Hyper-expression of Small Nucleolar RNAs (snoRNAs) in Female Inflorescences of Hazelnut (Corylus avellana L.) Supports rRNA Aggregation In vitro

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Under certain in vitro (salt and temperature) conditions rRNA aggregation occurs in female inflorescences but not in leaves or pollen RNA preparations from hazelnut (Corylus avellana L.), a species of economic interest. This paper describes experiments addressing an explanation of this phenomenon. The experiments demonstrate that: (i) trans-acting factors induce rRNA aggregate formation in female inflorescences RNA preparations; (ii) these factors support aggregation also of heterologous rRNA; (iii) aggregation is a function of temperature pre-treatment of rRNA and not of source 18S rRNA; (iv) the factors inducing rRNA aggregates are sensitive to RNase; (v) antisense small nucleolar RNAs (snoRNAs) participate in rRNA aggregate formation. snoRNAs are involved in pre-rRNA spacer cleavages, and are required for the two most common types of rRNA modifications: 2'-O-ribose methylation and pseudouridylation. Even though it is questionable whether rRNA aggregation really happens in female inflorescence in vivo, the phenomenon observed in vitro may reflect the abundance of snoRNAs in these reproductive structures. In fact the level of accumulation of three tested snoRNAs, R1, U14 and U3, is much higher in female inflorescence than in leaves or pollen of hazelnut. This finding opens the possibility of studying the role of snoRNAs in tissue development in plants.

Keywords: Corylus avellana — Female inflorescences — pre-rRNA processing — Ribosome biogenesis — rRNA – snoRNA.

Abbreviations: cv, cultivar; pre-rRNA, precursor ribosomal RNA; snoRNA, small nucleolar RNA.

Introduction

In all eukaryotes, ribosomes are assembled in the nucleolus where the long pre-rRNA is synthesised, associated with ribosomal proteins and processed into mature rRNA through a series of nucleolytic cleavages. Processing of pre-rRNA also involves the modification of specific rRNA nucleotides by either 2'-O-ribose methylation or base pseudouridylation. Modification of rRNA affects about 50–100 rRNA nucleotides and probably occurs co-transcriptionally. The different sites of rRNA modification, generally found within conserved domains of the mature rRNA sequences, do not show any obvious consensus structure likely to be recognised by a single methylase. Recent resurgence of interest in the study of pre-rRNA processing and ribosome biogenesis has been largely stimulated by the discovery that most sites of rRNA modification are targeted by guide small nucleolar RNAs (snoRNAs) which possess long tracts (10–21 nucleotides) of perfect complementarity to mature rRNAs (Brown et al. 2003, Terms and Terms 2002). The sites of pseudouridine formation and 2'-O-ribose methylation on pre-rRNA are selected by site-specific base pairing with snoRNAs (Bachellerie et al. 1995, Bachellerie and Cavaillé 1997, Barneche et al. 2000, Brown and Shaw 1998, Cavaillé et al. 1996, Kiss-László et al. 1996, Lafontaine and Tollervey 1998, Maxwell and Fourrier 1995, Nicoloso et al. 1996, Smith and Steitz 1997, Tollervey and Kiss 1997, Tycowski et al. 1996). Around 150 different species of snoRNA are predicted to be present in vertebrate nucleoli. All known snoRNAs, except for MRP-7.2 RNA, can be divided into two major classes, designated the box C/D snoRNAs and the box H/ACA snoRNAs, on the basis of conserved sequence elements and conserved predicted secondary structures (Balakin et al. 1996, Barneche et al. 2001, Ganot et al. 1997, Liang et al. 2002). In association with protein cofactors (Filipowicz and Pogacic 2002), the box C/D snoRNAs can bind to pre-rRNA at either one or two sites of 2'-O-ribose methylation, and, similarly, the box H/ACA snoRNAs can bind to pre-rRNA at either one or two sites of pseudouridine formation (Darzacq et al. 2002, Kiss 2001, Kiss 2002). Several boxC/D snoRNAs, including the most abundant U3, U8 and U14, are involved in pre-rRNA spacer cleavage (Brown et al. 2001, Maxwell and Fourrier 1995, Smith and Steitz 1997, Tollervey and Kiss 1997). In addition to their involvement in pre-rRNA processing, snoRNA are intriguing because of both their peculiar gene organization and their biosynthetic pathway (Maxwell and Fourrier 1995, Leverette et al. 1992, Sollner-Webb 1993). Although the role of snoRNAs in rRNA modification has been well defined, the biological significance of rRNA modifications remains unclear. 2'-O-ribose methylation and base pseudouridylation may be
involved in fine-tuning the folding of elongating pre-rRNA and in modulation of ribosomal protein binding (Maden 1990a, Maden 1990b, Maxwell and Fournier 1995, Bachellerie and Cavaillé 1997, Ofengand and Bakin 1997). However, in spite of these argumentations and of the phylogenetic conservation of the rRNA modifications, antisense snoRNAs tested in yeast are dispensable for growth.

Our understanding of pre-rRNA processing and ribosome biogenesis in plants lags behind that in animals and yeast. The recent interest in studying snoRNAs in plants is mostly due to discovery that plants contain more snoRNAs than other eukaryotes and have evolved novel expression and processing strategies. The increased number of rRNA modifications, which will influence ribosome function, and the novel modes of expression might reflect the environmental conditions to which plants are exposed (Brown and Shaw 1998, Brown et al. 2003). Polyploidy and chromosomal rearrangements have generated multiple copies of snoRNA genes, leading to the generation of new snoRNAs. The large snoRNA family in plants is therefore becoming an ideal model for investigation of mechanisms of evolution of gene families in plants (Brown et al. 2003).

In this paper we demonstrate that under certain in vitro (salt and temperature) conditions rRNA aggregation occurs in female inflorescences but not in leaves or pollen RNA preparations from hazelnut (Corylus avellana L.). Hazelnut, besides being a species of economic interest, represents a biological system particularly attractive for analysis of female structure differentiation and development, due to the peculiar features of its reproductive biology (Germain 1994, Thompson 1979).

This paper describes experiments addressing an explanation of rRNA aggregate formation in vitro. Evidence is provided that snoRNAs participate in rRNA aggregate formation and that level of accumulation of three tested snoRNAs, R1, U14 and U3, is much higher in female inflorescence than in leaves or pollen of hazelnut.

**Results**

**Presence of a factor inducing macromolecular rRNA aggregates in hazelnut female inflorescences**

In an attempt to identify novel transcripts involved in hazelnut floral development, total RNA was extracted from pollen grains, female inflorescences or leaves of two cultivars: cv. TR and cv. SG. The RNAs were electrophoresed either on agarose-formaldehyde denaturing gels according to standard procedures (Fig. 1A) or on non-denaturing agarose gels prewarming the samples to 65°C in a saline buffer (RN, see Materials and Methods) (Fig. 1B). In Fig. 1A the major rRNA species, 25S and 18S, are visible from all different tissues. In a non-denaturing gel, 25S and 18S rRNAs formed distinct bands from pollen grains (Fig. 1B, lanes 1 and 2) and leaves (Fig. 1B, lanes 3 and 5). In contrast, smear and accumulation of ethidium-bromide-stained material in the wells was detectable with the RNAs extracted from the female inflorescences instead of 25S and 18S bands (Fig. 1B, lanes 4 and 6). Because migration of small molecular weight RNAs did not appear to be affected in all samples we excluded that the anomalous migration of 25S and 18S was due to an artefact of RNA extraction.

To understand the nature of the phenomenon leading to changes in the migration patterns of 25S and 18S rRNA from the female inflorescences, we processed the samples in different ways before electrophoretic analysis on non-denaturing gels (Fig. 2). In Fig. 2, lanes 1 and 2, total RNAs extracted from the female inflorescences of cv. SG were resuspended in water and prewarmed to 37°C or 65°C, respectively, before loading. In Fig. 2, lanes 3 and 4, the RNA samples were resuspended in RN buffer, and prewarmed as above. Distinct bands corresponding to 25S and 18S rRNA were detectable in the RNA samples resuspended in water and prewarmed at either temperatures (Fig. 2, lanes 1 and 2) and in the RNA sample resuspended in RN buffer and prewarmed to 37°C (Fig. 2, lane 3). In contrast, a “shift” of RNA material in the well was visible upon prewarming to 65°C of the RN-resuspended sample (lane 4). This result suggested the existence of factor(s) responsible for
rRNA shift, which might be activated by prewarming to 65°C in the presence of salts.

In order to verify the activity of the factor(s) on rRNA derived from different organisms, the RNAs extracted from the female inflorescences of cv. SG were incubated with total RNAs purified from the yeast *Saccharomyces cerevisiae* in 1 : 1 molar ratio, using the above mentioned conditions (Fig. 2, lanes 5–8). Incubation at 65°C in RN buffer resulted in shift of *S. cerevisiae* 26S and 18S rRNA together with the plant major rRNAs (Fig. 2, lane 8). The shift did not occur with *S. cerevisiae* RNAs alone although in the presence of RN buffer and after prewarming to 65°C (Fig. 2, lane 9). Consistent with the above described results the rRNA shift was not apparent either in water-resuspended samples at 37°C or 65°C (Fig. 2, lanes 5–8). The shift did not occur with *S. cerevisiae* RNAs alone although in the presence of RN buffer and after prewarming to 65°C (Fig. 2, lane 9). Consistent with the above described results the rRNA shift was not apparent either in water-resuspended samples at 37°C or 65°C (Fig. 2, lanes 5–8, respectively), or in RN-resuspended samples at 37°C (Fig. 2, lane 7). Similar rRNA shift was observed upon incubation with total RNAs extracted from shoots of different plant species including *Daucus carota*, *Nicotiana tabacum* and *Zea mays* (data not shown).

Kinetics of the phenomenon responsible for rRNA shift was studied by time course experiments (Fig. 3). RNA samples from the cv. SG female inflorescences were resuspended in buffer RN and prewarmed to 65°C for different times (0 to 10 min, Fig. 3A, lanes 1–8) before loading onto a non-denaturing agarose gel. Formation of higher molecular weight rRNA aggregates resulting from shifting increased progressively with time. At later times most of rRNA material appeared to be trapped in the wells. Apparently, most of the small molecular weight rRNAs were not involved in the aggregate formation. To verify the specificity of rRNA aggregates, the RNAs were blotted onto a Nylon membrane and hybridised to a 25S-18S rRNA specific probe (B). Specific signals were detectable either in non-shifted or shifted rRNA. However, the intensity of hybridisation signals decreased with formation of progressively higher molecular weight rRNA aggregates (Fig. 3B, lanes 1–8). We verified that the aggregates blotted onto the membrane, although with decreased efficiency. Therefore, the dramatic decrease hybridisation efficiency of aggregated rRNA was possibly due to the sequestration of single-stranded regions. More unlikely, incubation at 65°C resulted in degradation of rRNA.

To rule out this possibility, we performed an experiment using an in vitro produced [32P]-labeled 18S rRNA (Fig. 4).
The labeled 18S rRNA (Fig. 4, lane 1) was incubated in buffer RN in the presence of equivalent amounts of total RNA from cv. SG female inflorescences, and prewarmed to 65°C for 30 s to 10 min (Fig. 4, lanes 2–6). Slow migrating 18S rRNA molecules and macromolecular aggregates in the wells accumulated with time. Aggregate formation was dependent on the presence of cv. SG RNA, as prewarming to 65°C for 10 min of the labeled 18S rRNA alone did not result in aggregate formation (Fig. 4, lane 1). This result confirmed that aggregate formation was not an intrinsic property of 18S rRNA, and, therefore, was not due to an artefact, subsequent to rRNA molecular refolding, but was dependent on the presence of (a) factor(s) in cv SG RNA preparation.

Characterisation of the factor(s) inducing macromolecular rRNA aggregates

To gain information about the nature of the factor(s) inducing macromolecular rRNA aggregates, the cv. SG female inflorescence RNA samples were pretreated either with proteinase K or pronase before performing the incubation with yeast S. cerevisiae RNA, according to the procedure followed in the experiment in Fig. 2. Pretreatment with pronase did not result in loss of activity of the factor(s) inducing aggregates (Fig. 5A, lanes 9–10). On the contrary, proteinase K partially disrupted the activity (Fig. 5A, lanes 7–8). This finding was due to minimal contamination of the proteinase K preparation with RNase (data not shown). The activity disappeared after treatment with either RNase T1 (Fig. 5B, lane 3) or RNase A (data not shown).

In an attempt to purify the rRNA-aggregate-forming factor(s), total RNA from the cv SG female inflorescence was extracted from female inflorescences of hazelnut cv SG was resuspended in buffer RN pretreated with either proteinase K or pronase before performing the incubation with yeast S. cerevisiae RNA, according to the procedure followed in the experiment in Fig. 2. Pretreatment with pronase did not result in loss of activity of the factor(s) inducing aggregates (Fig. 5A, lanes 9–10). On the contrary, proteinase K partially disrupted the activity (Fig. 5A, lanes 7–8). This finding was due to minimal contamination of the proteinase K preparation with RNase (data not shown). The activity disappeared after treatment with either RNase T1 (Fig. 5B, lane 3) or RNase A (data not shown).

In an attempt to purify the rRNA-aggregate-forming factor(s), total RNA from the cv SG female inflorescence was...
fractionated on a sucrose gradient (Fig. 6A). Collected fractions were assayed using yeast *S. cerevisiae* as above described (Fig. 6B). The activity was found in fractions migrating in lower density zone of the gradient, in the range of the small molecular weight RNAs. Because these fractions might, in principle, contain the snoRNAs, which are involved in rRNA processing and modification and possess long complementarities to mature rRNAs (Brown and Shaw 1998), we investigated the presence of a plant box C/D snoRNA, snoR1, in the collected fractions. snoR1 is involved in 2'-O-ribose methylation of a guanine residue (2781) of the mature 25S maize rRNA (Barneche et al. 2000, Leader et al. 1997). To this purpose, a Northern blot experiment was performed by analyzing the individual fraction on an acrylamide-urea denaturing gels using as a probe a 32P-labeled oligonucleotide complementary to snoR1 (Fig. 6C). A hybridisation signal was evident maximally in the fraction with the peak activity.

To obtain further evidence that snoRNAs were involved in formation of rRNA aggregates, we developed an alternative assay to the non-denaturing agarose gel, using sucrose gradients. In Fig. 7A, total RNA from the cv SG female inflorescence was fractionated after prewarming treatment to either 65°C for 10 min in buffer RN (open circles) or to 37°C (closed circles). The absorbance of collected fractions was determined at 260 nm as in Fig. 6A. (B) Pools of five consecutive fractions from the two gradients (before prewarming RNA samples to either 37°C or 65°C) were electrophoresed on a 6% acrylamide-8M urea denaturing gel, blotted onto Nylon membrane and hybridized to a [32P]-labeled oligonucleotide complementary to snoR1 or to tRNAVal. (Fig. 7B).
nals to either probes were maximally present in the same fractions corresponding to the lower density zone of the gradient. In contrast, in the sample prewarmed to 65°C, the hybridisation signal to snoR1 occurred in the single large peak in the higher density, whereas the hybridisation signal to tRNA Val remained in the fractions corresponding to the lower density zone. Similar results were obtained with other tested snoRNAs, U14 and U3, which are needed for the initial 5-external spacer cleavage and the subsequent cleavages that give rise to the 18S small subunit (Maxwell and Fournier 1995, Smith and Steitz 1997, Tollervey and Kiss 1997). These findings were consistent with the hypothesis that snoRNAs participate in rRNA aggregate formation.

As snoRNAs seem to play an essential role in the cell metabolism and are therefore ubiquitous, we believed that the phenomenon of rRNA aggregate formation was so pronounced in plant female inflorescences because of the presence of very high amounts of snoRNAs. To test this hypothesis, the amounts of several snoRNAs were measured by slot blot in different plant tissues. In Fig. 8 a representative experiment with a snoR1-specific probe is shown. Quantitative analysis confirmed the presence of snoR1, U14 and U3 in the cv. SG female inflorescence, in amounts 15- to 30-fold higher than in leaves and pollen grains (Table 1 and data not shown). This finding was consistent with lack of aggregate formation with RNA extracted from leaves and pollen grains.

**Discussion**

In this paper we demonstrate the existence of factor(s) inducing rRNA aggregate formation in hazelnut female inflorescences in vitro, and we have biochemical evidence that this factor(s) is associated with a spectacular accumulation of snoRNAs in these reproductive structures.

snoRNAs involved in rRNA modifications function through direct base-pairing interactions with pre-rRNA. These interactions are possibly responsible for the apparent shifting of the rRNA aggregates evidenced by us in hazelnut female inflorescences RNA preparations using non-denaturing agarose gels (Fig. 1, 2, 3A). Decreased hybridisation efficiency of aggregated rRNA to specific rRNA probes is likely due to the sequestration of single-stranded regions (Fig. 3B).

Further evidence that antisense snoRNAs are involved in formation of rRNA aggregates was obtained with sucrose gradients. Indeed, the rRNA aggregation factor(s) activity was recovered in the same collected fractions of the gradients where a box C/D antisense snoRNA, snoR1 was found (Fig. 6). Moreover, snoR1, U14 and U3 were detected in the rRNA aggregates (Fig. 7). It is reasonable that, in addition to these, other snoRNAs may participate in the formation of the aggregates. Likely candidates should be snoRNAs having complementarity to both 18S and 28S rRNA.

U14 and U3, which are involved in pre-rRNA spacer cleavage (Brown et al. 2001, Maxwell and Fournier 1995, Smith and Steitz 1997, Tollervey and Kiss 1997), accumulate in the hazelnut female inflorescences (Fig. 8, Table 1). The cells in the inflorescences are actively dividing, at variance with those of leaves and pollen grains. This could be easily tested, for instance by analyzing root tips. Therefore, the metabolic activity of inflorescences is likely to be much higher than in pollen or leaf cells. As a consequence, higher levels of these snoRNAs may reflect a demand for greater ribosome production. Tissue- and development-dependent regulation of snoRNAs has been also reported in other organisms, for instance in *Xenopus* oocytes (Cecconi et al. 1994). A nucleolar U3 ribonucleoprotein is highly expressed in the heart and neural tube by 36 h of avian development, at a time when these organs are rapidly developing (Adamson et al. 2001).

**Table 1 snoRNAs levels in different hazelnut tissues**

<table>
<thead>
<tr>
<th>Plant tissue</th>
<th>snoR1 *</th>
<th>U14 *</th>
<th>U3 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Pollen grains</td>
<td>0.35</td>
<td>0.42</td>
<td>0.39</td>
</tr>
<tr>
<td>Female inflorescences</td>
<td>14.5</td>
<td>23.70</td>
<td>19.64</td>
</tr>
</tbody>
</table>

* Relative amounts of each snoR1, U14 and U3 were determined in the different plant tissues by slot blot experiments. Plant RNAs (1 µg) were hybridized to [32P]-labeled oligonucleotides complementary to snoR1, U14 and U3 (Fig. 8 and Materials and Methods). Quantitative data were obtained by densitometric analysis of autoradiograms. Values obtained with leaves for each probe were arbitrarily set to 1.00.
The biological significance of accumulation of snoR1, an antisense snoR1 involved in 2'-O-ribose methylation of a conserved G residue in the 25S rRNA of plant species (Barneche et al. 2000, Leader et al. 1997) in the inflorescences (Fig. 8) is an open question. Addressing this question is difficult as the actual role of rRNA modifications in cell metabolism is unclear. On the basis of their postulated involvement in folding of elongating pre-rRNA and in modulation of ribosomal protein binding, it has been proposed that rRNA modification might provide an additional level of regulation of gene expression (Bachelierie and Cavaillé 1997, Brown et al. 2003, Maden 1990a, Maden 1990b, Maxwell and Fournier 1995, Mesi and Iwabuchi 1995, Ofengand and Bakin 1997). If this hypothesis was correct, differential accumulation of snoRNAs during the development of the reproductive structures might have functional consequences. In the light of this hypothesis, fluorescence in situ RNA hybridization experiments have been planned to identify the specific cellular types (somatic or germinal) in the hazelnut female inflorescences where the snoRNAs accumulation occurs and to define the precise timing of this phenomenon. Efficient procedures have been recently developed to detect and to quantify snoRNAs in living cells using linear fluorochrome-labeled 2'-O-methyl oligoribonucleotides (Molenaar et al. 2001). In addition, primer extension analyses as described by Maden et al. (1995) and Bakin and Ofengand (1993) will be used in the future to correlate snoRNAs accumulation to the pattern of rRNA 2'-O-ribose methylation and pseudouridylation. Examining the pattern of rRNA modifications in different conditions of cell growth and differentiation and/or in different cells (somatic or germinal cells) might help to clarify their actual physiological role.

Many plant snoRNA genes show a different mode of organisation and expression distinct from that of homologous animal genes. In Arabidopsis thaliana U3 is encoded by three different genes transcribed by RNA polymerase III, whereas in animals the U3 genes are transcribed by RNA polymerase II (Kiss et al. 1991). In maize U14 is encoded by multiple genes tightly clustered with other snoRNAs, including snoR1, U49, snoR2 and snoR3 (Leader et al. 1997). In spite of the different mode of expression of the U14 and U3 genes in plants, the increase of these snoRNAs in hazelnut female inflorescences was about of the same extent (Table 1). The molecular basis of the co-regulated expression of these snoRNAs during the hazelnut development will be investigated in future studies.

Materials and Methods

Plant material

Two cultivars of hazelnut (Corylus avellana L., cv. San Giovanni [cv. SG] and cv. Tonda Romana [cv. TR]) located in Caserta (Italy), were used as experimental material. Plant samples (pollen grains, female inflorescences and leaves) were collected during different stages of their development. Shoots from Daucus carota, Nicotiana tabacum and Zea mays respectively, were collected from 5- to 6-day-old seedlings at 23°C in plastic pots containing sterile quartz sand under a 12 h photoperiod (2,000-4,000 lux, TLD 18 W/33 Philips, Eindhoven, The Netherlands).

All material was frozen in liquid nitrogen immediately after collection and extensively washed, and stored at –80°C until used.

Strains and plasmids

The yeast strain S. cerevisiae Sc57 rho0 was derived from Sc57 rho+ (S. cerevisiae YM654 α ura3-52, his3-Δ200, ade2-101, lys2-801) gift from Mark Johnston after ethidium bromide mutagenesis (Del Giudice et al. 1997).

The Esherichia coli strain DH 5α was used for plasmid amplification.

The recombinant plasmid pBG35 (Goldsbrough and Cullis 1981) containing the 25S-18S rRNA genes from Linum usitatissimum plant species, was used to obtain the rRNA gene specific probe. The pBG35–27 recombinant plasmid was constructed by inserting the 1,543 bp 18S rDNA EcoRI/KpnI fragment subcloned from pBG35 plasmid into pGEM-Z3I (+) vector plasmid (Promega).

Bacterial transformation and plasmid DNA purification were performed as previously described (Del Giudice 1981).

Media and culture conditions

S. cerevisiae was grown in glucose complete medium (3% glucose, 1% yeast extract) at 28°C as previously described (Massardo et al. 1990). Bacteria were grown in L-broth (Miller 1972) under constant aeration at 37°C.

RNA procedures

Total plant RNA was isolated by a phenol-lithium chloride small-scale procedure (Verwoerd et al. 1989). 1–2 g of tissue (FW) were ground in a mortar with liquid nitrogen. After grinding, 2.5 ml of hot (80°C) extraction buffer (Verwoerd et al. 1989) were added. The mixture was homogenised by vortex for 1 min and 1.25 ml of chloroform-isooamyl-alcohol (24 : 1) was added and vortexed. After centrifugation for 5 min, the water phase was removed and RNA precipitated overnight at –20°C mixing with one volume of 4 M LiCl. After collecting by centrifugation, RNA pellets were dissolved in 2 ml sterile water and precipitated with 0.1 volume 3 M sodium acetate plus 2 volumes of ethanol. After centrifugation, the RNA pellets were washed with 70% ethanol, dried, dissolved in sterile water to a concentration of 2 mg ml–1 and tested, or stored at –80°C.

Total yeast RNA was extracted by the freeze-thaw method as previously described (Manna et al. 1996).

Gel electrophoresis of RNA samples was performed in horizontal slab-gels either on agarose-formaldehyde denaturing gels or on non-denaturing agarose gels according to standard procedure (Sambrook et al. 1989).

RNA gel mobility shift was carried out diluting aliquots total RNA in a 30 µl final volume RN saline buffer [RN: 10 mM Tris-HCl (pH 5.5) 10 mM MgCl2, 1 mM DTT, 50 mM KCl]. RNA mixtures were incubated for 5–10 min at 65°C, ice chilled, then loaded on 0.8% agarose gel and electrophoresed in a Tris-acetate buffer (40 mM Trizma Base, 20 mM sodium acetate, 1 mM EDTA, pH 8.2) with 1 µg ml–1 ethidium bromide added, at 40 V for 3–4 h at room temperature. RNA bands were visualised by long wave length UV and photographed with a Polaroid MP-3 camera.

Total plant RNA was fractionated on a sucrose RNase free gradient, dissolving it in sterile distilled water at a concentration of 400 µg ml–1 and 0.5 ml aliquots of it were layered on a 15% to 40% sucrose gradient in water. The gradients were run at 4°C for 16 h at 36,000 RPM in an SW40 Spinco rotor. Approximately 50 fractions were collected by puncturing the bottom of the tubes and assayed for...
absorbance at 260 nm. The RNA from each fraction was precipitated with sodium acetate and ethanol.

Northern blot, RNA slot-blot and RNA/DNA-hybridisation were performed according to Sambrook et al. (1989).

**DNA and RNA probes**

The 8,600 bp 25S-18S rDNA BamHI fragment from pBG35 recombinant plasmid, was electrophoresed on agarose gel, eluted from the gel at 80 V for 1 h, extracted three times with phenol-chloroform-isooamyl alcohol, ethanol precipitated and utilised as probe. Labeling of DNA fragments with α-[^32P]dCTP was done with the random-primer DNA labeling kit (Amersham).

The synthetic oligonucleotides: 5'-GGAGGCTCAGAGTGTC-3', CTAAGGAAGGA TTAAACAGCACAAGCTCTCAAGTGAATCTGCTCAACGGAGGGAACAAAGTCCCTGCTCATACAA TCCAAGGAAGGA TTAAACAGCACAAGCT were used in the hybridization procedures.

5'-end labeling of synthetic oligonucleotide with T4 polynucleotide kinase and γ-[^32P]ATP was performed according to Sambrook et al. (1989).[^32P]18S rRNA was obtained by in vitro transcription of KpnI linearised pBG35-27 recombinant plasmid with T7 RNA polymerase according to the instructions of the supplier (Promega).

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