Confirmation of the relative expression levels of the *Petunia* (Mitchell) rbcS genes

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ABSTRACT

We have mapped the transcription start sites of three petunia rbcS genes and reassayed the relative expression levels of the petunia rbcS genes using the technique of primer extension. This analysis was performed specifically to address the confusion in the literature concerning the relative expression levels of the two petunia rbcS genes, SSU301 and SSU11A. The primer extension analysis reported here confirms our previous results, specifically that the rbcS gene, which we term SSU301, gives significantly higher levels of steady state RNA than any of the other rbcS genes in leaf tissue from 10 week old petunia plants.

INTRODUCTION.

The enzyme ribulose bisphosphate carboxylase (RuBPCase) is composed of large and small subunits, eight of each assembling to give one molecule of RuBPCase. The large subunit (rbcL) is encoded on chloroplast DNA and is synthesized in the chloroplast (1). The small subunit (rbcS) is encoded by multiple nuclear genes in all the species which have been examined (2-6) and it is synthesized as a higher molecular weight precursor in the cytoplasm (7).

We have isolated and characterized seven of the eight rbcS genes from Petunia (Mitchell) genomic DNA. The eight genes can be divided into three subfamilies based on nucleotide sequence homology (2). These three subfamilies, previously called 71, 117 and 51, are referred to here for simplicity as A, B and C respectively. Subfamily A contains the SSU301 gene, subfamily B also contains one gene, SSU611 and subfamily C contains six genes: SSU491; SSU112; SSU911; SSU211; SSU231 and SSU511.

We have recently reported on the relative expression levels in leaf tissue for the eight petunia rbcS genes (8). The steady

state RNA levels of five of the genes were assayed directly using probes derived from the 3' untranslated regions of the rbcS genes (3' tail probes). Three of the probes were specific to the corresponding transcript and the fourth hybridized to two rbcS transcripts. Large differences in the steady state RNA levels of the different rbcS genes were observed when the 3' tail probes were labelled to the same specific activity and hybridized to Northern blots and slot blots of total RNA from petunia leaf tissue. It was not possible to construct 3' gene specific probes for every rbcS gene due to the high degree of sequence homology between six of the eight genes (C subfamily (2,9)). In order to establish the relative expression levels for all eight genes, in addition to the hybridization data, we measured the frequency of cDNA clones derived from each of the rbcS genes in a λ gt10 library constructed from petunia leaf poly A mRNA. Inherent in this method for measuring relative expression levels is the assumption that there is not differential cloning of the different mRNAs. In order to minimize the inaccuracies due to this assumption, we used this approach to estimate the relative expression of the rbcS genes only within the C subfamily. There is a very high level of sequence homology between the genes of this subfamily so differential cloning of the different mRNA's is unlikely to occur. Indeed when one compares the relative expression of the rbcS genes measured using either 3' tail probe hybridization to total RNA or by frequency of cloning in a cDNA library, the numbers correspond very well for the genes of the C subfamily. The relative expression levels that were assigned to each gene using the two methods described above are: SSU301 47%; SSU611 23.2%; SSU491 7%; SSU112 5.4%; SSU911 ,1%; SSU211 1.9%; SSU231 and SSU511 15.2% (8). The SSU511 gene is identical to SSU231 over the region of sequence which we have used to compare and identify the rbcS genes so individual expression level values could not be assigned to these two genes.

Tumer et al.(10) have recently reported on the relative expression levels of two of the petunia rbcS genes, SSU8 which corresponds to SSU301 in our terminology (from hereon referred to as SSU301), and SSU11A which corresponds to our SSU511 (from hereon referred to as SSU11A). They estimated the relative

expression levels of these two genes in leaf tissue using 5' and 3' specific oligonucleotide probes in hybridizations to Northern blots of total and poly A RNA. In contrast to our previous results they found that the SSU301 gene accounts for only 5% of the total rbcS expression and SSU11A accounts for 40%. They explained the discrepancy in the two sets of data by concluding that the measurement of the relative abundance of rbcS cDNA clones in a cDNA library was an inaccurate method to measure the relative expression levels of different rbcS genes.

We report in this paper additional data quantitating the expression levels of the rbcS genes, using the technique of primer extension (11). These data confirm our previous measurements on the expression levels of these genes and demonstrate again that the rbcS gene which we term SSU301, gives higher levels of steady state RNA in petunia leaf tissue than any of the other rbcS genes. Several explanations for the discrepancy between our data and the data of Tumer et al.(10) are discussed.

METHODS

Plant material

The *Petunia* (Mitchell) strain is a doubled haploid produced by anther culture from a hybrid between *Petunia hybrida* var Rose of Heaven and *Petunia axillaris* (12). The plants were grown under greenhouse conditions. RNA was extracted from the young leaves of 10 week old plants.

RNA isolation

Total RNA was isolated from the petunia leaf tissue as described in (8). The RNA was examined on a formaldehyde-agarose gel (13) to check that no RNA degradation had occurred during isolation.

Primer extension analysis

The three oligonucleotides 301T, 611T and 911T used in the primer extension analysis (11, adapted for use with plant RNA by D.Gidoni, in prep.) were synthesized on an Applied Biosystems DNA synthesizer. The oligonucleotides were purified on a 20% polyacrylamide/8M urea sequencing gel, eluted from the polyacrylamide and further purified on a Sep-pak (Millipore) C18

reverse phase column. The extinction coefficients of each oligonucleotide were determined by the addition of the extinction coefficient values of each of the individual nucleotides (E_{260nm} pH7 x 10^3: G 11.7; A 15.4; T 7.4; C 7.5). The oligonucleotides were radiolabelled with γ^{32}P-ATP (5000 Ci/mmol, Amersham) using T_4 polynucleotide kinase, at ATP concentrations which gave rapid and complete labelling of the oligonucleotide. This ensured that the primers were of equal specific activity. Equal pmoles of the three different oligonucleotides labelled to approximately (within 10%) the same extent showing that contaminants from the purification procedure were not significantly affecting the radiolabelling. The labelled oligonucleotide was separated from the free ATP by one ethanol precipitation in 2.25M ammonium acetate and a second ethanol precipitation in 0.3M Na acetate. The effectiveness of this separation was checked by chromatography on polyethyleneimine strips. 0.2 pmol of kinased oligonucleotide were annealed in 250mM KCl, 2mM Tris-HCl pH 7.9, 1mM vanadyl ribonucleoside complex with 10ug total petunia RNA. The annealing reaction was carried out in a volume of 10µl for 3h and the samples were centrifuged briefly in a microfuge every hour to reduce condensation problems. Each oligonucleotide was annealed to the total RNA at five different temperatures: 37°C; 43°C; 48°C; 55°C; 60°C to ensure the optimal annealing temperature for each oligonucleotide was reached. After the annealing, 23.5µl of 10mM $MgCl_2$, 5mM DTT, 20mM Tris-HCl pH8.3, 0,33mM of each dNTP, 100µg/ml actinomycin D, 1mM vanadyl ribonucleoside complex and 10 units of AMV reverse transcriptase (Life Sciences) were added and the mixture was incubated at 37C for 45min. The nucleic acids were precipitated by the addition of ethanol, the pellets were washed with 70% ethanol, dried and resuspended in 3µl 0.1M NaOH, 1mM EDTA. After 15min at 25C, 6µl of 98% formamide (deionized), 1mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue were added and the samples heated at 90°C for 3min. Approximately 25% of the sample was loaded and electrophoresed on an 8% polyacrylamide/8M urea sequencing gel which was dried and autoradiographed.
The X-ray films were scanned using an LKB 2202 scanning

densitometer. A serial dilution of one sample was also loaded onto a gel and scanned for use as a standard curve.

RESULTS

Due to the recent conflicting data of Tumer et al.(10) we have re-assayed the relative expression levels of the petunia rbcS genes in leaf tissue using the technique of primer extension. There are a number of advantages to using this technique for assaying the steady state level of individual genes: firstly, the oligonucleotides used in the analysis give two levels of specificity for each gene, one by virtue of their specific sequence and the other as a function of the length of the primer extended fragment that is generated. Secondly, the hybridization reaction takes place in solution and a large excess of oligonucleotide can be used to drive the annealing reaction to completion ensuring that all of the RNA molecules corresponding to an individual gene serve as templates for the primer extension reaction. Since the hydridization reaction is driven to completion, differences in the hybridization efficiency of different probes is not a complication in this analysis. Differential hybridization efficiencies of different probes is an issue which must be carefully considered when relative gene expression is assayed by hybridizing different probes to Northern blots.

We used a primer extension analysis to establish the transcription start sites for three of the petunia rbcS genes: SSU301; SSU611 and SSU911 (Fig.1B). The transcription start site determined by Tumer et al.(10) for the gene SSU11A is also indicated in Fig.1A. We assume the multiple primer extended fragments which we see for each gene (Fig.1B) reflect multiple transcription start sites rather than incomplete extension products since they are reproducibly observed with different RNA preparations. Knowledge of the transcription start sites for each of these genes allows the relative expression levels of SSU301, SSU611, SSU911 and SSU11A to be assayed by primer extension.

The oligonucleotides which we used in the primer extension reactions were designed to be specific to gene transcripts from

A

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SSU301  TATATAAAGGGTGTGGTGGGCAATACAAAAGtcAgTGTGAAGTGTTTAAAGGAAAAAGCTTTGGAAGA
SSU611  TATATATAGGACAGGAGTCATAGTGCAATGACCATcaTaAAGCA.......................
SSU491  TATAAATAGAAGTGGGACTAGGAAGCAAGGACCATTGACCCTCTTGAGAACAAAGCTCAAGGGAACCA
SSU911  TATAAATAGATATTGCAAATGTCAAGGGAAgcAATAGCAATTATATTTAGCA................
SSU112  TATAAATAGAGGGGGAAGACTACGTACCGCAATAACTTTTCTAAGGATATTTCAGCA...........
SSU231  TATAAATAGATATTGCAAAGTTCAAAGGAAGCAATAGCAATTATTTTTAGCA................
SSU11A  TATATATAGAAGGGGGCACTATACATCaatAACCCTCTTGAAGCAAAGGTGGGAAAGGGAAACACAAA

SSU301  AGCAAAAATCTTCTAACT.......ATGGCTTCCTCTGTGATTTCCTCT...GCAGCTGTTGCTACTC
SSU611  ........................ATGGCTTCCTCAGTTATCTCCTCG...GCAGCCGTTGCTACTA
SSU491  AGGATTTATTTTCAGAA........ATGGCTTCCTCAGTGATGTCCTCA...GCAGCAGTTGCCACAA
SSU911  ........................ATGGCTTCTTCAGTGATGTCCTCA...GCTGCAGTTGCCACAA
SSU112  ........................ATGGCTTCCTCAGTGATGTCCTCATCAGCAGCAGTTGCCACAA
SSU231  ........................ATGGCTTCCTCAGTGATGTCCTCA...GCTGCAGTTGCCACAA
SSU11A  AATATAAGCTAACGATTCTTTAGCAATGGCTTCCTCAGTGATGTCCTCA...GCTGCAGTTGCCACAA

SSU301  GCACTAATGTGGCTCAAGCTAGCATGGTTGCACCTTTTAATGGTCTTAAGTCTGCTGTCTCCTTCC
SSU611  GCAGCAATGCTGTTCAAGCTAGCATGGTTGCACCTTTCACTGGCCTAAAATCTGCCTCAGCTTTCC
SSU491  ACACCAATGCTGCTCAAGCTAGCATGGTTGCACCCTTCACTGGCCTCAAGTCTGCAGCCTCCTT
SSU911  GCACCAATGCTGCACAAGCCAGCATGGTTGCACCCTTCACTGGCCTCAAGTCTGCAGCCTCCTT
SSU112  GCACCAATGCTGCCCAAGCCAGCATGGTTGCACCCTTCACTGGCCTCAAGTCCGCAGCCTCCTT
SSU231  GCACCAATGCTGCTCAAGCCAGCATGGTTGCACCCTTCACTGGCCTCAAGTCTGCAGCCTCCTT
SSU11A  GCACCAATGCTGCTCAAGCCAGCATGGTTGCACCCTTCACTGGCCTCAAGTCTGCAGCCTCCTT
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B

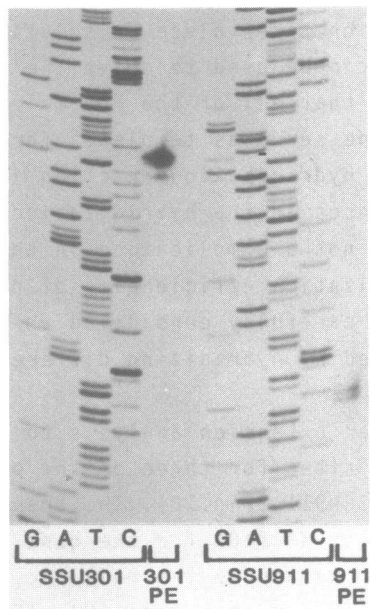


Figure 1. A. Nucleotide sequence of the 5' region of 7 petunia rbcS genes, showing the region of the gene complementary to the oligonucleotides which were used in the primer extension analysis.
Sequences from the TATA box (underlined in the figure) to the region of the gene complementary to the oligonucleotides (also underlined) are shown. The sequence has been arranged so that regions of homology are aligned (the dots reflect gaps created during the aligning procedure). The transcription start sites are indicated by lower case letters. The transcription start site for SSU11A is from Tumer et al.(10).

B. Mapping of the transcription start sites of the genes SSU301 and SSU911. The primer extended fragments of SSU301 and SSU911 are shown together with dideoxy sequencing ladders generated from the genes with the same oligonucleotides as used to give the primer extended fragments. The primer extended fragment shown for SSU911 was generated from a tobacco plant transformed with the petunia SSU911 gene (Dean et al. in prep.). This way cross hybridization of the oligonucleotide with the other members of the C subfamily could be avoided. The same transcription start sites are used in petunia (Fig.2).

each of the three rbcS subfamilies. This was achieved by making the oligonucleotides complementary to a region within the transit peptide coding region which is divergent between the subfamilies (Fig.1A). We also selected the three oligonucleotides so that the 3' terminal nucleotide was different in each case. A mismatch in the 3' nucleotide is assumed to prevent extension by reverse transcriptase of the oligonucleotide (14). This is also shown by the results, since the extension products from the different oligonucleotides are all different lengths. The nucleotide sequence of the three oligonucleotides, their calculated T_d (dissociation temperature) and the genes to which they correspond, are summarized in Table 1. The oligonucleotides 301T and 611T were designed to anneal and extend only from the genes SSU301 (subfamily A) and SSU611 (subfamily B) respectively, while the oligonucleotide 911T was designed to anneal and extend from all of the C subfamily genes: SSU491; SSU911; SSU231; SSU11A;

Table 1. Nucleotide sequence, gene specificity and calculated dissociation temperature of the three oligonucleotides used in the primer extension analysis.

oligo	gene subfamily	gene	sequence	calc T_d °C
301T	A	301	3' ATTCAGACGACAGAGGAAGG 5'	60
611T	B	611	3' TTTTAGACGGAGTCGAAAGG 5'	58
911T	C	911, 112 231, 491 211, 511(11A)	3' GTTCAGACGTCGGAGGAA 5'	56

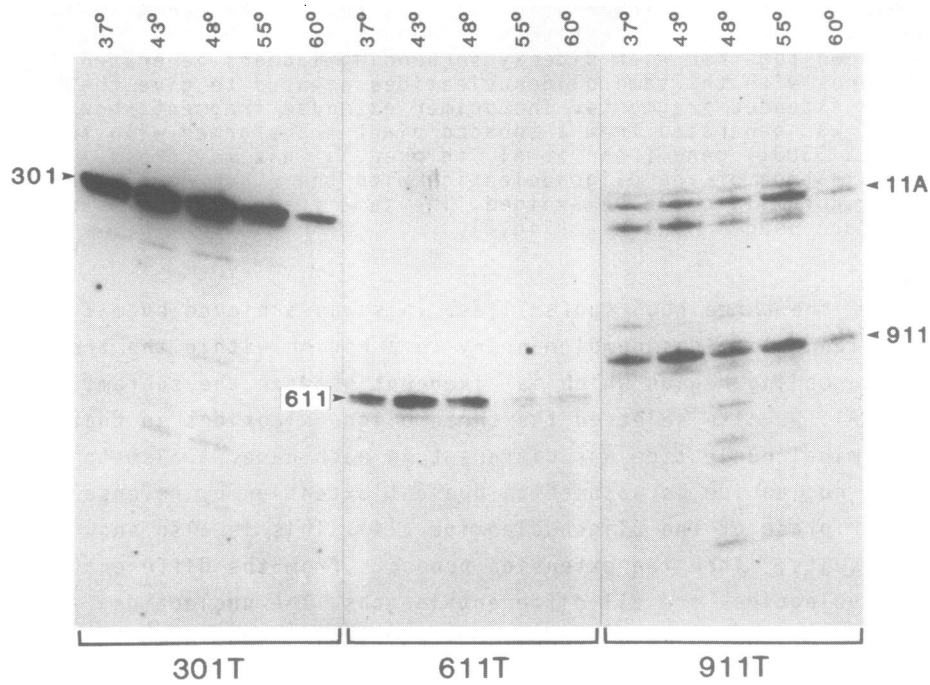


Figure 2. Primer extension analysis of the rbcS genes in petunia leaf tissue.
The three oligonucleotides 301T, 611T and 911T were annealed to total petunia RNA at several temperatures. The primer extended fragments corresponding to the genes SSU301, SSU611, SSU11A and SSU911 are indicated.

SSU112 and SSU211. The 911T oligonucleotide was synthesized as an 18-mer rather than a 20-mer (as 301T and 611T) so that the calculated T_d (from the formula 4^oC (G+C) + 2^oC (A+T) (15)) for the three oligonucleotides would be similar (Table 1).

The primer extension analysis using the oligonucleotides 301T, 611T and 911T with total RNA from petunia leaf tissue is shown in Fig.2. The length of the primer extended fragment generated from the 301T oligonucleotide is 161 nucleotides (Fig.2), which matches the predicted size of a primer extended fragment from the major transcription start site of the SSU301 gene. The much smaller fragments are due to a small amount of RNA degradation which occurred during the primer extension analysis. The rbcS mRNA was found to be highly sensitive to RNAses, an

observation which is discussed in more detail later. The length of the primer extended fragment generated from the 611T oligonucleotide is 115 nucleotides (Fig.2), which corresponds to the predicted length of the primer extended fragment for the major transcription start site of the SSU611 gene. The 911T oligonucleotide gave several distinct primer extended fragments with petunia leaf RNA (Fig.2) indicating that several of the genes within subfamily C have different length 5' untranslated leader regions. The predicted primer extended fragments for SSU911 are 125 and 126 nucleotides, and the predicted primer extended fragment for SSU11A is between 168-170 nucleotides, and these are indicated in Fig.2. Since we do not know the transcription start sites for every gene of the C subfamily we can only tentatively assign each of the observed primer extended fragments to individual genes based upon examination of the nucleotide sequences of the 5' regions of the genes (Fig.1A). The largest fragment (175 nucleotides) probably corresponds to SSU491, and we believe that the primer extended fragments for the genes SSU112, SSU231 and SSU911 all comigrate at the position marked for SSU911 in Fig.2. This prediction is supported by the observation of two separate annealing temperature optima (43 and 55°C) within this band. The gene SSU112 has one mismatch with the oligonucleotide 911T, and this centrally located mismatch could reduce the T_d of the oligonucleotide to mRNA by up to 15°C (16). If the above assignments are correct, one primer extended fragment (the third one from the top) remains unaccounted for. This fragment most probably corresponds to the SSU211 gene, for which we do not have the nucleotide sequence.

The experimentally determined optimal annealing temperatures of the three oligonucleotides 301T, 611T and 911T are 48, 43 and 55°C respectively which differ from the calculated T_d values for the oligonucleotides (60, 58 and 56°C respectively). Hence the general rule of performing annealing reactions of different oligonucleotides at 10°C below their calculated T_d may not give the optimal annealing conditions. If two oligonucleotides are to be used to assay relative expression of two different genes, then the optimal annealing temperature for each oligonucleotide must be empirically determined prior to quantitative analysis. This

empirical measurement was omitted in the analysis of Tumer et al.(10) which may partially explain the different results.

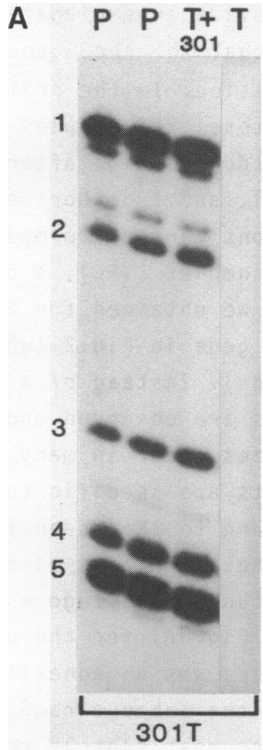
The most significant component of these primer extension data is the relative amount of signal from the extended fragments of the individual genes. Under the conditions of this experiment the amount of primer extended fragment is a direct measure of the level of steady state RNA corresponding to each gene in the petunia leaf total RNA. The results shown in Fig.2 therefore confirm our previous data, which showed that of all the petunia rbcS genes, the SSU301 gene gives the highest levels of steady state RNA. The SSU611 gene gives the next highest levels (from this analysis 4 fold lower than SSU301), followed by individual genes of C subfamily. The SSU11A gene gives 5 fold lower levels of steady state RNA than SSU301.

DISCUSSION.

We have used a primer extension analysis to map the transcription start sites of three of the petunia rbcS genes and also to measure the expression levels of the petunia rbcS genes in leaf tissue. This analysis has confirmed our previous data describing the relative expression levels of the different petunia genes (8). Although the absolute pool sizes of mRNA corresponding to the various genes differ slightly depending on the method of measurement the relative ranking of the mRNA pools from the genes that can be identified in the primer extension analysis, is identical irrespective of the method used to make the measurement. For example, SSU301 was measured as 2 fold stronger than SSU611 using a 3' tail probe hybridization to a Northern blot of total petunia leaf RNA, whereas it is measured as 4 fold stronger by the primer extension method.

The primer extension analysis described in this paper was performed specifically to determine the relative expression levels of the two rbcS genes assayed by Tumer et al.(10), namely SSU301 and SSU11A. These results clearly show that in the petunia plants which we assayed, the SSU301 gene is much more strongly expressed in leaf tissue than the SSU11A gene. These results are in contrast to the results of Tumer et al.(10).

One interesting observation which we made during the course



B SSU301

1 2 3 4 5

tcAgTGTGAAGTGTTTAAAGGAAAAAGCTTTGGAAGAAGCAAAAATCTTCTAACTATG

Figure 3. A. Primer extension analysis of the SSU301 gene in petunia and tobacco leaf tissue. The analysis using the oligonucleotide 301T, was performed without vanadyl ribonucleoside complex. The lanes marked P are two independent total RNA samples from petunia leaf tissue (the second one was also used in the analysis shown in Fig.2). The lane marked T+301 shows the primer extension analysis using total RNA from leaf tissue of a tobacco transformant containing the petunia SSU301 gene. The lane marked T shows the primer extension analysis using total RNA from leaf tissue of an untransformed tobacco plant. The lengths (in nucleotides) of the primer extended fragments are: 1 161; 2 145; 3 118; 4 108; 5 104.
B. The nucleotide sequence of the 5' untranslated leader of the SSU301 gene. The endpoints of the primer extended fragments (1-5) marked in Fig.3A are indicated. An inverted repeat structure (allowing for G-T pairing) is marked by the two arrows. The hatched line underneath the sequence marks the position of the 5' oligonucleotide used in the analysis by Tumer et al.(10).

of this analysis may provide an explanation for the conflicting results. We have found that the rbcS genes are particularly sensitive to RNA degradation. In the primer extension analysis shown in Fig.3 we used total RNA samples from petunia leaf tissue which showed no sign of degradation after electrophoresis on a formaldehyde-agarose gel, and from Northern blot hybridizations (8). The primer extensions were performed without the addition of vanadyl ribonucleoside complex (VRC), a competitive inhibitor of RNAses, and the results we obtained for SSU301 are very different to those shown for this gene in Fig.2 (where VRC had been included in the reactions). Instead of a major primer extended fragment, many fragments are observed and these fragments which are of very discrete sizes occur in many independent RNA samples. Furthermore the fragments are specific to the individual genes since an identical pattern to that seen for the oligonucleotide 301T with petunia leaf total RNA, is also seen in a tobacco plant transformed with the petunia SSU301 gene (Fig.3A) (Dean et al. in prep.). The blank lane (Fig.3A) for the untransformed tobacco total RNA shows that there was no annealing and extension of the 301T oligonucleotide to the tobacco rbcS genes under these annealing conditions (the most closely related tobacco gene has 2 mismatches with the 301T oligonucleotide (17)). Since the shorter primer extended fragments are absent when VRC is used in the procedure (with the same RNA sample) we conclude that the shorter fragments result from specific RNAse nicking. Using the same primer extension conditions (without VRC) with transcripts from several other genes and gene fusions and using the same primer extension conditions and solutions, we have never seen the specific shorter fragments that we observe with the rbcS transcripts. In the few cases where we have seen RNA degradation during the primer extension analysis the result is a smear of fragment sizes rather than specific size classes. We believe therefore that there are regions in the rbcS transcripts which are particularly sensitive to RNAse action.

The sizes of the shorter primer extended fragments for the SSU301 gene were measured and their endpoints are indicated on the nucleotide sequence (Fig.3B). One endpoint (fragment size 118 nucleotides, endpoint 3 Fig.3B) occurs in a series of 5 A

residues which is located in the loop region between an 8bp inverted repeat. This is a region which could be particularly sensitive to RNAse action. This is also the region of the 5' untranslated leader of the SSU301 transcript which was chosen by Tumer et al.(10) to construct the complementary 5' oligonucleotide (indicated in Fig.3B). Potentially, RNAse nicking at these specific sites during preparation of the Northern blot would remove the 5' region of the mRNA which is complementary to the 5' oligonucleotide, hence this would reduce the hybridization of this oligonucleotide to SSU301 transcripts, thereby underestimating the expression level of this gene. While the RNA samples analyzed by Tumer et al.(10) are clearly not degraded to any large extent as demonstrated by the sharpness of the bands on the Northern blots, we believe that the rbcS transcripts are more sensitive to RNAse action than many other leaf RNA species. Clearly, this is only one possible explanation for the different relative expression levels of the SSU301 and SSU11A genes as measured by the two different groups.

Tumer et al.(10) also used oligonucleotides complementary to the 3' end of the SSU301 and SSU11A transcripts to assay the relative expression levels of the rbcS genes. A major shortcoming of this analysis is that the oligonucleotide designed to anneal with the 3' untranslated region of the gene SSU11A is also perfectly complementary to one other gene of C subfamily, namely (SSU231) (9) and it may also anneal to some degree to several of the other genes within this subfamily. This 3' oligonucleotide cross-hybridization could overestimate the expression level of SSU11A.

Thirdly, the optimal annealing temperature of the 5' and 3' oligonucleotides to the leaf RNA was not empirically determined in the analysis of Tumer et al.(10). We have shown here that performing annealings of RNA to different oligonucleotides at a temperature 10°C below the calculated T_d of the oligonucleotide does not always give the most optimal annealing conditions. This may have led to inaccuracies in the comparison of the different oligonucleotides by Tumer et al.(10).

Finally, there is the obvious and quite likely possibility that the Petunia (Mitchell) plants analyzed by the two groups

were physiologically and developmentally different since they were grown under different environmental conditions. We are currently investigating whether the different rbcS genes are differentially expressed during development.

This analysis has confirmed our original data and at present we can only speculate as to the basis of the discrepancy in the results from the two different groups. We hope to resolve this confusion in the near future.

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