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Upstream sequences determine the difference in transcript abundance of pea *rbcS* genes

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Summary. A system was developed for the quantitative analysis of transcript levels in transgenic plants. It makes use of a T-DNA vector containing the intact pea *rbcS-3A* gene as a reference gene. At 5 kb distance from the reference gene a pseudo wild-type *rbcS-3A* gene was inserted with a 3′ tail derived from the related *rbcS-E9* gene. Transcript levels varied between plants but the two genes were similarly affected in all cases. Analysis of chimeric genes between *rbcS-3A* and *rbcS-E9* demonstrated that sequences upstream of −50 are responsible for the differences in transcript abundance between the two genes. A comparison between the *rbcS-3A* and *3C* genes showed that unlike the situation in pea, in tobacco the *rbcS-3C* gene is two- to threefold more highly expressed than *rbcS-3A*.

Key words: Multigene family – Pea *rbcS* genes – Transcription – Enhancer-like elements – Transgenic tobacco

Introduction

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase is composed of eight large and eight small subunits. Whereas the large subunit is highly conserved between organisms, the small subunit shows considerable interspecies sequence divergence. In all higher plants examined to date the small subunit is encoded by small multigene families (*rbcS* genes). For two plants, pea and petunia, complete nucleotide sequences of the genes and their flanking regions have been determined for each individual member of the families (Coruzzi et al. 1984; Cashmore 1983; Timko et al. 1985a; Fluhr et al. 1986b; Dean et al. 1985, 1987a, b; Tumer et al. 1986). From these analyses it has become clear that there is only minor intraspecies sequence variation in the coding regions. In fact, in the pea cultivar Progress No. 9 the DNA sequence data predict identical amino acid sequences for the mature polypeptides of all five genes (Fluhr et al. 1986b).

The high level of intraspecies sequence conservation leads to the interesting question of why multiple *rbcS* genes exist. Both in pea and petunia the individual members of the *rbcs* gene families are expressed differentially. In pea, the *rbcS-3A, 3C,* and *8.0* genes are the most highly expressed accounting for approximately 40%, 35%, and 18% of total *rbcS* steady state mRNA in leaves; the low-expressed *3.6* and *E9* genes make up 7% of total *rbcS* mRNA. Moreover, the *rbcS-3.6* and *E9* mRNAs are underrepresented in seeds and petals (Fluhr et al. 1986b). In petunia there are similar quantitative differences in the expression of individual genes, but there is continuing controversy with regard to which genes are the most highly expressed ones (Dean et al. 1985, 1987a, b; Tumer et al. 1986).

The differential expression of the pea *rbcS* genes could be the result of differences in transcription initiation or posttranscriptional processes. For instance, in the human histone gene family, upstream sequences mediate S-phase specific enhancement of transcription (Heintz and Roeder 1984). In addition, sequences in the 5′ and 3′ non-translated mRNA play a role in mRNA stability (Morris et al. 1986; Lüscher et al. 1985; Capasso et al. 1987).

To study the DNA sequences that confer the regulated expression of the pea *rbcS* genes we have previously introduced individual genes into tobacco or petunia cells and raised transgenic plants. From these studies it became clear that enhancer-like elements in the upstream region of both the *rbcS-E9* and *rbcS-3A* genes can mediate light-regulated and organ-specific expression (Nagy et al. 1985; Fluhr et al. 1986a; Kuhlemeier et al. 1987a). However, it is not obvious that these upstream elements determine the quantitative differences between these two genes, especially because they are highly conserved between the two genes. Therefore, it would not be unlikely if sequences downstream of the transcription initiation site would be of influence.

In order to compare the expression of individual genes in transgenic plants in a quantitative manner, we constructed agrobacterial vectors which contain one of the *rbcS* genes as a reference gene and have a cloning site for the simultaneous introduction of a second *rbcS* gene. Here we show, using the reference system, that the 20- to 50-fold difference in steady state mRNA between the *rbcS-3A* and *E9* genes is caused primarily if not exclusively by sequences upstream of the TATA box. In addition, a careful comparison of the expression of the *rbcS-3A* and *3C* genes indicates that, unlike the situation in pea, in tobacco the *rbcS-3C* gene is two to three times more highly expressed than the *rbcS-3A* gene.

Materials and methods

DNA cloning. The *rbcS-3A* gene (Fluhr et al. 1986b) was cloned into the *Bgl*II and *Eco*RI sites of the pMON200

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polylinker (Fraley et al. 1985) as a *Xho*II – *Eco*RI fragment. Subsequently the 3′ terminal 600 bp were deleted by double digestion with *Sal*I and *Eco*RI, followed by filling in with Klenow DNA polymerase. The unique *Sst*II site between the nopaline synthase (*nos*) gene and the T-DNA right border was modified to give *Bgl*II and *Cla*I sites, the *Cla*I site being closer to the right border. The construction of the *rbcS-3A* pseudo wild-type gene has been described (Kuhlemeier et al. 1987a).

Gene transfer and analysis. Triparental mating, leaf disc transformation of *Nicotiana tabacum* and generation of transgenic plants were performed as previously described (Kuhlemeier et al. 1987a). An average of eight to ten independently derived transgenic plants was individually assayed for each construct.

Total RNA was prepared as follows: between 0.5 and 3 g tissue was ground to a fine powder with a pestle and mortar in the presence of liquid nitrogen. Eight milliliters of a solution containing 50 mM Tris-Cl, pH 8.0, 300 mM NaCl, 5 mM EDTA, 2% SDS, 2 mM aurin tricarboxylic acid (Sigma A-1895), 14 mM 2-mercaptoethanol was added to the powder and the mixture was homogenized. One milliliter of 3 M KCl was then added and the mixture left on ice for 15 min. The suspension was centrifuged for 5 min at 5,000 rpm, and the supernatant was transferred to a Corex tube through four layers of miracloth. To precipitate the RNA, 2.5 ml 8 M LiCl was added to the supernatant and the mixture was kept overnight at 4° C. RNA was pelleted by centrifugation at 8,000 rpm for 20 min and redissolved in 3 ml H_2O + 2 mM aurin tricarboxylic acid. This preparation was phenol extracted, ethanol precipitated, washed with 70% ethanol, dried in a vacuum desiccator, resuspended in 0.1–0.5 ml 10 mM Tris-Cl pH 8.0 1 mM EDTA and stored at −80° C. RNA prepared by this method is suitable for S1 nuclease protection and Northern analysis and is stable at −80° C for at least 1 year.

S1 protection analysis was carried out as described by Fluhr et al. (1986b).

Protein binding. Preparation of pea nuclear extracts and gel retardation analysis were done as described by Green et al. (1987).

Results

The reference system

To compare the transcript levels of individual pea *rbcS* genes in pea reliably, a quantitative S1 protection assay was devised (Fluhr et al. 1986b; Fig. 1A). Using a single probe, individual members of the *rbcS* gene family can be distinguished due to the partial homology between the transcripts at their 3′ ends. This method eliminates possible errors originating from differences in the efficiency of probe labeling or hybridization conditions. To reduce errors resulting from positional variation, the vector depicted in Fig. 1B was constructed. It is based on the disarmed split-end vector pMON200 (Fraley et al. 1985). The genomic fragment containing the *rbcS-3A* gene including 410 bp of 5′ non-coding sequence (reference gene) was cloned into the pMON200 polylinker. An *Sst*II restriction site, located between the *nos* gene and the right border of the T-DNA, was modified to give *Bgl*II and *Cla*I sites. All constructs (test genes) were cloned into this site, always with the

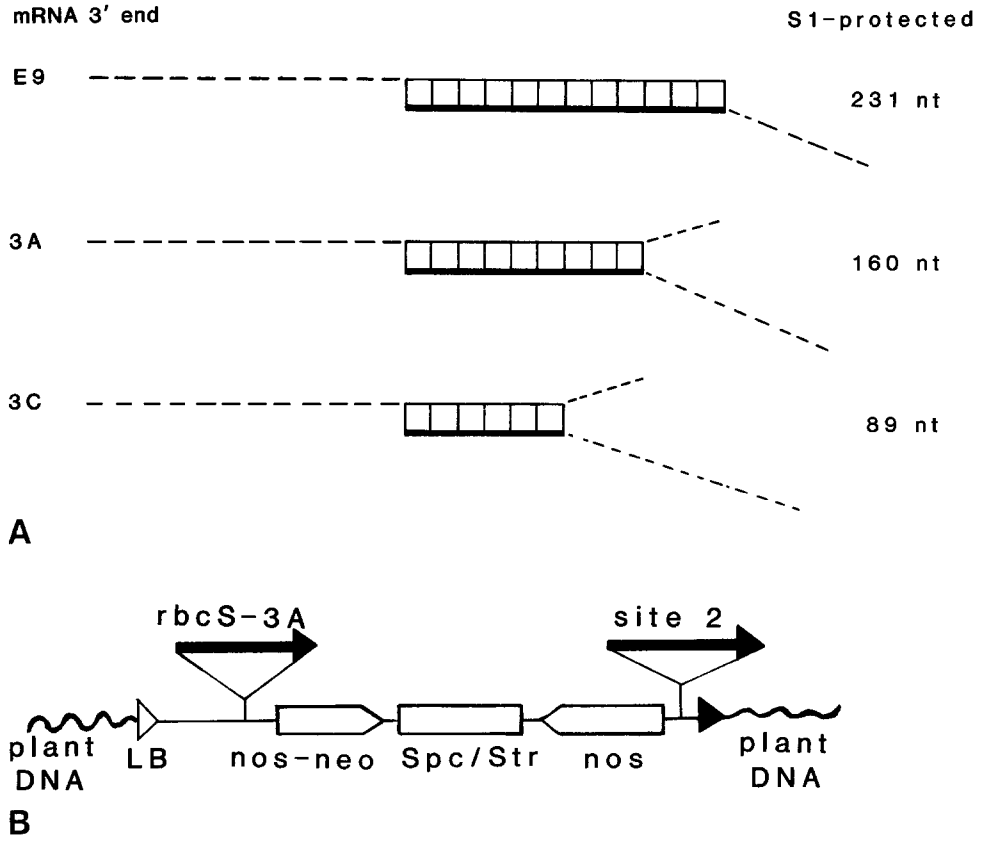

Fig. 1A, B. The reference/test gene system. **A** A 0.7 kb *Hin*dIII – *Cla*I fragment containing the 3′ end of the *rbcS-E9* gene and downstream non-transcribed DNA was end-labeled with Klenow DNA polymerase. The strands were separated and the coding strand was used as a probe in an S1 nuclease protection assay. This probe gives four major protected bands around 231 nucleotides with the *rbcS-E9* mRNA 3′ end, one band at 160 nucleotides with the *rbcS-3A* 3′ end and a set of bands around 89 nucleotides with the *rbcS-3C* 3′ end. The protected part of the probe is indicated by a *bold solid line*, the S1-sensitive parts of probe and mRNA are drawn as *dotted lines*. **B** Schematic representation of the transferred DNA after integration into the tobacco genome. The vector is derived from pMON200 (Fraley et al. 1985) and contains the intact *rbcS-3A* gene (reference gene) in the polylinker and the other *rbcS* gene (the test gene) in site 2, always in the orientation shown

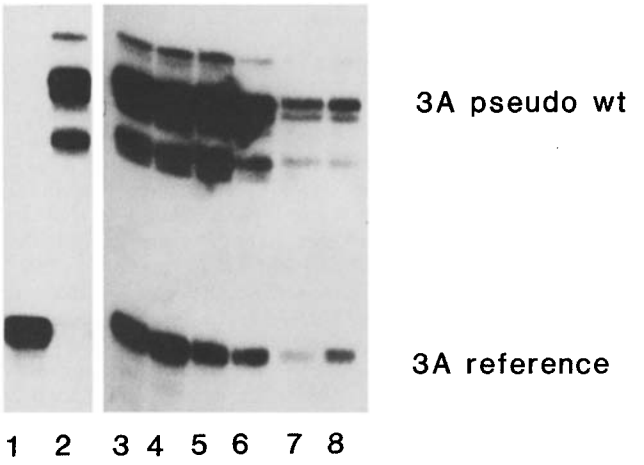

Fig. 2. Co-transfer of *rbcS-3A* wild-type and pseudo wild-type genes. S1 protection analysis of total RNA isolated from transgenic tobacco plants. Lane 1, *rbcS-3A* reference gene alone; lane 2, *rbcS-3A* pseudo wild-type gene alone; lanes 3–8, RNAs from six individual transgenic plants containing both *rbcS-3A* reference and pseudo wild-type genes. Twenty micrograms of total RNA was quantitatively analyzed with a single-stranded DNA probe derived from the 3′ end of the *rbcS-E9* gene

3′ ends, derived from the *rbcS-E9* gene, oriented towards the right border. Reference and test genes are separated by approximately 5 kb of DNA of non-plant origin. After transfer to tobacco the transcripts of reference and test genes can be resolved and quantitatively analyzed by S1 protection using a probe derived from the 3′ end of the *rbcS-E9* gene. This probe will protect 231 nucleotides of the *rbcS-E9* mRNA, 160 nucleotides of the *rbcS-3A* mRNA and 89 nucleotides of the *rbcS-3C* mRNA (Fluhr et al. 1986b; Fig. 1A).

To test the system a plasmid was constructed which contained in addition to the *rbcS-3A* reference gene, a *3A* pseudo wild-type gene in which the 3′ end was replaced by the 3′ end of the *rbcS-E9* gene. Control plants containing either the reference gene or the pseudo wild-type gene show exclusively the expected S1 protected bands of 160 and 231 nucleotides, respectively (Fig. 2, lanes 1 and 2). When both genes are present, the two signals can be observed (Fig. 2, lanes 3–8).

Fifteen transgenic plants were individually analyzed by this method and a representative sample of six plants is shown in Fig. 2, lanes 3–8. It can be seen that the absolute level of expression varies at least tenfold among individual plants (cf. lanes 3 and 8). However, in all cases examined, the ratio of the two signals varies only little. The most drastic independent variation, which is approximately twofold, can be observed between lanes 7 and 8. Thus, in this system positional variation can be controlled by calibrating the expression of the test gene with the expression of the reference gene. In addition, in all experiments eight to ten transgenic plants were individually analyzed.

The rbcS-3A *gene versus the* rbcS-E9 *gene*

Having ascertained the fidelity of the reference gene system we proceeded to localize the sequences that determine the differential expression between the *rbcS-3A* and *E9* genes. A possible determinant for high expression could be located in the 3′ non-coding region. An enhancer element downstream of the transcription termination signal has been observed in a chicken beta-globin gene (Choi and Engel 1986). Also, determinants for mRNA stability may reside in this region (Luscher et al. 1985; Capasso et al. 1987). However, when the *rbcS-3A* 3′ region was replaced by the corresponding segment from *rbcS-E9,* expression was not diminished (Fig. 2).

Another location for determinants of transcript abundance could be in the introns. Intron enhancers have been observed in several animal genes (Queen and Baltimore 1983; Picard and Schaffner 1984; Rossi and DeCrombrugghe 1987). The pea *rbcS* genes all have two introns at similar positions and there is considerable sequence homology among genes. Only the first intron of *rbcS-3A* is exceptional in that it is much larger and contains unique sequences (Fluhr et al. 1986b). To test whether the *rbcS-3A* introns could be involved in the high expression, the coding region between the *Sph*I and *Kpn*I sites from *rbcS-3A* was replaced by corresponding sequences from the pSS15 cDNA clone (Coruzzi et al. 1983, 1984; Fluhr et al. 1986b). In this region there are no nucleotide differences between pSS15 and the *rbcS-3A* or *rbcS-E9* exon sequences and therefore, the resulting construct is equivalent to an *rbcS-3A* gene with a precise deletion of both introns. As can be seen from Fig. 3, this construct is expressed at a level comparable to that of the intact *rbcS-3A* reference gene.

To determine which sequences are responsible for the different expression levels of *rbcS-3A* and *rbcS-E9,* a number of hybrid genes were constructed. Figure 4 (lanes 1 and 2) shows that the transcript level of the *rbcS-E9* gene is indeed considerably lower (at least 20-fold) than that of the *rbcS-3A* gene. Two chimeric genes were constructed using the conserved *Sph*I site at position +200 (Fig. 4, lanes 3, 4). The results show that the transcript level is determined by the 5′ part of the genes, that is, the *E9/3A* chimera is expressed as the *E9* gene, whereas the *3A/E9* construct is as highly expressed as the complete *rbcS-3A* gene.

Which sequences in the 5′ region are responsible for this expression? First, the effect of the TATA box region was assessed, as we (Morelli et al. 1985) and others (Timko et al. 1985b) have shown previously that the sequences in this region are involved in regulating the expression of the *rbcS* genes. Thus, the *rbcS-3A* sequence between −50 and +33, which contains the TATA box and transcriptional and translational initiation signals, was replaced by the corresponding sequence of *rbcS-E9*. Figure 4, lane 5 shows that this replacement does not alter the expression of the *rbcS-3A* gene.

Since neither the TATA box region nor downstream sequences appeared to determine transcript abundance, a chimeric gene was constructed, which contained the *rbcS-E9* sequence downstream of −50 fused to the *rbcS-3A* upstream region from −330 to −50. This construct is as highly expressed as the *rbcS-3A* reference gene (Fig. 4, lane 6). These results clearly show that sequences upstream of −50 determine the differences in transcript levels between *rbcS-E9* and *rbcS-3A*.

GT-1 binding sites in the rbcS-3A *and* E9 *upstream regions*

In vitro experiments have previously shown that there are at least four sequences in the *rbcS-3A* upstream region

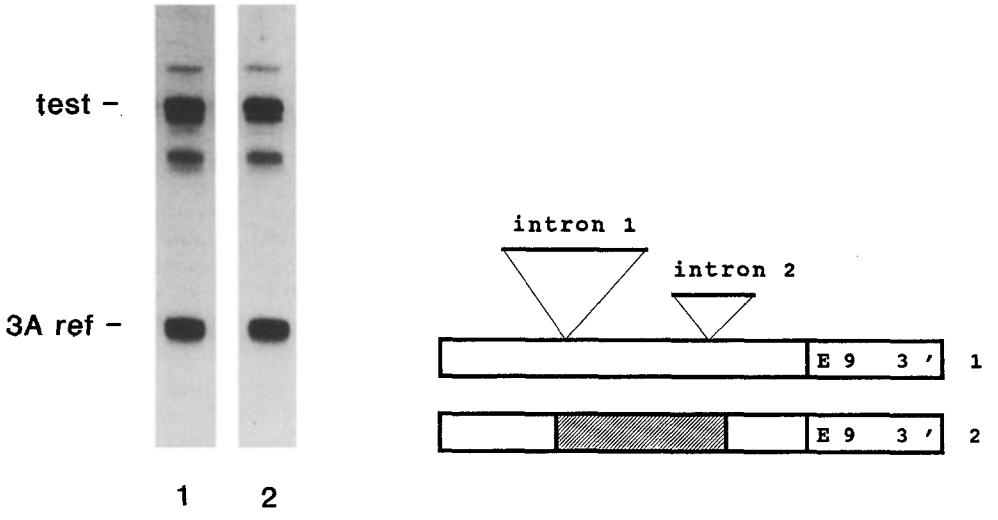

Fig. 3. The *rbcS-3A* introns are dispensable for high expression. Constructs were cloned into the *rbcS-3A* reference vector of Fig. 1. Lane 1, pseudo wild-type *rbcS-3A* gene. Lane 2, as lane 1, but with the *Sph*I – *Kpn*I fragment (+200 to +975 in the *rbcS-3A* sequence, *hatched* in the figure) replaced by the corresponding fragment from the cDNA pSS15 (Coruzzi et al. 1983). Ten micrograms of total RNA were analyzed by 3′ S1 protection

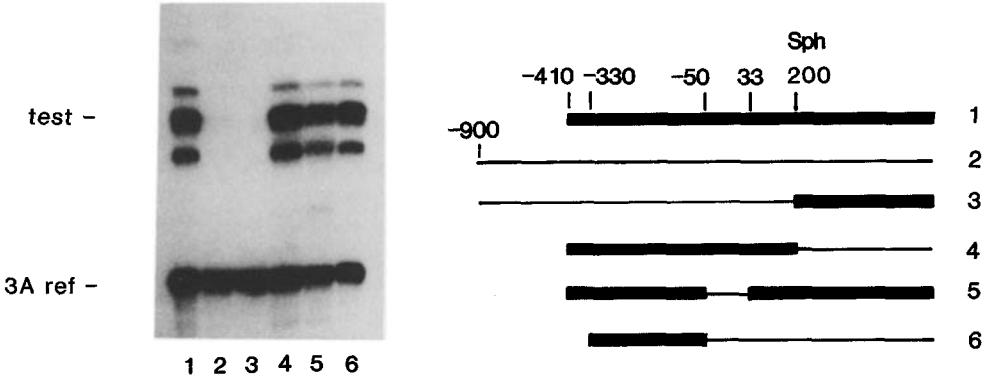

Fig. 4. S1 protection analysis of *rbcS-3A/E9* hybrid genes. Transgenic tobacco plants were generated using the vector depicted in Fig. 1 with the following constructs in site 2. Lane 1, *rbcS-3A* pseudo wild type, 10 μg RNA; lane 2, *rbcS-E9*, 30 μg RNA; lane 3, the *rbcS-E9/3A* hybrid fused at *Sph*I site, 30 μg RNA; lane 4, *rbcS-3A/E9* hybrid fused at *Sph*I site, 30 μg; lane 5, *rbcS-3A* gene with −50 to +33 region (*Bst*XI to *Eco*RV) substituted by corresponding fragment from *rbcS-E9*, 20 μg RNA; lane 6, *rbcS-3A* gene from −330 to −50 fused at *BstXI* site to *rbcS-E9* gene from −50 to 3′ end, 5 μg RNA. *Bold lines*, *rbcS-3A* sequences; *thin lines*, *rbcS-E9* sequences. All constructs have *rbcS-E9* 3′ ends

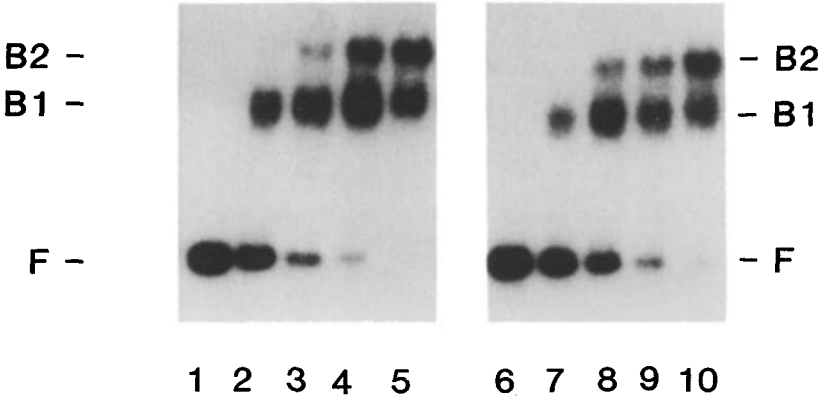

Fig. 5. Binding of nuclear protein GT-1 to the *rbcS-3A* and *E9* upstream regions. End-labeled *rbcS-3A* fragment (from −330 to −2) and *rbcS-E9* fragment (from −359 to −2) were incubated with increasing amounts of pea nuclear protein extract. Free DNA (F) and DNA-protein complexes (B1 and B2) were separated on a 1% agarose gel in T/E buffer. Lanes 1 and 6, no extract; lanes 2 and 7, 1 μg; lanes 3 and 8, 2 μg; lanes 4 and 9, 3 μg; lanes 5 and 10, 5 μg nuclear protein. Lanes 1–5, *rbcS-3A*; lanes 6–10, *rbcS-E9*

which serve as binding sites for the nuclear protein GT-1 (Green et al. 1987). Based on correlations between in vivo and in vitro data, we have speculated that GT-1 could be a positively acting transcriptional factor (C. Kuhlemeier et al., submitted). Conceivably, the difference in strength between the *rbcS-3A* and *E9* upstream regions could be caused by different affinities for GT-1. With this in mind, we carried out protein binding experiments using a pea nuclear extract and comparable fragments from the two genes. For *rbcS-3A* the −330 to −2 upstream element was chosen and for *rbcS-E9* the fragment between −359 and −2. A −359 deletion of *rbcS-E9* was shown in transgenic plants to direct transcript levels similar to those of the intact *rbcS-E9* gene (Nagy et al. 1985). The gel retardation experiments (Fig. 5) clearly show that GT-1 binds to the two fragments with similar affinities.

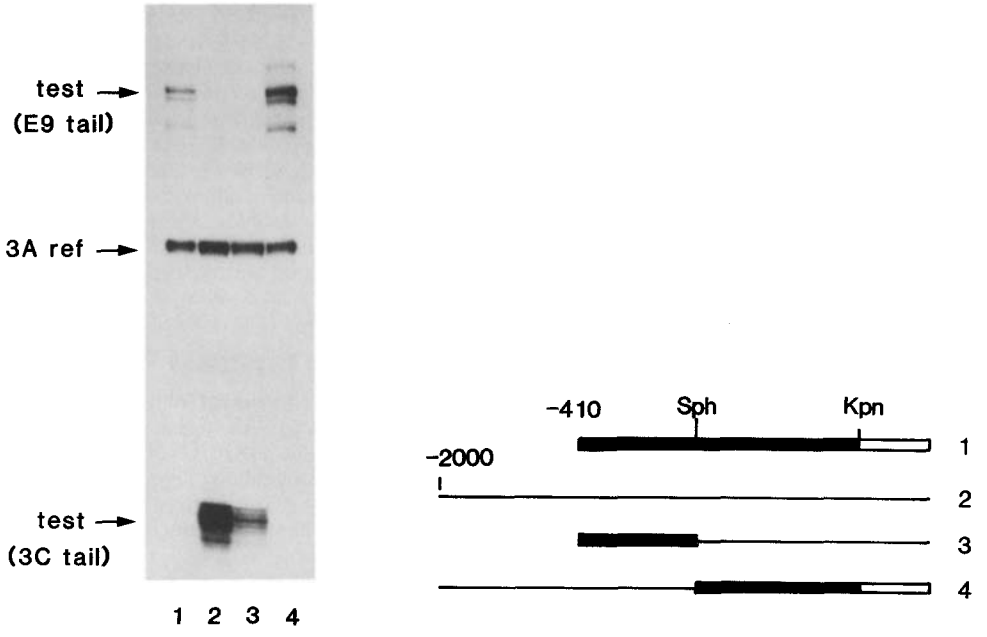

Fig. 6. Analysis of the transcript levels of *rbcS-3A/3C* hybrid genes by S1 protection. Transgenic plants were generated using the vector shown in Fig. 1, with the constructs described below in site 2. The amount of total RNA used per reaction was adjusted to give approximately equal intensities of the reference signals. Lane 1, *rbcS-3A* pseudo wild type, 10 μg RNA; lane 2, *rbcS-3C*, 20 μg RNA; lane 3, *rbcS-3A/3C* hybrid, fused at the conserved *Sph*I site, 30 μg RNA; lane 4, *rbcS-3C/3A* hybrid fused at the *Sph*I site, 20 μg RNA. *Thin lines*, *rbcS-3C* sequences; *filled bars*, *rbcS-3A* sequences, *open bars*, *rbcS-E9* 3′ ends

The rbcS-3A *gene versus the* rbcS-3C *gene*

In pea, the *rbcS-3A* and *3C* genes account for approximately 40% and 35% of total *rbcS* transcripts (Fluhr et al. 1986b). Therefore, we had expected that in transgenic tobacco plants also, these two genes would be expressed at about equal levels. To test this, the *rbcS-3C* gene was cloned into the test gene position (site 2 in Fig. 1 B) and the construct was transferred into tobacco plants. S1 analysis demonstrates that somewhat surprisingly, in transgenic tobacco the *rbcS-3C* transcript is two to three times more abundant than the *rbcS-3A* transcripts (Fig. 6, lanes 1 and 2). Chimeric genes were constructed between the *rbcS-3A* and *3C* genes and compared, as before, with the intact *rbcS-3A* reference gene. Figure 6 (lanes 3 and 4) shows that the higher expression of the *3C* gene appears to be determined by the 5′ part of the gene. We are presently investigating whether sequences upstream of −410 are capable of increasing *rbcS-3A* expression. However, we note that the differences in transcript levels observed for the chimeric genes are close to the accuracy limits of the assay. Therefore, we cannot exclude other regions of the *rbcS-3C* gene as important for the difference in transcript levels.

Discussion

The reference system

The five *rbcS* genes in pea are very similar both in their coding regions and in the flanking sequences. However, they display considerable differences in their modes of expression (Fluhr et al. 1986b). Upstream sequences are important for the regulated expression of these genes and such elements have been studied in considerable detail (see Kuhlemeier et al. 1987b, and references therein). So far, however, it has not been determined what causes the differences in transcript abundance between the genes. For such a study a gene transfer system is required that expresses the transferred gene in a quantitatively correct manner.

Whereas transgenes are generally expressed correctly, there is considerable variation in transcript abundance between individual transgenic plants. These variations can be due to the particular integration site in the host genome ('position effect'). Alternatively, the difference can be influenced by other parameters, such as: (1) the plants may not have been harvested under identical conditions; (2) mutations elsewhere in the genome may be induced by the tissue culture procedures (somaclonal variation); (3) the transgenic plant may in fact be a chimera in which only some of the cells contain the transgene; (4) small mutations in the transgene may escape detection by Southern analysis. A rigorous test for true positional variation is that an aberrantly expressing transgene must give rise to the normal scale of expression levels, when reisolated and reintroduced into other plants (Levis et al. 1985). Such an analysis has not yet been performed in plants.

The vector system used in this study allows for the co-transfer of two genes separated by 5 kb of intervening DNA. This approach strongly reduces possible position effects. In addition, since the two genes are analyzed in the same plant, they will be equally affected by somaclonal variation or chimerism. In this study, eight to ten plants were individually analyzed for each construct. We reason that if all these plants give similar results, the possibility of an undetected mutation is unlikely.

Although this system works quite well for the *rbcS* genes, we do not claim that our reference gene system is generally applicable. Especially weakly expressed genes may be much more sensitive to short-range position effects, that is, one of the genes may be more influenced than the other by an enhancer residing in the flanking host DNA. Independent variation between closely linked genes has also been observed for *nos-nptII* and *rbcS-E9* (Nagy et al. 1985) and *OCS* and *Cab* (Jones et al. 1985), and divergently transcribed *nptII* and T-DNA transcript *7* genes (Kay et al. 1987). An important difference between these systems and the reference gene system described here, is that in our case the reference gene and test gene have identical coding regions and very similar flanking sequences.

Upstream sequences determine high expression of rbcS-3A

The difference in transcript abundance of *rbcS-3A* and *E9* could be the result of sequence differences in any part of the genes. In pea, the most highly expressed gene, *rbcS-3A*, has an unusually large first intron (Fluhr et al. 1986b). Also in petunia, the highly expressed *SSU311* gene has an additional intron (Dean et al. 1985, 1987a, b). Previous work had located light regulatory elements in the vicinity of the TATA box (Morelli et al. 1985; Timko et al. 1985b) and further upstream (Timko et al. 1985b; Fluhr et al. 1986a; Kuhlemeier et al. 1987a).

The high expression of the *rbcS-3A* gene is mediated by sequences between −330 and −50. We cannot rigorously exclude the existence of determinants downstream of −50, but these must be of minor importance compared with the powerful *rbcS-3A* upstream enhancer. In general, upstream elements are thought to act by increasing the rate of transcription (Treisman and Maniatis 1986; Weber and Schaffner 1986). One construct (Fig. 4, lane 6) consists of *rbcS-E9* sequences from −50 down and produces a transcript that is identical to the *rbcS-E9* mRNA. However, the steady state mRNA is similar to *rbcS-3A*. This makes it very unlikely that differential processing or transcript stability play a role, but instead indicates that the high expression of *rbcS-3A* is due to a higher rate of transcription initiation. A sequence comparison between the 5′ non-transcribed regions of *rbcS-3A* and *E9* (Fluhr et al. 1986b) has revealed that in the first 200 bp there is over 90% sequence homology, but that upstream of −200 the two sequences are completely dissimilar. We have recently shown that a 5′ deletion of the *rbcS-3A* gene to −166 is still fully active when transcript levels are measured in transgenic tobacco plants (Kuhlemeier et al. 1987a). This could mean that despite the high level of sequence homology, the region from −166 to −50 is functionally dissimilar in the two genes. Alternatively, the *rbcS-E9* gene may contain silencing elements, upstream of −170, which are not present in *rbcS-3A*. Since GT-1, which we speculated to be more likely a positive transcription factor (C. Kuhlemeier et al., submitted), binds either upstream region with equal affinity (Fig. 5), we tend to favor the latter hypothesis. Detailed studies of the *rbcS-E9* upstream region are required to localize such negative *cis*-acting elements.

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