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Ontogenetic regulation and photoregulation of members of the *Phaseolus vulgaris* L. *rbcS* gene family

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Abstract. The *rbcS1, 2* and *3* genes of *Phaseolus vulgaris* are identical in coding sequence and we have studied their expression using gene-specific probes derived from their 3′ non-coding regions. The genes differ in their relative levels of expression but show only minor qualitative differences in their regulation. Transcripts of the three genes are undetectable in primary leaves in the imbibed seed, accumulate early in leaf expansion reaching a maximum 7–10 d after sowing and decrease to low levels by the time expansion is complete. Both dark-grown and light-grown primary leaves exhibit this ontogenetic pattern of expression, although the light-grown leaves have two to three times more *rbcS* transcripts. Light can over-ride the ontogenetic control of *rbcS* expression; for example, when 7-d-old dark-grown primary leaves are illuminated there is a 6- to 12-fold increase in the transcript levels of the *rbcS* genes. Transfer of illuminated leaves to darkness results in the loss of transcripts of all three genes, but *rbcS2* transcripts persist in the dark-adapted leaves. Possible physiological mechanisms of the ontogenetic regulation of expression are discussed.

Key words: Ribulose 1,5-bisphosphate carboxylase/oxygenase – *rbcS* genes – Leaf development – Photoregulation – *Phaseolus*

Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a very important and abundant enzyme in photosynthetic tissues. It is composed of two types of polypeptide subunit, a nuclear-encoded small subunit and a plastid-encoded large subunit. Genes encoding the small subunit (*rbcS* genes) are well characterised in terms of their structure, organisation and regulation of expression (reviewed by Manzara and Gruissem 1988; Dean et al. 1989). The *rbcS* genes encode a precursor of the mature small-subunit polypeptide and their sequences are well conserved across taxonomic groups. In those species studied to date the genes are present in small multigene families, members of which are sometimes closely linked in the genome. Expression of the *rbcS* genes is spatially-regulated between organs (Manzara and Gruissem 1988; Dean et al. 1989), and within tissues and cells of a given organ (Aoyagi et al. 1988), and correlates with the presence of chloroplasts (Taylor 1989). Expression is also subject to temporal regulation during development (Nelson et al. 1984; Greenland et al. 1987; Wanner and Gruissem 1991). In most species, expression of the *rbcS* genes is stimulated by light mediated by phytochrome (reviewed by Tobin and Silverthorne 1985) or a blue-light photoreceptor (Fluhr and Chua 1986; Clugston et al. 1991; Sawbridge et al. 1994) depending on the stage of leaf development. In addition, expression is regulated by a range of other environmental and endogenous signals, including by metabolites such as sucrose and glucose (Sheen 1990; Krapp et al. 1993). There is evidence of differential regulation, both quantitatively and qualitatively, among members of *rbcS* gene families in several species (Dean et al. 1985; Fluhr et al. 1986; Sugita and Gruissem 1987; Wanner and Gruissem 1991; Dedonder et al. 1993). To a major extent, but not exclusively, expression of the *rbcS* genes is controlled at the transcriptional level (Gallagher et al. 1985; Shirley et al. 1990; Wanner and Gruissem 1991; Silverthorne and Tobin 1992). The DNA sequence elements and transcription factors concerned with the regulation of *rbcS* transcription are being identified (Gilmartin et al. 1990; Manzara et al. 1991), but much less is known about the signal transduction pathways which converge on these transcription factors.

It is important to understand how developmental and environmental signals are integrated in the regulation of gene expression. We have chosen to investigate aspects of this problem using the *rbcS* gene family of French bean

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Abbreviations: *rbcS* = gene encoding the small subunit of Rubisco; Rubisco = ribulose 1,5-bisphosphate carboxylase/oxygenase

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(*Phaseolus vulgaris* L.). The primary leaves of *P. vulgaris* offer an excellent developmental system to study the regulation of expression. The primary leaves are visible in the seed and can be studied following germination and through to senescence over approximately a 40-d period. The *P. vulgaris rbcS* gene family is particularly interesting for studies of the molecular basis of differential expression. Present evidence indicates that *P. vulgaris* has three *rbcS* genes, linked at a single locus, and the nucleotide sequences of their entire coding regions are identical (Knight and Jenkins 1992; N.A.R. Urwin and G.I. Jenkins, unpublished data). The genes can be distinguished only in their divergent 3′ and 5′ non-coding sequences. This degree of conservation is the highest among *rbcS* gene families studied to date. In other species, the genes usually differ to some degree, particularly in the sequence of the N-terminal transit peptide, although two identical coding sequences are found, for example, in tomato (Sugita et al. 1987). Because the three *P. vulgaris rbcS* genes are probably recently diverged, they may offer a good system to identify DNA sequence elements concerned with quantitative and qualitative aspects of regulation.

In this paper we show that the members of the *P. vulgaris rbcS* gene family are subject to ontogenetic regulation and photoregulation in leaves and that while there are quantitative differences in expression, qualitatively their regulation is very similar.

Materials and methods

Plant material. Seeds of *Phaseolus vulgaris* L. cv. Tendergreen were germinated in compost and plants grown in controlled-environment rooms at 20°C. Some plants were grown in absolute darkness while others were grown under continuous white light (warm-white fluorescent tubes; Osram, München, Germany) at the photon fluence rates indicated. The fluence rates (400–700 nm) were measured with a LI-185B quantum sensor (LiCor, Lincoln, Neb., USA).

Primary leaves were harvested into liquid nitrogen at the times indicated and their age is given relative to day 0, the time of sowing. For the ontogenetic experiments, primary leaves pooled from five plants at each time point were used to prepare RNA. Similarly, in experiments involving illumination of dark-grown plants or dark-adaption of illuminated plants, four pairs of primary leaves were pooled at each time point.

Isolation of RNA and measurement of transcript levels. Total RNA was extracted from primary leaves, run on agarose-formaldehyde gels and blotted onto nylon filters as described by Sawbridge et al. (1994). Hybridisation to the cDNA insert of plasmid pPvSS191, which contains the complete coding sequence of the *rbcS3* gene (Knight and Jenkins 1992), was carried out under essentially the same conditions as described previously (Knight and Jenkins 1992). Equal amounts of RNA (normally 5–10 μg, but 20 μg for dark-grown plants) were loaded into the wells of each gel and loading was examined by ethidium bromide staining and/or hybridisation to an rDNA probe (Gerlach and Bedbrook 1979).

For measurements of transcript abundance corresponding to specific *rbcS* genes, gene-specific oligonucleotide probes were hybridised to RNA on the Northern blots. These 21-mer oligonucleotides of equal GC content were derived from the 3′ non-coding sequences of the genes. The oligonucleotide sequences are: rbcS-1, AGCTTTCAACAGAAGATAATC; rbcS-2, GAAGATGAAATCTCAAAATGC; rbcS-3, ATAAATTTCCTTACGCAGAAG. A further oligonucleotide (rbcS-G, AATCCAATGATACGGATGAAA), corresponding to a region of the coding sequence and of identical GC content to the gene-specific probes, was also used. Each oligonucleotide (200 ng) was labelled to high specific activity using 3.7 MBq [γ-^{32}P]dATP (111 TBq·mmol^{-1}) and T4 polynucleotide kinase, essentially as described by Maxam and Gilbert (1980). Filters were pre-hybridised for at least 4 h at 42 °C in 6 × SSC (20 × SSC is 3 M NaCl, 0.3 M sodium citrate), 0.05% (w/v) sodium pyrophosphate, 0.5% (w/v) SDS, 200 μg·ml^{-1} heparin (Binnie 1990). Hybridisation was carried out in the same solution containing equal amounts, normally 1.5×10^6 cpm·ml^{-1}, of the labelled oligonucleotide for 4 h. The filters were washed at 46 °C in 5 × SSC, 0.1% (w/v) SDS for 20 min and the wash was then repeated twice, reducing the salt concentration to 2 × SSC and then to 1 × SSC. The filters were autoradiographed at − 80 °C. When appropriate, autoradiographs were scanned with a laser densitometer (2202 Ultroscan; LKB, Bromma, Sweden).

Hybridisation of oligonucleotide probes to DNA. Plasmids pPvSS1672, pPvSS965 and pPvSS191 (Knight and Jenkins 1992) containing the *rbcS1*, *rbcS2* and *rbcS3* cDNAs respectively, were linearised with an appropriate restriction endonuclease. A solution of linearised plasmid DNA was heat-denatured and mixed with an equal volume of cold 20 × SSC. The plasmid DNA in a final volume of 220 μl was applied to a nylon filter soaked in 10 × SSC using a slot-blot manifold (Bio-Rad, Watford, UK). The filter was placed in 1.5 M NaCl, 0.5 M NaOH for 5 min followed by 0.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 1 min and then UV-irradiated for 90 s to fix the DNA to the filter. Equal cpm of the *rbcS* oligonucleotide probes, estimated to have specific activities within 10% of each other, were hybridised to the DNA. Hybridisation and washing were carried out as described for the hybridisation of the oligonucleotides to RNA. The relative efficiencies of hybridisation were estimated by densitometric scanning of the resulting autoradiographs.

Reproducibility of experiments. Each experiment to measure transcript abundance was repeated with several different batches of plants. Similar results were obtained in each set of experiments and representative data from single experiments are therefore presented.

Results

Ontogenetic regulation of rbcS transcript levels. To investigate the regulation of *rbcS* transcript levels during primary leaf ontogenesis, blots of total RNA isolated from leaves of different ages were hybridised to the *rbcS3* cDNA probe pPvSS191 (Knight and Jenkins 1992). This probe does not discriminate between transcripts of the three *rbcS* genes because their coding sequences are identical. Figure 1A shows *rbcS* transcripts in total RNA from primary leaves of plants grown from seed in either complete darkness or 50 or 100 μmol·m^{-2}·s^{-1} continuous white light. Approximately three times more RNA was loaded per track for the dark-grown plants to facilitate comparison of the ontogenetic profiles. This loading difference is compensated for in the graphical representation in Fig. 1B. The *rbcS* transcripts are undetectable in the primary leaves in the imbibed seeds 3 d after sowing but are detectable in 5-d-old primary leaves, which are just emerging above the compost and from the seed coat. There is a distinct peak in the *rbcS* transcript level on day 7 in primary leaves grown in darkness and thereafter transcripts decline to day 20. A similar profile is seen in 50 μmol·m^{-2}·s^{-1} white light; although the peak is broader the maximum is still on day 7. The maximum level of *rbcS* transcripts in 100 μmol·m^{-2}·s^{-1} white light is on day 10 and at this fluence rate *rbcS* transcripts in the 15- and 20-d-old primary leaves are below the limit of

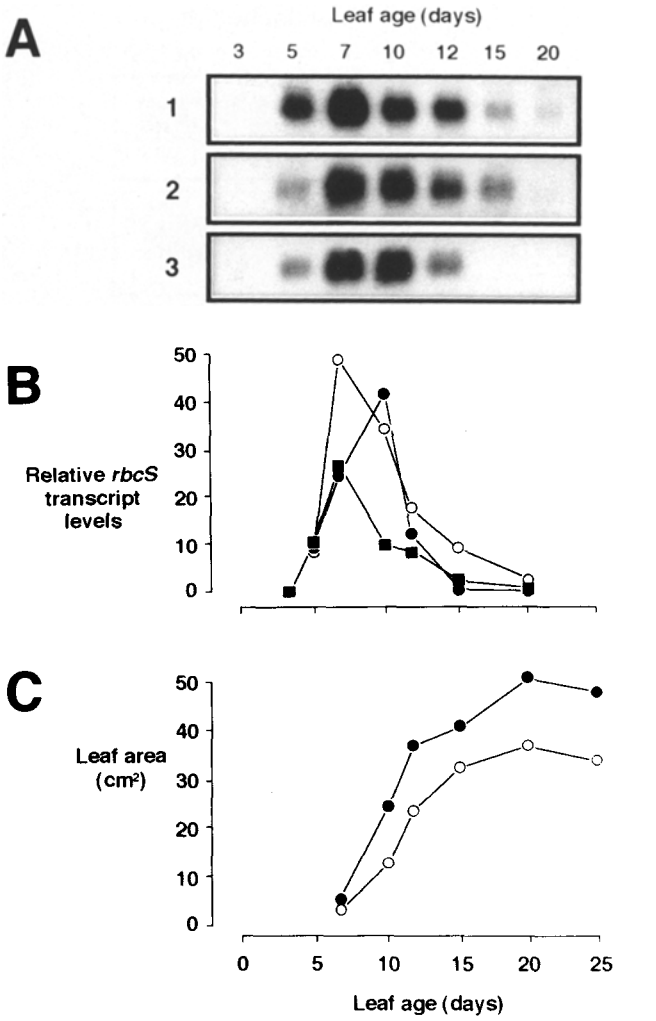

Fig. 1A–C. Ontogenetic regulation of *rbcS* transcript levels in *P. vulgaris* primary leaves. **A** Autoradiograph showing *rbcS* transcript levels in total RNA from primary leaves grown either in darkness (*panel 1*), or 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light (*panel 2*) or 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light (*panel 3*). Equal amounts of RNA were present in each lane (20 µg in *panel 1*; 7.5 µg in *2* and *3*). **B** Graphical representation of *rbcS* transcript levels from densitometric scans, compensated for RNA-loading differences, for plants grown in darkness (*closed squares*), or 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light (*open circles*), or 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light (*closed circles*). **C** Area of the primary leaves grown in 50 (*open circles*) and 100 (*closed circles*) $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light

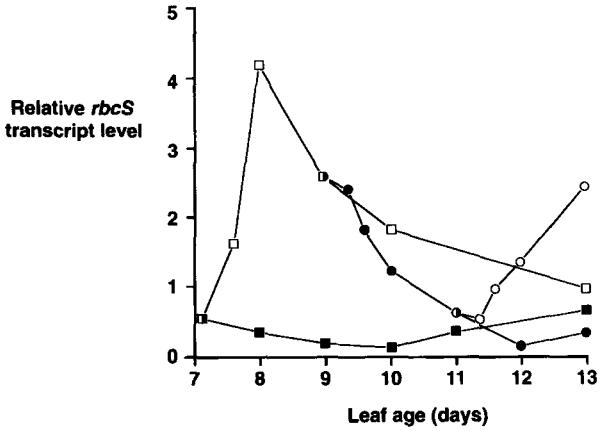

Fig. 2. Photoregulation of *rbcS* transcript levels during primary leaf ontogenesis in *P. vulgaris*. Plants were grown in darkness for 7 d. Some plants were left in the dark (*closed squares*) while others were transferred to continuous 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light (*open squares*). After 2 d some of the illuminated plants were returned to darkness (*closed circles*). After a further 2 d some of the dark-adapted plants were returned to the light (*open circles*). Northern blots were hybridised to the *rbcS* cDNA probe and the autoradiographs scanned to measure the relative *rbcS* transcript levels

detection. A shift in the ontogenetic peak of expression to slightly older leaves at higher fluence rates has been observed consistently in several experiments. It can be seen from Fig. 1C that the peak of *rbcS* expression occurs well before the completion of primary leaf expansion.

Photoregulation of rbcS transcript levels during primary leaf ontogenesis. We wished to investigate the light-regulation of *rbcS* expression in the context of the underlying ontogenetic regulation. Plants were grown from seed in darkness for 7 d and some were then transferred to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light. After 2 d some of these plants were returned to darkness and after 2 d in the dark, some of the plants were returned to white light. The levels of *rbcS* transcripts in the primary leaves were measured by hybridisation of the pPvSS191 cDNA to Northern blots of total RNA and the resulting autoradiographs were scanned with a densitometer (Fig. 2). Illumination of the 7-d-old dark-grown plants results in a sharp increase in the *rbcS* transcript level which peaks 24 h after the start of illumination. The level of *rbcS* transcripts reached is approximately eightfold higher than the 7-d dark level, whereas under continuous illumination at this fluence rate the maximum level is only approximately twofold greater (Fig. 1). In contrast, re-illumination of 11-d-old dark-adapted plants results in the level of *rbcS* transcripts increasing more slowly over 48 h and perhaps beyond. This relative level of transcripts is much greater than would normally be observed in a 13-d-old leaf. The decrease in *rbcS* transcripts in 9-d-old leaves returned to darkness is more rapid than in those left in the light but requires 48 h to reach the level in dark-grown plants.

Photoregulation of individual rbcS genes. Measurements of transcript levels of the individual *rbcS* genes were made by hybridisation of 21-mer oligonucleotides specific to the 3′non-translated sequences to Northern blots of total RNA. The specificity of these probes and their suitability for quantification of relative transcript levels is demonstrated in Fig. 3. Increasing amounts of plasmid DNA encoding the three different *rbcS* genes were slot-blotted onto four identical filters. Each filter was probed with either the non-discriminating, coding-sequence oligonucleotide, or one of the three gene-specific oligonucleotide probes. Figure 3 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 DNA. Nevertheless, measurements (not shown) of cpm hybridised versus amount of

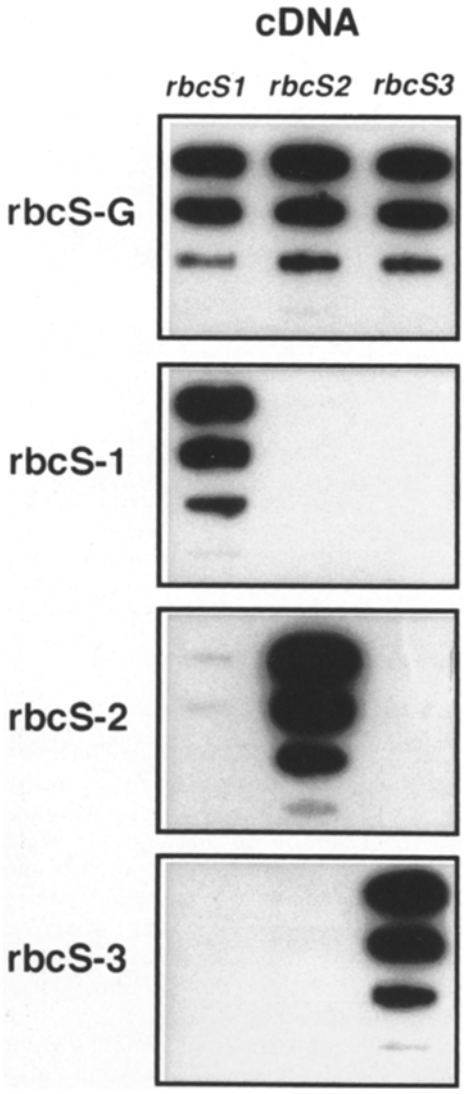

Fig. 3. Specificity of the gene-specific oligonucleotide probes. Plasmids encoding the three different *P. vulgaris rbcS* cDNAs were linearised and slot-blotted onto four replicate filters. Amounts of plasmid DNA on each filter were (from the top) 50, 25, 5 and 0.5 fmol. The filters were hybridised to equal cpm of either the coding-sequence oligonucleotide probe, rbcS-G, or one of the gene-specific oligonucleotide probes, rbcS-1, rbcS-2 and rbcS-3. The filters were washed and autoradiographed

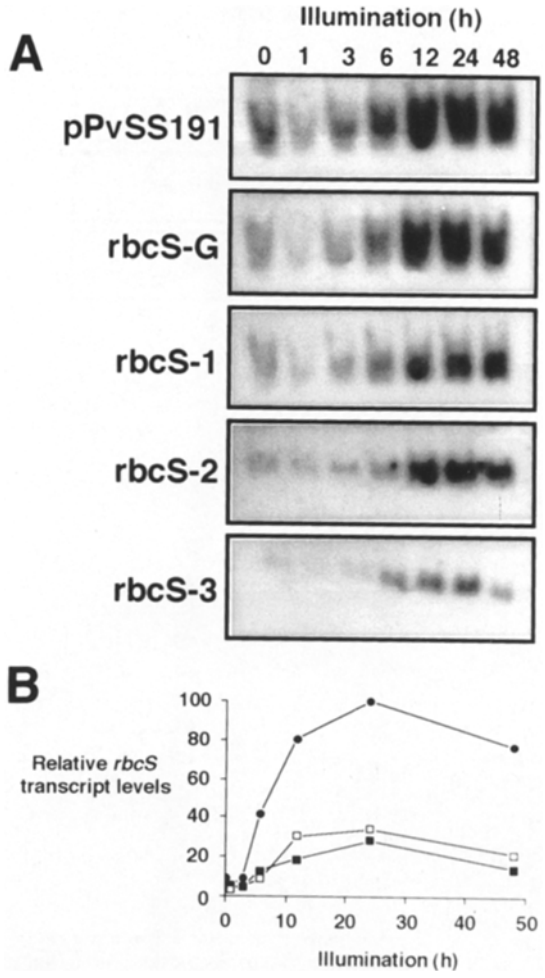

Fig. 4A, B. Expression of individual *rbcS* genes following illumination of dark-grown *P. vulgaris* plants. Plants grown in darkness for 7 d were placed in continuous 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light for up to 48 h. RNA was isolated from the primary leaves and replicate Northern blots were probed with either pPvSS191 cDNA, the non-descriminating oligonucleotide rbcS-G, or the gene-specific oligonucleotides rbcS-1, rbcS-2 or rbcS-3. **A** Autoradiographs of the filters. **B** Relative abundance of the transcripts of specific *rbcS* genes, based on densitometric scans and adjusted for the relative efficiency of hybridisation of the oligonucleotide probes. *Closed circles, rbcS1; open squares, rbcS2; closed squares, rbcS3*

target DNA with probes of equal specific activity under identical hybridisation conditions, show that the probes hybridise to their targets with different efficiencies. If the hybridisation efficiency of the rbcS-2 oligonucleotide is given a value of 100%, the mean relative hybridisation efficiencies of the rbcS-1 and rbcS-3 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. These different hybridisation efficiencies, calculated for each hybridisation, are taken into account in determining the relative transcript levels as indicated below.

The levels of transcripts of the three *rbcS* genes were measured following illumination of dark-grown plants. Plants were grown for 7 d in darkness prior to illumination with 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light. Three identical Northern blots were made for total RNA isolated from the primary leaves over a time course (Fig. 4A). One filter was probed with the pPvSS191 cDNA and re-probed with the non-discriminating oligonucleotide probe. As expected, these probes give very similar results. The *rbcS* transcripts are detectable in the dark-grown primary leaves and, after a lag of 1–3 h, increase with increasing time in the light for 24 h before starting to fall. These results are consistent with those presented in Fig. 2. This filter was then re-probed with the rbcS-1 oligonucleotide and the remaining two filters probed with the other two gene-specific probes. The blots were probed with equivalent amounts of labelled oligonucleotide. The results show that all three genes are expressed in the dark. Moreover, the transcripts of the

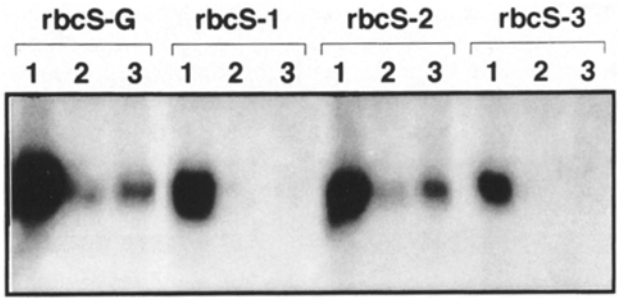

Fig. 5. Effect of prolonged darkness on transcript levels of the three *rbcS* genes. *Phaseolus vulgaris* plants were grown in darkness for 7 d and transferred to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light for 2 d. The plants were then returned to darkness for up to 4 d. Replicate Northern blots of total RNA were hybridised to the rbcS-G oligonucleotide or the gene-specific oligonucleotide probes rbcS-1, rbcS-2 and rbcS-3. *Lane 1*, 7 d dark plus 2 d light; *lane 2*, 2 d further dark treatment; *lane 3*, 4 d further dark treatment

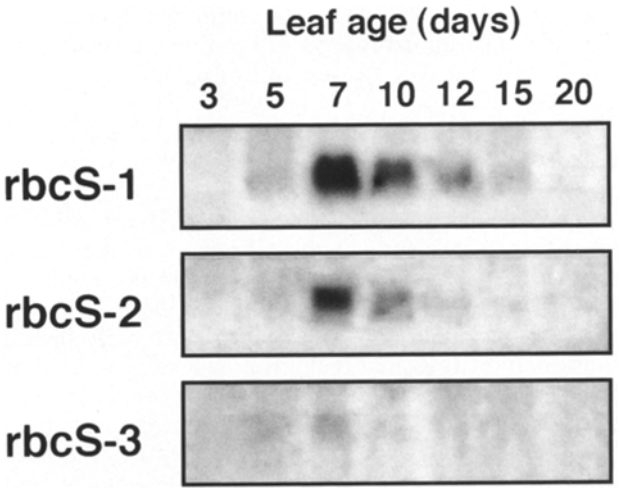

Fig. 6. Ontogenetic regulation of individual *rbcS* genes in light-grown *P. vulgaris* plants. Plants were grown in 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light and Northern blots of total RNA isolated from the primary leaves at the times indicated were hybridised to the gene-specific oligonucleotide probes rbcS-1, rbcS-2 and rbcS-3. The filters were washed and autoradiographed

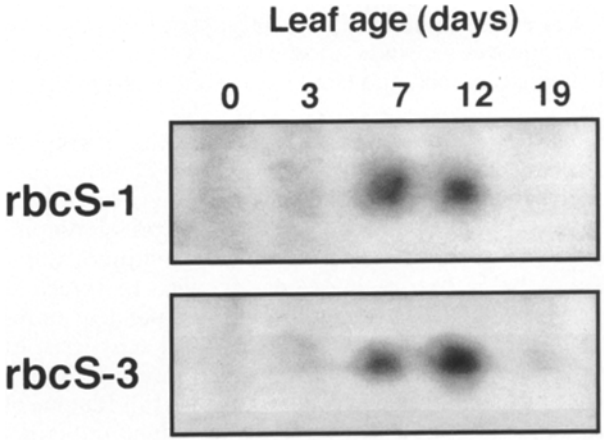

Fig. 7. Ontogenetic regulation of individual *rbcS* genes in dark-grown *P. vulgaris* plants. Plants were grown in darkness and Northern blots of total RNA isolated from the primary leaves at the times indicated were hybridised to the gene-specific oligonucleotide probes rbcS-1 and rbcS-3. The filters were washed and autoradiographed. The rbcS-3 autoradiograph is over-exposed relative to rbcS-1 to facilitate comparison of the expression profiles. The relative abundance of *rbcS1* and *rbcS3* transcripts in 7-d-old dark-grown leaves is shown in Fig. 4

genes increase in the light showing that all of them are photoregulated. The qualitative pattern of expression is very similar for all three genes with the main difference being in the amount of transcript accumulated at each time point. Densitometric scans of the autoradiographs were made and adjusted for the different relative hybridisation efficiencies of the three gene-specific oligonucleotide probes. These results (Fig. 4B) show that transcripts from *rbcS1* are the most abundant in both the dark-grown and illuminated plants, with those from *rbcS2* the next most abundant and *rbcS3* contributing the least to the *rbcS* transcript level. The contributions of the three genes' transcripts to total *rbcS* mRNA estimated after 24 h illumination in this experiment are: *rbcS1* 61%, *rbcS*2 21% and *rbcS3* 18%. Each of the genes has a substantial light-stimulation of its transcript level under these conditions, ranging from approximately 6-fold for *rbcS3* to 12-fold for *rbcS1*.

Further experiments were conducted to investigate whether the three *rbcS* genes showed similar regulation when illuminated plants were dark-adapted. Plants grown in darkness for 7 d and then illuminated for 2 d were returned to darkness for 2 or 4 d. Total RNA was hybridised to the gene-specific oligonucleotide probes as well as to the non-discriminating oligonucleotide probe. The results (Fig. 5) show that transcripts of all three genes fall rapidly on transfer to darkness, consistent with Fig. 2. However, transcripts of the *rbcS2* gene appear to account for the *rbcS* transcripts present in the dark-treated leaves and these transcripts increase slightly between 2 and 4 d in the dark.

Ontogenetic regulation of the individual rbcS genes. To investigate whether members of the *rbcS* gene family show differences in their ontogenetic regulation, gene-specific probes were hybridised to total RNA isolated from leaves of different ages grown in 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light. The ontogenetic pattern of expression observed in the light is very similar qualitatively for the three genes (Fig. 6) and corresponds to that observed with the cDNA probe (Fig. 1). The quantitative differences in expression are of the same order as seen in dark-grown and illuminated primary leaves (Fig. 4). We found it difficult to measure accurately the levels of transcripts of the individual genes in darkness even using a reverse transcriptase-polymerase chain reaction method (data not shown). However, reliable data were obtained for genes *rbcS1* and *rbcS3*, and these indicate that both show qualitatively similar ontogenetic regulation in darkness (Fig. 7), comparable to that shown in Fig. 1. The autoradiograph for *rbcS*3 in Fig. 7 has been over-exposed relative to *rbcS*1 so

that the expression profiles can be compared. The quantitative differences in expression of the three genes in 7-d-old dark-grown leaves are shown in Fig. 4.

Discussion

Ontogenetic regulation of rbcS transcripts. Expression of the *P. vulgaris rbcS* genes is subject to temporal regulation during primary-leaf ontogenesis. Transcripts are undetectable in the primary leaves in imbibed seeds but appear soon after germination. They reach a peak early in leaf expansion and subsequently fall to low levels by the time expansion is complete. Bate et al. (1991) reported that *rbcS* transcripts were at very low levels in senescent primary leaves. This pattern of regulation enables the plant to synthesise Rubisco very early in leaf development and hence contributes to the rapid production of fully functional chloroplasts in the light. A doubling of the fluence rate during leaf expansion appears to have little effect on the absolute level of *rbcS* transcripts in total RNA but extends the period over which transcripts accumulate, such that the peak in transcript level occurs later in ontogenesis. We reported previously that leaves which have completed the ontogenetic accumulation of *rbcS* transcripts are very sensitive to the fluence rate of light, and a large increase in *rbcS* transcripts is observed if primary leaves grown in a low fluence rate are transferred to a high fluence rate (Jenkins 1986; Sawbridge et al. 1994).

A feature of the ontogenetic control is that it is light-independent: essentially the same pattern of *rbcS* transcript accumulation is observed in dark-grown leaves. What light does is to amplify and extend expression upon this underlying pattern, although under continuous illumination the transcript level is only two- to threefold higher than in darkness. Similar light-independent ontogenetic regulation has been reported in other species. In cucumber (Greenland et al. 1987), *rbcS* transcripts appear soon after germination and follow a similar time course of increase and subsequent decrease in both light and darkness, with the light level approximately 2.5 times that in darkness. In the second leaves of maize, *rbcS* transcripts increase and subsequently decrease during leaf development in both light (Nelson et al. 1984; Loza-Tavera et al. 1990) and darkness (Nelson et al. 1984), although in the light the peak appears earlier than in darkness and the transcript level is approximately threefold higher (Nelson et al. 1984). The mechanisms responsible for light-independent temporal regulation are unknown. It is conceivable that levels of metabolites such as sucrose and/or plant growth regulators such as cytokinin modulate expression during early ontogenesis, but this is entirely speculative. Moreover, any hypothesis must explain both the initial increase and subsequent decrease in expression and how a similar pattern of regulation can occur in both light and darkness. Carbohydrate accumulation in leaves and cell cultures has been shown to depress *rbcS* transcript levels (Krapp et al. 1993) and sugars decrease *rbcS* promoter activity in protoplast transient expression assays in maize (Sheen 1990) and *P. vulgaris* (N.A.R. Urwin and G.I. Jenkins, unpublished data). However, the decrease in *rbcS* transcript levels during ontogenesis in the light is unlikely to be explained by carbohydrate repression because it coincides with the time when the leaf assumes the role of a major exporting organ rather than a 'sink'. Furthermore, the ontogenetic decrease in *rbcS* transcripts in darkness is unlikely to coincide with increased levels of carbohydrate in the leaves. It is also unclear whether changing carbohydrate levels could explain the initial ontogenetic increase in *rbcS* transcript accumulation. An alternative possibility is that changes in the levels of cytokinins, or responsiveness to cytokinins, could contribute to the ontogenetic regulation of *rbcS* expression. It is well known that exogenous cytokinins promote the accumulation of chloroplast components during leaf development, including in *P. vulgaris* (Naito et al. 1979), and Flores and Tobin (1986) have reported that exogenous cytokinin stimulates *rbcS* transcript accumulation in dark-grown *Lemna* fronds. Clearly the involvement of growth regulators and carbohydrates in regulating *rbcS* expression during leaf ontogenesis needs to be investigated.

Interaction of ontogenetic regulation and photoregulation. Although ontogenetic regulation is very important in establishing the level of *rbcS* transcripts, expression can be dramatically affected by sudden changes in the light environment. When dark-grown primary leaves of *P. vulgaris* are illuminated at the ontogenetic peak in expression, there is a sharp increase in the *rbcS* transcript level to a much greater extent, relative to the dark level, than normally occurs during development in continuous light. Similarly, when dark-adapted plants are re-illuminated there is a substantial, albeit slower, increase in the *rbcS* transcript level well above that normally seen at this stage in ontogenesis. Clearly light can over-ride the ontogenetic control. On a more subtle level, primary leaves increase or decrease their *rbcS* transcript levels when they are transferred to higher or lower fluence rates of light respectively (Jenkins 1986; Sawbridge et al. 1994). The plant has evolved mechanisms to optimise the levels of photosynthetic components in a given light environment via the regulation of gene expression. In the case of *rbcS*, phytochrome mediates the light-stimulation of expression when dark-grown seedlings are first illuminated (Tobin and Silverthorne 1985; Barnett et al. 1987) whereas a blue-light photoreceptor functions as the fluence-rate detector in mature leaves (Sawbridge et al. 1984). Krapp et al. (1993) have discussed the role of metabolic events associated with the carbohydrate status of the leaf in controlling *rbcS* transcript levels, the amount of Rubisco and hence photosynthetic capacity. This mechanism is likely to be important, in conjunction with light-regulation, in modulating *rbcS* expression throughout leaf development. A mechanism for sensing photosynthetic capacity may explain why the rate of increase in *rbcS* transcripts is slower upon re-illumination of dark-adapted plants (which will already contain some Rubisco) than it is when dark-grown plants are first illuminated. It is not the case that older leaves are less responsive to light. We have found that 25-d-old primary leaves of *P. vulgaris* grown in a low fluence rate of white light which have a very low *rbcS* transcript level can rapidly increase their transcript level when they are trans-

ferred to a higher fluence rate (data not shown). These observations further illustrate that plants have sensitive mechanisms for modulating the level of *rbcS* transcripts and hence Rubisco protein according to the prevailing light environment. Research is required to dissect the signal transduction pathways involved in metabolic regulation and light regulation and to define the molecular mechanisms through which they interact to regulate *rbcS* transcription.

Differential regulation of rbcS genes. We wished to investigate whether members of the *P. vulgaris rbcS* gene family were differentially regulated either quantitatively or qualitatively. The identity of the coding sequences suggests recent divergence and the possibility that promoter sequences may be relatively well conserved. It might therefore be easier to identify DNA sequence elements responsible for aspects of regulation than in some other species, although possible differences in the rates of evolution of *rbcS* coding sequences and promoter sequences have been noted (Dedonder et al. 1993).

It is well known that members of *rbcS* gene families in various species show large differences in their level of expression; for example, the *Petunia rbcS* genes differ 50- to 100-fold in the levels of their accumulated transcripts (Dean et al. 1985). We found that the *P. vulgaris rbcS* genes also show substantial quantitative differences in expression with *rbcS1* being most highly expressed and *rbcS3* least expressed. The relative transcript levels of the genes measured by hybridisation in several experiments correlate with the frequency of their cDNAs in a leaf cDNA library (Knight and Jenkins 1992). The three *rbcS* genes show very similar qualitative regulation both ontogenetically and in light-regulation. The only differences observed are in the extent of induction on illumination of dark-grown plants and the accumulation of the *rbcS2* transcripts in dark-adapted leaves. Much greater differences in regulation are seen in some other *rbcS* gene families. In *Arabidopsis*, the four *rbcS* genes show marked differences in their photoregulation under various conditions of illumination (Dedonder et al. 1993) and in tomato there are clear differences in the extent of light-responsiveness of the *rbcS* genes (Sugita and Gruissem 1987; Wanner and Gruissem 1991). More-subtle differences in photoregulation are seen among the pea *rbcS* genes (Fluhr et al. 1986). The degree of conservation of the coding sequence is not necessarily correlated with similarity in expression. In both tomato and *Arabidopsis*, some of the greatest differences in expression are between genes at the same locus with highly conserved coding sequences (Sugita and Gruissem 1987; Dedonder et al. 1993).

Analysis of the promoters of *rbcS* genes has revealed considerable complexity in the sequence elements concerned with photoregulation (Gilmartin et al. 1990), but relatively little information is available regarding *cis*-elements and transcription factors concerned with temporal control. In *rbcS* promoter analysis experiments, Kuhlemeier et al. (1988) and Ueda et al. (1989) noted differences in the relative levels of reporter-gene expression conferred by particular 5′ flanking sequences in immature and mature transgenic tobacco leaves. These observations suggest that particular promoter elements may be more important in regulating *rbcS* transcription at certain stages of leaf development than at others. However, little attention has been paid to this important aspect of regulation. Analysis of the promoters of the *P. vulgaris rbcS* genes may reveal whether specific sequence elements are involved in ontogenetic regulation and light-regulation.

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