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

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Abbreviations

Abbreviations not described in 'Instructions to Authors', The Biochemical Journal (1992) 281: 1-19 are listed below, with their definitions.

AMV	Avian Myeloblastosis Virus
cpm	counts per minute
CsCl	Caesium chloride
DEPC	diethyl pyrocarbonate
ds	double stranded
EtBr	Ethidium bromide
°C	degrees celcius
kb	kilobases
NPT	neomycin phosphotransferase
%	per cent
pH	hydrogen ion concentration, $-\log_{10}$ of
PHY	phytochrome protein
psi	pounds per square inch
S.E.	Standard Error of the Mean
TEMED	N,N,N',N',-tetramethylethylene diamine
(v/v)	(volume/volume)
(w/v)	(weight/volume)

Summary

The expression of the three known *rbcS* genes of *Phaseolus vulgaris* L. was examined under different growth conditions in the primary leaves using gene-specific oligonucleotide probes. These experiments demonstrated that the three *rbcS* genes had different quantitative levels of expression but very similar patterns of expression under the majority of conditions examined. The one exception to this was when plants that had been light-adapted were returned to darkness. Under these conditions, mRNA levels of one of the genes (*rbcS2*) increased after prolonged (4 days) dark adaptation. Whether this observation represents a real difference in the pattern of expression of the *rbcS2* gene relative to the other two genes is discussed.

The expression of the gene family was also examined using non-discriminating oligonucleotide and cDNA probes. These studies showed that there is an underlying ontogenetic control of *rbcS* expression in the primary leaves of dark-grown *Phaseolus vulgaris* plants. This pattern of expression is modified by the presence of light, and the extent of the 'modification' is dependent on the fluence rate of light with which the plant is illuminated.

The control of *rbcS* mRNA levels in the light-grown plant in response to an increased fluence rate of light was also examined. It was found that exposure of 16 day old plants grown under a low (15 $\mu mol/m^2/s$) fluence rate of white light to an increased fluence rate (150 $\mu mol/m^2/s$) of white light for 2 days resulted in a substantial increase in the *rbcS* mRNA level. Similar experiments using increased fluence rates of different light qualities (blue-enriched and red) indicated that a blue light photoreceptor was involved in mediating the increase in *rbcS* mRNA levels. Measurements of oxygen evolution and stomatal resistance showed that the different qualities of light did not result in differential rates of photosynthesis and stomatal opening under the different light qualities, supporting the idea that it was a blue light photoreceptor that was mediating the increase in *rbcS* expression. An increase in *rbcS* expression in response to an increased fluence rate of blue-enriched light, which was not apparent with an

identical fluence rate of red light, was found to occur in primary leaves of different ages, showing that the response was not limited to one developmental stage of the primary leaf.

The photoregulation of transcription of the *rbcS* genes was examined. Plants were grown in a similar manner to those used for experiments to measure the steady state mRNA level, and moved to an increased fluence rate of blue-enriched light. Leaves were harvested over a timecourse and nuclei extracted from them for use in run-on transcription assays. These experiments showed that the blue light treatment resulted in an increase in the transcription rate of the *rbcS* genes that peaked at around 12 hours and fell by 24 hours. The effect of other light qualities on the transcription of *rbcS* genes was also examined after a 12 hour treatment. These experiments indicated that, when equal fluence rates of light were used, an increased fluence rate of blue-enriched light was the most effective in increasing the transcription of *rbcS* genes. These results indicated that the increase in steady-state *rbcS* mRNA seen after treatment with an increased fluence rate of blue-enriched light occurred as a result of a blue photoreceptor mediating an increase in *rbcS* transcription. As expected from the steady-state mRNA data, an equal fluence rate of white light gave a smaller increase in *rbcS* transcription than the blue-enriched light, but, somewhat unexpectedly, an increased fluence rate of red light also gave a small increase in transcription of the *rbcS* genes, a response that was not evident when steady-state *rbcS* mRNA levels were studied. Possible reasons for this are discussed.

A method to determine the relative levels of the mRNA of the three *rbcS* genes in a single reaction was developed. This was done because such information could not be obtained from S1-nuclease analysis with a single probe due to the similarity between the sequences of the three genes. The method involved using the polymerase chain reaction with three gene-specific 3' primers, and a single non-discriminating 5' primer, following a reverse transcriptase reaction carried out with the three gene-specific 3' primers and total RNA. These primers should theoretically have allowed the amplification of three PCR products of different sizes. This was demonstrated to be the

case, and the method was shown to produce similar measurements of *rbcS* mRNA levels to hybridisation of probes to a northern blot. The results demonstrated that the method could prove useful with some further refinement which, unfortunately, time did not allow. One problem with the method is that the estimates of the three genes' relative mRNA levels differ from those found both through the use of northern analysis with gene-specific oligonucleotide probes and examination of the frequency of the corresponding cDNAs in a cDNA library. Possible reasons for this discrepancy are discussed.

Introduction

The reactions of photosynthesis, which fix carbon dioxide (CO_2) from the atmosphere using light from the sun as an energy source, provide the organic carbon necessary for animals such as ourselves to live. Thus the understanding of the mechanisms by which these organisms photosynthesise is of fundamental importance. The study of photosynthesis has been carried out for a long time by many of the biological disciplines. A point that is apparent in all the studies of photosynthesis is the fact that plants adapt their photosynthetic machinery to enable photosynthesis to occur as efficiently as possible. The way the plant achieves this adaptation involves a number of different mechanisms. One of these mechanisms is the control of the expression of the genes encoding the enzymes of the photosynthetic machinery so that their levels are appropriate for the light environment of the plant. In this study I examine the control of the genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase in the primary leaves of *Phaseolus vulgaris*. This enzyme catalyses the primary fixation of atmospheric carbon dioxide into a three carbon compound, and thus control of the genes encoding it is of vital importance to the plant.

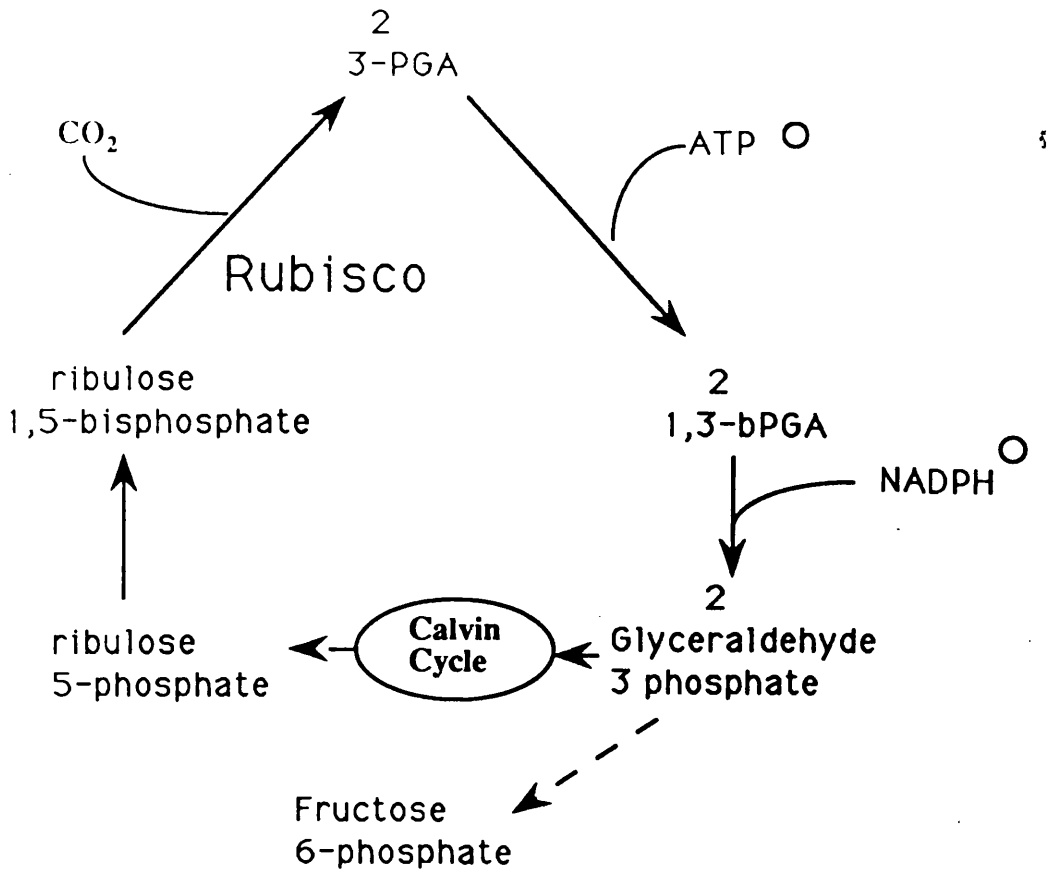
1.1 Ribulose 1,5-bisphosphate carboxylase/oxygenase : the enzyme

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most abundant enzyme in the leaves of light-grown plants. The enzyme catalyses two different reactions : i) the carboxylation of ribulose 1,5 - bisphosphate (RuBP) and ii) the oxygenation of RuBP (Jensen and Bahr, 1977). These reactions shown in Fig.1.1 are both catalysed at the same active site and can be said to be in competition with each other, their relative rates being dependent on the relative concentrations of CO_2 and O_2 and the ambient temperature (Lorimer and Andrews, 1981). The carboxylation of RuBP is the first step in the photosynthetic fixation of atmospheric CO_2, whereas the

Fig 1.1 The reactions catalysed by Rubisco

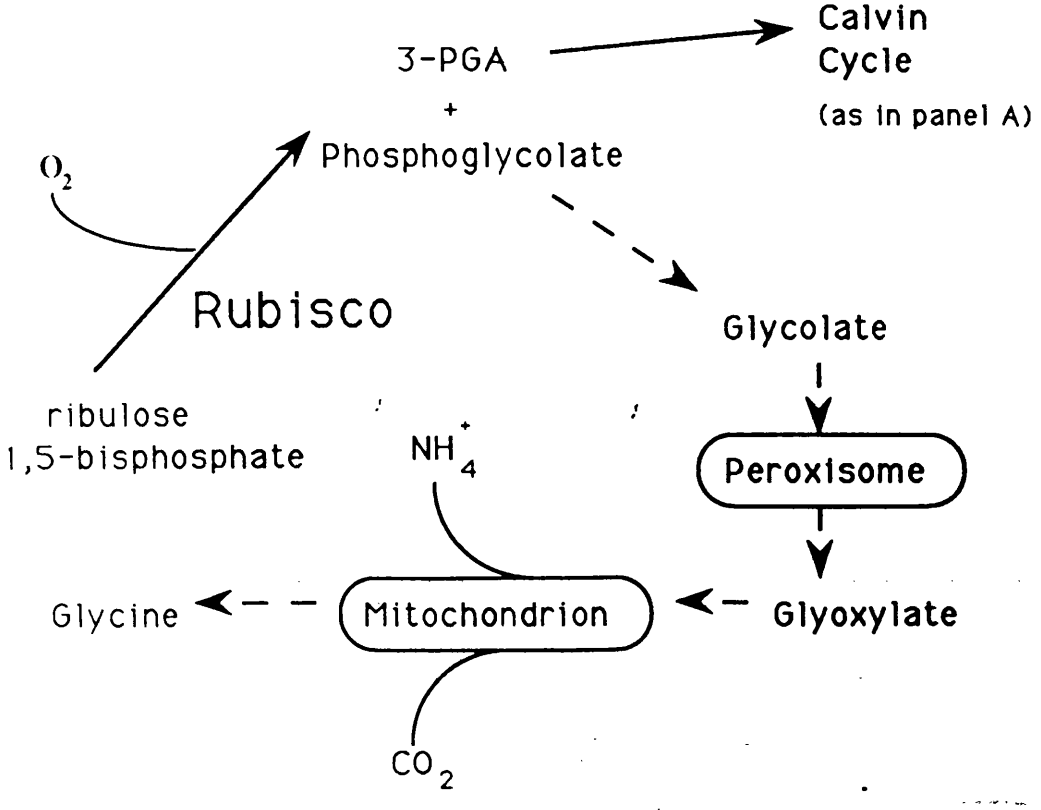
The reactions catalysed by Rubisco are shown. Panel A depicts the carboxylase reaction and panel B, the oxygenase reaction. The subsequent fate of the products from each reaction is also shown in 'cartoon' form.

A



○ : From the light reactions of photosynthesis

B



oxygenation of RuBP is the first step of the photorespiration pathway. The reactions of photorespiration salvage most of the oxygenated carbon products but the reactions consume O_2 and release CO_2 (Lorimer and Andrews, 1981). Under natural conditions (21% O_2 and 0.033% CO_2 at atmospheric pressure) the ratio of carboxylation to oxygenation is approximately 3 to 4 : 1 in plants using Rubisco as their primary CO_2-fixing enzyme (C 3 plants) (Farquar et al., 1980). Since the enzyme is so inefficient at fixing CO_2 a large amount of work has been done to elucidate the crystal structure of the enzyme and thus the mechanisms of both reactions with the eventual aim of improving the efficiency of the enzyme's carboxylation reaction.

1.1.1 The structure of Rubisco

The enzyme was purified as early as 1956 by Weissbach and his co-workers who showed that the protein formed PGA from CO_2 and RuBP. The enzyme has since been purified from a large number of higher plants, algae, and photosynthetic, chemosynthetic and blue-green bacteria. All of the plant and algal enzymes have been shown to have a mass of 560 kDa and to be composed of eight large subunits (L) (~ 55-56 kDa) and eight small subunits (S) (~ 12-15 kDa). The numbers and types of subunits present in the bacteria vary between species from L_2 in *Rhodospirillum rubrum* (*Rh. rubrum*) to L_8S_8 in *Thiocapsa roseopersiciana* (McFadden, 1980).

1.1.2 The large subunit (L)

The function of the large subunit of Rubisco has been known for a relatively long time. Nishimura et al. (1973 and 1974) carried out experiments on Rubisco from spinach dissociated into its subunits. They found that the large subunit separated from the small subunit could carry out the carboxylation reaction. Further evidence came

from the fact, mentioned in the previous section, that the enzyme from *Rh. rubrum* was found to be just a dimer of two large subunits and could still catalyse carboxylation (McFadden, 1974). The relative simplicity of the enzyme from *Rh. rubrum* has led to it being studied by X-ray crystallography and compared to enzymes from plants with which it has a 30% amino acid sequence identity (Nargung et al., 1984). Anderson et al. (1989) crystallised both the *Rh. rubrum* enzyme and that from spinach and described their active sites. The active site of the *Rh. rubrum* Rubisco is formed from amino acid side-chains from both large subunits. The spinach enzyme was made up of four pairs of *Rh. rubrum*-like L_2 dimers forming a cylindrical structure. The active sites were similar to those of the *Rh. rubrum* enzyme, again being composed of amino acid side-chains from two neighbouring large subunits. These sites faced away from the 'cylinder' into the solution.

1.1.3 The small subunit

Unlike the large subunit the specific role of the small subunit is not known. In the crystal structures described by Anderson et al. (1989) and Chapman et al. (1987) for the spinach and tobacco enzymes respectively the small subunits sit in crevices between the ends of the L_2 dimers, four at each end of the cylinder of four L_2 dimers. This is physically a long distance from the active site and no residues from the small subunits are present in the active site. This implies that the small subunits are not directly involved in the catalysis. Despite this, the carboxylase activity of the L_8 core is only 1% of the L_8S_8 holoenzyme (Ellis and Gray, 1986) implying that the small subunits' binding must at least have an indirect effect on the enzyme's activity.

1.2 Rubicso genes

Early evidence based on the genetic analysis of *Nicotiana* lines implied that the two types of Rubisco subunit were encoded in different genomes. The large subunit was found to be maternally inherited by Chan et al. (1972) and by Sakano et al. (1974) implying that it was encoded on the chloroplastic DNA, whereas the small subunit was biparentally inherited (Kawashima et al., 1972) implying that it was encoded by the nuclear DNA. This genetic evidence was confirmed by the cloning of the two different subunits from the expected genomes. A fragment of chloroplastic DNA encoding the large subunit was cloned by Coen and co-workers in 1977 from *Zea mays* and a cDNA clone encoding a small subunit gene was cloned by Bedbrook et al. in 1980. This cDNA clone was shown to be encoded by the nuclear DNA.

1.2.1 The large subunit genes (*rbcL*)

Since the initial cloning by Coen et al. the gene for the large subunit has been mapped on the chloroplast genome of a number of different higher plants (listed by Crouse et al., 1985). In all the species looked at the *rbcL* gene is present as a single copy per chloroplast DNA molecule. Taking into account the fact that each chloroplast contains between 10 - 100 copies of its DNA (depending on the species and the developmental stage of the leaf) and that each cell contains 10 - 200 chloroplasts per cell, the copy number of *rbcL* can be as high as several thousand copies per cell (Miziorko and Lorimer, 1983). This copy number enables a large amount of protein to be made over a relatively short period.

The *rbcL* gene has been cloned and sequenced from a number of different higher plants (for a review see Manzara and Gruissem, 1988). In all the genes sequenced so far the gene has been found to contain no introns, to be approximately 1.4 - 1.8 kbp in length, and to encode a peptide of around 475 amino acids. In some

species this single gene encodes two differently sized transcripts. In maize there is a larger 1.8 kb transcript which commences from the start of transcription and a smaller 1.6 kb transcript which may result either from processing of the larger transcript, or arise from a transcription start site further downstream (Crossland et al., 1984; Orozco et al., 1985). The coding region of *rbcL* genes is highly conserved between species. Zurawski et al. (1981) compared the *rbcL* sequences from spinach and maize and found 84% identity between the nucleotide sequence which encoded the 475 amino acid protein in both plants. Comparing the deduced amino acid sequences gave a higher identity of 90% due to silent base changes. If the amino acid sequences of two closely related species, petunia and tobacco, are compared the amino acid identity increases to 97% (Aldrich et al., 1986). This high amino acid conservation between species is not surprising considering the fundamental nature of the reaction that the enzyme catalyses and the nucleotide sequences of the 5' flanking regions and coding sequences have been used to predict evolutionary relationships (Ritland and Clegg, 1987).

1.2.2 The small subunit genes (*rbcS*)

In all of the higher plants studied the *rbcS* genes encode a transit peptide of 46-57 amino acids and a mature protein of 120-128 amino acids (Nagy et al., 1986). The mRNA is translated on cytoplasmic ribosomes and the transit peptide targets the protein to the chloroplast membrane (Keegstra, 1989). In fact the transit peptide contains enough information to direct a foreign protein, such the antibiotic resistance gene, NPT 2, to the chloroplast (Van den Broeck et al., 1986). The transit peptide is then cleaved by a chloroplastic protease during or just after the translocation of the peptide through the chloroplast membrane (Robinson and Ellis, 1984). Once inside the chloroplast the mature protein is assembled with the large subunit in a reaction involving the plastid chaperonin 60 to give the L_8S_8 holoenzyme (Ellis, 1990).

In all plants the *rbcS* genes are present as small nuclear gene families. Their structure and organisation has been reviewed by Dean et al. (1989) and Manzara and Gruissem (1988). The exception to this is cucumber which has been reported to have only one *rbcS* gene per haploid genome (Greenland et al., 1987). In pea, for example, there are 5 *rbcS* genes (Polans et al., 1985) as there are in tomato (Sugita et al., 1987). The much studied plant *Arabidopsis thaliana* has 4 *rbcS* genes (Krebbers et al., 1988), petunia 8 (Dean et al., 1987) and the aquatic monocot *Lemna gibba* (duckweed), around 12. It should be pointed out that duckweed is polyploid which accounts for the larger number of *rbcS* genes (Wimpee et al., 1983).

Genomic and cDNA *rbcS* clones have been obtained from these and other species and a large number of *rbcS* sequences are known. All of the *rbcS* genes isolated from higher plants have at least one intron. Genes isolated from dicots all have 2 or 3 introns (Dean et al., 1989) in positions conserved between species. In two-intron genes the first intron from the initiation codon is found between codons 2 and 3 of the mature polypeptide, and the second between codons 41 and 42. Tomato and petunia, both members of the *Solanaceae*, have a single gene with three introns. The first two introns are in the same positions as in the two-intron genes with the third intron being present within codon 65 (Manzara and Gruissem, 1988). Genes isolated from monocots often have only one intron. The genes sequenced from duckweed all have one intron sited in the same position as the second intron in dicots (Silverthorne et al., 1990). However, a single intron gene from wheat has its intron in the same position as the first dicot intron (Broglie et al., 1983) implying that none of the introns are essential for the function of *rbcS* genes.

Some of the *rbcS* genes have also been mapped to positions on chromosomes. The 5 *rbcS* genes of pea all map to the same chromosome (Polans et al., 1985) and are very closely linked. This is not true for tomato which also has 5 *rbcS* genes. These have been mapped to three separate loci on two different chromosomes. One locus encodes 3 two-intron *rbcS* genes, with the other loci encoding single *rbcS* genes, one being a three-intron gene (Pichersky et al., 1986; Sugita et al., 1987). A similar

arrangement of genes is also seen in petunia. The genes have been placed into sub-families by nucleotide homology. There is one sub-family which contains 6 two-intron genes, 5 of which are known to be closely linked, with the other gene of this family present at a different locus, and two sub-families which each contain a single gene, one of these being a three-intron gene, as in tomato. These observations indicate that some of the *rbcS* genes of small nuclear gene families are often found to be closely linked.

Comparisons of the sequences coding for the mature protein in higher plants have revealed at least 70% amino acid identity between them (Meagher et al., 1989). If the DNA sequences are compared within gene families it can be seen that there is greater conservation of sequence than between species. Within the tomato gene family of 5 genes the DNA sequence conservation varies from 86 % to 100 %. If the deduced amino acid sequences are compared the amount of conservation rises to 91 % to 100 %. Most of the amino acid changes occur within the transit peptide but nevertheless 5 different genes produce 3 different mature proteins (Manzara and Gruissem, 1988). That the majority of changes occur in the transit peptide is true for all of the *rbcS* genes studied (Dean et al., 1989; Meagher et al., 1989). If the sequences of the genes are looked at within a single genetic locus within a gene family, the conservation of sequence rises. The *rbcS* genes of pea which are encoded at a single locus vary by only 1 % at the amino acid level (Fluhr et al., 1986a). Thus the *rbcS* genes have been conserved relatively highly through evolution although at present the function of the protein is not clear.

1.2.3 *Phaseolus vulgaris* *rbcS* genes

RbcS cDNA clones were picked from a light-grown *P. vulgaris* primary leaf cDNA library (provided by Dr J.V.Cullimore, University of Warwick) screened with a pea *rbcS* cDNA probe (Bedbrook et al., 1980). The clones were classified by A-tracking (di-deoxynucleotide sequencing with only di-deoxyadenine present) of the 3'

end of the coding sequence and the 3' untranslated sequences (Knight and Jenkins, 1992). Comparison of the 3' untranslated sequences indicated that there were three classes of cDNA present in the library. These were named with regard to their abundance in the cDNA library with *rbcS1* type cDNAs representing 54% of the 42 cDNAs examined, *rbcS2* 36%, and *rbcS3* 10%. A clone representing each type of cDNA was sequenced revealing that the nucleotide sequence for both the transit peptide and the mature protein was identical for all three genes with differences only appearing in the 5' and 3' untranslated sequences. The *rbcS2* and *rbcS3* transcripts both had two different polyadenylation signals with the poly-A tail starting at two different sites in their 3' untranslated sequences. One of the *rbcS1* type cDNA clones was found to contain two intron sequences. These were present between codons 2 and 3 and codons 47 and 48 of the mature peptide as expected for a dicot two-intron gene. The presence of intron sequences within the cDNA clone was thought to be due to the cloning of an unprocessed sequence as none of the other *rbcS1* clones contained any intron sequences (Knight and Jenkins, 1992). Southern blot data has indicated that there are probably three *rbcS* genes present in *P. vulgaris* but does not rule out the possibility of there being a fourth gene which could contribute a small amount of transcript to the light-grown leaf *rbcS* mRNA pool (Knight and Jenkins, 1992). Further Southern blot analysis has shown that the *rbcS* genes may be encoded on a 25 kilobase piece of the *Phaseolus vulgaris* genome (N.A.R. Urwin and G.I. Jenkins, unpublished data).

1.3 Expression and control of *rbcS* and *rbcL* genes

The presence of the genes for the two subunits of Rubisco in different genomes leads to the obvious question of how the expression of the *rbcS* and *rbcL* genes is co-ordinated to produce stoiochemetric amounts of their respective proteins. To answer this the expression patterns of the two sets of genes and their points of control have been intensively studied. The answer, while still not clear, has been shown to involve

transcriptional, post-transcriptional, translational and post-translational control and to depend on the species under investigation.

1.3.1 Expression and control of *rbcL* genes

The level of *rbcL* mRNA has been shown to be light-regulated and to be under developmental control in a large number of species. These include barley (Klein and Mullet, 1990), *Vigna aconitifolia* (Keller et al., 1993), *Arabidopsis thaliana* (Chory et al., 1989), pea (Thompson et al., 1983; Sasaki et al., 1984, 1987), maize (Nelson et al., 1984) and *Amaranthus hypochondriacus* (Berry et al., 1985). The mechanism of the light induced increase in *rbcL* levels has been studied by a number of groups. Sasaki et al. (1984) showed that the increase in the expression of *rbcL* relative to total extractable RNA in response to light could be explained by the increase in the copy number of the chloroplastic DNA. Inamine et al. (1985) found that the increase in the large subunit protein as measured by the increase in Rubisco activity could not be explained by the 3-5 fold increase in the *rbcL* mRNA and proposed that the *rbcL* mRNA present in the plastids of dark grown peas was not being translated. Upon illumination the translation of *rbcL* mRNA increased in concert with the increase in *rbcL* transcription due to an increase in template copy number to give the large increase in protein. However, Sasaki et al. (1987) looked at the effect of using increasing light intensities on dark grown plants. They found that the increase in chloroplastic DNA copy number did not correlate with the increase in *rbcL* mRNA with increasing light intensity and proposed that there was also an increase in the transcription of the *rbcL* genes in response to increasing light.

The effect of the transition from light to dark on *rbcL* has been studied in *Amaranthus hypochondriacus* by Berry et al. (1988). They found that the association of *rbcL* mRNA with plastid polysomal fractions was the same in plastids from light grown or dark-adapted plants. However in the dark-adapted plants the rate of synthesis

of the large subunit dropped dramatically implying that the *rbcL* mRNA was not being translated in the dark-adapted plants. This showed that there is translational control of *rbcL* mRNA in response to dark adaptation in *Amaranthus hypochondriacus* .

Developmental control of *rbcL* mRNA levels was shown in barley. Klein and Mullet (1987) found that the level of *rbcL* mRNA and protein was highest in 4.5 days old dark grown barley seedlings implying that light was not required for its production. The level of mRNA then fell over the next four days. This pattern was followed in the light grown seedlings. The difference between pea, where there is a light induced increase in *rbcL* mRNA and protein, and barley is probably due to the fact that leaf and chloroplast development are much less dependent on light in barley (Robertson and Laetsch, 1974). However later work by Klein and Mullet (1990) on the illumination of 9 day old dark grown barley showed that, in this age of seedling, light did give an increase in the *rbcL* mRNA level. This increase was found to be due to a light-induced increase in plastid transcription showing that *rbcL* mRNA levels in barley are under both developmental and light regulation.

Despite the fact that there is a light-induced increase in transcription of chloroplast genes the mechanism bringing about this increase is not clear (for a review see Gruissem, 1989). The initiation rates from chloroplast promoters have been studied *in vitro* and been shown to be similar to the rates seen *in vivo* and the relative amounts of different RNA species can be explained in part by promoter strength (Deng and Gruissem, 1987). The increase in plastid transcription in response to light could be due to changes in the DNA topology as seen in *E. coli* (Wang, 1985). *In vitro* experiments with plasmid topoisomers of the linked *atpB* and *rbcL* genes in maize chloroplast extracts showed that the superhelical state of the DNA could affect transcription from these plasmids (Stirdivant et al., 1987). The topoisomerase II inhibitor novobiocin was also shown to affect the transcription of *atpB* and *rbcL* in pea chloroplast extracts differently, suggesting that this could be a method the chloroplast uses to modulate the expression of linked genes independently (Lam and Chua, 1987) but none of these effects have been shown *in vivo*.

Another way the chloroplast's genes could be differentially controlled could be through the presence of different RNA polymerases. Four open reading frames have been identified in chloroplast DNA which have homology to bacterial RNA polymerases (Gruissem, 1989). Antibodies raised against two of the proteins encoded by these genes, *rpo* A and *rpo* B, have been shown to partially inhibit chloroplastic transcription implying that they are functional (Little and Hallick, 1988). There is also evidence of a nuclear encoded chloroplastic RNA polymerase. Falk et al. (1993) have shown that lysed plastids from barley plants grown from seed at 34°C, a temperature which stops chloroplastic translation through the inhibition of 70S ribosomes, can still carry out transcription at roughly the same rate as those from control leaves. This evidence is supported by the work of Hess et al. (1993) who showed that there was transcription in the white chloroplasts of the *albostrians* mutant of barley. This mutant lacks plastid ribosomes and thus, plastid translation products, including the plastid encoded RNA polymerase. This implies that the RNA polymerase responsible is nuclear encoded and the expression of this/these gene(s) would enable some nuclear control of chloroplast transcription.

1.3.2 Expression of *rbcS* genes

The expression of *rbcS* genes and its control has been the subject of more research than the *rbcL* genes. One reason for this is that the plants response to one of its major stimuli, namely light, was found to be an increase in the transcription of nuclear genes encoding chloroplastic proteins. Gallagher and Ellis (1982) extracted transcriptionally competent nuclei from light-grown and etiolated peas. They found that the transcription of *rbcS* genes and the genes encoding the major chlorophyll a/b binding protein (*cab* genes) was increased 18 and 9 fold respectively in the light-grown nuclei when compared to nuclei isolated from dark-grown plants. Transcription of genes encoding ribosomal RNA increased only two-fold as a consequence of growth in

light, thus showing that all transcription was not up-regulated by the same extent. This implied that analysis of the *rbcS* promoter region should elucidate some of the mechanism of control. The fact that the technology of transgenic plants, reporter genes, and promoter analysis was developing rapidly at approximately the same time meant that the study of steady state *rbcS* mRNA levels could be integrated with analysis of *rbcS* promoters both *in vitro* and also *in vivo* in transgenic plants. These techniques have been applied and, despite the complexity that has been revealed, the process of 'switching' on and off of *rbcS* genes in response to light and other stimuli is beginning to be elucidated.

1.3.2.1 Expression patterns of *rbcS* genes

A large amount of the early work on the expression of *rbcS* genes was done on the expression of the *rbcS* gene family as a whole in various organs of various plants. One common feature is that the *rbcS* genes are always expressed to their maximum in leaves, and there is always very little expression in the roots (Dean et al., 1989), which is unsurprising given the role of the *rbcS* protein. For example, if the level of expression of *rbcS* genes in the light-grown leaf is given a value of 100%, the expression levels in tomato plant stems, etiolated seedlings and green fruit were found to be 3.2%, 4.6%, and 6.5% of this value respectively (Sugita and Gruissem, 1987). Similar values were also found for pea (Coruzzi et al., 1984) and for petunia (Dean et al., 1985) but the large difference in expression of *rbcS* between light-grown leaves and tissue from etiolated seedlings is not always seen. In barley the *rbcS* expression levels are found to be approximately the same in light and dark-grown seedlings during early development (Batschauer et al., 1986) and in cucumber the level of *rbcS* expression in dark-grown cotyledons is 30% of that in light-grown seedlings (Greenland et al., 1987).

The differences in expression in different organs led to the question of whether different members of the gene families which encode *rbcS* were expressed differentially to give the different expression patterns. The expression of various individual members of *rbcS* gene families has been studied in pea (Coruzzi et al., 1984; Fluhr et al., 1986b), petunia (Dean et al., 1985; Tumer et al., 1986; Dean et al., 1987), tomato (Sugita and Gruissem, 1984; Wanner and Gruissem, 1991), duckweed (Silverthorne et al., 1990), maize (Sheen and Bogorad, 1986) and soybean (Shirley et al., 1990) among other species.Various methods have been used to study the expression of individual members of *rbcS* gene families. These include S1 analysis, the counting of different types of genes in a cDNA library, northern analysis with oligonucleotide probes, and primer extension. All these methods exploit the differences between the nucleotide sequences of the individual *rbcS* genes and the relative merits of these methods are discussed by Dean et al. (1987).

The results of these investigations are generally similar to those examining the expression of the *rbcS* families' transcript as a whole, in that there are differences between species in the way that they express the individual members of their *rbcS* gene families. In petunia, for example, the gene most expressed in light grown leaves , SSU 301, is also the most expressed gene in all the other organs studied. The remaining genes' ranking by expression levels also remains the same in all the organs examined (Dean et al., 1985). In pea the *rbcS-3A* gene is the most expressed gene in all the tissues except petals and the pericarp where it is expressed at 90% and 98% of the level of *rbcS-3C*, the second most expressed gene in light grown leaves. The other three genes are expressed at a much lower level (Fluhr et al., 1986b).

In contrast, in tomato, there are greater developmental differences between the individual members of the *rbcS* gene family. In the immature leaf *rbcS-3B* is the most expressed gene whereas in the mature leaf *rbcS-3C* is most expressed, the level of *rbcS-3B* falling to 50% of this level. In the fruit the *rbcS-1* gene is the most expressed and in etiolated tissue yet another gene, the *rbcS-2* gene, is the most expressed. Also in etiolated seedlings of tomato, the two genes that are expressed at the highest level in

light grown leaves, *rbcS-3B* and *rbcS-3C*, are undetectable at the level of transcript accumulation. Thus in tomato the individual genes are differentially expressed through development and in different organs whereas in petunia, and to some extent in pea, the ranking of the genes with regards to expression remains the same.

The responses of the individual members of gene families to dark to light transitions also change with development. In pea the exposure of etiolated seedlings to light resulted in an increase of the *rbcS-3A* and *rbcS-3C* genes' transcripts to high levels after 24 hours but the level of transcripts from two other genes, *rbcSE-9* and *rbcS 8.0* barely rose at all (Kuhlemeier et al., 1987). In contrast, if an already green leaf was dark adapted and then returned to the light the levels of all four genes rose to a high level after 24 hours (Kuhlemeier et al., 1987). This implies that there is a developmental control over the response of the *rbcSE-9* and *rbcS 8.0* genes to light.

The response of tomato *rbcS* genes to light/dark transitions is also interesting. The two genes that are not expressed in etiolated seedlings, *rbcS-3B* and *rbcS-3C*, have a lag of between 3-9 hours prior to their transcript levels increasing on exposure of the seedling to light. If a light grown leaf is dark adapted these two genes' transcripts disappear within 3 hours as does the transcript from the *rbcS-1* gene (Manzara and Gruissem, 1988). This is interesting because the *rbcS-1* gene is the gene that is most expressed in etiolated seedlings implying that there is a developmental control over the control of *rbcS-1* expression in the dark; the plant has developed the ability to repress *rbcS-1* expression in the dark.

If the response of an *rbcS* gene family is looked at in a C4 plant another interesting effect of light can be seen. Nelson et al. (1984) showed that the *rbcS* mRNA was present in dark-grown maize at approximately 30 % of the light-grown level after 7 days and that it would increase to the light-grown level upon illumination. These observations were taken further by Sheen and Bogorad (1986). They showed that illumination of etiolated maize seedlings caused a decrease in *rbcS* expression in the mesophyll cells but a transient increase in its expression in the bundle sheath cells. This is because C4 plants fix CO_2 in the mesophyll cell using phospho*enol* pyruvate

carboxylase, an enzyme with a much higher affinity for CO_2 than Rubisco. This CO_2 is then released in the bundle sheath cells where it is fixed again by Rubisco. The action of light on etiolated maize leaves leads to the expression of *rbcS* in a cell specific manner consistent with the C4 adaptation. Recent work on *Amaranthus hypochondriacus*, a C4 dicot, has shown that in the very young leaves there is *rbcS* expression in both the mesophyll cells and the bundle sheath cells (Wang et al., 1993). They found that the expression of *rbcS* becomes limited to the bundle sheath cells when the leaf becomes a "source" of carbon source through photosynthesis, rather than a carbon "sink" which it is while it is developing.

The pattern of *rbcS* expression, as measured at the level of accumulated mRNA, was studied during the growth of dark-grown maize by Nelson et al. (1984). They found that there was a light independent expression of *rbcS* which peaked on the seventh day after sowing and then falls to a level 0.5% of that of light grown plants by the ninth day. Despite this low level, the nine day old dark-grown plants contain 50 % of the amount of Rubisco protein found in nine day old light grown plants. This implies that the protein made in the dark is stable.

A similar pattern of expression is seen in cucumber (Greenland et al., 1987). The *rbcS* mRNA levels measured in the hypocotyls of dark grown cucumber plants reached a peak after 6 days growth, prior to falling to a lower level. If the cucumber was grown in the light there was still a peak of *rbcS* expression on the sixth day after planting, but the level of expression was higher than that found in the dark-grown cotyledons. This implies that light acts to modulate the pattern of expression seen in the dark. Peaks of expression of *rbcS* are also seen in dark-grown *Arabidopsis* (Dedonder et al., 1993), and Amaranth (Berry et al., 1985).

The level of expression of *rbcS* in light-grown tissue has also been seen to change during the development of the plant. As I have mentioned, there is a peak of expression in the cotyledons of cucumber on the sixth day after planting in the light (Greenland et al., 1987). A similar pattern of expression is seen in 7 day old etiolated

pea seedlings that are illuminated. The level of *rbcS* mRNA rises until the fourth day of illumination and then starts to fall to a lower level (Sasaki et al., 1985).

The pattern of *rbcS* expression was also followed during the development of maize leaves (Loza-Tavera et al., 1990). They found that the level of *rbcS* expression rose during the period of leaf expansion. Once the leaf had reached its expanded size the level of *rbcS* started to decline, and was undetectable by the time the leaf was starting to senesce. This implied that there was an underlying developmental control of *rbcS* expression. The expression of *rbcS* has also been followed through the senscence of the primary leaves of *Phaseolus vulgaris* (Bate et al., 1991).They found that the mRNA levels of *rbcS* were highest in the youngest leaves they examined (7 days after planting), and that after 21 days there was no *rbcS* mRNA detectable. This was paralleled by a decline in the amount of the small subunit of Rubisco as measured by western blotting. These results again showed that *rbcS* expression is switched off developmentally during senescence of the leaf.

1.3.2.2 Control of *rbcS* expression

As the differences between the steady-state levels of *rbcS* mRNA between etiolated and light grown tissue indicate, there is a light induced induction of *rbcS* mRNA levels which in most species is greater than that shown for the *rbcL* genes. This response had been shown to be at the transcriptional level by Gallagher and Ellis (1982) by using run-on transcription analysis with nuclei from light and dark grown pea. Subsequent research has compared the rate of transcription, as measured in this way, with the steady state levels of various *rbcS* genes to look for other points of control.

Shirley and Meagher (1990) examined the transcription rates in nuclei isolated from the leaves of light and dark-grown soybean seedlings. They found that there was a 32-fold increase in the level of transcription between the light and dark-grown

seedlings but that at the level of *rbcS* mRNA as measured by northern analysis there was only an 8-fold increase between light and dark. This difference between observed *in vitro* transcription rate and steady state mRNA level was not seen when these parameters were studied in a soybean *actin* gene whose steady state mRNA level paralleled its rate of transcription. They concluded that the action of light made the *rbcS* mRNA more unstable than it was in the dark and that this post-transcriptional regulation led to the discrepancy.

Other work from the same group examined the contribution of post-transcriptional regulation to the control of *rbcS* mRNA levels in mature soybean leaves. They found that the transfer of green, mature soybean leaves to the dark caused a 16-fold decrease in the *in vitro* rate of transcription but a 64-fold decrease in the amount of steady state mRNA, whereas again, the rate of *actin* transcription and its mRNA level both declined to the same extent (4-fold). This showed that the *rbcS* mRNA in mature soybean seedlings is more stable in the light than in the dark and this implies a developmental change in the post-transcriptional regulation (Thompson and Meagher, 1990). Changes in the stability of *rbcS* transcripts with increasing age have also been shown in tomato (Wanner and Gruissem, 1991). Comparisons of mRNA levels and *in vitro* transcription from leaves of different ages showed that as the leaf aged the stability of *rbcS* transcripts decreased.

Thompson and Meagher (1990) also presented results on the control of petunia *rbcS* gene families. On placing light grown petunia plants in the dark for 2 days the rate of transcription of the *rbcS-A* and *rbcS-C* gene families was paralleled by a similar fall in the steady state mRNA from each family. On the return of the plants to light the transcription rates of the two gene families returned to the light levels after 2 hours with the *rbcS* mRNA from the *rbcS-C* gene family returning to its light level after 24 hours. The level of *rbcS-A* mRNA increased to 4 times its light-grown level indicating that it is more stable on returning to light. Evidence of differential stability contributing to *rbcS* control is also found in light-grown soybean where the transcript

with the highest steady state level is transcribed at half the rate of the transcript which is present at a quarter of its abundance (Shirley et al.,1990).

Another level of regulation of *rbcS* has been implied in *Amaranthus hypochondriacus*. During the last stages of development of the leaves in this species there is a specific drop in the synthesis of both subunits of Rubisco. If the levels of RNA are examined by northern analysis the levels are only seen to drop modestly and not enough to account for the large drop in protein synthesis. The authors (Nikolau and Klessig, 1987) say that this implies that there is a translational control of both *rbcS* and *rbcL* mRNA. This conclusion was also arrived at when the synthesis of *rbcS* protein was studied in dark grown Amaranth. The level of *rbcS* mRNA was high from 2 to 8 days after planting but *rbcS* protein was only synthesised between days 2 and 5, again implying a translational control of *rbcS*.

One area where post-transcriptional regulation has also been shown is in organ specific expression. In duckweed there is expression of *rbcS* genes in the roots because, as an aquatic plant, its roots are exposed to light. When the transcription of two of the duckweed's *rbcS* genes is examined both SSU1 and SSU5B are transcribed in roots and fronds, but at the steady state mRNA level the mRNA for SSU5B is undetectable in roots. This indicates that post-transcriptional events control the organ specific expression of SSU5B (Silverthorne and Tobin, 1990).

1.4 Expression from *rbcS* promoters in a transgenic environment

All of the work described in Section 1.4, despite showing that there is regulation of *rbcS* mRNA by post-transcriptional mechanisms, also shows that the primary mechanism involved in the regulation of *rbcS* mRNA levels is the control of transcription itself. In all of the cases in which light brings about an increase in *rbcS* mRNA steady-state levels, the basis of this increase has been found to be an increase in transcription. The post-transcriptional controls then provide a secondary modulation of

rbcS gene expression. Thus it is not surprising that there has been intensive study of the regions of the *rbcS* genes that control the rate of transcription in both transformed callus and transgenic plants.

1.4.1 Expression from *rbcS* promoters in transformed calli

The early work on the promoters of *rbcS* genes was carried out before the production of transgenic plants was commonplace. Therefore, to examine expression directed by defined *rbcS* promoter sequences, experiments were carried out by transforming protoplasts with the required construct and then culturing the transformed tissue and looking at the effects of light and dark growth on these tissues. Broglie et al. (1984) transformed petunia protoplasts with the whole of a pea *rbcS* gene along with a kilobase of sequence 5' to the transcription start site. They found that the gene was transcribed in the transformed callus, was correctly processed, and produced a small subunit protein that could be distinguished from the native petunia small subunit protein. Furthermore, they also found that the level of the pea *rbcS* mRNA would fall if green callus was placed in the dark for 4 weeks and would rise if the callus was returned to the light for 2 weeks. These effects were not seen on the mRNA from a co-transformed kanomycin resistance gene under the control of a nopaline synthase promoter and 3' regions, which was equally expressed in light and dark grown callus. Thus the pea gene was behaving somewhat like a pea gene would in a pea leaf and this experiment showed that the mechanisms of photoregulation via 5' sequences were sufficiently conserved to allow experiments in heterologous environments.

That it was likely to be the upstream region that was conferring this light regulation on the pea *rbcS* gene in the petunia callus was shown by Herrera-Estrella et al. (1984). They used the 5' sequences from another pea *rbcS* gene and fused it to a coding region containing the bacterial gene, chloramphenicol acetyl transferase (CAT). This gene encodes an enzyme whose product is easily assayable and has been much

used as a reporter gene. This construct was transformed into tobacco protoplasts and transformed tissue was cultured. They found that light-grown callus contained significantly more CAT activity and RNA than dark-grown callus. Also the level of CAT activity in dark-grown calli that were placed in the light for 18 hours rose 5 to 15 times that present in the dark-grown callus. This effect was much less evident with a CAT gene which was fused to the 5' region of a nopoline synthase gene. The CAT activity from this gene increased only twofold on transfer of dark-grown callus to light and similar results were found when nopaline synthase activity was measured, there also being an intact nopaline synthase gene present in the T-DNA. The increase in CAT activity seen from the pea *rbcS* promoter was correlated with an increase in level of CAT mRNA in light-grown callus.

These two groups then started to define regions of the upstream region that were responsible for light induction. Morelli et al. (1985) showed that a *rbcS* gene derived from the pea *rbcS-E9* gene with only 35 bp upstream from the transcription start site (including the TATA box) was sufficient to confer light regulation on the *rbcS* coding sequence in transformed petunia callus. They also showed that the deletion of the sequences from -1052 to -352 (- indicates the number of bases 5' to the start of transcription) resulted in a drop in the level of transcription to 15 to 20% of that from the full promoter. Timko et al., also in 1985, showed that the -92 fragment of the pea *rbcS cc3.6* promoter was sufficient to confer light induction on a CAT gene in tobacco callus. They also showed that the remaining part of this 5' region (-973 to -90) would restore the level of expression from the -92 promoter to that of the full -973 promoter when fused to it in either orientation. This is one of the properties of enhancers in animal systems and the -973 to -90 sequence was called "enhancer-like" because it didn't function 3' to the coding sequence.

The major problem with these early experiments was that they were carried out on callus produced by transformation with oncogenic Ti plasmids. These plasmids, as well as transferring the engineered *rbcS* genes, also transferred genes for phytohormones in their T-DNA to induce callus formation. This meant that the callus

contained artificially high levels of phytohormones which wouldn't be found in the leaves of plants. Another problem with the use of callus was that complex media were needed to culture them and these media differed between light and dark growth conditions. One major constituent of the media was sucrose which is an end product of photosynthesis and has been shown to inhibit *rbcS* expression. These problems may explain the long times taken for light responses in callus when compared to those in plants (weeks as opposed to hours/days).

1.4.2 Expression from *rbcS* promoters in transgenic plants

The answer to the problems of studying the expression of *rbcS* genes in callus was solved by the use of non-oncogenic Ti plasmids to transform plant tissue. The lack of phytohormones allowed transgenic plants to be recovered from transformed tissue by manipulating exogenous hormone conditions. Nagy et al. (1986) transformed tobacco and petunia protoplasts with the full *rbcS-E9* promoter and coding sequence and also with a construct containing a -352 promoter. Transgenic plants obtained from these experiments expressed the pea *rbcS* gene in leaves in a light dependent manner. The pea *rbcS* transcript was not present in roots and stems implying that the information for organ-specific and light-induced transcription was contained within the -352 bases from the transcription start site.

Fluhr et al. (1986b) examined whether the cognate TATA box region of the *rbcS-3A* gene was required for this control. They fused a -327 to -48 fragment of the *rbcS-3A* promoter, in both orientations, to a -46 to +8 fragment of the Cauliflower Mosaic Virus (CaMV) 35S promoter and used this to transform plants. The expression of these constructs was compared to other constructs. The full -327 to 0 *rbcS-3A* promoter drove CAT expression in a light and organ-specific manner and this pattern of CAT expression was seen with both of the hybrid promoters. A construct containing just the CaMV 35S -46 to +8 promoter fragment fused to CAT was not expressed at all.

This implied that the -327 to -48 fragment contained all the information to confer light and organ-specific expression on a heterologous promoter. The paper also aligned the 5' upstream sequences of the *rbcS-3A* and *rbcS E9* genes and found three conserved 'boxes' of sequence upstream from the TATA box which was itself part of a conserved sequence. One of these sequences, Box II, resembled the core sequence of SV40 mammalian virus enhancer.

It was on the function of these boxes (present between bases-169 and -112) that research effort next focused. Kuhlemeier et al. (1987) synthesised a 58 base oligonucleotide that contained the three conserved boxes (I, II, and, III) and placed it in front of the promoter fragment from CaMV 35S (-48 to +6) driving a CAT gene. The correct distance between the three boxes and the TATA box of the CaMV promoter was maintained by inserting a synthesised DNA stuffer fragment. This promoter construct was not able to express the CAT gene in transgenic tobacco implying that the three boxes on their own could not function as an enhancer. However, if the CaMV 35S enhancer fragment was placed upstream from this promoter construct the expression from this promoter was seen to be down regulated in the dark when compared to a promoter without the 58 base insert. The light levels from these two promoters were the same implying that the three boxes functioned as a negative element in this context. It was also found that if three copies of box II were present instead of the three different boxes there was still a down regulation in the dark. Box III also proved to have negative regulatory qualities in the dark in a different promoter context. The paper also described the presence of a positive regulatory element (an element which when removed caused loss of light stimulation) between bases -166 and -149 which was found by sequential deletions of the *rbcS-3A* promoter. This positive element overlapped the sequence containing the three boxes which had been shown to be a negative element.

The importance of each of the three boxes was then examined by mutating each of them in turn in a -166 *rbcS-3A* promoter and comparing the expression from the mutated gene to a standard unmutated construct transformed into the plant in the same

plasmid. This showed that the loss of box I had no effect on transcription, but that the loss of boxes II and III caused transcription to drop to near undetectable levels in light grown leaves (Kuhlemeier et al., 1987b).

I have previously mentioned that the similarity of the box II sequence to the core sequence of the SV40 virus had been noticed. This core sequence required the presence of 2 guanine residues in the sequence for it to be transcriptionally active. If the analagous guanine residues in the box II sequence were changed to cytosines expression was lost. These mutations didn't have an effect if they were present in the context of a -410 *rbcS-3A* promoter implying that there were redundant sequences present in the -410 to -166 fragment that could carry out the same functions as the mutated boxes. In fact there were sequences similar to the box II and III sequences in this part of the promoter and these were called box II* and III* (Kuhlemeier et al., 1988a). Subsequent developmental experiments with the -166 and -410 promoters showed that the -166 promoter could not support expression in young tobacco leaves, in contrast to the -410 promoter, implying that development is important in determining which parts of the promoter are used (Kuhlemeir et al., 1988b). It was also demonstrated that the -410 to -50 *rbcS-3A* enhancer, when fused to the -46 to +6 CaMV 35S promoter fragment, was capable of specifying the correct cell type for expression of a CAT gene (Aoyagi et al., 1988).

These experiments in transgenic plants showed that the *rbcS-3A* enhancer was capable of endowing organ-specific and light regulated expression on a CaMV 35S promoter-driven reporter gene. The experiments in calli had indicated that a *rbcS-E9* promoter fragment (from -35 to 0) was capable of conferring light regulation on its coding sequence. Experiments to examine the promoter in transgenic plants proved difficult because no expression was seen with a -50 to +15 *rbcS-3A* driven CAT gene. However if a heat shock element enhancer was used (which did not confer light regulation on a construct with the CaMV 35S promoter) light regulation of the CAT gene was seen in leaves at 40°C. In these plants expression was also seen in the roots

indicating that although the promoter can confer light regulation it may not confer organ specificity.

These types of experiments have also been done using the *rbcS* promoters from other plants. Poulsen and Chua (1988) examined the architecture of the *Nicotiana plumbaginifolia rbcS-8B* gene's 5' region in transgenic tobacco plants. They found that the -1038 to -102 region contained two enhancer-like elements. The element nearest the TATA box (-312 to -102) confers light regulation on the -90 to +6 CaMV 35S promoter/CAT gene construct and also limits its expression to chlorophyll-containing tissue, like the pea enhancers. In contrast, the element further upstream only confers tissue specific expression on this construct, the level of CAT in the leaves being the same in the light and dark. This implied that, in some cases, tissue specific and light regulation can be separated into different DNA control sequences.

Similar experiments have also been undertaken with a tomato *rbcS* promoter (Ueda et al., 1989), which also revealed the presence of positive and negative elements. Schaffner and Sheen (1991) compared the expression of CAT from different *rbcS* promoters in maize and tobacco protoplasts. They found that maize and wheat *rbcS* promoters would drive expression of the CAT gene in maize protoplasts, but that pea, tobacco, and *Arabidopsis rbcS* promoters did not function in this context. The opposite was true if tobacco protoplasts were used implying that the control of *rbcS* genes by their promoters has evolved differently in monocots and dicots.

Another question that was examined using the transgenic plant system was whether the steady state level of *rbcS* mRNA from the individual members of a *rbcS* gene family was due to differing promoter 'strengths' or to differential processing of transcripts. This was examined in pea by Kuhlemeier et al. (1988b). They used a transformation vector which contained the full copy of the *rbcS* gene (promoter+coding sequence+3' non-coding sequence) to which another gene was to be compared. This gene was placed in the same vector in a cloning site about 5 kilobases away, thus ensuring that both genes are present in the same position in the host plant's genome. When they compared the *rbcS-3A* gene to the *rbcS-E9* gene (present in its

entirety) they found that exchanging the 3' non-coding region of the *rbcS-3A* gene with that from *rbcS-E9* gene had no effect on the chimeric *rbcS-3A* gene's expression relative to the *rbcS-E9* gene. Deletion of the *rbcS-3A* gene's introns also had no effect on its relative expression as did placing the -50 to +33 region from the *rbcS-E9* gene into the *rbcS-3A* gene either with *rbcS-E9* coding sequence or with the *rbcS-3A* coding sequence. These results implied that it was the *rbcS-3A* enhancer sequence (upstream from -50) that determined the expression level of the *rbcS-3A* gene.

Similar experiments were done by Dean et al. (1989a and b) with the most abundantly expressed petunia *rbcS* gene (SSU301), and the least expressed petunia *rbcS* gene (SSU911). By replacing fragments of the 911 promoter with the equivalent region of the 301 promoter they found two regions of the 301 promoter (-258 to -178 and -291 to -204) that would raise the expression of the chimeric 911 gene 25-fold. These fragments had this effect in either orientation and had the same effect on expression levels of the 911 transcript level as replacing its native promoter with the whole of the 301 promoter (Dean et al., 1989a).

However, unlike the situation in pea, the placing of the 911 coding region under the expression of the 301 promoter didn't raise the expression of the 911 transcript to the level of the 301 transcript driven by its own promoter. Also, placing the coding region of the 301 gene under the control of the 911 promoter resulted in it being expressed at a level only 15 times greater than the 911 gene. This implied that, as well as the promoter affecting the expression level, there were elements in the coding or 3' non-coding regions of the two genes that had an effect. Experiments with chimeric 911 genes consisting of the 911 promoter fused to more and more of its coding sequence and less and less of the coding sequence and 3' non-coding sequences of the 301 gene were undertaken to see which part of the 301 coding sequence gave increased level of expression from the 911 promoter. These experiments revealed that the 3' non-coding sequence of the 301 gene fused to the 911 coding sequence was as effective in raising the level of expression from the 911 promoter as using all of the 301 coding sequence

and 3' non-coding sequence. This implied that the 3' non-coding sequences were important in regulating *rbcS* mRNA levels in petunia.

To examine whether the effect of the 3' sequences was exerted at the transcriptional level run-on transcription assays were performed with the transgenic plants. It was found that the presence of 3' sequences from 301 increased the transcription rate from the 911 promoter, when compared to the intact 911 gene. This indicated that the 3' sequences of the 301 gene were increasing transcription from the 911 promoter which would result in the increase in steady state levels that were seen. However, the possibility of post-transcriptional regulation was not ruled out.

1.4.3 The involvement of DNA binding proteins in *rbcS* transcription

The identification of DNA sequences that are important in the regulation of *rbcS* genes raised the obvious question as to how these sequences were responsible for the regulation that they were able to endow upon a promoter. In animal systems promoters, such as those of the *Drosophila* heat shock genes, had been shown to bind specific trans-acting protein factors. Thus it was for trans-acting protein binding factors that researchers began searching.

The work on the pea *rbcS* genes described in the previous section had identified the importance of the box II and box III sequences for light regulation. Green et al. (1987) used gel retardation assays to examine whether these sequences bound protein factors. Using the -330 to -90 pea *rbcS-3A* promoter, which contains these sequences, as a labelled probe they found two band shifts indicative of two protein DNA complexes formed on the probe. These bands could be decreased if cold probe was added as a competitor to the binding reaction but were unaffected if a cold CaMV 35S promoter fragment was added, implying that the bands were due to binding specific to the pea *rbcS* promoter. If the -330 to -90 sequence was divided into two pieces, -330 to -171 and -170 to -50, it was found that both the fragments would form

a DNA/protein complex and could compete against the full -330 to -50 fragment. Experiments with -170 to -50 promoters with the I, II, and III boxes deleted indicated that boxes II and III were essential for formation of the DNA/protein complex. These boxes were also protected in DNAase 1 footprinting experiments indicating that there was a protein(s) bound to them specifically. This protein was called GT-1.

DNA methylation experiments indicated that the 2 guanine residues at the centre of the box II motif were essential for binding of the factor *in vitro* and a mutant promoter containing these guanines changed to cytosines was examined by Kuhlemeier et al. (1988a) in transgenic plants and was found to be transcriptionally inactive (see previous section). This use of *in vitro* experiments to provide ideas for mutations to be tested in transgenic plants was to be the model for future dissection of the regulation of *rbcS* expression.

That there were likely to be other DNA binding proteins involved in the light response was indicated by the fact that the binding activity to boxes II and III was present in nuclear extracts from plants grown in the dark and in the light (Green et al., 1987). This implied that the light response was not going to be simply a matter of the presence or absence of this binding activity. Another nuclear binding activity to *rbcS* genes' promoters was found by Giuliano et al. (1988). Using fragments of a tomato *rbcS-3A* promoter they found that a fragment from -411 to -242 bound a protein in tomato nuclear extracts. This binding could be competed against by *rbcS* promoter sequences from pea, *Arabidopsis* and the other tomato *rbcS* genes. Comparisons of the sequences of these promoters showed that there were three conserved sequences in the promoters, called the L, I and G boxes. DNAase footprinting showed that it was the G box that was being protected by the binding activity and an oligonucleotide composed of the 12 nucleotides of the G box competed for binding as efficiently as the whole *rbcS* promoter against a labelled -411 to -242 fragment.

Subsequent work on the G and I box sequences showed that they were able to drive expression of a reporter gene in yeast. Donald et al. (1990) fused an *Arabidopsis rbcS-1A* promoter to a truncated iso-1-cytochrome C gene and found that expression

was dependent on the I and G boxes being intact. A fragment of the promoter that contained the G box was found to bind a factor in yeast nuclear extracts. Methylation interference studies were also carried out, using both yeast and *Arabidopsis* nuclear extracts to protect the promoter fragment. The patterns obtained from bound and unbound DNA were indistinguishable between the plant and yeast, and showed that the presence of a methyl group on the guanine residues in the G box prevented formation of the DNA/protein complex. This study demonstrated conservation between nuclear binding proteins between plants and yeast.

That the G and I boxes were also important for the expression of the *rbcS-1A* promoter in *Arabidopsis* was shown by Donald and Cashmore in 1990. They showed that the mutation of the G box within the *rbcS-1A* promoter reduced the steady state level of the reporter gene's mRNA 40-fold; I box mutations, in the same context, gave a 10-fold reduction. The reduction in expression seen with G box mutations was not as great if the promoter fragment was fused to a CaMV 35S -89 or -167 promoter fragment, implying that the function of G box binding could be mimicked to some degree by CaMV 35 S promoter binding proteins.

The ability of CaMV 35S DNA binding proteins to mimic the functions of some of the factors that bind to *rbcS* promoters has also been seen in experiments undertaken by Chua's group. Lam and Chua (1990) fused a tetramer of box II sequences to a -90 CaMV 35S promoter and found that the reporter gene under the control of this promoter was expressed in a light dependent and tissue specific manner. However, if the same tetramer of box II sequences was fused to a -50 *rbcS-3A* promoter no expression was seen. This implied that the binding of GT 1 on its own was not sufficient for expression and that some of the sequence between -50 and -123 (the start of box III) bound a factor(s) that was also required, along with GT 1, for expression from the promoter (Cuozzo-Davis et al., 1990).

Cuozzo-Davis et al. (1990) also studied the ability of the -330 to -170 sequence of the pea *rbcS-3A* promoter to control expression from the -50 *rbcS-3A* promoter. This sequence, which contains the boxes II* and III* was able to confer light regulated

and tissue specific expression on a reporter gene both with and without the box II* sequence intact. This is in contrast to the -166 promoter in which intactness of the box II sequences is a pre-requisite for expression, perhaps implying that the method of controlling expression is different between the two parts of the promoter.

The way the box II and III sequences were involved in controlling *rbcS-3A* expression was examined in more detail by Gilmartin and Chua (1990a). They looked at a wild-type -166 (box II-box III) promoter and compared it to two engineered forms, one containing two box II sequences, and one containing two box III sequences. They found that the box II-box II derivative would bind GT 1 *in vitro* as efficiently as the wild type -166 promoter whereas the binding of GT 1 to the box III-box III derivative was much less efficient. When analysis of the promoters was carried out in transgenic plants it was found that the expression levels of the *rbcS-3A* coding sequence, as measured at the steady state RNA level, mimicked these binding affinities, with expression from the box II-box II promoter being indistinguishable from the wild-type -166 promoter and expression from the box III-box III promoter being 20-fold less. This demonstrated a correlation between *in vitro* binding ability and expression.

The result of changing the spacing between the two boxes was also studied by Gilmartin and Chua (1990b). They found that insertion of as few as 2 bases between box II and box III (wild-type distance : 12 base pairs) would significantly reduce expression from a promoter containing this mutation in transgenic plants. Deletion of bases between the sites had a lesser effect with a deletion of 8 bases reducing expression slightly. However a 10 base pair deletion would completely abolish expression. These mutants would all bind GT 1 *in vitro* implying that it was the relative conformation of the bound GT 1's that was important.

The fact that deletion of box III abolished expression when deleted from the -166 promoter, yet only bound GT 1 weakly led to an in depth study of box III and its surrounding sequences. Sarokin and Chua (1992) studied the effect of scanning 2 base pair mutations through the box II binding site. They found that mutations in the 5' and 3' flanking sequences which did not affect GT 1 binding *in vitro* caused a significant

drop in transcription when assayed in transgenic plants. This suggested that there was loss of binding of factors other than GT 1 which caused the drop in expression. Using oligonucleotides containing the flanking sequences of box III two separate binding activities were identified, one specific to each end of box III. The sequences bound by the factors contained the GATA motif, previously identified in the CaMV 35S promoter (as-2 element : Lam and Chua, 1989) among others. The as-2 sequence could compete with the sequence at the 3' end of box III for binding implicating this motif as important in binding the 3 ' factor (named 3AF3). This was proved when mutants of this motif were unable to bind the 3' factor when introduced into the 3' oligonucleotide probe.

The degree of band shift seen was different for both factors when nuclear extracts were prepared from light and dark grown plants. In both cases the probe was retarded more by the 'light-grown' extract. Treatment of the light-grown extract with phosphatase resulted in the production of the faster moving complex seen when 'dark-grown' extract was used. This implied that the factors were phosphorylated in the light-grown extract. Phosphatase treatment of the dark-grown extracts revealed differences in the effect of phosphorylation on the two binding activities. The binding activity to the 3' oligonucleotide is lost in phosphatase treated dark-grown extract suggesting that phosphorylation is essential for the binding activity of the factor. In contrast, binding to the 5' oligonucleotide is unaffected by phosphatase treatment of the dark-grown extract perhaps indicating a difference in the method of control of the two factors in response to changing light conditions.

The involvement of phosphorylation in the regulation of a plant DNA binding protein had previously been shown by Datta and Cashmore (1989). They found that a 12 base pair A and T containing consensus sequence (the AT-1 box) found in the pea *rbcS-3.6* , tomato *rbcS-3A* , and *cab-E* promoters would bind a factor from pea nuclei. Deletion of part of this sequence from the 5' end of the tomato *rbcS-3.6* promoter resulted in loss of expression from the promoter demonstrating the importance of the sequence. The binding activity of the factor was found to be dependent on the protein

being dephosphorylated, the opposite scenario to that seen with 3AF3. The effect of dark treatment on the binding activity was not presented.

The presence of a light regulated DNA binding activity has been shown in *Lemna gibba*. Buzby et al. (1990) found a binding activity in nuclear extracts prepared from light-grown plants which was much reduced in plants that had been dark adapted for upto 7 days. The activity also increased two to threefold if light grown plants which had been placed in the dark for one day were returned to the light. The involvement of phosphorylation in the activity was not investigated although mixing of light extracts with dark extracts was said to give an additive effect, perhaps suggesting that something in the light extract was able to activate binding activity in the dark extract.

There is also a protein present in tobacco nuclear extracts that was identified by its ability to bind a tetramer containing the GATA motif. This protein, GAF-1, was shown to be more abundant in nuclear extracts from light-grown plants than those prepared from dark-grown plants (Gilmartin et al., 1990). The GATA motif has been found in the promoters of cab genes (Gidoni et al., 1989), *rbcS* genes (Dean et al., 1985) and also in the CaMV 35S promoter (Lam and Chua, 1989). In this context it binds a protein, ASF-2, which is present in equal amounts in both light and dark adapted plants. This implies that different proteins bind to motifs containing the sequence GATA depending on the 'context' of the sequence. However, the presence of more GAF 1 in light grown tobacco compared to dark adapted tobacco implies that the GATA motif probably has a role in light regulation.

That the modification of DNA binding proteins is important in light regulation of *rbcS* genes has been shown by Manzara et al. (1991). They examined the DNAase 1 'footprints' of the five tomato *rbcS* promoters. They found that there was no difference in the footprints seen with nuclear extracts prepared from dark-grown cotyledons and those prepared from cotyledons exposed to the light for 6 hours which implies that the changes in transcription seen during this period must be due to modification of these factors. In contrast, the footprints from nuclear extracts prepared from the different organs of the tomato plants were found to differ, indicating that organ specific

regulation may be controlled by the presence or absence of transcription factors (Carrasco et al.,1993; Manzara et al., 1993).

1.5 Analysis of the photoreceptors involved in photoregulation of *rbcS* genes

In the work described in the previous sections of this Introduction I have described differences in the expression of *rbcS* genes between light-grown and dark-grown plants. In all these cases the light sources used were white light sources. Plants have evolved to use the light received efficiently, to maximise photosynthesis in different light environments. To do this plants have evolved various photoreceptors to measure the presence, quantity, quality, duration and direction of the light that they are receiving. These photoreceptors include phytochromes, a family of molecules that absorb mainly in the red/far-red end of the spectrum, blue UV-A and UV-B photoreceptor(s) and also the photosynthetic pigments themselves. Various combinations of these photoreceptors have been shown to control, for example, gene expression and enzyme activities in order to adapt the plants to the light they are receiving.

1.5.1 The Phytochromes

The presence of a photoreceptor which, when it absorbed red light, would initiate a response which could be negated by a subsequent far-red light treatment was first shown by Borthwick et al. in 1952. They were studying the action spectra for the initiation of germination in lettuce seeds and found that the red wavelengths of the spectrum were the most effective in producing germination. They also found that if the red light treatment (R) was followed by treatment of the seeds with far-red wavelengths

(FR) the effect of the red light was reduced (R : R followed by FR (R/FR), 98% germination compared to 54% germination). They also found that it was the last light treatment that was important in that a R/FR/R treatment gave 100 % germination whereas a R/FR/R/FR treatment gave 43 % germination. This led them to propose a photoreceptor which, when it absorbed red light, would convert to an active far-red absorbing form (P_{fr}). When this form absorbed far-red light it would reconvert to the inactive red absorbing form (P_r) This red / far-red photoreversibility, now known as the low fluence response (LFR), is a classic test for the involvement of phytochome in a response. Other responses shown to have the same action spectra were the inhibition of flowering in short-day plants, the promotion of flowering in long-day plants and photomorphogenesis in etiolated peas (for references see Colbert, 1988). Thus a single photoreceptor could be seen to be involved in a large number of different responses.

The knowledge of the action spectra for both the induction and the negation of responses led to the development of a spectrophotometric assay for this receptor which became known as phytochrome (Butler et al., 1959). The same paper described the partial purification of a protein detected by this assay. This was extracted from etiolated tissue which contained very little pigmentation, such as chlorophyll, to 'cloud' the assay. However, the purification of an undegraded phytochrome, which had the same spectral properties *in vitro* as *in vivo*, was not achieved until 1983 when Vierstra and Quail purified a 124 kDa phytochrome from etiolated wheat.

Prior to this, partially degraded phytochrome had been used to raise antibodies against phytochrome. When these were used for radio-immunoassays on extracts from etiolated and light-grown tissue it was shown that phytochrome was 50 to 100-fold more abundant in the etiolated tissue (Hunt and Pratt, 1979). This was shown to be due to the preferential degradation of the P_{fr} formed on illumination (Schäfer et al., 1975) and the down-regulation of the mRNA for phytochrome on illumination (Colbert et al., 1983). This down-regulation was shown to be under the control of phytochrome itself (Colbert et al., 1983).

The fact that the phytochrome mRNA was present at a higher level in etiolated tissue, and the existence of antibodies against phytochrome enabled the cloning of a phytochrome cDNA from wheat by differential screening (Hershey et al., 1984). Subsequently phytochrome cDNAs and genes have been cloned from a number of species including pea (Sato, 1988), rice (Kay et al., 1989), and *Arabidopsis* (Sharrock and Quail, 1988). Southern blot analysis of the *Arabidopsis* genome implied that there were five phytochrome genes (Sharrock and Quail, 1989). Three of these have been shown to encode different proteins through the cloning of three different cDNA sequences (*phyA, phyB* and *phyC*) for phytochrome from *Arabidopsis* (Sharrock and Quail, 1989), and the presence of the other two genes (*phyD* and *phyE*)has been shown through cloning of their sequences from *Arabidopsis* (Quail 1991). The phytochrome that is prevalent in the etiolated tissue and is light labile, has been called Type I phytochrome and is encoded by the *phyA* gene. The type II phytochromes, which predominate in green tissue, are encoded by the other phytochrome genes (*phyB-E*), although it has yet to be unequivocally shown that *phyD* and *phyE* encode functional polypeptides (Quail, 1991). The Type II phytochromes were thought to be stable in the light but studies on the three phytochromes of wheat (Wang et al., 1991) have shown that while the Type I phytochrome (124 kDa) is much more prevalent in etiolated wheat seedlings than in light grown seedlings, the levels of the other two phytochromes (Type II phytochromes) also fall on illumination (Wang et al., 1993).

The existence of a family of phytochrome molecules has led to attempts to assign different roles to the different types of phytochrome. Smith and Whitelam (1990) suggest that the high level of Type I phytochrome present in etiolated seedlings may act as an 'antenna' for the presence of light. This would enable a rapid switch from the etiolated mode of growth to the suppression of hypocotyl extension and the promotion of leaf expansion, chlorophyll synthesis and development of chloroplasts that is required for photosynthesis to occur. The roles put forward for the other phytochromes present in the light grown plant include shade avoidance, photoperiodism, and control of flowering among other functions (Smith and Whitelam,

1990). These ideas are now being tested with the use of phytochrome mutants and phytochrome over-expressing transgenic plants.

Despite absorbing light primarily in the red/far-red region of the spectrum, phytochrome also absorbs in the blue. The absorbance of blue light by phytochromes is usually involved in the high irradiance reactions (HIR) of phytochrome. The HIR are apparent when plants are subjected to a long continuous irradiation of a relatively low fluence rate (Smith et al., 1991) and are characterised by the failure of the response to conform to the Bunsen-Roscoe law of photochemical equivalence (Mâcinelli, 1983). This law (the reciprocity law) states that the action of photochemically active light is solely dependent on the dose (the intensity of the light (I) multiplied by the duration of the light treatment (t)). If this is not seen in the response studied it implies that either; 1) the concentration of the photoreceptor is changing with I and/or t, or that 2) the response is due to the interaction between the products of two photochemical reactions. The spectra of HIR have peaks in the blue/UV-A, far red and to a lesser extent in the red. These responses have been explained as being solely a phytochrome response. The concentration of phytochrome A falls on exposure of etiolated tissue to light when P_{fr} is formed and preferentially degraded. The HIR is though to occur under a continuous, relatively low irradiance because the amount of P_{fr} formed is enough to initiate the response, but not enough to be degraded rapidly . If a higher fluence rate is used for a shorter length of time to give the same dose of light, then the P_{fr} formed is degraded more rapidly and thus the response is smaller. This explains the failure of the response to conform to the law of reciprocity (Smith et al., 1991).

1.5.2 Blue/UV-A photoreceptors

In contrast to phytochrome, a molecule, or molecules, which match the action spectra found for blue/UV-A initiated responses has yet to be identified. Examples of these spectra can be found in The Blue Light Syndrome (ed. Senger, 1980) and Briggs

and Iino (1983). The action spectra for different responses can be seen to have different absorption maxima. Comparisons of these action spectra with various pigments found in plants have lead to suggestions that carotenoids, flavins and/or pterins could be candidates for the photoreceptor (De Fabo, 1980; Shropshire Jr., 1980; Galland, 1992). Briggs and Short (1991) found that the blue light induced phosphorylation of a protein from etiolated pea stems is blocked when flavin is held in the reduced state by treatment with phenylacetic acid. They concluded that the photoreceptor in this case was a flavin. Responses shown to involve light perception by the blue/UV-A photoreceptors include chloroplast movement in response to high or low fluence rates of light (Haupt and Scheurlein, 1990), stomatal opening (Zeiger, 1990), inhibition of hypocotyl elongation (Young et al., 1992), phototropism (Steinitz and Poff, 1986) and leaf movement in *Phaseolus vulgaris* (Nishizaki, 1990) among other responses.

One of the problems in showing that a response involves a blue/UV-A photoreceptor is that phytochrome also absorbs in the blue end of the spectrum (Viestra and Quail, 1983), as does chlorophyll (Salisbury and Ross, 1978) and absorption in this region of the spectrum is often seen in the HIR described earlier. The problem of deciding whether a response with a blue action spectrum is due to the action of a blue/UV-A photoreceptor, or to the absorption of blue light by phytochrome was addressed by Schäfer and Haupt (1983). A simultaneous irradiation with blue and far-red light that produces a response that is lower than the response with blue alone shows that phytochrome is involved in the response, but does not prove that it is the sole photoreceptor operating. The action of P_{fr} may be required for the blue photoreceptor to produce a response. Another way to look at the problem is to use the fact that phytochrome is a photoreversible pigment. In such a pigment the two forms are present in equilibrium with each other dependent on the incident light; i.e. under a given light condition the photochemical reactions P_r--k1-->P_{fr} and P_{fr}--k2-->Pr lead to an equilibrium between P_r and P_{fr}. The 'light equivalence principle' (Schäfer, 1983) states that if the values for k1/k1+k2 and k1+k2 are the same under two different light regimes for the same photoreceptor then that light will be perceived as the same if only

that photoreceptor is used in the response measured. As blue light is much less effective at photoconverting phytochrome (Schäfer, 1981) than red wavelenghths then the effect of blue light on a response would be equivalent to an irradiance of 10 to 100 fold lower fluence rate between 690 and 700 nm (Schäfer and Haupt, 1983). An example of this kind of analysis can be seen in the analysis of the inhibition of hypocotyl extension in *Sinapis alba* (Holmes and Schäfer, 1981).

1.5.3 Photoregulatory mutants

The existence of different photoreceptors has been postulated through photobiological experiments. Direct evidence has come from the study of mutants in photomorphogenic responses. These studies have shown that mutants can be found in both red and blue light responses, indicating that the receptor systems for these responses can be separated genetically. The action of light on dark-grown plants has also been shown to act by activating 'light-like' development which is repressed in dark-grown plants. This has been demonstrated through the isolation of mutants that have lost this ability to repress 'light -like' development in dark-grown plants.

1.5.3.1 Phytochrome mutants and transgenic plants

Some of the first photoregulatory mutants were found by looking for mutant plants that had elongated hypocotyls in white light; i.e. plants that did not show de-etiolation type responses. These types of mutants include the *hy* mutants of *Arabidopsis* (Koornneef et al., 1980; Chory et al., 1989; Parks and Quail, 1993), the cucumber *lh* mutant (Adamse et al., 1987) and the *aurea* mutant of tomato (Adamse et al., 1988). The majority of these mutants have been shown to be deficient in active phytochrome. Parks et al. (1989) showed that *hy-1* and *hy-2* had wild type levels of

phytochrome apoprotein despite their phenotype. The lack of phytochrome function in these mutants was shown to be due to mutations in chromophore synthesis (Parks and Quail, 1991). The phenotype of *hy-3* has been shown to be due to a deficiency in the protein encoded by the *phyB* gene (Somers et al., 1991) and this has subsequently been shown to be due to a lesion in *phy B* (Reed et al., 1993). The *aurea* mutant of tomato has been shown to be deficient in the light labile PHY A phytochrome (Lopez-Juez et al., 1991).

Analysis of the photomorphogenic responses of these mutants has shown that there are different roles for the different phytochrome species. McCormac et al. (1992) examined anthocyanin synthesis and hypocotyl elongation in wild type and mutant *Arabidopsis*, tomato and tobacco as well as mustard. They found that, on transfer to white light, supplemental far-red light caused an increase in anthocyanin synthesis and a suppression of hypocotyl elongation in all the wild type plants. After a period of adaptation to the white light, which varied between species, addition of far-red light to the white light caused an increase in hypocotyl elongation and a decrease in anthocyanin synthesis. Thus the perception of far-red in etiolated tissue exposed to light can be seen to increase the responses typical of de-etiolation, whereas in the green plant supplemental far-red caused reactions involved in shade avoidance. Comparing the responses of the *phyB* -deficient *hy-3* mutant of *Arabidopsis* to those of the wild type, it could be seen that the initial suppression of hypocotyl elongation with supplemental far-red was seen on transfer of the etiolated plants to light (McCormac et al., 1992) However, the addition of far-red to white light-irradiated, light-adapted *hy-3* plants did not cause an elongation of the hypocotyl. This implies that PHY B type phytochrome is involved in the shade avoidance reactions seen in green plants. In contrast, *aurea* (the PHY A deficient mutant of tomato) behaved differently in the etiolated state, as expected because PHY A is the type of phytochrome that is predominant in etiolated seedlings. The etiolated *aurea* mutant of tomato showed immediate elongation of the hypocotyl on illumination with supplemental far-red light, a response that only manifested itself after three days in the wild type. This was interpreted as the *aurea*

mutant behaving immediately as a light adapted plant due to its PHY A pool not needing to be depleted on illumination.

Despite the fact that PHY A is not able to control the responses usually controlled by PHY B in the light-grown plant as shown in the *hy-3* mutant, PHY A is able to function in light-grown plants. This has been shown in transgenic plants containing a *phy A* gene under the control of the CaMV 35 S promoter. Oat *phy A* has been expressed in transgenic tomato (Boylan and Quail, 1989), and tobacco (Keller et al., 1989). These plants have a phenotype that is opposite to that of the phytochrome deficient mutants, being dwarfed with dark green foliage. The dwarfism can be interpreted as being due to Type I (PHY A) phytochrome responses (de-etiolation type responses) being present in the light-grown plant due to the excess of that phytochrome due to its expression from the strong constitutive promoter.

1.5.3.2 Blue light response mutants

A number of mutants in blue light responses have been identified. Koornneef et al. (1980) found that in one of their *hy* mutants, *hy-4* , hypocotyl elongation was supressed by red and far-red light but was not supressed by blue light, implying that the mutation in *hy-4* was involved in responses mediated by a blue light receptor. The supression of hypocotyl extension under blue light, and not far-red, was used by Liscum and Hangarter (1991) who isolated three mutants they called *blu 1*, *2*, and *3*. These plants, like *hy-4*, did not show supression of hypocotyl elongation in blue light. These plants, unlike phytochrome mutants, did not show a mutant phenotype in the mature plants indicating that the mutated products may only be important during seedling development.

Young et al. (1992) compared the effect of different wavelengths of light on the inhibition of hypocotyl extension in the *blu 1* (Liscum and Hangarter, 1991) and *hy-6* (Chory et al., 1989) mutants, as well as a *blu 1 hy-6* double mutant. They found that

the wild type plants showed some inhibition under continuous low fluence rate light of all wavelengths, and maxima under high fluence UV-A, blue and far-red light. The *hy-6* plants lost sensitivity to all these wavelengths at low fluences, whereas they retained the ability to respond to high fluence rate blue and UV-A light. The *blu 1* plants were shown to have lost the ability to respond to the blue wavelengths at the higher fluence rate, but retained their responses to red, far-red and UV-A light. The double mutants were found to only respond to the UV-A light. These results imply that in *Arabidopsis* seedlings there are three different photoreceptors involved in the photoregulation of supression of hypocotyl elongation and that they are ecoded by different genes.

1.5.3.3 Mutants in the control of de-etiolation

The previous two sub-sections discuss mutations in specific photoreceptors or their signal transduction chains. Recently there have been a number of studies on mutant plants that develop similarly to a light-grown plant in total darkness. Chory et al. (1989) screened a mutant population of *Arabidopsis* in the dark and looked for plants that had a de-etiolated appearance with a reduction in hypocotyl length. These plants were called *det 1* and showed chloroplast and leaf development, anthocyanin accumulation and transcription of several nuclear genes for chloroplastic proteins, including *rbcS*, that are normally only expressed in the light-grown tissue. Interestingly, the *det 1* plants, once illuminated, would switch off transcription of their nuclear chloroplastic genes if returned to darkness. This implies that the *det 1* mutation does not affect light / dark transitions once the plant is green, but does affect the initial switch between dark and light development. They suggested that in the wild type plant the *det 1* gene product acts to repress 'light' type development and that in the mutant this repression is not present allowing de-etiolation to occur.

A second *det* mutant (*det-2*) has been isolated by Chory et al. (1991). This mutant had a similar phenotype to that of *det-1* except that *det-2* plants did not initiate

chloroplast development in the etiolated plants. The two *det* mutants were crossed with the phytochrome deficient *hy* mutants, *hy -1*, *hy -2*, and *hy -6*. These mutants had the phenotype of the *det* mutants except that the *det-2 hy* double mutants had the pale green leaf colour of the *hy -1* mutants. These results imply that the *det* products function partially epistatically to phytochrome (Chory, 1992). This means that the loss of DET activity partially compensates for the loss of phytochrome in the double mutants.

A number of mutants with similar phenotypes to the *det* mutants have been isolated. Deng et al. (1991) isolated a mutant *Arabidopsis* that showed a constitutive photomorphogenic phenotype and called it *cop-1*. This mutant was shown to be genetically distinct from the *det* mutants and, like *det* , acted partially epistatically to phytochrome as shown by *cop-1 hy-1* double mutants having a similar phenotype to the *cop-1* mutant (Deng and Quail, 1992). Further *cop* mutants have since been isolated. Wei and Deng (1992) isolated *cop-9*, a distinct locus from *cop-1*, which conferred a very similar phenotype to *cop-1* mutations on the plant. Like *cop-1*, *cop-9* plants still showed phytochrome control over germination, a process which is lost in the *det-1* plants, implying that these mutations act in different signal transduction pathways. A recent study (Hou et al., 1993) screened for mutant plants that had enlarged cotyledons when dark grown, but still had the long hypocotyls typical of the etiolated plant. They found three different genes called *cop 2, 3,* and *4* and found that mutations of these genes would partially suppress the long hypocotyls of *hy-1* and *hy-3* in the light suggesting that, like the other mutants of this type, their gene products acted downstream of phytochrome. These mutants also showed that photomorphogenesis of different organs of the plant could be controlled by different genes.

This type of mutant has also been found in pea. Frances et al. (1992) found a mutant of pea (*lip 1*) that would develop like an etiolated plant in the dark. The plastids developed in the dark and the dark-grown mutant had 10-fold less spectrally detectable phytochrome than the wild type. This was attributed to a reduction of PHY A in the dark grown plant. The phenotype of the mutant was very similar to *det 1*.

1.5.4 Photoregulation of *rbcS* transcription in etiolated plants

In common with most photobiological systems, the initial examination of the photoreceptors controlling *rbcS* expression was done in etiolated seedlings where the light labile PHY A is the predominant phytochrome. Filner and Klein (1968) showed that a red light pulse would result in an increase the level of Rubisco activity in dark grown *Phaseolus vulgaris* seedlings. Far-red light would stop further accumulation of Rubisco activity up to 48 hours after the initial red pulse implying that the increase was mediated by the presence of P_{fr}. A similar effect on translatable *rbcS* mRNA was shown by Tobin (1981) in *Lemna gibba*. This indicated that phytochrome, the photoreceptor involved in mediating the increase, acted to increase the steady state level of *rbcS* mRNA. Silverthorne and Tobin (1984) carried out run-on transcription assays with nuclei isolated from dark grown plants treated with red, and red followed by far-red light. These experiments showed that the increase in *rbcS* mRNA levels in response to red light was mediated at the transcriptional level.

The phytochrome control of the initial increase in *rbcS* transcript levels on illumination of etiolated seedlings has been demonstrated in a number of different species including pea (Jenkins et al., 1983; Sasaki et al. 1983; Thompson et al.,1983), soybean (Berry-Lowe and Meagher, 1985) mustard (Oelmuller et al., 1986), and *Arabidopsis* (Dedonder et al., 1993). The control of *rbcS* expression by phytochrome in etiolated pea is interesting in that there are two separate responses seen at the level of *rbcS* mRNA transcript level when the plant is illuminated with white light. Gallagher et al. (1985) showed that there is an initial peak in *rbcS* transcription within 5 hours, and then a subsequent rise in the transcription of *rbcS* mRNA over the next 36 hours. Jenkins (1986) showed that there was a lag in the accumulation of *rbcS* mRNA when etiolated pea plants were illuminated with white light. He found that this lag was reduced if the plants were illuminated with five minutes of red light 24 hours prior to the continuous white light illumination. This reduction was not seen if the red light pulse was followed by a far-red pulse implying that phytochrome was the receptor

involved in reducing the lag. These responses were shown to occur at the level of transcription through the use of run-on transcription assays (Barnett et al., 1987).

The amount of red light required to induce transcription of the pea *rbcS* genes was studied by Kaufman et al. (1984) who compared it to the amount required to induce the *cab* genes.They found that the *rbcS* genes required about 10,000 fold more light (10 μmol/m^2) than the *cab* genes implying that the increase in *rbcS* transcription is mediated by the low-fluence responses of phytochrome (defined in Smith et al., 1991) ; i.e. the classical red/far-red reversible response. In contrast, the *cab* genes responded in the range of light typical of the very-low fluence response of phytochrome (defined in Smith et al., 1991) and this was supported by the fact that far-red light was sufficient to induce their transcription. That the very low fluence response had very little effect on the expression of the *rbcS* genes of etiolated pea was confirmed by Yoshida et al. (1989).

The regulation of the four *rbcS* genes of *Arabidopsis* in etiolated seedlings was examined by Dedonder et al. (1993). They found that a red light pulse would induce *rbcS* expression of three of the four genes 3 to 4 fold, although one gene (*rbcS 1B*) appears unresponsive. A far-red light treatment also results in an increase in *rbcS* mRNA level but to a lower level than that seen with red light. A similar level of induction is seen if a red pulse is followed by a far-red pulse, or if a blue light pulse is used. The response of the genes to different fluences of red light was also examined. The four genes did not respond identically; *rbcS-1A* and *rbcS-3B* were both induced by a fluence of 10 μmol/m^2, whereas rbcS-2B reqired a fluence of 10,000 μmol/m^2 for an equivilant level of induction, and *rbcS-1B* was not induced at all. This data showed that the individual genes of a gene-family could be rgulated differentially by phytochrome.

1.5.5 Photoregulation of *rbcS* expression during greening and in light grown plants

The photoregulation of *rbcS* genes during greening and in light-grown plants is more complicated that that seen in etiolated seedlings given pulses of light due to the need to adapt the development of the chloroplast to the prevailing light environment. Fluhr and Chua (1986) studied the photoregulation of two pea *rbcS* genes (*rbcS-3A* and *rbcS-3C*). They found that a pulse of red light would induce the accumulation of mRNA from these genes in etiolated pea seedlings to a level almost equivalent to that seen with a 24 hour white light treatment of these seedlings. The effect of the red light pulse could be negated by a subsequent far-red pulse, implying that phytochrome was the photoreceptor involved in mediating this response. However, if 21 day old light-grown pea plants were dark adapted for 4 days, a red light pulse had very little (*rbcS-3A*) or no (*rbcS-3C*) effect. Despite this, a white light treatment would return the *rbcS* mRNA level to light grown levels.

This developmental change in the photoreceptor usage was examined in transgenic petunia plants, transformed with both the pea *rbcS* genes examined. Mature petunia plants which had been dark adapted for 4 days were given a red light pulse, and the level of the *rbcS-3A* transgene measured. They found that this treatment did not raise the level of the *rbcS-3-A* gene, implying that the control of the pea *rbcS-3A* gene was conserved between species. A response to a red light pulse was seen if the transgenic plants were repeatedly cut back to promote the growth of axilliary buds. If these plants were given a red pulse of light an increase in the level of the pea *rbcS* mRNA was seen, and this could be negated by a far-red pulse. This phytochrome control was said to be apparent because the auxiliary buds were more immature and thus expressed the phytochrome control seen in the etiolated peas.

The wavelengths of light responsible for the white light mediated increase in *rbcS* expression seen in dark adapted mature pea leaves was examined by placing the dark adapted in continuous red or blue light regimes of equal fluence rates (10

μmol/m^2/s) for eight hours. These experiments showed that the blue fluences resulted in a 5 fold increase in *rbcS* mRNA levels relative to that seen with the continuous red treatment. Both the red and blue induced increases were not seen if supplemental far-red light was used, implying that the response induced by the blue light, presumably through a blue light receptor, required the presence of phytochrome for the response to manifest itself.

The plants used by Fluhr and Chua for their experiments on expression in mature pea leaves had been extensively dark adapted (4 days), and this may well change the balance of the phytochromes and may also result in loss of chlorophyll. Clugston et al. (1990) examined the effect of different qualities of light on pea plants that had been dark adapted for 5 hours, which should avoid these difficulties. They found that continuous red light of 120 μmol/m^2/s was 65 % as effective as white and blue light in raising *rbcS* transcription rates and mRNA levels. The difference between this study and that of Fluhr and Chua may also be due to the different fluence rates used, but it again showed the greater effectiveness of blue light relative to red light in green tissue.

Dedonder et al (1993) illuminated 9 day old etiolated *Arabidopsis* plants with constant red, far-red and blue light for twenty four hours. They found that the three different wavelengths of light were approximately equivalent to 24 hours of white light in their ability to induce *rbcS* mRNA levels. If continuous irradiation with white and red light was used, the *rbcS* mRNA levels were higher after ten days, than the levels seen with a 24 hour light treatment of 9 day old etiolated seedlings. In these experiments a blue light pulse was also given to 9 day old red light-grown plants prior to another 24 hours of red light. In this treatment the *rbcS* levels of three of the genes increased slightly over those seen with continuous red and white light. This implies the involvement of a blue photoreceptor.

Despite the fact that blue light appears more effective at regulating the level of *rbcS* mRNA in green tissue than in etiolated tissue, in some species, the presence of some phytochrome in its P_{fr} form appears to be required for the expression of *rbcS*

genes. Thompson and Meagher (1990) studied the effect of a 15 minute far-red light pulse on the expression of *rbcS* genes in light-grown petunia plants transferred to darkness. They found that such a far-red treatment would result in a much more rapid decrease in the transcription of the petunia *rbcS-3C* gene and its mRNA levels than just a dark treatment. However, the effect of a far-red treatment on the *rbcS-3A* gene was indistinguishable from that produced by dark alone. Similar effects of a far-red treatment on *rbcS* gene expression during dark adaptation were seen in soybean (Shirley and Meagher, 1990). These effects were shown to be mediated by the promoter regions of the soybean *rbcS* genes, as similar effects of a far-red treatment prior to a dark treatment were seen when these regions were used to drive the expression of a neomycin phosphotransferase gene in transgenic petunia plants (Shirley et al., 1987).

Despite the fact that P_{fr} appears to be required for expression of *rbcS* genes, that ratio of P_{fr} to the total phytochrome pool appears not to be important. Jenkins and Smith (1985) examined the effect of changing the ratio of red light to far-red light on hypocotyl extension and *rbcS* and *cab* mRNA levels. The differing ratios of red to far-red light were calculated to give *in vivo* P_{fr} to total phytochrome ratios of 0.78 to 0.37. The lowering of the P_{fr} to total phytochrome ratio caused the expected increase in hypocotyl length that is thought to be a shade avoidance response, but the levels of *rbcS* and *cab* mRNA were the same in all the treatments. This implies that the phytochrome controlled response to shading does not control the *rbcS* expression level.

1.5.6 The regulation of *rbcS* expression by increased irradiance

The responses of etiolated and dark-adapted light grown plants to light can be seen as the plant adapting itself to carry out photosynthesis. However, the light environment the plant finds itself in in nature is not constant and many factors of the

light environment may change, including the duration of the light period, and the quantity and quality of the light it receives. It has been known for some time that the quantity of light incident upon a plant can be seen to have profound effects on the morphology of the plant's leaves and chloroplasts (Boardman, 1977; Bjorkman; 1981, Anderson, 1986). Plants that are able to grow under high irradiances (sun plants), are able to adapt their chloroplasts such that the photon fluence rate required to saturate photosynthesis increases with growth under increasing irradiance. This also means that the maximum rate of photosynthesis the plants are able to sustain increases with increasing irradiance (Bjorkman, 1981). This process involves controlling the relative amounts of the components of the electron transfer chain of the thylakoid, the light harvesting complexes and the enzymes involved in carbon dioxide fixation (Boardman, 1977; Bjorkman, 1981, Anderson, 1986).

As Rubisco is the first of the enzymes that are involved in the fixation of carbon dioxide, it is unsurprising that it should be regulated in response to changes in the fluence rate of the incident light. Blenkinsop and Dale (1974) showed that growth of barley in increasing irradiances of light resulted in increasing amounts of Rubisco. They also showed that shading of high-light grown barley leaves resulted in a drop in the level of Rubisco, and that the effect of shading on the amount of Rubisco could be reversed by removing the shading. Interestingly, the effect of shading could be mimicked by placing a high light-grown leaf in a stream of carbon dioxide-free air, perhaps implying that metabolic feedback was involved in the shading response.

The effect of a drop in the irradiance incident upon a plant on the levels of the small subunit of Rubisco was also shown by Jenkins (1986). He showed that moving a *Phaseolus vulgaris* plants grown for 10 days in 300 $\mu mol/m^2/s$ to a lower fluence rate of white light (50 $\mu mol/m^2/s$) for three days resulted in a drop in the level of the small subunit in the primary leaves to 70% of that in the primary leaves of plants left at the higher fluence rate for the same length of time. If the experiment was repeated with plants grown at the lower fluence rate and moved to the higher fluence rate, the level of the small subunit in the high fluence treated plants' primary leaves was 161% of that in

the untreated plants. This increase in the level of small subunit protein was accompanied by an increase in the level of *rbcS* mRNA in the high fluence treated leaves approaching threefold that present in the 13 day old untreated plants. This suggested that the increase in the small subunit polypeptide was mediated by an increase in the *rbcS* mRNA level.

The increase in *rbcS* mRNA levels in response to an increased irradiance of light has also been seen in pea (Jordan et al., 1992) and tobacco (Prioul and Reyss, 1987 and 1988). Indeed, the work of Prioul and Reyss (1988) has shown that differing irradiances on the same tobacco leaf can result in different levels of *rbcS* mRNA and Rubisco within the same leaf. The plants in their study were grown under 60 $\mu mol/m^2/s$ white light for 90 to 95 days prior to some of the adult leaves being part shaded to give an incident fluence rate of 25 $\mu mol/m^2/s$. The other part of the leaf was given an increased fluence rate of 360 $\mu mol/m^2/s$. The leaves were sampled over the next two days, and they found that the amount of *rbcS* mRNA increased in the part of the leaf subjected to the higher irradiance and decreased in the shaded portion over the two days. These changes in *rbcS* mRNA were paralleled by changes in the amount and activity of Rubisco protein.

The photoreceptor involved in sensing the amount of light for these irradiance controlled responses is not yet known. Leong and Anderson (1986) found that growth of peas in red, far-red enriched and far-red deficient light environment did not alter the composition of the thylakoid membranes of the chloroplasts relative to a control light source over a range of light intensities. This suggested that the phytochrome photoequilibrium was not involved in the control of thylakoid composition with increasing irradiance.

Later work by Chow et al. (1990) showed an effect of continuous supplementary far-red light over a continuous white light background on the composition of the thylakoid membranes of the chloroplasts of 14 day old peas, when compared to those of plants that had not received the continuous supplemental far-red. They found that the supplemental far-red light caused an increase in the photosynthetic

capacity of the leaf similar to that seen when a leaf was given an increased irradiance. They suggested that the far-red light which could only be absorbed by photosystem one caused an increase in cyclic or pseudo-cyclic electron flow around this photosystem and that the ATP produced would result in an imbalance of metabolites in the chloroplast. The sensing of this imbalance would then lead to adaptation of the components of the chloroplast relative to each other such that the imbalance was corrected by the adaptation.

Another candidate for the detection of irradiance in light-grown plants is a blue light photoreceptor. Blue wavelengths of light have been shown to be more effective in increasing the mRNA level of *rbcS* in a number of plants as has been discussed. In the green alga *Chlorogonium elongatum* the amount of Rubisco and its mRNAs have been shown to increase with increasing fluence rate of incident light. The same study showed that the blue wavelengths were the most effective at inducing Rubisco implying that a blue photoreceptor is responsible for controlling the amount of Rubisco present (Roscher and Zetsche, 1986).

Drumm-Herrel and Mohr (1988) studied the effect of red and UV-A light on the appearence of Rubisco and *rbcS* mRNA in etiolated *Sorghum vulgare*. A blue light source had similar effects to the UV-A light source in initial experiments but the phytochrome equilibrium produced under the UV-A light source was identical to that obtained under the red light source, whereas the blue light produced a different phytochrome photoequilibrium. For this reason they used the UV-A source for further investigation. They found that the effect of UV-A was greater than that of red at the same fluence rate on both the increase in protein and *rbcS* mRNA. They also showed that increasing the fluence rate of the UV-A light caused an increase in the amount of Rubisco, a phenomenon that was not seen with the red light used. This implied that a UV-A photoreceptor was involved in measuring the amount of light the plant was receiving.

1.6 Conclusions

The research presented in this Introduction has shown that the *rbcS* genes of higher plants are usually present as small multi-gene families. They are expressed in the nucleus and encode a protein that is a larger molecular weight precursor. The transit peptide is cleaved on import of the protein into the chloroplast where the mature polypeptide forms the holoenzyme with the chloroplastically encoded large subunit to give the L_8S_8 Rubisco holoenzyme. This protein catalyses the primary fixation of atmospheric CO_2.

The *rbcS* genes are regulated, primarily at the level of transcription via a complicated set of protein DNA interactions. This regulation is tissue specific, developmental, and also environmental. One of the major environmental factors controlling *rbcS* expression is light. The perception of light by plants involves the use of a number of different photoreceptors which act primarily at different wavelengths of the spectrum, and their existence has been shown by a number of different experimental techniques. These include physiological experiments under different light regimes and the isolation of mutant plants. The analysis of expression using transgenic plants containing *rbcS* promoter sequences along with photomorphogenic mutants is leading to a greater understanding of the processes involved in photo-regulation.

1.7 The aims of this study

Phaseolus vulgaris was chosen as a system to study the expression of *rbcS* genes for a number of reasons. Firstly, work on the expression of the *rbcS* genes of *Phaseolus vulgaris* had shown that they were expressed to a significant level in the dark grown primary leaf, and S1 nuclease assays were unable to resolve whether each of the *rbcS* genes was photoregulated (Knight and Jenkins, 1992). The possibility that the least expressed of the three *rbcS* genes was less photoregulated than the other two

genes was suggested by preliminary dot-blot analysis of total RNA from light and dark grown plants hybridised to gene-specific oligonucleotide probes (Knight, 1990). One aim of this project was to see if this was in fact the case, because as can be seen from the experiments described in the Introduction, the control of *rbcS* expression is largely at the level of transcription. Thus, if one of the *rbcS* genes of *Phaseolus vulgaris* proved to be non-photoregulated, comparisons of the 5' sequences of this gene with those of the photoregulated genes would be instructive in the elucidation of the mechanisms of photoregulation. Similarly, any other differences in the expression of the three genes could perhaps be correlated to differences in their 5' flanking sequences.

Another reason for using *Phaseolus vulgaris* was the fact that its primary leaves present a very good system for the study of the ontogenetic expression of *rbcS* genes. This is because the primary leaves can be dissected out of the imbibed seed, have a morphology distinct from that of the other leaves of *Phaseolus vulgaris*, and can thus be studied from the seed to their senescence over approximately 30-35 days. The physiology of the primary leaves has been studied for a relatively long time, and it is known that the cell division is completed early in the development of the primary leaf and that most of the leaf growth is due to cell expansion (Volkenburg and Cleland, 1979; 1980; 1981). Thus changes in *rbcS* expression during the development of the primary leaf can be correlated to known changes in leaf physiology.

Previous work had also shown that the expression of *rbcS* genes in *Phaseolus vulgaris* can be controlled by changes in the fluence rate of the light environment of the primary leaves (Jenkins, 1986). The nature of the photoreceptor that controls this response is not known. Experiments were to be carried out using light sources of different qualities, but the same fluence rates, to examine whether the photoreceptor was phytochrome, the photosynthetic pigments, or a blue light photoreceptor. The level of control of this response was also to be examined through the use of run-on transcription experiments in isolated nuclei. These experiments would show whether the increased fluence rate caused an increase in the transcription of the *rbcS* genes. If

this was not the case then another mechanism, such as changes in the transcript stability, could perhaps be involved. If the response was shown to be transcriptionally regulated, elements of the promoter that control the response could perhaps be dissected from the whole promoter region in subsequent studies.

Finally, a method of measuring the relative amounts of the individual *rbcS* genes in a given RNA sample in a single reaction using a method based on the polymerase chain reaction was to be developed. This was carried out because the similarity of the 3' non-translated region of the three *rbcS* genes did not permit identification of their respective transcripts through the use of S1 analysis with a single probe (Knight and Jenkins, 1992). A method of this type should have the advantage that all three genes are being examined in the same reaction and therefore differences between reactions should not affect the estimation of the individual genes' transcripts. This factor should be taken into account when comparing different northern blots, and the two methods of measuring *rbcS* transcript abundance were to be compared.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study were obtained from BDH (AnalaR® grade ; Poole, Dorset) unless otherwise indicated.

2.1.2 DNA modifying enzymes

All restriction enzymes were purchased from Gibco-BRL, Paisley, Strathclyde together with their reaction buffers, which were provided at a 10 x concentration. T 4 Polynucleotide kinase was supplied by Boehringer Mannheim Gmbh.(Germany).

2.1.3 Radiochemicals

The radiochemicals used in this study were supplied by Amersham International plc. (Amersham, U.K.).

2.1.4 Plasmids and the bacterial strain used

The plasmids used are described in Table 2.1. *E.coli* TG 1 (Messing et al., 1981) was used as the recipient bacteria to enable amplification of the plasmids for plasmid DNA preparations.

Table 2.1 The plasmids used in this study.

The plasmids used in this study and their sources are described.

Plasmid	Description	Reference.
pUC 9	Same sequence as pUC 8 except for a different multiple cloning site	Viera and Messing (1982)
pPvSS 191	'*rbcS3* type' cDNA cloned into pUC 8	Knight and Jenkins, (1992)
pPvSS 1672	'*rbcS 1* type' cDNA cloned into pUC 8	Knight and Jenkins, (1992)
pPvSS 965	'*rbcS 2* type' cDNA cloned into pUC 8	Knight and Jenkins, (1992)
pCF 42	*Chlamydomonas reinhardtii* α tubulin cDNA cloned into pBR322	Donated by Dr. J. Schloss

2.1.5 The oligonucleotide probes used

Figure 2.1.1 (after Knight and Jenkins, 1992) shows the partial sequences of three cDNA clones, representating each of the three different *rbcS* genes known in *Phaseolus vulgaris*. The 3' non-translated sequences to which the three gene-specific oligonucleotide probes are designed are marked. Two different, non-discriminating, coding sequence oligonucleotide probes were used in this study. One is of the same length, GC content, and sense as the three gene-specific oligonucleotides and was used for the northern hybridisation experiments. The other is longer (25 nucleotides compared to 21) and is designed anti-sense to the oligonucleotides used for northern analysis. This olignucleotide was used for the RT-PCR experiments. The sequences to which these two oligonucleotides were designed are also marked on Fig 2.1.1. The sequences of all the oligonucleotides are shown in Fig 2.1.2.

2.1.6 Liquid and solid bacterial growth media

L-broth (1 % Bacto®-tryptone (w/v), 0.5 % Bacto® Yeast extract (w/v), (both supplied by Difco Laboratories, Michigan, USA) 1 % NaCl (w/v)), supplemented with the appropriate antibiotic, was used for the culture of *E.coli* . For the plating out of *E. coli* L-agar was prepared by adding 1.5 g/l agar (Difco) to L-broth prior to sterilisation. The appropriate antibiotic was added after sterilisation and prior to pouring the plates.

2.1.7 Antibiotics

E. coli containing the plasmid strains shown in Table 2.1.1 could be selected using ampicillin. This was supplied by the Sigma Chemical Co. (Poole) and was stored at 25 mg/ml in filter sterilised water. The antibiotic was added to the media after

Fig 2.1.1 The sequences of the 3' regions of the the three types of *Phaseolus vulgaris rbcS* genes and the regions of these sequences to which oligonucleotides were designed

The sequences of three *Phaseolus vulgaris* pPvSS cDNA clones, one representative of each of the three types of *rbcS* genes, are shown from nucleotide 601 to nucleotide 1000. The sequences to which oligonucleotides were designed are highlighted.

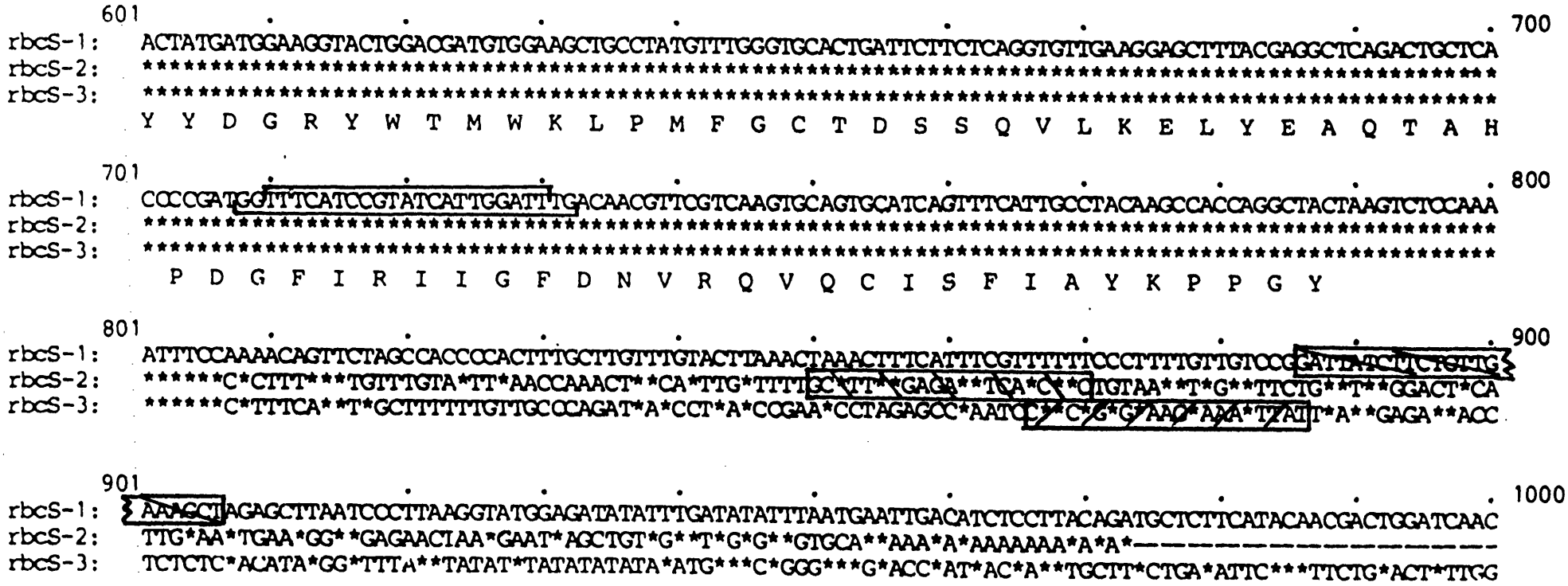

601
700
rbcS-1: ACTATGATGGAAGGTACTGGACGATGTGGAAGCTGCCTATGTTTGGGTGCACTGATTCTTCTCAGGTGTTGAAGGAGCTTTACGAGGCTCAGACTGCTCA
rbcS-2: **********
rbcS-3: **********
Y Y D G R Y W T M W K L P M F G C T D S S Q V L K E L Y E A Q T A H
701
800
rbcS-1: CCCCGATGGTTTCATCCGTATCATTGGATTTGACAACGTTCGTCAAGTGCAGTGCATCAGTTTCATTGCCTACAAGCCACCAGGCTACTAAGTCTCCAAA
rbcS-2: **********
rbcS-3: **********
P D G F I R I I G F D N V R Q V Q C I S F I A Y K P P G Y
801
900
rbcS-1: ATTTCCAAAACAGTTCTAGCCACCCCACTTTGCTTGTTTGTACTTAAACTAAACTTTCATTTCGTTTTTTCCCTTTGTTGTCCGGATTATCTCTGTTG
rbcS-2: ******C*CTTT***TGTTTGTA*TT*AACCAAACT**CA*TTG*TTTTGC*TT**GAGA**TCA*C**CTGTAA**T*G**TTCTG**T**GGACT*CA
rbcS-3: ******C*TTTCA**T*GCTTTTTTGTTGCCCAGAT*A*CCT*A*CCGAA*CCTAGAGCC*AATCT**C*G*G*AAC*AAA*TTATT*A**GAGA**ACC
901
1000
rbcS-1: AAAGCTAGAGCTTAATCCCTTAAGGTATGGAGATATATTTGATATATTTAATGAATTGACATCTCCTTACAGATGCTCTTCATACAACGACTGGATCAAC
rbcS-2: TTG*AA*TGAA*GG**GAGAACIAA*GAAT*AGCTGT*G**T*G*G**GTGCA**AAA*A*AAAAAAA*A*A*----------
rbcS-3: TCTCTC*ACATA*GG*TTTA**TATAT*TATATATATA*ATG***C*GGG***G*ACC*AT*AC*A**TGCTT*CTGA*ATTC***TTCTG*ACT*TTGG

Fig 2.1.2 The sequences of the five oligonucleotide probes used in this study

The sequences of the oligonuleotide probes used in this study are shown (Knight and Jenkins, 1991; Simpson, Sawbridge, Jenkins and Brown, 1992)

Oligonucleotide	5' Sequence 3'
rbcS1 specific	AGCTTTCAACAGAAGATAATC
rbcS2 specific	AATCCAATGATACGGATGAAA
rbcS3 specific	ATAAATTTCCTTACGCAGAAG
non-discriminating, (for northern blots) *rbcSG*	AATCCAATGATACGGATGAAA
non-discriminating, (for RT-PCR)	GGTTTCATCCGTATCATTGGATTTG

sterilisation when the temperature of the media had fallen to 50°C to a final concentration of 50 μg/ml.

2.2 Growth and illumination of plants

2.2.1 Growth and harvesting of plant material

Seeds of *Phaseolus vulgaris* cv Tendergreen (supplied by Sharps International, Sleaford) were germinated in water soaked potting compost (I.C.I. Agrochemicals, Farnham) at 20°C. The number of seeds per pot was varied depending on the experiment and is given in the results section for each experiment. The primary leaves were harvested into liquid nitrogen and stored at -70°C prior to their use. For dark grown tissue the top of the plant was harvested into liquid nitrogen in darkness and the primary leaves dissected from the frozen tissue.

2.2.2 Illumination of plants

White light was provided by Osram 45 W 'warm white' fluorescent tubes, red light by surrounding these tubes with orange cinnemoid (Kodak), and blue-enriched light by Sylvania 40 W T12 blue fluorescent tubes. The required fluence rate was achieved by varying the number of tubes in the growth area, and adjusting the distance of the plants from the light source. The fluence rate used for each experiment is defined in the appropriate results section as are the spectra of the light sources (Fig 3.3.1).

2.2.3 Light measurement

2.2.3.1 Fluence rate measurement

The photon fluence rates of the light sources used was measured using a Li-Cor Li-185B quantum sensor. The sensor was held at the level of the primary leaves and the plane of the sensor was parallel to the light source.

2.2.3.2 Spectral photon distribution measurement

The spectral photon distributions of the light sources was measured with a spectroradiometer (Macam Instruments) linked to a chart recorder.

2.3 General preparatory procedures

2.3.1 pH measurement

The pH of solutions other than phenol was measured using a Corning pH meter 220 and combination electrode. The pH of phenol-containing solutions was measured using Whatman Narrow Range pH paper (pH 6.0 - pH 8.0).

2.3.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 minutes. Small batches were sterilised in a Prestige High Dome pressure cooker, with larger batches being done in a Laboratory Thermal Equipment Autoclave 225E.

2.3.3 Filter sterilisation

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

2.3.4 Glassware

Glassware was sterilised by baking in an oven at 180°C for 12 hours.

2.3.5 Solutions and equipment for RNA work

Solutions for RNA work were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma Chemical Co.) overnight and then autoclaved. Solutions containing Tris were made with DEPC treated distilled water. Sterilised glassware and plasticware was used throughout.

2.4 Preparation of total RNA from primary leaves

2.4.1 Preparation of phenol reagent

Five hundred grams of detached crystals of phenol (Analytical reagent, Fisons, Loughborough) was melted at 65 °C and an aliquot (5 ml) was examined to see if there was any colouration indicating oxidation. If the solution was clear 0.58 g of 8 hydroxyquinoline was added prior to the phenol cooling. The warm solution was then saturated with 10 mM Tris-HCl pH 7.6. The aqueous phase was then aspirated off, replaced with 100 ml of 100 mM Tris-HCl pH 8.0 and the solution mixed vigorously for 15 minutes. The pH of the aqueous phase was measured and the aspiration and

washing with 100 mM Tris-HCl pH 8.0 was repeated until the pH of the aqueous phase was greater than 7.6. Once this pH was reached the aqueous phase was replaced with 100 ml of 10 mM Tris-HCl pH 7.6 and the solution stored at 4 °C.

2.4.2 Preparation of Kirby reagent (Parish and Kirby, 1966)

Six grams of 4-aminosalycilic acid (4-AS, Na salt : Sigma Chemical Co.) were dissolved in 10 ml of DEPC-treated distilled water. Twenty-five ml of 40 mM Tris-HCl, 200 mM KCl pH 7.6 was added followed by 10 ml of 10% (w/v) tri-iso propylnapthelenesulphonic acid, (TNS; Na salt: Eastman / Kodak Chemicals) which rendered the solution cloudy. The addition of 8 ml of phenol reagent redissolved the precipitate and the solution was made up to 100 ml with DEPC-treated distilled water. The solution could be stored at 4 °C for two weeks.

Kirby reagent : 6% (w/v) 4-AS
10 mM Tris-HCl pH 7.6
50 mM KCl
1% (w/v) TNS
8% (w/v) Phenol reagent.

2.4.3 Isolation of total plant RNA (modified from Parish and Kirby, 1966)

Approximately 1 g of frozen plant tissue was ground to a fine powder under liquid nitrogen in a mortar. The powder was then transferred to a 15 ml Corex ® tube containing 5ml of Kirby reagent and 5 ml of phenol reagent and mixed with a spatula. This tube was kept on ice as were those used in all other steps. The tube was then spun at 3000 rpm for 10 minutes in a MSE Mistral 2l centrifuge using an 8 x 50 ml swing-out rotor at 10°C. Following the spin the upper aqueous phase was taken off with a pasteur pippette and transferred to a 15 ml Corex ® tube containing 2.5 ml of phenol

reagent. These solutions were then mixed prior to the addition of 2.5 ml of chloroform. The solutions were mixed again and the tube was then centrifuged as described previously except that the temperature was 4°C. After this spin the aqueous phase was again mixed with phenol reagent and chloroform as described above and centrifuged again at 4 °C. After this spin the aqueous phase was transferred to an empty 15 ml Corex ® tube and 8 ml of ice cold ethanol was added and the solutions mixed. This tube was then placed at -20°C for 2-12 hours. The tube was then centrifuged as before at 4 °C and the supernatant discarded. The pellet was washed twice with 80% (v/v) ethanol, 50 mM NaCl and re-pelleted after each wash by centrifugation The resulting pellet was dried *in vacuo* and dissolved in DEPC-treated distilled water and stored at -80 °C until use. An aliquot of the RNA solution was usually run on an agarose gel to see if the RNA was undegraded and the concentration of the RNA was estimated by spectrophotometry (Section 2.6).

2.4.4 DNAase treatment of RNA preparations

If the RNA preparation was to be used for RT-PCR analysis the final dry pellet from the extraction described in Section 2.4.3 was dissolved in 100 µl of RQI DNAase buffer (40 mM Tris-HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$). Two to five units of RQI RNAase-free DNAase (1 unit / µl, Promega) was added to the solution and the reaction mixture was incubated at 37°C for 10 minutes. The reaction volume was made up to 2 ml with DEPC-treated distilled water, 2 ml of phenol reagent was added and the phases mixed. The tube was then centrifuged at 3000 rpm for 10 minutes in a MSE Mistral 2l centrifuge using an 8 x 50 ml swing-out rotor at 4°C. The aqueous phase was then taken off with a pasteur pipette and transferred to a 15 ml Corex® tube containing 1 ml of phenol reagent. The phases were mixed and then 1 ml of chloroform was added prior to mixing again. The tube was then spun as before. The aqueous phase was removed into an empty 15 ml Corex® tube and 0.2 ml of 3 M sodium acetate pH 5.2 was added followed by 6 ml of ethanol. The tube was inverted

to mix the phases and then placed at -20°C for 2 - 12 hours. The tube was then treated as the ethanol-containing tube in Section 2.4.3.

2.5 The amplification and purification of plasmid DNA

2.5.1 Preparation of competent cells (essentially as described in Sambrook et al., 1989)

E. Coli were grown up overnight in 10 ml of L-broth at 37°C. Twenty ml of fresh L-broth was inoculated with 1 ml of the overnight culture and was shaken at 37°C for 100 minutes. The culture was transferred to sterile 15 ml centrifuge tubes. The cells were then re-pelleted by centrifugation for 5 minutes at 2500 rpm at 4°C in a Beckman J2-HS centrifuge using a JA 20 type 8 x 50 ml fixed angle rotor, and the supernatant decanted. The cells were then resuspended in a total of 10 ml of ice-cold 50 mM $CaCl_2$ and left on ice for 20 minutes. The suspension of cells was then spun as before and the pellet resuspended in a total of 0.6 ml of 50 mM $CaCl_2$. These cells could then either be stored at -80°C after flash freezing in liquid nitrogen or left on ice until used.

2.5.2 Transformation of competent cells

The competent cells prepared as in Section 2.5.1 were divided into 0.2 ml aliquots in 7 ml sterile plastic bijous. One bijou was used per transformation. Two hundred ng of plasmid DNA in a volume of 1-10 µl was added to the cells and the suspension was mixed by gentle shaking prior to leaving on ice for 15 - 20 minutes. After this period the cells were heat-shocked at 37°C for 5 minutes and then returned to ice for a further 15 minutes. Then 0.6 ml of L-broth was added to the cells and the cells were placed at 37°C for 90 minutes to allow expression of the drug resistance encoded

on the plasmid. One hundred µl serial 1 in 10 dilutions of the cell suspension were plated out on agar plates with the appropriate antibiotic. The plates were allowed to dry and then placed at 37°C overnight. Controls carried out were transformed cells plated on a plate containing no antibiotic and competent cells which had been treated as described except that no DNA was added, plated on antibiotic-containing plates.

2.5.3 Large scale plasmid DNA preparation

This method is essentially as described by Birnboim and Doly (1979).

A single colony of bacteria containing the plasmid was picked off an agar plate containing the appropriate antibiotic and used to inoculate 10 ml of L-broth also containing the appropriate antibiotic. This was shaken at 37°C overnight. The following day the culture was placed at 4°C until required. One ml of the culture was used to inoculate 1 litre of L-broth (with antibiotic) which was shaken at 37°C for approximately 18 hours. The culture was decanted into sterile screw top 250 ml centrifuge bottles and centrifuged at 7000 rpm for 10 minutes at 4 °C in a Beckman J2-HS centrifuge with a 6 x 250 ml fixed angle rotor to pellet the cells. The supernatant was decanted off and the cells were resuspended with a total volume of 20 ml of GET (50 mM glucose, 25 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0). Two hundred mg of lysozyme (48,000 units/mg solid; Sigma Chemical Co.) was dissolved in 2 ml of GET and this was added to the resuspended cells and they were incubated at room temperature for 10 minutes. Then 40 ml of 0.2 M NaOH:1 % SDS (1:1 v/v) was added, the suspension mixed gently, and then left on ice for 10 minutes. After this incubation 20 ml of 3 M potassium acetate pH 4.8 was added and the suspension was mixed by inverting prior to being returned to ice for a further 30 minutes. The mixture was then centrifuged at 7000 rpm for 10 minutes at 4°C as above. The supernatant was poured into another 250 ml centrifuge bottle through 1 layer of muslin and the pellet and precipitate discarded. Fifty ml of propan-2-ol was added to the filtrate, the phases

mixed and left at room temperature for 5 minutes. The DNA and RNA was then pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the centrifuge bottle inverted to allow the pellet to dry. The dried pellet was dissolved in 7.1 ml of TE pH 8.0 (10 mM Tris, 1 mM Na_2EDTA, pH to 8.0 with HCl), and transferred to an ultracentrifuge tube containing 6.72 g of CsCl. The tube was inverted and 425 µl of 10 mg/ml EtBr was added and the tube left at room temperature for 30 minutes to allow all the CsCl to dissolve. The tube was then centrifuged against a balanced tube at 45,000 rpm in a Beckman Ti 70.1 rotor in a Beckman L7-55 Ultracentrifuge for 48 hours at room temperature. After centrifugation the plasmid band was removed with a sterile pasteur pipette into a 15ml Corex® tube. An equal volume of CsCl-saturated isoamyl-alcohol was added and the phases mixed. The phases were allowed to separate and the upper phase was removed. This isoamyl-alcohol extraction was repeated until the aqueous phase contained no pink colour. The aqueous phase was then dialysed against a 1000 x volume of TE pH 8.0 in dialysis tubing (Medicell International Ltd.) for two hours at room temperature and then overnight against fresh TE pH 8.0 at 4°C the DNA solution was then stored in aliquots at -20°C.

2.6 Quantification of DNA and RNA

An aliquot of the nucleic acid solution to be quantified (usually 5 - 10 µl) was made up to 1 ml with DEPC treated distilled water. The absorbance of the nucleic acid containing solution was measured between 220 nm and 300 nm against a DEPC treated

distilled water blank. An absorbance at 260 nm (A_{260}) of 1 was taken to indicate the following concentrations :

Form of nucleic acid	Concentration (μg/ml)
Double stranded DNA	50
Single stranded DNA and RNA	40
Oligonucleotides	20

2.7 Agarose gel electrophoreseis

All agarose (*ultra* PURE™) was supplied by Life Technologies.

2.7.1 Electrophoresis of DNA

The appropriate concentration of agarose (0.5 - 2.0 % w/v) was added to the volume of 1 x TBE (0.09 M Tris-borate, 2 mM Na_2EDTA) required for the gel being cast. The agarose suspension was heated in a microwave oven until all the agarose had dissolved. The agarose solution was then allowed to cool to around 60°C at which point 10 mg/ml EtBr was added to a final concentration of 1 μg/ml. The gel solution was then poured into the electrophoresis apparatus and allowed to set for 30 minutes. Enough 1 x TBE running buffer was then added to just submerge the gel. The samples to be loaded were mixed with one-tenth volume DEPC treated loading buffer (50% glycerol (v/v), 1 mM Na_2EDTA pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF), and loaded with an automatic pipette. The gels were run at 40 to 100 mA until the bromophenol blue had migrated two-thirds of the way down the gel.

2.7.2 Non-denaturing electrophoresis of RNA

Non-denaturing electrophoresis of RNA was carried out as described in Section 2.7.1 for DNA.

2.7.3 Denaturing electrophoresis of RNA

This method was used for gels that were to be northern blotted for northern hybridisation analysis. The appropriate amount of agarose (1.3 - 1.5 g) was added to 10 ml of 10 x MOPS buffer (0.2 M MOPS; Sigma Chemical Co., 0.05 M sodium acetate, 0.01 M Na_2EDTA, pH 7.0) and 73 ml of DEPC treated distilled water. The agarose suspension was then heated in a microwave oven until all the agarose had dissolved. Once the agarose solution had cooled to 60°C 17 ml of formaldehyde (37% v/v; Sigma Chemical Co.) was added and the gel was mixed by swirling prior to pouring into the electrophoresis apparatus. After a period of 30 minutes to allow the gel to set the gel was just covered with 1 x MOPS running buffer. The RNA (5 - 20 µg) was prepared in a solution of 50% (v/v) formamide (Fluka Biochemicals, BDR), 1 x MOPS, 5.92% formaldehyde to a volume not greater than 50 µl. This solution was heated to 65°C for 15 minutes and then placed on ice where a one-tenth volume of running buffer and 2 µl of 2.5 mg/ml EtBr was added. The sample was then loaded onto the gel with an automatic pipette and the gel was run at 50 to 100 mA until the bromophenol blue had migrated two thirds of the distance down the gel. The gel was then washed in DEPC treated distilled water for 15 minutes and then visualised under UV light.

2.8 Digestion of DNA with restriction endonucleases

The restriction enzymes and buffers used were supplied by Gibco-BRL. The DNA to be restricted was prepared in a solution of 1 x the appropriate buffer and 1-20 units of restriction enzyme were added ensuring that its concentration didn't exceed 10% (v/v). The reactions were incubated at 37°C for 2 to 6 hours and the reactions were monitored by running an aliquot of the reaction on an agarose gel against uncut DNA.

2.9 Blotting of nucleic acids

2.9.1 Northern blotting

A denaturing agarose gel was run to separate the RNA as described in Section 2.7.3. A support was prepared onto which was placed a wick of Whatman 3 MM paper which was then soaked with 20 x SSC (3 M NaCl, 0.3 mM trisodium citrate). The wick was also dipped into a reservoir of 20 x SSC. The gel was placed on top of the wick with the well side of the gel touching it, ensuring that there were no air bubbles between the wick and the gel. A piece of nylon membrane (Hybond-N, Amersham International) was cut to the same size as the gel and placed on top of the gel, again ensuring that there were no air bubbles between the nylon and the gel. Two pieces of Whatman 3 MM paper cut to the size of the gel were then soaked in 2 x SSC and placed on top of the nylon membrane. The area of the wick within a few mm of the gel was then covered with cling film to prevent a short circuit and a large quantity of paper towels was then placed on top of the 3 MM paper. A glass plate was placed on top of the stack of towels and a 250 g weight paced on top. The blot was left overnight and

then disassembled. The filter was placed RNA side down on a UV transilluminator for 90 s to fix the RNA. At this point the transfer of the RNA could be assessed as the RNA on the filter is still stained with EtBr.

2.9.2 Slot blotting of DNA

The plasmid to be slot-blotted was first treated with restriction enzymes to linearise the plasmid. The success of the restriction was tested by running a small aliquot of the plasmid on an agarose gel against an uncut plasmid. The DNA solution was then made up to such a volume so as to give a final volume of 100 µl per slot to be blotted. This DNA solution was heated to 95°C for 2 minutes and then cooled on ice. An equal volume of ice-cold 20 x SSC was added giving a final DNA volume per slot of 200 µl. A Hybond-N nylon filter was cut to 130 mm by 90 mm and soaked in distilled water for 10 minutes followed by 10 x SSC for 20 minutes. The filter was then set up in a Bio-Rad Bio-Dot® SF slot blot apparatus with sufficient suction to pull through a 200 µl volume in 30 seconds. The wells were washed twice with 200 µl of 10 x SSC prior to the sample being loaded. Once the sample had been loaded and sucked through, the apparatus was disassembled and the filter soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes prior to being soaked in neutralizing solution (0.5 M Tris-HCl, 1.5 M NaCl, pH 8.0) for one minute. The filter was fixed by being placed DNA side down on a UV transilluminator for 90 seconds.

2.10 Radio-labelling of DNA

2.10.1 Preparation of the DNA to be labelled

The DNA sequence to be labelled was cut out of its host plasmid using the appropriate restriction enzyme(s). After the digestion had occured the whole restriction reaction was loaded on a low-melting point agarose gel along side different amounts of cut plasmid. The amount of insert DNA was estimated by comparing it to the known amounts of DNA loaded and the insert band was cut out of the gel and placed into a pre-weighed Eppendorf® tube. Water was added at the ratio of 3 ml per g of gel and the tube boiled for 7 minutes to dissolve the agarose and denature the DNA. The DNA was then aliquotted and could be stored at -20°C.

2.10.2 Random priming of dsDNA

DNA was labelled with [α ^{32}P]dCTP using the Megaprime DNA labelling kit supplied by Amersham International.

Twenty five ng of DNA prepared as above was made up to a volume of 10 µl and 5 µl of primer solution (random nona-nucleotides) was added. The solutions were mixed in a screw top Eppendorf®and then heated to 95°C for 5 minutes. The tube was put on ice and 10 µl of buffer solution (dATP, dGTP, dTTP), 50 µCi [α^{32}P]dCTP (3000 Ci / mmol), 2µl of enzyme solution (2 units of DNA polymerase 1 'Klenow' fragment) and 18 µl of distilled water were added. The tube was incubated at 37°C for 60 minutes. After this time the reaction was stopped by the addition of 5 µl of 0.5 M Na_2EDTA and a further 45 µl of distilled water was added.

2.10.3 Polynucleotide kinase labelling of oligonucleotides

This method is a slight modification of that described by Maxam and Gilbert (1980).

The following amounts of the following solutions were mixed in a screw top Eppendorf® tube : 10 µl 50 mM $MgCl_2$

5 µl 1M Tris-HCl pH 7.6

5 µl 200 mM β-mercaptoethanol (Koch-Light)

200 ng of oligonucleotide

100 µCi [γ^{32}P]ATP (3,000 Ci / mol)

1 µl T4 polynucleotide kinase (10 units)

Distilled water to 50 µl.

The reaction was incubated at 37°C for 1 hour before it was stopped by heating to 65°C. Then a further 50 µl of distilled water was added.

2.10.4 Separation of labelled DNA from un-incorporated radio-nucleotides using spin columns

A sterile 1 ml syringe barrel was plugged with siliconised glass wool and filled with Sephadex TESN. The Sephadex TESN was made by autoclaving Sephadex G 50 medium (Pharmacia, Milton-Keynes) in a 20 fold volume of TESN (10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA, 0.1 M NaCl, 0.05% SDS (w/v)). The column was spun in a 15 ml Corex® tube at 3,000 rpm for 4 minutes at room temperature in MSE Mistral 2L centrifuge with a swing out 8 x 50 ml rotor. This process was repeated until the column volume was 1 ml. Once this column volume was achieved 100 µl of TESN was loaded onto the top of the column and the column centrifuged as before. This wash with TESN was repeated once more and then the solution of labelled DNA and unincorporated nucleotides was loaded onto the column and a clean screw-top Eppendorf® tube was

placed under the column to collect the eluate from the column. The column was then centrifuged as before and the DNA solution in the Eppendorf® tube was stored at -20°C until used. The column was then disposed of.

2.11 Measurement of the incorporation of radionucleotides into DNA probes

Two µl of the probe solution to be measured was spotted one cm above one end of a piece of Whatman DE 81 paper. This end of the paper was then suspended into 0.3 M ammonium formate solution pH 8.0 ensuring that the liquid level didn't cover the area where the probe was spotted. The ammonium formate solution was allowed to migrate up the piece of paper. When the liquid front had travelled approximately 5 cm past the origin the paper was removed from the liquid, wrapped in cling film and autoradiographed for 10 minutes at room temperature (Fig 2.2). The paper was cut in half so that the spot at the origin, which represents the incorporated label, was separated from the smear, which represents the unincorporated label. The two halves of the paper were then placed in 4 ml of Ecoscint A (National Diagnostics) in a scintillation vial and counted. The percentage incorporation could then be worked out as: (the number of counts at the origin / the number of counts in the smear plus the number of counts at the origin) multiplied by 100. Specific activities of labelled probes (i.e. cpm incorporated / µg of DNA could be calculated given that the amount of radiolabelled deoxy-nucleotide, template DNA and percentage incorporation of the radiolabel were known. A typical random priming reaction with an incorporation of 60% would yield a probe with a specific activity of 1.7×10^9 cpm/µg.

Fig 2.2 Autoradiograph of a DE-81 chromatogram of an aliquot of a DNA random priming reaction.

Two µl of a completed DNA random priming reaction was spotted onto DE-81 paper and a chromatogram run in 0.3 M ammonium formate pH 8.0 as described in Sections 2.11. The chromatogram was then autoradiographed. The areas on the autoradiograph which represent the incorporated and unincorporated nucleotides are indicated.


← Unincorporated
← Incorporated

2.12 Hybridisation analysis of RNA

2.12.1 Hybridisation analysis of RNA using cDNA probes

Nylon filters, prepared as in Section 2.9.1, were pre-hybridised for four hours at the appropriate temperature in a shaking water bath. Just enough pre-hybridisation solution was used (5 x SSC, 50% formamide (v/v), 5 x Denhardt's solution (v/v; 0.1% Ficoll 400 (w/v), 0.1% PVP 360 (w/v), 0.1% BSA (w/v) (all Sigma Chemical Co.)), 100-200 μg denatured, sonicated salmon sperm DNA, 0.1% SDS (w/v)) to cover the filter(s) in the bottom of a tupperware box. The cDNA probe was denatured by heating to 95°C for 2 minutes then cooling on ice and then added to the pre-hybridisation solution using an automatic pipette. Normally 1 to 2 million cpm of labelled probe was added per ml of hybridisation solution. The hybridisation reaction was carried out at the same temperature as the pre-hybridisation for 16 hours. The hybridisation solution was then replaced with an excess of the wash solution and these washes were repeated at the chosen temperature and salt concentration to give the stringency required. Details are given in the appropriate figure legends. After washing the filters were wrapped in cling film and autoradiographed at -80°C.

2.12.2 Hybridisation analysis of RNA using oligonucleotide probes

Filters to be probed with oligonucleotides were pre-hybridised in the same way as for experiments with cDNA probes except that the pre-hybridisation solution used was 6 x SSC, 0.05% sodium pyrophosphate (w/v), 0.5% SDS (w/v), 200 μg heparin (Binnie, 1990). The oligonucleotide was added to the pre-hybridisation solution with an automatic pippette after 4 hours pre-hybridisation. When comparative hybridisations between the three gene-specific probes were being made, the probes were only used used if the difference in the labelling efficiency (percentage incorporation) between

them was less than 10 percent. If this was the case equal cpm of labelled oligonucleotide (normally 1-2 million cpm/ml hybridisation solution) were added to each hybridisation reaction. The hybridisation reaction continued for 4 hours after which the filters were washed at the temperature and salt concentration to give the required stringency. Details are given in Section 3.1.4. After washing the filters were wrapped in cling film and autoradiographed at -80°C.

2.12.3 Autoradiography

Filters to be autoradiographed were wrapped in cling film and exposed to Fuji X-ray film type RX in a film cassette at -80°C for the appropriate length of time. Wet acrylamide gels to be autoradiographed were left on one gel plate and wrapped in cling film. The other gel plate was then placed on top of the film, and the gel plate, film, gel, gel plate sandwich wrapped in two layers of aluminium foil and placed inside a light tight cupboard at room temperature for the appropriate length of time.

2.12.4 Stripping filters of bound radioactive probes and blocking agents

After hybridisation and autoradiography the bound probe and blocking agents were removed by boiling a solution of 0.1% (w/v) SDS and pouring the boiling solution over the filter. The solution was then allowed to cool to room temperature, and the process was repeated a further two times. The filter was then wrapped in cling film and autoradiographed overnight to check that the bound probe had been removed.

2.12.5 Staining of filter-bound RNA/DNA with Methylene Blue (Herrin and Schmidt, 1988)

The filter containing the bound RNA/DNA was placed in 0.04% Methylene Blue (w/v), 0.5 M sodium acetate pH 5.2 for 5 to 10 minutes. The filter was then washed in DEPC-treated, distilled water until the RNA bands appeared as blue bands on a white background. The stain could be removed by washing the filter in 20% (v/v) acetic acid until the bands were no longer visible.

2.13 Scanning of Autoradiographs

The absorbances of bands representing *rbcS* mRNA were measured using an LKB 2202 Ultroscan laser desitometer. An area of the film that had been exposed to the filter but which did not contain any hybridising bands was used to set a reference absorbance. The absorbances of the tracks of the film containing RNA were measured relative to this absorbance. The output from the scanner representing a band is a peak, the area under which is recorded. This area is representative of the density of the band which is itself representative of the amount of hybridisation.

2.14 Preparation of nuclei from *P. vulgaris* primary leaves

This method is a modification of that described by Gallager and Ellis (1982).

2.14.1 Percoll gradient preparation

Percoll (Sigma Chemical Co.) was mixed with 5 x gradient buffer (GB) (2.2 M sucrose, 125 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$) and sterile distilled water to give solutions of 40, 60, and 80% percoll (v/v). These all contained a final concentration of 1 x GB. The gradient was formed by layering the solutions in the following order in a 15 ml Corex® tube. At the bottom of the tube 2.5 ml of sucrose pad (85% sucrose (w/v), 25 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$) was overlain with 2.5 ml of the 80% percoll solution prepared as above, followed by 2.5 ml of the 60% percoll solution and 2.5 ml of the 40% percoll solution. The integrity of the gradient could be checked by holding it up to the light and looking for the interfaces between the layers which should be visible.

2.14.2 Isolation of nuclei

Eighteen leaves were harvested and cut into strips with scissors prior to being placed into ice-cold ether. The leaves were then infused with ether under vacuum in a fume-hood for 30 seconds. All manipulations after this point were carried out on ice and all centrifugation steps were done at 4°C with the brake off. The ether was then poured off and replaced with 30 ml of nuclei wash buffer (NWB) (0.44 M sucrose, 25 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 0.5 % (v/v) triton X 100*(Sigma Chemical Co.), 10 mM β mercaptoethanol* (* = added after autoclaving)). The leaves were washed by swirling for 2 minutes and then the NWB was poured off, some fresh NWB added, and the process repeated. After the second wash the leaves were washed with 30 ml of nuclei isolation buffer (NIB) (0.44 M sucrose, 25 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 2.5% ficoll 400 (w/v), 5.0% dextran T 40 (w/v) (Sigma Chemical Co.), 10 mM β-mercaptoethanol*). This was poured off and replaced with 20 ml of fresh NIB. The leaves were then homogenised in a System Technik PCU 2 polytron (Lucerne, Switzerland). After approximately 2 minutes intermittent homogenisation at

four tenths of full speed Triton X 100 was added to a concentration of 0.5% (v/v) and the leaves were homogenised in the same way for another 2 minutes. The crude extract was then filtered through 8 layers of muslin, to filter off leaf debris, into a 30 ml Corex® tube. This was spun at 3000 rpm for 5 minutes in a Beckman GS-6 R centrifuge, using a 4 bucket swing out rotor, to pellet the nuclei. The pellet was resuspended in 2 ml of NIB + 0.5 % (v/v) Triton X 100 and loaded onto the top of a Percoll step gradient set up as described in Section 2.14.1. The gradient was spun at 3000 rpm for 30 minutes as above. After centrifugation the nuclei appeared as a white/greenish band at the interface of the 80% percoll and the sucrose pad. This band was removed with a sterile plastic pasteur and pipetted into a clean 15 ml Corex® tube. The nuclei were washed with 10 ml of nuclei resuspension buffer (NRB) (0.44 M sucrose, 50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol*, 20% glycerol (v/v)) and then centrifuged at 3000 rpm for 5 minutes as above to re-pellet the nuclei. This wash with NRB and the pelleting step were repeated and the nuclei resuspended in 500 µl of NRB. They were then stored at -80°C until use.

2.14.3 Estimation of the number of nuclei prepared

A 50 µl aliquot of the nuclei prepared as in Section 2.14.3 were mixed with 5 µl of 10 mg/ml EtBr in an Eppendorf® tube. They were then pipetted onto a haemocytometer with a depth of 0.1 mm and a defined area of 1/400 mm^2. The nuclei were examined under UV illumination at a magnification of 40 x on a Leitz compact Microscope Photometer (Wetzlar, Germany). The nuclei showed red fluorescence and the number in the defined square could be counted and thus the number of nuclei per ml estimated (see Section 3.4.1).

2.15 Run-on transcription assays

2.15.1 Reaction mixture

Run on transcription reactions were carried out using 2 million nuclei per reaction. The volume of stock nuclei containing this number was pipetted into an Eppendorf® tube and the nuclei were pelleted by centrifugation at slow speed (6,500 rpm) in a MSE Microcentaur.centrifuge for 30 seconds. The pelleted nuclei were resuspended in 20 µl of NRB. Seventy five µl of transcription assay buffer (50 mM Tris-HCl pH 8.0, 75 mM ammonium acetate, 10 mM $MgCl_2$, 0.5 mM CTP, GTP, ATP, 0.8 mM aurintricarboxylic acid (an inhibitor of initiation of transcription), 10% (v/v) glycerol) and 50 µCi of [α^{32}P] UTP (3000 Ci / mmol) were added and the solutions mixed by pipetting. The tube was incubated at 27°C for 20 minutes and then placed on ice to stop the reaction.

2.15.2 Extraction and purification of labelled RNA from nuclei

To the run-on transcription reaction which had been left on ice 1 ml of TNT (50 mM Tris-HCl pH 7.8, 10 mM NaCl, 2% (w/v) tri-iso-propylnathalenesulphonic acid, sodium salt) was added and the solutions mixed by pipetting. This mixture was added to a 15 ml Corex® tube containing 1 ml of TNT, 1 ml of phenol reagent and 1 ml of chloroform. The contents of the tube were mixed by pipetting them up and down in a sterile plastic pasteur pippette (Sigma Chemical Co.). The tube was then centrifuged at 3,000 rpm at 4 °C for 5 minutes in a MSE Mistral 2L centrifuge with a swing out rotor. After centrifugation the upper aqueous phase was taken off and mixed with 2 ml of phenol reagent in a fresh 15 ml Corex® tube. This tube was left on ice (all subsequent manipulations were carried out at 4°C) while 1 ml of TNT was added to and mixed with the first phenol phase. This tube was then spun as before and the aqueous phase

taken off and mixed with that from the first extraction. This second tube was then spun as before. The aqueous phase was then taken off and added to another 15 ml Corex® tube containing 2 ml of phenol reagent. The solutions were mixed and the centrifugation repeated. The aqueous phase was taken off and added to a 15 ml Corex® tube containing 2 ml of chloroform. The phases were mixed and the tube centrifuged as previously described. The aqueous phase was then transferred to an empty 15 ml Corex® tube where 10 µl of *E. coli* tRNA (100 µg/ml), 10 µl 0.1 M UTP (both from Sigma Chemical Co.), and 40 µl of 5 M NaCl were added. Then 2.5 volumes of ethanol was added, the solutions mixed, and the tube placed at - 20°C for 30 minutes to precipitate the nucleic acids.

The nucleic acids were pelleted by centrifugation as before and the supernatant discarded. Two ml of 80% (v/v) ethanol were added, the pellet resuspended, and the tube spun as before. The supernatant was discarded and the pellet was dried *in vacuo*. The pellet was then dissolved in 0.1 ml of RQI DNAase 1 buffer and 2.5 µl of RQI DNAase 1 (RNAase free) was added (Section 2.4.4). The tube was incubated at 37°C for 10 minutes and the reaction was stopped by putting the tubes on ice. The RNA solution was loaded on top of a spin column prepared as in Section 2.10.4. The eluate from the column was collected in a screw-top Eppendorf® tube and 10 µl of UTP, 10 µl of 5 M NaCl, 10 µl of *E. coli* tRNA (100 µg/ml) and 0.4 ml of ethanol were added. The tube was stored at -20°C for 16 to 20 hours prior to being spun in a MSE Microcentaur centrifuge at high speed (12,00 rpm) for 5 minutes. The supernatant was poured off and 100 µl of 80% (v/v) ethanol was added to the pellet and the tube vortexed before being centrifuged as before. The supernatant was taken off with an automatic pipette and the pellet dried *in vacuo*.

2.15.3 Hybridisation of RNA from run-on transcription reactions

The dried RNA pellet prepared as in Section 2.15.2 was dissolved in 750 µl of RNA hybridisation solution (RNA hyb.) (50% formamide (v/v), 40 mM PIPES pH

6.5, 0.5 M NaCl, 1 mM Na_2EDTA, 0.4% SDS (w/v), 100 µg/ml polyadenylic acid (Sigma Chemical Co.)) and 5 µl of this solution was chromatographed (Section 2.11) to estimate the incorporation of radio-label into the RNA. Each RNA solution was hybridised to a nylon filter containing 2 slots of 5 µg of a *rbcS* encoding cDNA (pPvSS 191; Knight and Jenkins, 1992) and 2 slots of 5 µg of a control plasmid (pUC 9) which is the host plasmid for the *rbcS* cDNA. Each filter was pre-hybridised in 3 ml of the RNA hyb. in one well of a six well sterile tissue culture dish (Corning, New York) for 4 hours at 41°C. After pre-hybridisation the RNA hyb. was replaced with 1.5 ml of RNA hyb. containing the dissolved run-on transcripts. The same number of incorporated cpm was added to each hybridisation (1-3 x 10^6 cpm) for comparative hybridisations between different treatments. The RNA hyb. was then overlain with 3 ml of paraffin oil and the hybridisation reaction was carried out at 41°C for 48 hours. After this period the filters were washed briefly in chloroform and then in 1.4 X SSC, 0.1% SDS (w/v), followed by two washes in 0.1 x SSC, 0.1% SDS (w/v). These washes were carried out at 60°C for 30 minutes per wash after which the filters were autoradiographed at -80°C. The filters were then cut up into individual slots and the pieces were added to 4 ml of Ecoscint® in scintillation vials. These were counted for 10 minutes in a LKB 1209 rackbeta scintillation counter.

2.16 Reverse transcriptase-polymerase chain reaction (RT-PCR) measurement of *rbcS* transcript levels

2.16.1 First strand cDNA synthesis

This reaction was carried out using the Promega reverse transcription system kit. Any enzymes and reagents used were supplied with this kit unless noted otherwise. One to five µg of RNA prepared as described in Sections 2.4.3 and 2.4.4 was used in a volume not greater than 11 µl in a 0.5 ml Eppendorf® tube. To this was added 0.5 µg

of the three 3' gene specific oligonucleotides (Table 2.1.2). The tube was heated to 92°C for 2 minutes prior to cooling on ice. Then 2.5 µl of a solution of dATP, dCTP, dGTP, dTTP, (dNTPs, each at a concentration of 10 mM), 2.5 µl of 10x reverse transcriptase buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 1 %Triton X-100 (v/v)), 0.6 µl of rRNasin® ribonuclease inhibitor (40 units/µl), 15 units of AMV reverse transcriptase and a volume of DEPC treated distilled water to give a final reaction volume of 25 µl. In one tube the AMV reverse transcriptase was replaced with DEPC treated distilled water as a control. The reaction was incubated at 42°C for 75 minutes. The reaction was then stored on ice.

2.16.2 Gel purification of the labelled 5' oligonucleotide

The 5' oligonucleotide (25mer, Table 2.1.2) was labelled as described in Section 2.10.3 except that 1 µg of oligonucleotide was used and no water was added after the reaction was complete. Instead 2 µl of *E. coli* tRNA (1 mg/ml, Sigma Chemical Co.), 80 µl of 2.5 M ammonium acetate, and 300 µl of ethanol were added to precipitate the oligonucleotide. The tube was stored at -20°C for 20 minutes prior to being spun for 15 minutes in a MSE Microcentaur centrifuge at high speed (12,000 rpm) for 15 minutes at 4°C. The supernatant was discarded and the pellet dried *in vacuo* prior to being suspended in 1 µl of DEPC-treated distilled water and 1 µl of stop solution from a Sequenase® sequencing kit (United States Biochemical, Cleveland : 95% formamide(v/v), 20 mM Na_2EDTA, 0.05% (w/v) Bromophenol Blue, 0.05% (w/v) Xylene Cyanol FF). This was then loaded on a 16% polyacrylamide / urea sequencing gel (Section 2.16.4) which had been pre-run for 15 minutes at 1750 volts. The gel was run until the Bromophenol Blue had migrated half the length of the gel. After running, the top plate of the sequencing gel was removed and the gel wrapped in cling-film. The gel was autoradiographed for 30 minutes (Fig 2.3) and the area of the gel corresponding to the major labelled band that had migrated the least distance was

Fig 2.3 Autoradiograph of an oligonucleotide preparation polyacrylamide / urea gel

The general oligonucleotide used for PCR work was labelled and loaded on a 16 % polyacrylamide / urea gel as described in Section 2.16.2. The gel was wrapped in cling film and autoradiographed as described and an autoradiograph from one such experiment is shown. The largest major labelled band (indicated by an arrow) was then cut out and the oligonucleotide eluted.

excised from the gel and placed in a clean Eppendorf® tube. Four hundred µl of oligoelution buffer (0.3 M ammonium acetate, 10 mM Tris-HCl pH 7.5) was then added to the gel slice and the gel slice mashed up with the melted end of a 1 ml automatic pipette tip. The tube was shaken overnight in a lead pot on an orbital shaker. After this period 200 µl of phenol reagent and 200 µl of chloroform were added to the tube and the phases mixed by pipetting. The tube was spun in a MSE Microcentaur centrifuge at high speed for 5 minutes to separate the phases. The upper aqueous phase was taken off and added to an Eppendorf® tube containing 400 µl of chloroform and mixed and spun as before. The upper aqueous phase was again taken off and placed in a clean Eppendorf® tube to which 5 µl of *E. coli* tRNA (1 mg/ml), 240 µl of 2.5 M ammonium acetate, and 900 µl of ethanol was added. The tube's contents were mixed by inversion and the tube placed at -20°C for 40 minutes before being centrifuged for 15 minutes in a MSE Microcentaur centrifuge at high speed (12,000 rpm) for 15 minutes at 4°C. The supernatant was discarded and the pellet dried *in vacuo*. The dried pellet was then counted by scintillation counting, and allowing for a counting efficiency of 30%, DEPC treated distilled water was added to give a probe concentration of approximately 300,000 cpm per µl. This was stored at - 20°C until use.

2.16.3 Polymerase chain reaction

The following solutions were added to the 25 µl reverse transcriptase reaction carried out as described in Section 2.16.1 : 10 µl of 10 x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 16 µl of a solution of dNTPs (dATP, dCTP, dGTP, dTTP all 1.25 mM), 5 µl of each 3' primer (each 20 µM solutions), 5 µl of 5' primer (approximately 1.5 x 10^6 cpm), 0.5 µl of Taq DNA polymerase (Promega : 40 units / µl) and DEPC-treated distilled water to a final volume of 100 µl. One hundred µl of paraffin was then layered on top of the reaction and the tubes were placed in a Perkin Elmer Cetus DNA Thermal Cycler. The step cycles used were a 1 minute 94°C denaturing step, a 2 minute 55°C annealing step, and a 3 minute 72°C extension

step, after an initial 3 minute 94°C delay file. These cycles were repeated the requisite number of times before a sink cycle of 72°C for ten minutes followed by the temperature being lowered to 4°C. Aliquots of these samples were then mixed with an equal volume of Sequenase® stop solution and run on a 6% acrylamide / urea gel.

2.16.4 Acrylamide / urea gel electrophoresis of DNA

A gel mix containing the appropriate percentage of acrylamide was made by mixing the appropriate amounts of the following two stock solutions to a final volume of 50 ml : Acrylamide mix (20% (v/v) acrylamide (30 : 1, acrylamide : bis-acrylamide), 1 x TBE (Section 2.7.1), 7 M urea), and Urea mix (7 M urea, 1 x TBE). The gel mix was then degassed and 150 µl fresh ammonium per sulphate (10% w/v) and 75 µl of TEMED (Sigma Chemical Co.) were added. The gel was poured between two 40 cm glass sequencing plates, the notched one of which has been pre-treated with dimethydichlorosilane solution (Fisons), separated by 2mm thick spacers ensuring that there were no air bubbles. Once set, the gel is set up in the electrophoresis equipment (the running buffer used was 1 x TBE) the gel is pre-run at 1750 V prior to loading the samples to heat up the gel. After pre-running the wells were flushed with running buffer to wash out urea and the samples loaded in a volume of 2-10 µl. The gel was then run at the required voltage for the required length of time. Once the gels had run the siliconised notched plate was removed and the gel wrapped in cling film prior to autoradiography at room temperature. The gel was left wet so that the gel slices containing the DNA corresponding to the three *rbcS* genes could be cut from the gel and placed in Ecoscint® overnight and counted. If knowledge of the incorporation into each product was not required, the gel was placed in 10% (v/v) acetic acid, 10% (v/v) methanol for 20 minutes, once the upper silanised plate had been removed. The fixed gel was then transferred onto Whatman 3MM paper and dried on on a slab gel drier at 80°C for 30 minutes. The gel was then autoradiographed for the required time.

2.17 The measurement of oxygen evolution from *Phaseolus vulgaris* leaf discs

A leaf disc oxygen electrode of the type described by Delieu and Walker (1981) was used to measure the oxygen evolution from illuminated leaf discs. The oxygen electrode was calibrated by passing nitrogen gas through the chamber until there was no change in the voltage reading (zero oxygen). The maximum response to oxygen was then set by passing air through the chamber, again until there was no further change in the voltage, to give a value for the voltage for 21% oxygen (0.25 μmol O_2/ml at 20°C). These calibrations were carried out with the $NaHCO_3$ / Na_2CO_3 (19/1 v/v of M solutions) soaked capillary mat, leaf support and representative leaf disc in position to ensure that the calibration was done with the volume of the chamber approximately the same as when the measurements would be taken. A fresh leaf disc was then placed in the oxygen electrode and placed in darkness until a steady rate of oxygen consumption was seen for 5 minutes. The leaf was then illuminated with the light source required and the oxygen evolution was measured for 10 minutes once a steady rate had been established. If different light sources were used the leaves were returned to darkness between light sources and the order of illumination was varied. The rate of oxygen evolution in nmol/min/cm^2 for each leaf disc was then calculated.

2.18 Measurement of the stomatal resistance of *Phaseolus vulgaris* primary leaves

The stomatal resistance of *Phaseolus vulgaris* primary leaves was measured using a Li-Cor Li-60 diffusive resistance meter. The meter was calibrated using the resistance test plate supplied with the meter. The calibration was carried out at 16°C (as

measured using the meter) and at this temperature the diffusive resistance of water in air (α) is 0.2431. The following formula was used to work out the diffusive resistance of each of the positions on the resistance test plate at this temperature (Li-Cor Li 60 manual) :

$$r = \frac{L}{\alpha} = \frac{4A(L_o + \pi * d/8)}{\alpha * n * \pi d^2}$$

where L = effective diffusion path length (cm)

α = diffusivity of water vapour in air at the given temperature (s / cm)

L_o = length of test plate hole (cm)

A = aperture area of vapour cup (cm^2)

n = number of holes in test plate exposed to vapour cup apperture

$\pi * d / 8$ = end effect correction.

For the resistance test plate supplied, d = 0.1 cm, L = 0.1524 cm, and A = 2.0 cm^2. Table 2.3 shows the resitance values for each of the resistance plate positions and the times taken for the needle of the resitance meter to move from 30 mA to 70 mA on the HUM 2 setting, the time taken for 4 repeats, the mean time taken, and the mean time taken corrected to a temperature of 25 °C using the correction factor supplied in the manual. These figures were used to plot a calibration curve for the time taken for the reading to increase from 30 mA to 70 mA on the HUM 2 setting (corrected to 25 °C) against the diffusive resistance at which that reading was taken (Fig 2.4). The meter was then used to measure the diffusive resistance of *Phaseolus vulgaris* primary leaves. To do this the plants were left under the light environment under which the diffusive resistance was to be measured for 30 minutes prior to the vapour cup being placed on the under surface of the leaf whose diffusive resistance was to be measured. The air in the vapour cup was then replaced with dried air until the reading had reached 0 mA. The pumping of dry air was stopped and the reading allowed to increase. The time taken for the reading to increase from 30 to 70 mA (on the HUM 2 setting) was then recorded. Three readings were taken for each leaf but each leaf's readings were not taken consecutively. Five leaves' diffusive resistance was measured for each light

treatment examined and the order of the readings between leaves was varied for each of the three readings. The times were then converted to times at 25 °C and the diffusive resistances read off the calibration curve shown in Fig 2.4. The temperature of the vapour cup was measured before and after each batch of readings and the mean temperature used. Stomatal resisatance was expressed as s^{-1}.

Table 2.2 The values obtained when calibrating the Li-Cor Li-60 diffusive resistance meter.

The diffusive resistances of the apertures on the calibration plate, described by hole no, is tabulated along with the times taken for the ampage to increase from 30 mA to 70 mA on the HUM 2 setting at each of these resistances. Also tabulated is the mean time taken for each resistance value, and this value corrected to a temperature of 25 °C for each resistance value.

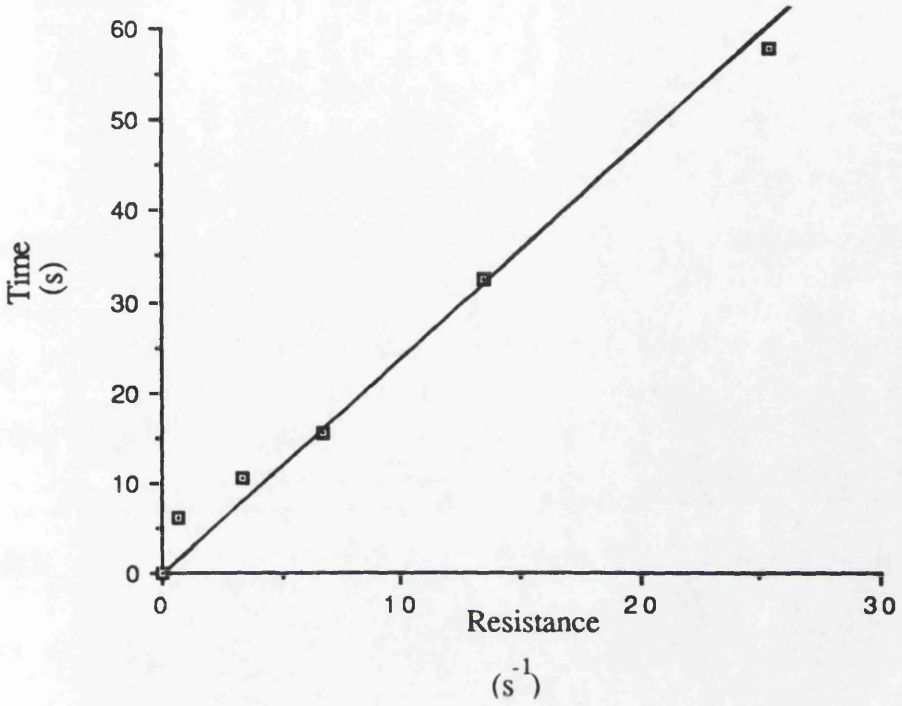
No. of holes open	Resistance (s^{-1})	Times at 16 °C (s)	Mean Time (s)	Time corrected to 25°C
Open	0.63	10, 10, 10, 11	10.25	6.02
60	3.36	18, 18. 19, 20	18.75	10.50
30	6.73	28, 28, 28, 27	27.75	15.54
15	13.46	62, 57, 57, 56	58.00	32.48
8	25.25	112, 102, 101, 99	103.50	57.96

Correction factor to convert values to 25 °C = 0.56

(from calibration curve supplied with meter)

Fig 2.4 Graph showing the times taken for the increase in current from 30 to 70 mA on the HUM 2 setting of the Li-Cor Li 60 diffusive resistance meter at different defined diffusive resistances

The vapour cup of the Li-Cor Li 60 difusive resistance meter was placed over various apertures of known diffusive resistances on a calibration plate with a wick of soaked 3MM paper (Whatman). The time taken for the ampage to increase from 30 mA to 70 mA was measured four times at each diffusive resistance. This graph plots the mean time taken, corrected to a temperature of 25°C, against the diffusive resistance.


Time
(s)
60
50
40
30
20
10
0
0
10
20
30
Resistance
(s^{-1})

Chapter 3 RESULTS

3.1 Validity of the methods used to measure *rbcS* transcript levels in this study

The experiments in this section establish the validity of the methods used in this study for the analysis of *rbcS* mRNA levels as related to a given amount of total RNA. These methods were used for all the northern blots in the following sections unless a specific difference in approach is described in the figure legends and text. Descriptions of the methods can be found in Section 2.

3.1.1 Experiments to demonstrate the quality of the total RNA used in this study

Figure 3.1.1 shows a scan of the absorbance of 5 µl of a total RNA sample extracted from a light grown primary leaf in 995 µl of water. The absorbance was measured between 220 nm and 300 nm. The absorbance at 260 nm (A_{260}) gives a measure of the concentration of the RNA, and the absorbance at 280 nm (A_{280}) an indication of the amount of contaminating protein. The ratio of these two absorbances (A_{260} / A_{280}) is used as an indicator of the purity of the RNA preparation. The absorbances at these two wavelengths, the A_{260} / A_{280} ratio and the concentration of the RNA sample scanned in Fig 3.1.1 are given in Table 3.1.1. A reasonably pure RNA sample is taken to be one whose A_{260} / A_{280} ratio is greater than 1.8. It can be seen that this RNA fullfils this criterion and all the RNA used for experiments in this study had an A_{260} / A_{280} ratio of greater than 1.8.

3.1.2 Formaldehyde gel electrophoresis and northern blotting of *P. vulgaris* total RNA

Increasing amounts of the RNA examined in Fig 3.1.1 were run on a formaldehyde gel (Section 2.7.3) against RNA size markers. A photograph of this gel following ethidium bromide staining and partial destaining (Section 2.7.3) is shown in Fig 3.1.2. It can be seen that with an increasing amount of total RNA loaded there is an increasing amount of ethidium bromide fluorescence present. Especially visible are the bands of ribosomal RNA, and the presence of these bands without smearing being visible is a good indicator of the lack of degradation of the RNA. For this study the relative amounts of fluorescence visible in these ribosomal bands was used as an indicator of whether the gel was evenly loaded.

Following partial destaining such that the ribosomal bands were visible the gels were then northern blotted (Section 2.9.1). The photograph in Fig 3.1.3 shows the northern blot from the gel shown in Fig 3.1.2. The ethidium bromide which stained the RNA in the gel remains bound in the northern blotting procedure and enables the efficiency of transfer to be examined. It can be seen that in this case the RNA has transferred well. The loading of RNA was also examined after hybridisation experiments had been undertaken. To do this the filter was stripped of radioactive material and blocking agents (Section 2.12.4) and then stained with methylene blue (Section 2.12.5). This resulted in the bound RNA being stained blue and gave an estimate of the RNA still bound to the filter. This method gave very similar results to ethidium bromide staining (data not shown).

3.1.3 Experiments to estimate the sizes of the transcripts hybridised to the *P.vulgaris* pPvSS191 *rbcS* cDNA probe and a *Chlamydomonas reinhardtii* α tubulin cDNA probe

The blot shown in Fig 3.1.3 was pre-hybridised and hybridised as described in Section 2.12.1 with the pPvSS191 *rbcS* probe (Knight and Jenkins, 1992). This probe encodes a cDNA copy of the type 3 *rbcS* gene of *P. vulgaris* but as the coding sequences of the three *P. vulgaris rbcS* genes are identical this probe is non-discriminating and will hybridise to the transcripts of all three genes. This probe hybridised to two different sizes of transcript (1.0 and 0.9 kb) as can be seen in autoradiograph A in Fig 3.1.4. The estimation of the size of the transcripts is based on the mobility of the RNA size markers. These values were used to plot the calibration curve shown in Fig 3.1.5. The sizes estimated (given in Table 3.1.2) for the *rbcS* transcripts agree with estimates made from cDNA sizes (Knight and Jenkins, 1992). The presence of two different transcript sizes is probably due to different processing of the transcripts from different genes as described in Section 1.2.3 (also see Discussion, Section 4.1). Some hybridisation is also apparent to a transcript of larger size. This binding may be non-specific (see Discussion, Section 4.1).

The blot shown in panel A of Fig 3.1.4 was then stripped of bound probe (Section 2.12.4) and then pre-hybridised and hybridised as described in Section 2.12.1 except that for the *Chlamydomonas reinhardtii* α tubulin probe the hybridisation was carried out at 30°C and the washes were much less stringent. This was because the probe being was presumably not identical to the *P.vulgaris* α tubulin sequence. Despite this, a signal was obtained using this probe and this signal increases with the increasing amount of total RNA loaded (Panel B in Fig 3.1.4). That the *Chlamydomonas reinhardtii* α tubulin probe hybridised to *Phaseolus vulgaris* α tubulin sequences was not unexpected as comparisons between an *Arabidopsis* α tubulin gene and that from *Chlamydomonas reinhardtii* revealed great similarity in amino acid sequence (Ludwig et al., 1987). The size of transcript (1.45 kb; Table

3.1.2) agrees with that expected for a higher plant α tubulin transcript (Ludwig et al., 1987).

3.1.4 Determination of the washing conditions used for the *rbcS* gene-specific oligonucleotide probes

A formaldehyde gel was run using three identical 10 μg samples of RNA extracted from light grown primary leaves. The gel was blotted and replicate filters with one lane per filter were made. These filters were pre-hybridised and hybridised as described in Section 2.12.2 with the non-discriminating *rbcSG* oligonucleotide probe. The filters were then washed differently as described in the legend to Fig 3.1.6 which shows the autoradiographs from the three washed filters. As can be seen the washes at 50°C gave a reduction in the background hybridisation but also a significant reduction in the specific signal. Therefore the washing conditions described for Panel A of Fig 3.1.6 were used for the susequent oligonucleotide blots in this study. The GC-content of the three gene-specific oligonucleotides is identical to that of the *rbcSG* oligonucleotide and thus their melting conditions should be similar to those of the *rbcSG* probe.

3.1.5 Demonstration of the specificity of the *rbcS* gene-specific oligonucleotide probes for the sequences to which they were designed

Increasing amounts of three plasmid DNAs, each encoding a cDNA representing one of the three different *P. vulgaris rbcS* genes, were slot blotted onto four identical filters. The amounts of each plasmid and their loading pattern are given in Fig 3.1.7. Each filter was probed with either the non-discriminating, coding sequence oligonucleotide, or one of the three gene-specific oligonucleotide probes. Equal cpm of

labelled oligonucleotide were added to each hybridisation, which was carried out as described in Section 2.12.2. The filters were then washed as described for filter A in Fig 3.1.6 and autoradiographed. The autoradiographs from this experiment are shown in Fig 3.1.7. The autoradiograph shows that each gene-specific oligonucleotide hybridises solely to its corresponding plasmid and that the amount of hybridisation increases with an increasing amount of plasmid up to 50 fmoles. These autoradiographs were scanned and the data from these scans is shown in Fig 3.1.8. The amount of hybridisation for each oligonucleotide can be seen to increase with an increasing amount of plasmid, with this increase tailing off at higher plasmid amounts. It can also be seen that the oligonucleotides do not hybridise with equal efficiency despite their having the same GC content. The *rbcS2* specific oligonucleotide hybridises more efficiently than that specific for *rbcS1* transcripts, which in turn hybridises more efficiently than the *rbcS3* specific oligonucleotide. If the hybridisation efficiency of the *rbcS2* oligonucleotide is given a value of 100% the mean relative hybridisation (mean of the relative hybridisation measured at each point on Fig 3.1.8) of the *rbcS1* and *rbcS3* oligonucleotides are 71% and 54% respectively. These figures compare well to the values of 75% and 53% found by Knight (1989) who used slightly different washing conditions.

Fig 3.1.1 Absorbance of *P. vulgaris* primary leaf total RNA between 220 nm and 300 nm

5 µl of a total RNA sample prepared as described in Section 2.4.3 from a *Phaseolus vulgaris* primary leaf was dissolved in 995 µl of distilled water and its absorbance measured from 220 nm to 300 nm against a distilled water blank (see Section 2.6).

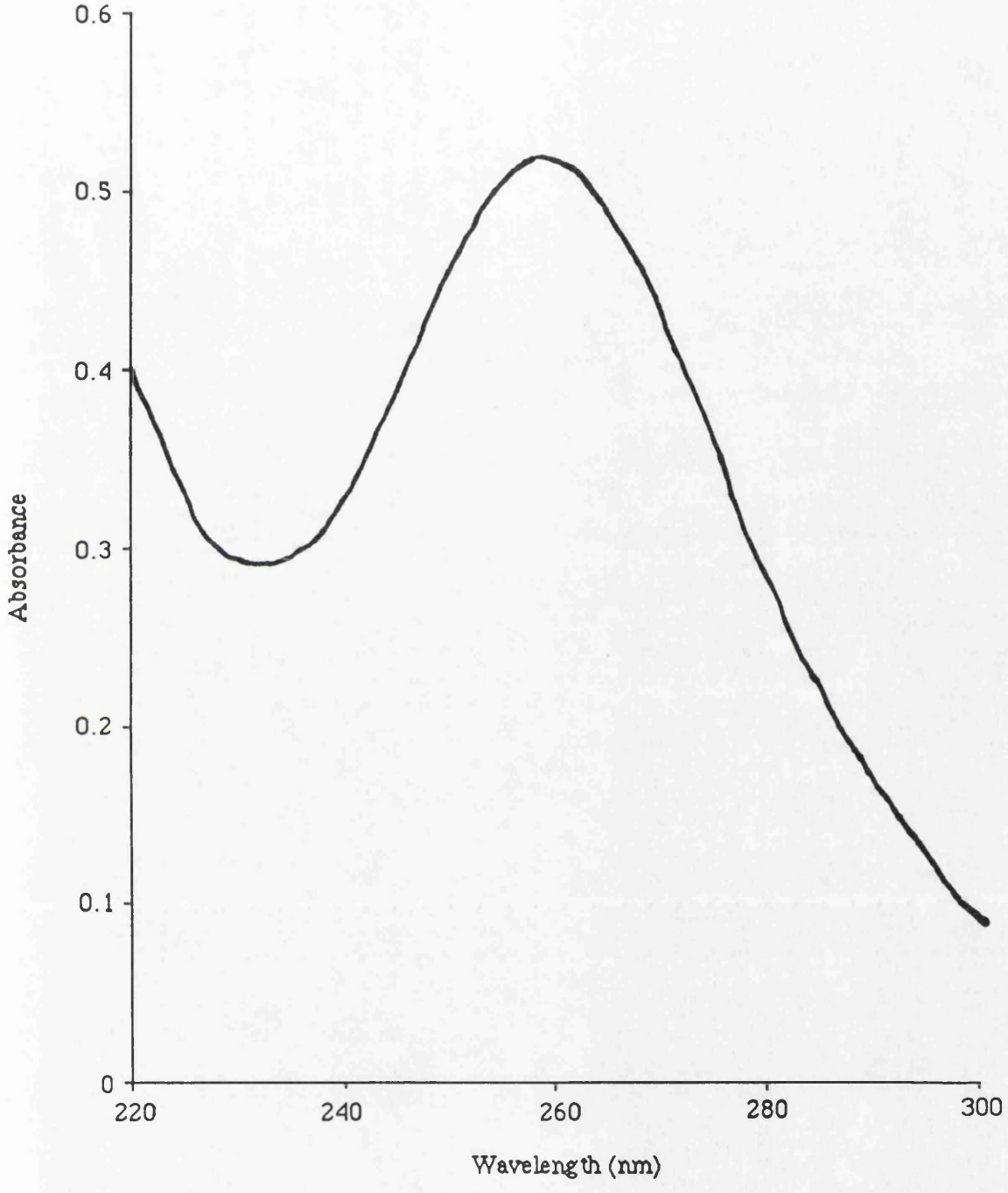

0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
220
240
260
280
300
Wavelength (nm)

Table 3.1.1 The absorbance of Phaseolus vulgaris primary leaf total RNA at 260 nm and 280 nm, its quality as measured by the ratio of these absorbances, and its concentration.

The absorbance peak shown in Fig.3.1.1 is at 260 nm and this absorbance value (A_{260}) was measured along with that at 280 nm (A_{280}). These values are given along with the ratio of these absorbances (A_{260} / A_{280}) and the calculated concentration of this particular RNA sample.

Wavelength (nm)	Absorbance	Ratio A_{260} / A_{280}	RNA Concentration (mg/ml)
260	0.512	1.86	4.1
280	0.275		

Fig 3.1.2 Photograph of an ethidium bromide stained formaldehyde gel showing increasing amounts of *P. vulgaris* primary leaf total RNA and RNA markers

Different amounts of the RNA described in Fig.3.1.1 were run on a formaldehyde gel against 5 µl of 1.77 kb - 0.16 kb Gibco-B.R.L. RNA markers. Staining and destaining were carried out as described in Section 2.7.3. The ribosomal RNA bands and the RNA size markers are indicated. The amount of RNA in each lane is indicated below. The markers were run in lane 1.

Lane	1	2	3	4	5	6	7
Amount of RNA (µg)	5.0	2.5	5.0	7.5	10.0	12.5	15.0

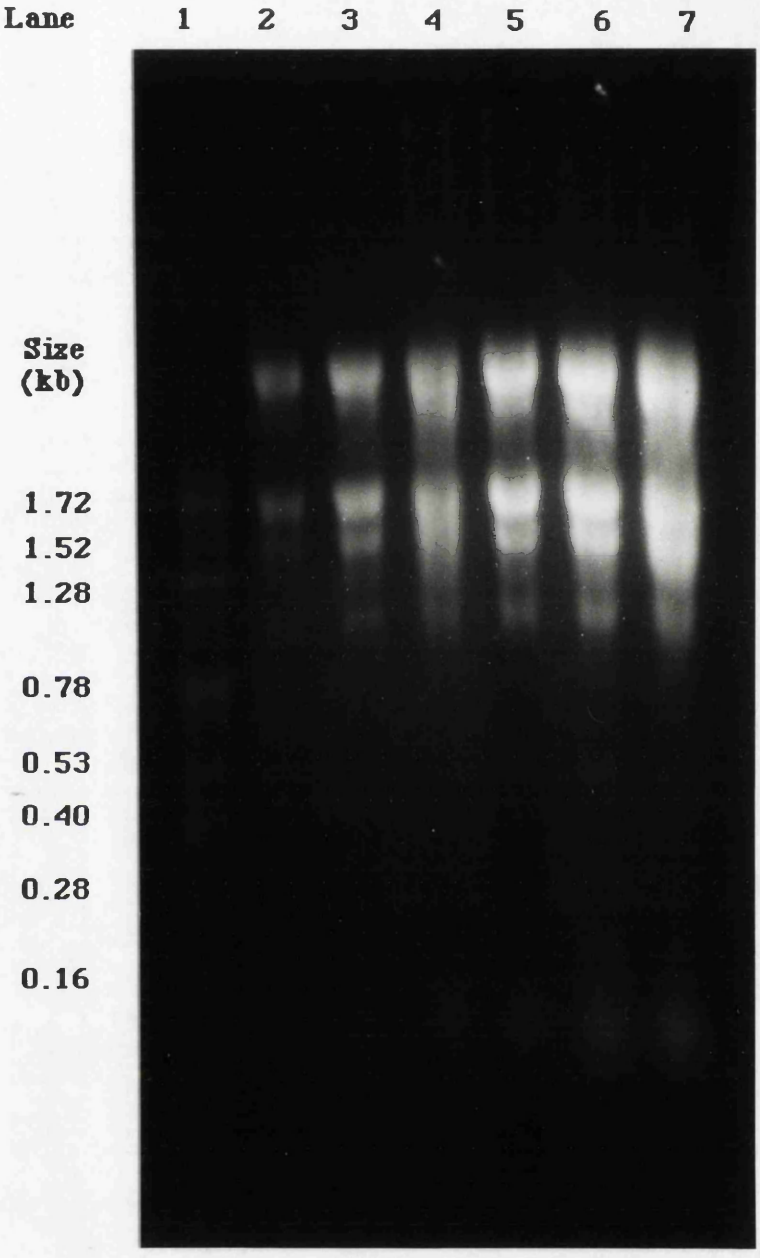

Lane
1
2
3
4
5
6
7
Size
(kb)
1.72
1.52
1.28
0.78
0.53
0.40
0.28
0.16

Fig 3.1.3. Photograph of an ethidium bromide stained northern blot from a formaldehyde gel showing increasing amounts of *Phaseolus vulgaris* primary leaf total RNA and RNA markers

The formaldehyde gel shown in Fig 3.1.2 was blotted onto a nylon filter by the method described in Section 2.9.1. The lane numbers refer to those described in the legend to Fig 3.1.2.

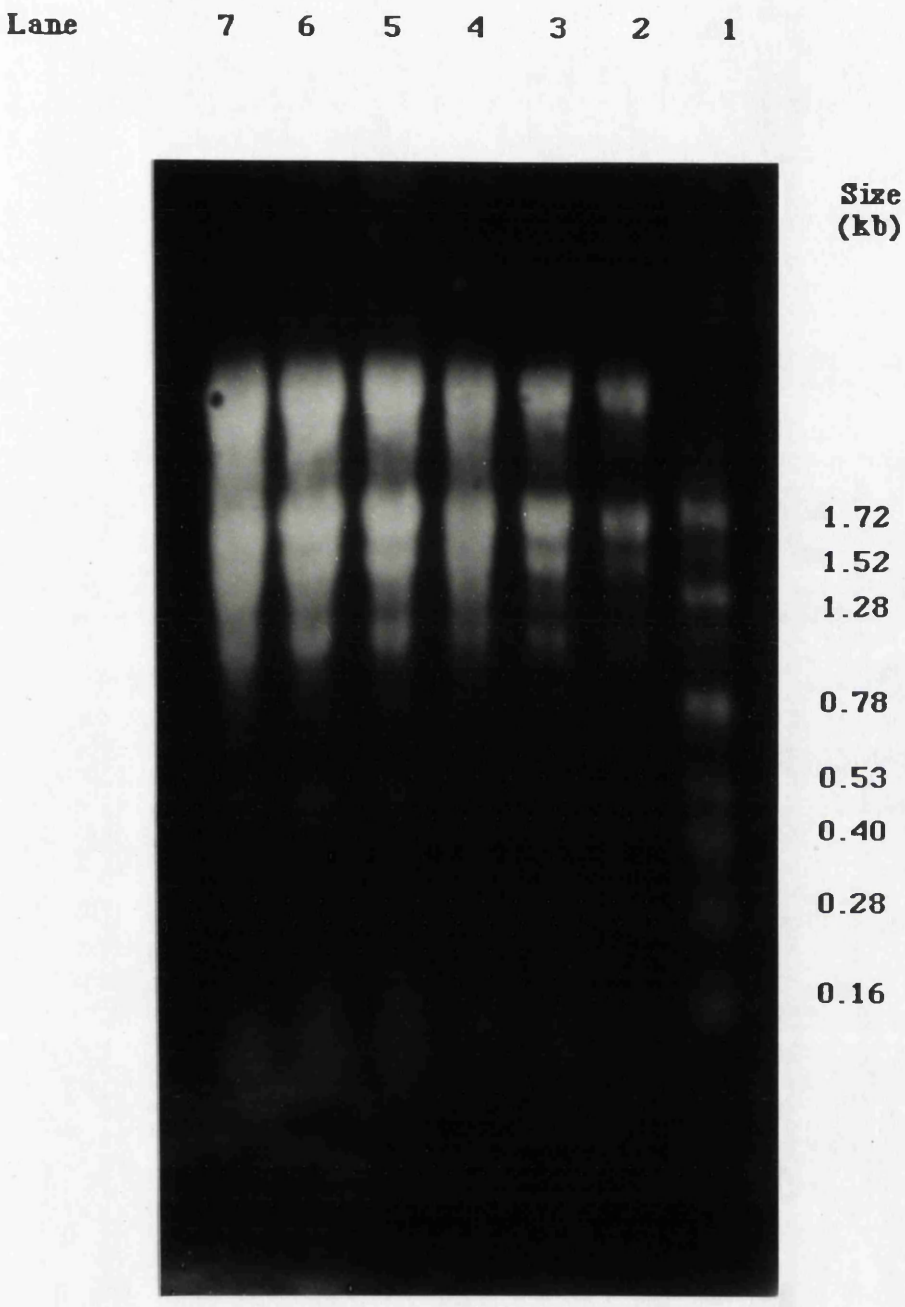
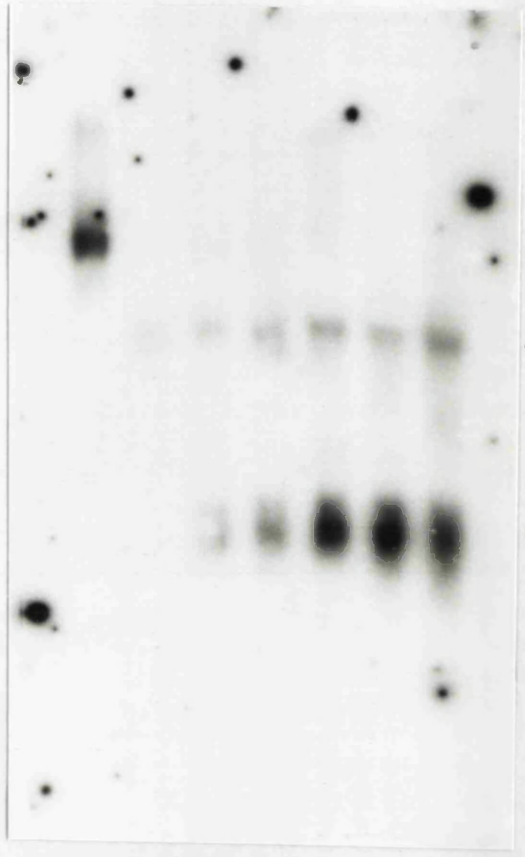

Lane
7
6
5
4
3
2
1
Size
(kb)
1.72
1.52
1.28
0.78
0.53
0.40
0.28
0.16

Fig 3.1.4 Sizes of transcripts in *Phaseolus vulgaris* RNA hybridising to *Phaseolus vulgaris rbcS* and *Chlamydomonas rheinhardti* α tubulin cDNA probes

A northen blot of increasing amounts of *Phaseolus vulgaris* RNA was pre-hybridised and hybridised as described in Section 2.12.1 to the *Phaseolus vulgaris rbcS* cDNA probe pPvSS191. 1 x 10^6 cpm of labelled probe was added per ml of hybridisation solution. The blot was washed at 65°C for 20 minutes per wash. The initial wash was in 2 x SSC, 0.1% (w/v) SDS, the second in 0.2 x SSC, 0.1% (w/v) SDS, and the third in 0.1 x SSC, 0.1% (w/v) SDS. After washing the blot was autoradiographed as described in Section 2.12.3 and the resulting autoradiograph is shown in panel A. The lane numbers refer to those described in the legend to Fig 3.1.2.

This filter was then stripped of bound radioactivity and blocking agents (Section 2.12.4) and then pre-hybridised and hybridised as described in Section 2.12.2 except that the temperature was maintained at 30°C. The washes were carried out a 35°C for 20 minutes per wash. The first two washes were carried out in 5 x SSC, 0.1% SDS (w/v), and the final wash in 2 x SSC, 0.1% SDS (w/v). After washing the blot was autoradiographed as described in Section 2.12.3 and the resulting autoradiograph is shown in panel B. The lane numbers refer to those described in the legend to Fig 3.1.2. rRNA, *rbcS* and α tubulin transcripts are indicated.

Panel A



Panel B

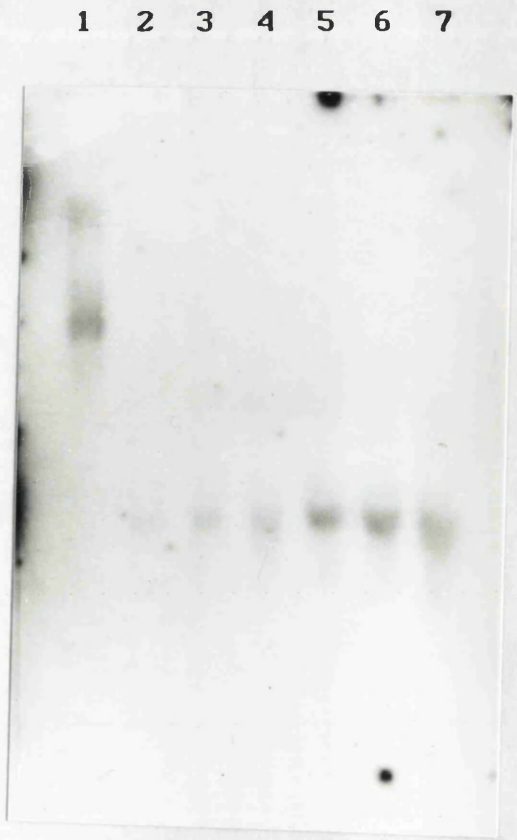


Fig 3.1.5 Graph of $\log_{10}$ of the size of the RNA markers plotted against the distance moved by the RNA markers

The distance each of the RNA markers migrated in the gel (Fig 3.1.2) is plotted on the x-axis against the $\log_{10}$ of each markers' size (bp) on the y-axis.

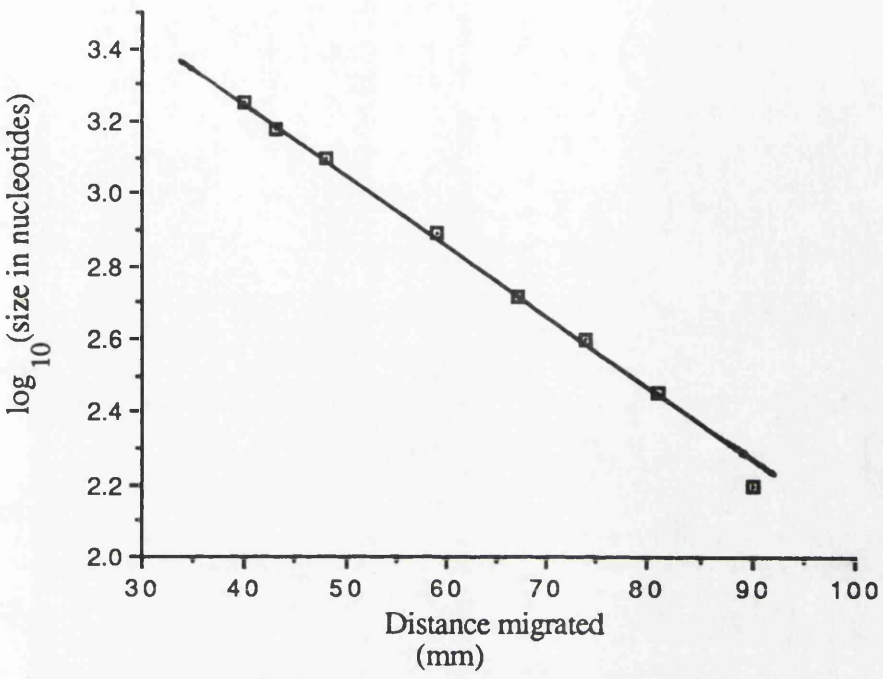

log 10 (size in nucleotides)
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
30
40
50
60
70
80
90
100
Distance migrated
(mm)

Table 3.1.2 Table showing the calculated size of the transcripts hybridised by the *rbcS* and α tubulin probes

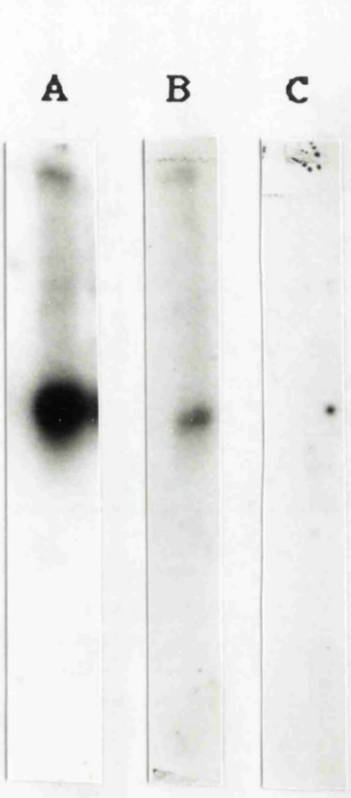
The distances migrated by the bands hybridised by the α tubulin probe and the *rbcS* probe were measured from the autoradiographs shown in Fig 3.1.4. These values were then plotted on Fig 3.1.5 to give values for the $\log_{10}$ of the sizes of the *Phaseolus vulgaris* α tubulin, and *rbcS* transcripts and hence estimates of the sizes of the transcripts.

Probe used	Distance migrated (mm)	Log_{10} size (from Fig 3.1.5.)	Size of hybridising transcripts (nucleotides)
α tubulin	45	3.16	~ 1,400
rbcS	53	3.00	~ 1,000
	56	2.95	~ 900

Fig 3.1.6 The effects of different washing conditions on the hybridisation of the non-disciminating *rbcS* oligonucleotide probe to light grown leaf total RNA

Identical 10 μg samples of total RNA extracted from light grown leaves were run on a formaldehyde gel and blotted. The blot was cut up into three pieces each containing one lane of RNA. These blots were then pre-hybridised and hybridised as described in Section 2.12.2 with the non-discriminating *rbcS* oligonucleotide probe (*rbcSG* oligonucleotide probe) shown in Section 2.1.5. These blots were then washed as follows:

All panels : 15 minutes in 6 x SSC at 46°C.
15 minutes in 2 x SSC at 46°C.
Panel A : 15 minutes in 1 x SSC at 46°C.
Panel B : 15 minutes in 1 x SSC at 50°C.
Panel C : 15 minutes in 0.1 x SSC at 50°C.



Oligonucleotide Probe Used

rbcSG

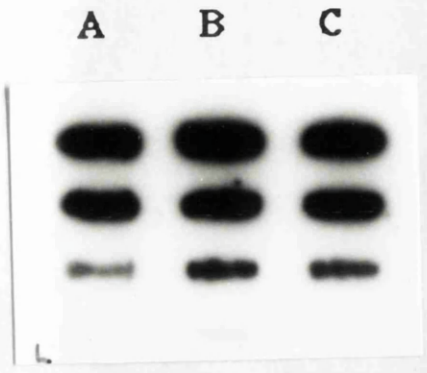
Fig 3.1.7 Demonstration of the specificity of the gene-specific oligonucleotide probes for the sequences for which they were designed by hybridisation to plasmid DNAs containing sequences representative of the three *rbcS* genes

Three plasmid DNAs encoding cDNAs representing the 3 different *Phaseolus vulgaris rbcS* genes were linearised and slot blotted onto a filter as described in Section 2.9.2. Plasmid A (pPvSS 191) contained a sequence from the *rbcS 3* cDNA, Plasmid B (pPvSS 965) contained a sequence from the *rbcS 2* cDNA, and Plasmid C (pPvSS1672) contained a sequence from the *rbcS 1* cDNA. The plasmids were blotted as follows :

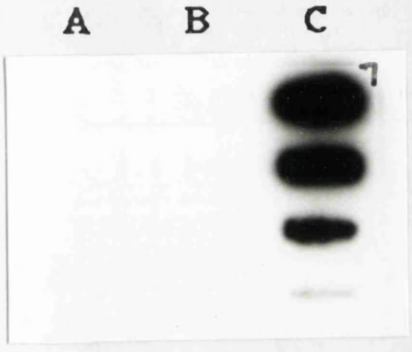
Plasmid	A	B	C
DNA	50	50	50
amount	25	25	25
(fmoles)	5	5	5
	0.5	0.5	0.5

Four replicate filters were hybridised as described in Section 2.12.2, each filter being probed with either the non-discriminating coding sequence oligonucleotide probe (*rbcSG*), or one of the gene-specific oligonucleotide probes designed to the 3' non-translated regions of the three types of cDNA (*rbcS1*, *rbcS2*, *rbcS3*) The same number of cpm of labelled oligonucleotide per ml of hybridisation solution were added for each oligonucleotide with the specific activity of the probes estimated to be within 10% of each other. The filters were washed as described in Fig.3.1.6 (Panel A) and then autoradiographed.

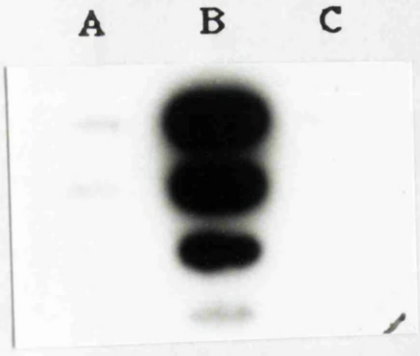
Oligonucleotide Probe Used



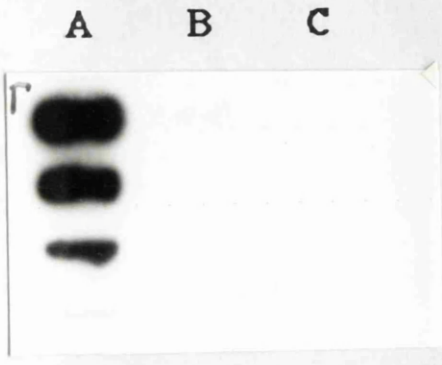
rbcSG



rbcS1



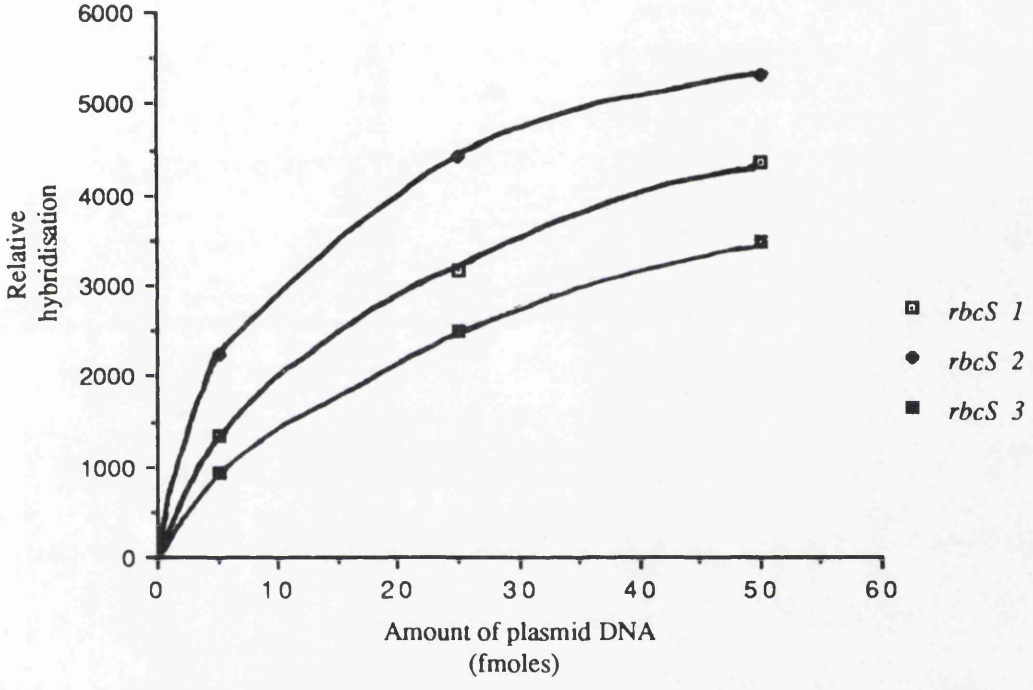
rbcS2



rbcS3

Fig 3.1.8 Hybridisation efficiency of three gene-specific oligonucleotide probes

The three blots probed with the three gene specific oligonucleotide probes shown in Fig 3.1.7 were scanned with a laser densitometer (Section 2.13). The relative hybridisation (as measured by peak area) for each oligonucleotide is plotted against the amount of target plasmid.


6000
5000
4000
3000
2000
1000
0
Relative hybridisation
0
10
20
30
40
50
60
Amount of plasmid DNA (fmoles)
rbcS 1
rbcS 2
rbcS 3

3.2 Ontogenetic and light regulation of *rbcS* mRNA level

The aim of the experiments described in this section was to investigate the regulation of expression of *P. vulgaris rbcS* genes through the development of the primary leaf under various different conditions. The regulation of the gene family as a whole in primary leaves is examined. Whether there is differential regulation of individual members of the gene family in primary leaves is examined when dark grown plants are illuminated to ascertain whether all three genes are light regulated. The regulation of the three *rbcS* genes in plants that have been illuminated for two days which are returned to the dark is also studied in the primary leaves. The conditions used for the hybridisation experiments are as described in Section 2.12, and the washing conditions used for the oligonucleotide hybridisations are as described for Panel A of Fig 3.1.6.

3.2.1 Ontogenetic control of *rbcS* gene expression in darkness and two different fluence rates of white light

Plants were grown 10 to the pot from seed under complete darkness and also under two different continuous white light treatments. These were 50 μmol/m^2/s white light and 100 μmol/m^2/s white light. Primary leaves were harvested from 5 different plants at the times indicated in Fig 3.2.1 and pooled. RNA was extracted from these leaves and equal amounts of total RNA (7.5 μg) were used for northern analysis with the light grown samples. For the dark grown treatment 20 μg of total RNA was used for each sample because of the lower abundance of *rbcS* mRNA in dark grown plants. The autoradiographs from this experiment are shown in Fig 3.2.1. In each case *rbcS* transcripts are undetectable in 3 day old primary leaves, but are apparent after 5 days. There is a peak at the level of message accumulation on day 7 of the timecourse in primary leaves grown under both darkness and 50 μmol/m^2/s white light. The peak of expression in the primary leaves grown under 100 μM/m^2/s white light is on day 10.

The level of transcripts in the 20 day old primary leaves is at the limit of detection in these experiments. Figure 3.2.2 shows a graphical representation of the data in 3.2.1. The absorbance of the autoradiographs was measured using a laser densitometer (Section 2.13). The values obtained for the dark grown plants were multiplied by 0.375 to compensate for the greater amount of total RNA loaded on the gel. This figure illustrates that the pattern of expression in the primary leaves of plants grown under 50 $\mu mol/m^2/s$ white light is very similar to that seen in those of the dark grown plants. The pattern appears to be different in the primary leaves of plants grown under 100 $\mu mol/m^2/s$ white light in that the peak of expression occurs at around 10 days from planting. This has been seen in two other experiments (data not shown) and is therefore likely to be significant.

3.2.2 Leaf area measured over time in the two light treatments used in Fig 3.2.1

The area of three primary leaves was measured at each of the timepoints in Fig 3.2.1 when primary leaves were harvested for RNA extraction and also at 25 days after planting. This was done for both light treatments. The mean leaf area is plotted for both light treatments against time in Fig 3.2.3. It can be seen by comparing this figure to Fig 3.2.2 that the peak of *rbcS* expression occurs well before the completion of primary leaf expansion.

3.2.3 The changes of *rbcS* mRNA steady state levels in response to light and dark adaptation of primary leaves

Plants were grown from seed 10 to the pot in darkness and some were then transferred to the light. Some of these plants were returned to the dark after two days in the light and after two days in the dark some of these dark adapted plants were returned to the same white light regime as used for the first treatment. The purpose of the

experiments was to investigate the interaction between ontogenetic regulation and light-regulation of *rbcS* expression. A diagramatic description of the growth regime is given in Table 3.2.1. Primary leaves were harvested at the times given in Fig 3.2.4.and the extracted RNA used for northern analysis. The autoradiographs from the experiment, which are shown in Fig 3.2.4, were scanned using a laser densitometer (Section 2.13) and these values were used to plot the graph showing the changes in the steady state *rbcS* RNA levels with time through the experiment (Fig 3.2.5). This graph illustrates a number of interesting observations. The response of the *rbcS* genes to illumination, at the level of accumulated RNA, is seen as an increase in the proportion of the *rbcS* message relative to total RNA. This increase peaks 24 hours following illumination after which the level of *rbcS* mRNA starts to decline. Also illustrated is the fact that, while the peak in *rbcS* mRNA levels in response to the initial illumination occurs after 24 hours of illumination, reillumination of 11 day old dark adapted plants results in the level of *rbcS* mRNA increasing over 48 hours and perhaps beyond. This observation may indicate that there is also a developmental element in the response of the *rbcS* genes to light i.e. the kinetics of the increase in *rbcS* transcript level in response to illumination of a dark grown plant changes with the age and state of development of the plant. Also apparent is the fact that returning 9 day old plants that have been illuminated to darkness, results in the level of *rbcS* expression declining at a greater rate than in those that had been left under illumination. This implies that although the level of *rbcS* mRNA is declining in the illuminated plant, the light must be still 'supporting' the level of *rbcS* since its level falls more quickly in darkness. These observations have been seen in a number of different experiments.

3.2.4 Control of expression of the three *rbcS* genes on illumination of seven day old dark grown plants

The contribution of the three genes' individual transcripts to the increase in *rbcS* mRNA level on illumination was examined by northern blot analysis. Plants were

grown 10 to the pot for 7 days in darkness prior to being illuminated with 150 μmol/m^2/s white light. Four pairs of primary leaves were harvested at the timepoints given in Fig 3.2.6. Total RNA was extracted from the pooled primary leaves and used for northern analysis. Ten μg of total RNA was loaded onto the gel for each timepoint and three identical filters were made and these were used for hybridisation experiments. They were probed with both the pPvSS191 cDNA probe and the non-disciminating and gene-specific oligonucleotide probes. The autoradiographs from these experiments are shown in Fig 3.2.6. Panel A, an autoradiograph probed with the cDNA probe, shows that the *rbcS* mRNA level is detectable in the seven day old dark grown primary leaves. Its level relative to total RNA increases with increasing time in the light for 24 hours and then starts to fall by 48 hours. These results are consistent with those seen in Fig 3.2.5. Panel B is an autoradiograph of the same blot re-probed with the non-discriminating oligonucleotide probe. It can be seen that both probes give the same result, implying that both probes recognise the same transcripts. This filter was then reprobed with the *rbcS1* oligonucleotide probe, and the autoradiograph from this experiment is shown in panel C. The other two autoradiographs are from hybridisations with the other two gene-specific probes; *rbcS2* is shown in panel D and *rbcS3* is shown in panel E. These three blots were probed with equivalent amounts of labelled oligonucleotide, and they show that all three genes are expressed in the dark. Also evident is the fact that the three genes' transcripts all increase with increasing time in the light showing that all three genes are photoregulated. Indeed the pattern of expression is very similar for all three genes with the main difference being in the amounts of mRNA accumulated at each time point. This is emphasised in Fig 3.2.7, a plot of the scans of the three autoradiographs C, D and E, adjusted for the different relative hybridisation efficiencies of the three oligonucleotide probes, against time. These results show that transcripts from *rbcS1* are the most abundant in the light grown plant with those from *rbcS2* the next most abundant and *rbcS3* contributing the least to the *rbcS* mRNA level. The relative amounts of the three genes' transcripts estimated after 24 hours illumination are : *rbcS1* 61%, *rbcS2* 22%, and *rbcS3* 17%.

3.2.5 The regulation of the three *rbcS* genes in the primary leaves of nine day old light adapted plants given a prolonged dark treatment

Experiments were conducted to investigate whether the three *rbcS* genes showed similar regulation when 7 day old plants that had been illuminated for 2 days were returned to darkness. The experiment shown in Fig 3.2.5 indicated that there was a slight increase in the *rbcS* mRNA levels in primary leaves of plants that had been dark adapted for 4 days. This experiment was done to examine whether this was due to one or all of the genes' transcript levels increasing. The initial harvesting of 4 pairs of primary leaves was done after the two days of light treatment and the plants were then returned to the dark. Four pairs of primary leaves were harvested after 2 days and then after a further 2 days. Total RNA was extracted from the pooled leaves and four replicate blots were made. These were probed with the gene specific oligonucleotide probes as well as with the non-discriminating oligonucleotide probe and the autoradiographs from these experiments are shown in Fig 3.2.8. These show that all three genes' transcript levels fall on transfer to darkness. The figure also shows that the transcripts from the *rbcS2* gene appear to account for the mRNA present in the dark treated leaves and indeed appears to increase between two and four days in the dark. This particular experiment was not repeated but the level of *rbcS* transcript can be seen to be increasing after 4 days dark adaption of plants treated in similar manner to those in Fig 3.2.5, which suggests that the level of the *rbcS* transcripts does increase after 4 days dark treatment. Whether this increase is due to the expression of the *rbcS2* gene alone is considered in the discussion (Section 4.2.3). Ethidium bromide staining of the gel, and blot suggests that the difference is not due to loading differences between the samples (data not shown).

Fig 3.2.1 The steady state level of *rbcS* mRNA in *Phaseolus vulgaris* primary leaves as a fraction of the total extractable RNA measured over time in three different light conditions

Plants were grown at approximately 10 plants to the pot as described in Section 2.2.1. Three different light treatments were used, these being :

A) Complete darkness.

B) 50 μmol/m^2/s white light.

C) 100 μmol/m^2/s white light.

Five pots were used for each treatment and five primary leaves were harvested at the time points shown below. Total RNA was extracted from these pooled leaves and examined by northern analysis as described in Section 2.12.1. For the light grown treatments 7.5 μg of total RNA was loaded in each track, and for the dark grown treatment 20 μg of total RNA was used. The resulting blots were probed with the *rbcSG* oligonucleotide probe at a concentration of 2 x 10^6 incorporated cpm per ml of hybridisation solution in the same hybridisation reaction. The blots were washed as described for Panel A in Fig 3.1.6.

Lane no.	1	2	3	4	5	6	7
Time from planting. (Days)	3	5	7	10	12	15	20

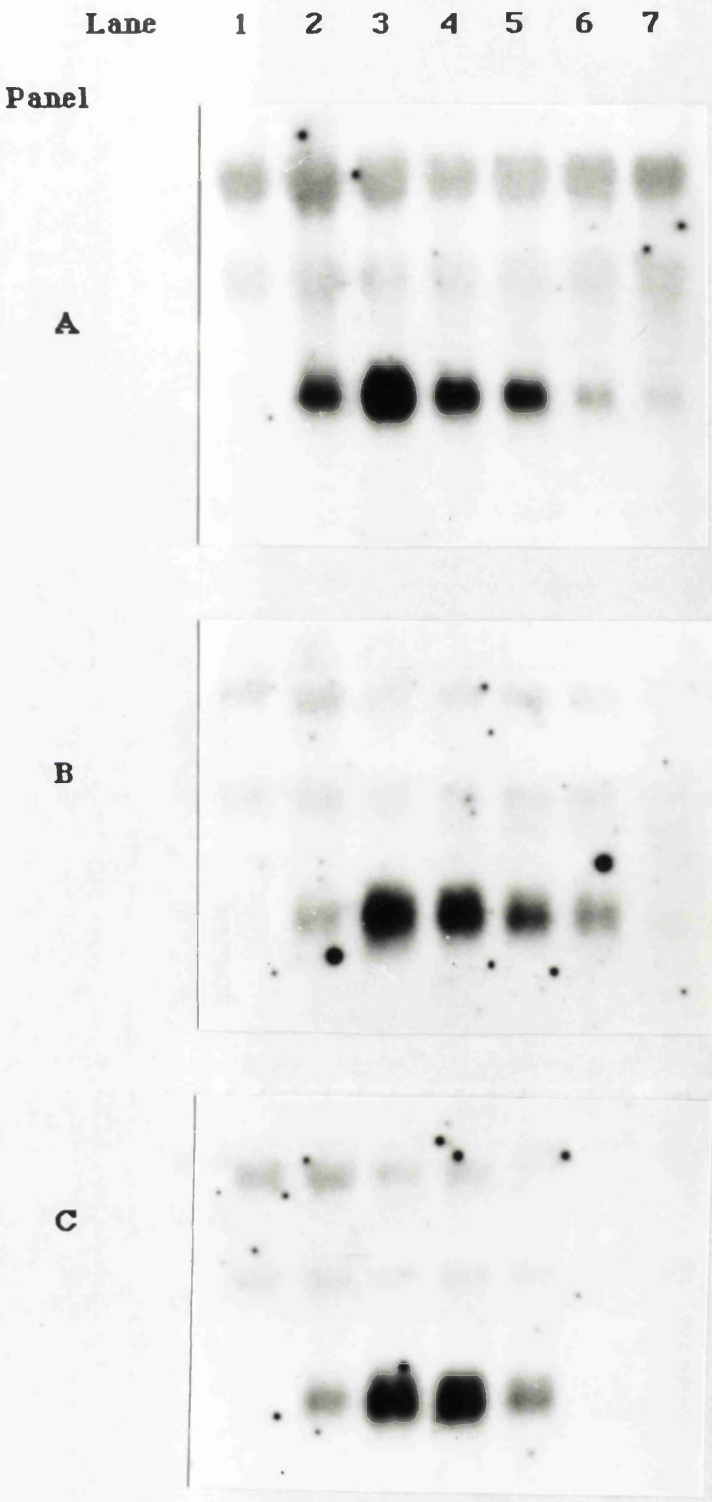

Lane 1 2 3 4 5 6 7
Panel
A
B
C

Fig 3.2.2 A graph showing estimates of the relative amounts of *rbcS* transcripts in the three treatments used in Fig 3.2.1

The autoradiographs presented in Fig 3.2.1 were scanned using a scanning laser densitometer as described in Section 2.13. The areas of the peaks for the dark grown treatment were multiplied by .375 to compensate for the different amounts of total RNA loaded on gels for dark-grown and light-grown samples. The values obtained are plotted against the number of days from planting that each sample was taken.

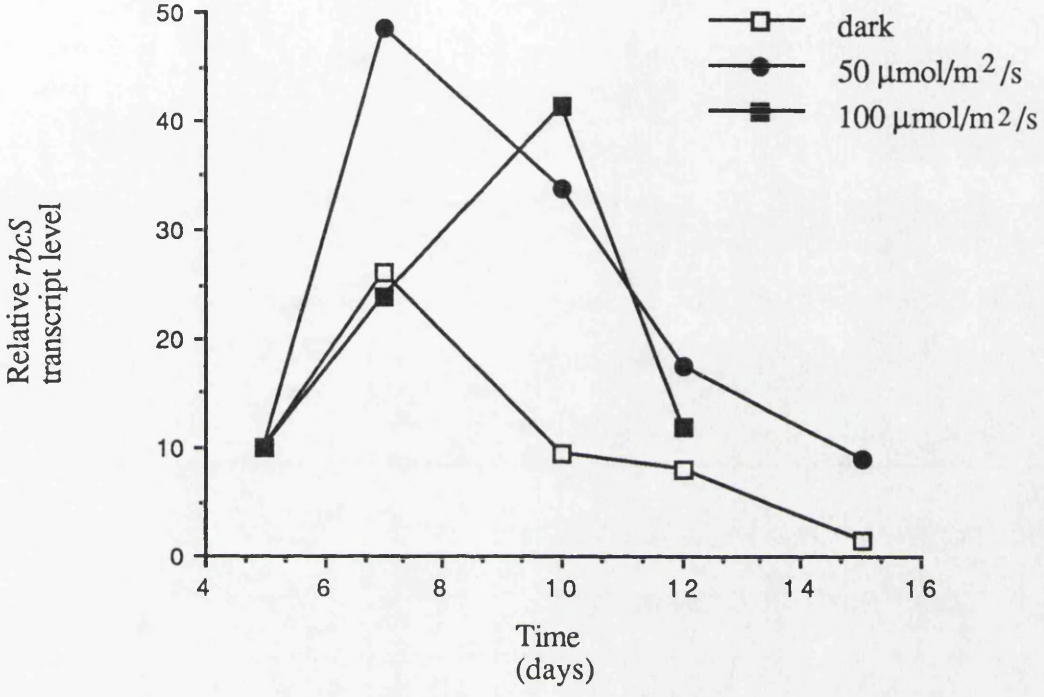

Relative rbcS transcript level
50
40
30
20
10
0
4
6
8
10
12
14
16
Time (days)
dark
50 µmol/m2/s
100 µmol/m2/s

Fig 3.2.3 The area of primary leaves under two different light conditions

The area of the primary leaves used in the experiment described in Section 3.2.1 was measured for each of the timepoints at which RNA was extracted. The mean leaf area is plotted against time for plants grown under 100 $\mu mol/m^2/s$ and 50 $\mu mol/m^2/s$ white light.

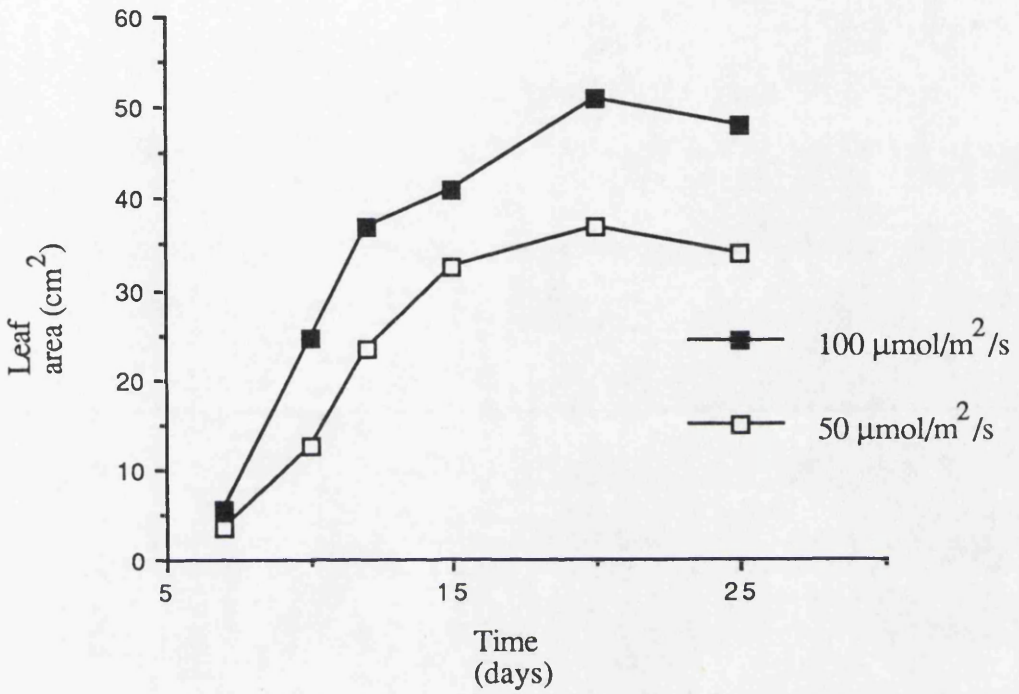
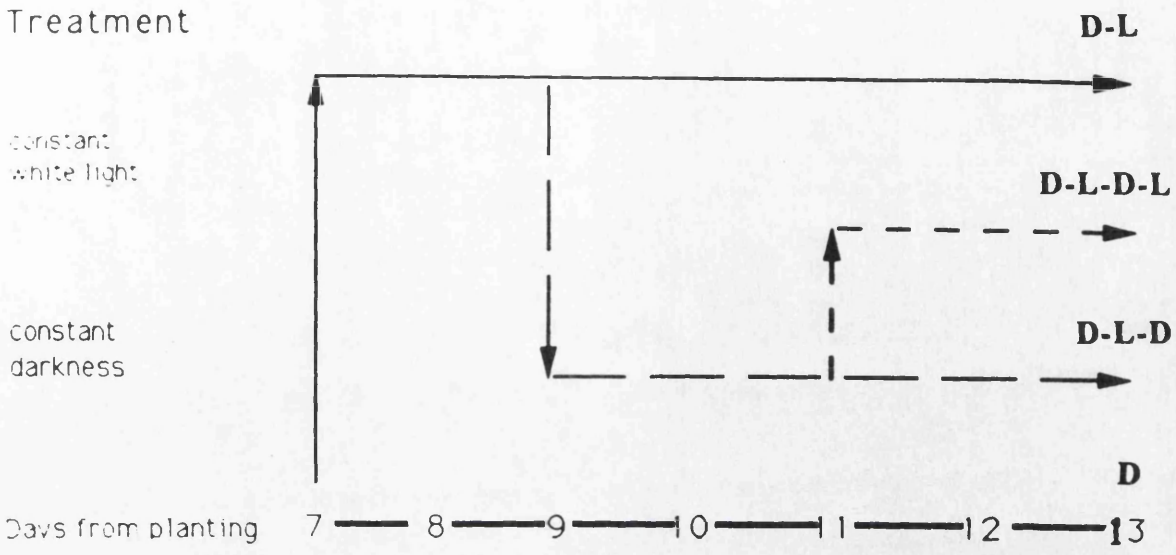

60
50
40
30
20
10
0
Leaf area (cm^2)
5
15
25
Time (days)
100 µmol/m^2/s
50 µmol/m^2/s

Table 3.2.1 A diagramatic representation of the experimental treatments used to examine the regulation of *rbcS* levels in response to changes in the light environment

Plants were grown, 10 to the pot, in darkness for 7 days. Some plants were left in the dark while others were transferred to continuous 100 μmol/m2/s white light. After two days some of these light treated plants were returned to the dark while the rest were left in the light. After a further two days some of those plants returned to the dark were returned to the light.



Plants moved from:		to:	Treatment
Constant darkness	———	Constant light	Light **D-L**
Constant light	—— ——	Constant darkness	Returned to Darkness **D-L-D**
Constant darkness	— — — -	Constant light	Returned to Light **D-L-D-L**
Plants left in constant darkness	———	Plants left in constant darkness	Kept in constant Darkness **D**

Fig 3.2.4 Autoradiographs showing the *rbcS* mRNA levels as a fraction of the total primary leaf RNA over time in response to the treatments described in Table 3.2.1

Plants were grown in the manner described in Table 3.2.1 and 5 leaves were harvested, pooled and used for RNA extractions at the times indicated. The RNA was used for northern analysis with 10 µg of total RNA used for each timepoint. The blots produced were hybridised in the same solution with the *P.vulgaris* pPvSS191 *rbcS* cDNA probe at a concentration of 1.5 x 10^6 incorporated cpm per ml of hybridisation solution.

Treatment	Dark Grown (D)						Moved to light (D-L)					
Lane No.	1	2	3	4	5	6	1	2	3	4	5	6
Days from sowing.	7	8	9	10	11	13	7	8	9	10	11	13
Time from start of treatment. (hours)	0	24	48	72	96	144	12	24	48	72	96	144

Treatment	Returned to Darkness (D-L-D)						Returned to Light (D-L-D-L)			
Lane No.	1	2	3	4	5	6	1	2	3	4
Days from sowing.	9		10	11	12	13	11		12	13
Time from start of treatment. (hours)	6	12	24	48	72	96	6	12	24	48

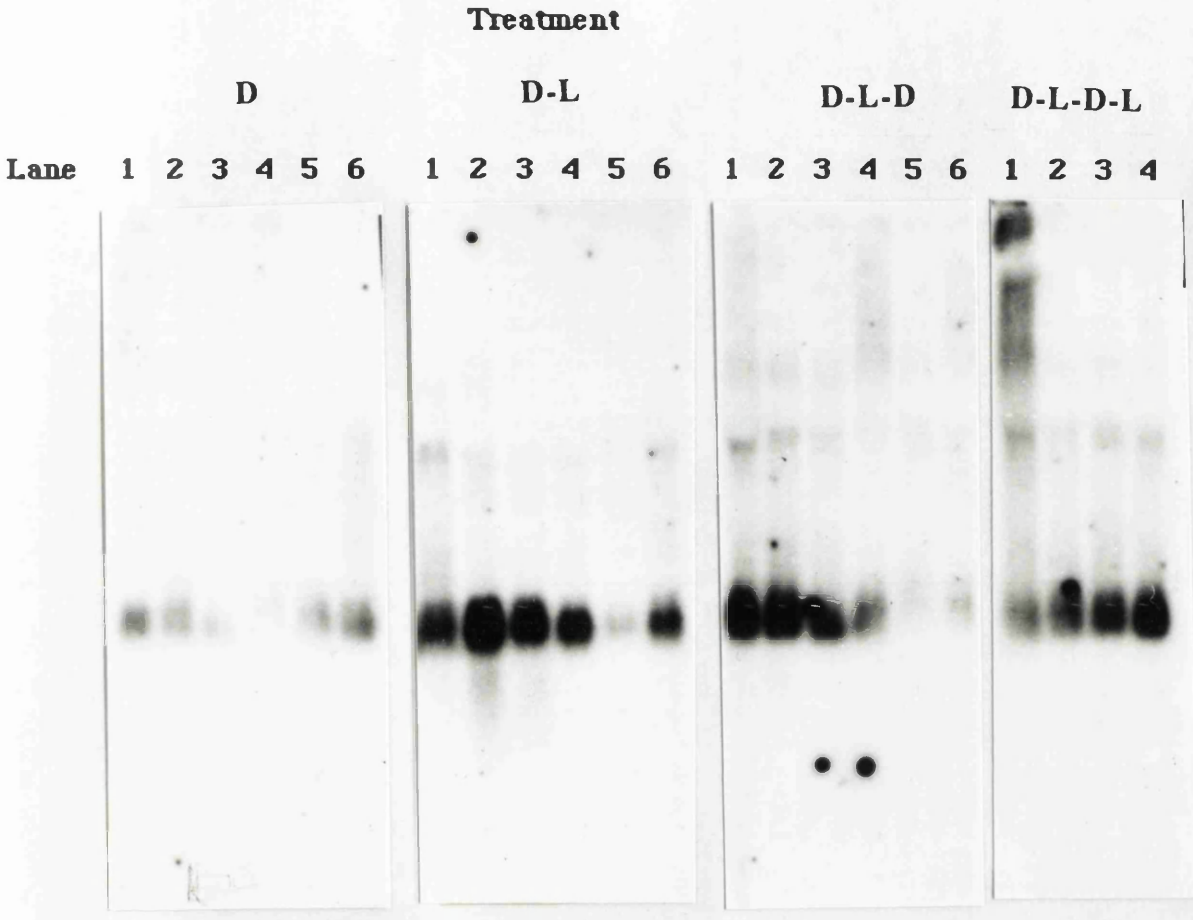

Treatment
D
D-L
D-L-D
D-L-D-L
Lane 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4

Fig 3.2.5 Graph showing estimates of the relative amount of *rbcS* mRNA present for each treatment examined in the experiment described in Fig 3.2.4

The autoradiographs shown in Fig 3.2.4 were scanned using a laser densitometer (Section 2.13). The absorbances obtained were used to plot a graph showing the relative amounts of *rbcS* mRNA present in 10 μg of total RNA at each of the timepoints examined in each of the treatments used. These treatments were :

D : Continuous darkness.
D-L : Dark-grown plants transferred to light.
D-L-D : Illuminated plants returned to dark.
D-L-D-L : Dark adapted plants returned to light.

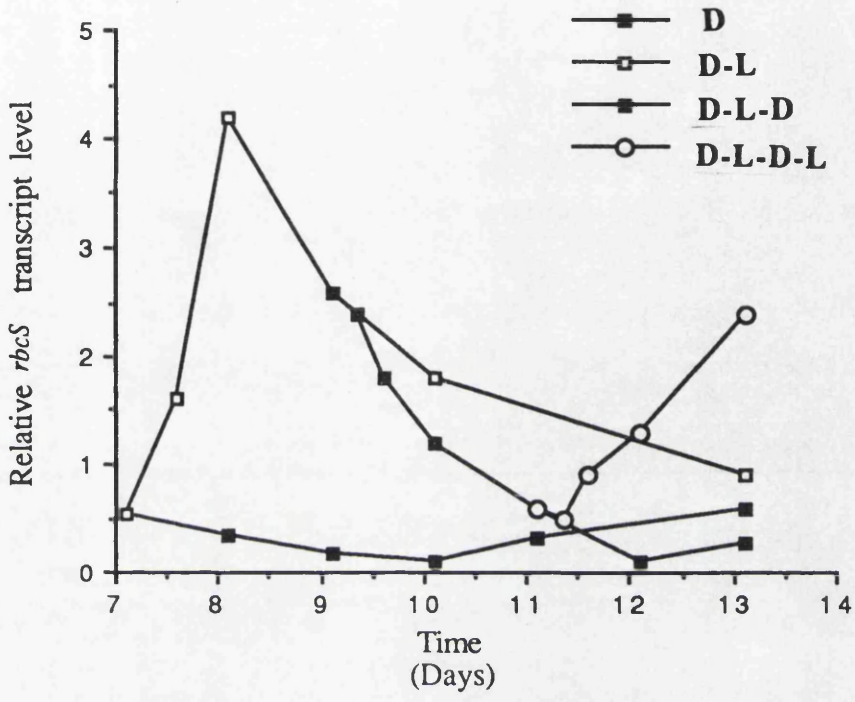

D
D-L
D-L-D
D-L-D-L
Relative rbcS transcript level
0
1
2
3
4
5
7
8
9
10
11
12
13
14
Time
(Days)

Fig 3.2.6 Autoradiographs showing the expression of the three *rbcS* genes following illumination of 7 day old dark grown plants

Plants were grown 10 to the pot in the dark for 7 days. They were then placed in continuous 150μM/m^2/s white light for 2 days. Four pairs of primary leaves were harvested at the times indicated and total RNA was extracted for use in northern analysis. Ten μg of total RNA were used for each timepoint and three replicate filters were used. The autoradiographs from this experiment are described below :

Panel	Probe used
A	pPvSS191 cDNA; 1.5 x 10^6 cpm per ml hybridisation solution.
B	*rbcSG* oligonucleotide ; 2 x 10^6 cpm per ml hybridisation solution.
C	*rbcS1* oligonucleotide ; 2 x 10^6 cpm per ml hybridisation solution.
D	*rbcS2* oligonucleotide ; 2 x 10^6 cpm per ml hybridisation solution.
E	*rbcS3* oligonucleotide ; 2 x 10^6 cpm per ml hybridisation solution.

Lane No.	1	2	3	4	5	6	7
Time under illumination (hours)	0	1	3	6	12	24	48

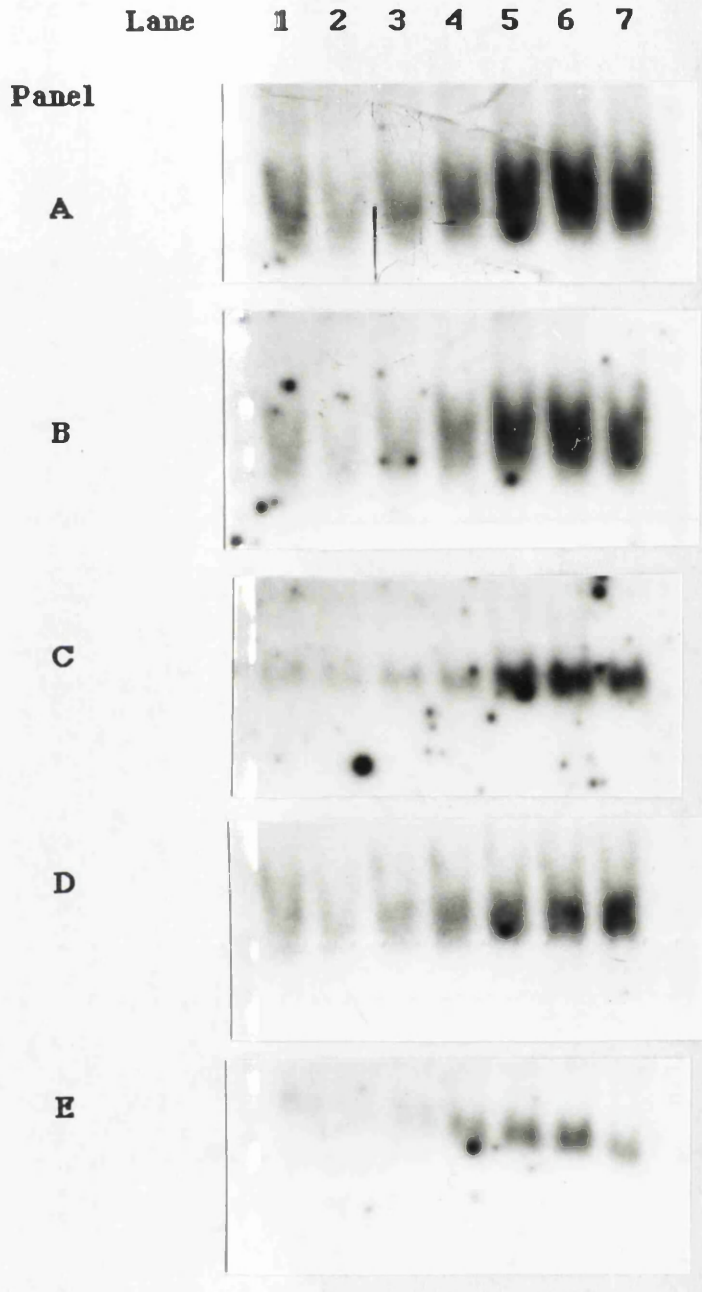

Lane 1 2 3 4 5 6 7
Panel
A
B
C
D
E

Fig 3.2.7 A graph showing the relative transcript levels of the three *rbcS* genes in 7 day old dark grown plants transferred to light for 48 hours

The autoradiographs shown in panels C, D, and E of Fig 3.2.6 were scanned as described in Section 2.13 to give an estimate of the relative amount of transcript present for each of the three *rbcS* genes at each of the timepoints examined. The absorbance values for *rbcS1* and *rbcS3* were multiplied by 1.41 and 1.85 respectively to adjust the values for the hybridisation efficiency of their probes relative to that of the *rbcS2* oligonucleotide (see Section 3.1.8). These values were plotted against the time of harvest in white light.

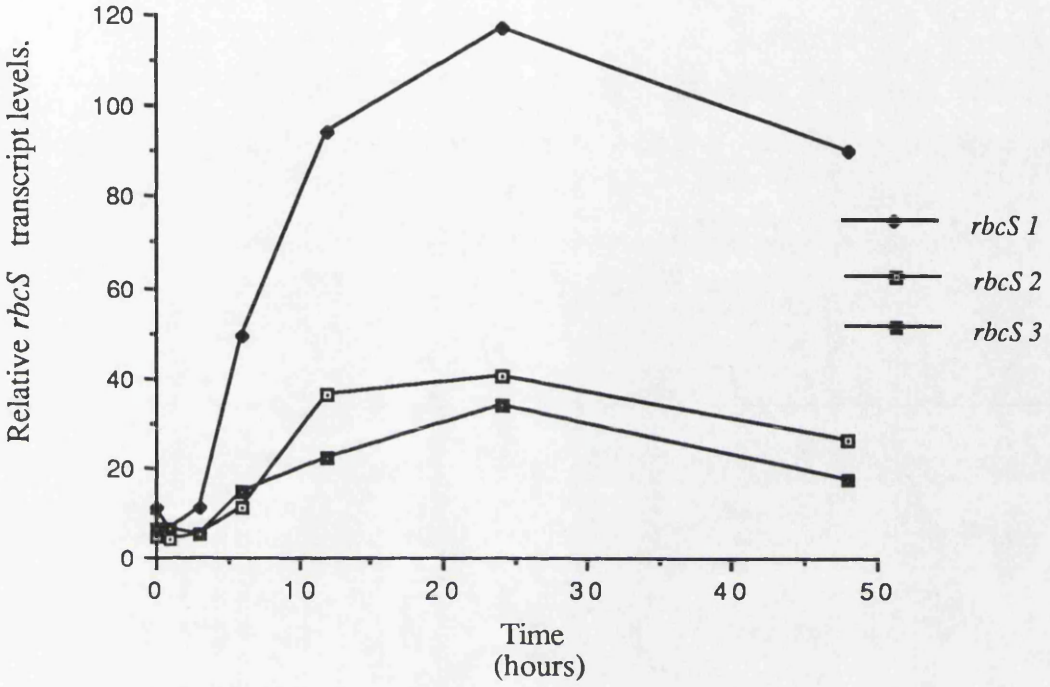
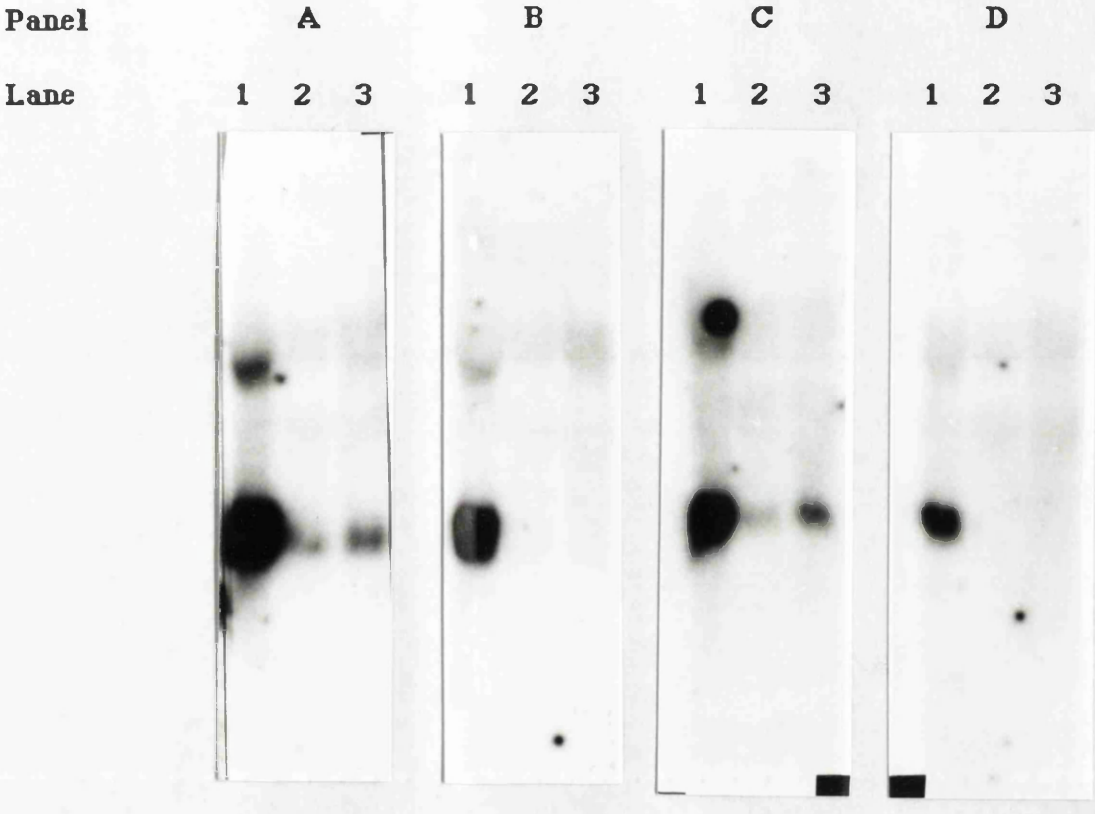

Relative rbcS transcript levels.
120
100
80
60
40
20
0
0
10
20
30
40
50
Time
(hours)
rbcS 1
rbcS 2
rbcS 3

Fig 3.2.8 Autoradiographs showing the effect of prolonged darkness on the three *rbcS* genes in light-adapted 9 day old plants

Plants were grown 10 to the pot in complete darkness for 7 days and then transferred to 150μM/m^2/s white light for 2 days. The plants were then returned to the dark for four days. Four pairs of primary leaves were harvested just prior to the return to the dark and then 2 days and 4 days into the dark treatment. Total RNA was extracted from the pooled leaves and examined by northern analysis. Four replicate blots were made with 10 μg of total RNA per lane. Each blot was probed with one of the four oligonucleotide probes at a probe concentration of 1.5 x 10^6 incorporated cpm per ml of hybridisation solution. In each autoradiograph the tracks are :

1) Day 9 i.e. 7 days dark grown then 2 days light grown.
2) Day 11 i.e. 2 days dark treatment after day 9.
3) Day 13 i.e. 4 days dark treatment after day 9.

Panel	A	B	C	D
Oligonucleotide probe used	*rbcSG*	*rbcS1*	*rbcS2*	*rbcS3*


Panel
A
B
C
D
Lane
1 2 3
1 2 3
1 2 3
1 2 3

3.3 The identity of the photoreceptor responsible for the response of *rbcS* genes in *P. vulgaris* primary leaves to an increased fluence rate of light

The *rbcS* genes of *P. vulgaris* have previously been shown to respond to an increased irradiance of white light in such a way that the steady state level of their transcripts, as a proportion of the total RNA, is increased after 2 days treatment with the increased irradiance (Jenkins, 1986). The experiments described in this section confirm such a response and examine the identity of the photoreceptor(s) responsible for the measurement of the fluence rate of light perceived and which lead to the increase in the *rbcS* mRNA level. The regulation of the mRNA levels of the three *rbcS* genes and the α–tubulin gene(s) in response to an increased fluence rate of blue-enriched light is also examined.

3.3.1 The light regimes used for these experiments

The fluence rates of the light regimes used for these experiments are described for each experiment. Three different qualities of light were used in this study; these being white, red and blue-enriched (blue). The spectral distribution of photons in each of these light regimes at a fluence rate of 150 μmol/m^2/s was measured as described in Section 2.2.3.2 and are shown in Fig 3.1.1. An important fact to notice is that the red light contains no detectable light under 500 nm , i.e. it has no blue light. The phytochrome photoequilibria under each of these light regimes and the cycling rate between P_{fr} and P_r was estimated using the method of Gardener and Graceffo (1982) by E. López-Juez. These values are given in Table 3.3.1. It can be seen that the estimated photoequilibria under the red and white light sources are the same and that the cycling rate in the red is approximately five times that in the blue.

3.3.2 The response of *rbcS* genes to an increased fluence rate of white light in light grown plants

Plants were grown, one to the pot, from seed under 15 μmol/m²/s white light for 16 days. Under these conditions the plants are approximately twice the height of plants grown under high white light for the same length of time implying the action of a shade avoidance response. Some of these plants were then transferred to 150 μmol/m²/s white light and their primary leaves held perpendicular to the incident light to ensure an equal irradiance across the leaf, as the studies of Prioul and Reyss (1987) found that shading of one part of a tobacco leaf resulted in a lower *rbcS* mRNA level in that part of the leaf. After 2 days the primary leaves were harvested from both the 150 μmol/m²/s white treated plants and those left under the 15 μmol/m²/s white regime. These were used for RNA extraction and the *rbcS* mRNA level examined by northern blot analysis. The autoradiograph from this experiment is shown in Fig 3.3.2. This shows that a treatment with an increased fluence rate of white light results in an increase in the level of *rbcS* mRNA relative to the total RNA extractable from the primary leaves. This finding is consistent with that of Jenkins (1986).

3.3.3 The effect of increased fluence rates of different qualities of light on the steady state transcript level of *rbcS*

This experiment was performed to examine which photoreceptor(s) was/were involved in the regulation of the *rbcS* genes in response to giving the plant an increased fluence rate of light. To this end plants were grown under 15 μmol/m²/s light for 16 days. Then 8 plants at the same developmental stage (as estimated by height and leaf area) were chosen for movement to different light regimes and their primary leaves held perpendicular to the incident light to ensure an equal fluence rate across the leaf. The light regimes used are described in Fig 3.3.3. The primary leaves were harvested after 2 days treatment and used for RNA extraction. The RNA extracted was used for

northern analysis and a representative autoradiograph from one of the five experiments undertaken independently is shown in Panel A of Fig 3.3.3. This autoradiograph shows that increasing the fluence rate of the blue light (lanes 6 ,7 and 8) results in an increase in the amount of *rbcS* mRNA present as a proportion of the total RNA extractable from the primary leaves. In contrast, increasing the fluence rate of red light (lanes 3 ,4 and 5) has no such effect. The figure also illustrates that the high white light treatment (lane 2) does not give as large an increase in *rbcS* mRNA level as the high blue light (lane 8) probably because it is the blue component of the white light that is responsible for the effect. The amount of light below 500 nm in the white light accounts for approximately 8 % of its fluence rate whereas the proportion rises to 68 % in the blue enriched light (Table 3.3.1). These results imply the involvement of a blue photoreceptor in fluence rate detection for if the photoreceptor responsible for mediating the increase in *rbcS* mRNA levels with increasing irradiance was measuring the red end of the spectrum the red light regime would give an increase in *rbcS* mRNA levels with the increasing fluence rates of red light. As light from both the red and blue end of the spectrum is absorbed with approximately equal efficiency by the photosynthetic pigments, this result implies that these pigments, such as chlorophyll, could not be responsible for the response. Also, the phytochrome photoequilibria under the red and white light regimes is the same (Table 3.3.1) but the *rbcS* mRNA levels are not the same under these two light regimes at the same fluence rate. This implies that the phytochrome photoequilibrium is not the controlling factor. Futhermore, the phytochrome cycling rate is five times higher in the red than in the blue. If this were the controlling factor then the expression of *rbcS* genes would be higher in the red than in the blue. If the lower cycling rate in the blue resulted in the increase in expression then an increase in expression would also be expected to be seen in the 15 $\mu mol/m^2/s$ white light as compared to the 150 $\mu mol/m^2/s$ white light whereas the opposite is the case (Fig 3.3.3, lanes 1 and 2 respectively).

Panel B of Fig 3.3.3 shows a similar autoradiograph except that it contains 10 μg of total RNA per lane from each light treatment. This blot was probed with the α

tubulin sequence from *Chlamydomonas reinhardtii* at a greater probe concentration (see Legend, Fig 3.3.3). The autoradiograph shows that the level of α tubulin mRNA is the same in all of the light treatments. This indicates that the effect of the different light treatments on the level of *rbcS* mRNA is not seen when other RNA polymerase II transcripts are examined, implying that this effect is not an increase in the mRNA levels of all RNA polymerase II transcribed genes.

3.3.4 The efficiency of utilisation of the light regimes used in this experiment by the photosynthetic light harvesting pigments as measured by oxygen evolution

The effect of blue light on the *rbcS* mRNA level could have been due to the particular blue light used being utilised more effectively by the light harvesting apparatus in the light reactions of photosynthesis than the red light. If this was the case there would be more reducing power available for the dark reactions in the blue light and this could produce a greater demand for ribulose 1,5-bisphosphate carboxylase and thus increased *rbcS* expression. To examine this possibility plants were grown as described in Fig 3.3.2 under 15 μmol/m^2/s white light for 16 days. Several 10 cm^2 leaf disks were cut out from the centre of leaves at the same developmental stage and these were used in a leaf disk oxygen electrode to measure the rate of photosynthesis. The leaf disks were illuminated with 77 μmol/m^2/s blue light and with 71 μmol/m^2/s red light and the oxygen evolution was measured under both light treatments. The results obtained are shown in Table 3.3.2. The order of illumination was varied and the respiration rate (oxygen uptake) was checked after each illumination (by returning the leaf to darkness). The results show that the rates of oxygen evolution were the same under both the red and the blue light sources. This implies that differential efficiency of usage of the light sources by the photosynthetic pigments cannot explain the increase in *rbcS* expression under the blue light regime. The figure also shows values for the oxygen evolution obtained from leaf disks cut from plants grown under 120 μmol/m^2/s

white light. These values are higher than those seen with plants grown under 15 μmol/m^2/s white light indicating that growth under a higher fluence rate of light results in an increase in the photosynthetic rate. These values are also approximately the same under both the red and blue light regimes implying that the similarity in oxygen evolution rates under the two light sources seen with leaves grown under 15 μmol/m^2/s white light was not particular to chloroplasts grown under low light conditions.

3.3.5 The opening of stomata under the different light regimes used in this study

Another possible way the blue light could affect the level of the *rbcS* mRNA could be through the stomata being more open in the blue light than in the red. The stomata are the pores in the under side of the bean leaf that are the entry point for the atmospheric carbon dioxide that is fixed by Rubisco. If the stomata were preferentially closed under the red light source then the leaves would be deprived of the substrate for the reaction catalysed by Rubisco and this could result in the low level of expression seen under red light.

Plants were grown in the same way as described in Section 3.3.3. After 16 days 5 plants at the same developmental stage were placed under the red source described in the previous Section. The plants were left to equilibrate for 15 minutes and then three different readings were taken for 5 primary leaves in a random order using the Li-Cor diffusive resistance meter (Section 2.18). The plants were returned to low white light and left for one hour prior to being placed under the same blue source as used in the previous Section. The same number of readings were taken on the same leaves after another 15 minute equilibration period. The raw data and the corrected data for the blue and red treatments are presented in Table 3.3.3, I and II respectively. The mean stomatal resistance for the plants in the blue regime can be seen to be slightly less than that for the red implying that the stomata are slightly more open in the blue light,

but this difference is unlikely to be large enough to explain the large difference seen in the *rbcS* mRNA levels between the two light treatments.

3.3.6 The effect of increasing the fluence rate of blue light on the transcript levels for the three *rbcS* genes

These experiments were carried out to examine whether the increase in *rbcS* transcript levels seen with an increasing fluence rate of blue light was a result of the levels of transcripts from all the *rbcS* genes increasing or whether there were differences between the genes. Plants were grown as described in Section 3.3.3 for 16 days and then transferred to 15, 50, and 150 μmol/m²/s blue light and also to 150 μmol/m²/s white light for two days. The primary leaves were held perpendicular to the incident light to ensure an equal irradiance across the leaf. The primary leaves were then harvested and used for northern analysis. Four replicate blots were made and three of the blots were probed with the gene specific oligonucleotide probes indicated in Fig 3.3.4. The other blot was probed with the *Chlamydomonas reinhardtii* α–tubulin cDNA probe (also shown in Fig 3.3.4). The three blots probed with the gene specific oligonucleotide probes show that the level of each gene's transcript increases relative to the total RNA with increasing irradiances of blue light. The *rbcS3* gene transcript contributes the least to the *rbcS* transcript pool with the other two genes appearing to contribute roughly equally. As the *rbcS2* specific oligonucleotide hybridises with greater efficiency than the *rbcS1* specific oligonucleotide the level of the *rbcS1* transcript is probably greater. The α-tubulin probe on the other hand shows that the α-tubulin transcript(s) do not increase with increasing irradiances of blue light with approximately the same level being present in all the light treatments. This again implies that the effect of blue light on *rbcS* mRNA is not a non-specific increase in the amount of mRNA as a whole relative to total RNA. In these experiments the level of *rbcS* transcripts in the 150 μmol/m²/s white treated plants can be seen to be approximately the same as that seen for the 15 μmol/m²/s blue treated plants. This again emphasises

the fact that it is probably blue light that results in an increase in the *rbcS* transcript levels.

3.3.7 The effect of increasing the fluence rate of red and blue-enriched light on the *rbcS* transcript levels in older primary leaves

These experiments were performed to ascertain whether the effect of blue light on the *rbcS* transcript levels was apparent at a later stage in the development of the primary leaf. Plants were grown one to the pot for 25 days in 15 μmol/m^2/s white light. Plants of the same developmental stage (second pair of trifoliate leaves just opening) were then transferred to 150 μmol/m^2/s blue or 150 μmol/m^2/s red light or left in 15 μmol/m^2/s white light. The primary leaves were harvested and used for RNA extraction. The RNA was then examined by northern analysis and a representative autoradiograph from one of two experiments is shown in Fig 3.3.5. The results show that even at this late stage in the primary leaf's life it is able to respond to an increased fluence rate of blue light with an increase in *rbcS* mRNA relative to total RNA. This response is still not brought about by an increase of red light. This implies that the blue response is not limited to one period of the primary leaf's development.

Table 3.3.1 The properties of the light environments

The calculated phytochrome photoequilibrium (øc) and the rate of cycling between Pr and Pfr forms is shown for each of the light qualities used in this study.

Light Quality (See Fig 3.3.1)	Fluence rate (400-700 nm) $\mu mol/m^2/s$	øc	Phytochrome cycling rate s^{-1}
White (8% <500 nm)	50	0.71	2.98×10^5
Red (>500 nm; no blue)	50	0.72	4.93×10^5
Blue-enriched (68% < 500nm)	50	0.62	0.92×10^5

Fig 3.3.1 The spectral distributions of photons in each of the three light qualities used

Three different light qualities were used in this study, namely white, red and blue enriched. The spectral distributions of photons in each of these qualities was measured using a Macam Instruments spectroradiometer at a light intensity of 150 $\mu mol/m^2/s$ in each case. The number of quanta are plotted against the wavelength measured.

Light Quality: Blue-Enriched
Red
White

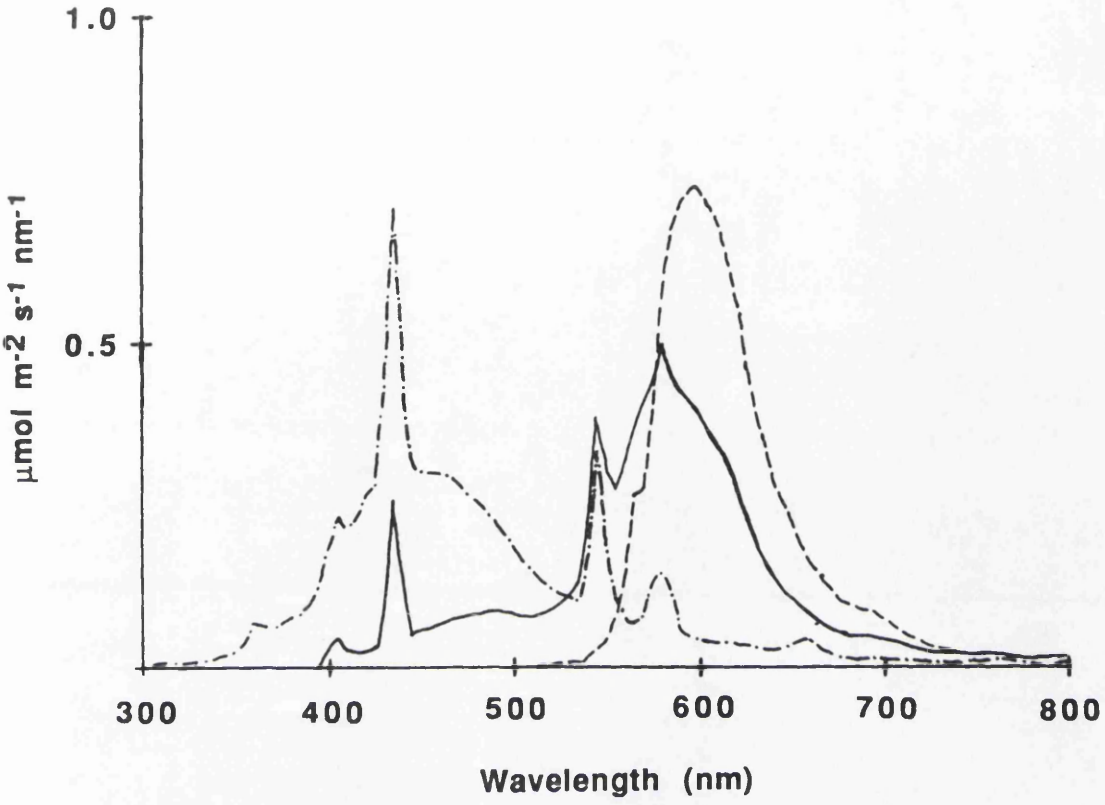

1.0
0.5
µmol m-2 s-1 nm-1
300
400
500
600
700
800
Wavelength (nm)

Fig 3.3.2 Autoradiograph showing the effect of two days increased fluence rate of white light on the *rbcS* mRNA level in the primary leaves of 16 day old 15 μmol/m²/s white light grown plants

Three 16 day old 15 μmol/m²/s white light grown plants were moved to 150 μmol/m²/s white light for two days. Their primary leaves were held so that the incident light was the same intensity over the leaf (i.e. the leaves were perpendicular to the light source). After this time the primary leaves from plants under both light regimes were harvested and used for RNA extraction. Equal amounts of total RNA (7.5 μg) from each treatment were used for northern analysis.

Lane 1 : 15 μmol/m²/s for 16 days, 150 μmol/m²/s white light for 2 days.

Lane 2 : 15 μmol/m²/s for 18 days.

The blot was probed with the pPvSS191 *rbcS* cDNA probe at a concentration of 1.5 x 10^6 cpm per ml of hybridisation solution.

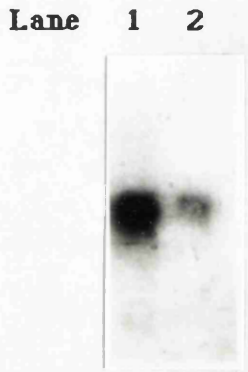

Lane 1 2

Fig 3.3.3 The effect of different light qualities and fluence rates on the level of *rbcS* and α tubulin mRNA in the primary leaves of 16 day old low-white grown plants

Forty plants were grown one to the pot for 16 days under low-white light. Eight plants that were at the same developmental stage (as estimated by height and leaf area) were then chosen. One plant was moved to each light regime for 2 days and their primary leaves were held so that the incident light was the same intensity over the leaf (i.e. the leaves were perpendicular to the light source). The light regimes are described below :

Light quality (Fig 3.3.1)	Fluence Rate ($\mu mol/m^2/s$)	Lane
White	15	1
	150	2
Red	15	3
	50	4
	150	5
Blue	15	6
	50	7
	150	8

The primary leaves were harvested and total RNA was extracted for use in northern analysis. This autoradiograph in Panel A is representative of those obtained from 5 repeat experiments. Five μg of total RNA from each treatment was used. The blot was probed with the pPvSS191 *rbcS* cDNA probe at a concentration of 1.5×10^6 incorporated cpm per ml of hybridisation solution.

The autoradiograph shown in panel B is from the same experiment except that 10 μg of total RNA was used for each treatment and the blot was probed with the *Chlamydomonas reinhardtii* α tubulin cDNA probe at a concentration of 2×10^6 incorporated cpm per ml of hybridisation solution and washed as appropriate for this probe (Panel B, Fig 3.1.4).

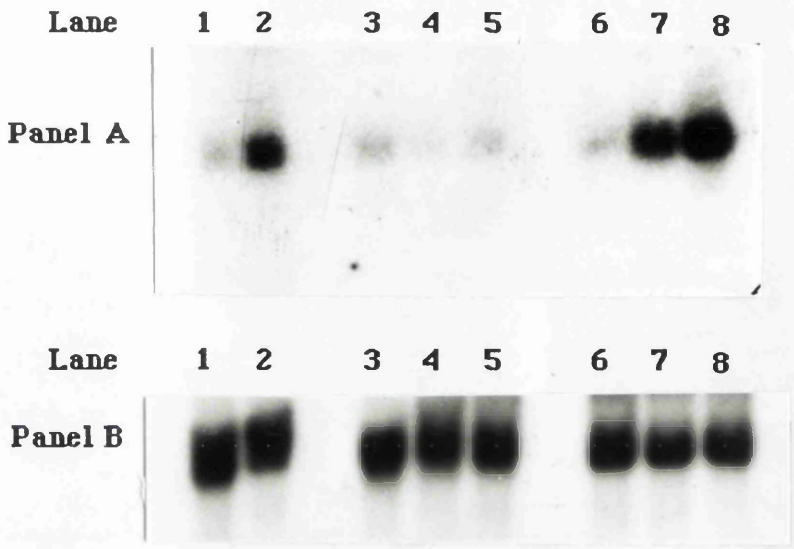

Lane 1 2 3 4 5 6 7 8
Panel A
Lane 1 2 3 4 5 6 7 8
Panel B

Table 3.3.2 Photosynthetic oxygen evolution in leaf disks measured under the blue and red light sources used in this study

Plants were grown, one to the pot, under the following light regimes : A,15 $\mu mol/m^2/s$ white light, and B, 120 $\mu mol/m^2/s$ white light, for 16 days. Leaf disks ($10cm^2$) were then taken from the centre of the primary leaves of those plants at the same developmental stage (as estimated by height and leaf area) and these were used in a leaf-disk oxygen electrode to measure the photosynthetic oxygen evolution. The method is described in Section 2.17. Two different light regimes were used for these experiments : 77 $\mu mol/m^2/s$ blue enriched light.

71 $\mu mol/m^2/s$ red light. (see Fig 3.3.1 for spectra)

Four or five different leaf disks were used for each experiment and the order of illumination was varied. The values measured are presented together with the means for each treatment for each leaf type, and the standard errors.

A) Leaf discs from plants grown under 15 μmol/m^2/s white light

	Light source used	
	Blue-enriched	Red
Leaf Disc number	Oxygen Evolution	
	mmol O_2 /min per leaf disc	mmol O_2 /min per leaf disc
1	0.022	0.020
2	0.019	0.023
3	0.027	0.023
4	0.023	0.029
	Mean	**Mean**
	0.024	**0.024**
	S.E.+/- 0.0016	S.E.+/-0.0018

B) Leaf discs from plants grown under 120 μmol/m^2/s white light

	Light source used	
	Blue-enriched	Red
Leaf Disc number	Oxygen Evolution	
	mmol O_2 /min per leaf disc	mmol O_2 /min per leaf disc
1	0.039	0.036
2	0.026	0.026
3	-------	0.030
4	0.031	0.042
5	0.046	0.039
	Mean	**Mean**
	0.036	**0.035**
	S.E.+/- 0.0038	S.E.+/-0.0030

Table 3.3.3 The stomatal resistance of 16 day old low-white grown plants measured under red and blue light regimes

Plants were grown, one to the pot, for 16 days under 15 $\mu mol/m^2/s$ white light. Five plants at the same developmental stage (see Table 3.3.2) were then placed under 71 $\mu mol/m^2/s$ red light and left for 15 minutes. Three readings of the time taken for the required increase in ampage were then taken for each leaf using the Li-Cor diffusive resistance meter at the HUM 2 setting as described in Section 2.18. The readings were taken in a random order. The plants were returned to 15 $\mu mol/m^2/s$ white light for one hour and then illuminated with 77 $\mu mol/m^2/s$ blue enriched light and left for 15 minutes prior to measuring the diffusive resistance of the leaves as for the red experiment. The temperature of the measuring cup was taken for each treatment and used to correct the resistances measured to values at 25°C. The raw data, corrected data, diffusive resistances and the mean diffusive resistances are presented for each treatment.

I. Light source used : Blue light 77 μmol/m²/s.

Leaf used	Time taken (s)	Mean time taken (s)	Mean time.corrected to 25°C (s)	Stomatal diffusive resistance. (s/cm)
1	42	37.6	27.1	11.0
	37			
	34			
2	39	41.3	29.7	12.6
	42			
	43			
3	37	33.7	24.3	10.0
	31			
	33			
4	60	61.3	44.1	20.2
	53			
	71			
5	30	28.2	20.4	8.0
	27			
	28			
		Mean Diffusive	**Stomatal Resistance**	**12.4**

II. Light source used : Red light 71 μmol/m²/s

Leaf used	Time taken (s)	Mean time taken (s)	Mean time.corrected to 25°C (s)	Stomatal diffusive resistance. (s/cm)
1	53	44.0	29.0	12.0
	38			
	41			
2	57	55.3	36.5	15.6
	56			
	53			
3	41	38.3	25.3	10.4
	38			
	36			
4	42	60.3	39.8	17.2
	71			
	68			
5	29	37.0	24.4	10.0
	42			
	39			
		Mean Diffusive	**Stomatal Resistance**	**13.0**

Fig 3.3.4 The effect on increasing fluence rates of blue-enriched light on the transcript levels of the three *rbcS* genes

Plants were grown, one to the pot, under low-white light for 16 days. Plants at the same developmental stage (see Fig.3.3.2) were then moved to the following light environments for 2 days :

1) 150 $\mu m/m^2/s$ blue-enriched
2) 100 $\mu m/m^2/s$ blue-enriched
3) 15 $\mu m/m^2/s$ blue-enriched
4) 150 $\mu m/m^2/s$ white

The primary leaves were then harvested and used for RNA preparations. Ten µg of total RNA were used per treatment. The numbers above are used to identify the lanes on each blot. The three blots probed with the oligonucleotide probes were each probed with 1.7×10^6 cpm of incorporated probe per ml of hybridisation solution. The blot probed with the α tubulin cDNA probe was probed with 1.0×10^6 cpm incorporated probe per ml of hybridisation solution.

Panel	A	B	C	D
Probe used.	*rbcS1*	*rbcS2*	*rbcS3*	α–tubulin

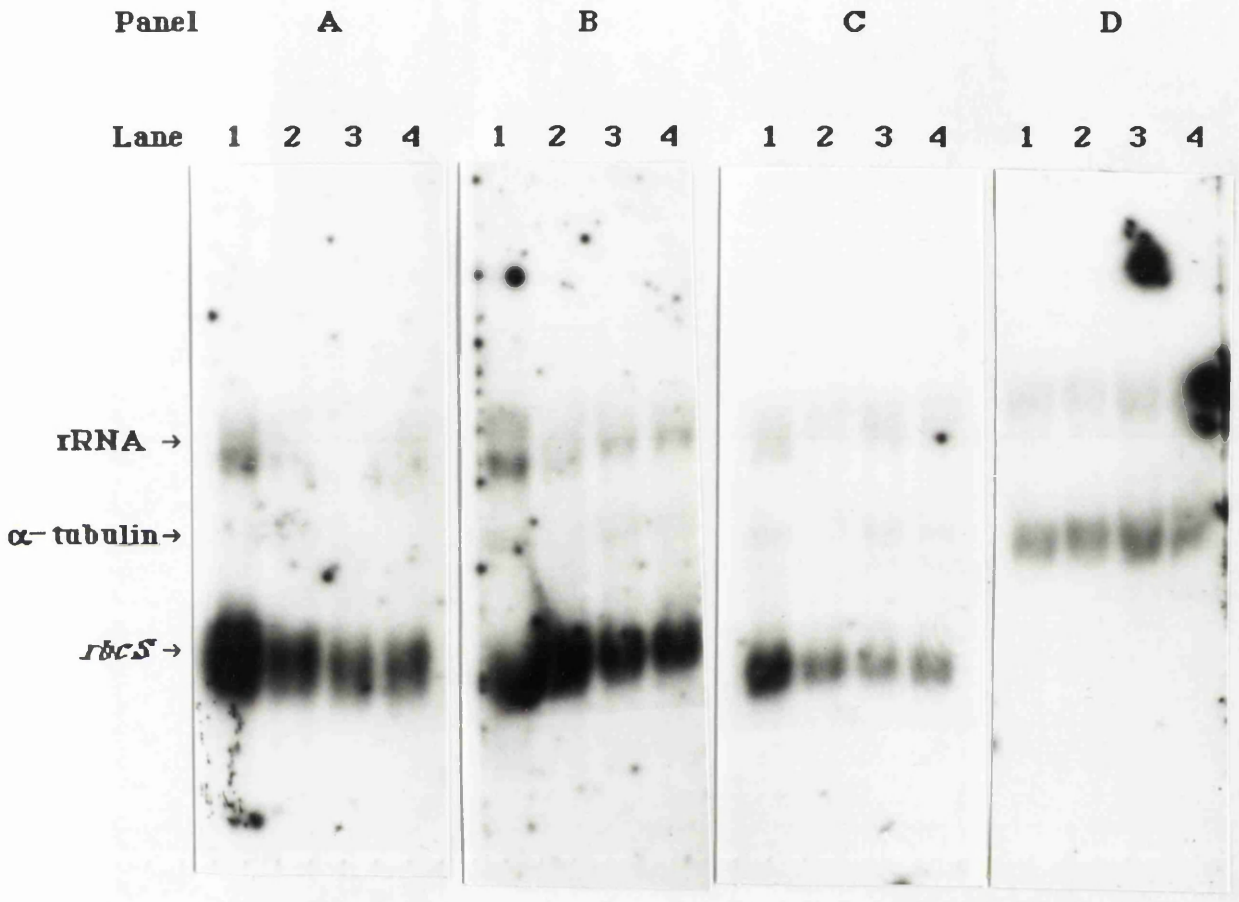
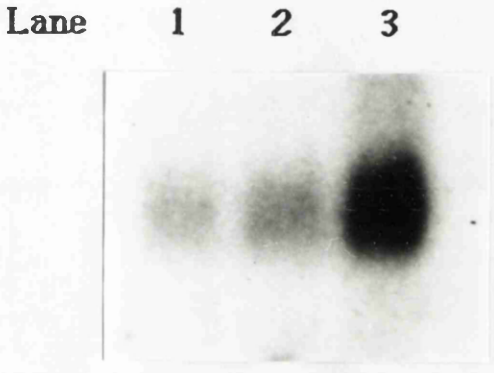

Panel A B C D
Lane 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
rRNA →
α– tubulin →
rbcS →

Fig 3.3.5 The effect of increased fluence rates of red and blue-enriched light on the *rbcS* transcript levels in older plants

Plants were grown, one to the pot, for 25 days under 15 μmol/m^2/s white light. Plants at approximately the same developmental stage (second set of trifoliates just opening) were moved to 150 μmol/m^2/s blue-enriched or 150 μmol/m^2/s red light, or left under 15 μmol/m^2/s white light for two days. The primary leaves were harvested and the total RNA extracted used for northern analysis. Each lane contains 7.5 μg of total RNA and the blot was hybridised with the pPvSS191 *rbcS* cDNA probe at a concentration of 2 x 10^6 incorporated counts per ml. of hybridisation solution.

Lane	1	2	3
Treatment	red	white	blue


Lane 1 2 3

3.4 The regulation of *rbcS* transcription in *P.vulgaris* nuclei isolated from primary leaves treated with different qualities of light

The *rbcS* transcript level as measured by its relative amount in total RNA has been seen to increase in response to increasing irradiances of blue light in the previous section (Section 3.3). This could have been caused by an increase in the rate of transcription of the *rbcS* genes and/or an increase in the post-transcriptional stability of the *rbcS* mRNA. The experiments described in this section examine the first of these possibilities. The method chosen to examine the transcription of the *rbcS* genes was the method of run-on transcription in isolated nuclei described for pea plants by Gallagher and Ellis (1982).

3.4.1 Assessment of the nuclei using visualisation with ethidium bromide and UV microscopy

Plants were grown under 15 μmol/m^2/s white light for 16 days and then transferred to 100 μmol/m^2/s white light for 12 hours prior to isolation of the nuclei as described in Section 2.14. Once isolated an aliquot of the nuclei was stained with ethidium bromide as described in Section 2.14.3 and examined under UV illumination at a magnification of forty times. Photographs of these nuclei are shown in Fig 3.4.1, both under UV illumination and under bright field illumination. Comparison of these two pairs of photographs shows that the majority of the objects seen under bright field illumination are fluorescing under UV illumination, implying that they are nuclei. The number of nuclei in the preparation was estimated by counting the fluorescent nuclei in an area defined by a haemocytometer.

3.4.2 The size of transcripts obtained by carrying out a run-on transcription reaction with isolated nuclei

Two million of the nuclei shown in Fig 3.4.1 were used in a run-on transcription reaction (Section 2.15). The nuclear RNA was then purified as described in Section 2.15.2 except that the final pellet was dissolved in DEPC-treated distilled water. Ten µg of this sample was run on a formaldehyde gel which was then blotted. This blot was autoradiographed and this can be seen in Fig 3.4.2. The position of the wells has been marked and the bottom of the autoradiograph is where the smaller marker dye (bromophenol blue) in the RNA loading mix has reached. The labelled RNA in lane 3 can be seen to start from just below the wells and to form a streak to the bottom of the gel. This implies that a wide range of sizes of RNA has been labelled in the run-on transcription reaction. The darker area at the foot of the gel is not unincorporated label as the incorpation into RNA as measured by the DE-81 chromatography method (Section 2.11) was greater than 95%. These labelled transcripts are therefore probably small labelled RNA molecules.

3.4.3 The relationship between the amount of labelled RNA added to the hybridisation reaction and the specific hybridisation to the *rbcS*-encoding pPvSS191 plasmid

One million purified nuclei were used in a run-on transcription reaction and the labelled RNA purified as described in Sections 2.14-2.15. The RNA concentration in cpm per ml of hybridisation solution was measured by DE-81 chromatography and increasing numbers of cpm were used in a hybridisation reaction (Section 2.15.3). The number of cpm added to each reaction is given in Table 3.4.1 as are the resulting number of cpm hybridised to the pUC 9 control plasmid (background hybridisation) and to the *rbcS* -encoding pPvSS191 plasmid (specific hybridisation). Also shown are the net cpm hybridised (background hybridisation minus specific hybridisation) and

these are plotted against the number of cpm added to each hybridisation reaction in Fig 3.4.3. This Figure shows that there is a linear relationship between the number of cpm added to the hybridisation reaction and the amount of net hybridisation implying that the plasmid DNA is in excess in the reaction for up to 3.5 x 10^6 cpm of RNA added.

3.4.4 Timecourse of the increase in *rbcS* steady state mRNA levels following transfer from 15 μmol/m^2/s white light to an increased fluence rate of blue light

To examine whether the effect of an increased fluence rate of blue light on the steady state *rbcS* mRNA level was mediated at the transcriptional level, knowledge of the time at which the transcription rate was likely to be increased was required. To this end plants were grown, 10 to the pot, for 16 days in 15 μmol/m^2/s white light. This number of plants per pot was required to produce enough primary leaf tissue for nuclei isolation. After 16 days some pots were transferred to 100 μmol/m^2/s blue light. Primary leaves were harvested at the times given in Fig 3.4.4 and used for RNA extraction. The RNA was then used for northern blot analysis and the autoradiograph from this experiment is shown in Fig 3.4.4. This was scanned and the graph in Fig 3.4.5 shows the relative amount of hybridisation for each timepoint. It can be seen that the *rbcS* mRNA level increases as a proportion of the total RNA for 24 hours after transfer and the level has fallen by 48 hours. This observation was seen in three repeats of this experiment. This pattern is reminiscent of that seen when 7 day old etiolated plants are illuminated; that is a peak of *rbcS* mRNA levels after 24 hours followed by a decline. The autoradiograph also shows that the increase seen in *rbcS* steady state transcript level on transfer from 15 μmol/m^2/s white light to 100 μmol/m^2/s blue light is not as great as that seen in the experiments described in Section 3.3.3. This may be due to the fact that the leaves of the plants in this experiment were not supported and were also in close proximity to those of the other plants in the pot. This may have reduced the effective fluence rate of light perceived by the leaves.

3.4.5 The effect of transfer from 15 μmol/m²/s white light to an increased fluence rate of blue light on the rate of *rbcS* transcription

Plants were grown under 15 μmol/m²/s white light for 16 days in the same way as described in Section 3.4.4. After 16 days some pots were moved to 100 μmol/m²/s blue light and nuclei were harvested at the time points given in Table 3.4.2 in both the blue and white light regimes. These leaves were harvested, cut into strips and placed into ice-cold ether in the light regimes in which they were grown and nuclei isolated from them. Labelled RNA was prepared from these nuclei after run-on transcription reactions had been carried out and equal numbers of cpm were hybridised to 5 μg of the *rbcS*-encoding pPvSS191 plasmid and to the same amount of pUC 9 plasmid as a control (Sections 2.14 and 2.15). These experiments were carried out under the conditions previously determined as for DNA excess hybridisations (Section 3.4.3). The net cpm hybridised specifically to the pPvSS191 plasmid for each timepoint were expressed as a percentage of the net cpm specifically hybridising from RNA prepared from leaves harvested in 15 μmol/m²/s white light at time zero. This was done because these nuclei were prepared from plants that had not been moved and these values should be similar between experiments. The values obtained from three experiments are shown in Table 3.4.2 as are the mean values which are plotted in Fig 3.4.6. This figure shows that the rate of transcription increases within 6 hours of transfer from 15 μmol/m²/s white light to 100 μmol/m²/s blue light and has fallen by 24 hours and nearly returns to the 15 μmol/m²/s white light level after 48 hours despite being in 100 μmol/m²/s blue light. These kinetics fit in with those shown for the *rbcS* steady state mRNA level (Fig 3.4.5) with the peak of transcription occuring prior to the peak seen at the accumulated mRNA level. This implies that the increase in the steady state level of *rbcS* mRNA in response to transfer from 15 μmol/m²/s white light to a higher fluence rate of blue light is primarily controlled at the transcriptional level.

3.4.6 The effect of transfer from 15 μmol/m²/s white light to increased fluence rates of lights of different qualities on the rate of *rbcS* transcription

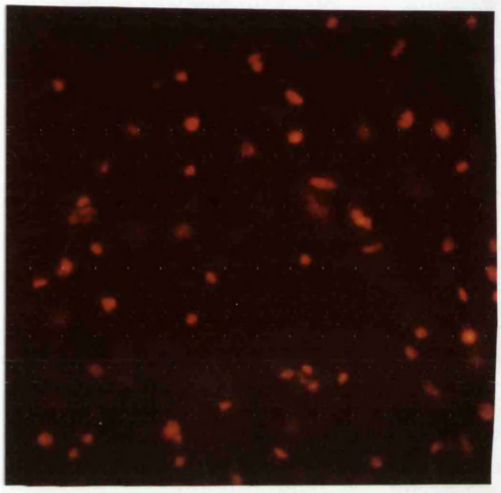
The experiments described in the previous Section (3.4.5) established that the transfer of plants from 15 μmol/m²/s white light to an increased fluence rate of blue-enriched light resulted in an increase in the rate of *rbcS* gene transcription. It was also important to examine whether the effects of an increased fluence rate of other light qualities on the transcription of the *rbcS* genes were consistent with their previously observed effects on the steady state *rbcS* mRNA level (Section 3.3.3). Plants were grown, 10 to the pot, for 16 days under 15 μmol/m²/s white light. Two pots were left in 15 μmol/m²/s white light and two pots each were then transferred to 100 μmol/m²/s blue-enriched, red, and white light, or placed in darkness. The plants were left in these regimes for 12 hours as that was the time when the maximum transcription rate was observed following transfer to an increased fluence rate of blue-enriched light. After this period the primary leaves were harvested, cut into slices, and placed into ice-cold ether in the light regimes in which they had been illuminated. These leaves were used for nuclei preparations and the nuclei were used for run-on transcription reactions. The labelled RNA which was then prepared was hybridised under DNA excess conditions to 5 μg of the *rbcS* encoding pPvSS191 plasmid and to the same amount of pUC 9 plasmid as a control (Sections 2.14 and 2.15) with equal cpm per ml of hybridisation solution being used for each treatment. A representative autoradiograph from one of the three experiments is shown in Fig 3.4.7. The net cpm hybridised specifically to the pPvSS191 *rbcS* -encoding plasmid for each treatment are expressed as a percentage of those hybridised in the 15 μmol/m²/s white light treatment for each experiment and these values are presented in Table 3.4.3. The mean values for each treatment are presented in Fig 3.4.8. These show again that a 100 μmol/m²/s blue-enriched light treatment results in an approximate doubling of the transcription rate of *rbcS* genes when compared to 15 μmol/m²/s white light grown plants. The use of 100 μmol/m²/s

blue enriched light is more effective than an equivalent fluence rate of white light although this also gives an increase in the transcription rate. Not surprisingly, placing the plants in the dark results in a decrease in the *rbcS* transcription rate. A surprise is that the 100 μmol/m^2/s red light also gives an increase in the rate of transcription of *rbcS* genes. This is in contrast to the similar levels of accumulated *rbcS* mRNA seen in low white and red light. This could imply that there is some post-transcriptional destruction of *rbcS* mRNA under red light.

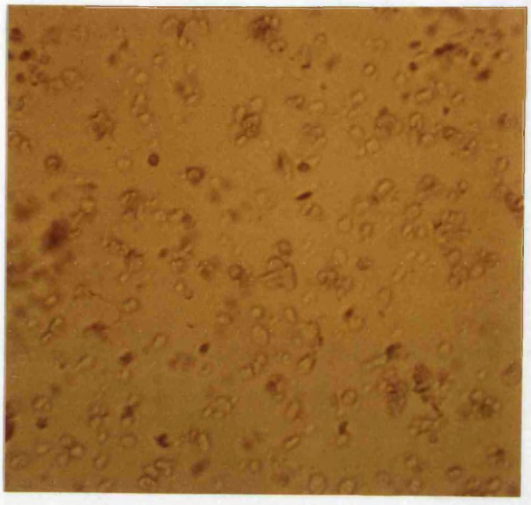
Fig 3.4.1 Photographs of ethidium bromide stained nuclei from *P.vulgaris* primary leaves under UV and white light illumination

Plants were grown, 10 to the pot, under 15 μmol/m^2/s white light for 16 days and then transferred to 100 μmol/m^2/s white light for 12 hours. The primary leaves were then harvested, cut into strips and placed in ice-cold ether. Nuclei were then prepared and stained as described in Section 2.14. They were examined by UV microscopy and photographed at a magnification of 40 times. This photograph is shown in Panel A. The same field of view is also shown under bright field illumination (Panel B). Panel C shows a dilution of the nuclei under UV illumination at a magnification of 40 times, with the bright field illumination shown in panel D.

A



B



C

D

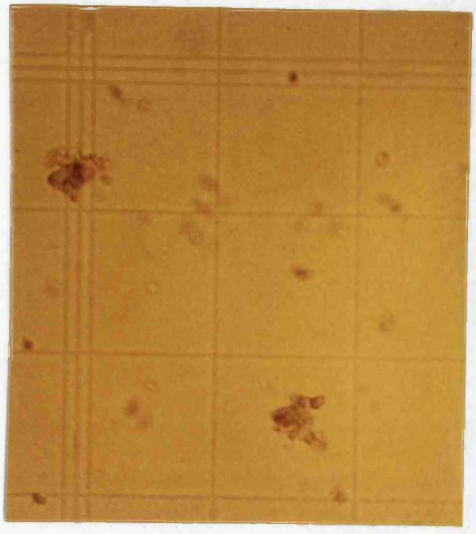


Fig 3.4.2 An indication of the size of the run-on transcripts obtained from purified *Phaseolus vulgaris* nuclei

One million purified nuclei, prepared as in Section 2.14 were used in a run-on transcription reaction as described in Section 2.15.1 and the RNA purified as in Section 2.15.2 with the exception that the final pellet was dissolved in 50 µl of DEPC-treated distilled water. The concentration of the sample was measured spectrophotometrically at 260 nm and three identical 10 µg samples were run on a formaldehyde gel. The gel was then blotted onto a nylon membrane and the resulting blot autoradiographed. The position of the wells is marked. The differences between the lanes is presumably due to blotting differences.


Lane 1 2 3
Wells →
← Wells

Table 3.4.1 The linearity of the specific hybridisation to the pPvSS191 *rbcS* plasmid with increasing amounts of labelled RNA added to the hybridisation reaction

One million purified nuclei, prepared as in Section 2.14 were used in a run-on transcription reaction, and RNA prepared as in Section 2.15. The resulting RNA, dissolved in hybridisation solution, was chromatographed and the incorporation measured (Section 2.11). Increasing amounts of incorporated cpm were then used in separate hybridisation reactions to immobilised, linearised pPvSS191(*rbcS*) and pUC (control) 9 plasmids (5 μg each). These hybridisations and the following washes were performed as described in Section 2.15.3 and the filters autoradiographed. The hybridising bands were then cut out and counted by liquid scintillation counting. The cpm hybridised to each plasmid together with the net cpm hybridised specifically to pPvSS191 (cpm hybridised to pPvSS191 minus cpm hybridised to pUC 9) are tabulated for each amount of labelled RNA used.

Cpm of ^{32}P-labelled RNA added (x 10^{-6})	Cpm hybridised to pPvSS191 *rbcS* plasmid (Specific cpm)	Cpm hybridised to pUC 9 control Plasmid (Background cpm)	Net cpm. (Specific cpm- Background cpm)
3.5	166	97	73
2.0	101	70	31
1.0	73	56	17
0.5	55	49	6
0	35	35	0

Fig 3.4.3 Demonstration that the hybridisation of labelled RNA occurs under DNA excess conditions

The net number of cpm specifically hybridised to the pPvSS191 *rbcS* plasmid is plotted against the number of incorporated cpm of RNA added to each hybridisation reaction. The values are derived from Table 3.4.1.

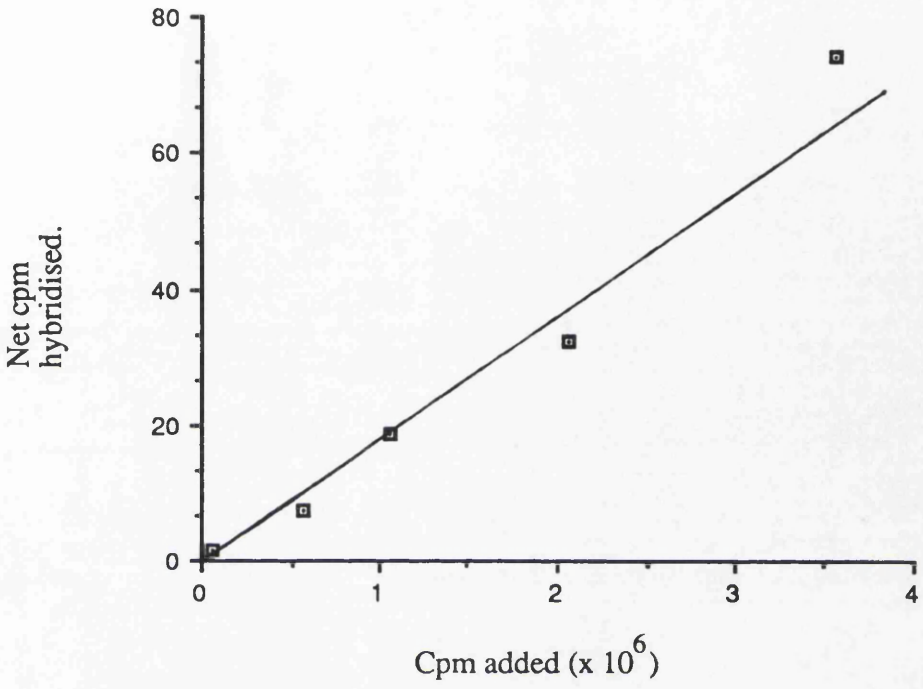

Net cpm hybridised.
80
60
40
20
0
0
1
2
3
4
Cpm added (x 10^6)

Fig 3.4.4 Timecourse of the increase in *rbcS* steady state mRNA levels following transfer from 15 μmol/m²/s white light to an increased fluence rate of blue light

Plants were grown, 10 to the pot, under 15 μmol/m²/s white light for 16 days. At this point two pots were moved to a fluence rate of 100 μmol/m²/s blue light. Three pairs of primary leaves were harvested at the time points given below :

Light regime	Timepoint (hours)	Lane
White	6	1
(15 μmol/m²/s)	12	2
	24	3
	48	4
Blue	6	5
(100 μmol/m²/s)	12	6
	24	7
	48	8

The primary leaves were used for RNA extraction and 10 μg of this RNA was examined on a northen blot. This blot was probed with the pPvSS191 *rbcS* cDNA probe at a concentration of 2 x 10^6 incorporated cpm per ml of hybridisation solution. This filter was washed and subjected to autoradiography.

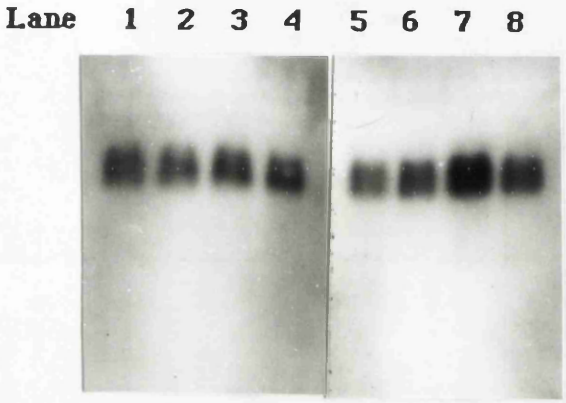

Lane 1 2 3 4 5 6 7 8

Fig 3.4.5 Graphical representation of *rbcS* transcript levels in primary leaves following transfer of *Phaseolus vulgaris* plants from 15 μmol/m²/s white light to 100 μmol/m²/s blue-enriched light

The bands on the autoradiograph shown in Fig 3.4.4 were scanned using a laser densitometer as described in Section 2.13. The absorbance values for each timepoint which are representative of the amount of hybridisation are plotted against time.

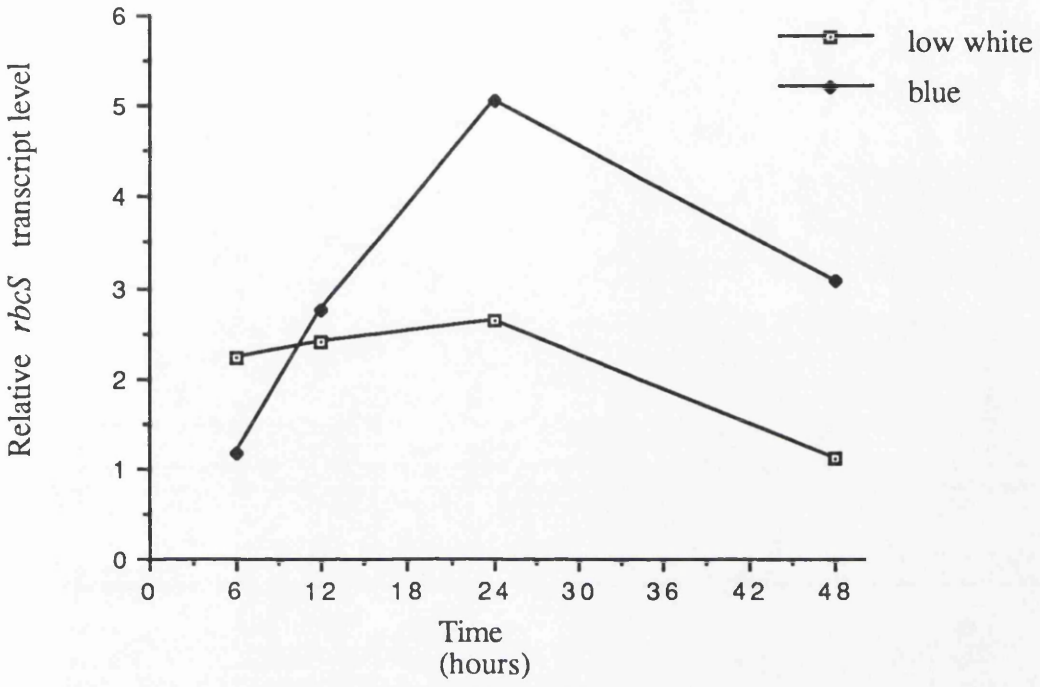

Relative rbcS transcript level
6
5
4
3
2
1
0
0
6
12
18
24
30
36
42
48
Time
(hours)
low white
blue

Table 3.4.2 Timecourse of the change in transcription rate of *rbcS* genes in the primary leaves of plants moved from 15 μmol/m²/s white light to 100 μmol/m²/s blue-enriched light

Plants were grown, 10 to the pot, under 15 μmol/m²/s white light for 16 days. Some plants were then transferred to 100 μmol/m²/s blue light for 2 days. Primary leaves were harvested, cut into strips, and placed into ice-cold ether in the light environments under which they were growing at the times given in the table. One million purified nuclei were prepared from these leaves as described in Section 2.14 and used in run-on transcription reactions as described in Section 2.15.1. The RNA purified from the reactions (Section 2.15.2) was hybridised under DNA excess conditions to 5 μg of pPvSS191 and pUC 9 plasmid DNAs (Section 2.15.3). Cpm specifically hybridised to pPvSS191 were calculated. The values from three separate experiment experiments are shown. Each value is expressed as a percentage of the net cpm hybridised from the 15 μmol/m²/s white light time zero treatment for each experiment. The mean values and the standard error for each timepoint are also presented.

Treatment	Time from start of treatment (hours)	Net hybridisation (see legend)	Mean net hybridisation +/- standard error
15 µmol/m^2/s white light	0	100 100 100	100 +/-6.5
	48	— 85 121	103 +/- 8.3
100 µmol/m^2/s Blue-enriched light	6	172 150 194	172 +/- 6
	12	130 253 184	189 +/- 18.6
	24	76 145 166	129 +/- 12.6
	48	— 122 113	117 +/- 3

N.B. The standard error given for the time zero treatment is the standard error of the cpm hybridised for that timepoint between the three experiments.

Fig 3.4.6 Graphical representation of the change in transcription rate of *rbcS* genes in the primary leaves of plants moved from 15 μmol/m²/s white light to 100 μmol/m²/s blue-enriched light

The mean values for the transcription rate of the *rbcS* genes at each of the timepoints examined in Table 3.4.2 is plotted against time. The standard errors for each timepoint are presented.

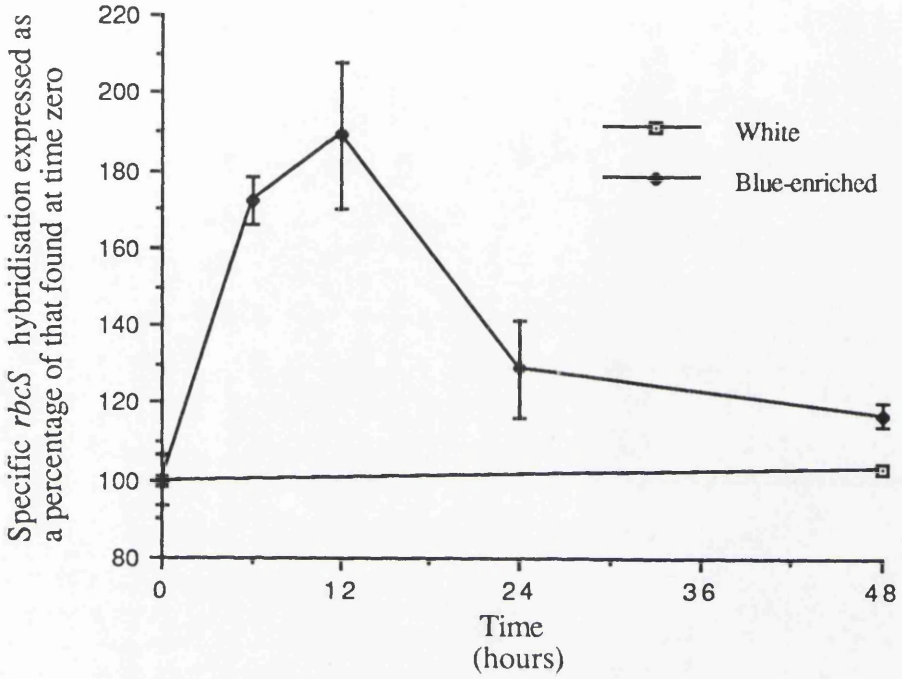

Specific rbcS hybridisation expressed as a percentage of that found at time zero
220
200
180
160
140
120
100
80
0
12
24
36
48
Time (hours)
White
Blue-enriched

Fig 3.4.7 A representative autoradiograph from a run-on transcription RNA hybridisation experiment to examine the effect of different qualities of light of the same fluence rate on the rate of transcription of *rbcS* genes in primary leaves

Plants were grown, ten to the pot, under 15 μmol/m^2/s white light for 16 days before being transferred to one of the following light regimes for 12 hours :

Panel	Light Quality	Fluence rate (μmol/m^2/s)
A	Blue	100
B	Red	100
C	White	100
D	White	15
E	Darkness	0

After the 12 hour light treatment the primary leaves were harvested and run-on transcription analysis carried out as described in the legend to Table 3.4.2. Labelled RNA was hybridised to the pPvSS191 (*rbcS*) and pUC 9 (control) plasmids. The autoradiograph obtained from one such analysis is shown.

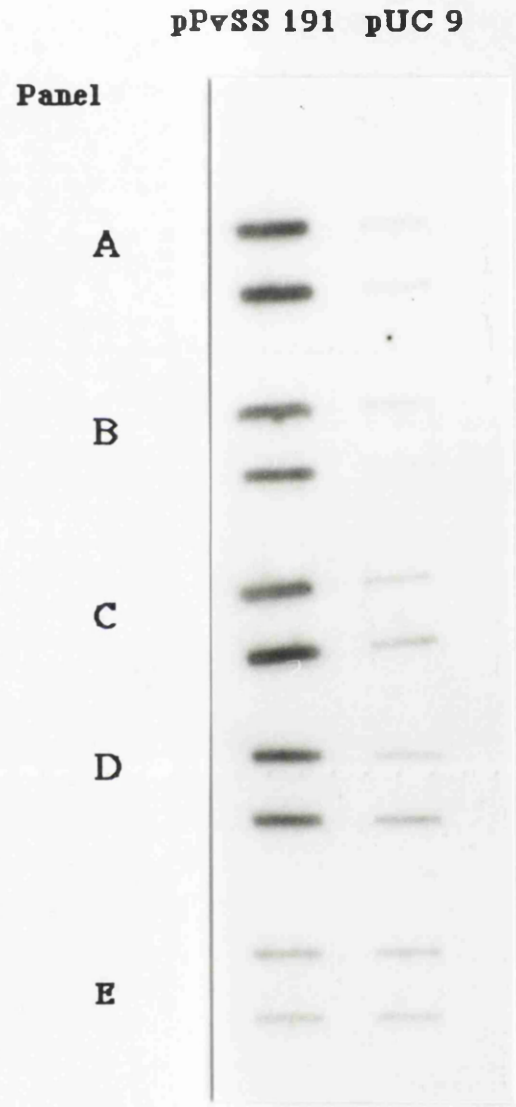

pPvSS 191
pUC 9
Panel
A
B
C
D
E

Table 3.4.3 The net hybridisation to the pPvSS191 *rbcS* plasmid in three separate experiments to examine the effect of different qualities of light of the same fluence rate on the rate of transcription of *rbcS* genes in primary leaves

Plants were grown and treated as described in the legend to Fig 3.4.7 and nuclei prepared and run-on transcription analysis carried out as described in the legend to Table 3.4.2. The net hybridisation to the *rbcS* encoding pPvSS191 plasmid for each of the treatments is expressed as a percentage of that from the 15 μmol/m^2/s white light grown plants for each experiment. These values are presented along with the mean value for each treatment. The standard error is also presented for each mean value.
Plants grown in 15 μmol/m^2/s white light for 16 days were transferred for 12 hours to:

A) 15 μmol/m^2/s white light,
B) 100 μmol/m^2/s blue-enriched light,
C) 100 μmol/m^2/s red light,
D) 100 μmol/m^2/s white light,
E) darkness.

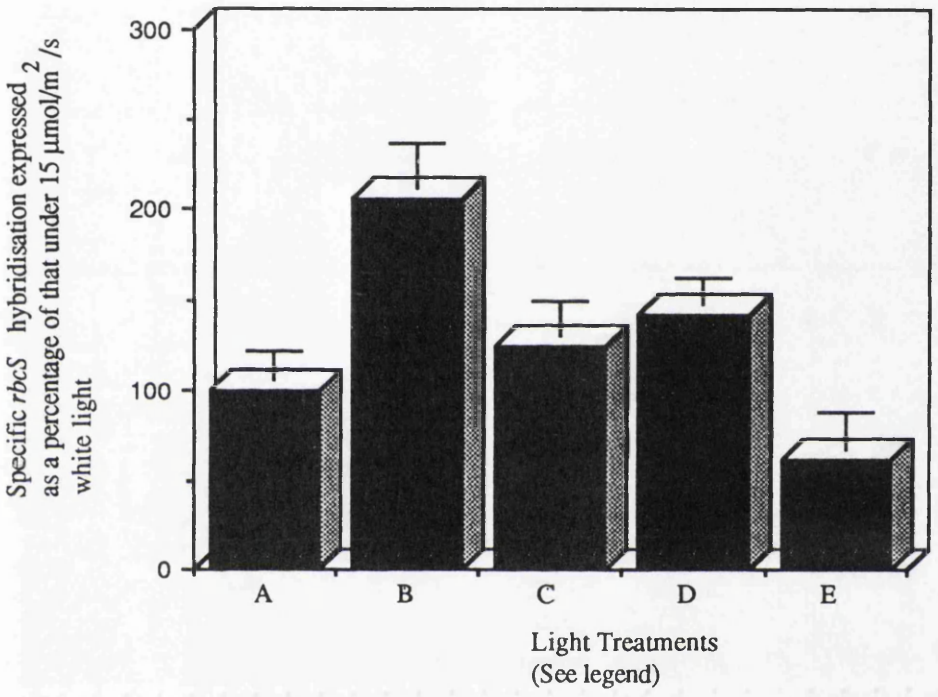
Light Treatment ($\mu mol/m^2/s$)	Net hybridisation	Mean net hybridisation +/- standard error.
a) White	100	100
15	100	+/- 6.5
	100	
b) Blue	150	207
100	253	+/- 14
	218	
c) Red	88	125
100	167	+/-10.6
	121	
d) White	109	142
100	172	+/- 8.3
	145	
e) Darkness	—	61
	92	+/-15.5
	30	

N.B. The standard error given for the time zero treatment is the standard error of the cpm hybridised for that timepoint between the three experiments.

Fig 3.4.8 A graphical depiction of the relative rates of transcription of the *rbcS* genes in primary leaves of 16 day old 15 μmol/m²/s white light grown plants transferred to different light qualities for 12 hours

The mean values for each light treatment and their standard errors from Table 3.4.4.are shown. The light treatments were :

A) 15 μmol/m²/s white light,
B) 100 μmol/m²/s blue-enriched light,
C) 100 μmol/m²/s red light,
D) 100 μmol/m²/s white light,
E) darkness.


Specific rbcS hybridisation expressed as a percentage of that under 15 µmol/m 2 /s white light
300
200
100
0
A
B
C
D
E
Light Treatments
(See legend)

3.5 Measurement of the levels of individual *rbcS* transcripts using the reverse transcriptase polymerase chain reaction (RT-PCR)

The fact that the three *rbcS* genes have different 3' non-coding sequences was used to design oligonucleotides for use as probes for northern analysis. These oligonucleotides could also be used as gene-specific 3' primers for use in a polymerase chain reaction. The advantage of using a PCR system to measure the amount of the three transcripts is that the reaction for all three transcripts can be done in a single tube, thus avoiding the difficulty of comparing results from different hybridisation reactions. This cannot be achieved through the use of S1 analysis with a single probe because the 3' sequences are too similar to allow the three different genes to be resolved. For such a PCR to work a non-discriminating 5' oligonucleotide needed to be designed to hybridise to the DNA strand produced from the 3' oligonucletide primers by reverse transcriptase. This oligonucleotide is therefore in the opposite 'sense' to the non-discriminating oligonucleotide used for the northern blots. These oligonucleotides are illustrated in Figs 2.1.1 and 2.1.2. Hence the purpose of the experiments described in this Section was to develop and use a PCR-based system to measure the levels of the different *rbcS* transcripts.

3.5.1 Measuring the size of the fragments produced by RT-PCR using the oligonucleotides described in Section 2.1.5

To carry out the reaction the 5' oligonucleotide was end labelled and gel purified as described in Sections 2.10.3 and 2.16.2. The reverse transcription reaction was performed as described in Section 2.16.1 with 4 μg of DNAased RNA from either light or dark grown primary leaves and the three gene-specific oligonucleotide primers in one tube for each RNA sample (Section 2.4.4). A control tube was set up with RNA extracted from light grown leaves and the same contents as for the reverse transcription reaction except that the reverse transcriptase was not added. The PCR was carried out

using 1.5 x 10^6 dpm of 5' labelled non-discriminating primer per tube (Section 2.16.3) with the tubes described above. The step cycles used were a 1 minute 94°C denaturing step, a 2 minute 55°C annealing step, and a 3 minute 72°C extension step, after an initial 3 minute 94°C delay file. Twenty four cycles were carried out prior to the 10 minute 72°C extension step to allow any unfinished transcripts to be transcribed to their full length (Section 2.16.3). Five µl aliquots of each sample were mixed with an equal volume of Sequenase® stop buffer and 4 µl of each sample was added to a 6% acrylamide/urea sequencing gel along with a sequencing reaction to provide size markers (donated by C.Simpson, Scottish Crop Research Institute, Invergowrie). The gel was run and autoradiographed as described in Sections 2.16.4 and 2.12.3 respectively. The autoradiograph of this gel can be seen in Fig 3.5.1. The arrows point to the bands of the predicted size for each *rbcS* gene and there is no obvious difference between light and dark RNA for these PCR conditions and amount of RNA. The lane which had the products from the control (no reverse transcriptase) is empty. This demonstrates that there was no contaminating DNA present in the DNAased RNA for this would be the only way for there to be a complementary sequence for the 5' non-discriminating oligonucleotide to hybridise to to allow the amplification of a labelled band. The bands identified by an asterix in Fig 3.5.1 were only apparent if the products of the RT-PCR were run immediately after the PCR and disappeared if the reactions were stored at 4°C for 24 hours (see lane 7 of Fig 3.5.5).

3.5.2 Ascertaining the correct conditions for quantitative RT-PCR

To be able to use the RT-PCR method for quantitative measurement of the three *rbcS* genes' transcript levels the PCR must be stopped when the amount of product for each *rbcS* gene is still increasing in a logarithmic fashion. To do this reverse transcriptase reactions were carried out using 4 µg of RNA. These were then used for the PCR with the cycle conditions described in Section 3.5.1. Five µl samples were taken after the extension step (72°C for 3 minutes) of cycles 16, 18, 20, 22, and 24.

These samples were placed at 72°C for 10 minutes to allow the new DNA to be extended to its full length. The samples were then run out on a 6% acrylamide/urea sequencing gel and the gel autoradiographed as described in Section 2.12.3. The bands corresponding to each genes' PCR product were cut out of the gel and placed in 4 ml of Ecoscint® overnight prior to scintillation counting. The $\log_{10}$ of the number of cpm from each genes' amplified product is plotted against cycle number in Fig 3.5.2. The gradient of the line is indicative of the efficiency of the *Taq* polymerase and as the coding sequence of the three *rbcS* genes is identical this efficiency should be approximately the same for each of the genes, allowing for differences in the 3' non-coding sequence. In this case the plot of the $\log_{10}$ of cpm aganst cycle number for each gene is not parallel and thus using this range of cycle number and 4 µg of RNA for the reverse transcriptase reaction will not give a quantitative assay of relative transcript abundance. This may explain why there is no difference between the light and dark RNA samples shown in Fig 3.5.1.

To attempt to get conditions where a quantitative comparison of light and dark RNA samples could be undertaken the amount of starting RNA was reduced to 1 µg and the range of cycle numbers reduced to cycles 10 through to 18. The reverse transcriptase reaction and the PCR was carried out as described above and 10 µl samples were taken from the reaction after the extension steps (72°C for 3 minutes) of the cycles given in Fig 3.5.3. These samples were placed at 72°C for 10 minutes to allow the new DNA to be extended to its full length. These samples were run out on a 6% acrylamide/urea sequencing gel and the gel autoradiographed as described in Section 2.12.3. The autoradiograph from this experiment is shown in Fig 3.5.3. This autoradiograph was then placed over the gel and the bands corresponding to the three *rbcS* genes' PCR products cut out and placed in Ecoscint® overnight prior to scintillation counting. The $\log_{10}$ of cpm is plotted against the cycle number in Fig 3.5.4. It can be seen that in this case the $\log_{10}$ of cpm against cycle number is a straight line up to cycle 15 implying the amount of product produced for each gene is increasing

in a logarithmic fashion. This is not true after this cycle as the lines for each gene start to tail off. The increase in product for each gene between cycles 10 and 15 can also be seen to be occurring at the same rate for each gene because the plots for each gene are parallel.

3.5.3 Examination of the differences in *rbcS* mRNA levels for each gene in light and dark samples

A comparison between RT-PCR and northern blot analysis was undertaken. To do this, the levels of *rbcS* transcripts for each gene in RNA samples from light and dark grown primary leaves of different ages were examined. The samples used had been studied previously by northern analysis (Figs 3.2.1 and 3.2.2). Total RNA samples prepared from primary leaves harvested 5, 7, and 10 days after sowing were used for RT-PCR analysis. These samples were chosen because the pattern of expression of the *rbcS* gene family as a whole was shown to peak at seven days in a northern analysis. If the conditions for the RT-PCR reaction described in Section 3.5.2 were suitable for comparative quantitative RT-PCR then this pattern of expression should be seen in the RT-PCR products from these samples. One μg of DNAased RNA was used for the reverse transcriptase reaction for each sample and then PCR was carried out for 14 cycles with the cycle conditions described in Section 3.5.1. The reactions were then placed at 72°C for ten minutes prior to being placed on ice. Five μl samples of each reaction were mixed with an equal volume of Sequenase® stop solution and run out on a 6% acrylamide/urea sequencing gel. The gel was autoradiographed as described in Section 2.12.3 and the autoradiograph from this experiment can be seen in Fig 3.5.5. The samples from the light grown primary leaves do show the expected peak of steady state *rbcS* mRNA in the 7 day sample. This is shown in Fig 3.5.6 which shows a plot of the expression from each gene in the light grown samples and the sum of the three genes' expression. The pattern shown by the sum of the three *rbcS* PCR products is very similar to that seen in Fig 3.2.2, a scan of

the blot of the RNA samples from light grown primary leaves hybridised to a probe that recognises all of the *rbcS* transcripts (Fig 3.2.1). These results suggest that this method could be useful for measuring the level of *rbcS* expression when the system is finely tuned. It can be noticed that all three genes show a peak in their expression on the seventh day after planting in the light grown plant. This again shows that the regulation of the three genes is very similar.

The pattern of expression shown in the RNA samples from the dark grown plants does not show the expected peak on the seventh day. The reason for this is unclear and may be due to inherent differences between the measurement of transcript levels by RT-PCR and northern blots. This is unlikely because the RNA samples from the light grown primary leaves gave the same result when studied by both methods. It is more likely that the difference is due to the RT-PCR not being fully optimised, and possible differences in the amount of input RNA between samples. However the level of expression seen in the dark grown samples is lower than that seen in the light grown samples (compare to Fig 3.5.1) implying that the conditions are near to being optimised.

Fig 3.5.1 The sizes of the transcripts obtained from RT-PCR of RNA from light and dark grown *Phaseolus vulgaris* primary leaves

RT-PCRs were performed with 4 μg of total DNAased RNA from light and dark grown primary leaves (Sections 2.16.1 and 2.16.3). One RT-PCR was also performed with 4 μg of RNA from light grown primary leaves with no reverse transcriptase added as a control for contaminating DNA. 5 μl from each reaction was mixed with an equal amount of Sequenase® Stop solution and run on a 6% polyacrylamide / urea gel until the bromophenol blue reached the bottom of the gel (Section 2.16.4). Sequencing reaction size markers (donated by Craig Simpson, S.C.R.I., Invergowrie, Scotland) were also run on the gel. The gel was fixed, dried and autoradiographed (Sections 2.16.4). The contents of each lane are given below:

Lane	1	2	3	4	5	6	7
Contents:	Sequencing reaction				RT-PCR reactions		
	A	C	G	T	Light RNA	Dark RNA	Control No reverse transcriptase

The gene-specific PCR products are indicated, and non-specific bands are indicated by an *.

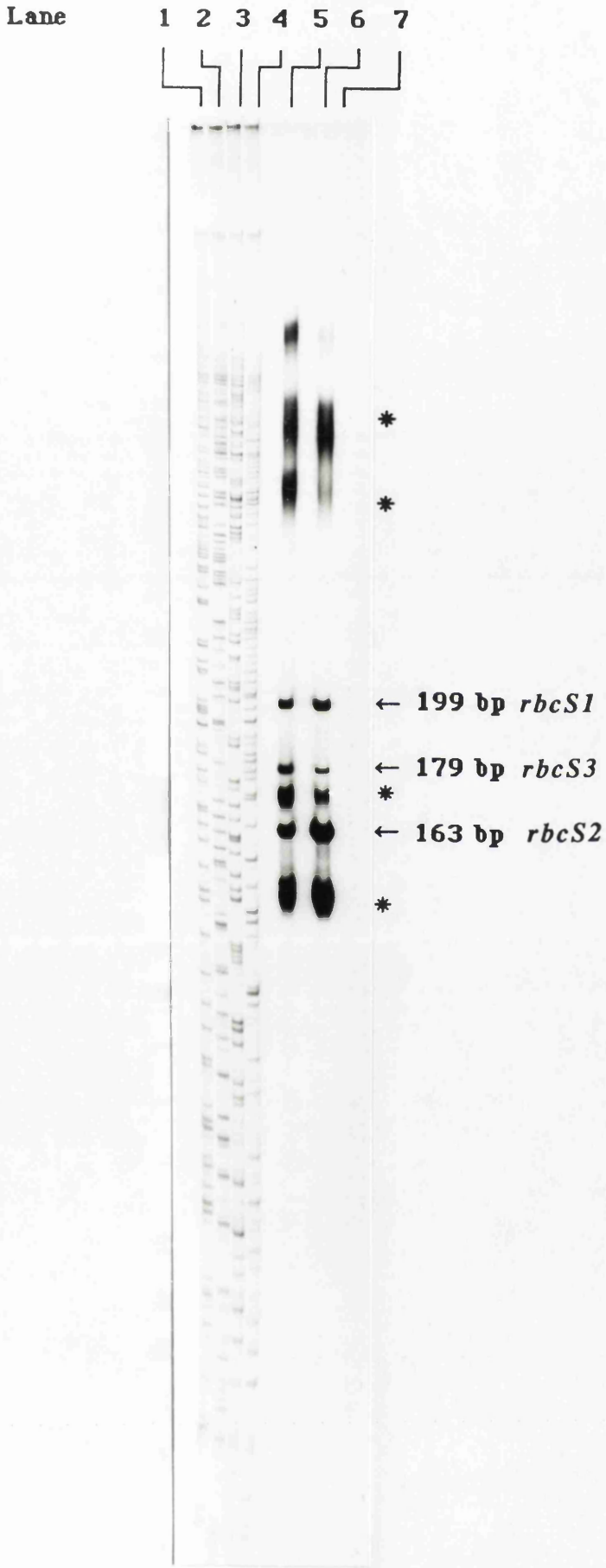

Lane
1 2 3 4 5 6 7
*
*
← 199 bp rbcS1
← 179 bp rbcS3
*
← 163 bp rbcS2
*

Fig 3.5.2 The amount of radiolabel incorporated into each genes' PCR product per cycle when 4 μg of RNA is used as starting material

RT-PCR (Sections 2.16.1 and 2.16.3) was performed with 4 μg of total primary leaf RNA as starting material. 10 μl samples were taken after the extension step of cycles 16, 18, 20, 22 and 24 and placed at 72°C for ten minutes. The samples were kept on ice and 5 μl of each sample was mixed with an equal volume of Sequenase® Stop solution and run on a 6% polyacrylamide / urea gel until the bromophenol blue reached the bottom of the gel (Section 2.16.4). The gel was autoradiographed wet, and the areas of the gel corresponding to each genes' PCR product (as indicated by the autoradiograph) excised from the gel. These slices were placed in 4 ml of Ecoscint® overnight and the amount of radioactivity present in each slice measured by scintillation counting. The cpm in each genes' PCR product is plotted against the cycle number at which the sample was taken.

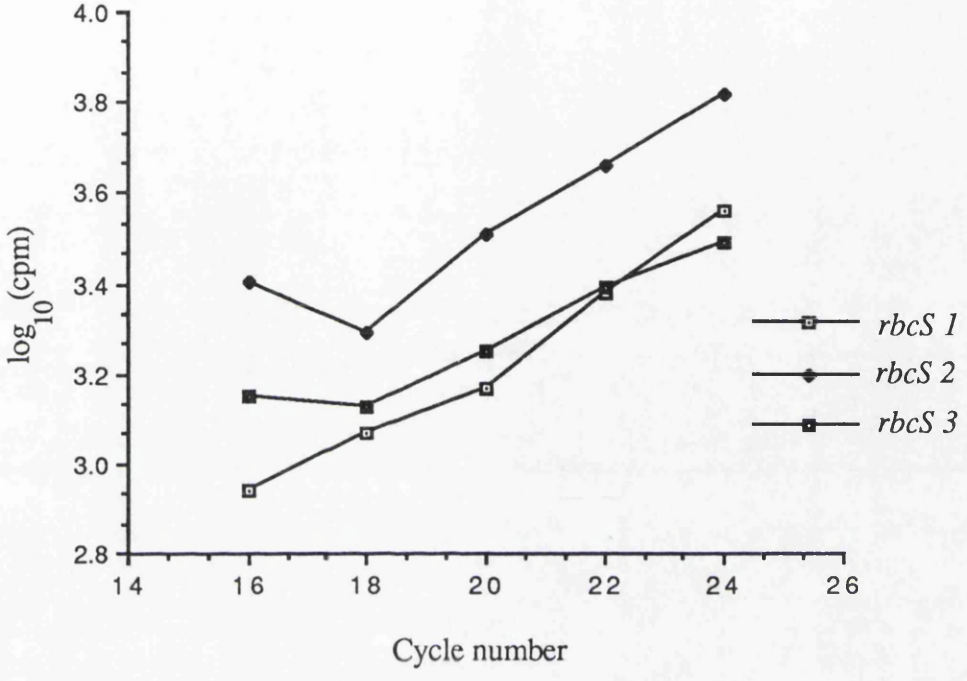

4.0
3.8
3.6
3.4
3.2
3.0
2.8
log10 (cpm)
14
16
18
20
22
24
26
Cycle number
rbcS 1
rbcS 2
rbcS 3

Fig 3.5.3 Illustration of the increase in incorporation into the *rbcS* gene-specific PCR products with increasing cycle number

RT-PCR (Sections 2.16.1 and 2.16.3) was performed with 1 μg of total primary leaf RNA as starting material. 10 μl samples were taken after the extension step of cycles 10, 11, 12, 13, 14, 15, 16 and 18 and placed at 72°C for ten minutes. The samples were kept on ice and 5 μl of each sample was mixed with an equal volume of Sequenase® Stop solution and run on a 6% polyacrylamide / urea gel until the bromophenol blue reached the bottom of the gel (Section 2.16.4). The gel was autoradiographed wet. The cycle number is given for each lane and the gene-specific PCR products are indicated.

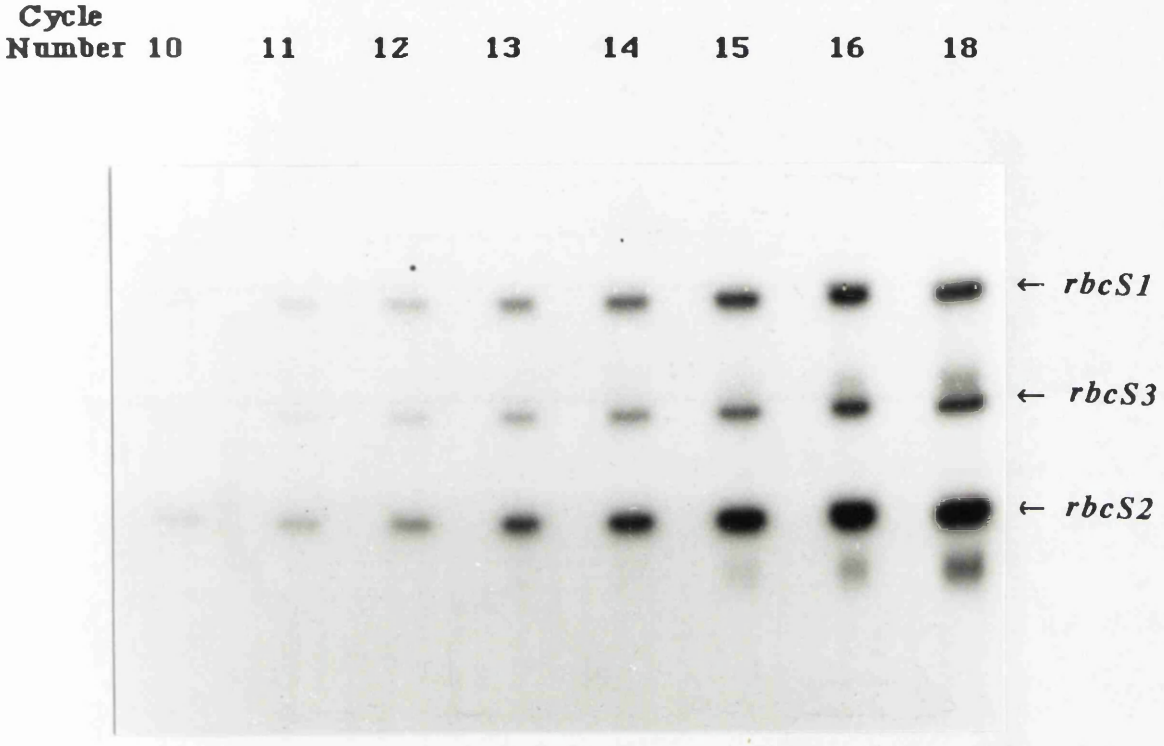

Cycle
Number 10
11
12
13
14
15
16
18
← rbcS1
← rbcS3
← rbcS2

Fig 3.5.4 The amount of radiolabel incorporated into each genes' PCR product per cycle when 1 μg of RNA is used as starting material

RT-PCR (Sections 2.16.1 and 2.16.3) was performed with 1 μg of total primary leaf RNA as starting material (see legend to Fig 3.5.3). The area of the gel corresponding to each genes' PCR product (as indicated in the autoradiograph in Fig 3.5.3) was excised from the gel. These slices were placed in 4 ml of Ecoscint® overnight and the amount of radioactivity present in each slice measured by scintillation counting. The cpm in each genes' PCR product is plotted against the cycle number at which the sample was taken.

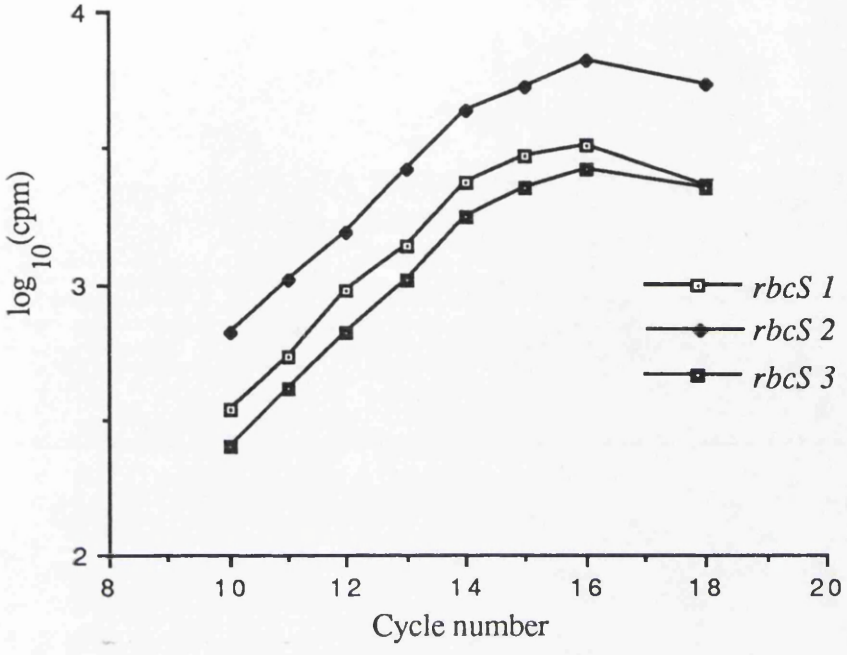

4
3
2
log 10(cpm)
8
10
12
14
16
18
20
Cycle number
rbcS 1
rbcS 2
rbcS 3

Fig 3.5.5 Measurement of the levels of individual *rbcS* transcripts in different total RNA samples using RT-PCR

RT-PCR was performed with 1 μg of total RNA samples from light and dark grown primary leaves of different ages. These samples had previously been examined by northern analysis (Figs 3.2.1 and 3.2.2). The samples are described below :

Lane	1	2	3	4	5	6
Light	+	+	+	-	-	-
Day harvested (after sowing)	5	7	10	5	7	10

The cycle conditions were as described in Section 2.16.3 and 14 cycles were performed prior to the sink cycle. 5 μl of each sample was mixed with an equal volume of Sequenase® Stop solution and run on a 6% polyacrylamide / urea gel until the bromophenol blue reached the bottom of the gel (Section 2.16.4). The gel was autoradiographed wet. The gene-specific PCR products are indicated.
N.B. The sample in lane 7 is 5 μl of the 'light' sample examined in Fig 3.5.1.

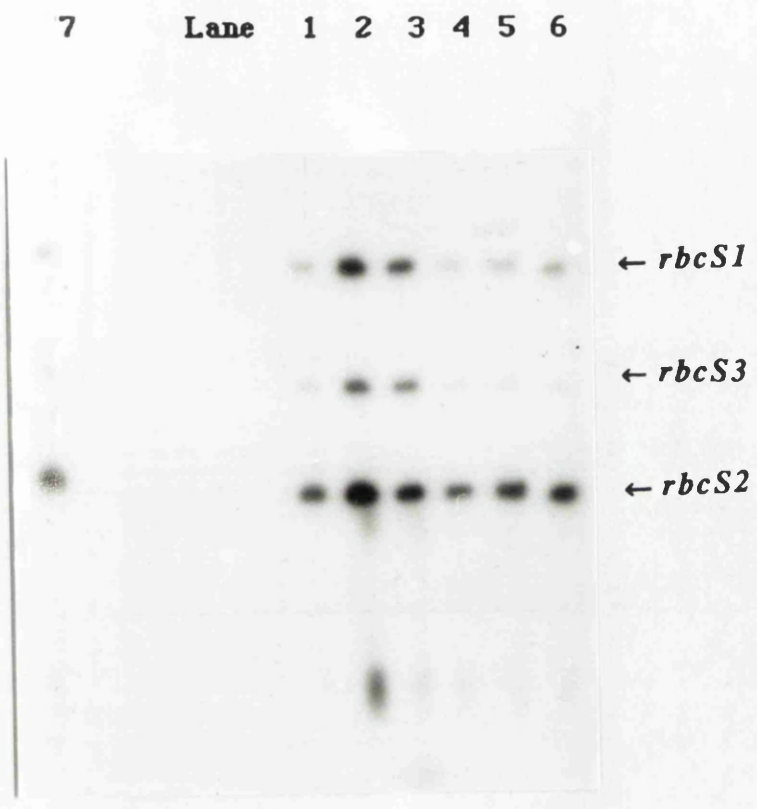
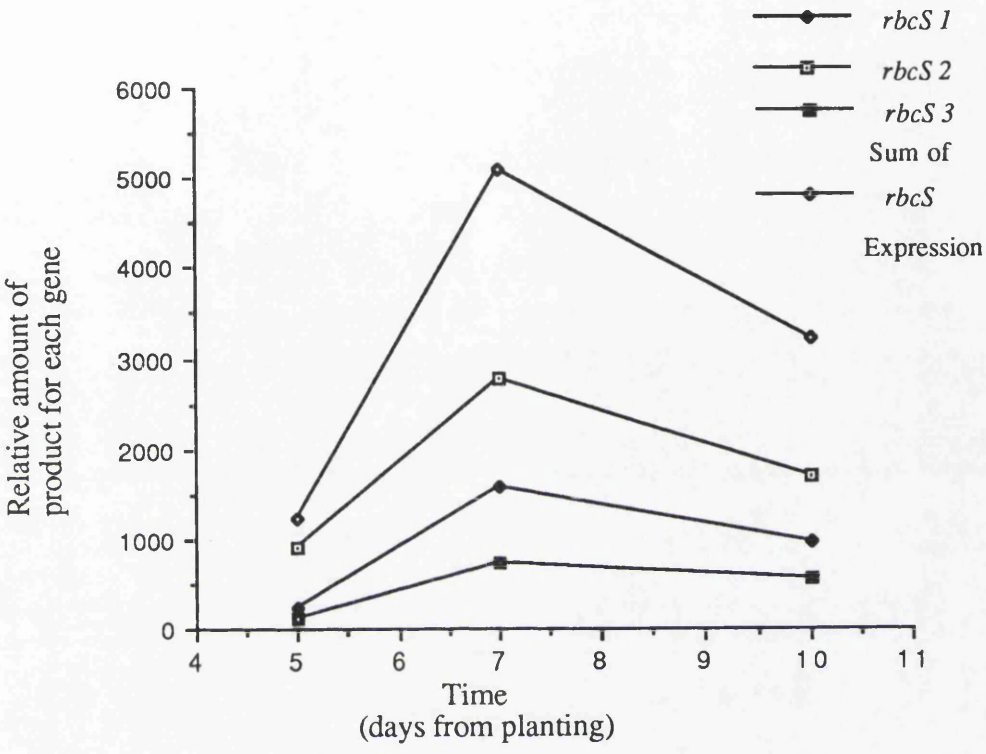

7
Lane 1 2 3 4 5 6
← rbcS1
← rbcS3
← rbcS2

Fig 3.5.6 Illustration of the changes in *rbcS* transcript levels with increasing age of light-grown primary leaves, as measured by RT-PCR

The autoradiograph shown in Fig 3.5.5 was scanned with a laser densitometer (Section 2.13). The area of the band corresponding to each genes' PCR product is plotted against the time the primary leaves were harvested. The sum of the areas of the three genes' PCR products is also plotted for each time point examined.


rbcS 1
rbcS 2
rbcS 3
Sum of
rbcS
Expression
Relative amount of
product for each gene
6000
5000
4000
3000
2000
1000
0
4
5
6
7
8
9
10
11
Time
(days from planting)

Discussion

4.1 The validity of the methods used to examine *rbcS* transcript levels

The experiments described in Section 3.1 were carried out to establish the validity of the hybridisation analyses carried out in this study. The experiments described in Sections 3.1.2 and 3.1.3 show that if the same total RNA sample is used in increasing amounts, for a northern blot, the amount of ethidium bromide fluorescence increases with an increasing amount of RNA loaded. There are parallel increases in the amounts of the *rbcS* transcript and α tubulin transcript as measured by a hybridisation experiment. This indicates that, as expected, increasing the amount of total RNA loaded results in a similar increase in the amount of mRNA that is loaded if the same sample of RNA is used. The implication from this is that if two different RNA samples, which show the same fluorescence of their ribosomal RNA bands, and the same level of α tubulin transcript, show different levels of *rbcS* transcript, then the amount of *rbcS* mRNA has been regulated differently from both total RNA and constituitively expressed RNA polymerase II transcribed genes.

The sizes of the *rbcS* transcripts are measured to be 0.9 and 1.0 kb (Table 3.1.2). Knight (1989) studied a number of *rbcS* cDNAs and found that there were differences in size between them. Thus the appearance of two differently sized transcripts is not unexpected. The fact that there are different sizes of transcript can be explained by the presence of different polyadenylation sites in the 3' sequences of the *rbcS2* and *rbcS3* genes which would result in different lengths of the 3' non-coding sequences (Knight and Jenkins, 1992). The precise size of the poly-A tails is not known and may differ between the genes and this again would result in different sizes of transcript. The different sizes of transcripts are not resolved in all of the experiments described in this thesis, probably because of differences in the length of time the gels were run and the exact percentage (w/v) of agarose present in the gels.

There is also hybridisation visible to a transcript of much higher molecular weight when total RNA is probed with the *rbcS* cDNA pPvSS191 (Fig 3.1.3). This band has been shown, by Toluidine Blue staining of the filter after hybridisation, to correspond to a ribosomal RNA band. The strength of this signal can sometimes be seen to be proportional to the specific hybridisation to *rbcS* transcripts (see Fig 3.2.5) and this would imply that the hybridisation may be due to specific hybridisation to *rbcS* transcripts complexed in the ribosomal RNA. However the hybridisation to this higher molecular weight band can also be seen to be proportional to the amount of total RNA loaded (see Fig 3.2.1). Therefore another explanation may be that the probe hybridises non-specifically to this band because of the large amount of RNA present at this position. This may be a more likely proposal because the *rbcS* probe also hybridises to the largest RNA molecular weight marker, which is also bound by the α tubulin probe (Fig 3.1.3) which recognises a different specific transcript to that hybridised to by the *rbcS* probe.

The determination of the washing conditions to be used for the oligonucleotide probes was carried out using the non-discriminating oligonucleotide probe (*rbcSG*). This was done because this probe would recognise the transcripts from all three genes. Thus the signal obtained with this probe would be the strongest obtainable with an oligonucleotide probe. As the GC content of the four oligonucleotides was identical the hybridisation and washing conditions required for an optimum signal from each oligonucleotide should be similar (Sambrook et al., 1989). The experiment to determine whether the oligonucleotide probes were specific to the sequences they were designed to be complementary to, while showing that this was the case (Fig 3.1.7) also showed that, under the hybridisation and washing conditions used, the oligonucleotides did not hybridise with the same efficiency to their respective sequences (Fig 3.1.8). The *rbcS2* oligonucleotide hybridised with the most efficiency, with the *rbcS1* and *rbcS3* oligonucleotides hybridising at 71% and 54% of the efficiency of the *rbcS2* oligonucleotide respectively. This is presumably due to their different sequences (Table 2.1.2). While their GC content is identical in terms of numbers of G and C per

oligonucleotide, the distribution of the G and C residues probably has an effect on the strength of the bonds formed between the oligonucleotides and their target sequences resulting in the different hybridisation efficiencies seen. These differences may also have had an effect on the results obtained from the reverse-transcriptase, polymerase chain reaction experiments (Section 3.5) and this will be discussed later.

4.2 Ontogenetic and light regulation of *rbcS* mRNA levels

4.2.1 Regulation of *rbcS* mRNA levels in conditions of constant light and darkness

The regulation of *rbcS* expression has been shown to involve many factors, including light and the developmental state of the leaf (see Section 1.3.2.1). The experiment described in Section 3.2.1 shows that there is a peak of *rbcS* expression in the dark grown primary leaf, seven days after planting. A peak of *rbcS* expression in dark grown plants is also seen in a number of different species. Examples include the hypocotyls of cucumber (Greenland et al., 1987), maize leaves (Nelson et al., 1984) and *Arabidopsis* cotyledons (Dedonder et al., 1993). Thus ontogenetic control of *rbcS* expression in dark grown primary leaves is not unexpected. Filner and Klein (1968) examined the changes in extractable Rubisco activity in etiolated seedlings of a different cultivar of *Phaseolus vulgaris* (Black valentine). They found that there was a peak of enzyme activity 8 days after planting. Bradbeer et al. (1974) studied the development of the plastids in another cultivar of *Phaseolus vulgaris* (Alabaster) during dark growth. They found that there was a peak of Rubisco activity after 12 days of growth. This data implies that there is ontogenetic control of the appearance of Rubisco activity. The data presented here suggests that the control of this appearance may have been by *rbcS* mRNA level.

The presence of Rubisco activity in dark-grown leaves (Filner and Klein, 1968; Bradbeer et al., 1974) implies that *rbcS* mRNA is translated into the small subunit polypeptide and that this is processed, transported into the chloroplast, and combines with the large subunit to form the holoenzyme, since only this form of the enzyme is active. Whether the enzyme is fully functional in the dark grown plant is not known. In light grown *Phaseolus vulgaris* primary leaves which have been kept in the dark overnight the amount of Rubisco activity measurable is 10% of that of a leaf that was not dark treated (Seemann et al., 1985). The loss of activity was not due to loss of Rubisco protein but was found to be due to an inhibitor of Rubisco that bound to the catalytic site of the enzyme (Seemann et al., 1985). Interestingly, the drop in activity due to dark treatment was also seen in cucumber, another species that has a relatively large amount of Rubisco protein in its dark-grown tissue (Greenland et al., 1987).

The inhibitor was purified from dark treated *Phaseolus vulgaris* leaves and found to be 2-carboxyarabinitol 1-phosphate (CA1P) by comparisons of its inhibitory properties with those of synthetically prepared analagous compounds (Berry et al., 1987). The structure of the inhibitor was very similar to a known Rubisco transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate. The level of this inhibitor in *Phaseolus vulgaris* decreases with increasing photon fluence rates and disappears within 10 minutes if a saturating photon fluence rate is used (Portis Jr., 1992). However, *Phaseolus vulgaris* is the only species studied so far in which this inhibitor is present at levels sufficient to account for all the inhibition of Rubisco seen in dark treated plants. There is a possibility that this compound may be present in the dark grown primary leaves and that the Rubisco holoenzyme present in these leaves is thus in an inactive form. The fact that the level of *rbcS* mRNA peaks on the seventh day after planting may be due to the fact that the primary leaves emerge from the seed coat at around six days after planting. The presence of some Rubisco in the dark grown plastid may enable photosynthetic carbon fixation to proceed earlier than if the Rubisco mRNA had to be transcribed and translated after the onset of illumination.

The pattern of expression seen in primary leaves grown under 50 μmol/m^2/s peaks on the seventh day after planting as seen for plants grown in continuous darkness (Fig 3.2.2). There is, however, an increase in the amount of *rbcS* mRNA present in the 50 μmol/m^2/s light-grown leaves compared to those grown in the dark, presumably because more Rubisco protein is required in the light grown plant than in the dark grown plant. If a greater fluence rate of white light is used (100 μmol/m^2/s) the peak in *rbcS* mRNA occurs later (10 days after planting). This can be attributed to the plant requiring more Rubisco in a higher fluence rate to make the most efficient use of the extra light energy. The accumulation of a greater amount of Rubisco may take longer and thus require a longer period of high *rbcS* mRNA levels to enable the greater amount of protein to be made. This pattern of expression is again similar to that seen in cucumber (Greenland et al., 1987).

The leaf area of the primary leaves of plants grown under the two different light regimes was measured (Fig 3.2.3). This figure shows that the peak of *rbcS* mRNA levels occurred well before the completion of leaf expansion. The expansion of *Phaseolus vulgaris* primary leaves has been extensively studied (Van Volkenburg and Cleland, 1979; 1980; 1981;1990; Van Volkenburgh et al 1990). They have shown that a primary leaf grown under 4 μmol/m^2/s light would complete cell division by 10 days and that the rest of the increase in leaf size was due to cell expansion. It would thus appear that the increase in *rbcS* mRNA levels may occur during the period of cell division, and well before the completion of the cell expansion phase of primary leaf growth during growth under continuous light.

4.2.2 Regulation of *rbcS* mRNA levels in the primary leaves of plants subjected to changes in their light environments.

The experiments described in Section 3.2.3 indicate that the level of *rbcS* mRNA is not solely under ontogenetic control. Plants were grown in darkness for 7 days and then illuminated. The level of *rbcS* mRNA increases after illumination for 24

hours and then begins to fall. If these light treated plants are returned to the dark after 2 days illumination the level of *rbcS* mRNA falls more rapidly than if the plants were left under the illumination. This implies that, despite the levels of *rbcS* mRNA falling in the light grown plant over this time, its level must be maintained in some fashion, relative to the plants returned to the dark.

Thompson and Meagher (1990) studied the effect of light dark transitions on the expression of *rbcS* genes in soybean and petunia. They found that the *rbcS* mRNA levels of two of petunia's *rbcS* genes fell 4-fold after 24 hours dark adaptation and that similar effects were seen on the transcription of the genes. In soybean there was a 16-fold drop in the level of transcription after 48 hours of dark-adaptation and a 64-fold drop in the *rbcS* mRNA level, implying that the dark treatment caused a decrease in the stability of the RNA relative to its light grown level. Similar experiments could be carried out with *Phaseolus vulgaris* primary leaves to examine whether the decline in *rbcS* mRNA levels in the dark is solely due to a decrease in transcription, or whether there is also a post-transcriptional control.

The graph in Fig 3.2.5 shows that primary leaves dark adapted for two days would increase their *rbcS* levels on re-illumination. This increase may be still continuing after 48 hours under illumination and shows different kinetics to the initial increase in *rbcS* levels on illumination. This may be due to the age of leaf, the fact that it has already been under illumination, changes in the balance of photoreceptors or a combination of these factors. This experiment also shows that an increase in *rbcS* mRNA levels in response to illumination in *Phaseolus vulgaris* primary leaves is not limited to a particular stage of the leaf's development. This is in accordance with many other studies on *rbcS* expression (for example; Fluhr and Chua, 1986; Thompson and Meagher, 1990).

Another point to notice is the fact that the level of *rbcS* mRNA increases slightly on the fourth day after dark adaptation (Fig 3.2.5 and Fig 3.2.8). This small increase has also been found in separate experiments undertaken by Dr.N.A.R. Urwin in our laboratory and it is likely to be a significant observation. One can only speculate

as to why there is an increase in the level of *rbcS* mRNA present in primary leaves that have been dark adapted for a prolonged period. It is possible it is a preparatory response to enable the plant to respond to a return of light but why this should happen after four days is not clear. Another possible explanation is that prolonged dark adaptation results in a reiteration of the developmental programme seen in dark grown seedlings.

The graph also illustrates the fact that, in primary leaves that have developed chloroplasts, the response to a change from a dark environment to a light environment (or *vice versa*) at the *rbcS* mRNA level is not seen during the first 6 hours in the changed environment. This may reflect the time taken for the response to be detectable at the level of accumulated mRNA. A similar lack of response at the accumulated mRNA level to similar changes in light environments was seen by Shirley and Meagher (1990) in soybean primary leaves during the early timepoints examined after transfer of the plants. Despite this, changes in the rate of transcription (measured by run-on transcription assays) were detected at these early timepoints. Their data showed substantial discrepancies between the mRNA level and the transcription rates that led them to propose a role for post-transcriptional regulation of *rbcS* mRNA. Again these types of experiments could be carried out in *Phaseolus vulgaris*.

4.2.3 Regulation of the three *rbcS* genes' mRNA levels during dark to light, and light to dark transitions.

Experiments were also undertaken to examine the regulation of the three individual *rbcS* genes when 7 day old etiolated plants were illuminated (Section 3.2.4). These experiments showed that the three genes were expressed in the etiolated leaf and that the mRNA level from all three genes rose on illumination, to a peak level after 24 hours. There are some slight differences in the pattern of expression of the three genes but these are probably not significant since the overall pattern of the three genes' expression is similar. If the relative amounts of the transcripts to the sum of the three

transcript levels are estimated at their peak level (after 24 hours of illumination) the amounts are: *rbcS1* 61%, *rbcS2* 28%, *rbcS3* 13%. These can be compared to the levels estimated from the frequency of the corresponding cDNAs in a cDNA library: *rbcS1* 55%, *rbcS2* 35%, *rbcS3* 10% (Knight and Jenkins, 1992). These figures are quite close to each other and the differences could be interpreted as being due to the different methods being employed to estimate the transcript levels.

The control of expression of the three *rbcS* genes in dark adapted plants was also examined (Section 3.2.5). Plants that had been grown in the dark for 7 days prior to being illuminated for 2 days were returned to the dark for 4 days and samples taken over this period. The level of all three genes dropped after two days in darkness to undetectable levels in the case of *rbcS1* and *rbcS3* (Fig 3.2.8). The non-discriminating probe showed that there was some *rbcS* mRNA present after 2 days dark adaptation and that this level increased after 4 days of darkness. This pattern of expression was also seen for the *rbcS2* gene. The increase in transcript levels from the *rbcS2* gene in dark adapted leaves may be a pattern of expression peculiar to that gene. However the possibility remains that transcripts from the other two *rbcS* genes may be below the level of detection because of the lower hybridisation efficiency of their specific probes.

4.3 The regulation of *rbcS* expression by an increased fluence rate.

The results discussed in the previous Section showed that the three *rbcS* genes of *Phaseolus vulgaris* are light regulated. That is, their respective mRNA levels increase on exposure to light and fall when the plant is placed in darkness. Work described in Section 1.5.6 showed that the *rbcS* mRNA level in *Phaseolus vulgaris* is also controlled by the fluence rate of the light environment that the plant is growing in. The work presented in Section 3.3 was undertaken to examine the photoreceptor(s) controlling the regulation of *rbcS* mRNA levels in response to an increased fluence rate of light.

The strategy used to examine the photoreceptor(s) involved in the control of *rbcS* mRNA levels in response to an increase in the fluence rate of incident light was to grow the plants under 15 μmol/m^2/s white light and then move them to increased fluence rates of different qualities of light (Section 3.3.1). These light regimes would enable conclusions to be made about the particular photoreceptor(s) involved in the response (see Section 1.5.2). The experiment described in Section 3.3.2 shows that an increased fluence rate of white light, over 2 days, results in an increase in the level of *rbcS* mRNA when compared to 18 day old plants that had not been moved to the higher fluence rate. These results are similar to those of Jenkins (1986) who found a similar increase in the *rbcS* mRNA level after a three day, high fluence rate treatment of 10 day old *Phaseolus vulgaris* plants.

Jenkins (1986) also showed that there was an increase in the small subunit protein with an increased fluence rate. The increase in *rbcS* mRNA levels may be the method the plants use to control the amount of Rubisco holoenzyme in relation to the amount of light the plant receives. In sections of tobacco leaves given an increased fluence rate of white light the level of *rbcS* mRNA rose over the two days of the treatment (Prioul and Reyss, 1987). There was a parallel increase in the amount of Rubisco protein. The level of *rbcL* mRNA did not increase with the increased fluence rate of light, implying that it was probably the increase in *rbcS* mRNA which was resulting in the increase in the amount of Rubisco protein.

The view that the level of *rbcS* mRNA is important in regulating the amount of Rubisco protein has been shown in a number of studies. Rodermel et al. (1988) showed that tobacco plants expressing antisense *rbcS* DNA sequences had reduced *rbcS* mRNA levels, and small subunit protein. There was a similar decrease in the amount of large subunit protein despite its mRNA levels being unaffected. This demonstrated that, while the level of *rbcS* had little or no effect on the level of *rbcL* mRNA, there was a large effect on the amount of large subunit protein and thus holoenzyme. A similar effect was shown in pea, where treatment with α amanitin, an inhibitor of eukaryotic mRNA synthesis, would result in lower amounts of *rbcS*

mRNA, small subunit protein, and large subunit protein without having any effect on the level of *rbcL* mRNA (Sasaki, 1986). This again implies that the level of *rbcS* mRNA and the small subunit protein control the amount of large subunit protein and consequently the holoenzyme. Hobbs et al. (1990) found that genetic variability in the levels of Rubisco protein in different pea genotypes correlated with the amount of *rbcS* mRNA present, providing more evidence for the idea that the amount of small subunit protein may control the amount of Rubisco holoenzyme. A decrease in *rbcS* mRNA levels sometimes can have an effect on the *rbcL* mRNA levels, and *vice versa.* Studies on rye (Winter and Feierabend, 1990) have shown that inhibitors that specifically inhibit either cytoplasmic or plastidic transcription also affect the level of the Rubisco mRNA transcribed in the other compartment.

The experiments described above indicate that the control of *rbcS* mRNA levels in relation to the fluence rate of light may well an be an important control point in the metabolism of the plant, affecting the amount of Rubisco protein. Possible candidates for the photoreceptor mediating this response are proposed in Section 1.5.6. The experiments described in Sections 3.3.3, 3.3.4, and 3.3.5 demonstrate that the photoreceptor that carries out this function in the primary leaves of *Phaseolus vulgaris* is a blue light photoreceptor. The experiment described in Section 3.3.6 demonstrate that all three of the *rbcS* genes' RNA levels increase with an increasing fluence rate of blue-enriched light, showing that the increase in the total level of *rbcS* mRNA is not the result of one gene's mRNA level being differentially regulated, but of an increase in the level of mRNA from all the genes.

The involvement of one of the phytochromes as the primary photoreceptor involved in controlling *rbcS* mRNA levels as a result of an increase in the fluence rate is discounted (see Section 3.3.3) but there may well be a requirement for the presence of some P_{fr} for the response to occur. Attempts to illuminate the plants simultaneously with blue-enriched and far-red light to lower the amount of P_{fr} present during a blue-enriched light treatment gave results that were not reproducible, although in one case such a simultaneous illumination resulted in there being no increase in *rbcS* mRNA

levels (data not shown). A study with tomato showed that a greater increase in *rbcS* mRNA levels was seen with a blue irradiation over a red light background, than that seen with an equal fluence of red light alone, implying that there was a blue light photoreceptor involved in the response (Oelmüller et al., 1989). This difference was not seen if *aurea*, the PHY A deficient mutant of tomato was used, indicating that in this case the blue light photoreceptor may require phytochrome to be present to be functional.

Withers et al. (1991) studied the photosynthetic acclimation of tomato plants to an increased irradiance of different qualities of light. They found that an acclimation to an increased irradiance of light given over seven days, as measured by the maximum photosynthetic rate(P_{max}), amount of chlorophyll a and b, and dark respiration rate, would occur without an increase in the irradiance of blue light. This light regime, in which the increased irradiance was produced by low pressure sodium lights and thus did not contain an increased irradiance of blue light, would result in an acclimative response that was not as great as that seen with an equal increased fluence rate containing increased levels blue light (P_{max} 0.572 as compared to 0.677mg/m^2/s CO_2). An increase in acclimation was also seen if an increased irradiance was given which contained no blue light, although again the response was not as great as that seen with an equal fluence rate of light containing the blue part of the spectrum. The authors found that green light also resulted in an acclimative response and that if an increased fluence rate of green light was given such that the green treatment gave the same photosynthetic rate as the light containing the blue region of the spectrum the acclimative responses shown by the plants were the same in the green, no blue and blue containing light regimes. This lead the authors to propose that it was the photosynthetic light harvesting pigments that were responsible for the acclimation to an increased irradiance.

The experiments described above (Withers et al., 1991) studied the whole leaf over a longer acclimative period and use a higher fluence rate than those described in Section 3.3. The leaves Withers et al. (1991) were studying were only 20% expanded

at the start of the light treatment and thus it is possible that a different response is being examined, for the 16 day old primary leaves examined in this study, were almost fully expanded (Fig 3.2.2). The different ages of the leaves between the two studies may well result in different photoreceptor usage (see later in the discussion). The action of the photosynthetic pigments as the fluence rate detector responsible for the regulation of *rbcS* mRNA levels in *Phaseolus vulgaris* at higher fluence rates than those examined in this study cannot be ruled out. At the fluence rates examined however, the experiments described in Sections 3.3.4 and 3.3.5, along with the experiment shown in Fig 3.3.3, indicate that the photosynthetic pigments cannot be the photoreceptors controlling *rbcS* expression in the *Phaseolus vulgaris* primary leaf in response to an increased fluence rate. Both the red and blue-enriched light sources are able to drive photosynthesis at the same rate (Table 3.3.2) which is as expected given that the quantum absorption efficiency of chlorophyll is the same in the red and blue regions of the spectrum. The stomata were also found to be open to approximately the same extent under both the red and blue-enriched light sources (Table 3.3.3). This is presumably because the chloroplasts in the stomata are photosynthesising equally well under both light sources and are thus keeping the stomata open to the same extent. This would ensure that the primary leaves have the same level of CO_2 for the dark reactions of photosynthesis and thus rule out an effect of differential stomatal opening between the two different light sources on *rbcS* mRNA levels.

The experiment described in Section 3.3.7 demonstrates that moving older plants whose leaves have fully expanded to a higher fluence rate of blue-enriched and red light still results in a higher level of *rbcS* mRNA under the high fluence of blue-enriched light. This shows that the blue photoreceptor and the neccessary signal transduction components are still active in the older fully expanded leaves and that the blue light regulation of *rbcS* mRNA levels is not linked to a particular phase of primary leaf development.

Changes in photoreceptor usage during development have been seen for a number of genes and enzymes. Frohnmeyer et al. (1992) studied the photoreceptors

that controlled the expression of the genes encoding chalcone synthase (*CHS*) in parsley and mustard. The authors found that *CHS* expression in etiolated plants was primarily under the control of phytochrome. However, in the leaves of mature plants dark adapted for 32 hours, a UV-B photoreceptor was the predominant controlling photoreceptor. This pattern of changes in photoreceptor usage is similar to that seen by Fluhr and Chua (1986b) for the regulation of *rbcS* mRNA levels in pea and petunia transformed with a pea *rbcS* gene, where *rbcS* expression is under phytochrome control in the etiolated plant and under the control of a blue light photoreceptor in re-etiolated mature leaves. Elmlinger and Mohr (1991) studied the appearance of the plastidic ferredoxin-dependent glutamate synthase (an enzyme involved in ammonium assimilation) in Scots pine seedlings. They found that the appearance of the enzyme was strongly induced by white light. In etiolated seedlings younger than 10 days the white light could be replaced by a red light treatment implying the action of phytochrome. In older seedlings blue/UV-A light was required for full induction of the enzyme, although this effect was not seen if a simultaneous far-red light treatment was given with the blue light. Thus in older seedlings the enzyme's appearance is controlled by a blue/UV-A photoreceptor which needs the presence of some P_{fr} for its action. This pattern of photoregulation has also been seen for two other enzymes involved in nitrogen metabolism, alanine aminotransferase (Otter et al., 1992) and glutamine synthetase (Elmlinger and Mohr, 1992).

As previously stated, the photoreceptor that controls the *rbcS* mRNA levels in the green primary leaves of *Phaseolus vulgaris* is a blue photoreceptor. Work by Knight (1990) had shown that in etiolated *Phaseolus vulgaris* seedlings a red light pulse (5 minutes) would lead to higher levels of *rbcS* mRNA than a 24 hour white light treatment. It has been shown recently that a 24 hour red light treatment also results in lower *rbcS* mRNA levels than a 5 minute red pulse (S. Robertson and G.I. Jenkins, unpublished data). It would seem that there is a similar change in photoreceptor usage, from phytochrome in the etiolated plant, to a blue/UV-A photoreceptor in the green plant, in the *Phaseolus vulgaris* primary leaves, to those described in the previous

paragraph. The action of the high levels of type 1 phytochrome in the etiolated plant has been proposed to be as a light 'antenna' (Smith and Whitelam, 1990) which detects the presence of light early to allow the plant to adapt its development to growth under a light environment. The initial peak in *rbcS* mRNA seen in etiolated *Phaseolus vulgaris* primary leaves after a red light pulse may be a manifestation of this. The fact that a 24 hour red or white light treatment results in a lower *rbcS* mRNA level may reflect the development of a different control of *rbcS* mRNA levels once the plant is green. Such a control may involve the blue light receptor.

4.4 The regulation of *rbcS* transcription by light of different qualities.

4.4.1 Run-on transcription assays.

The experiments described in Section 3.3 demonstrated that the *rbcS* mRNA level is regulated by an increase in fluence rate and that this regulation is mediated by a blue photoreceptor. The experiments described in Section 3.4 examined whether the increase in steady state mRNA was mediated by an increase in the transcription of *rbcS* genes. The method chosen was run-on transcription analysis which was initially used by Gallagher and Ellis (1982) who showed that nuclei isolated from dark-grown pea leaves showed much lower expression of *rbcS* and *cab* genes than those isolated from illuminated leaf tissue. This method has been used by a number of groups in concert with measurements of the amount of steady state mRNA to examine the role of transcriptional and post-transcriptional controls in the expression of *rbcS* (e.g. Silverthorne and Tobin, 1984; Ernst et al., 1984; Shirley and Meagher,1990) and also *cab* (e.g. Mösinger et al., 1985; Marrs and Kaufman, 1989) and phytochrome genes (Colbert et al., 1983).

Thompson and White (1991) express reservations about the use of run-on transcription assays in quantitative work, because of a number of factors. The assay works on the assumption that, because initiation of transcription does not seem to occur

in isolated nuclei, the radiolabelled transcripts that are made are 'run-on' from those already initiated. The amount of these transcripts thus gives an indication of how many RNA polymerase complexes were elongating transcripts at the moment the nuclei were harvested. Work on animal systems has shown that treatment of nuclei with agents such as high salt, heparin and sarkosyl dissociates chromosomal protein, but not active transcription complexes (e.g. Rougvie and Lis, 1988). When barley nuclei were treated with heparin overall RNA transcription increased 5 fold and *cab* and protochlorophyll reductase gene transcription was no longer affected by a prior *in vivo* red light treatment. This implied that the effect of phytochrome was on the chromatin structure, and that these changes could be seen in run-on assays (Mösinger et al, 1987). This, along with problems of background hybridisation in the analysis of run-on RNA led Thompson and White to question the usefulness of run-on transcription assays as a quantitative measure of transcription.

However, work carried out using run-on transcription assays to analyse transcription of *rbcS* genes can be compared to experiments using transgenic plants containing *rbcS* promoters driving reporter genes. For example, the lower levels of *rbcS* transcription seen in dark grown nuclei (Gallagher and Ellis, 1992) can be correlated with a low level of expression of a pea *rbcS* gene in dark grown transgenic petunia (Nagy et al., 1986). Comparisons of this sort show that data from run-on transcription analysis of *rbcS* genes usually correlates with that from transgenic plants examining transcription from *rbcS* promoters. They can therefore be useful in assessing whether the control of a particular response seen at the level of accumulated *rbcS* mRNA is regulated by changes in the level of transcription prior to examining the promoter(s) of the *rbcS* genes in a transgenic environment, which is a time consuming business.

The photographs shown in Fig 3.4.1 show nuclei isolated from *Phaseolus vulgaris* primary leaves that were 16 days old. The nuclei fluoresce under UV illumination when stained with ethidium bromide because it stains the DNA present. These nuclei were shown to be capable of carrying out run-on transcription at rates

similar to those in the literature and produced a relatively wide size range of transcripts (Fig 3.4.2). The hybridisation conditions used were shown to be conditions of DNA excess (Fig 3.4.3) which implies that the hybridisation signal seen is related to the amount of labelled sequence of interest added to the hybridisation reactions.

4.4.2 Analysis of the effect of transferring a plant from 15 μmol/m²/s white light to 100 μmol/m²/s of blue-enriched light on *rbcS* transcription.

The experiments described in Section 3.4.4 show that the steady state *rbcS* mRNA level increases over the 24 hours following transfer from a 15 μmol/m²/s white light environment to a 100 μmol/m²/s blue-enriched light environment and then starts to fall. This implies that there are two points of control in the response to an increased fluence rate of blue light. The first results in the initial increase in the *rbcS* mRNA level, but there must also be a control which stops this increase after 24 hours. The cessation of an increase in mRNA is probably because the plant has made enough new Rubisco protein to utilise the extra energy provided by the increased fluence rate and that this increase in the amount of Rubisco protein feeds back in some way on the rate of *rbcS* transcription.

The experiment shown in Fig 3.4.5 shows that the increase and decrease in steady state *rbcS* mRNA levels are preceded by similar changes in the level of *rbcS* transcription. The level of *rbcS* transcription appears to peak after 12 hours in the increased blue-enriched fluence rate of light, 12 hours prior to the peak at the level of steady state *rbcS* mRNA. This large time difference may well be due to the fact that the rate of transcription was not measured between 12 and 24 hours, and the rate of *rbcS* transcription could start to decrease closer to the 24 hour timepoint than the 12 hour timepoint at which it was measured.

The increase in transcription is probably as a result of excitation of a blue photoreceptor (for discussion see Section 4.4.3). The swiching off of transcription

could possibly result from metabolic feedback. Quick et al. (1991) used the tobacco plants expressing an *rbcS* antisense gene, generated by Rodermel et al. (1988) to examine the control exerted by Rubisco over photosynthesis. In mutant plants with up to 60% of their wild-type levels of Rubisco, photosynthesis was only reduced by 6%. This was due to there being an increase in the activation of Rubisco in the mutant plants (wild type 60%, mutant 90% activated) to compensate for the lower amount of Rubisco. Quick and his co-authors concluded that wild-type plants had approximately twice as much Rubisco protein as they required to avoid direct limitation of photosynthesis by Rubisco and that this resulted in the plants investing 15% more protein in Rubisco than was strictly necessary.

Therefore an increase in irradiance may possibly be dealt with by the plant by increasing the activation state of its Rubisco. However, as a plant normally has twice as much Rubisco as required, it may then increase *rbcS* transcription to make more Rubisco protein until it again has such a level of Rubisco that it can reduce its Rubisco activation state to 60%. The increased activation of Rubisco in the antisense *rbcS* mutants was accompanied by an increased ATP/ADP ratio and an increased thylakoid energisation (ΔpH) which may be required to maintain the increased ATP/ADP ratio (Quick et al., 1991). If in a wild type plant, an increase in Rubisco activation occurs with an increase in irradiance with a concomittant increase in the ATP/ADP ratio and thylakoid energisation, the ATP/ADP ratio and thylakoid energisation may return to their previous levels over the same time period as the Rubisco protein increases. Such a return to a previous state as this could be a possible candidate for the mechanism that results in the switching off of *rbcS* transcription. This hypothesis could be tested by correlating the timecourse in changes in transcription and mRNA levels with measurable changes in chloroplast status such as Rubisco activation state and thylakoid energisation.

It is noticeable that the pattern of *rbcS* expression seen when the *Phaseolus vulgaris* plants are moved from a 15 μmol/m^2/s environment to an increased fluence rate of blue-enriched light is similar to that seen when dark grown *Phaseolus vulgaris*

plants are illuminated (compare Fig 3.4.5 to Fig 3.2.6). It is possible that a similar control of the amount of *rbcS* transcription as that discussed above may result in the decrease in *rbcS* mRNA levels after illumination of dark grown *Phaseolus vulgaris* plants.

4.4.3 Analysis of the effect of transferring a plant from 15 μmol/m²/s white light to 100 μmol/m²/s light of different qualities on *rbcS* transcription

The effect on *rbcS* transcription of moving plants grown under 15 μmol/m²/s white light for 16 days to different qualities of 100 μmol/m²/s light for 12 hours was examined in Section 3.4.6. A 12 hour treatment was chosen because this was the time that transcription peaked under an increased fluence rate of blue-enriched light with these growing conditions. This experiment showed that the largest increase in transcription was seen with the blue-enriched light which resulted in a doubling in the *rbcS* transcription rate when compared to the transcription rate in 15 μmol/m²/s white light (Table 3.4.4 and Fig 3.4.8). An identical increased fluence of white light also resulted in an increase in the *rbcS* transcription rate but this increase was under half that seen with blue-enriched light, which was consistent with the effects of increased fluence rates of blue-enriched and white light at the level of steady state mRNA (Fig 3.3.3). However an increased fluence rate of red light also gave an increase in the rate of *rbcS* transcription which was not evident at the steady state mRNA level.

There are a number different possible reasons why the increase in transcription under red-light does not result in an increase in *rbcS* steady state mRNA. Direct comparisons between the two experiments (Section 3.3.3 and Section 3.4.6) must be made with care because the plants used for the nuclei isolations were grown 10 to the pot whereas the plants used for the experiment described in Section 3.3.3 were grown in individual pots. The increase in plant density was required because of the large amount of tissue that was required for the nuclei preparations. The proximity of other

plants is known to be perceived by phytochrome and to induce a shade avoidance reaction (Casal and Smith, 1989) and this may have occurred in some of the plants used for nuclei preparations. Another effect of the presence of other plants in the same pot in the experiment described in Section 3.4.6 may be that the spectrum of the incident light was altered slightly through reflection and absorption of some of the light, leading to different results than those expected from Fig 3.3.3.

Another difference between the two sets of experiments is that the experiments shown in Fig 3.3.3 measure the steady state mRNA level after a 48 hour treatment. The increase in transcription seen under red light after 12 hours may result in an early peak in steady state *rbcS* mRNA and a subsequent fall to the 15 $\mu mol/m^2/s$ white light level after 48 hours. This is unlikely because, even though the level of transcription under blue-enriched light peaked after 12 hours in the experiments described in Section 3.4.5, there was still an increase in the steady state *rbcS* mRNA level detectable after 48 hours under blue enriched light in the experiment described in Section 3.3.3. Therefore it is likely that if the only difference between the red and the blue-enriched treatment was the relative transcription rate, an increase in *rbcS* mRNA would still be detectable after 48 hours in the red environment. There is a possibility that the red light treatment induces an *rbcS* mRNA destruction system. This could account for the level of steady state *rbcS* mRNA present in the red treated plants' primary leaves being similar to those present under 15 $\mu mol/m^2/s$ white light despite there being an increase in transcription.

Despite the reservations expressed above the blue-enriched light regime does induce a greater increase in transcription than a similar fluence rate of white, which is in itself more effective then red light and these observations explain the effects of the increased fluence rates of blue-enriched and white light on *rbcS* mRNA levels. Marrs and Kaufman (1989) demonstrated that a brief (30 minutes) blue irradiation of red light grown pea seedlings resulted in an increase in *cab* mRNA level over controls that were left in the red light. The increase in *cab* mRNA levels was shown to be caused by an increase in *cab* transcription through the use of run-on transcription assays. This increase was detectable after 3 hours. Effects of blue light on transcription have also

been seen for the chalcone synthase (*chs*)gene in isolated, cultured parsley cells (Ohl et al., 1989). It was shown that a blue treatment prior to a UV treatment resulted in a more rapid increase in transcription of *chs* than was seen if no blue pre-treatment was given.

The effect of blue light on *rbcS* transcription has also been seen on the *rbcS* genes of pea. A 30 minute blue light treatment of dark treated, light adapted pea plants has been shown to increase *rbcS* transcription to levels similar to those obtained with a treatment with white light for the same duration and fluence rate (Clugston et al., 1990). A red treatment of the same duration and fluence rate resulted in a lesser rise in transcription. These data is similar to those obtained using *Phaseolus vulgaris* plants in this study in that the red treatment was not as effective as the blue and white treatments. The fact that the white and the blue were equally effective in pea could be due to the different species and light sources being used, the fact that a 5 hour dark treatment was used for the studies on pea, and/or that the pea plants were younger (8 days) as compared to the 15 day old *Phaseolus vulgaris* plants used in this study. The younger pea plants may not have reached the stage in their development at which the blue photoreceptor is fully active (See Section 4.3).

4.5 RT-PCR examination of the three *rbcS* genes' mRNAs

The reverse transcriptase-polymerase chain reaction (RT-PCR) is a PCR performed on a population of first strand cDNA molecules produced by undertaking reverse transcription of a total RNA sample. The technique has been used to study alternative splicing reactions where relatively small amounts of RNA are present (e.g. Golde et al., 1990). The advantage of the method for the analysis of the expression of the *rbcS* gene family of *Phaseolus vulgaris* is that the level of each of the transcripts can be measured in one reaction, thus avoiding possible differences, for example, between hybridisation reactions. The *rbcS* genes of *Phaseolus vulgaris* are particularly suitable for this type of analysis in that their coding sequence is identical. This allows

the design of a non-discriminating 5' oligonucleotide primer and also ensures that the efficiency of the *Taq* polymerase will be identical over this portion of the three cDNAs during amplification. The differences present in the 3' non-coding sequence allow the amplification of distinct products for each of the three *rbcS* genes' transcripts through the use of gene specific 3' oligonucleotide primers. The extra bands (visible in Fig 3.5.1) are only present when the products of the PCR are run out immediately. If the reactions are stored at 4°C for 24 hours these bands disappear (Lane 7, Fig 3.5.5). What the bands represent is unknown, but they do not correspond in size to the expected products from *rbcS* transcripts.

The experiment shown in Fig 3.5.1 shows that there were three amplification products of the predicted size when RT-PCR was carried out on RNA from both light and dark grown plants. The fact that there was very little difference between the amounts of each of the amplification products between the samples from the light and dark grown plants probably reflects the fact that the PCR had been allowed to proceed for too many cycles. As each reaction was carried out with the same amounts of oligonucleotide primers, if the PCR was allowed to continue until these were becoming limiting, the amount of product generated in each reaction would be start to become proportional to the amount of primers added to each reaction, not to the amount of input target RNA. This may explain why similar amounts of product were seen in both the samples.

That this was likely to have been the explanation was shown in the experiments described in Section 3.5.2. Reducing the number of cycles while still using 4 μg of total RNA in the initial reverse transcriptase reaction still did not result in conditions where the PCR amplified each genes' cDNAs with equal efficiency (Fig 3.5.2). Reducing the amount of initial input total RNA to 1 μg and decreasing the cycle number still further resulted in the products from the PCR for each gene increasing in a logarithmic fashion between cycles 10 and 14 (Fig 3.5.4). Under these conditions the $\log_{10}$ of the amount of product from each gene plotted against the cycle number results

in a parallel plot for each gene. This implies that, as expected, the *Taq* polymerase is amplifying each product with equal efficiency.

These conditions were then used to examine the three *rbcS* genes' mRNA levels in samples taken during the growth of plants in the dark and under 50 μmol/m^2/s white light. These RNA samples had previously been examined using northern analysis with the non-discriminating oligonucleotide probe and the *rbcS* mRNA level in both sets of samples was shown to peak on the seventh day after sowing. RT-PCR with these samples using the conditions suggested by the experiments described in Section 3.5.2 proved a qualified success. There was a greater amount of product for each of the genes in the RNA samples from the light grown leaves compared to the dark grown leaves (Fig 3.5.5). This suggests that the conditions are approximately correct for comparisons between RNA samples. Scanning the autoradiograph of the products from the RNA samples extracted from light grown leaves and plotting the total amount of product from the three genes against the time they were taken gives a pattern similar to that seen from scanning the northern blot of the RNA samples (compare Fig 3.5.6 to Fig 3.2.2) again implying that the conditions used were correct.

The peak amount of product from the RNA samples extracted from dark grown plants was found in the samples extracted from the 10 day old leaves. This is different to the peak seen when these RNA samples were examined by northern analysis. An explanation for this could be that the RT-PCR is not optimised for the lower amount of RNA present in the samples from dark grown plants. Another possibility is that the amounts of RNA input into the reaction may not have been identical. To test for this, an internal control in the reactions would be useful. This would need to be an RNA sequence whose concentration does not vary over the conditions examined. Another possibility is to manufacture a sequence which has the same primer sequences, but which has a different size of product, which could be added in known concentrations to the reactions to compete with the unknown amounts of test sequence. This would however complicate the analysis, requiring a larger number of reactions. The simplest

answer may be to run a sample of the RNA on an agarose gel and EtBr stain the gel to see if similar amounts of RNA are present in each sample to be used for the RT-PCR.

If the relative amounts of the transcripts are examined using the RT-PCR method it can be seen that the product from the *rbcS2* gene is the most abundant. This is contrary to the results found using oligonucleotide hybridisation, which found the transcript from the *rbcS1* gene to be the most abundant. The data from the oligonucleotide probing of RNA on northern blots is also in approximate agreement with data from the classification of *rbcS* cDNAs (Knight and Jenkins, 1992). The S1-analysis of total RNA in the same study (Knight and Jenkins, 1992) also suggested that the *rbcS1* transcript is the most abundant. A possible reason for the discrepancy between the RT-PCR data and the other sets of data is the differences between the hybridisation efficiencies of the 3' oligonucleotide probes. The data shown in Fig 3.1.8 shows that the *rbcS1* and *rbcS3* specific oligonucleotides hybridise at 71% and 54% of the efficiency of the *rbcS2* specific oligonucleotide. This could mean that there is more likelihood of an *rbcS2* primer binding to its complementary sequence than an *rbcS1* or *rbcS3* primer binding to its sequence. This would lead to preferential synthesis of the *rbcS2* specific product and the difference would be amplified through the reaction, leading to the *rbcS2* specific product being over-represented. This could be examined by using different gene-specific primers and measuring their hybridisation efficiency prior to using them for RT-PCR. If a *rbcS2* specific primer with a different hybridisation efficiency gave the same result, with regards to transcript level, as the present *rbcS2* oligonucleotide then the factor of differing hybridisation efficiencies could be ruled out as a cause for the discrepancy between the different methods.

4.6 Conclusions.

The work presented in this study enables several conclusions to be drawn about the expression of the three *rbcS* genes in *Phaseolus vulgaris*. Experiments examining

the regulation of the three genes in dark grown plants that have been illuminated shows that all three genes are photoregulated. Furthermore, under most of the conditions that the expression of the three genes was studied, their pattern of expression at the accumulated transcript level was similar, with the major difference being in the amount of accumulated transcript for the three genes. A possible explanation for the similarity of the qualitative expression patterns of the three genes is the likelyhood that the three *Phaseolus vulgaris rbcS* genes have only relatively recently been duplicated. This is suggested by the fact that the coding sequences of the three genes are identical (Knight and Jenkins, 1992) and the possibility that the genes may be linked (N.A.R. Urwin and G.I.Jenkins, unpublished data). Thus the promoter regions of the three genes may not have altered very much relative to each other, resulting in the similar qualitative patterns of expression.

The expression of the *rbcS* gene family as a whole was shown to be under developmental regulation in dark grown and light grown plants and this pattern of expression was modified by light/dark transitions. This pattern of expression, which is similar to that of the single cucumber *rbcS* gene (Greenland et al., 1987), indicates that there is a control of *rbcS* expression which is independent of light. The interactions between the ontogenetic and light regulated control of *rbcS* expression shown represent an interesting avenue for further work.

The study of the regulation of *rbcS* expression when the fluence rate of the light environment increases has shown that the photoreceptor that senses this change is a blue detecting photoreceptor. Perception of blue-enriched light results in an increase in *rbcS* mRNA levels which is dependent on the fluence rate of the blue-enriched light that the plant is receiving, in the range of fluence rates that was studied. The increase in *rbcS* steady state mRNA levels in response to an increased fluence rate was found to be mediated by an increase in the transcription rate of the *rbcS* genes which was greatest under the blue-enriched light. There was, however, an increase in transcription under an increased fluence rate of red light which was not apparent in studies of the steady state *rbcS* mRNA level under this light regime.

The regulation of the *rbcS* genes at the level of steady state mRNA in response to moving the plants from a low fluence rate to a higher fluence rate of blue-enriched light, and indeed in response to moving plants from growth in darkness to light, was shown to involve an increase in *rbcS* mRNA followed by a decrease. In the case of moving the plants to an increased fluence of blue-enriched light this was shown to be transcriptionally controlled. This demonstrates that there is also a control mechanism that turns off *rbcS* transcription. There is a possibility that this turning off of *rbcS* transcription may involve metabolic feedback, and this could represent another intersting area for future research.

An RT-PCR method was developed to measured the mRNA levels from the three *rbcS* genes. This method produced products of the expected size for each of the three genes, but the relative abundance of the three products was not the same as the relative abundance of the three *rbcS* genes' transcripts as measured using gene specific oligonucleotide probing of northern blots, and also the frequency of the complementary cDNAs in a cDNA library. This discrepancy remains to be resolved, but the method was shown to produce a similar result to a northern blotting experiment and the method may be useful for studying the pattern of transcription of the three genes in dark grown material which proved problematic with gene specific oligonucleotide probing of northern blots.

4.7 Future work.

The promoter region of the *rbcSβ2* gene has been cloned in our laboratory, and work is being undertaken to examine the regulatory properties of the promoter. One could try to define a region of the promoter that is responsible for the regulation of transcription in response to an increased fluence rate of light, and see if this part of the promoter is regulated by blue light. If such a region could be defined, it could then be compared to the regions of other *rbcS* promoters that have already been characterised.

DNA binding proteins that would presumably bind to the promoter would also be another target for future research. The mechanisms of the ontogenetic regulation of the *Phaseolus vulgaris rbcS* genes could also be examined and compared to the mechansims involved in light regulation.

Another area for research could be the relation of the regulation of *rbcS* expression to regulation of the Rubisco protein by biochemical means via the Rubisco activase protein. It would seem likely that there is some integration of control between Rubisco protein and its genes such that the plant has the 'right' amount of protein for the light conditions. Factors such as the balance of metabolites could feedback to the *rbcS* genes and this area of *rbcS* expression has not been extensively studied. Both of the areas of research mentioned would be of great interest in terms of integrating biochemical control of metabolism with the signal transduction involved in gene regulation which is an expanding area of research.

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