The EMBO Journal vol.13 no.9 pp.2211-2217, 1994

A novel RNA gene in the tobacco plastid genome: its possible role in the maturation of 16S rRNA

Antonio Vera[1] and Masahiro Sugiura[2]

Center for Gene Research, Nagoya University, Nagoya 464-01, Japan
[1]Present address: Departamento de Biología Vegetal, Universidad de Alcalá, Alcalá de Henares, 28871 Madrid, Spain
[2]Corresponding author

Communicated by R.G.Herrmann

A small plastid-encoded RNA (spRNA, 218 nt) has been detected in tobacco. The corresponding locus (*sprA*) does not contain any open reading frame and is actively transcribed from its own promoter, as shown by ribonuclease protection assays using *in vitro* capped RNAs. Gel-shift and UV-crosslinking experiments showed the formation of a specific complex between spRNA and chloroplast polypeptides. The mobility of the complex was further shifted when a transcript bearing part of the 16S rRNA leader sequence was added to the incubation mixture. Glycerol gradient fractionation of a chloroplast lysate indicated a preferential sedimentation of spRNA at 15-20S and 70S. These observations, and the potential base-pairing with the leader sequence of pre-16S rRNA, suggest a role for spRNA in chloroplast ribosome biogenesis, i.e. 16S rRNA maturation. By sequencing of tomato plastid DNA and heterologous northern hybridizations, the presence of *sprA* homologs and their expression in a number of dicot plants have also been shown.

Key words: in vitro capping/plastid gene expression/rRNA processing/small plastid RNA/tobacco

Introduction

Photosynthesis in higher plants takes place in specialized organelles of the plastid type known as chloroplasts which possess their own DNA and genetic system. The expression of chloroplast genes is regulated at the transcriptional and post-transcriptional levels [reviewed in Mullet (1988), Gruissem (1989), Igloi and Kössel (1992), Rochaix (1992) and Gruissem and Tonkyn (1993)]. The plastid genetic system appears to be very complex in terms of post-transcriptional modifications of the primary message. For example, extensive 5′ and 3′ end maturation, *cis* and *trans* splicing and RNA editing have been observed in chloroplasts [reviewed in Sugiura (1989, 1992), Rochaix (1992) and Gruissem and Tonkyn (1993)]. However, the molecular mechanisms underlying these processes are poorly understood. In general, post-transcriptional RNA modifications are complicated events accomplished by a number of protein and RNA factors. In eukaryotic nuclei, polyadenylation and splicing necessitate the participation of small nuclear ribonucleoprotein (snRNP) complexes, heterogeneous nuclear ribonucleoprotein (hnRNP) complexes and poly(A)-binding proteins [reviewed in Dreyfuss *et al.* (1988)]. Many of them contain a highly conserved motif termed ribonucleoprotein consensus sequence (RNP-CS) [reviewed in Mattaj (1989)]. Likewise, a group of nuclear-encoded chloroplast ribonucleoproteins containing the RNP-CS have been characterized in tobacco (Li and Sugiura, 1990; Ye *et al.*, 1991) that are likely to be involved in plastid RNA metabolism. Similar proteins identified in chloroplasts from *Chlamydomonas* (Danon and Mayfield, 1991) and spinach (Schuster and Gruissem, 1991) have been correlated with translational regulation of the *psbA* mRNA and mRNA 3′ end processing, respectively. Recent investigations have revealed the existence of a sizeable family of chloroplast ribonucleoproteins (Ye *et al.*, 1991; Mieszczak *et al.*, 1992) which may reflect the diversity of the functions performed inside the organelle.

In addition to protein factors, a vast array of RNA processing reactions require the participation of small RNAs. Uridin-rich small nuclear RNAs (U-snRNA) present in the snRNP complexes and small nucleolar RNAs (snoRNA) are essential for nuclear pre-mRNA splicing (Guthrie, 1991) and pre-rRNA maturation (Beltrame and Tollervey, 1992), respectively. In prokaryotes, a growing number of small RNAs with diverse regulatory and metabolic functions are being reported [reviewed in Inouye and Delihas (1988)]. In organelles, small guide RNAs (gRNA) are known to mediate RNA editing in mitochondria of kinetoplastid protozoa (Simpson, 1990). Similarly, an RNA factor has been suggested to be part of the plant mitochondrial editing system (Araya *et al.*, 1992). In chloroplasts, conservation of sequences around several editing sites has suggested that chloroplast gRNAs may also be considered (Maier *et al.*, 1992b). Moreover, for some chloroplast introns having boundary sequences resembling those of nuclear gene introns (Shinozaki *et al.*, 1986), a similar splicing mechanism has been postulated (Li and Sugiura, 1990) which eventually requires *trans*-acting RNA factors. An example of chloroplast *trans*-acting RNA has been documented in the green alga *Chlamydomonas reinhardtii*. A small transcript (~400 nt) encoded in the plastid *tscA* locus was found to be necessary for *trans* splicing of pre-mRNAs from the divided *psaA* gene (Goldschmidt-Clermont *et al.*, 1991). Aside from the *tscA* RNA, the occurrence of structural (non-polypeptide-coding) RNAs other than tRNA or rRNA in the chloroplast genetic system, although conceivable, remains to be demonstrated.

In this report, we describe a distinct RNA gene (*sprA*) located in the small single copy (SSC) region of tobacco chloroplast DNA. This gene is transcribed independently producing a small plastid RNA (spRNA) of 218 nt. A function of spRNA in chloroplast ribosome formation, possibly as a *trans*-acting factor in rRNA maturation, is discussed.

Results

spRNA is transcribed monocistronically

In the tobacco chloroplast genome, the DNA region between *trnL* and *ndhF* is 1869 bp in length and, aside from *rpl32*, no other putative gene structures could be found (Figure 1A; Shinozaki *et al.*, 1986). However, Northern blot analysis revealed the existence of a small (~220 nt) and stable RNA complementary to a region downstream from *rpl32* (Figure 1B).

Primer extension results indicate that the 5′ end of spRNA corresponds to a position 69 bp downstream from the *rpl32* termination codon (Figure 1C). Interestingly, likely prokaryotic-type promoter motifs were found upstream from the mapped position. To verify this possibility, we carried out highly sensitive ribonuclease protection assays of *in vitro* capped RNA (Vera and Sugiura, 1992). Unlike transcripts whose 5′ ends are originated by post-transcriptional processing, chloroplast primary transcripts retain a triphosphate group; thus they can be selectively labeled using the capping enzyme, guanylyltransferase, and [α-^{32}P]GTP (Christianson and Rabinowitz, 1983). Total leaf RNA was capped and subjected to ribonuclease protection assay with an antisense RNA probe overlapping the 5′ region of *sprA* (Figure 1A). According to our expectations, a specific protected band of 160 nt was detected (Figure 1D, *sprA* lanes), confirming that the position obtained by primer extension analysis corresponds to a real transcription initiation site. A positive control for the primary transcript (370 nt) from *rpl32* (Vera *et al.*, 1992) was run in parallel (Figure 1D, *rpl32* lanes). An unspecified band of 200 nt appears even in the negative control without the riboprobe (Figure 1D, C lanes) and probably corresponds to a self-complementary sequence present in the capped RNA samples. The 3′ end position of the transcript was calculated through independent mung bean nuclease and ribonuclease protection assays (data not shown). Taken together, a size of 218 ± 5 nt was assigned to spRNA from position 115 199 in the tobacco chloroplast genome (Shinozaki *et al.*, 1986), consistent with our Northern blot results.

Several stop codons in the three frames (eight, seven and six times, respectively) and the absence of initiation codons preclude this transcript from being the message for a polypeptide. RNA editing has been found in tobacco chloroplasts (Kudla *et al.*, 1992; T.Hirose, T.Wakasugi, M.Sugiura and H.Kössel, unpublished results) and editing of spRNA cannot be excluded. However, we found the possible reading frames of only 11 codons or less in spRNA assuming the C to U transitions observed so far in chloroplasts (Hoch *et al.*, 1991; Kudla *et al.*, 1992; Maier *et al.*, 1992a,b). This result seems to be against the idea of conversion of spRNA into a translatable message. Hence it can be concluded that this locus (designated *sprA*) is a new RNA gene in tobacco chloroplast DNA.

Association of spRNA with chloroplast proteins

Generally, small RNAs appear complexed with proteins and/or other RNAs in RNP particles (Reddy and Busch, 1988). To examine its association with chloroplast proteins, uniformly-labeled spRNA was produced *in vitro* and binding experiments were carried out with an S28 fraction from purified chloroplasts. Migration of free spRNA in a native gel was retarded when incubated with the chloroplast fraction

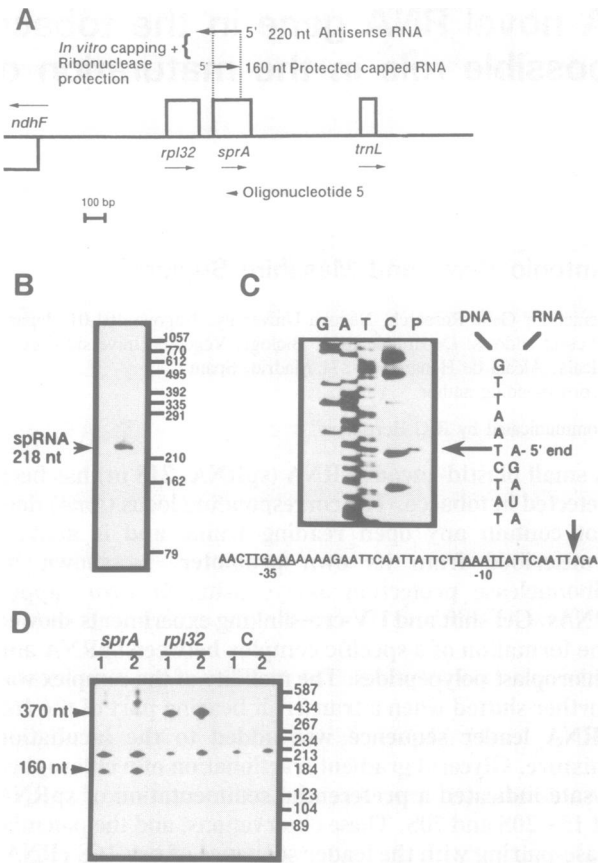

Fig. 1. Identification and mapping of *sprA* on the tobacco chloroplast genome. (**A**) Schematic view of *sprA* and neighboring genes (accession number Z00044). Open boxes represent coding regions. The position of oligonucleotide 5 is indicated by a small arrowhead. Thin arrows indicate the direction of transcription. (**B**) Detection of spRNA by Northern blot analysis. Total leaf RNA was fractionated in a 5% polyacrylamide gel, transferred to a nylon membrane and hybridized with [^{32}P]oligonucleotide 5. On the right are the positions (nt) of *Hin*cII ϕx174 RF-DNA size markers. (**C**) Precise 5′ end mapping of the *sprA* transcript by primer extension. The oligonucleotide 5 was utilized as a primer. G, A, T and C are dideoxy sequencing ladders. P indicates the extended product. (**D**) Determination of the transcription initiation site of *sprA* by *in vitro* capping and ribonuclease protection assay. Capped total leaf RNA (24 μg) was annealed to the antisense riboprobe depicted in (A) and digested with 20 U RNase T1/0.1 U RNase A (lanes 1) or 40 U RNase T1/0.2 U RNase A (lanes 2). A positive control with the characterized primary transcript from *rpl32* (Vera *et al.*, 1992) (*rpl32* lanes) and a negative control without riboprobe (C lanes) were run in parallel. For control experiments, 12 μg capped RNA were used. Positions (nt) of size markers (*Hae*III digest of pBR322) are shown on the right.

(Figure 2A, lanes 1 and 2). This is most probably caused by complex formation with polypeptide(s) present in the mixture because pretreatment with proteinase K prevented the mobility shift (see Figure 3B). The polyanion, heparin, was included in the incubation as a non-specific competitor to displace proteins attached by charge interactions. In this case, the extent of the shift was diminished presumably as a consequence of the removal of non-specifically-bound polypeptide(s). However, substantial gel retardation was still clearly visible (Figure 2A, lane 3). Moreover, addition of ribonuclease T1 prior to heparin resulted only in a slight reduction of the mobility shift (Figure 2A, lane 4). The permanence of a complex under the described conditions of incubation implies the specific interaction of spRNA with

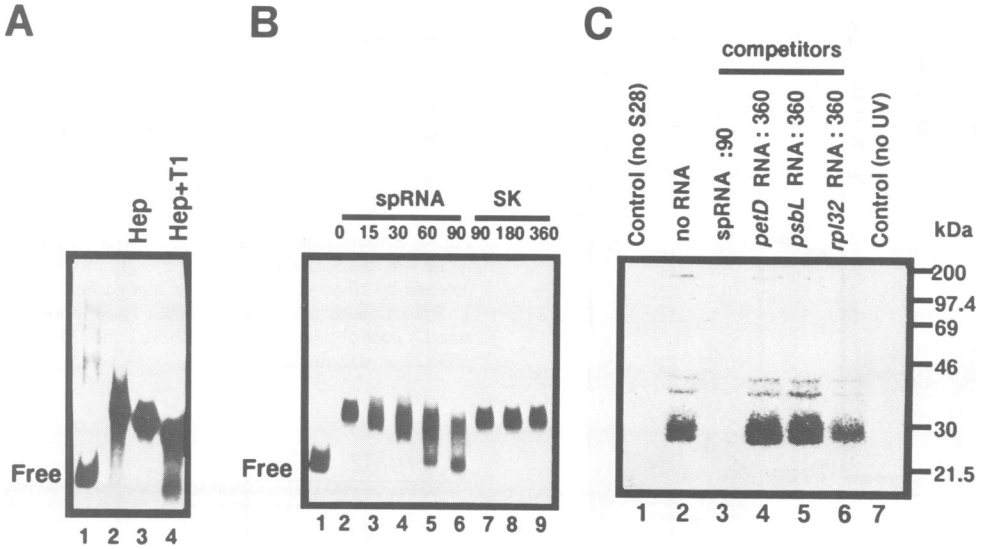

Fig. 2. Formation of complexes between spRNA and chloroplast proteins. (**A**) [^{32}P]spRNA was incubated with the S28 fraction of chloroplast lysates. The products were analyzed by electrophoresis in native gels (4% polyacrylamide) either as free RNA (lane 1), after incubation with the S28 fraction only (lane 2), after heparin treatment (lane 3) or after sequential addition of RNase T1 followed by heparin (lane 4). (**B**) Competition experiments. Binding reactions were carried out as in lane 3 (heparin) of (A), except that 5 min before the inclusion of [^{32}P]spRNA, unlabeled competitor RNAs were added. Competitors were spRNA (lanes 3–6) and a 120 nt transcript from the polylinker region of Bluescript SK$^+$ (SK, lanes 7–9). Numerals on the top indicate molar excess. (**C**) UV-crosslinking of spRNA. Binding reactions were performed as in (A), lane 2. Crosslinked products were subjected to SDS–PAGE (12.5%). [^{32}P]spRNA was incubated with water (lane 1), with the S28 fraction (lanes 2–7) in the absence (lanes 1, 2 and 7) or presence of unlabeled RNA competitors. Competitors were spRNA (lane 3), the 3′ untranslated region of *petD* RNA (lane 4), *psbL* RNA (lane 5) and the 5′ leader of the *rpl32* RNA (lane 6). Molar excess was 90 times for unlabeled spRNA and 360 times for the rest of competitors. Lane 7, no UV irradiation. Positions of the molecular mass markers (Rainbow markers, Bio-Rad) are shown on the right.

chloroplast polypeptide(s), hence protecting it against nuclease attack. This notion was reinforced by competition experiments. Increasing molar excess of an unlabeled transcript corresponding to the polylinker region of the cloning vector did not affect the band shifting even in the presence of 10 mg/ml heparin (Figure 2B, lanes 7–9), whereas self-competition with much lower amounts of unlabeled spRNA had a considerable effect on the position of the retarded complex that gradually displaced labeled spRNA to the free RNA position (Figure 2B, lanes 1–6). Other competitors, such as synthetic ribohomopolymers and several chloroplast gene-specific transcripts, did not affect the mobility shift (data not shown).

After UV irradiation and ribonuclease A treatment of the incubation mixture containing spRNA and the chloroplast fraction, a number of crosslinked polypeptides were resolved by SDS–PAGE with apparent molecular masses of 170, 42, 36, 33, 31 and 28 kDa (Figure 2C, lane 2). These bands could not be visualized without UV irradiation or S28 fraction (Figure 2C, lanes 1 and 7), indicating that the polypeptides were really bound to the RNA. No polypeptide band was observed after incubation with a molar excess of unlabeled spRNA (Figure 2C, lane 3). However, the inclusion of a huge molar excess of transcripts from *petD*, *psbL* and *rpl32* genes did not affect the electrophoretic pattern (Figure 2C, lanes 4–6), suggesting again specific binding with spRNA. One exception was the 170 kDa polypeptide whose binding to spRNA seems to be inhibited by *psbL* and *rpl32* transcripts (Figure 2C, lanes 5 and 6). Because this polypeptide does not appear when incubation takes place in the presence of heparin (not shown), it is reasonable to think that this polypeptide binds non-specifically. This could explain the increase in migration observed after heparin treatment (Figure 2A).

spRNA may be a trans-acting factor in ribosome biogenesis

Supercomplex structures are often formed between RNP particles and their target RNA substrates (Reddy and Busch, 1988; Tyc and Steitz, 1989). Because direct analysis is difficult due to the absence of proper plant *in vitro* systems (Goodal *et al.*, 1991), a supershift strategy was designed to explore this phenomenon in spRNA. A variety of tobacco chloroplast transcripts, including introns, 5′ and 3′ untranslated regions, rRNA, tRNA and polypeptide-coding sequences, were produced *in vitro*. Binding reactions were performed, as stated previously, with labeled spRNA and then each of the above unlabeled transcripts was supplemented after 5 min incubation. The formation of a supercomplex (supershift) was monitored under native electrophoresis conditions. Only a transcript bearing the leader sequence adjacent to the mature 16S rRNA produced an additional band above the main shift (Figure 3A, lane 6), suggesting an interaction of this transcript with the spRNA–protein complex. Limitations due to electrophoresis conditions (Lane *et al.*, 1992) might be responsible for the small proportion of labeled spRNA supershifted.

Most interestingly, a portion of spRNA (positions 33–42) exhibits complementarity to a highly conserved pre-16S rRNA sequence immediately before the processing site (Figure 3C). However, labeled spRNA was not supershifted when incubated with pre-16S rRNA in the absence of the chloroplast fraction (Figure 3B, lane 3). When most of the potentially base-pairing nucleotides (corresponding to

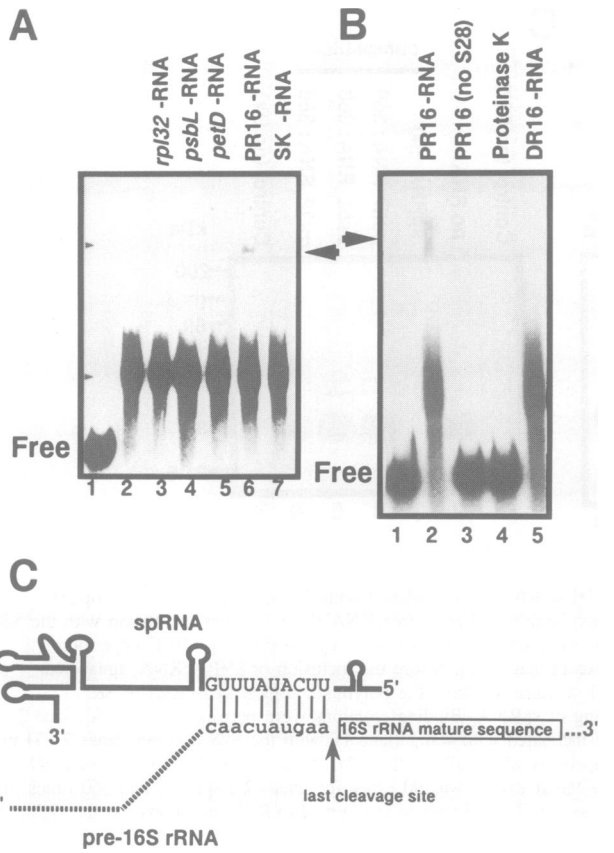

Fig. 3. Formation of a supercomplex among spRNA, chloroplast proteins and pre-16S rRNA. (**A**) [^{32}P]spRNA was incubated with the 28S fraction and separated by native gels either as free RNA (lane 1) or after incubation with S28 only (lane 2). A second unlabeled RNA was added after 5 min incubation with [^{32}P]spRNA (lanes 3–7). *rpl32*, *psbL* and *petD* RNAs are as in Figure 2C. SK RNA is as in Figure 2B. PR16 contains the last 77 nt of the leader and the first 203 nt of 16S rRNA (Vera *et al.*, 1993). Supershift is indicated by a big arrowhead. (**B**) spRNA was separated either as free RNA (lane 1), after incubation with S28 and PR16 as in (A) (lane 2), or by replacing S28 with water (lane 3) or with proteinase K-digested S28 (500 μg/ml, 37°C, 1 h) (lane 4). Lane 5, as in lane 2 replacing PR16 by a deletion construct DR16 (47 nt shorter, from −50 to −4 with respect to the mature 16S rRNA). (**C**) Possible interaction between spRNA and pre-16S rRNA. The secondary structure is drawn according to the Squiggles program. Complementarity is marked by vertical bars. Mature 16S rRNA sequence is boxed and the boundary with the leader is marked with an arrow. The length of stem–loop regions is drawn arbitrarily.

positions 42–36 in spRNA) were deleted from the pre-16S rRNA sequence, supershift of spRNA was not observed (Figure 3B, lane 5). These results argue that the protein factor(s) present in the chloroplast fraction is necessary for the appropriate folding and specific interaction between spRNA and pre-16S rRNA.

To further substantiate our hypothesis, purified tobacco chloroplasts were lysed and fractionated in glycerol gradients. The resulting fractions were tested for the presence of spRNA by ribonuclease protection experiments. The distribution of spRNA along the gradient indicates major sedimentations at 15–20S and ~70S (Figure 4). The two particle populations may be a reflection of the two complexes identified *in vitro* (see above) and preferential detection of spRNA at 70S is in concordance with a role in ribosome biogenesis.

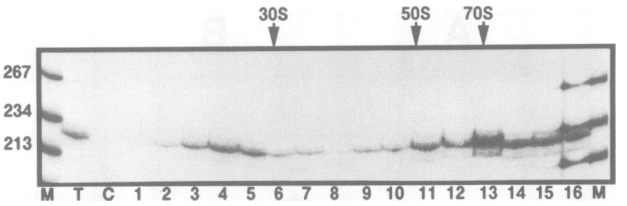

Fig. 4. Sedimentation of spRNA in a glycerol gradient. A chloroplast lysate was fractionated on a 10–30% glycerol gradient. spRNA was detected by ribonuclease protection assay using antisense spRNA. Lane T, RNA isolated from the chloroplast lysate prior to centrifugation; lane C, control with total yeast RNA. Lanes M, *Hae*III digest of pBR322 as size markers (nt).

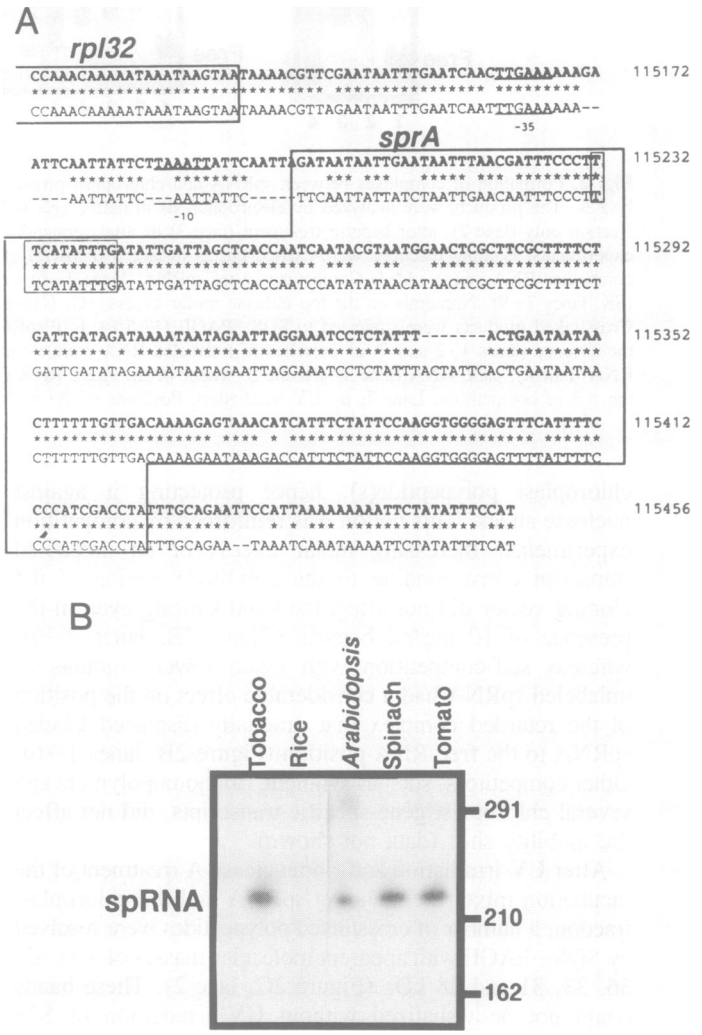

Fig. 5. The presence of *sprA* homologs in other plant species. (**A**) DNA sequence comparison of the *sprA* region from tobacco and tomato chloroplast genomes. Upper bold letters indicate the tobacco sequence. Lower normal type letters correspond to the tomato DNA. Coding regions are boxed. The tomato *sprA* boundaries are tentatively assigned by comparison with the tobacco sequence. Asterisks indicate nucleotide identity and gaps have been introduced to maximize homology. Prokaryotic promoter motifs are underlined and the potential base-pairing sequence with pre-16S rRNA is shadowed. Numbers on the right correspond to nucleotide positions in the tobacco chloroplast genome (Shinozaki *et al.*, 1986). (**B**) Northern blot analysis. Total leaf RNA from tobacco, rice, *Arabidopsis*, spinach and tomato hybridized with labeled oligonucleotide NBO as a probe. Size markers (nt) are a *Hinc*II digest of φx174 RF-DNA.

SprA is expressed in other plant species

No significant sequence homology to *sprA* could be found in the DNA databases. Apparently, it is absent from the liverwort (Ohyama *et al.*, 1986), rice (Hiratsuka *et al.*, 1989), maize (R.M.Maier, G.L.Igloi and H.Kössel, unpublished data) and black pine (T.Wakasugi, J.Tsudzuki, S.Ito, K.Nakashima, T.Tsudzuki and M.Sugiura, manuscript in preparation) chloroplast genomes. However, we sequenced the corresponding region in tomato and found a high level of identity (90%) between both species (Figure 5A). This conservation includes the nucleotides involved in the potential base-pairing with pre-16S rRNA depicted in Figure 3C.

Unexpectedly, several deletions in the upstream region of the tomato *sprA* homolog disrupt the putative −10 motif present in tobacco, but not the −35 element. Tomato *sprA* might lack its own promoter, being cotranscribed with *rpl32*, or alternatively a distinct promoter structure may be active for this gene.

To verify the expression of the tomato *sprA* homolog, tomato leaf RNA was analyzed by Northern blots hybridized to a labeled synthetic oligoprobe complementary to a conserved region in both tobacco and tomato *sprA* genes. We also searched *sprA* homologs in rice, *Arabidopsis* and spinach. RNAs similar in size to tobacco spRNA are detected in all dicots analyzed (Figure 5B). On the other hand, no positive result could be obtained with rice RNA, suggesting the absence of *sprA* in monocots or sequence divergence with respect to dicot plants. Further experimentation with different monocot species is required to substantiate this hypothesis.

Discussion

A new type of chloroplast RNA gene

We described *sprA*, a new locus in the chloroplast genome. RNA mapping and sequence analysis indicate that the gene product, spRNA, is a small RNA different from rRNA and tRNA. *In vitro* capping assays proved that spRNA is not a processed product but a primary transcript. Therefore *sprA* is a bona fide gene whose product represents a new type of structural (non-polypeptide-encoding) RNA in the chloroplast. This finding *per se* is very interesting because it indicates that higher plant chloroplast genomes may contain additional RNA genes other than those coding for tRNA and rRNA.

A possible role for spRNA in ribosome biogenesis

We have provided evidence for the specific association of spRNA with chloroplast soluble protein(s) *in vitro*. This may reflect the existence *in vivo* of an spRNA−protein particle. A growing body of knowledge suggests that the formation of cytoplasmic ribosomes requires a set of snoRNAs for pre-rRNA processing and subsequent accumulation of the mature products (Kass *et al.*, 1990; Li *et al.*, 1990). Base-pairing of U14 snoRNA (Maxwell and Martin, 1986) and U3 snoRNA (Beltrame and Tollervey, 1992) to 18S rRNA and the 5′ external transcribed spacer (ETS), respectively, has been demonstrated. Complementarity of sequences between snoRNAs and pre-rRNA has been suggested to be important in processing of the precursor and/or the assembly of the processing complex (Kass *et al.*, 1990). The formation of a supercomplex from the spRNA−RNP particle and pre-16S rRNA is also suggestive of a similar function of spRNA in the chloroplast RNA processing machinery.

Since chloroplast rRNA gene sequences and operon arrangement are of the prokaryotic type (Delp and Kössel, 1991; Sugiura, 1992), current ideas about chloroplast rRNA processing are based on the bacterial model. This remains speculative because of the lack of experimental evidence (Delp and Kössel, 1991). For example, the flanking regions of 16S and 23S rRNAs are predicted to form duplex structures susceptible to a RNase III-like activity, as shown in *Escherichia coli*. This activity could accomplish 16S rRNA maturation. However, in both bacteria (Srivastava and Schlessinger, 1990) and the chloroplast predicted model (Delp and Kössel, 1991), a small stretch of nucleotides pertaining to the leader sequence still remains even after RNase III digestion. In *E.coli*, the final maturation takes place within pre-70S ribosomes (Srivastava and Schlessinger, 1990). We detected previously the presence of 5′ pre-mature 16S rRNA in 70S plastid ribosomes (Vera *et al.*, 1993). This is probably related to the sedimentation of spRNA with the 70S fraction. Interestingly, a small RNA of unknown function found in a variety of bacterial species also cofractionates with 70S ribosomes (A.Muto, personal communication) which could be involved in their biogenesis. Therefore we propose that spRNA is part of an RNP complex involved in 16S rRNA maturation in chloroplast 70S ribosomes. It remains to be resolved whether spRNA belongs to a catalytic core and/or it acts as a sort of guiding RNA for the processing machinery.

Occurrence of sprA in other species

We found *sprA* homologs in a number of dicots but not in monocots. This is puzzling because of the strong conservation observed among monocots and dicots in the 16S rRNA 5′ leader sequence. A possible explanation might be a high degree of sequence divergence in monocots which precludes the detection of *sprA* by heterologous probes. Very short sequences corresponding to the putative base-pairing site are found in the rice chloroplast genome in some spacer regions. Preliminary data seem to indicate the presence of some small transcripts corresponding to these regions (not shown) whose further characterization is currently under investigation.

Alternatively, spRNA might be useful but not essential, thus allowing other factor(s) to take over its function in other plant groups. It is noteworthy that several enzymatic activities involved in the formation of prokaryotic rRNA fall into this category. For instance, RNase III is a key enzyme in pre-rRNA processing, but it is dispensable because *E.coli* RNase III$^-$ mutants can accumulate mature rRNA species (Srivastava and Schlessinger, 1989, 1990), perhaps because alternative ways for processing exist in the cell. This is also likely to occur in the plastid compartment. Wolfe *et al.* (1992) have suggested a difference in processing of pre-16S rRNA in the plastids of the parasitic plant *Epiphagus virginiana*, where mature rRNA is produced despite deletions in the flanking regions which do not allow the formation of an RNase III substrate structure. Likewise, a proximal promoter for the rRNA operon in tobacco chloroplasts produces a transcript with a very short leader so that much of the duplex structure cannot be formed (A.Vera and M.Sugiura, manuscript in preparation). Barkan (1993) has also shown normal maturation of 23S rRNA in a maize

nuclear mutant defective in 16S rRNA processing which implies a factor distinct from RNase III. The development of a chloroplast *in vitro* system capable of rRNA processing will be of invaluable help in deciphering the mechanisms involved and also the role of factors like spRNA.

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* var Bright Yellow 4) plants were grown for 2 months at 24°C under an 18 h photoperiod. Two week-old seedlings of spinach, *Arabidopsis* and tomato, and 1 week-old rice seedlings were also grown under the same conditions.

Plasmid constructions and in vitro transcription

The plasmid pTBa2 contains almost the whole SSC region of tobacco chloroplast DNA (Sugiura *et al.*, 1986). The plasmid pBB14, used to generate the antisense RNA for ribonuclease protection experiments, was obtained after subcloning of a 286 bp *Hpa*II fragment from the insert of pTBa2 into pBluescript SK+ (Stratagene). The entire *sprA* sequence was amplified from pTBa2 by PCR with Amplitaq DNA polymerase (Perkin Elmer Cetus). The PCR primers used were (restriction sites created for cloning are in bold): SPR1, 5′-GC**GACGTC**AGATAATAATTGAA-TAATTTAA-3′; and SPR2, 5′-GC**GGATCC**TAGGTCGATGGGGAA-AATGAAA-3′.

Thirty cycles were performed, each of 1 min at 94°C, 2 min at 50°C and 1 min at 72°C (for 10 min in the last cycle). The resultant fragment was cloned between the *Sac*I and *Bam*HI sites of pBluescript SK+ producing the pSPR plasmid. For PSBL plasmid construction, a 140 bp fragment (the first 118 bp of the *psbL* coding region and the adjacent 22 bp of the 5′ untranslated region) was PCR amplified as above from pTS9 (Sugiura *et al.*, 1986) and then inserted between the *Bam*HI and *Hin*dIII sites of pBluescript SK+. The PCR primers used were: PSBL1, 5′-GC**GGATCC**ACTTAATCCGAATTATAGAGCTACG-3′; and PSBL2, 5′-GC**AAGCTT**CTTAATTGAAGAAATAATTGG-3′. pDIR (constructed by M.Sugita) includes the last 75 bp of *petD* exon II and the following 226 bp 3′ untranslated region, which can form a stem–loop structure. The plasmids pBB12 containing *rpl32* (Vera *et al.*, 1992) and PR16 containing 16S rDNA (Vera *et al.*, 1993) were described. DR16 was constructed by digestion of PR16 with *Hha*I followed by gel purification (Geneclean, Bio 101) and religation. The resultant plasmid lacks 47 bp from PR16 corresponding to positions −4 to −50 with respect to the mature 16S rRNA.

The plasmid pBB14 was cut with *Bam*HI, pDIR was cut with *Ssp*I, PSBL was digested with *Hin*dIII and pBB12 was cut with *Spe*I or with *Bam*HI when the opposite strand was transcribed. Similarly, pSPR was linearized with *Bam*HI to produce spRNA or with *Sac*I when antisense spRNA was synthesized. Digestion of PR16 and DR16 was carried out with *Hin*dIII. The vector pBluescript SK+ without insert was cut with *Kpn*I to produce the SK transcript. 1 μg each of linearized plasmid DNAs were used as template for *in vitro* transcription with 10 U T7 or T3 RNA polymerase as described (Vera *et al.*, 1992, 1993). All transcripts were gel purified.

Northern blot analysis and RNA mapping

Total RNA was extracted from leaves as described (Li and Sugiura, 1990). For Northern blot, RNA was separated in denaturing (7.5 M urea) 5% polyacrylamide gels in 0.5 × TBE (0.45 M Tris–borate, 1 mM EDTA, pH 8). After transfer in 8 mM Na_2HPO_4 and 6 mM sodium citrate, the blotted nylon filters (Hybond N, Amersham) were UV irradiated. Prehybridization (2 h) and hybridization (overnight) were at 50°C in 6 × SSC, 5 × Denhardt's, 0.1% SDS and 50 μg/ml salmon sperm DNA. The filters were washed at the same temperature under increasingly stringent conditions (twice in 6 × SSC, 0.1% SDS for 20 min; twice in 3 × SSC, 0.1% SDS; twice in 1 × SSC, 0.1% SDS for 20 min). Oligonucleotide probes were labeled at their 5′ ends with polynucleotide kinase following a standard method (Sambrook *et al.*, 1989). The sequence of the oligonucleotide 5 used in Figure 1 has been described (Vera *et al.*, 1992). The oligonucleotide NBO used in Figure 5 is 5′-CTCCCCACCTTGGAA-TAGAAATG-3′. Oligonucleotides were prepared with a DNA synthesizer (Applied Biosystems 380 A). Primer extension, *in vitro* capping and ribonuclease protection procedures were as reported (Vera and Sugiura, 1992; Vera *et al.*, 1992, 1993).

Chloroplast protein extract, gel-shift and UV-crosslinking assays

Intact chloroplasts were prepared from tobacco leaves by Percoll gradient centrifugation as described (Li and Sugiura, 1990). Chloroplasts from 300 to 400 g of tobacco leaves were homogenized in 20 mM Tris–HCl (pH 8.2), 2 mM DTT. After centrifugation at 28 000 g_{max} and 2°C for 30 min, the supernatant was collected (S28 fraction) and its protein content was determined by the Bio-Rad assay according to the instructions of the manufacturer. The fraction was divided into aliquots and stored at −70°C until use.

Binding reactions were carried out at 25°C for 20 min with the S28 fraction (100 μg protein, 5 mg/ml) and 15 000 c.p.m. of [^{32}P]spRNA (0.5–1.0 ng) in the presence of 1 U Inhibit-ACE (5 Prime→3 Prime, Inc) and RNA binding buffer, RB (10 mM HEPES–NaOH, pH 7.6, 40 mM KCl, 5% glycerol, 1 mM DTT) in a total volume of 30 μl. Heparin was supplemented for the last 5 min of incubation at a final concentration of 10 mg/ml. For ribonuclease treatment of protein-complexed RNA, 1 U of ribonuclease T1 (Ambion) was added after 10 min of incubation followed by heparin treatment as above. Unlabeled competitor RNA species were supplied to the reaction mixture 5 min before the addition of [^{32}P]spRNA. For supershift assays, a second target RNA (unlabeled) was added after 5 min of incubation with [^{32}P]spRNA. The complexes were resolved in non-denaturing 4% polyacrylamide gels, as in Leibold and Munro (1988), except that 0.5 × TBE was used as the buffer system.

For UV-crosslinking assays, binding reactions were performed as described above without ribonuclease inhibitor. After incubation the reaction mixtures were UV irradiated (360 mJ/cm^2) in a UV crosslinker (Funa, FS-1500). Subsequent digestion with ribonuclease A (final 75 μg/ml) was undertaken for 20 min at 37°C. A standard procedure (Sambrook *et al.*, 1989) was followed to separate the labeled polypeptides by SDS–PAGE (12.5% polyacrylamide).

Glycerol gradient sedimentation

Chloroplasts were isolated as indicated above and homogenized in 10 mM HEPES–NaOH (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$ and 1 mM DTT. The homogenate (10 mg/ml protein) was loaded onto linear glycerol gradients (10–30%) containing the same buffer and centrifuged in a Hitachi SRP28SA rotor at 26 000 r.p.m. (121 000 *g*) for 5 h at 4°C. Gradients were divided into 16 fractions. RNA was extracted with phenol:chloroform:isoamylic alcohol (25:24:1), ethanol precipitated and analyzed by ribonuclease protection as above.

Sequencing of tomato chloroplast DNA

1 μg of tomato total root DNA (provided by M.Sugita) was PCR amplified with Taq polymerase and oligonucleotides complementary to highly conserved sequences from tobacco *ndhF* and *trnL*: SP1B, 5′-GCGGAT-CCCATATTCATATGTCTGTTCCAT-3′; and SP2B, 5′-GCAAGCTTT-GCCGCCACTCGGACTCGAACC-3′. The PCR profile was 1.5 min at 94°C, 2 min at 50°C and 3 min at 72°C during 30 cycles (the last cycle, 72°C for 10 min). A nearly 2 kbp amplified fragment was gel purified (Geneclean II, Bio 101) and used as template for direct sequencing with a dsDNA cycle sequencing system (BRL) following the recommendations of the manufacturer. An internal primer equivalent to the conserved 5′ end of *rpl32*, (ST2, 5′-ATGGCAGTTCCAAAAAAACG-3′) was used in a quick cycle programme (20 cycles, 10 s at 94°C, 10 s at 65°C). The sequence of the tomato *sprA* region will appear in the EMBL sequence database (accession number D17805).

Acknowledgements

We thank Drs M.Sugita, T.Wakasugi, Y.Li and T.Hirose for helpful discussions and Dr S.Kapoor for critical reading of the manuscript. Tomato DNA and pDIR plasmid were kind gifts from Dr M.Sugita. We wish to thank Dr R.Herrmann (Munich) for valuable suggestions and Drs A.Muto (Hirosaki) and H.Kössel (Freiburg) for communicating unpublished results. A.V. was funded by the E.C. Scientific Exchange Programme in Japan and the Japan Society for the Promotion of Science. This work was supported in part by a Grant-in-Aid (number 91040) from the Ministry of Education, Science and Culture (Japan).

References

Araya,A., Domec,C., Begu,D. and Litvak,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1040–1044.

Barkan,A. (1993) *Plant Cell*, **5**, 389–402.

Beltrame,M. and Tollervey,D. (1992) *EMBO J.*, **11**, 1531–1542.

Christianson,T. and Rabinowitz,M. (1983) *J. Biol. Chem.*, **258**, 14025–14033.

Danon,A. and Mayfield,S. (1991) *EMBO J.*, **10**, 3993–4001.

Delp,G. and Kössel,H. (1991) In Bogorad,L. and Vasil,I.K. (eds), *The Molecular Biology of Plastids*. Academic press, San Diego, CA, pp. 139–167.
Dreyfuss,G., Swanson,M.S. and Piñol-Roma,S. (1988) *Trends Biochem. Sci.*, **13**, 86–91.
Goldschmidt-Clermont,M., Choquet,Y., Girard-Bascou,J., Michel,F., Schirmer-Raire,M. and Rochaix,J.D. (1991) *Cell*, **65**, 135–143.
Goodal,G.J., Kiss,T. and Filipowicz,W. (1991) *Oxford Surv. Plant Mol. Cell Biol.*, **7**, 255–296.
Gruissem,W. (1989) *Cell*, **56**, 161–170.
Gruissem,W. and Tonkyn,J.C. (1993) *Crit. Rev. Plant Sci.*, **12**, 19–55.
Guthrie,C. (1991) *Science*, **253**, 157–163.
Hiratsuka,J. *et al.* (1989) *Mol. Gen. Genet.*, **217**, 185–194.
Hoch,B., Maier,R.M., Appel,K., Igloi,G.L. and Kössel,H. (1991) *Nature*, **353**, 178–180.
Igloi,G.L. and Kössel,H. (1992) *Crit. Rev. Plant Sci.*, **10**, 525–558.
Inouye,M. and Delihas,N. (1988) *Cell*, **53**, 5–7.
Kass,S., Tyc.,K., Steitz,J.A. and Sollner-Weber,B. (1990) *Cell*, **60**, 897–908.
Kudla,J., Igloi,G.L., Metzlaff,M., Hagemann,R. and Kössel,H. (1992) *EMBO J.*, **11**, 1099–1103.
Lane,D., Prentki,P. and Chandler,M. (1992) *Microbiol. Rev.*, **56**, 509–528.
Leibold,E.A. and Munro,H.N. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 2171–2175.
Li,Y. and Sugiura,M. (1990) *EMBO J.*, **9**, 3059–3066.
Li,H., Zagorski,J. and Fournier,M.J. (1990) *Mol. Cell. Biol.*, **10**, 1145–1152.
Maier,R.M., Hoch,B., Zeltz,P. and Kössel,H. (1992a) *Plant Cell*, **4**, 609–616.
Maier,R.M., Neckerman,K., Hoch,B., Akhmedov,N.B. and Kössel,H. (1992b) *Nucleic Acids Res.*, **20**, 6189–6194.
Mattaj,I.W. (1989) *Cell*, **57**, 1–3.
Maxwell,E.S. and Martin,T.E. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 7261–7265.
Mieszczak,M., Klahre,R., Levy,J.H., Goodall,G.J. and Filipowicz,W. (1992) *Mol. Gen. Genet.*, **234**, 390–400.
Mullet,J. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **39**, 475–502.
Ohyama,K. *et al.* (1986) *Nature*, **322**, 572–574.
Reddy,R. and Busch,H. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, Berlin, Germany, pp. 1–37.
Rochaix,J.D. (1992) *Annu. Rev. Cell Biol.*, **8**, 1–28.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schuster,G. and Gruissem,W. (1991) *EMBO J.*, **10**, 1493–1502.
Shinozaki,K. *et al.* (1986) *EMBO J.*, **5**, 2943–2049.
Simpson,L. (1990) *Science*, **250**, 512–513.
Srivastava,A.K. and Schlessinger,D. (1989) *Nucleic Acids Res.*, **17**, 1649–1664.
Srivastava,A.K. and Schlessinger,D. (1990) In Hill,W.E., Dahlberg,A., Garret,R.A., Moore,P.B., Schlessinger,D. and Warner,J.R. (eds), *The Ribosome. Structure, Function and Evolution*. American Society for Microbiology, Washington, DC, pp. 426–434.
Sugiura,M. (1989) *Annu. Rev. Cell Biol.*, **5**, 51–70.
Sugiura,M. (1992) *Plant Mol. Biol.*, **19**, 149–168.
Sugiura,M., Shinozaki,K., Zaita,N., Kusuda,M. and Kumano,M. (1986) *Plant Sci.*, **44**, 211–216.
Tyc,K. and Steitz,J.A. (1989) *EMBO J.*, **8**, 3113–3119.
Vera,A. and Sugiura,M. (1992) *Plant Mol. Biol.*, **19**, 309–311.
Vera,A., Matsubayashi,T. and Sugiura,M. (1992) *Mol. Gen. Genet.*, **233**, 151–156.
Vera,A., Yokoi,F. and Sugiura,M. (1993) *FEBS Lett.*, **327**, 29–31.
Wolfe,K.H., Katz-Downie,D.S., Morden,C.W. and Palmer,J.D. (1992) *Plant Mol. Biol.*, **18**, 1037–1048.
Ye,L., Li,Y., Fukami-Kobayashi,K., Go,M., Konishi,T., Watanabe,A. and Sugiura,M. (1991) *Nucleic Acids Res.*, **19**, 6485–6490.

Received on October 26, 1993; revised on February 22, 1994