 - Hybridisation of P. vulgaris rbcS cDNA probe to dots of RNA isolated from P. vulgaris primary leaf tissue harvested after various light treatments - phytochrome experiment

Dark-grown plants were exposed either to 3 min red-light (R), 3 min far-red light (FR), 3 min red light followed by 3 min far-red light (R/FR). Following these illuminations plants were grown for a further 24h in darkness. Controls used for comparison were plants not illuminated prior to 24h in the dark (24D) and dark-grown plants transferred to continuous white light for 24h. RNA dots were prepared, pre-hybridised and hybridised exactly as described for figure 25, and were then autoradiographed.

(a) Autoradiogram of dots after hybridisation to a P. vulgaris rbcS cDNA probe.

(b) Plot of cpm over background for each treatment expressed as a percentage relative to cpm obtained from RNA from leaves grown in continuous white light for 24h (table 11).

TABLE 12 - Hybridisation of P. vulgaris rbcS cDNA probe to RNA dots isolated from various P. vulgaris tissue harvested at particular stages of development (experiment A).

See section 3.6.1.2.3 for a description of the tissues used in this experiment.
Experimental conditions were as described in the legend to figure 25.

TISSUE	Average cpm per dot	Average cpm -background	Relative percentage(%)
Light-grown leaves (48h)	181	141	100
Dark-grown leaves	65	25	18
Light-grown cotyledons	46	6	4
Dark-grown cotyledons	49	9	6
Roots	53	13	10
Stem (Light-grown)	49	9	6
Immature pods	52	12	8
BACKGROUND	40	0	-

various stages of development: dark-grown leaves and dark-grown stems were harvested from 6 day-old dark-grown plants ; light-grown leaves (24h) were collected after exposure of these dark grown plants to continuous white light for 24h; light-grown leaves (48h) and light-grown stems were collected after a further 24h exposure to white light; roots were harvested from seeds germinated hydroponically on 3MM paper in the dark. Hybridisation is represented as a relative proportion of light-grown leaves (48h) (table 13). These results were consistent with those obtained in experiment A. Levels of rbcS transcripts in dark-grown stems were very low even in comparison to other non-leaf tissue.

3.6.1.2.4 RNA concentration series

Experiments A and B.
A series of RNA dots was prepared from the RNA sample of primary leaves of plants germinated and grown in continuous light for 14 days, so as to construct a RNA concentration series over the range 0.0-3.0µg.
Results are given as average cpm per dot with background subtracted (tables 14 and 15). Background in this case was taken as the average cpm for dots without RNA (0.00µg). Figures 27a and 28a show the autoradiograms of the dots after hybridisation from experiments A and B respectively. Figures 27b and 28b show graphs of log[average cpm per dot] against log[amount of RNA in µg] from experiments A and B respectively. A linear relationship between hybridisation and RNA input was observed in both experiments.

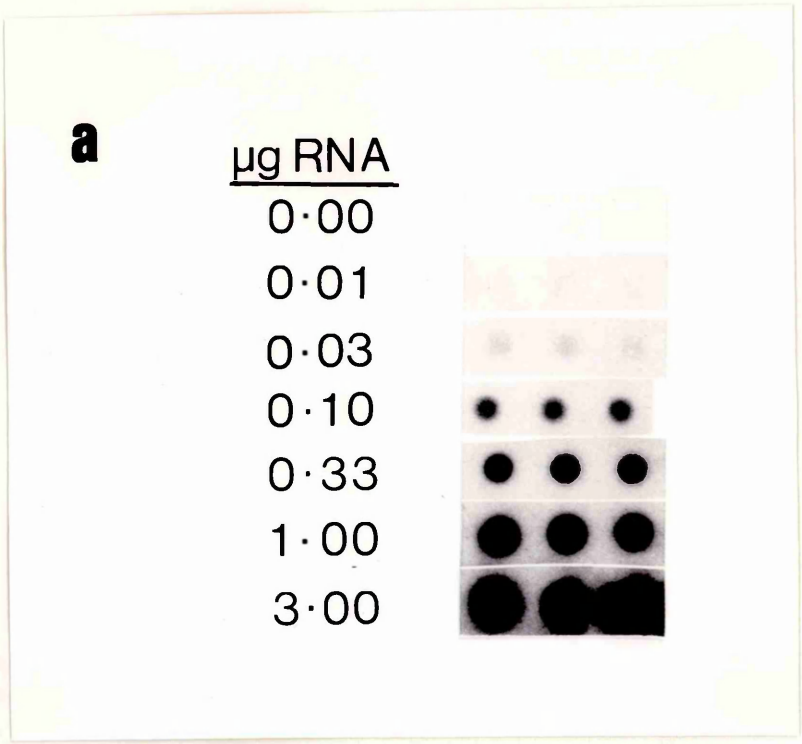
3.6.2 Using oligonucleotide probes

Dot blots of total RNA were hybridised to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG so as

TABLE 13 - Hybridisation of P. vulgaris rbcS cDNA probe to RNA dots isolated from various P. vulgaris tissue harvested at particular stages of development (experiment B).

See section 3.6.1.2.3 for a description of the tissues used in this experiment.
Experimental conditions were as described in the legend to figure 25.

TISSUE	Average cpm per dot	Average cpm -background	Relative percentage(%)
Light-grown leaves (24h)	166	92	54
Light-grown leaves (48h)	307	233	100
Dark-grown leaves	109	35	11
Dark-grown stems	76	2	1
Light-grown stems	100	26	8
Roots	89	15	5
BACKGROUND	74	0	-



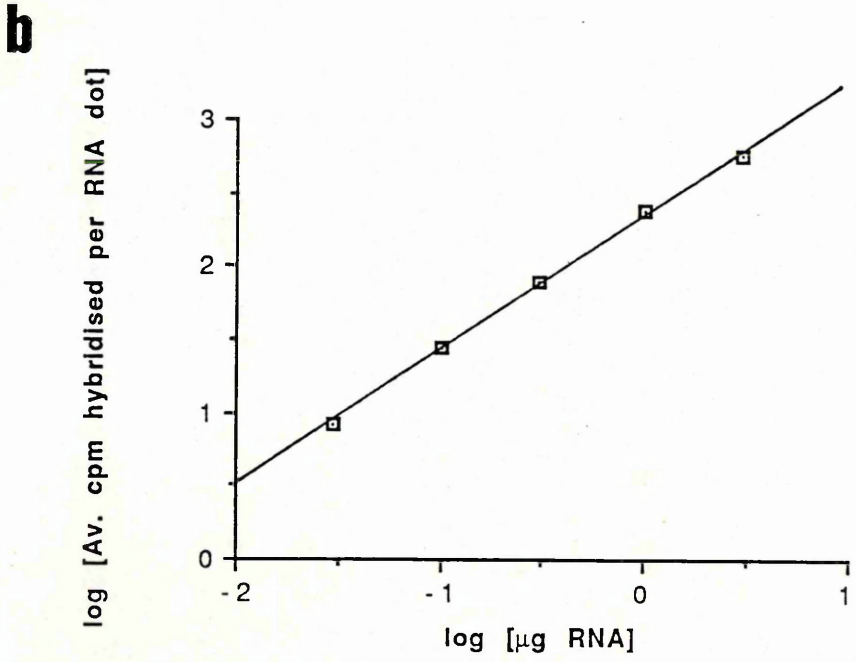
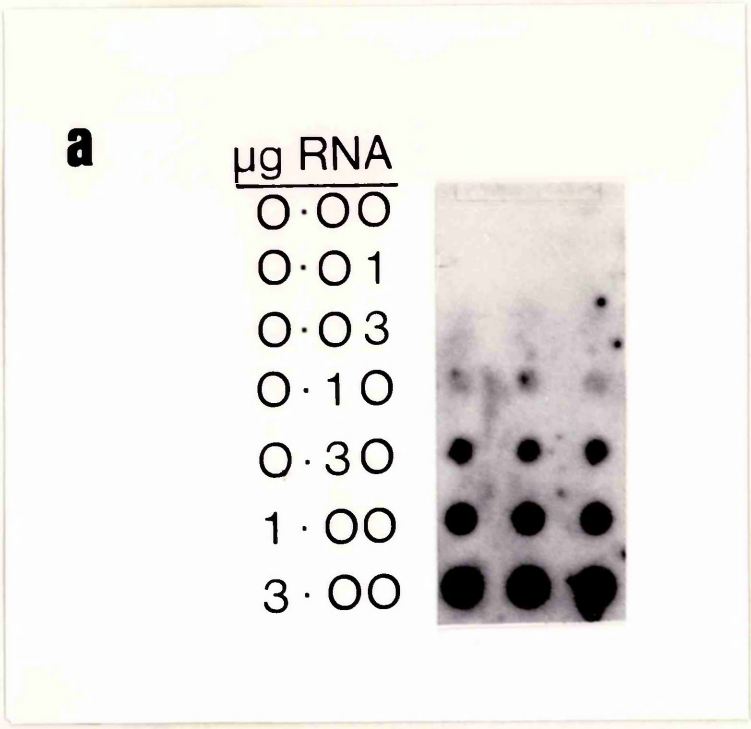


Figure 27 - Hybridisation of a P. vulgaris rbcS cDNA probe to a concentration series of RNA dots (experiment A).

A series of RNA dots was prepared from the RNA sample of primary leaves of plants germinated and grown in continuous light for 14 days, so as to construct a concentration series over the range 0.0-3.0µg. Hybridisation and washing of filters was exactly as described in figure 23.

(a) Autoradiogram of RNA dots after hybridisation.

(b) Plot of log[average cpm per dot] against log[amount of RNA in µg] (table 14).



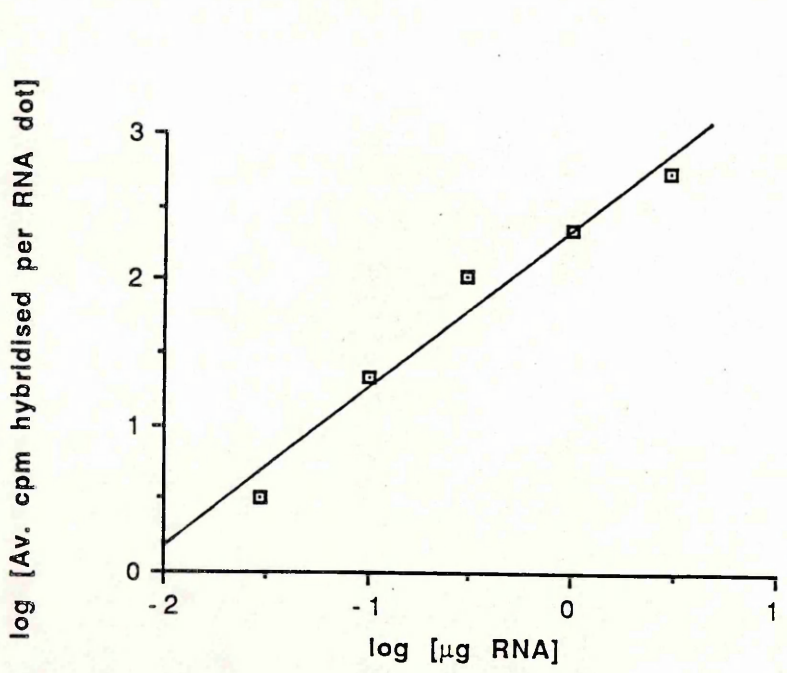


Figure 28 - Hybridisation of a P. vulgaris rbcS cDNA probe to a concentration series of RNA dots (experiment B)

A series of RNA dots was prepared from the RNA sample of primary leaves of plants germinated and grown in continuous light for 14 days, so as to construct a concentration series over the range 0.0-3.0µg. Hybridisation and washing of filters was exactly as described in figure 25.

(a) Autoradiogram of RNA dots after hybridisation.

(b) Plot of log[average cpm per dot] against log[amount of RNA in µg] (table 15).

TABLE 14 - Hybridisation of P. vulgaris rbcS cDNA probe to dots containing an increasing series of RNA amount (experiment A).

Experimental conditions were as described in the legend to figure 27.

ug RNA per dot	Average cpm -background
0.00	0
0.01	0
0.03	8
0.10	28
0.30	80
1.00	238
3.00	572

TABLE 15 - Hybridisation of P. vulgaris rbcS cDNA probe to dots containing an increasing series of RNA amount (experiment B).

Experimental conditions were as described in the legend to figure 28.

ug RNA per dot	Average cpm -background
0.00	0
0.01	0
0.03	3
0.10	22
0.30	104
1.00	220
3.00	524

to obtain measurements of the relative steady-state levels of individual P. vulgaris rbcS transcripts.

3.6.2.1 Specificity of hybridisation of gene-specific oligonucleotide probes to rbcS cDNA clones

To test the specificity of the gene-specific oligonucleotide probes rbcS1, rbcS2 and rbcS3, they were hybridised to dot blots of the plasmid DNA of the three rbcS cDNA clones from which they were designed.

Triplicate dot blots (70fmol DNA each) of linearised pPvSS1672, pPvSS965, pPvSS191 and pUC19 plasmid DNA were prepared as in section 2.17.3 on four filters so that each filter carried all four plasmids and was identical to the others. The four oligonucleotide probes (rbcS1, rbcS2, rbcS3 and rbcSG) were end-labelled using T4 polynucleotide kinase (section 2.19.1.2) to approximately $1x10^9$dpm/µg. The dissociation temperature of the oligonucleotide probes was calculated using the formula:

$Td = 2^{o}C(A+T) + 4^{o}C(G+C)$ (Tumer et al., 1986).

Hybridisation was carried out at Td-10°C (46°C) as in section 2.19.1.2, and the filters were then washed and autoradiographed (figure 29). Hybridisation to individual dots was then estimated by scintillation counting. Table 16 shows the results obtained, expressed as average cpm per DNA dot. It was observed that each gene-specific oligonucleotide probe (rbcS1, rbcS2 and rbcS3) was specific to its cognate cDNA clone. The general probe rbcSG was seen to hybridise similarly to all three types of rbcS cDNA clone.

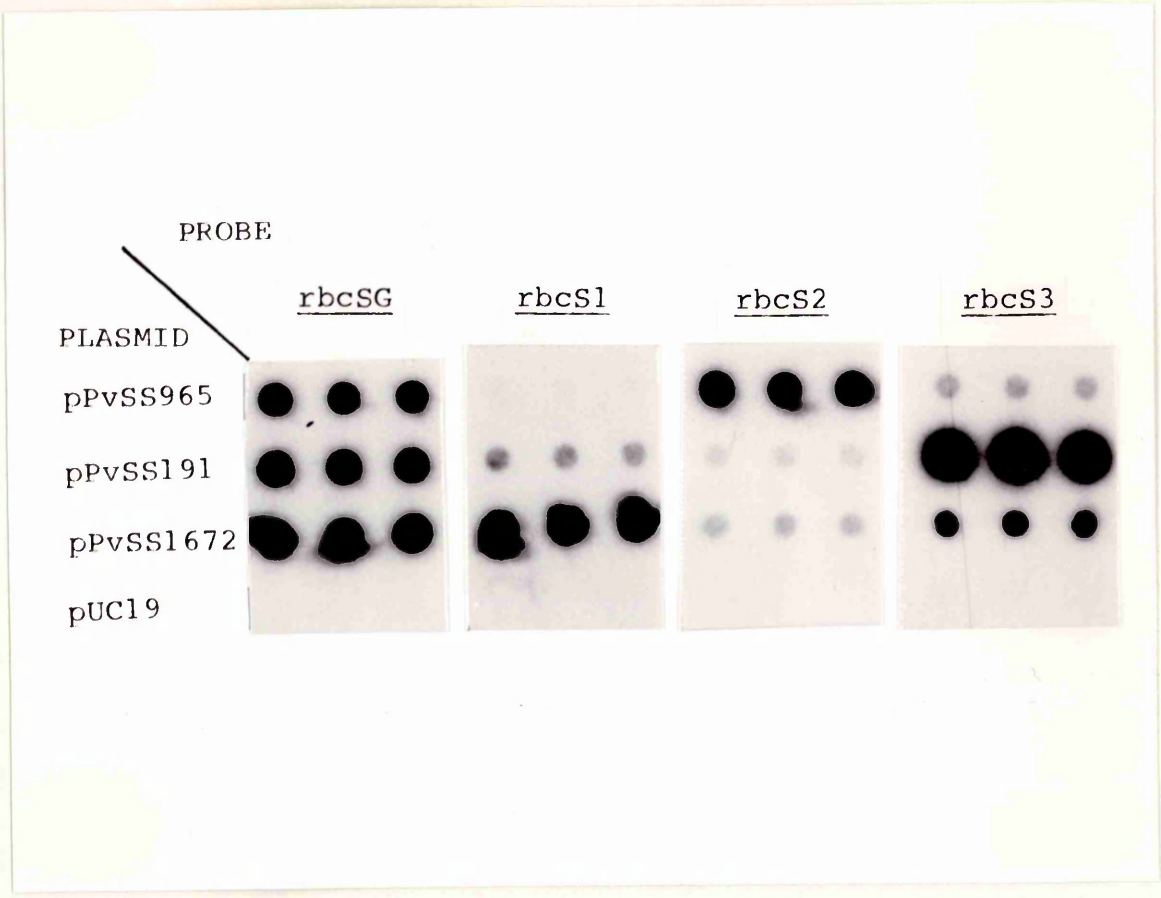


Figure 29 - Specificity of hybridisation of oligonucleotide probes to the plasmid DNA of P. vulgaris rbcS cDNA clones.

Triplicate dot blots (70fmol DNA each) of linearised pPvSS1672, pPvSS965, pPvSS191 and pUC19 plasmid DNA were prepared as in section 2.17.3 on four filters so that each filter carried all four plasmids and was identical to the others. The 4 oligonucleotide probes were end-labelled using T4 polynucleotide kinase (section 2.19.1.2) to approximately $1x10^9$cpm/µg. Hybridisation was carried out at 46°C as in section 2.19.1.2 in hybridisation solution containing approximately $6x10^6$cpm/ml. The filters were washed at room temperature twice for 15 min followed by twice at 52°C for 15 min and then autoradiographed.

Table 16 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbsS3 and rbcSG to dots of the plasmid DNA of pPvSS1672, pPvSS965, pPvSS191 and pUC19.

Experimental conditions were as described in the legend to figure 29.

	P L A S M I D S			
	pPvSS1672	pPvSS965	pPvSS191	pUC19
PROBES	Average cpm/dot	Average cpm/dot	Average cpm/dot	Average cpm/dot
rbcSG	3985	1857	2834	85
rbcS3	783	264	11818	111
rbcS2	215	2804	134	37
rbcS1	3524	62	261	94

3.6.2.2 Specificity of hybridisation of gene-specific oligonucleotide probes to rbcS transcripts

Four identical Northern blots of total RNA from light- and dark-grown primary leaves were individually hybridised to the oligonucleotide probes rbcSG, rbcS1, rbcS2 and rbcS3 (section 2.18.1.2). After washing, the filters were autoradiographed (figure 30). Sizes of hybridising bands were estimated from the migration of single-stranded RNA size markers (supplied by BCL) loaded on the same gel from which the blot was made (figure 31). The different mobilities of the rRNA bands on the gel in figure 31 were most likely due to the presence of different concentrations of salt in the original RNA samples due to differences in their preparation.

All four oligonucleotide probes bound non-specifically to the rRNA bands on the blot, and the rbcS3 probe also bound non-specifically to the tRNA. Probes rbcS2 and rbcS3 bound to both the 25S and 18S rRNA bands whereas probes rbcS1 and rbcSG bound predominantly to the 25S rRNA band. The hybridisation of all 4 oligonucleotide probes produced a hybridising band located between the 0.6 and 1.0 kbp marker fragments.

3.6.2.3 Relative hybridisation signals of gene-specific oligonucleotide probes

To use the oligonucleotide probes for quantitative estimates of the relative steady-state levels of different rbcS transcripts, the relative hybridisation signal for each probe to complementary sequence was calculated for a given experiment, so that results could be normalised with respect to the different probes. The relative hybridisation signal is determined by the specific activity of the probe and its efficiency of hybridisation to its complementary sequence.

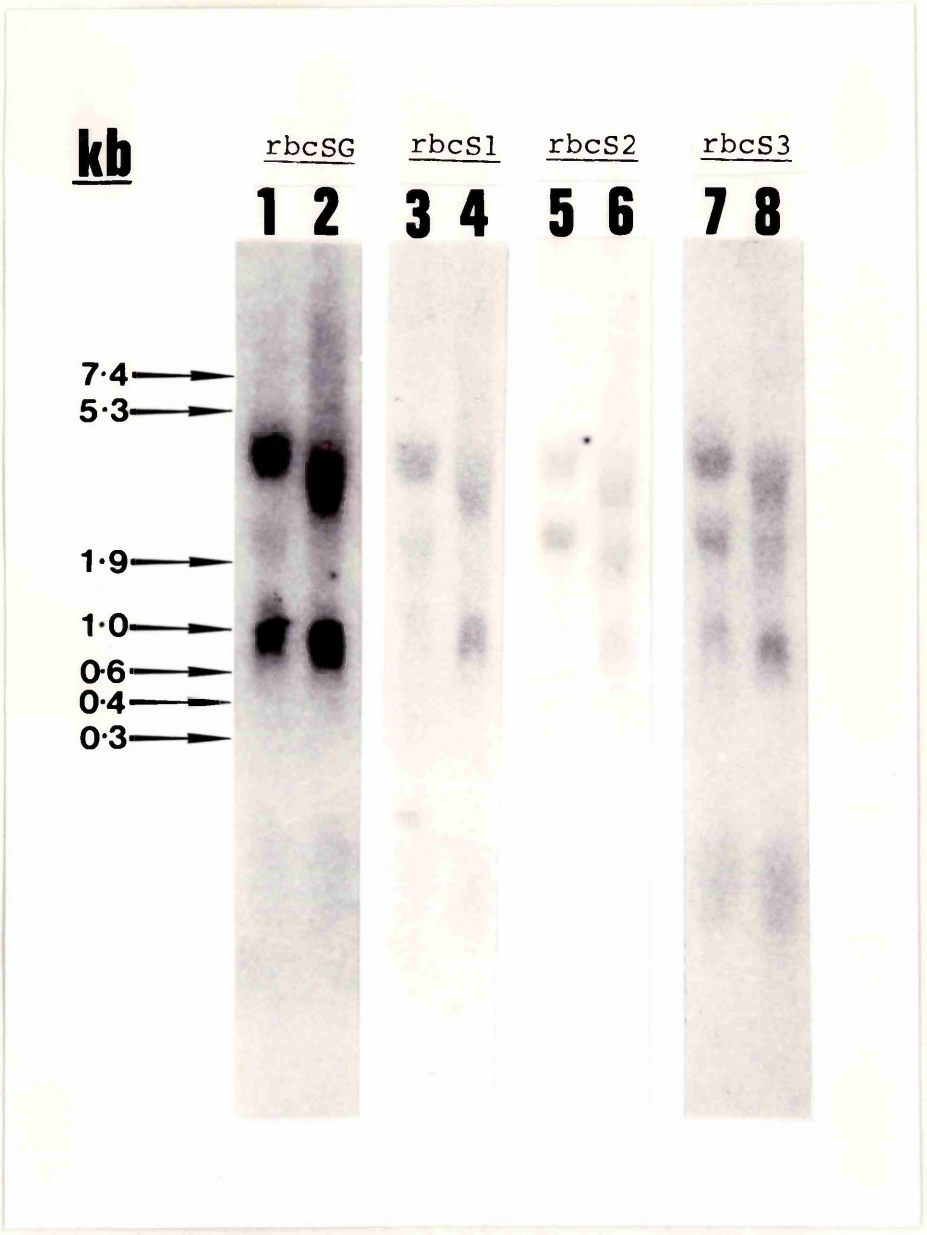


Figure 30 - Specificity of hybridisation of oligonucleotide probes to total RNA from P. vulgaris.

RNA was isolated from light- and dark-grown primary leaves (approximately 5µg/track). Samples were prepared, denatured and run on a 1.2% agarose/formaldehyde gel as in section 2.13.2 and northern blots made as in section 2.17.2. Labelling of oligonucleotide probes was as described in section 3.6.2.3. Hybridisation was carried out at 46°C as in section 2.19.1.2 in hybridisation solution containing approximately $1x10^6$cpm/ml. The filters were washed at room temperature three times in 6xSSC for 20 min and then autoradiographed. Autoradiograms are shown of 4 identical blots after hybridisation to oligonucleotide probes rbcSG, rbcS1, rbcS2 and rbcS3 (section 2.18.1.2).

Tracks 1, 3, 5 and 7: dark-grown primary leaves; tracks 2, 4, 6 and 8: light-grown primary leaves.

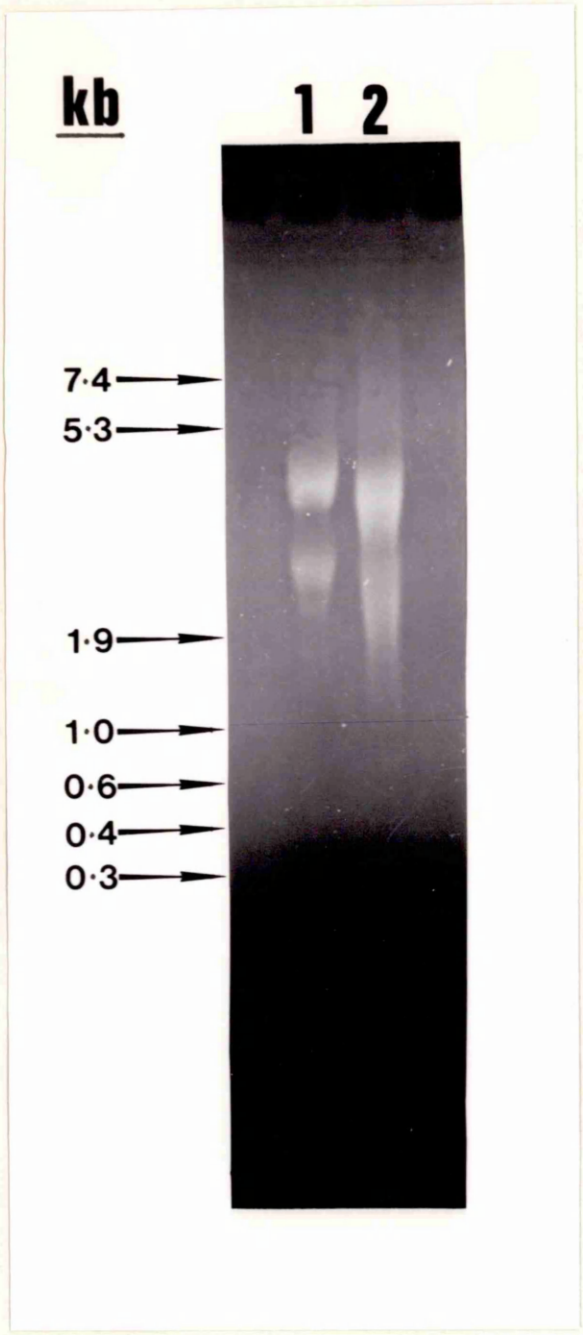


Figure 31 - Gel electrophoresis of P. vulgaris total RNA

RNA was prepared from dark- and light-grown primary leaves and 5µg per track was run on a 1.2% agarose/formaldehyde gel as described in section 2.13.1. Single-stranded RNA markers (BCL) were run alongside. These gels were blotted as in section 2.17.2. This figure shows two of the eight tracks run on the same gel to provide four identical filters for hybridisation (figure 30). Track 1: dark-grown primary leaves; track 2: light-grown primary leaves.

An oligonucleotide labelling reaction was carried out as in section 2.18.4 with all the components reduced to a half (ie. 60ng oligonucleotide was labelled). Upon completion of the labelling reaction the final volume was made up to 500µl and 5µl from each labelling reaction was spotted onto two separate DE81 paper circles. Incorporated radioactivity was then estimated by scintillation counting (section 2.18.7.2). Table 17 shows the incorporation obtained for each individual oligonucleotide probe.
Triplicate dot blots (70fmol DNA each) of linearised plasmids pPvSS1672, pPvSS965 and pPvSS191 were prepared as in section 2.17.3 on four filters so that each filter carried all three plasmids and was identical to the others. Hybridisation was carried out at 46°C as in section 2.19.1.2, the filters were washed and then autoradiographed (figure 32). Hybridisation to individual dots was then estimated by scintillation counting. Table 18 shows the results obtained expressed as average cpm per DNA dot. As before, each oligonucleotide probe was found to hybridise specifically to its cognate rbcS cDNA clone.

The relative hybridisation signals for probes rbcS1, rbcS2 and rbcS3 were calculated relative to probe rbcSG:

Hybridisation signal of probe rbcS3 relative to probe rbcSG =

$$\frac{1159.74}{2357.18} \times 100\ \% = \underline{49\%}$$

Hybridisation signal of probe rbcS2 relative to probe rbcSG =

$$\frac{1934.27}{2122.95} \times 100\% = \underline{91\%}$$

TABLE 17 - Counts incorporated in 0.6 ng of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG.

Experimental details were as described in section 3.6.2.3.

PROBES	unwashed disc (cpm)	washed disc (cpm)	sp.act. (dpm/ug)	% incorp.
rbcSG	593 825	452 603	7.54×10^8	76
rbcS3	655 032	539 792	9.00×10^8	82
rbcS2	574 199	445 315	7.42×10^8	78
rbcS1	510 883	464 563	7.74×10^8	91

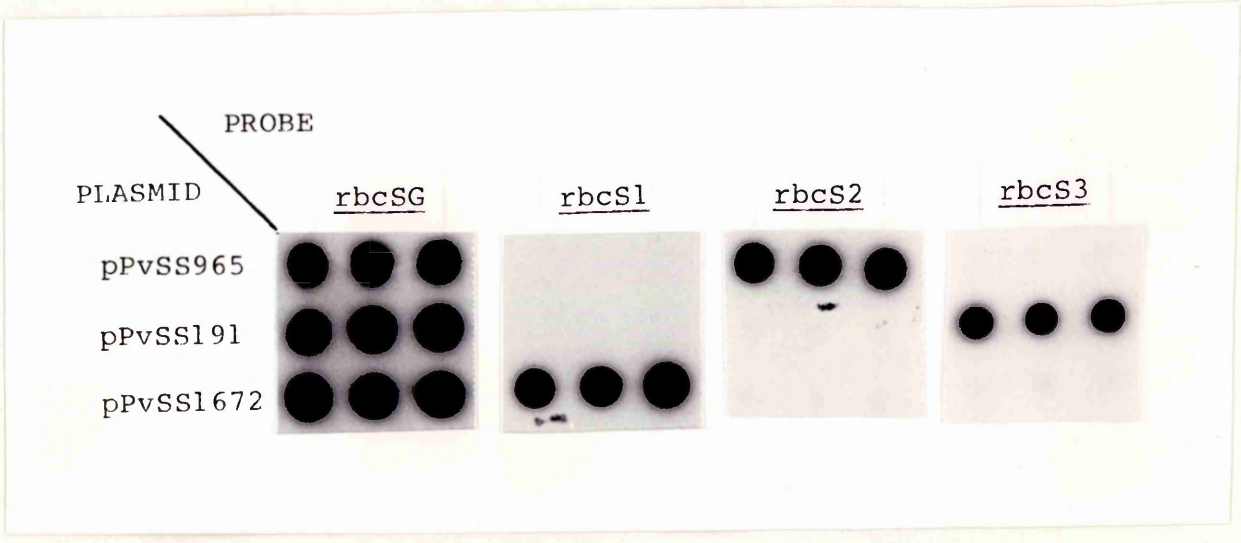


Figure 32 - Hybridisation of oligonucleotide probes (rbcS1, rbcS2, rbcS3 and rbcSg) to the plasmid DNA of P. vulgaris rbcS cDNA clones. Triplicate dot blots (70fmol DNA each) of linearised plasmids pPvSS1672, pPvSS965 and pPvSS191 were prepared as in section 2.17.3 on four filters so that each filter carried all three plasmids and was identical to the others. Hybridisation was carried out at 46°C as in section 2.19.1.2 in hybridisation solution containing approximately $1x10^6$cpm/ml. The filters were washed at room temperature three times in 6xSSC for 20 min and then autoradiographed.

Table 18 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbsS3 and rbcSG to dots of the plasmid DNA of pPvSS1672, pPvSS965 and pPvSS191.

Experimental conditions were as described in the legend to figure 32.

	P L A S M I D S		
	pPvSS1672	pPvSS965	pPvSS191
PROBES	Average cpm/dot	Average cpm/dot	Average cpm/dot
rbcSG	2705	2123	2357
rbcS3	40	57	1160
rbcS2	60	1934	45
rbcS1	1872	40	61

Hybridisation signal of probe rbcS1 relative to probe rbcSG =

$$\frac{1871.94}{2705.12} \times 100\% = \underline{69\%}$$

These values for the relative hybridisation signals were used to normalise the values obtained for cpm above background in section 3.6.2.4 relative to rbcSG. The normalised values were termed 'relative hybridisation', and were calculated using the following equation:

Relative hybridisation=

$$\frac{100}{\%\text{hybridisation signal}} \times \text{cpm above background}$$

3.6.2.4 Analysis of RNA

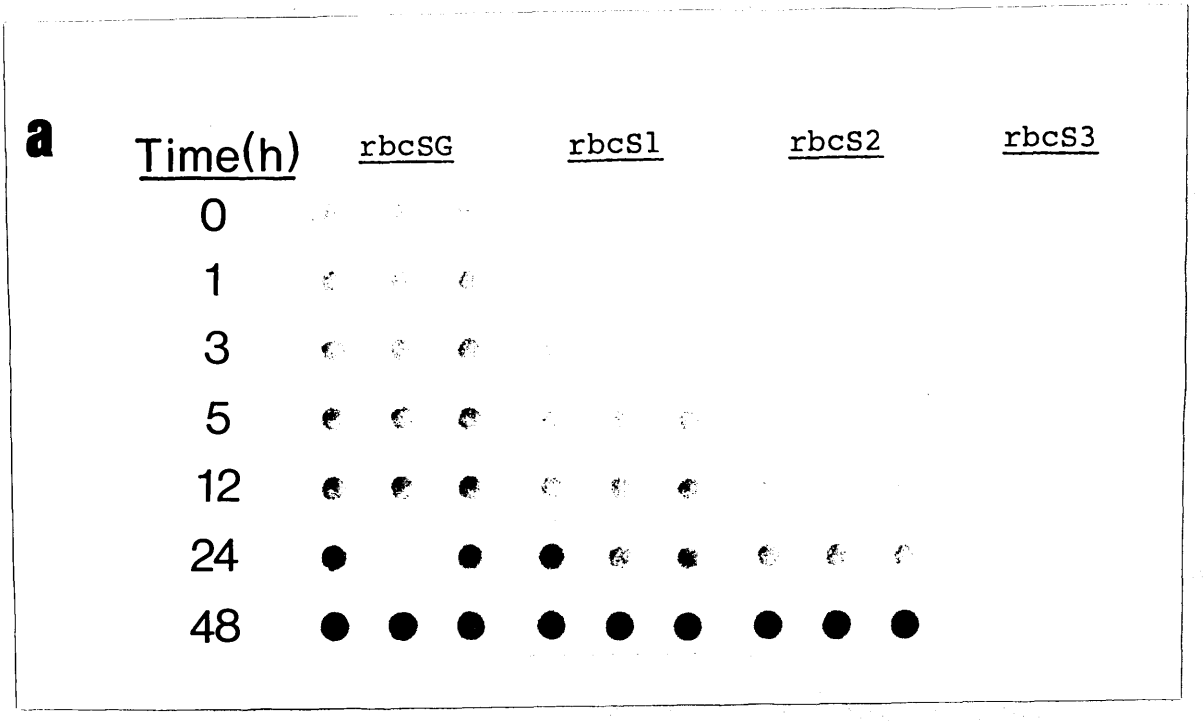
One batch of plants and one preparation of labelled oligonucleotides was used for all the experiments described in this section. RNA dot blots (10µg) were set up in triplicate for the different treatments as in section 2.17.4, RNA having been prepared from the primary leaves of at least 8 different plants per treatment. Labelling of the oligonucleotide probes, pre-hybridisation and hybridisation of the filters and washing conditions were exactly as described in the legend to figure 32. After autoradiography individual dots were cut from the filters and hybridisation was estimated by scintillation counting. Throughout, the results are given as average cpm per dot after background is subtracted. The cpm for background is the radioactivity hybridised to 10µg total rat liver RNA (gifted by Dr. S. Griffifths of this department), which takes into account non-specific hybridisation as well as natural background. The background counts for rbcSG, rbcS1, rbcS2 and rbcS3 were 59, 45, 40 and 49 cpm respectively.

3.6.2.4.1 Greening time course

Table 19 shows results obtained with dark-grown P. vulgaris seedlings (6 days old) which were illuminated with continuous white light at 200µmol $m^{-2}s^{-1}$ as in section 2.8 and from which samples of primary leaves were taken after 0, 1, 3, 5, 12, 24 and 48h. Table 19 expresses the results as average cpm per dot over background and as relative hybridisation calculated using the relative hybridisation signals of the different oligonucleotide probes (section 3.6.2.2). Figure 33a shows the autoradiograms of the dots after hybridisation. Figure 33b shows a plot of relative hybridisation against time in white light, obtained using the individual oligonucleotide probes. Figure 34 shows a plot of the total relative hybridisation values for rbcS1, rbcS2 and rbcS3 along with a plot of the cpm hybridised using rbcSG. The results showed that rbcS1 and rbcS2 transcript levels increased steadily over the 48h time course. The levels of rbcS3 transcripts remained stable over 48h. The kinetics of the totalled relative hybridisations of rbcS1, rbcS2 and rbcS3 were different to those of the hybridisation of rbcSG over the 48h time course, the former showing a more marked increase than the latter.

3.6.2.4.2 Dark time course

Table 20 shows the results obtained with dark-grown P. vulgaris seedlings (6 days old) which were grown in continuous darkness as in section 2.8 and from which samples of primary leaves were taken after 0, 48, 96 and 192h. Table 20 expresses the results as average cpm per dot over background and as relative hybridisation calculated using the relative hybridisation signals of the different oligonucleotide probes (section 3.6.2.2). Figure 35a shows the autoradiograms of the dots after



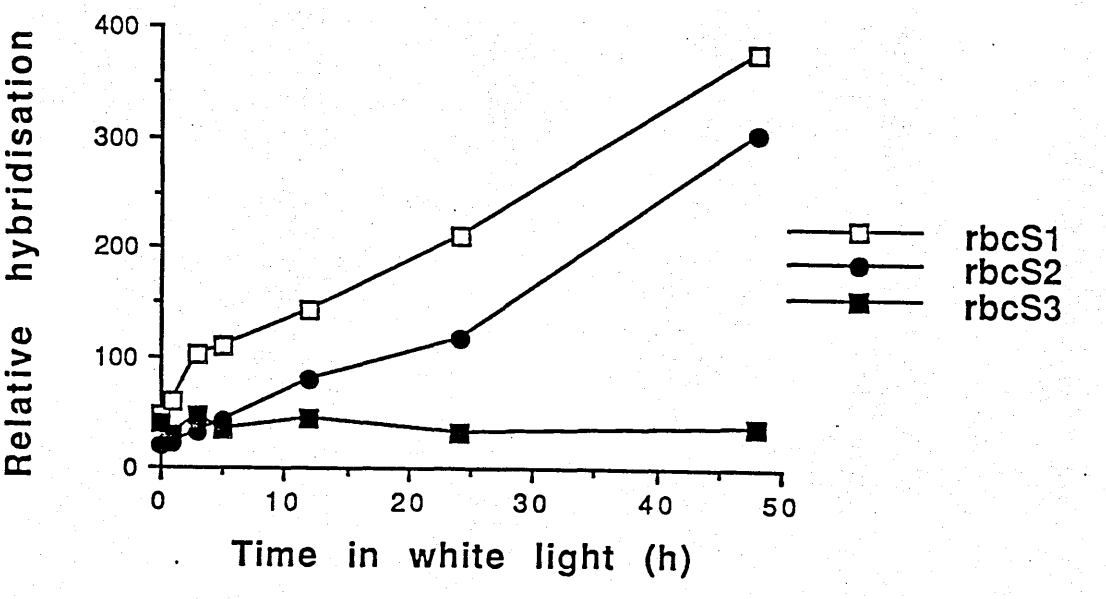


Figure 33 - Hybridisation of oligonucleotide probes to dots of RNA isolated from P. vulgaris primary leaf tissue harvested during a 48h time course in continuous white light

RNA dot blots (10µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from the primary leaves of at least 8 different plants at each time point. Labelling of the oligonucleotide probes, pre-hybridisation and hybridisation of the filters and washing conditions were exactly as described in figure 32.
(a) Autoradiogram of RNA dots after hybridisation to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG.
(b) Plot of the relative hybridisation of rbcS1, rbcS2 and rbcS3 against time in white light (table 19).

TABLE 19 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG to RNA dots isolated from P. vulgaris tissue harvested during a 48h time course in continuous white light.

Experimental conditions were as described in the legend to figure 33.

	rbcSG	rbcS1		rbcS2		rbcS3	
Time	Average cpm/dot	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.
0h	**98**	33	**48**	18	**19**	19	**40**
1h	**109**	43	**62**	21	**23**	15	**31**
3h	**133**	71	**102**	29	**32**	23	**47**
5h	**146**	76	**110**	39	**42**	17	**34**
12h	**159**	100	**145**	73	**80**	23	**46**
24h	**211**	146	**211**	108	**119**	16	**32**
48h	**327**	261	**377**	276	**303**	19	**38**

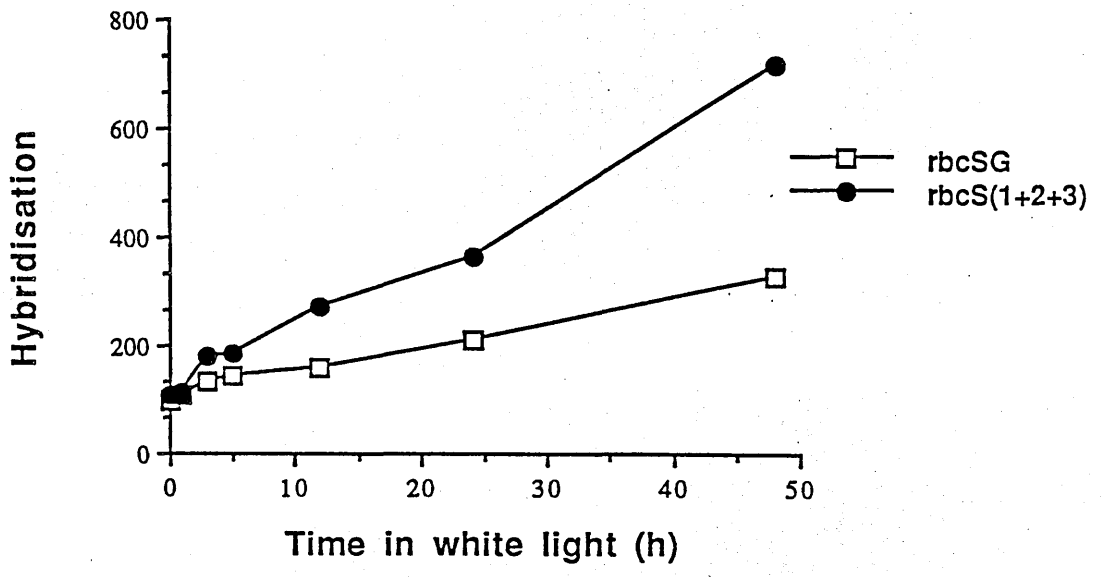
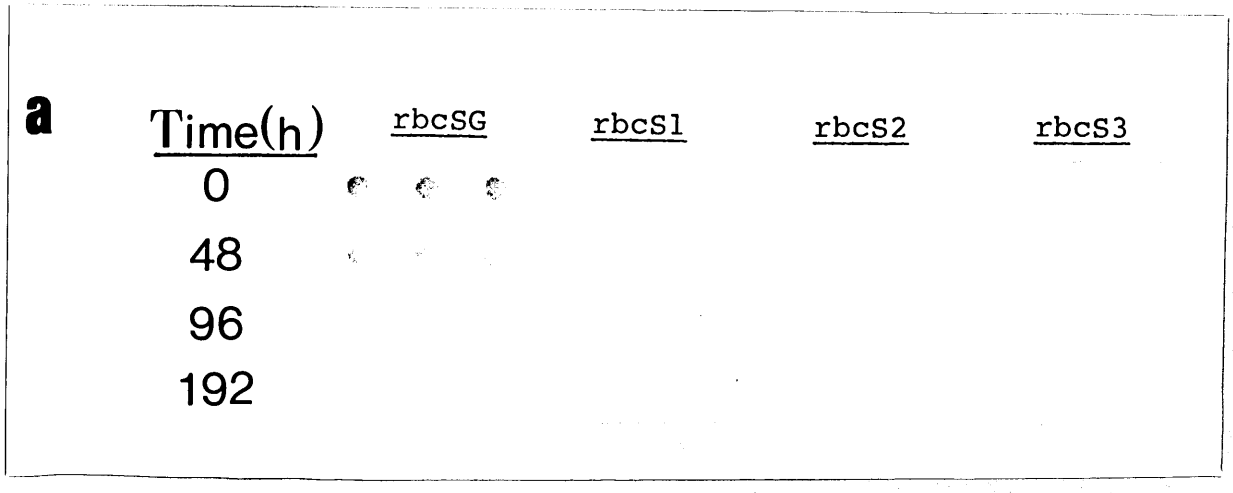


Figure 34 - Comparison of the combined relative hybridisation values of rbcS1, rbcS2 and rbcS3 to primary leaf total RNA during a greening time course with the average cpm hybridised to rbcSG.
This figure shows a plot of the combined relative hybridisations of rbcS1, rbcS2 and rbcS3 and a plot of the cpm above background of rbcSG against time in white light.



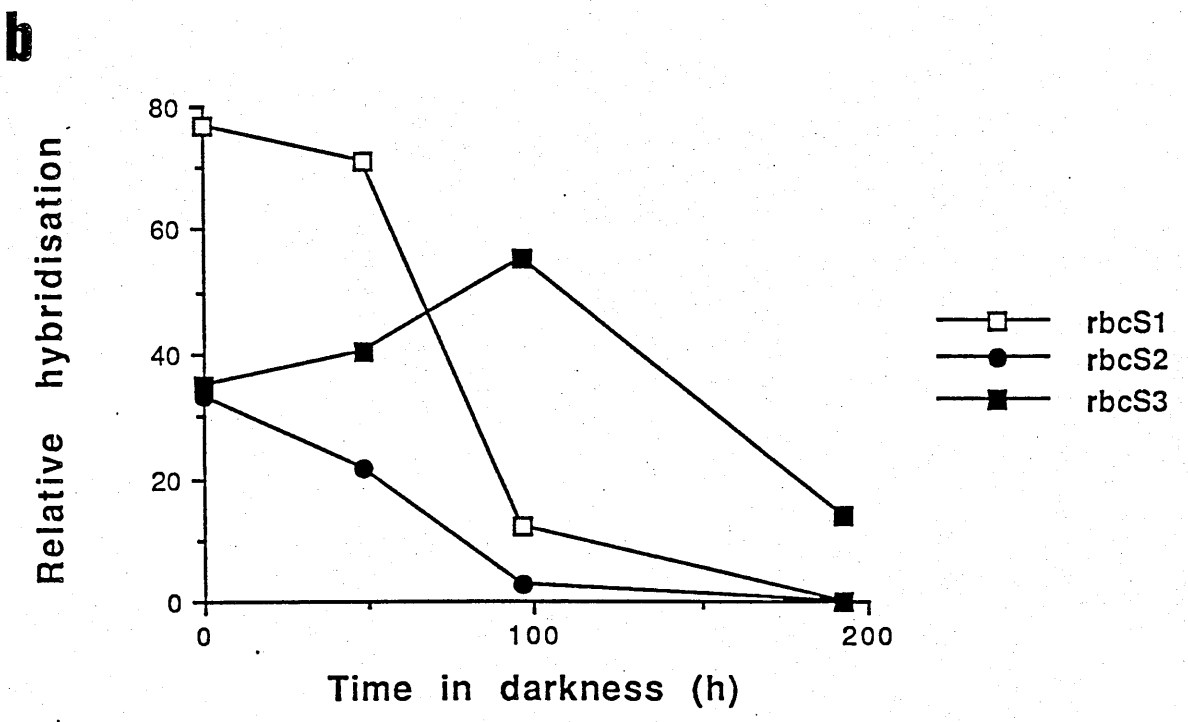


Figure 35 - Hybridisation of oligonucleotide probes to dots of RNA isolated from P. vulgaris primary leaf tissue harvested during a 192 h time course in the dark

RNA dot blots (10µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from the primary leaves of at least 8 different plants at each time point. Labelling of the oligonucleotide probes, pre-hybridisation and hybridisation of the filters and washing conditions were exactly as described in figure 32.
(a) Autoradiogram of RNA dots after hybridisation to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG.
(b) Plot of the relative hybridisation of rbcS1, rbcS2 and rbcS3 against time in darkness (table 20).

TABLE 20 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG to RNA dots isolated from primary leaf tissue harvested during a 192h time course in continuous darkness.

Experimental conditions were as described in the legend to figure 35.

	rbcSG	rbcS1		rbcS2		rbcS3	
Time	Average cpm/dot	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.
0h	**130**	53	**77**	30	**33**	17	**35**
48h	**85**	49	**71**	20	**22**	20	**40**
96h	**42**	8	**12**	3	**3**	27	**56**
192h	**11**	-3	**0**	-1	**0**	7	**15**

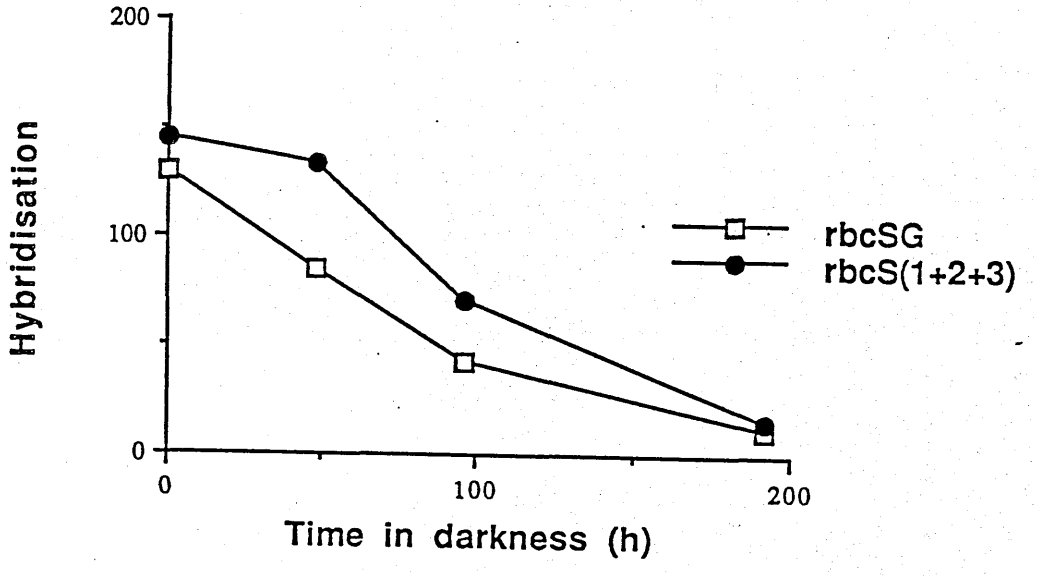


Figure 36 - Comparison of the combined relative hybridisation values of rbcS1, rbcS2 and rbcS3 to primary leaf total RNA during a time course in the dark with the average cpm hybridised to rbcSG.
Figure shows a plot of the combined relative hybridisations of rbcS1, rbcS2 and rbcS3 and the average cpm hybridised for rbcSG against time in the dark.

hybridisation. Figure 35b shows a plot of relative hybridisation against time in darkness, obtained using the individual oligonucleotide probes. Figure 36 shows a plot of the total relative hybridisation values for rbcS1, rbcS2 and rbcS3 along with a plot of the cpm hybridised using rbcSG. The results show differences in the levels of rbcS1, rbcS2 and rbcS3 transcripts in the dark. rbcS1 and rbcS2 levels were different in magnitude but shared similar kinetics. rbcS3 transcript levels showed different kinetics to rbcS1 and rbcS2 transcripts over the time course. The correlation between the totalled values for rbcS1, rbcS2 and rbcS3 with rbcSG was better in this experiment than in the greening time course (section 3.6.2.4.1).

3.6.2.4.3 Transfer of plants with greened primary leaves into darkness

Table 21 shows the results obtained with P. vulgaris seedlings (6 days old) which had been illuminated with continuous white light at 200µmol $m^{-2}s^{-1}$ as in section 2.8 for 48h and then transferred to darkness. Samples of primary leaves were taken after 0, 12, 24, 48 and 96h following transfer to darkness. Table 21 expresses the results as average cpm per dot over background and as relative hybridisation calculated using the relative hybridisation signals of the different oligonucleotide probes (section 3.6.2.2). Figure 37a shows the autoradiograms of the dots after hybridisation. Figure 37b shows a plot of relative hybridisation against time in darkness, obtained using the individual oligonucleotide probes. These results show that the levels of rbcS1 and rbcS2 transcripts dropped drastically over the 96h timecourse with similar kinetics. Decrease in the levels of rbcS3 transcripts was much less marked.

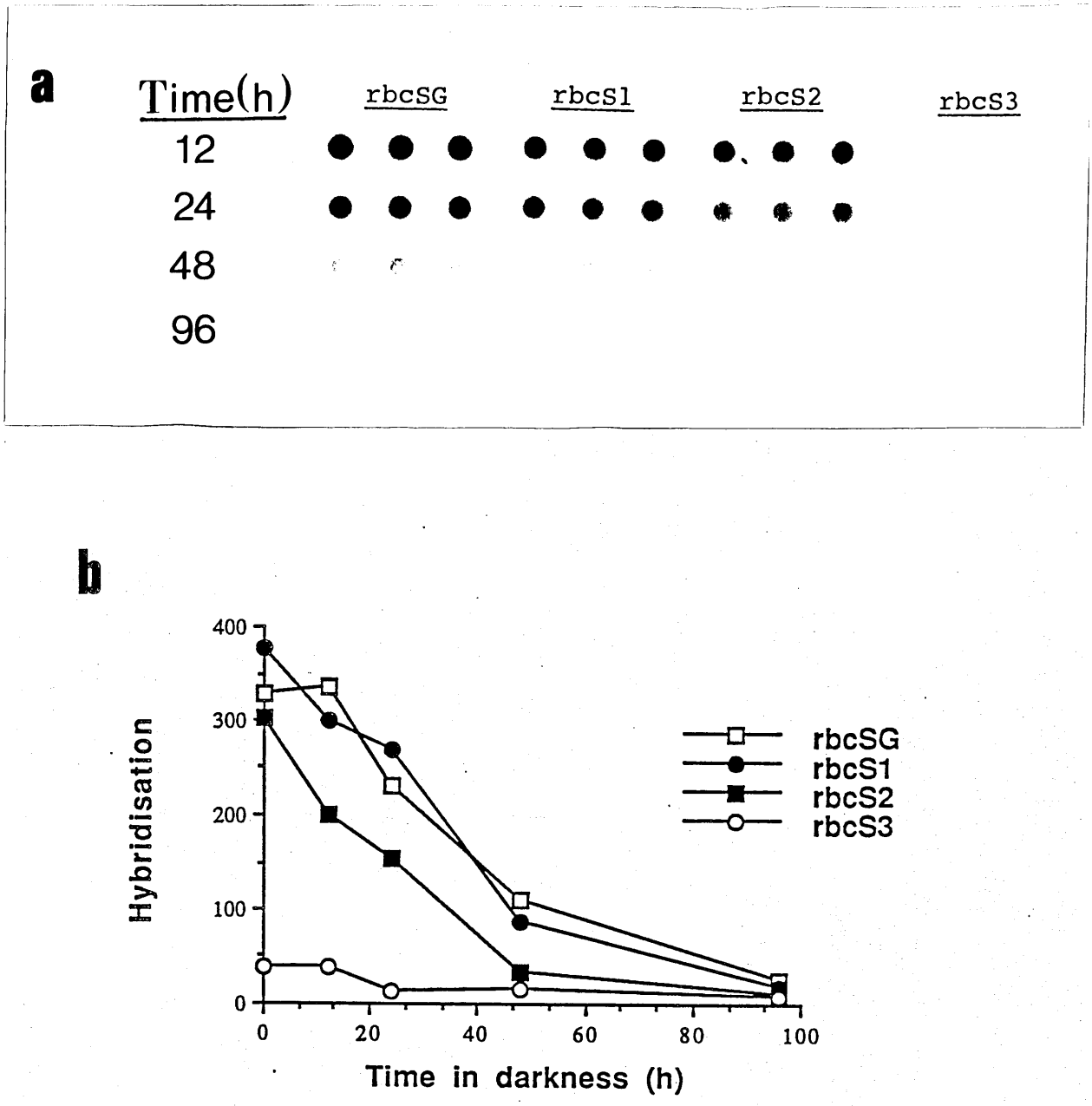


Figure 37 - Hybridisation of oligonucleotide probes to dots of RNA isolated from P. vulgaris primary leaf tissue harvested during a 96h time course in the dark after 48h continuous white light.

RNA dot blots (10µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from the primary leaves of at least 8 different plants at each time point. Labelling of the oligonucleotide probes, pre-hybridisation and hybridisation of the filters and washing conditions were exactly as described for figure 32.

(a) Autoradiogram of RNA dots after hybridisation to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG.

(b) Plot of the relative hybridisation of rbcS1, rbcS2 and rbcS3 and average cpm of rbcSG hybridisation against time in darkness (table 21).

TABLE 21 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG to RNA dots isolated from primary leaf tissue harvested from light-grown plants after transfer to continuous darkness.

Experimental conditions were as described in the legend to figure 37.

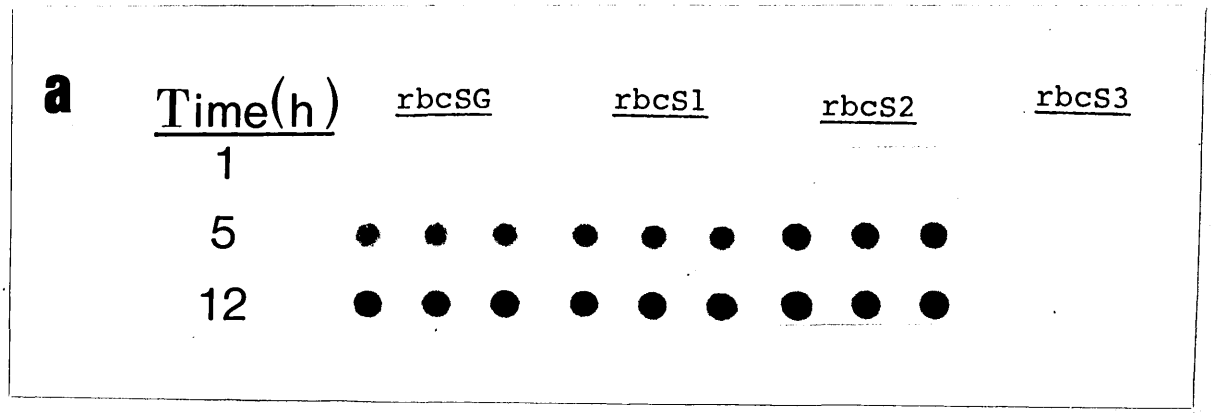
	rbcSG	rbcS1		rbcS2		rbcS3	
	Average cpm/dot	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.
Time							
12h	**336**	208	**301**	182	**199**	19	**39**
24h	**231**	186	**268**	139	**153**	7	**14**
48h	**111**	59	**86**	30	**33**	8	**16**
96h	**26**	13	**19**	9	**10**	4	**8**

3.6.2.4.4 Kinetics of rbcS transcript increases following re-illumination of greened primary leaf tissue.

Table 22 shows the results obtained with greened P. vulgaris seedlings which were taken from the 96h time-point of the dark time course in section 3.6.2.3.4 and which were illuminated with continuous white light at 200µmol $m^{-2}s^{-1}$ as in section 2.8. Samples of primary leaves were taken after 1,5 and 12h of re-illumination. Results are expressed as average cpm per dot over background and as relative hybridisation calculated using the relative hybridisation signals of the different oligonucleotides probes (section 3.6.2.2). Figure 38a shows the autoradiograms of the dots after hybridisation. Figure 38b shows a plot of relative hybridisation against time in white light, obtained using the individual oligonucleotide probes. The results show that rbcS1, rbcS2 and rbcS3 transcripts showed increases in abundance over the 12h time course. In the case of rbcS1 and rbcS2 these increases were very marked and their kinetics were very similar.

3.6.2.4.5 Phytochrome experiment

Table 23 shows the results obtained with dark-grown P. vulgaris seedlings (7 days old) which were given light treatments as in section 2.8 and replaced in the dark before primary leaves were harvested. The treatments were red light for 3 min (treatments 'R'), far-red light for 3 min (treatment FR 24) and red light for 3 min followed immediately by 3 min far-red light (treatment R/FR 24). The numbers following the treatment letters are the hours in darkness after which samples were taken. Treatment 'D24' represents a control which was not illuminated prior to the 24h incubation in the dark, and 'L24' represents the sample taken at the 24h time-point in a



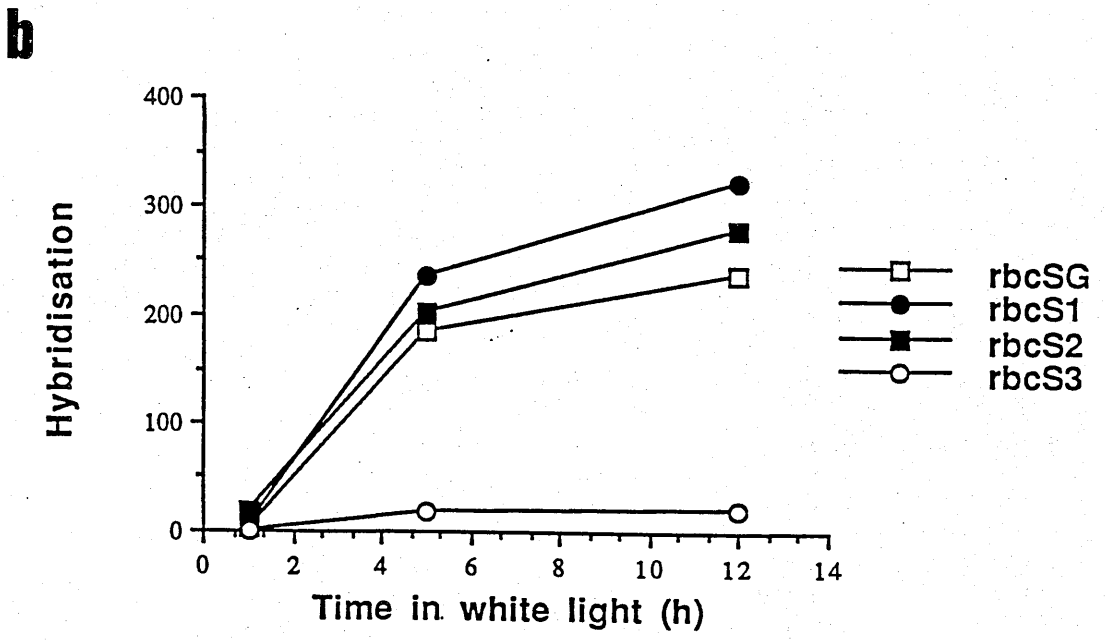


Figure 38 - Hybridisation of oligonucleotide probes to dots of RNA isolated from P. vulgaris primary leaf tissue harvested during a 12h time course in continuous white light after 4 days in the dark.

RNA dot blots (10µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from the primary leaves at least 8 different plants at each time point. Labelling of the oligonucleotide probes, pre-hybridisation and hybridisation of the filters and washing conditions were exactly as described for figure 32.

(a) Autoradiogram of RNA dots after hybridisation to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG.

(b) Plot of relative hybridisation of rbcS1, rbcS2 and rbcS3 and average cpm of rbcSG hybridisation against time in white light (table 22).

TABLE 22 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG to RNA dots isolated from primary leaf tissue harvested from plants after re-illumination with continuous white light.

Experimental conditions were as described in the legend to figure 38.

	rbcSG	rbcS1		rbcS2		rbcS3	
	Average cpm/dot	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.
Time							
1h	**2**	4	**5**	16	**18**	-3	**0**
5h	**184**	167	**235**	184	**202**	9	**18**
12h	**238**	223	**322**	254	**279**	10	**21**

TABLE 23 -Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG to RNA dots isolated from primary leaf tissue harvested after various light treatments.

Dark-grown plants were given the following treatments prior to the harvesting of primary leaf tissue:
L24: 24h illumination with continuous white light; R12, R24, R48 and R96: 3 min illumination with red light followed by 12, 24, 48 and 96h darkness respectively; FR24 3 min illumination with far-red light followed by 24h darkness; R/FR24: 3 min illumination with red light followed by 3 min with far-red light and 24h darkness; D24: 24h darkness.
RNA was extracted, 10µg dots were prepared and these were hybridised to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG as described in the legend to figure 39.

	rbcSG	rbcS1		rbcS2		rbcS3	
	Average cpm/dot	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.	Average cpm/dot	Rel. Hybn.
Treatmt							
L24	**211**	261	**211**	108	**119**	16	**38**
R24	**264**	156	**226**	79	**87**	36	**74**
FR24	**141**	55	**80**	20	**22**	19	**39**
R/FR24	**179**	98	**142**	48	**52**	23	**47**
D24	**104**	59	**85**	17	**19**	23	**47**
R12	**164**	86	**124**	52	**57**	21	**43**
R48	**200**	128	**186**	56	**62**	33	**67**
R96	**58**	24	**35**	7	**7**	12	**25**

greening experiment from the same batch of plants (section 3.6.2.3.1). Table 23 expresses the results as average cpm per dot over background and as relative hybridisation calculated using the relative hybridisation signals of the different oligonucleotide probes (section 3.6.2.2). Figure 39a shows the autoradiograms of the dots after hybridisation. Figure 39b shows a plot of the relative hybridisation against time in darkness following illumination with red light, obtained using the individual oligonucleotide probes. Figure 40 represents the results as percentages relative to the result obtained for the 'L 24' treatment for each individual probe. These figures show that rbcS1, rbcS2 and rbcS3 transcript levels are increased as a result of illumination with red light, and these effects are partially reversible by a subsequent illumination with far-red light. The kinetics of these responses are different in each case. The results obtained using the rbcSG probe were consistent with those obtained using rbcS cDNA probes (section 3.6.1).

3.6.2.4.6 Concentration series

An RNA concentration series from 0-15µg was prepared from a single RNA sample used in the above experiments. The RNA sample used was that of the 48h time point in the greening experiment described in section 3.6.2.4.1. Since the same batch of plants and the same labelled oligonucleotides were used for the experiments described in sections 3.6.2.4.1 to 3.6.2.4.5, this concentration series was valid for each of these experiments. Table 24 gives results as average cpm per dot with background subtracted. Background in this case was taken as the average cpm for dots without RNA (0.00µg), as using cpm obtained with rat liver RNA would have produced negative numbers. Figure 41 shows the autoradiograms of the dots after hybridisation. Figure 42 shows plots of log[average

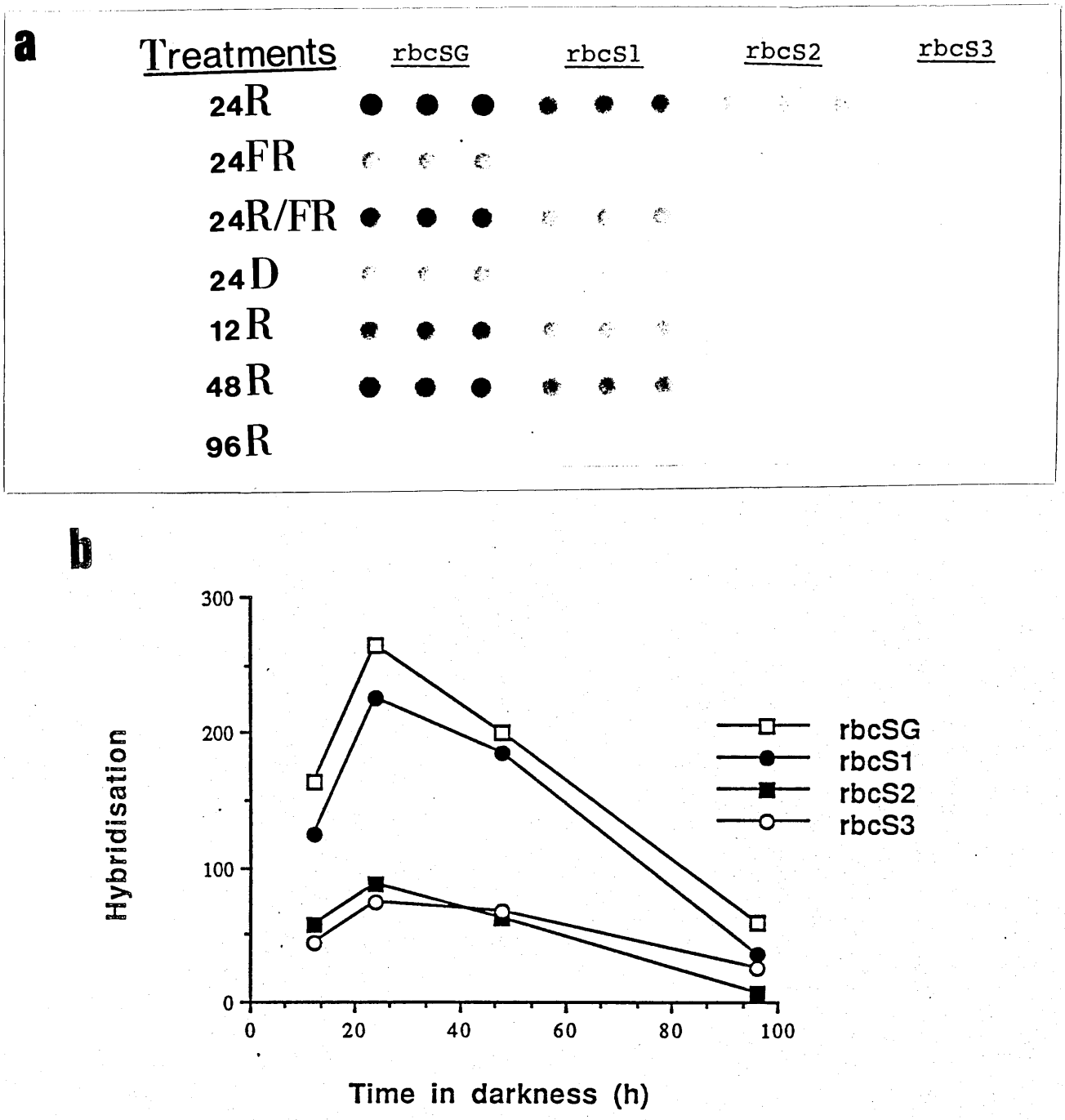


Figure 39 - Hybridisation of oligonucleotide probes to dots of RNA isolated from P. vulgaris primary leaf tissue harvested after different light treatments

Light treatments were as described for table 23. Following harvesting RNA dot blots (10µg) were set up in triplicate for each treatment as in section 2.17.4, RNA having been prepared from at least 8 different plants. Labelling of the oligonucleotide probes, pre-hybridisation and hybridisation of the filters and washing conditions were exactly as described for figure 32.

(a) Autoradiogram of dots after hybridisation to the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG.

(b) Plot of relative hybridisations of rbcS1, rbcS2 and rbcS3 and cpm hybridised for rbcSG against time in darkness after illumination with red light (table 23).

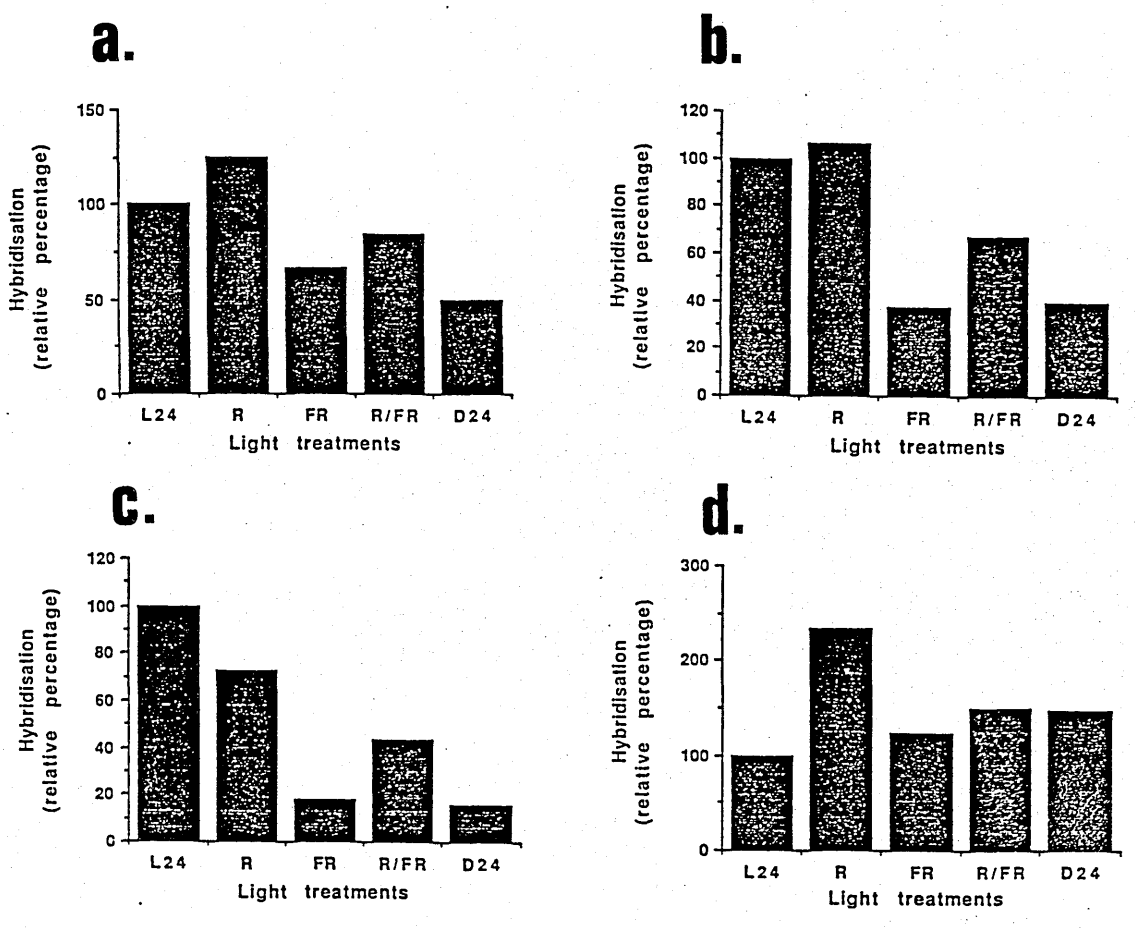


Figure 40 - Phytochrome experiment (see legend to figure 39)
Plots of cpm over background for each light treatment (described for table 23) expressed as percentages relative to cpm obtained from RNA from dark-grown primary leaves illuminated with continuous white light for 24h. Results shown were obtained using the rbcSG probe (a), rbcS1 probe (b), rbcS2 probe (c) and the rbcS3 probe (d).

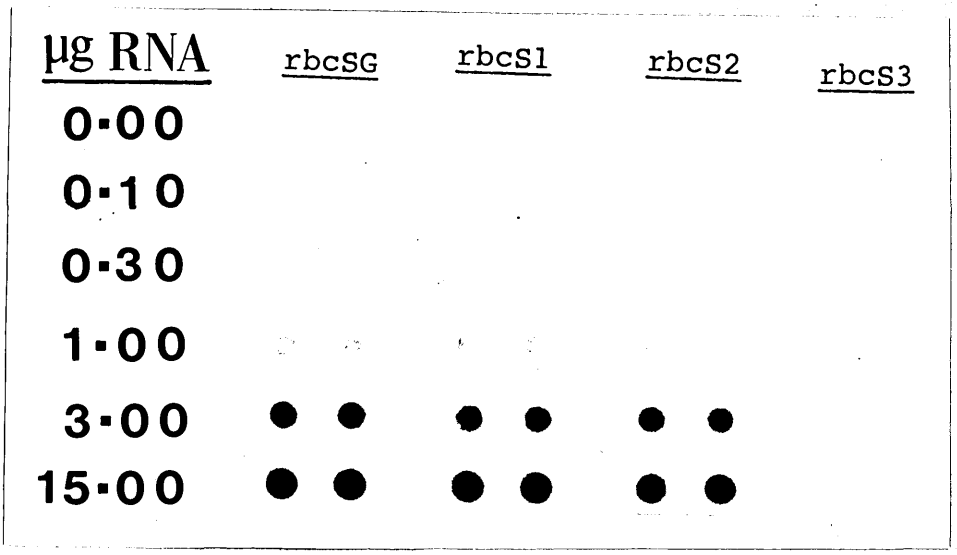


Figure 41 - Hybridisation of oligonucleotide probes to a concentration series of RNA dots.

A series of RNA dots was prepared from the RNA sample of from the 48h point from the greening time course in the same experiment (section 3.6.2.3.1), so as to construct a concentration series over the range 0.0-15.0µg. Hybridisation and washing of filters was exactly as described for figure 32.

Figure shows autoradiograms of dots after hybridisation to the oligonucleotide probes rbcS1 and rbcS2, rbcS3 and rbcSG.

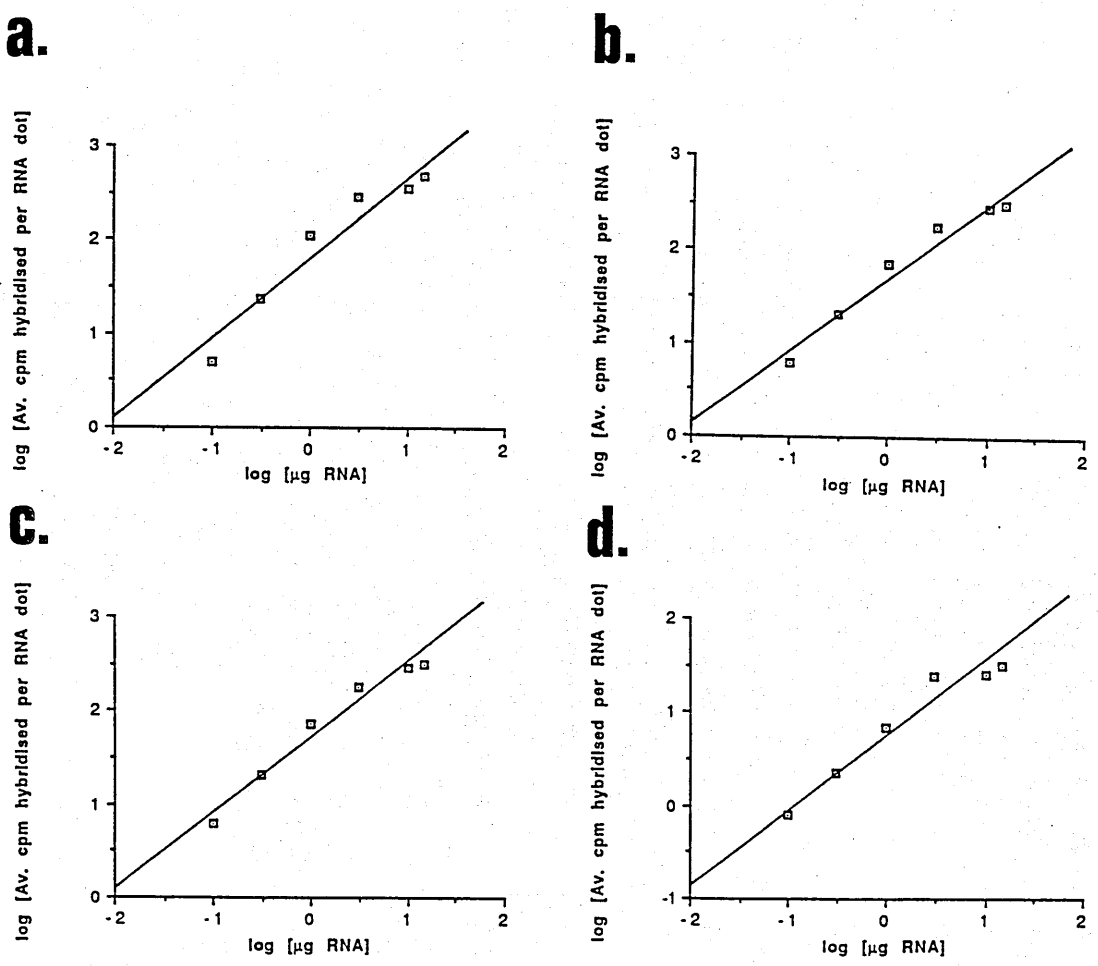


Figure 42 - Hybridisation of oligonucleotide probes to a concentration series of RNA dots.

A series of RNA dots was prepared from the RNA sample of from the 48h point from the greening time course in the same experiment (section 3.6.2.3.1), so as to construct a concentration series over the range 0.0-15.0µg. Hybridisation and washing of filters was exactly as described for figure 32.

Figure shows plot of log[average cpm per dot] against log[amount of RNA in ug] from using the rbcSG (a), rbcS1 (b), rbcS2 (c) and rbcS3 (d) oligonucleotide probes (table 24).

Table 24 - Hybridisation of the oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG to dots containing an increasing series of RNA amount.

Experimental conditions were as described in the legend to figure 41.

ug RNA/dot	PROBES			
	rbcSG	rbcS3	rbcS2	rbcS1
	av.cpm	av.cpm	av.cpm	av.cpm
0.0	0	0	0	0
0.1	5	1	6	8
0.3	23	2	21	20
1.0	112	7	71	64
3.0	290	24	179	229
10.0	349	25	283	270
15.0	475	31	307	384

cpm per dot] against log[amount of RNA in µg] for each individual oligonucleotide probe. These show that a linear relationship was obtained between amount of radioactivity hybridised and amount of RNA applied for all 4 oligonucleotide probes.

4. DISCUSSION

4.1 The structure of rbcS genes in P. vulgaris

Hybridisation of rbcS probes to Southern blots of genomic DNA was carried out to obtain an estimate of the number of copies of rbcS coding region sequences in the genome of P. vulgaris (section 3.1). Analysis of the DNA sequences of the three rbcS cDNA clones, pPvSS1672, pPvSS965 and pPvSS191 (section 3.4), showed that there were no recognition sites for BamH1 and HindIII within the coding regions of the three different types of rbcS cDNA clone identified, nor in the intronic sequences of the rbcS cDNA clone pPvSS1672. This indicates that there are no BamH1 or HindIII recognition sites within the coding regions of the rbcS genes to which these cDNA clones are related. This does not discount the possibility that such sites exist in rbcS intronic sequences not yet determined. Moreover, if additional, uncharacterised rbcS genes are present in P. vulgaris, it is conceivable that such genes could contain BamH1 or HindIII sites within their coding regions.

If it is assumed that no BamH1 or HindIII recognition sites exist in any of the rbcS genes of P. vulgaris, then complete digestion of genomic DNA by either of these two enzymes would produce DNA fragments containing any number of whole rbcS coding regions. A recognition site for EcoR1, on the other hand, was present in all three types of rbcS cDNA clone identified (at a conserved position spanning the codons for the 43rd and 44th amino acid residues of the rbcS mature polypeptide), and again the presence of further EcoR1 recognition sites within rbcS intronic sequences not yet determined cannot be discounted. If it is assumed that all the rbcS genes of P. vulgaris have only one EcoR1 recognition site, and that the position of this site is conserved, then a complete

digest of genomic DNA with EcoR1 would result in the cleavage of all the rbcS coding region sequences into two unequal halves. Hence a particular DNA fragment from EcoR1-digested genomic DNA could contain only part of the full sequence of a particular rbcS gene, and at most it could contain partial sequences from two rbcS genes.
The probes hybridised to the Southern blots of digested genomic DNA were the HindIII-HindIII inserts of pSSU61 and pSSU161 (figure 1), the EcoR1-EcoR1 insert of pPvSS1672 (figure 15) and the oligonucleotide probes rbcSG, rbcS1, rbcS2 and rbcS3 (figure 22). Theoretically all these probes should hybridise to different specific stretches of the rbcS gene sequences. The insert of pSSU61 contains sequence encoding the last 27 amino acids (approx. 80 bp) of the coding region of a pea rbcS gene as well as approximately 250 bp of 3'-untranslated sequence. The insert of pSSU161, on the other hand, contains sequence encoding amino acids 1 to 84 of the mature rbcS polypeptide (approx. 250 bp). In a Southern blot of genomic DNA digested with EcoR1, a probe made from these two inserts would be expected to hybridise to DNA fragments containing either or both halves of a particular rbcS gene cleaved by an internal EcoR1 recognition site. The EcoR1-EcoR1 insert from pPvSS1672 contains sequence from the 44th amino acid 3' downstream to the polyA tail (approx. 700 bp) and would be expected to hybridise only to the downstream half of a particular rbcS gene cleaved by an internal EcoR1 recognition site. The oligonucleotide probe rbcSG would hybridise only to the half of the gene downstream of the conserved EcoR1 site (see figure 16, 17 and 18). Similarly the oligonucleotide probes rbcS1, rbcS2 and rbcS3, would be expected to hybridise to the DNA fragments containing the 3' downstream half of a particular rbcS gene cleaved by an internal EcoR1 recognition site, these probes having been designed to hybridise to specific 3' untranslated

sequences of the three types of rbcS cDNA clones isolated. With BamH1- and HindIII-digested genomic DNA, all the above probes would be expected to hybridise to similarly-sized fragments, assuming that none of the rbcS genes had BamH1 or HindIII recognition sites within their coding regions or intronic sequences.

When the EcoR1-EcoR1 insert from pPvSS1672 was hybridised to genomic DNA digested with EcoR1, three hybridising fragments were detected. Assuming that there is only one EcoR1 site per gene, this would suggest that there are at least three rbcS genes in the genome of P. vulgaris. When the experiment was repeated using a probe made from the combination of the HindIII-HindIII inserts of pSSU61 and pSSU161 a further 3 hybridising fragments were detected, these fragments hybridising less relative to the other three. These 'extra' three hybridising fragments could result from the hybridisation of pSSU61 and pSSU161 to the 5' upstream half of the rbcS genes cleaved at an internal EcoR1 recognition site. The lower intensity of these 'extra' hybridisation fragments could be accounted for by the fact that a shorter stretch of the rbcS coding region was involved in these hybridisation events than with the hybridisation to the downstream halves of the rbcS genes, and also because the homology between the pea and P. vulgaris rbcS sequences is more divergent towards the 5'-end of the coding regions than towards the 3'-end. Thus the result using this probe was consistent with that obtained using the EcoR1-EcoR1 insert of pPvSS1672 as a probe.

When using the EcoR1-EcoR1 insert of pPvSS1672 as a probe one of the three EcoR1-hybridisation fragments hybridised more strongly relative to the other two. However, comparison with the same Southern blot using pSSU61 and pSSU161 as probe shows that the three hybridisation fragments common to the two hybridisations in this case hybridised to very similar extents. Thus it seems

probable that the fragment hybridising most strongly to the EcoR1-EcoR1 insert of pPvSS1672 contains the 3' downstream half of the rbcS gene (cleaved at its internal EcoR1 recognition site) related to the cDNA clone pPvSS1672, the increased hybridisation being due to hybridisation of complementary 3'-untranslated sequences (accounting for an extra 350 bp). Hybridisation of these 3'-untranslated sequences would be more limited in other rbcS genes due to decreased homology (figure 22).

With genomic DNA digested with BamH1, four hybridising fragments were obtained whether the HindIII-HindIII inserts of pSSU61 and pSSU161 or the EcoR1-EcoR1 insert of pPvSS1672 were used as probe. One of these fragments showed a lower level of hybridisation than the other three. If it is assumed that none of the rbcS genes contain a BamH1 recognition site then this would suggest that at least four rbcS genes were present in the genome of P. vulgaris, the fragment with lower intensity corresponding to a fragment possibly containing one rbcS gene and the other three containing two or more rbcS genes. An alternative explanation would be that the three stronger hybridising fragments contained one whole copy of a rbcS gene or a sufficient length of sequence to give a strong signal with the EcoR1-EcoR1 insert of pPvSS1672. The weaker hybridising fragment would then contain part (perhaps only a small fragment) of a rbcS gene which resulted from an uncharacterised BamH1 site. Such a site would most likely be in an intron or, less likely, in the coding sequence of any uncharacterised rbcS gene, and would have to be 3' downstream of the internal EcoR1-site in order for both fragments to hybridise to the EcoR1-EcoR1 insert of pPvSS1672. The latter would seem to be the more probable explanation as comparison between the intensities of the three more intense hybridisation fragments obtained from BamH1-digested genomic DNA with those from EcoR1-digested DNA shows them to be no more

intense and, since at least three of the rbcS genes of P. vulgaris have an internal EcoR1 site, most of the hybridising fragments in the EcoR1-digested DNA should contain only one whole or two partial rbcS sequences.
Of the three fragments hybridising most strongly in the BamH1-digested DNA, one was again stronger than the other two when using the EcoR1-EcoR1 insert of pPvSS1672 as a probe. Once again the three hybridisation fragments showed very similar relative amounts of hybridisation when the HindIII-HindIII inserts of pSSU61 and pSSU161 were used as probe. The higher relative signal obtained for this one fragment when hybridised to the EcoR1 insert of pPvSS1672 could again be accounted for by increased hybridisation to the 3'-untranslated region of the rbcS gene related to pPvSS1672.
With genomic DNA digested with HindIII, the pattern was the same when either pSSU61 and pSSU161 or the EcoR1-EcoR1 insert of pSS1672 was used as probe. Three hybridising fragments were obtained, one of which was substantially weaker than the other two. Again this weaker fragment could have resulted from the hybridisation of a fragment of a rbcS gene containing an uncharacterised HindIII recognition site. Since only 2 other strongly hybridising fragments were observed, one of these HindIII fragments could contain one whole plus a part of another rbcS gene.
The possibilty that the weakly hybridising fragments in BamH1- and HindIII-digested DNA are due to hybridisation to DNA sequences with some homology to rbcS sequences but which are not functional rbcS genes cannot be ruled out.
The rbcS gene-specific oligonucleotide probes rbcS1, rbcS2 and rbcS3 were hybridised to identical Southern blots of digested genomic DNA to assign specific rbcS genes to specific hybridising fragments. The oligonucleotide probe rbcSG was used as a general rbcS probe to confirm the results obtained using the probes produced from rbcS cDNA sequences (as described above) and also to provide a

comparison with the results obtained with the gene-specific oligonucleotide probes rbcS1, rbcS2 and rbcS3.

The oligonucleotide probe rbcSG hybridised to fragments of similar size and intensity in BamH1- and HindIII-digested genomic DNA to those obtained when using rbcS cDNA probes (described above). Fragments were detected in the digests which were in the same relative positions as the weakly hybridising fragments detected when rbcS cDNA probes were used (discussed above). As before, the 8 kbp BamH1 fragment was considerably less intense than the other three in the same track. If this hybridising fragment was due to the hybridisation of rbcSG to a rbcS gene, then the other three hybridising fragments in BamH1-digested DNA must contain more than one copy of a rbcS coding region. This would mean that the genome of P. vulgaris contains at least 7 rbcS coding region sequences. On the other hand, this hybridising fragment may have been produced as a result of the hybridisation to non-rbcS sequences with sufficient homology to allow hybridisation under these conditions. In this latter case the similarity in size between this and the fragment detected using rbcS cDNA probes would be coincidental. The presence of a larger number (at least 7) of rbcS genes seems an unlikely explanation because the relative amounts of hybridisation to DNA fragments in BamH1-digested DNA was no greater than to those in EcoR1- and HindIII-digested DNA. Hence these results are consistent with a minimum of three rbcS coding region sequences in the genome of P. vulgaris and a much greater number seems unlikely.

The lack of hybridisation of rbcSG to a fragment in HindIII-digested genomic DNA in the same relative position to the most weakly hybridising fragment detected using the rbcS cDNA probes indicates that this fragment does not contain a region of sequence homologous to rbcSG. If this fragment contained part of a rbcS coding region sequence

cleaved at an internal HindIII recognition site as described above, then this fragment must contain a region 5' upstream to the site to which rbcSG would be expected to hybridise. The fact that only 2 strongly hybridising bands are observed supports the notion that one of these HindIII fragments may contain part or all of two rbcS genes. EcoR1-digested DNA resulted in the production of 3 of the hybridising fragments corresponding to those obtained in blots using rbcS cDNA probes. The other two main hybridising fragments observed may have been due to non-specific hybridisation as discussed below.

When P. vulgaris genomic DNA digested with BamH1, EcoR1 and HindIII was hybridised to the oligonucleotide probes rbcS1, rbcS2 and rbcs3, more than one main hybridisation fragment was obtained in all but one case. Main hybridisation fragments which did not correspond in size to those obtained with rbcSG could have been produced as a result of the presence of 21bp sequences homologous to the oligonucleotide probes rbcS1, rbcS2 or rbcS3 in the genomic DNA which were not within a rbcS gene. On the other hand, the stringency at which these blots were washed was relatively low (see section 4.3), so it is possible that these extra hybridisation fragments were due to the hybridisation of the probes to 21bp sequences with one or more nucleotide mismatches. Hybridisation fragments of a lower intensity than these main ones could have resulted by the hybridisation of the oligonucleotide probes to 21bp sequences with increasing amounts of mismatches, still detected due to the relatively low stringency at which the blots were washed.

When the oligoncleotide probe rbcS1 was hybridised to BamH1- and HindIII-digested genomic DNA, one main hybridisation fragment was observed in each track corresponding to a hybridisation fragment obtained with the oligonucleotide probe rbsG. In neither case was the relative hybridisation to these fragments greater than to

the other fragments in the same track. These fragments were of approximate size 25 kbp in HindIII-digested genomic DNA and 12 kbp in BamH1-digested genomic DNA. This suggests that the sequence of the rbcS gene related to pPvSS1672 may be contained in these fragments. As these hybridisation fragments were not the most strongly hybridising in each case this conclusion has to be made tentatively. However, the correlation of the 12 kbp BamH1 fragment to pPvSS1672 is consistent with the results obtained using the EcoR1-EcoR1 insert of pPvSS1672 as probe as discussed above. In the case of EcoR1-digested genomic DNA hybridised to the same probe, one fragment hybridised far more strongly than any of the others in the same track. As the size of this fragment did not correspond to any obtained with EcoR1-digested DNA hybridised to rbcSG and as the amount of hybridisation of this fragment was far greater than any of those obtained from the hybridisation of BamH1- or HindIII-digested genomic DNA to the same probe, it is possible that this hybridisation fragment contained repeated sequences of sufficient homology to rbcS1 to allow hybridisation under these conditions. It was not possible to assign a particular hybridisation fragment to the rbcS gene related to pPvSS1672 in this case.

When the oligonucleotide probe rbcS2 was hybridised to either BamH1-, EcoR1- or HindIII-digested genomic DNA, several main hybridisation fragments were obtained in each case. However, one hybridisation fragment corresponding in size to one obtained using rbcSG was detected in genomic DNA digested with each individual enzyme. In this case the relative hybridisation to each of these hybridisation fragments was greater than to other hybridising fragments in the same track. Thus it seems likely that the sequence of the rbcS gene related to pPvSS965 is located on a fragment of approximate size 25 kbp in both BamH1- and HindIII-digested genomic DNA and on

a fragment of approximate size 1.1 kbp in EcoR1-digested genomic DNA. The situation was the same when the oligonucleotide rbcS3 was used. The sequence of the rbcS gene which is related to pPvSS191 is likely to be located on a fragment of approximate size 28 kbp in BamH1-digested genomic DNA, 5.0 kbp in HindIII-digested genomic DNA and 30 kbp on EcoR1-digested genomic DNA. Some caution has to be exercised in drawing these conclusions because the sizing of DNA fragments above 5 kbp was approximate, particularly with larger fragments.

Thus the results obtained from the use of the rbcS gene-specific oligonucleotide probes in conjunction with the general rbcS oligonucleotide probe rbcSG suggest that, in all but one case, the genes related to pPvSS1672, pPvSS965 and pPvSS191 were contained within different fragments of genomic DNA resulting from digestion with BamH1 and HindIII. The exception to this is a fragment of approximate size 25 kbp in HindIII-digested genomic DNA which apparently contains the sequences of the rbcS genes related to pPvSS1672 and pPvSS965. As the hybridisation to this fragment relative to the one of approximate size 5.0 kbp is very similar, this would suggest that this second hybridisation fragment may also contain 2 rbcS genes. Hybridisation to rbcS3 suggests that the rbcS gene related to pPvSS191 is one of these. If this is the case then this hybridisation fragment may also contain another as yet unidentified rbcS gene. This would mean that the rbcS multigene family in P. vulgaris contains at least 4 members. However, due to the factors discussed above (stringency, possible non-rbcS hybridising sequences etc.), this cannot be held as a firm conclusion. An improvement of the washing conditions for the oligonucleotide probes is necessary to resolve this question and additional restriction enzymes could be used. Nevertheless, it is clear that the rbcS multigene family contains more than one gene. The weight of evidence

favours 3, though this should be considered as a minimum value and the presence of additional genes cannot be ruled out. Questions of linkage and the presence of psuedogenes are yet to be resolved.

Having ascertained that there was more than one rbcS gene in P. vulgaris, a study of their individual expression was undertaken (discussed in section 4.2). The need for gene-specific probes required the determination of the nucleotide sequence of different rbcS cDNA clones. These clones were identified by screening many rbcS cDNA clones to obtain representatives of different P. vulgaris rbcS genes. Several different methods were used for this screening. First of all restriction mapping was used in an attempt to divide several rbcS cDNA clones into different restriction groups (section 3.3.1). These results showed that the rbcS cDNA clones pPvSS328, pPvSS381, pPvSS386, pPvSS1451, pPvSS2314 and pPvSS1672 showed similarities in their restriction patterns, notably they all had a fragment of approximate size 230bp after digestion with Alu1. As the partial nucleotide sequence of pPvSS1672 had been determined prior to this analysis, a 230 bp Alu1-Alu1 DNA fragment was expected from the digestion of rbcS cDNA clones of the same type as pPvSS1672. Thus pPvSS328, pPvSS381, pPvSS386, pPvSS1451 and pPvSS2314 were provisionally classed as being of the same type of rbcS cDNA clone as pPvSS1672. This result was subsequently confirmed by 'A-track' sequencing analysis, discussed below. However, pPvSS1672 was the only rbcS cDNA clone for which sequence information was available prior to screening, which made the classification of other different rbcS cDNA clones from restriction analysis difficult. Thus, even though pPvSS814, pPvSS1066, pPvSS1380, pPvSS1483, pPvSS2284 and pPvSS2285 had an apparently conserved fragment size of 500 bp after digestion with Alu1, subsequent 'A-track' sequencing analysis showed that amongst these 6 rbcS cDNA

clones there were representatives of three different types of rbcS cDNA clone. The high degree of homology of nucleotide sequence between the coding regions of rbcS genes in the same family means that even different rbcS cDNA clones have very similar restriction patterns. Added to this the rbcS cDNA clones had variably-sized G-C tails, the portion of sequence representing the polyA tail varied in length between clones of the same type (determined by 'A-track' analysis) and not all of the cDNA clones were full-length. This means that any restriction fragment which is not produced from two separate restriction sites from within the cDNA insert is subject to be of variable size between rbcS cDNA clones of same type. Thus it was concluded that restriction analysis would be of very limited use for the screening of rbcS cDNA clones.

The second method used to screen the rbcS cDNA clones was by S1 nuclease analysis. Once S1 nuclease analysis had been carried out on the total RNA of P. vulgaris (discussed in section 4.2), the transcripts of different rbcS genes were identified from their protection fragments. The sizes of these protected fragments were characteristic of a given rbcS transcript. In theory then, if the same sequences which were used as a probe in the analysis of total RNA were used as a probe in an S1 nuclease analysis of the rbcS cDNA clones, protection fragments of the same sizes as obtained with total RNA should be obtained. In this way a cDNA clone could be directly matched to a particular rbcS transcript. The first method attempted to obtain S1-protected fragments from cDNA clones was identical to the S1 nuclease analysis of RNA. However this was not successful, most probably due to the fact that this method had been optimised for the hybridisation of single-stranded DNA probe and single-stranded test RNA, whereas the plasmid DNA from the rbcS cDNA clone in this assay was double-stranded. Under these conditions the plasmid DNA would be more likely to

re-anneal than to hybridise to the probe, explaining why no detectable protected fragments were obtained.
The second method used involved the hybridisation of the rbcS cDNA clones in single-stranded form to the probe sequence also in single-stranded form. This was acheived by inserting both the probe sequence and the test rbcS cDNA clone sequence into M13 vectors and subsequently preparing single-stranded templates from these ligated DNA molecules. The probe insert and rbcS cDNA clone insert were orientated within the vectors in such a way that when the single-stranded templates were made the sequences of both inserts would be complementary to each other. When the BamH1-HindIII insert of pPvSS1672 was hybridised to the BamH1-EcoR1 insert of pBS1 a protection fragment of approximately 220 bp was obtained. This was very close to the 225 bp protection fragment expected from the analysis of nucleotide sequence data. When the BamH1-EcoR1 insert of pBS1 was hybridised to itself a protection fragment of approximate size 298 bp was obtained. This was close to the 285 bp protection fragment expected (due to the hybridisation of 225 bp of rbcS sequence + 60 bp of complementary M13mp18/mp19 polylinker sequence). As this method was successful it was used to analyse the completely uncharacterised rbcS cDNA clones pPvSS636, pPvSS1483, pPvSS2284 and pPvSS2227, along with the characterised rbcS cDNA clone pPvSS1672 as a control. Once again pPvSS1672 gave a protected fragment whose size was close to the expected 225 bp, but also a second protection fragment approximately 40 bp shorter. This illustrates the problems caused by the production of spurious protection fragments as a result of S1 nuclease analysis (discussed in detail in section 4.2). Of the other 4 rbcS cDNA clones pPvSS636 and pPvSS1483 gave protection fragments near to the 131 bp expected with the hindsight of 'A-track' analysis and nucleotide sequence data. On the other hand pPvSS2284 and pPvSS2227, which

should also have been expected to produce protected fragments of 131 bp, did not produce detectable protection fragments. Thus it was concluded that this method would be of limited use for the screening of rbcS cDNA clones. Firstly this method was inconsistent, secondly the gel system used to run the protected fragments would not enable the detection of small differences in their sizes and, finally, as more than one type of rbcS cDNA clone could have the same size of protection fragment (as is bourne out by the subsequent sequence data analysis) this method would not have been able to discriminate between them.

The final method used to screen the rbcS cDNA clones was 'A-track analysis'. This method overcame the disadvantages encountered using the other two methods discussed above. At the stage when this method was used partial nucleotide sequence data was available for pPvSS1672. The oligonucleotide sequencing primer no. 360 was designed from 18 bp of the coding region of this rbcS cDNA clone (section 3.4). The region from which this oligonucleotide primer was designed encodes the 71^{st} to the 76^{th} amino acids of the mature rbcS polypeptide. This region was chosen as the sequence of these amino acids is completely conserved in 7 plant species with which the comparison was made (figure 21), meaning that there was a high probability that the sequence of the oligonucleotide primer would be conserved between the different rbcS genes of P. vulgaris. This would allow the use of the primer for the 'A-track' analysis of any of the rbcS cDNA clones from P. vulgaris. The primer was designed to prime the sequencing synthesis reaction from the coding sequence through to the 3'-untranslated sequences of the rbcS cDNA clones. An additional check was made to ensure that the oligonucleotide primer had limited homology with the sequence of the M13 vector, so as to ensure that it would initiate the sequencing reaction at the single, defined

intended site.

A-track analysis of 42 rbcS cDNA clones identified 3 different types. The different types, 'type 1', 'type 2' and 'type 3', accounted for approximately 55, 36 and 9% of rbcS cDNA clones in this particular cDNA library respectively.

This analysis also allowed the detection of two different polyadenylation sites in both the 'type 2' and 'type 3' rbcS cDNA clones. In both these cases the rbcS cDNA clones with polyA tails starting further downstream were relatively more abundant. The sequences for polyadenylation signals are usually found between 11 and 30 bp of the start of the polyA tail itself (Fitzgerald et al., 1981). pPvSS1672 had the hexanucleotide sequence 'AATAAT' 18 bp upstream from the start of its polyA tail which most closely resembles the eukaryotic 'AAUAAA' consensus sequence (Montell et al., 1983). The other two rbcS cDNA clones pPvSS965 and pPvSS191 had 2 and 1 copies of the hexanucleotide sequence 'AATGAA' respectively. These are identical in sequence to the polyadenylation signals of 4 of the 5 pea rbcS genes described by Fluhr et al. (1986b). However, whereas these signals in pea were all at a conserved distance from the start of polyadenylation, this was not the case in P. vulgaris. The two polyadenylation signals in pPvSS965 were 12 bp and 24 bp upstream of the two polyadenylation sites respectively. The polyadenylation signal in pPvSS191 was within 3 bp of the second polyadenylation site. No possible polyadenylation signal for the first polyadenylation site of pPvSS191 was detected.

Once the three types of rbcS cDNA clone were identified, three rbcS cDNA clones (pPvSS1672, pPvSS965, pPvSS191) were chosen as representatives of each type and their nucleotide sequences were determined. All three types showed absolute 100% nucleotide sequence homology in the coding regions of the mature rbcS polypeptide. This 100%

homology extended into the coding regions of the transit peptides whose full sequences were present in pPvSS1672 and pPvSS191 and partial sequence in pPvSS965. Such a high degree of homology has only been reported in tomato (Sugita and Gruissem, 1987a) where the nucleotide sequence of all three exons and two of the introns of two separate rbcS genes showed 100% homology. The homology was very much lower in the 3'-untranslated sequences of all three rbcS cDNA clones as well as in the 5'-untranslated sequences present in pPvSS1672 and pPvSS191. The differences in the nucleotide sequences of these non-coding regions confirmed that these three rbcS cDNA clones were related to three separate rbcS genes.

The rbcS cDNA clone pPvSS1672 had short intronic sequences at two sites. The position of these two sites is conserved in all dicot and monocot species so far studied (section 1.2.2). The possibilty that other 'type 1' rbcS cDNA clones in the cDNA library also have introns cannot be discounted as restriction analysis (section 3.3.1) shows that clones like pPvSS328 and pPvSS381 would be long enough to contain them. However the variability of the G-C tails and polyA tails of these clones does not allow a firm conclusion. The Alu1 fragment containing the 5' end of the gene up to after the second intron is approximately 700 bp in pPvSS1672. pPvSS328, pPvSS381 and pPvSS1451 also had restriction fragments of around this size and so could possibly contain sequences for two introns. The presence of introns in so many cDNA clones brings into question whether sequences involved in splicing of this rbcS gene are aberrant. However comparison of the sequences around the splice sites (section 3.4) of the introns of pPvSS1672 and those from soybean (Berry-Lowe et al., 1982) and also eukaryotic consensus sequences do not indicate any major differences.

Comparison of the deduced amino acid sequence of the mature rbcS polypeptide with 7 other plant species shows

that it is highly conserved (figure 21). The sequences show the most homology to soybean (the most closely related of the species chosen for comparison) with 86% conservation of residues. Pea (another legume) shows the second highest homology, followed by other dicots petunia and tobacco. Surprisingly the sequence from P. vulgaris shows more homology to Lemna gibba (a monocot) than to spinach (another dicot). The high degree of similarity of Lemna rbcS amino acid sequence to soybean has been reported by Stiekema et al. (1983), which is consistent with this result. Wheat (a monocot) shows the least homology with 69% conservation of amino acid residues.

The most marked divergence in the amino acid sequence of P. vulgaris rbcS mature polypeptide relative to the other species is at the N-terminal methionine which becomes isoleucine in P. vulgaris. This involves a single nucleotide base change of ATG to ATT. In all other species studied the first amino acid of the mature rbcS polypeptide is invariably a methionine. This is the first amino acid after the cleavage site of the transit peptide during transport of rbcS precursor into chloroplasts. Broglie et al. (1983) point out that as the amino acids proximal to this cleavage site in both the mature and transit peptides are conserved, they may be involved in the cleavage process itself. Assuming that any of the genes related to the three rbcS cDNA clones identified in P. vulgaris produce functional polypeptide which is processed normally, this would imply that the first amino acid of the mature polypeptide does not need to be methionine for correct processing. However, similarity between methionine and isoleucine does not negate the involvement of either of these amino acids in processing. Van den Broek et al. (1985) found that the fusion of rbcS transit peptide sequences to bacterial sequences allowed proper targetting and processing. This suggests that the amino acids of the transit peptide are sufficient for

correct targetting and processing. However, the first amino acid of the bacterial polypeptide used in this experiment was a methionine, which does not clarifiy the involvement of the first amino acid of the mature polypeptide. These same workers suggest that the processing is specific to the Cys-Met site between the transit and mature polypeptides of rbcS. In the case the of P. vulgaris rbcS precursor, the enzyme involved in processing would need to be able to recognise a Cys-Ile site.

4.2 The expression of rbcS genes in P. vulgaris

The expression of rbcS genes in P. vulgaris was monitored by the measurement of the steady-state levels of rbcS transcripts. Total rbcS transcript levels were measured by RNA dot blot analysis. The levels of rbcS transcripts from individual rbcS genes were measured by S1 nuclease analysis and RNA dot blot analysis of total RNA using oligonucleotide probes. A critical examination of these two methods is presented in section 4.3.

The levels of total rbcS transcripts present in the primary leaves and some other tissues of P. vulgaris were estimated by the hybridisation of probes of rbcS cDNA clones from both pea and P. vulgaris to dot blots of P. vulgaris total RNA. When six day-old dark-grown seedlings were exposed to white light over 48h an approximate 11-fold increase was detected using the pea rbcS probe and an approximate 6-fold increase was seen using the P. vulgaris rbcS probe. As well as this difference in the extent of accumulation after 48h exposure to white light, observed with the different probes, the kinetics of these increases were different. The levels of rbcS transcripts showed a marked transient increase between 0-12h when the P. vulgaris rbcS probe was used to estimate them. This transient increase was not detected when pea rbcS was used

as probe. The levels of rbcS transcripts did show a greater rate of accumulation in the first 3h than at any other time in the time-course using the pea rbcS probe, but it was not as marked as with the P. vulgaris rbcS probe. Clearly, though, both probes indicated that the initial accumulation of rbcS transcripts in this time-course was relatively more rapid than later on. It is possible that the transient increase varied between the different experiments, as occurs in pea (Ellis et al., 1984). In this case the variable transient increase was attributed to unstable transcripts produced by pre-engaged RNA polymerases stimulated by light to proceed with RNA synthesis. Subsequent accumulation of rbcS transcripts, in this case, is attributed to the re-initiation of the RNA polymerases again stimulated by light. This process produces a slower rate of accumulation of rbcS transcripts with higher stability. It is possible that similar mechanisms are responsible for the light-mediated increases in rbcS transcripts in P. vulgaris.

Both cDNA probes indicated that the level of rbcS transcripts levelled-off between 24-48h. This is in contrast to species such as pea where accumulation of rbcS transcripts continues steadily up to 48h (Gallagher et al., 1985). Since the amount of white light has been shown to be the most important factor in determining the level of rbcS transcripts in P. vulgaris (Jenkins, 1986), it seems possible that the levels acheived were the maximum possible under these irradiance conditions.

The steady-state levels of rbcS transcripts at 0h (ie. without illumination with white light) were 9.0 and 17.8% of those at 48h using the pea and P. vulgaris probes to estimate them respectively. These relative levels were substantially higher than those reported for some other species, such as pea (Jenkins et al., 1983) where levels of around 1% would be expected under the same conditions. This indicates that the light-requirement for expression

of rbcS genes in P. vulgaris is not as strict as in some other species. On the other hand, in species such as barley (Batschauer et al., 1986) and cucumber (Greenland et al., 1987), photoregulation of rbcS genes is minimal. The levels of rbcS transcripts in tissues such as stems and roots were between 5-10% of those in light-grown primary leaves. These levels are higher than in other species such as pea (Coruzzi et al., 1984), but similar to those in tomato (Sugita and Gruissem, 1987). This suggests that the tissue-specific component of the regulation of rbcS gene expression is either less important in P. vulgaris and tomato than in some other species, or that control of expression in non-leaf tissue by tissue-specific factors (eg. plastid factors) allows a relatively high level of rbcS expression in these tissues. If plastid factors were involved, this would imply that the plastids in non-leaf tissues of P. vulgaris and tomato are more developed than in species such as pea so as to allow the production of these factors (see section 1.3.1.3).

A brief illumination of dark-grown seedlings with red light produced a considerable accumulation of rbcS transcripts in the primary leaves. After illumination and transfer to the dark for 24h, levels of rbcS transcripts were estimated at 1.8 (pea rbcS probe) and 1.4 (P. vulgaris rbcS probe) times greater than those in plants illuminated with continuous white light for 24h. This degree of stimulation was much higher than that obtained from pea seedlings under the same conditions. The level of rbcS transcripts in pea following red-illumination only reaches 10% of that reached after exposure to white light for 24h (Jenkins, 1986). In pea, red light induces two effects in etiolated seedlings. Firstly it stimulates the small accumulation of rbcS transcripts described above, and secondly, it renders the seedlings competent to respond to subsequent light treatments. This competence

is required for maximal expression in white light (Barnett et al., 1987; Jenkins, 1988). Since in P. vulgaris red light stimulates the accumulation of rbcS transcripts to levels equivalent to those obtained in white light, it seems likely that P. vulgaris etiolated seedlings do not require phytochrome-mediated competence to allow maximal rbcS expression. This might also explain the more rapid accumulation of rbcS transcripts during greening of etiolated P. vulgaris seedlings with respect to pea. Again a plastid factor may be involved in these different responses. If it were assumed that a plastid factor were required for maximal rbcS expression, it is possible that in pea the red light-mediated competence effect may occur by stimulating plastid development to a stage when it is able to produce plastid factor and subsequent maximal rbcS gene expression in light conditions. By the same argument it would have to be assumed that the plastids in dark-grown P. vulgaris are sufficiently developed to provide plastid factor after a single illumination with red light.

The effect of red-light on the accumulation of rbcS transcripts was partially reversed by a subsequent brief illumination with far-red light, reducing levels to 17% (pea rbcS probe) and 57% (P. vulgaris probe) of those obtained with red-light alone. Plants illuminated only with far-red light produced levels of around 16% (pea rbcS probe) and 36% (P. vulgaris rbcS probe) of those obtained with red-light. Again there were substantial differences between the experiments using the two different probes, but they were consisitent enough to deduce that red-light, acting via phytochrome, was capable of inducing the accumulation of rbcS transcripts to levels at least equivalent to those obtained from illumination with continous white light over the same time-scale. This suggests that phytochrome is the most important

photoreceptor involved in the control of the light-mediated accumulation of rbcS transcripts in young de-etiolating seedlings of P. vulgaris. As the levels of rbcS transcripts in the green primary leaves of P. vulgaris plants are controlled primarily by the fluence rate of white light (Jenkins, 1986), the importance of phytochrome must decrease, or at least its role must change, as the leaves and plastids develop.

As discussed above, there were differences in the results obtained from similar experiments using cDNA probes from pea and P. vulgaris. One reason for these differences might be that as the P. vulgaris probe used contained substantial 3'-untranslated sequences (350 bp of the total 700 bp) and that hybridisation and washing of filters where this probe had been used was at relatively high stringency. This would have the effect of producing results biased to the type of rbcS transcripts corresponding to pPvSS1672 from which the probe was made. Figure 22 shows that the homology of the 3'-untranslated regions of three rbcS genes of P. vulgaris is very much lower than in the coding region. This suggests that the probe would only hybridise substantially to the 350 bp or so of coding region sequence in transcripts not cognate to pPvSS1672. On the other hand, transcripts of 'type 1' (cognate to pPvSS1672) would hybridise to the full 700 bp of the probe, thus creating a biased result. The pea probe used had similar homologies to the 3'-untranslated regions of all three rbcS cDNA clones and so may have served as a more reliable indicator of total rbcS transcript levels.

Further estimation of the levels of total rbcS transcripts was made using the oligonucleotide probe rbcSG, which was designed to be a general rbcS probe. The results obtained with this probe, however, were not in complete agreement with those obtained using the rbcS cDNA probes. For instance, when a greening time-course was carried out the

level of total rbcS mRNA at time 0h was estimated as being 30% of that detected at 48h (table 19). This was a relatively greater figure than obtained with either of the rbcS cDNA probes. Furthermore this particular time-course was performed at fluence rate twice as high as that used in the experiments with the two cDNA probes. The effect of the higher fluence rate can be seen when comparing levels of rbcS transcripts at 24h and 48h with rbcSG as probe, the level of rbcS transcripts at 48h of illumination in white light is 1.5 times greater than at 24h, contrasting the situation described above when there was relatively little difference between the two. This is in agreement with the hypothesis that the irradiance level becomes the most important factor determining rbcS transcript levels in mature, green P. vulgaris primary leaves. Thus, there was probably a relatively higher level of rbcS transcripts at 48h in the expreriment at the higher fluence rate (using oligonucleotide probes) than in that at the lower fluence rate (using cDNA probes). However, since the level of rbcS transcripts at time 0h should have been relatively constant between the different experiments, then a much lower percentage, relative to 48h in white light, than obtained with the rbcS cDNA probes would have been expected using the rbcSG probe. This clearly was not the case, the level being much greater. The reasons how these results may have arisen are discussed in section 4.3.

The effect of red and far-red light was also studied using the rbcSG probe. After a short illumination with red light and transfer to the dark for 24h, levels of rbcS transcripts were estimated at 1.3 times higher than those in plants illuminated with continuous white light for 24h. The effect of red-light on the accumulation of rbcS transcripts was partially reversed by a subsequent brief illumination with far-red light, reducing levels to 68% of those obtained with red-light alone. Plants illuminated

only with far-red light produced levels of around 53% of those obtained with red-light. Once again there were discrepencies between the results from this experiment and those performed with the rbcS cDNA probes, even though the conditions, as far as could be determined, were identical. The possible reasons for these discrepencies are discussed in section 4.3.

In addition to the above experiments, the oligonucleotide probe rbcSG was used to estimate levels of rbcS transcripts during the development of seedlings in the dark, after transfer of light- grown seedlings to darkness and after their subsequent re-illumination. The kinetics of the red-light induction of rbcS transcript accumulation were also studied. Levels of rbcS transcripts in dark-grown seedlings at 5, 7, 11 and 15 days after germination were 40, 26, 13 and 3.5% respectively of those obtained after 48h illumination with white light. As levels of rbcS transcripts in dark-grown P. vulgaris varied with time it is possible that rbcS transcript aacumulation is controlled by developmental factors aside from the external influence of light. It is possible that these levels represent a transient increase in the level of rbcS transcripts prior to 5 days, possibly in readiness for the emergence of the primary leaves through the soil.

When plants illuminated for 48h in continuous white light were transferred to the dark, the relative levels of rbcS transcripts after 0, 12, 24, 48 and 96h were 100, 103, 71, 34 and 8%. This decline could be due to the combination of RNA degradation and of decreased rbcS gene transcription. If it is assumed that RNA degradation proceeds at a similar rate in darkness as in the light, these results indicate that there is a lag time of at least 12h when the level of transcription is little affected by transfer to darkness. However, if transcription stops promptly upon transfer to darkness, as it does in pea (Gallagher et al., 1985), then rbcS

transcript degradation would appear to be significant only after 12h in the dark. It is not possible to distinguish between these two possibilities without a direct study of rbcS transcription by performing, for instance, an in vitro transcription assay on isolated P. vulgaris nuclei. Subsequent re-illumination of these 'dark-adapted' plants produced a rapid accumulation of total rbcS transcripts. After 12h re-illumination levels were as high as in plants exposed for 24h in the same light during initial greening of the de-etiolated seedlings. Such rapid rises in rbcS transcript levels upon re-illumination have been reported in pea (Fluhr and Chua, 1986).

Levels of rbcS transcripts increased between 0-24h in darkness after a brief illumination with red light. These levels subsequently declined between 48-96h. This decline was relatively less rapid than that detected when light-grown seedlings were transferred to darkness. This is similar to the situation in pea (Barnett et al., 1987). These results suggest that there are differences in either or both transcriptional rates and RNA stability in white- and red-light illuminated primary leaves after transfer to darkness. The maximum level of rbcS transcripts after illumination with red light was presumably reached between 24-48h.

The levels of the transcripts of individual rbcS genes present in the primary leaves of P. vulgaris were estimated by S1 nuclease analysis of total RNA and by the hybridisation of the gene-specific oligonucleotide probes rbcS1, rbcS2 and rbcS3 to dot blots of total RNA. The relative merits of these two methods are discussed in section 4.3.

S1 nuclease analysis was performed on total RNA from light- and dark-grown primary leaves using a subclone of pPvSS1672 (pBS1) as probe. Four protected fragments were obtained in each case. The sizes of these fragments were the same in both cases. This would initially suggest that

at least 4 rbcS genes are expressed in both the light and the dark. However, if the number of the different rbcS cDNA clones appearing in the cDNA library is taken as an indication of the number of rbcS genes expressed in light-grown primary leaves, only three genes would appear to be expressed. Of the three types of rbcS cDNA clones, transcripts from 'type 1' would be expected to produce a protected fragment of 225 bp and the transcripts of 'type 2' and 'type 3' would both be expected to both produce protected fragments of 131 bp. These theoretical protection fragment sizes most closely match up to the experimental protection fragment sizes of 230 and 125 bp, leaving the protected fragments with sizes 220 and 115 bp unaccounted for. The relative abundance of the protected fragment of size 220 bp was similar to that of 230 bp in light-grown RNA. Thus, if the protected fragment of size 220 bp had resulted from the hybridisation of a distinct rbcS transcript, cDNA produced from it should be present in the cDNA library to a similar frequency as 'type 1' rbcS cDNA clones. However, even though 'type 1' rbcS cDNA clones represent 55% of the 42 rbcS cDNA clones analysed, no representatives of a cDNA corresponding to a transcript which would produce a protected fragment of 220 bp was isolated. Similarly the protected fragment with size 125 bp theoretically arose from the hybridisation of both 'type 2' and 'type 3' rbcS transcripts which together accounted for 45% of the 42 rbcS cDNAs analysed. Although the protected fragment of size 115 bp was present at a slightly lower abundance than that of 125 bp, representatives would still have been expected to have been detected in the cDNA library if it had resulted from the hybridisation of a distinct rbcS transcript. Thus it seems likely that the protected fragments of sizes 220 and 115 bp were artifacts resulting from the hybridisation to the three already characterised rbcS transcripts. DNA-RNA hybrids are known to be suceptible to S1 nuclease

at A-T rich regions, especially near the end of the duplexes, where 'breathing' can relax the double-stranded nature of the duplexes leading to artifacts (Dr. M. Evans, University of Durham, personal communication). Fluhr et al., 1986b describe such artifacts using a similar system to measure rbcS transcript levels in pea. The nucleotide sequence data for the rbcS cDNA clones pPvSS965 and pPvSS191 show that there is a 7bp A-T rich region starting 11 bp upstream from the theoretical 3'-untranslated end of the duplex. If this region were susceptible to attack by S1 nuclease then a protected fragment of approximately 120 bp would be produced, correlating well with the 115 bp fragment size observed in this experiment. However no A-T rich regions are found near to 'either' end of the theoretical duplex produced from 'type 1' transcripts (from the sequence data of pPvSS1672). The presence of A-T rich regions might not therefore be involved in the appearance of the 220 bp protected fragment. However, it was noted that when the digestion with S1 nuclease was performed at 37°C rather than at room temperature, the ratio of the 220bp protected fragment to the 230 bp protected fragment increased, suggesting that the artifact was somehow dependant on the incubation temperature. This observation 'does not' therefore rule out the involvement of the 'breathing' of DNA-RNA hybrids.

It was concluded that despite having certain advantages (described in section 4.3), S1 nuclease analysis was not particularly suitable for studying the accumulations of the different rbcS transcripts. No single probe could be designed from any of the three rbcS cDNA nucleotide sequences which would produce three separate protected fragments of different sizes from each separate type of rbcS transcript. The homologies of the 3'-untranslated regions of the three rbcS cDNA clones were such that any two rbcS transcript types which were not cognate to the probe used would produce protection fragments of the same

size. This meant that unless two separate probes were used in two separate experiments, the individual expression of the different rbcS genes could not be estimated. The use of two separate probes would have meant that many of the advantages of using this method would have been offset. Errors would also be introduced in the calculation of relative efficiencies due to the difficulty of obtaining accurate estimates of the specific activities of the probes used and the difficulty of comparison between two separate experiments. Thus the study concentrated on the use of gene-specific oligonucleotide probes hybridised to RNA dot blots to estimate of the relative abundances of the individual rbcS transcripts. Nevertheless, the S1 nuclease analysis of P. vulgaris total RNA suggests differential expression of the different rbcS genes in response to white light. Furthermore this analysis showed that transcripts cognate to pPvSS1672 accumulated to a greater extent in response to illumination with white light than the combined transcripts of pPvSS965 and pPvSS191.

The oligonucleotide probes rbcS1, rbcS2, rbcS3 and rbcSG were designed to hybridise to unique 21 bp sequences. The rbcS gene-specific probes (rbcS1, rbcS2 and rbcS3) were designed to hybridise specifically to each of the different rbcS transcript types (type 1', type 2' and type 3' respectively). The general rbcS oligonucleotide probe rbcSG was designed to contain sequence from within the coding region of the mature rbcS polypeptide which was totally conserved in all three rbcS cDNA clones. As well as being designed from unique sequences, all of the probes were designed with the same G-C content (7 out of 21 nucleotides were either G or C). This was in an attempt to ensure that the efficiencies of the hybridisations of the different probes would be as similar as possible for a given set of conditions. The specificity of hybridisation of these oligonucleotide probes was tested by their

hybridision to both the plasmid DNA of different rbcS cDNA clones and to total P. vulgaris RNA. Each of the gene-specific rbcS probes (rbcS1, rbcS2 and rbcS3) hybridised with similar efficiencies to their respective cDNA clones, with relatively little hybridisation to the other rbcS cDNA clones (section 3.6.2). The small amount of non-specific hybridisation calculated was possibly due to the contamination of the linearised plasmid DNA of the rbcS cDNA clones with plasmid DNA from the other types of rbcS cDNA clone during their extraction from the same agarose gel. This argument is supported by the fact that the second time this experiment was carried out (section 3.6.2.3) with plasmid DNA extracted more carefully, the 'non-specific' hybridisation was a lot lower. Furthermore, pUC19 DNA which showed the lowest 'non-specific' hybridisation in all cases had been extracted separately. The general rbcS oligonucleotide probe rbcSG hybridised to all three types of rbcS cDNA clone with similar efficiency, with very little hybridisation to the pUC19 control. Thus it was concluded that the gene-specific probes rbcS1, rbcS2 and rbcS3 were specific to the three types of rbcS cDNA clones, and the general rbcS oligonucleotide probe hybridised equally to all three types of cDNA clones.

These probes were then hybridised to total RNA from P. vulgaris on a Northern blot to ensure that they were specific to rbcS mRNA within the polyA$^+$ RNA population. Figure 30 shows that all 4 oligonucleotide probes hybridised to RNA species which were between 0.8 and 1.0 kbp in length. This size range is consistent with the sizes of transcripts expected from the analysis of the three rbcS cDNA clones isolated. Unless any of these probes had hybridised to other mRNA species in the same size range they were all specific to rbcS mRNA.

Even though the different oligonucleotide probes had been designed to have similar hybridisation efficiencies,

controls included with the RNA dot blot hybridisations indicated that the hybridisation efficiencies of the different oligonucleotide probes were quite different. The relative hybridisation signals of the probes rbcS1, rbcS2 and rbcS3 relative to rbcSG (100%) were calculated as 69, 91 and 49% respectively (section 3.6.2.3). These signals included two components; the efficiency of labelling and the efficiency of hybridisation. The labelling efficiencies seemed similar with similar rates of incorporation, but estimation of incorporation is prone to error as it cannot be certain that equal amounts of oligonucleotide are present in the reaction mixture and the estimation of incorporation using DE81 paper can only be used as a rough estimate. Taking these facts into consideration, though, it seems unlikely that labelling efficiency alone was responsible for the almost two-fold difference in overall hybridisation signal between rbcS2 and rbcS3. It seems, therefore, that the actual efficiency of hybridisation of the individual oligonucleotide probes was different even though they all had identical G-C content values. The values of signal relative to rbcSG were used to normalise values obtained with rbcS1, rbcS2 and rbcS3 so that comparison of the relative proportions of the different rbcS transcripts could be made.

Illumination of dark-grown seedlings with continuous white light for 48h produced a steady eight-fold increase of rbcS1 transcripts and a 16-fold increase of rbcS2 transcripts. The level of rbcS3 transcripts remained low and roughly constant over 48h. The relative proportions of the rbcS1, rbcS2 and rbcS3 transcripts also changed over 48h from 45, 18 and 37% respectively at 0h to 53, 42 and 5% respectively at 48h. These results imply that although white light is an important factor involved in the accumulation of rbcS1 and rbcS2 transcripts, it plays little or no role in the accumulation of rbcS3 in

greening, etiolated seedlings.
The levels of rbcS1 and rbcS2 transcripts in dark-grown seedlings declined over an 8 day time-course to undetectable levels. The level of rbcS3 transcripts showed an increase over at least the first 4 days, after which it declined to less than half its level at the start of the time-course. Changes in the levels of rbcS transcripts in the dark must presumably be due to developmental factors separate from the external influence of light. If this is the case then these results suggest that such factors control the accumulation of rbcS transcripts differentially in P. vulgaris.
Assuming that only the three rbcS transcript types characterised are expressed in any appreciable quantity in P. vulgaris, and that the estimates of the relative hybridisation signals of the different oligonucleotide probes are correct, then the combined levels estimated in these experiments for the individual rbcS transcripts should mimic the total rbcS transcript level detected by rbcSG. However, comparison of these values obtained from the time-courses in the light and dark discussed above shows that this is not the case (figures 34 and 36). Figure 34 shows quite substantial differences in the kinetics of the increase of total rbcS transcripts estimated using rbcSG compared to the increase calculated for the three rbcS gene-specific probes combined. The increase in rbcS transcripts over 48h is more marked when the combined hybridisations of rbcS1, rbcS2 and rbcS3 were used to estimate it than when rbcSG was used (a 7-fold increase compared to a 3-fold increase). A better correlation was seen in the time course in the dark. Apart from miscalculation of the relative hybridisation signals of the probes, another source of error might be in calculating the levels attributable to non-specific hybridisation. Both these sources of error are discussed in section 4.3. Aside from these two factors, the

involvement of other rbcS genes which have not been characterised could also conceivably explain the discrepencies. For instance, a fourth rbcS gene producing transcripts could possibly be detected using the rbcSG probe but not by the three rbcS gene-specific probes. If this gene produced rbcS transcripts early in the time-course, this could account for the flatter rise in total rbcS transcripts detected using rbcSG. However, as only three types of rbcS transcripts were detected in a cDNA library from light-grown primary leaves, this fourth gene would have to be very greatly reduced in its expression after longer illuminations with white light in order for it not to have been detected in the cDNA library. A final possibility which must be considered is that the relative hybridisation signals calculated to normalise the values for the levels of the different rbcS transcripts detected using rbcS1, rbcS2 and rbcS3 might be incorrect. As this experiment was only performed once it is not possible to test whether the same ratios of the levels of the different rbcS transcripts are obtained between experiments, and it remains a possibility that the relative hybridisation signals calculated resulted in biasing towards either the rbcS1 or rbcS2 transcripts to produce a sharper increase. Nevertheless, the relative abundances of rbcS1, rbcS2 and rbcS3 transcripts after 48h illumination with white light (53, 42 and 5% respectively) compare favourably with the frequencies (55, 36 and 9%) of the different rbcS cDNA clones from a library of light-grown tissue which provide an estimate of the levels of the different rbcS transcripts in this tissue.

Dark-grown plants illuminated with continuous white light for 48h were transferred to darkness to estimate the extent of RNA stability on the accumulation of different rbcS transcripts. Comparison of the levels of rbcS1 and rbcS2 transcripts following the transfer of light-grown plants to darkness for 96h shows that both decline to 3-5%

of their original levels at 0h. As the kinetics of these declines are very similar, it is likely that the differences in the accumulation of these two rbcS transcripts when illuminated with light are more likely as a result of different rates of transcription than differences in transcript stabilty. However it cannot be assumed that transfer into darkness affects the transcription of rbcS1 and rbcS2 equally. Therefore without a study involving a trancription assay of nuclei from tissue taken at the same stages, this conclusion can only be made tentatively.

Upon illumination with continuous white light for 48h, transfer to darkness for 96h and then re-illumination with continuous white light all three rbcS transcripts showed increases, although rbcS3 only slightly. In the case of rbcS1 and rbcS2 transcripts the levels acheived after 12h were near to the levels obtained after a 48h illumination with white light of etiolated seedlings (greening time-course). As discussed above, the rate of accumulation of rbcS transcripts may be limited by factors such as re-initiation of RNA synthesis. If this is the case then the higher rate of accumulation observed in re-illuminated seedlings could be due in part to the elongation of already initiated rbcS transcripts, in a situation similar to the transient increase observed in etiolated seedlings (see above). Alternatively more factors which are needed to re-initiate RNA synthesis may be present in the primary leaves of plants at this stage. A plastid factor might be involved in this rapid initiation. Plastids will have developed into chloroplasts by this stage and so might be capable of producing more of such a factor than the plastids of etiolated seedlings, accounting for the higher rate of rbcS transcript accumulation seen in re-illuminated plants.

The relative proportions of rbcS1, rbcS2 and rbcS3

transcripts after 12h were 52, 45 and 3% respectively, which were very similar to the ratios at the 48h time-point of the original greening time-course. This suggests that white light is an important factor in maintaining the relative levels of the different rbcS transcripts in more mature rbcS seedlings.

A brief illumination with red light induced an increase in the levels of all three rbcS transcripts over the dark control. Levels increased between 0 and 24h in darkness and declined between 48 and 96h, presumably reaching a maximum level between 24 and 48h. The kinetics of the accumulation of rbcS2 and rbcS3 transcripts in darkness were very similar, both reaching levels of 43-48% that of rbcS1 transcripts after 24h. Although rbcS1 reached higher levels than rbcS2 and rbcS3 transcripts, its level declined relatively more rapidly and reached levels similar to rbcS2 and rbcS3 after 96h. A greater relative decrease in the rate of transcription of rbcS1, or a greater degree of rbcS1 transcript degradation, would explain this more rapid decline.

Comparison of the levels of the different rbcS transcripts in darkness 24h after illumination with red light with those after 24h continuous white light show that there are substantial differences in the types of response. The levels of rbcS1, rbcS2 and rbcS3 transcripts were 107, 73 and 234% of those obtained after 24h in white light respectively. These results seem to indicate that red light plays a far more important role in the induction of rbcS3 transcript accumulation than white light. However, the magnitude of the difference must be considered with caution. It has to be pointed out that levels of hybridisation of rbcS3 were very much lower than for rbcS1 and rbcS2 and the difference between background and signal was very low thus allowing greater errors in the estimation of the amount of rbcS3 transcripts. Far-red light was less effective in inducing the accumulation of

all three rbcS transcripts. The levels obtained after a short illumination with far-red light of rbcS1, rbcS2 and rbcS3 transcripts were 35, 25 and 53% of those obtained with red light respectively. Far-red light also partially reversed the red-light mediated response in all three rbcS transcripts. A short illumination with far-red light directly after a red light illumination produced levels of rbcS1, rbcS2 and rbcS3 transcripts 63, 60 and 68% respectively of those obtained with red-light alone. This suggests that the accumulations induced by red light are mediated by phytochrome. Phytochrome-mediated accumulation of rbcS transcripts in etiolated seedlings is a common feature of most higher plant species studied (Dean et al., 1989).

4.3 Critical analysis of the methods used to study rbcS mRNA levels in P.vulgaris.

Using S1 nuclease analysis and dot blot analysis of total RNA to estimate the levels of individual rbcS transcripts both presented several problems. Many of these problems were purely technical, and were overcome by modifications of the procedures, but others affected the final interpretation of results.

S1 nuclease analysis was attempted using both double- and single-stranded DNA probes. The use of double-stranded probes requires the optimisation of hybridisation conditions for each individual probe. For a given probe at a given salt and formamide concentration, the hybridisation temperature must be sufficiently low to allow hybridisation of the probe to the RNA, but must not be so low as to favour the re-naturation of the probe. As the range of temperatures which can be used effectively is very narrow, the experimental hybridisation temperature must be calculated carefully. In this study the optimal temperature of hybridisation was calculated by two

different methods. In the first case the minimum temperature at which the DNA probe was sensitive to S1 nuclease was determined by performing an S1 nuclease assay with double-stranded DNA probe alone. This temperature is essentially the 'melting' temperature (Tm) of the DNA probe under these conditions. At this temperature the majority of the probe is single-stranded and has not re-annealed. Tm for the probe used was determined as 52°C. As the optimum for the hybridisation of RNA to DNA is 3-5°C higher than the Tm of the DNA probe (Hopwood et al., 1985) the experimental hybridisation temperature used was 56°C. The second method involves determining the optimal experimental hybridisation temperature from the G-C content of the probe, calculated from sequence data. Using both these methods, however, problems were encountered due to the predominent renaturation of the double-stranded DNA probe, suggesting in both cases that the hybridisation temperature was under-estimated. In hindsight a more efficient way of calculating the optimal hybridisation temperature would have been by setting up several identical hybridisation reactions of total RNA and labelled double-stranded probe and performing hybridisations at different temperatures. In this way the amount of renaturation of the DNA probe could be monitored in context of the amount of hybridisation to the test RNA. Other than the problems encountered in this study, the use of end-labelled double-stranded probes has certain advantages over the use of single-stranded probes prepared by primer extension. Probes are much easier to prepare, not requiring ligation into M13 and gel-purification, are more stable and can be produced in greater quantities thus allowing more samples to be processed. On the other hand, the use of such probes for detecting transcripts of low abundance would be limited by their lower relative specific activities. The use of single-stranded probes for S1 nuclease analysis has the advantage that

renaturation of the probe is not a problem and so the range of hybridisation temperatures available for use is much wider. This means that the hybridisation temperature does not need to be critically determined. Problems arose, however, due to the nature of the probe synthesis and in its isolation. Several methods were used to isolate the single-stranded probes for use in S1 nuclease analysis. Where the probe had been precipitated prior to running on a gel problems arose due to the poor resuspension of the probe. The difficulty in resuspending single-stranded DNA is usually remedied by the use of siliconised tubes. However, this did not appear to improve resuspension of the single-stranded probes used in this study. This meant that a lot of the labelled probe was not in solution when the gel was run and so stayed in the wells. Another problem was that many of the gel systems did not maintain the probe denatured long enough to allow the strands to separate in the gel. Both these problems resulted in very low yields as well as high background, often making location of the probe band on the gel difficult. Furthermore, the synthesis of the probe itself provided problems. Due to the high percentage of incorporation of labelled nucleotide in this method, the phosphate backbone of the single-stranded probe was very vulnerable to scission by radioactive decay. Calzone (1987) discusses these 'scission-rates' and states that using labelled nucleotide at 400 Ci/mmol allowed the use of the single-stranded probes for 5 days. This would mean that probe labelled using nucleotide at 3000Ci/mmol would become unusable after 16 hours. The full sequence of events from the synthesis of the probe to the loading of the protected fragments on a gel often took this amount of time. This would account for the poor results using labelled nucleotide at 3000Ci/mmol and the far better results using labelled nucleotide at 400Ci/mmol. However Burke (1984) does not report any difficulty using probes

labelled with nucleotide at 3000Ci/mmol. This seems to suggest that there is a further factor involved in the decay of single-stranded probes, possibly decay by chemical action. As the method described by Calzone was used to isolate the single-stranded probes in this study it is possible that this particular gel system produces a higher level of chemical degradation of the probe than the method described by Burke.

Two major problems were encountered when using oligonucleotide probes to calculate rbcS levels. The first was due to the apparent differences in hybridisation efficiencies of the different probes. The second problem was due to the difficulty of obtaining a satifactory estimate of non-specific hybridisation in each case. The difficulties arising due to the use of the relative hybridisation efficiencies calculated for each oligonucleotide probe to normalise the data are apparant when comparing results obtained using rbcSG against the combined results of rbcS1, rbcS2 and rbcS3 (figures 34 and 36). If it is assumed that the total rbcS transcript component in P. vulgaris at any given time consists solely of transcripts from the genes related to the three rbcS cDNA clones isolated, then the discrepencies between the results must be due to errors in the calculation of the relative hybridisation signals. On the other hand, if the relative hybridisation signals were correctly calculated then the differences could be explained by the involvement of rbcS transcripts from one or more rbcS genes other than those related to the 3 cDNA clones isolated. As the experiment was only performed once it is not possible to separate these two possibilities. However the involvement of other rbcS genes seems to be a less likely possibilty as discussed in section 4.2. A repeat of the experiment would show whether the relative hybridisation signals were incorrectly calculated or not. If the hybridisation efficiencies had been correctly calculated then it would

be expected that the repeat experiment would produce consistent results even though the relative hybridisation signals might be different between experiments. This would support the idea that other rbcS genes were expressed. On the other hand, if the relative hybridisation signals had not been calculated correctly then a repeat experiment would be expected to show a better match between total rbcS transcript levels calculated from using rbcSG and the level for rbcS1, rbcS2 and rbcS3 combined.

Difficulties also arise when correcting the data for counts attributable to non-specific hybridsation of the oligonucleotide probes. The result in figure 30 clearly shows that there is substantial hybridisation to rRNA for all four oligonucleotide probes and to tRNA in the case of rbcS3. Although direct comparison cannot be drawn between the RNA dot blots and Northern blots (due to the transfer process of the latter which does not guarantee equal transfer of RNA molecules over the whole range of size) this result does indicate substantial non-specific hybridisation. However, an accurate calculation of the non-specific component of the hybridisation to the RNA dot blots is not possible. The problem arises from the fact that control blots of rat liver total RNA were used to estimate the level of non-specific hybridisation for each oligonucleotide probe. This clearly does not take into account the possibility that total RNA from P. vulgaris might contain sequences of greater homology to the probes than the total RNA from rat. If this were the situation then the level of non-specific hybridisation would be under-estimated. If it is assumed that hybridisation of a given oligonucleotide to rRNA was roughly the same in all treatments then its effect would be to increase all the values obtained for the abundances of rbcS transcripts by the same amount. The fact that when rbcSG was used as a general rbcS probe the level of rbcS transcripts in

dark-grown leaves was about 30% that of light-grown leaves (table 19), whereas the use of rbcS cDNA probes estimated these levels at between 9-17%, is in agreement with this possibility. Furthermore, if the non-specific hybridisation of all the gene-specific rbcS probes was incorrectly estimated to different degrees, this could to some extent explain the discrepensies between the level of total rbcS transcripts calculated using the result from rbcSG with that calculated by the addition of the results from the three gene-specific probes.

Clearly, in the case of the Northern blot analysis described in section 3.6.2.2, the stringency at which the filters were washed was not high enough to optimise the ratio of specific to non-specific hybridisation. As these were the same conditions used for the RNA dot blot analyses, a significant component of non-specific hybridisation must have been present. The conditions of washing stringency used in these experiments were determined empirically because the optimal washing conditions calculated from theory using the formula described in section 3.6.2.1 had failed to provide a signal. However, this method was crude, involving the monitoring of the counts bound to the filters after each washing stage using a hand-held mini-monitor. The filters were autoradiographed when the counts were as low as possible but judged to be still high enough to provide an adequate signal overnight. These conditions were clearly not stringent enough to remove non-specific hybridisation to insignificant levels. As there is no reliable method to estimate the level of non-specific hybridisation these conditions need to be optimised in future experiments so as to reduce its levels to a minimum, thus reducing the level of error in calculating rbcS transcript levels. This could be done by hybridising the oligonucleotide probes to identical Northern blots of total RNA followed by washing at different temperatures at a constant salt

concentration. The temperature which most reduced non-specific hybridisation of each probe but still maintained an adequate signal from specific hybridisation to rbcS transcripts would be chosen as the optimal hybridisation temperature in each case for subsequent experiments.

Having discussed the shortcomings of the use of the oligonucleotide probes in this study, the limitations of the results obtained from using them need to be clearly defined. Clearly the level of non-specific hybridisation of the oligonucleotide probes to the RNA of P. vulgaris is in doubt. This means that the values for the relative proportions of different rbcS transcripts at a given stage can only be put forward tentatively. Furthermore, levels of rbcS mRNAs in a particular treatment expressed as a percentage of levels in an other treatment may be inaccurate. On the other hand, the qualitative aspects of the results can be held to be more reliable. For instance the kinetics of the increases of rbcS1 and rbcS2 mRNAs in illuminated etiolated seedlings should be uaffected by the limitations discussed. In this case the basal level of rbcS mRNA at 0h may be inaccurate, but the increases relative to it will be the same regardless of the level of non-specific hybridisation. The results obtained using rbcS cDNA probes must be held to be more accurate than those obtained using rbcSG to estimate total rbcS mRNA levels. The rbcS cDNA probes will have been less prone to non-specific hybridisation so will not have had the same limitations as rbcSG. Additionally, the experiments using two different rbcS cDNA probes produced reasonably consistent results. A further important limitation imposed on the results obtained using the oligonucleotide probes is the fact that the experiment was only performed once. Clearly these experiments need to be repeated several times before reliable quantative data can be obtained.

4.4 Conclusions

The small subunit of ribulose-1,5-bisphosphate carboxylase/oygenase is encoded by a multigene family of at least three members in P. vulgaris. The present weight of evidence suggests that there may only be 3 rbcS genes. Three of the members of this family show 100% conservation of nucleotide sequence in the coding region of the rbcS mature polypeptide. Two members show 100% conservation of nucleotide sequence in the coding region of the rbcS transit peptide. The complete transit peptide sequence for the third rbcS gene has not yet been determined. All three rbcS genes encoded an isoleucine residue at the N-terminus of the mature rbcS polypeptide instead of the methionine present in other species. Two regions of intronic sequences were present in the cDNA clone of one rbcS gene at conserved sites. Sequences from the 5' - and 3' -untranslated regions of these genes show significant divergence in nucleotide sequence. At least three members of the rbcS multigene family of P. vulgaris are expressed. Total rbcS transcripts accumulate to a readily detectable level in dark-grown primary leaves as well as in non-photosynthetic tissues such as the roots, suggesting a relatively less stringent regulation of rbcS transcript synthesis by light and by tissue-specific factors. Light-mediated accumulation of rbcS transcripts can be induced by both white and red light. The response to red light is partially reversible by far-red light implying the involvement of phytochrome. The patterns of the accumulation of transcripts from three specific rbcS genes show differences. Upon illumination with continuous white light the levels of transcripts of 2 rbcS genes increase substantially in primary leaves, whereas those of a third are much less affected. A similar situation occurs upon re-illumination of primary leaves after a period in

darkness. Red light induces accumulation of all three rbcS transcripts but to different extents. For all three genes these responses are partially reversible by far-red light implying the involvement of phytochrome. The level of rbcS transcripts in dark-grown primary leaves varies with time suggesting a developmental component to the control of rbcS trancript levels in P. vulgaris. All three rbcS transcripts show different patterns of accumulation in dark-grown plants, suggesting differential control of the individual rbcS genes by developmental factors.

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6. ABBREVIATIONS

The following list contains abbreviaitions other than SI units not described in main text.

DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
ssDNA	Single-stranded DNA
RF DNA	Replicative form DNA
DNase	Deoxyribonuclease
RNA	Ribonucleic acid
mRNA	Messenger RNA
tRNA	Transfer RNA
rRNA	Ribosomal RNA
RNase	Ribonuclease
dNTP	Deoxynucleoside triphosphate
ddNTP	Dideoxynucleoside triphosphate
bp	Base-pairs
kbp	Kilo base-pairs (1 000 bp)
U	enzyme units
kU	kilo enzyme units (1 000 U)
min	Minutes
h	Hours
psi	Pounds per square inch
rpm	Revolutions per minute
krpm	Kilo revolutions per minute (1 000 rpm)
cpm	Counts per minute
Cardice	Solid carbon dioxide (dry ice)
Rel. Hybn.	Relative hybridisation
Na_2EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
Tris	Tris[hydroxymethyl]aminomethane
UV	Ultra-violet
PVP	Polyvinylpolypyrrolidone
TLC	Thin-layer chromatography
TCA	Trichloroacetic acid

DTT	Dithiothreitol
PEG	Polyethyleneglycol
SDW	Sterile distilled water
SDS	Sodium dodecyl sulphate
DEPC	Diethyl pyrocarbonate
MOPS	3-[N-Morpholino]propanesulphonic acid
MES	2[N-Morpholino]propanesulphonic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2 -ethanesulphonic acid
PIPES	Piperazine-N,N'-bis-2-ethanesulphonic acid
BSA	Bovine serum albumin
TEMED	N,N,N',N'-Tetramethylethylenediamine
IPTG	Isopropyl- -D-thiogalactopyranoside
X-GAL	5-bromo-4-chloro-3-indolyl- -D-galactoside
EtBr	Ethidium bromide
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
NaCl	Sodium chloride
$CaCl_2$	Calcium (II) chloride
NH_4Cl	Ammonium chloride
CsCl	Caesium chloride
$MgCl_2$	Magnesium (II) chloride
$MgSO_4$	Magnesium (II) sulphate
$ZnSO_4$	Zinc (II) sulphate
Na_2HPO_4	Disodium hydrogenphosphate
NaH_2PO_4	Sodium dihydrogenphosphate
KH_2PO_4	Potassium dihydrogenphosphate
NaOAc	Sodium acetate
MgAc	Magnesium (II) acetate
NH_4AC	Ammonium acetate
ZnAc	Zinc acetate