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Plant U13 orthologues and orphan snoRNAs identified by RNomics of RNA from Arabidopsis nucleoli

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ABSTRACT

Small nucleolar RNAs (snoRNAs) and small Cajal body-specific RNAs (scaRNAs) are non-coding RNAs whose main function in eukaryotes is to guide the modification of nucleotides in ribosomal and spliceosomal small nuclear RNAs, respectively. Full-length sequences of Arabidopsis snoRNAs and scaRNAs have been obtained from cDNA libraries of capped and uncapped small RNAs using RNA from isolated nucleoli from Arabidopsis cell cultures. We have identified 31 novel snoRNA genes (9 box C/D and 22 box H/ACA) and 15 new variants of previously described snoRNAs. Three related capped snoRNAs with a distinct gene organization and structure were identified as orthologues of animal U13snoRNAs. In addition, eight of the novel genes had no complementarity to rRNAs or snRNAs and are therefore putative orphan snoRNAs potentially reflecting wider functions for these RNAs. The nucleolar localization of a number of the snoRNAs and the localization to nuclear bodies of two putative scaRNAs was confirmed by *in situ* hybridization. The majority of the novel snoRNA genes were found in new gene clusters or as part of previously described clusters. These results expand the repertoire of Arabidopsis snoRNAs to 188 snoRNA genes with 294 gene variants.

INTRODUCTION

In eukaryotes and archaeobacteria, small nucleolar RNAs (snoRNAs) form an abundant group of non-coding

RNAs (ncRNAs) which act as guide RNAs to determine the sites of 2'-O-ribose methylation and pseudouridylation of ribosomal RNA (rRNA), spliceosomal small nuclear RNAs (snRNAs) and tRNAs (1–3 for reviews). There are two major structurally different families of snoRNAs: box C/D snoRNAs responsible for 2'-O-ribose methylation and box H/ACA snoRNAs responsible for pseudouridylation (1–3). Box C/D snoRNAs contain conserved sequences: box C (RUGAUGA) and D (CUGA) near their 5' and 3' ends, respectively, and one or two regions of complementarity to their cognate RNAs. Box H/ACA snoRNAs can be folded into stem-loop structures in the 5' and 3' halves of the RNA which are followed by the conserved internal box H (ANANNA) and the 3'-terminal box ACA (ACANNN). One or both of the stem-loops contain an internal loop sequence with two regions of complementarity to their target RNA flanking a uridine residue which is modified to pseudouridine (1–3). Each class of mature snoRNA is associated with four different core proteins required for stability and function of the snoRNP, although other proteins are required in snoRNP assembly (3,4). The core proteins fibrillarin (box C/D) and NAP57/Cbf5p (box H/ACA) are thought to confer methylase and pseudouridylation activities, respectively. A related class of RNAs are the small Cajal-body-specific RNAs (scaRNAs) which target modification of spliceosomal snRNAs. ScaRNAs contain conserved sequences and structures of box C/D and H/ACA snoRNAs but can have complex combinations of box C/D and H/ACA sequences and are retained in Cajal bodies (CBs) by virtue of CAB box sequences (5–8).

Processing of pre-ribosomal RNAs (pre-rRNAs) into 18S, 5.8S and 25/28S rRNAs involves a series of

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cleavage reactions and exonucleolytic trimming (9). A small number of snoRNPs are required for pre-rRNA cleavage reactions in humans and yeast: U3, U8, U14, U17, U22, MRP, snR10 and snR30 but the majority of snoRNPs determine the sites of nucleotide modifications of rRNA. Most of the ~200 2'-O-ribose methylation and pseudouridylation sites in higher eukaryotes are in the active site of the ribosome and are thought to influence the efficiency of the ribosome and protein translation (10–12). The introduction of modifications in non-natural positions impaired cell growth in yeast through less efficient processing and increased turnover of pre-rRNAs, and reduced ribosome activity (13,14).

In the majority of higher eukaryotes studied to date 'orphan' snoRNAs have been described which are expressed but do not have complementarity to rRNA or snRNAs (15–26). Many of these orphan snoRNAs have potential mRNA targets suggesting other functions besides modifying rRNAs and snRNAs. Of particular interest are tandem arrays of orphan snoRNAs in a maternally imprinted region (IC-SNURF-SNRPN) of the human genome, many of which are conserved in other mammals. Some of these snoRNAs have brain-specific expression and loss of paternal expression of genes in this region is associated with developmental and behavioural problems (15,16). One of the genes, HBII-52, has complementarity to a brain-specific serotonin 2C receptor (5HT_{2C}R) and may affect an RNA editing event in the mRNA (16,27). In addition, HBII-52 influences alternative splicing of the serotonin 2C receptor by blocking a splicing silencer sequence to promote inclusion of an alternative exon (28). Computational analysis of putative mRNA targets of human orphan snoRNAs shows a significant preference to exon sequences and association with alternatively spliced genes suggesting a role in modulating alternative splicing of many mRNAs (25). More recently, two examples of snoRNAs being processed to microRNAs (miRNAs) have been described (29,30). A human box H/ACA snoRNA is processed by Dicer to generate small RNAs which were associated with Argonaute proteins and caused reduced expression of gene targets (29). Similarly, snoRNAs in the ancient eukaryote, *Giardia lamblia*, were processed to miRNAs capable of translational repression of target mRNAs (30). Finally, mining of small RNA libraries from different organisms including *Arabidopsis* has identified numerous snoRNA-derived small RNAs which are associated with components of RNA silencing pathways (31). Thus, orphan snoRNAs can modulate mRNA expression by directly affecting alternative splicing or being processed to miRNAs or sRNAs with the potential to base-pair with specific target mRNAs.

Identification of snoRNAs in plants has concentrated on the model species *Arabidopsis* and rice. Computational prediction of plant box C/D snoRNAs showed a high degree of sequence diversity in primary gene sequence and in the frequency of gene variants, and a number of snoRNAs specific to plants were identified (17,18,22,32). The gene variants usually show some sequence diversity outside of the conserved box C and D sequences and the

regions of complementarity to rRNA and snRNAs (18,33). The occurrence of gene variants reflects either major chromosomal duplication or rearrangements following hybridization or polyploidization (17,18,32,34). In contrast, very few box H/ACA genes have been identified by prediction (18) and our current knowledge of *Arabidopsis* box H/ACA snoRNAs is based largely on the molecular cloning of small ncRNAs from *Arabidopsis* seedlings (21). This RNomics approach identified 39 box H/ACA genes as well as novel box C/D snoRNAs, and the first plant scaRNAs (21). Recently, mining of *Arabidopsis* small RNA sequences identified 31 new snoRNA genes including scaRNAs (35).

One of the main features of plant snoRNA genes is that the majority are organized into polycistronic gene clusters (17,18,22,32,34,36). Plant polycistronic clusters are transcribed as a polycistronic precursor snoRNA (pre-snoRNA) which is then processed to release mature snoRNAs. Processing is thought to involve endonucleolytic activity followed by exonucleolytic trimming to produce the mature snoRNA (32,36). The detection of polycistronic precursor snoRNAs in CBs and the nucleolus by *in situ* hybridization suggests that processing occurs in both locations and/or that pre-snoRNAs traffic to the nucleolus via CBs (37).

RNomics approaches for analysing small RNA constituents have been successful in a number of species and provide a means of validating computational identification of small RNAs (19,21). We have previously used isolated nucleoli from *Arabidopsis* cell cultures to show the presence of exon junction complex proteins and mRNAs, and aberrant mRNAs in the nucleolus (38,39). Due to the nucleolar localization of snoRNAs and the processing patterns of plant pre-snoRNAs, we have used RNA from purified nucleoli to construct cDNA libraries to identify capped and uncapped snoRNAs and expressed orphan snoRNAs. The majority of the cDNAs isolated were full-length snoRNAs and allowed the identification of 31 novel box C/D and box H/ACA snoRNA genes including a U13 orthologue and eight putative orphan snoRNAs.

MATERIALS AND METHODS

cDNA libraries for small RNAs from the *Arabidopsis* nucleoli

Nucleoli were isolated from *Arabidopsis* Col0 cell cultures as described previously (38). Total RNA was extracted from isolated nucleoli using an RNeasy kit (Qiagen). The RNA was then 3' polyadenylated using the poly(A) kit (Invitrogen) according to the manufacturer's instructions. In brief, 1 µg RNA was incubated in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM EDTA, 1 mM DTT, 1 mM ATP and 5 units *Escherichia coli* poly A polymerase (Invitrogen) for 8 min at 37°C. Three different cDNA libraries were generated using 250 ng polyadenylated RNA using the GeneRacer system (Invitrogen) following the manufacturer's instructions. (i) RNA was dephosphorylated by calf intestinal alkaline phosphatase

and then 5' decapped by tobacco acid pyrophosphatase prior to ligation to RNA oligonucleotide adaptor (5'CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA3') by *E. coli* RNA ligase to specifically enrich for capped RNAs. (ii) RNA was 5' decapped prior to oligo adaptor ligation as above to clone both capped and uncapped RNAs. (iii) RNA was directly ligated to the RNA oligonucleotide adaptor to enrich for uncapped RNAs. First-strand cDNA was generated using an oligo(dT)-adaptor primer [5'GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)₁₈₋₂₄] and Superscript III RT. The second strand cDNA was generated using primers specific to 3' and 5' oligonucleotide adaptor sequences and Platinum Taq polymerase High Fidelity (Invitrogen). cDNA fragments smaller than 400 bp were eluted from agarose gels, inserted into the pCR4 TA-cloning vector (Invitrogen) and sequenced.

Northern analysis and RT-PCR for snoRNA clusters

Total RNA was extracted from cultured cells and *Arabidopsis* rosette leaves using TRI-reagent (Invitrogen) according to the manufacturer's protocol. Twenty micrograms RNA was fractionated on 8% polyacrylamide gels containing 8 M urea, transferred to nylon membrane and probed by DIG-uridine-labelled riboprobes. The membrane was washed and visualized by chemiluminescence (Roche). For RT-PCR, first-strand cDNA was produced using 5 µg of whole-cell RNA and oligo-dT₂₀ primer and Superscript III RT. One-twentieth of the mixture was taken for generation of second-strand cDNA by cycling reactions using Platinum Taq polymerase High Fidelity (Invitrogen) and the primers specific to up- and downstream snoRNA clusters. The reaction mixture was fractionated on 1.2% agarose gel and analysed.

Determination of putative modification sites

Putative modification sites were determined for novel box C/D snoRNAs by searching for complementarity of sequences upstream of the D or D' boxes to *Arabidopsis* rRNA and snRNA sequences using BLAST (40). For box H/ACA genes, the sequences were folded using MFOLD (41) and putative pseudouridylation pockets compared against rRNA and snRNA sequences. Orthologues of snoRNA genes were identified by BLAST, displayed and aligned using Clustal on Jalview (42) and conserved sequences identified.

RNA probe preparation for *in situ* hybridization

Templates for *in vitro* transcription were generated by PCR using T3-5' and T7-3' primers to add promoter sequences for T3 and T7 polymerases, respectively, to the sequences of interest. Primer sequences were: T3-5' adapter—GAATTAACCCCTCACTAAAGGGAGGACA CTGACATGGACTGAAGGAGTA and T7-3' adapter—TGTAATACGACTCACTATAGGGCGCTACGTAAC GGCATGACAGTG. Probes were prepared by *in vitro* transcription (43). PCR was performed with the following

cycles: 94°C for 3 min; then 30 cycles of 94°C for 45 s, 63°C for 45 s and 72°C for 1.5 min; and a final extension of 72°C for 6 min. *In vitro* transcription, using 1 in 10 dilution of the PCR product, was performed for 2 h at 37°C in the presence of digoxigenin-UTP nucleotides (0.35 mM) (43). Hydrolysis was performed immediately in 100 mM carbonate buffer, pH 10.2, at 60°C for 30 min and the products were precipitated in 2.5 M ammonium acetate and three volumes of 100% ethanol for 1 h at 48°C. The product was pelleted by centrifugation at 4000 rpm for 30 min, and the pellets were resuspended in 30 µl of 100 mM Tris, 10 mM EDTA buffer. Probe label incorporation was checked by dot-blotting (43).

Specimen preparation and *in situ* hybridisation

Four-day-old *Arabidopsis* Col0 seedlings were fixed in 4% formaldehyde solution, freshly prepared from paraformaldehyde (Sigma-Aldrich, Gillingham, UK, Gillingham, UK) in TBS (TBS: 10 mM Tris, 140 mM NaCl, pH adjusted to 7.4 with HCl) containing 0.1% Igepal CA-630 (Sigma-Aldrich, Gillingham, UK) for 1 h at room temperature. Penetration of fixative throughout the root was ensured by vacuum infiltration, prior to the incubation. Seedlings were washed several times in TBS to remove fixative, then the root tips were laid across wells of multi-well slides pre-treated with aminopropyltri-ethoxysilane (APTES—Sigma-Aldrich, Gillingham, UK), and the rest of the seedling was excised with a razor blade. The root tips were allowed to dry for several hours or overnight before being treated with a cell-wall degrading enzyme mixture consisting of 1% driselase (Sigma-Aldrich, Gillingham, UK), 0.5% cellulase (Onozuka R10, Yakult, Japan) and 0.025% pectolyase Y23 (Duchefa Biochemie, Haarlem Netherlands) in TBS for 15 min at room temperature. Then the roots were washed several times with TBS alone.

The digoxigenin-labelled RNA probes were diluted (1 in 20) into a hybridisation mix containing 50% formamide (Sigma-Aldrich, Gillingham, UK), 10% dextran sulphate (Sigma-Aldrich, Gillingham, UK), 1 mg/ml tRNA (Sigma-Aldrich, Gillingham, UK), 1× Denhardtts solution (Sigma-Aldrich, Gillingham, UK), 0.33 M NaCl, 0.01 M Tris-HCl, 0.01 M NaPO₄ and 5 mM EDTA (pH 6.8), then denatured at 80°C for 2 min, before being cooled on ice. Twenty microlitres of probe was applied to each well and slides incubated in a humid chamber at 37°C overnight. Slides were washed sequentially in 2× SSC (SSC: 20× stock solution consists of 3 M sodium chloride and 300 mM trisodium citrate, adjusted to pH 7.0 with HCl) at room temperature for 10 min, 2× SSC/50% formamide at 45°C for 15 min, 1× SSC/50% formamide at 45°C for 15 min and 2× SSC at room temperature for 5 min. A blocking solution of 3% Bovine Serum Albumin (Sigma-Aldrich, Gillingham, UK) in TBS was applied for 15 min, followed by a primary antibody solution containing monoclonal mouse anti-digoxin (Sigma-Aldrich, Gillingham, UK) diluted 1 in 5000 in blocking solution. Slides were incubated at room temperature for 90 min, washed 3 × 5 min in TBS

and a second antibody solution containing goat anti-mouse IgG Alexa Fluor 488 (Invitrogen) diluted 1 in 200 in blocking solution was applied and incubated for 90 min at room temperature. Slides were washed in TBS, 3 × 10 min and counterstained with DAPI (4',6-diamidino-2-phenylindole: Sigma-Aldrich, Gillingham, UK) (1 µg/ml TBS) for 5 min. Finally slides were washed for 5 min in TBS and mounted in Vectashield (Vector Laboratories Ltd, Peterborough PE2 6XS, UK).

Microscopy

Slides were viewed using a 60× objective (NA 1.4, oil immersion) on a Nikon Eclipse 600 epifluorescence microscope equipped with a Hamamatsu Orca ER cooled CCD digital camera, a motorized xy stage and a z-focus drive. Raw data stacks were deconvolved using AutoDeBlur and Autovisualise software version 9.3 (Autoquant, MediaCybernetics, Marlow, Buckinghamshire, UK). The deconvolved data stacks were then analysed with ImageJ (a public domain program by W. Rasband available from <http://rsb.info.nih.gov/ij/>). Final figures were prepared using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

RESULTS

Isolation of full-length snoRNAs from total RNA of isolated nucleoli

The mode of expression of different small RNAs determines whether transcripts are likely to be capped or uncapped. For example, plant snRNAs, and U3 and MRP snoRNAs are transcribed from their own promoters by RNA polymerase II or III and are capped (44). Most plant snoRNAs are transcribed as polycistronic pre-snoRNAs which are processed to generate mature box C/D and H/ACA snoRNAs and are expected to be uncapped (34,36). Similarly, dicistronic tsnoRNAs (tRNA-snoRNA) are processed to generate uncapped variants of snoR43 (45,46). To identify capped and uncapped snoRNAs and other small RNA transcripts from nucleoli, three different small RNA cDNA libraries were constructed from total RNA isolated from purified nucleoli. The first library was enriched for capped RNAs by treating the total nucleolar RNA with phosphatase to remove 5' phosphates before treating with decapping enzyme prior to first strand synthesis with reverse transcriptase and cDNA production. The second library was enriched for uncapped RNAs by carrying out cDNA synthesis directly on the total RNA. The third library was designed to contain a mixture of both capped and uncapped RNAs by treating the total nucleolar RNA with decapping enzyme before proceeding to cDNA synthesis. The three libraries are called 'capped', 'uncapped' and 'capped-uncapped' and 380, 254 and 604 clones were fully sequenced from them, respectively (total of 1238 clones sequenced) (Figure 1).

The main RNA constituents of the capped library were small nuclear RNAs (U1, U2, U4 and U5snRNA variants) and capped snoRNAs (Figure 1A). The capped snoRNA population contained a single U3snoRNA

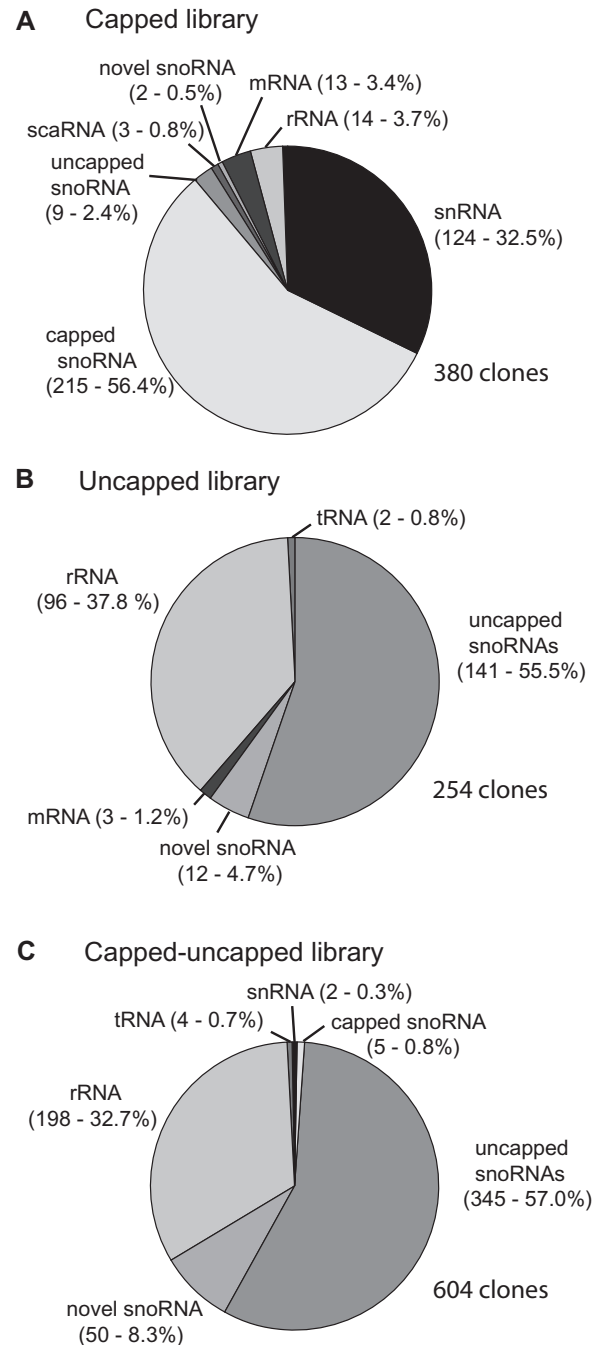


Figure 1. Classes of small RNAs isolated in cDNA libraries from nucleolar RNA. The number of clones and relative abundance (expressed as a percentage) are given in brackets for the different classes of small RNAs isolated in the capped (A), uncapped (B) and mixed capped-uncapped (C) libraries.

variant and multiple copies of three other snoRNAs (snoR105, snoR108 and snoR146). The latter three snoRNAs made up more than half of the clones from the capped library suggesting that these snoRNAs are capped. In addition, 63.3% of the clones were snoR146, 33.3% were snoR108 and only 3.3% were snoR105 suggesting that the abundance of these snoRNAs varies greatly with snoR146 being the most abundant in

Arabidopsis cell cultures. The low recovery of U3snoRNAs may reflect cloning bias due to the RNA size fractionation. The capped library also contained two clones of snoR102 and one of snoR109. SnoR102 is a scaRNA and snoR109 is an orphan snoRNA with no known target RNA, identified by Marker *et al.* (21). Only a small number of uncapped snoRNAs (including some novel snoRNAs) and rRNA fragments were isolated confirming the enrichment of this library for capped RNAs (Figure 1A).

The composition of the uncapped RNA library was distinctly different from that of the capped library (Figure 1B). The two major fractions were uncapped snoRNA variants and rRNA fragments. The rRNA fragments were from many different regions of the rRNA suggesting that they are breakdown products of rRNA degradation or artefacts of cloning. No capped small RNAs were isolated in the uncapped library. One clone each of variants of two orphan snoRNAs, snoR28-1c (C/D) and snoR110-1 (H/ACA) were obtained. To be able to compare the capped with the uncapped library, we also generated a mixed library by decapping before cDNA synthesis. This capped-uncapped library also had uncapped snoRNAs and rRNA fragments as the two major fractions, but contained two clones of capped snRNAs and five of capped snoRNAs including MRP (Figure 1C). Thus, although the capped snoRNAs are the major constituent of the capped library, their abundance on the basis of clone representation in the capped-uncapped library is probably of a similar order to the majority of uncapped snoRNAs. Taking all three libraries together, 134 snoRNA variants of 90 different snoRNA genes were cloned in this study. The majority of these variants are expressed from polycistronic gene clusters and thereby form a major fraction of the uncapped and capped-uncapped libraries. The vast majority of the snoRNA clones of previously identified snoRNAs were consistent with the sizes of computationally predicted gene sequences such that most sequences generated here represent full-length snoRNA sequences.

Novel snoRNAs

From the analysis of nucleolar RNAs we have identified 31 new Arabidopsis snoRNA genes (38 variants) and 15 new variants of known snoRNA genes (Table 1). Of the 31 new genes, 19 were obtained by direct cloning from nucleolar RNA of which 16 represented novel snoRNAs and a further three represented full-length clones of previously identified small RNA fragments with no known sequence motifs [(21) Supplementary Table S1]. By analysing the genomic sequences flanking all of these genes and determining their gene cluster organization, we were also able to predict the full-length sequences of 12 other new genes of which four were novel snoRNA genes and eight corresponded to partial sequences of small RNAs to which no function was assigned (21) (Table 1). We also isolated full-length clones of 22 snoRNA genes previously identified as partial sequences (21) for which only partial sequences were available, and by BLAST

analyses identified 14 previously unidentified variants of these genes. For example, snoR145 was cloned here and corresponded to the unknown sequence, Ath-122 (21). In the flanking regions of snoR145, other genes—snoR68, snoR159 and two copies of snoR135 were identified (Figure 2A). SnoR159 and snoR135 also corresponded to short RNA sequences with no known motifs, Ath-319 and Ath-118 (21), and upstream of snoR68 another putative box H/ACA gene (snoR157) was identified (Figure 2A). Similarly, two snoR88 gene variants were found upstream of two previously identified gene clusters containing snoR19, snoR20 and snoR38Y (18) (Figure 2B). In addition, snoR64 was found in the first cluster and between snoR19-1 and snoR64, another putative box H/ACA gene was predicted (snoR136). Thus, the cloning of novel snoRNAs and examination of flanking sequences has defined novel gene clusters or extended previously described gene clusters (18) (Figure 2A and B; Supplementary Figures S1 and S2). Although the majority of the new genes were in gene clusters, snoR151 and three variants of snoR155 were found in introns of ribosomal protein genes (Figure 2C; Supplementary Figure S3), and four of the novel genes appeared to be single genes. Recently, 31 new Arabidopsis snoRNA genes (44 gene variants) were identified from assembly of high throughput short sequence reads (35). Comparison of these genes with the 31 novel genes (38 variants) and 15 new variants of known snoRNAs obtained here from direct cloning and predictions from gene organization, only 12 genes (15 variants) were common to both studies (Table 1). In addition, we identified a second variant of snoR137 (35). We have adopted the snoRNA gene numbers of Chen and Wu (35) and used the next consecutive numbers for our novel genes. From previous studies (17,18,21,32) along with this study and that of Chen and Wu (35), to date, Arabidopsis contains 188 different snoRNA/scaRNA genes with 294 gene variants.

The majority of the novel snoRNAs identified here had orthologues in other plant species (identified by BLAST searches of plant ESTs and genomic sequences) providing evidence that the cloned sequences represented *bona fide* snoRNAs (Table 1). The alignment of orthologous gene sequences and secondary-structures predicted by MFOLD (Supplementary Figure S4) aided the identification of putative modification sites in rRNA and snRNAs for the majority of the novel box C/D and H/ACA snoRNA genes (Table 1; Supplementary Figures S5 and S6, respectively). Similarly, putative modification sites in 25S rRNA and U6snRNA were found for snoR111 and snoR112, respectively (Supplementary Figure S6) which were identified previously as orphan box H/ACA snoRNAs (21). The modification site for snoR112 (U6 position 35) corresponds to the site modified by ACA12 and HBI-100 in human U6 (position 40). However, we were unable to identify complementarity to rRNA or snRNA for the box C/D snoRNAs—snoR149 and snoR133, and the box H/ACA snoRNAs—snoR145, snoR157-snoR159, snoR163 or snoR164. These eight snoRNAs therefore represent putative orphan snoRNAs (Table 2).

Table 1. Novel snoRNAs and snoRNA gene variants

snoRNA ^a	Size (nt)	RNA class	Modification site	Rep. clone ^b	Gene copy	Chr ^c	Yeast/human ^d	Organization	Orthologues—representative ESTs
<i>Novel box C/D snoRNAs</i>									
AtsnoR146 (U13)	127	capped C/D	Unknown	At304	1	1	—	Single gene	ESTs: monkey flower
AtsnoR147	83	C/D	D 25S Cm1471 13bp	At502	1	1	Not modified	Cluster: snoR163/snoR147/snoR95	ESTs: tobacco, chicory, oat
AtsnoR148	84	C/D	D 25S Cm2673 18bp	At649	1	5	Not modified	Single gene	Genomic: Poplar ESTs: tomato, <i>Nicotiana benthamiana</i> , Asparagus, etc.
AtsnoR149	80	C/D	Unknown	At870	1	2	—	Cluster: snoR158a/snoR39BYa/snoR21a/snoR149	ESTs: poplar, safflower, barley, cocoa
AtsnoR116	84	C/D	D' 18S Um123 11bp	At938	1	1	Mgh18S-121; Z17B	Cluster: snoR117/snoR116/snoR85a/snoR85b	ESTs: apple, Jerusalem artichoke, dandelion
AtsnoR117	72	C/D	D' 25S Um2103 12bp	At359	1	1	Not modified	Cluster: snoR117/snoR116/snoR85a/snoR85b	ESTs: lettuce, grape, cotton, etc.
AtsnoR118	98	C/D	D' 25S Cm2187 12bp	Ath-19b— predicted	1	3	snR76(Y); HB-II 180A,B,C	Cluster: snoR161/snoR66/snoR118	Genomic: poplar; ESTs: lettuce, Jerusalem artichoke
AtsnoR120	72	C/D	D 25S Um 44 12bp	At838; Ath-475 Predicted	1	5	Not modified	Cluster: snoR133/snoR120	None found
AtsnoR133	101	C/D	Unknown	Predicted	1	5	—	Cluster: snoR133/snoR120	ESTs: Columbine, rice, maize
<i>Novel box H/ACA snoRNAs</i>									
AtsnoR150	150	H/ACA	P1:25S 2174	At213	1	1	Not modified	Cluster: snoR150/snoR138	Genomic: poplar; ESTs: Poplar, Melon, Papaya, etc.
AtsnoR151	151	H/ACA	P1: 25S 973	At549	1	4	snR43(Y); ACA9	Intronic snoRNA: in intron 2 of <i>At4g17380.1</i> (RPL15B)	None found
AtsnoR152a	163	H/ACA	P2: 18S 1531	At675	2	2	Target sequence not conserved	Cluster: snoR152a/snoR152b	Genomic: poplar; EST: poplar, cotton, orange, etc.
AtsnoR153	155	H/ACA	Unknown	At740	1	5	—	Cluster: snoR153/snoR12-2/AtU24-2	ESTs: cocoa, poplar, orange
AtsnoR154	156	H/ACA	P1: 18S 1479	At696	1	4	ACA54	Cluster: AtU19-2/snoR154	ESTs: orange, papaya
AtsnoR155a	95	H/ACA	P1: 25S 397 P2: 18S 56	At801	3	1	P1: target sequence not conserved; P2: not modified	Intronic H/ACA cluster in intron 2 of <i>At1g04480.1</i> (RPL23A): snoR155a/snoR155b/snoR155c	Genomic: poplar EST: orange, tobacco, apple, etc.
AtsnoR155b	156	H/ACA	P1:25S 1465	At1140	1	5	Not modified (Y); C in human	Single gene	ESTs: globe artichoke, barnadesia, oil palm
AtsnoR157	122	H/ACA	Unknown	Predicted	1	2	—	Cluster: snoR157/snoR68/snoR159/snoR145/snoR135a/snoR135b	None found
AtsnoR158a	226	H/ACA	Unknown	Ath-143— predicted	2	2	—	Clusters: snoR158a/snoR39BYa/snoR21a/snoR149; snoR158b/snoR39BY/snoR21b	ESTs: cow pea, red clover, asparagus
AtsnoR159	225	H/ACA	Unknown	Ath-319— predicted	1	2	—	Cluster: snoR157/snoR68/snoR159/snoR145/snoR135a/snoR135b	ESTs: melon, American sweet flag, wild radish
AtsnoR160	139	H/ACA	P2: 18S 1306	Ath-362— predicted	1	1	Not modified	Cluster: snoR160/snoR16-2/AtU43-2	ESTs: orange, apple, wild radish
AtsnoR161	155	H/ACA	P2: 18S 1188	At395; Ath-382 Ath-408— predicted	1	3	snR36 (Y); ACA36; ACA36B	Cluster: snoR161/snoR66/snoR118	ESTs: red sage, cowpea, runner bean
AtsnoR162	146	H/ACA	P1: 25S 654	Ath-408— predicted	1	1	Not modified	Intron 6/exon 7 of <i>AT1G30970</i> (Suppressor of <i>FRIGIDA4</i>)	Genomic: poplar; ESTs: eucalyptus, poplar, etc.
AtsnoR163	194	H/ACA	Unknown	Ath-424	1	1	—	Cluster: snoR163/snoR147/snoR95	ESTs: cowpea, orange, cassava
AtsnoR164	142	H/ACA	Unknown	Ath-601— predicted	1	2	—	Single gene in 3' UTR of <i>At2g37250</i> Adenosine kinase	Genomic: poplar; ESTs: globe artichoke, Russian dandelion
AtsnoR134-1	144	H/ACA	P1: 18S 1104; P2: 18S 1192	At511	3	1;1;4	P1: not modified; P2: snR35(Y); ACA13	Clusters: snoR134-1/AtU36-1/AtU38-1/snoR6-1/snoR97-1; snoR134-2/AtU36a-2/AtU38-2/snoR6-2/snoR97-2; snoR134-3/AtU36a-3/AtU38-3/snoR6-3	Genomic: poplar; ESTs: poplar, tomato, morning glory, etc.
AtsnoR134-2									
AtsnoR134-3									
AtsnoR139	152	H/ACA	P1: 18S 762	At654	1	5	snR80 (Y); ACA28	Cluster: snoR82/snoR139	Genomic: poplar; ESTs: <i>Aristolochia fimbriata</i>
AtsnoR138	116	H/ACA	P1: 25S 2445	Predicted	1	1	ACA3	Cluster: snoR150/snoR138	ESTs: apple, orange, papaya, Columbine
AtsnoR137-1	158	H/ACA	P1: 18S 1208	At1253	2	3; 4	Not modified	Single genes	Genomic: poplar; ESTs: Russian dandelion, papaya, Tomato, etc.
AtsnoR137-2									
AtsnoR136	128	H/ACA	P2: 25S 2833	Predicted	1	3	E3	Cluster: snoR88-2/snoR19-1/snoR136/snoR64/snoR20-1/snoR38Y-1	ESTs: soya, tomato

Capped box C/D snoRNAs are U13 orthologues

Of particular interest was the demonstration that three snoRNA species were highly abundant in the capped library. SnoR105, snoR108 and snoR146 are related monocistronic box C/D snoRNAs. SnoR105 and snoR108 were identified previously as partial sequences

which contained recognized promoter elements of plant snRNA genes [an upstream sequence element (USE) at ~-90 and a TATA-box at ~30 bp upstream of the transcription start site—ref. (44)] in the upstream region of their genomic sequences (21). Here, we obtained full-length sequences of snoR105 and snoR108 as well as the related snoR146 (Figure 3). The genomic sequences upstream of all three genes have USE and TATA promoter elements in the RNA polymerase II configuration (44). The presence of snRNA promoter elements as well as their efficient isolation from the capped library strongly suggest that these snoRNAs are capped while the vast majority of Arabidopsis snoRNAs are processed from polycistronic snoRNA precursors and are uncapped. The three genes contain conserved C and D boxes except that the box C sequence is internal in the coding sequence lying ~30 nt from the 5' end (Figure 3). Like most box C/D snoRNAs, short inverted repeats are present directly up and downstream of the box C and D sequences, respectively, which may facilitate the formation of a K-turn in the C/D motif to which the core p15.5 kDa protein binds as the first stage in box C/D snoRNP assembly (47). Alignment of the three snoRNA sequences show two regions of high conservation: the first 24 nt which shows only a single nucleotide change and positions 83–96 (Figure 3A). The structural features of these snoRNAs were reminiscent of animal U8 and U13 snoRNAs which contain complementarity to rRNA sequences (48). Alignment with the human U13 sequence (49) clearly showed similarity in the two most highly conserved regions (above) (Figure 3A). The Arabidopsis sequences were complementary to the 3'-end of 18S rRNAs and formed similar putative base-pairing interactions (Figure 3B). A number of other plant orthologues of the U13 snoRNAs have been identified in EST libraries and all have complementarity to the 3'-end of 18S rRNA (results not shown).

Expression of novel snoRNAs and U13 orthologues

The cloning of the U13 orthologues and novel snoRNAs provides direct evidence of expression. To further demonstrate expression of some of the novel genes, we detected snoRNAs by northern analysis (Figure 4). RNAs of predicted size were obtained with antisense probes to the

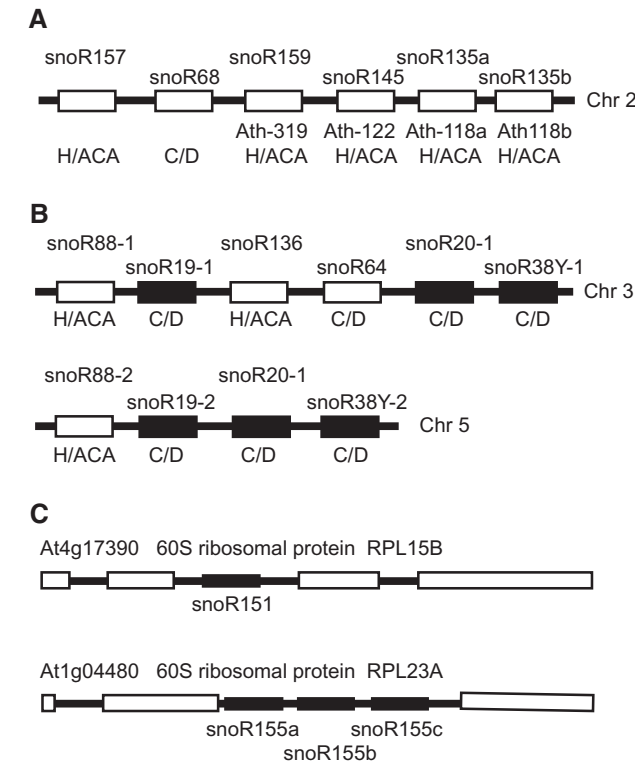


Figure 2. Gene organization of novel snoRNA gene clusters. (A) Novel snoRNA gene cluster: cloning of snoR145 (Ath-122) and examination of flanking sequences identified snoR68 and three snoRNA genes for which partial sequences had been identified previously but were not recognized as snoRNAs (Ath-319, Ath-122, Ath-118a and Ath-118b) (21). (B) Extended snoRNA gene cluster: two clusters containing gene copies of snoR19, snoR20 and snoR38Y (black boxes) were identified previously (18). Further three genes were added to the first cluster: snoR88-1 and snoR64 (21) and snoR136 was predicted. (C) Intronic snoRNA genes and gene cluster: two of the novel H/ACA snoRNAs were found in introns of mRNAs with snoR155 having three variants. White boxes, exons; black boxes, snoRNAs.

Table 2. Orphan snoRNAs in Arabidopsis

Orphan snoRNA	Type	Expression	Variants	Orthologues to date	Reference
snoR6	C/D	No	3	No	(18)
snoR28	C/D	Yes	6	Yes	(18, present study)
snoR106	C/D	Yes	2	No	(21)
snoR107	C/D	Yes	1	No	(21)
snoR110	H/ACA	Yes	3	No	(18, 21, present study)
snoR149	C/D	Yes	1	Yes	Present study
snoR133	C/D	No	1	Yes	Present study
snoR157	H/ACA	No	1	No	Present study
snoR145	H/ACA	Yes	1	Yes	(21, present study)
snoR158	H/ACA	Yes	1	Yes	(21, present study)
snoR159	H/ACA	Yes	1	Yes	(21, present study)
snoR163	H/ACA	Yes	1	Yes	(21, present study)
snoR164	H/ACA	Yes	1	Yes	(21, present study)

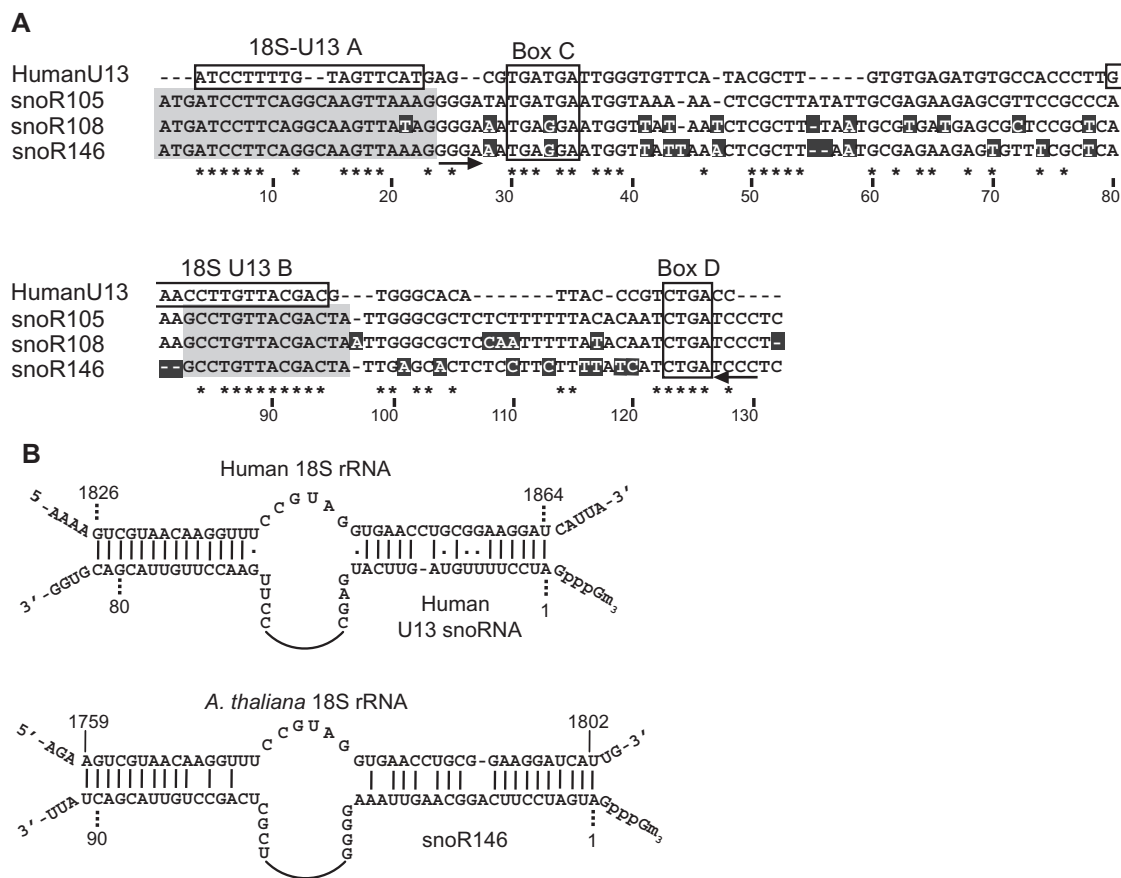


Figure 3. Sequence alignment of U13 snoRNAs. (A) The three plant U13 orthologues are aligned with human U13. Identical sequences are indicated by asterisks; for the three plant genes, sequence differences from snoR105 are highlighted white on black. Box C and D sequences are boxed; the two highly conserved regions in the plant genes are shaded grey; the two regions of complementarity to 18S rRNA in human U13 are boxed and labelled A and B [following human model: ref. (48)] and putative inverted repeats adjacent to boxes C and D are shown by arrows. (B) Putative base-pairing interactions between human U13 snoRNA and Arabidopsis U13 (snoR146) with the 3' regions of their cognate 18S rRNAs.

novel box C/D snoRNAs (snoR117, snoR147–149) and to the novel box H/ACA snoRNAs (snoR134, snoR150–156) (Figure 4A). Expression of the U13 orthologues (snoR105, snoR108 and snoR146), other orphan snoRNAs (snoR28-1c, snoR109, snoR110) and the scaRNA, snoR102, all of which were cloned in the nucleolar libraries, was also confirmed by northern analysis (Figure 4B). SnoR102 showed two bands on the northern analysis of ~350 and 150–170 bp. The clones of snoR102 obtained from the capped library were 365 bp long. SnoR102 was originally cloned as a 133 bp sequence from the 3' UTR of a protein-coding gene which gave a product on northern analysis of 185 bp (21) and corresponds to the 3' half of the clones obtained here and based on the positions of the C/D boxes would have an expected size of ~156 bp. A BLAST search identified our snoR102 sequence as the antisense of an annotated protein coding gene, At1g68945, of unknown function. It is highly likely that this gene encodes the 365 bp snoR102 transcript and is mis-annotated. The 5' half of this snoR102 transcript contains some sequences similar to C and D boxes but is not clearly identifiable as a snoRNA. Chen and Wu (35) identified a 166 nt variant of snoR102 (snoR102-2) which is

derived from the 3' UTR of an unknown protein gene, At4g30993. However, there is extensive sequence similarity upstream of the snoR102-2 gene with the 365 bp snoR102 sequence cloned here. Thus, both At1g68945 and At4g30993 contain related sequences with the snoR102 variants in their 3' halves. Thus, it appears that there are two variants of snoR102 which are each processed from either a longer precursor (cloned here and visible on Northern) or from the 3' UTR of the mRNA transcript from At4g30993. SnoR102 is therefore reminiscent of doublet guide RNAs which contain a box C/D snoRNA domain in their 3' halves and a novel box C/D-like domain in their 5' halves and where only the capped doublet RNA or the 3'-most box C/D RNA are stable (8). Whether the 5' half of snoR102 contains a functional domain guiding modification of snRNAs or rRNAs is unknown.

Many of the novel uncapped snoRNAs were part of polycistronic gene clusters (for example—Figure 2A and B). To confirm that the new and updated clusters are indeed transcribed as polycistronic pre-snoRNAs, we performed RT-PCR with primers to genes in various clusters using total RNA from Arabidopsis cell culture cells. Polycistronic precursor snoRNA transcripts of the expected sizes were detected for all clusters tested (Figure

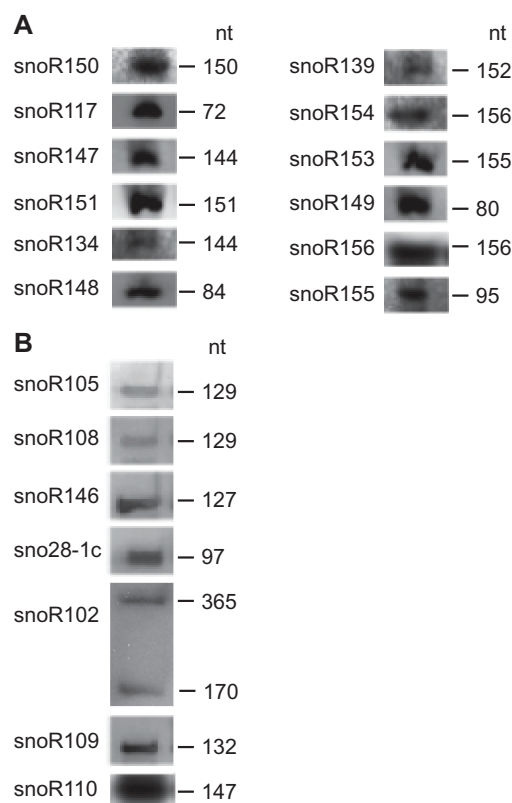


Figure 4. Northern analysis of novel snoRNAs, U13 orthologues and scaRNAs. (A) Northern analysis of novel snoRNAs and (B) northern analysis of the U13 snoRNAs (snoR105, snoR108 and snoR113), scaRNA (snoR102) and orphan snoRNAs (snoR28-1c, snoR109 and snoR110). Expected sizes of the transcripts are indicated and are consistent with markers (data not shown).

5A–C and Supplementary Figure S7). In addition, four of the novel genes and some variants appeared to be single genes and expression of snoR148 and U19-1 (intronic) was also confirmed by RT-PCR (Figure 5A and D).

Sub-cellular localization of novel snoRNAs and U13 orthologues

SnoRNAs are expected to be nucleolar. To examine the sub-cellular localization of the new box C/D and H/ACA snoRNAs identified here, antisense RNA probes were generated from some of the cDNA clones and hybridized to *Arabidopsis* Col-0 cell culture cells. The novel box C/D snoRNA (snoR117) and H/ACA snoRNAs (snoR151, snoR152 and snoR156) localized to the nucleolar region in the nuclei (Figure 6A–D). Similarly, the U13 orthologues also localized to the nucleolus (Figure 7A–C). Finally, to date, few *Arabidopsis* scaRNAs have been described (21,35) but the subcellular location of plant scaRNAs has not been addressed previously. Therefore, we determined the sub-cellular localization of the scaRNA, snoR102, which was cloned from the capped library. In contrast to the various novel box C/D and H/ACA snoRNAs, snoR102 did not localize to the whole nucleolar area but instead to two intensely labelled foci in and on the periphery of

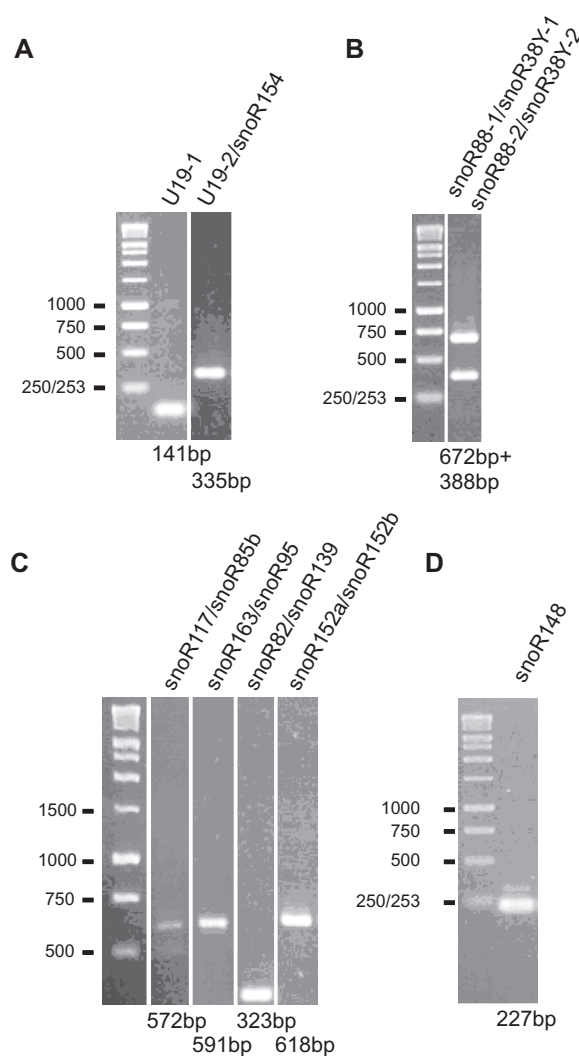


Figure 5. RT-PCR expression analysis of novel and extended polycistronic snoRNA gene clusters. (A–C) RT-PCR of polycistronic snoRNA clusters. Primers were positioned in the genes indicated above the lanes (see also Supplementary Figures S1 and S2) and the expected RT-PCR product sizes are indicated below the lanes and are consistent with size markers. Some primers pairs were able to amplify more than one related precursor RNA (B). (A and D) Transcripts from single genes were amplified using primers to the 5' and 3' ends of the coding sequences.

the nucleolus, perfectly consistent with CB labelling (Figure 8A). We also analysed the localization of the orphan snoRNA, snoR109 (21) which had again been cloned in the capped library. snoR109 localized to a single intense spot near the nucleolar periphery (Figure 8B). The labelling of snoR102 and snoR109 were therefore very similar and given that these cells usually show between 1 and 3 CBs, snoR109 may represent a novel scaRNA.

DISCUSSION

The eukaryotic nucleolus is involved in many aspects of RNA biogenesis and metabolism. The use of isolated

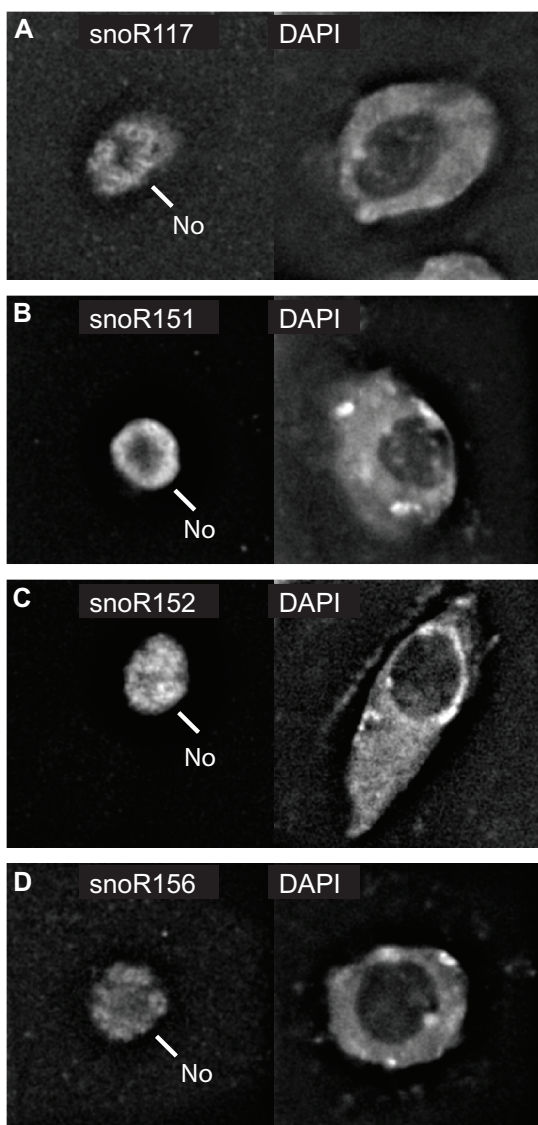


Figure 6. (A–D) Nucleolar localization of novel snoRNAs. *In situ* hybridizations with antisense probes of four novel snoRNAs labelled the nucleolus (left panel) compared to nuclear staining with DAPI (right panel). No: nucleolus.

plant nucleoli has led to the demonstration that mRNAs are present in nucleolar RNA and aberrantly spliced mRNAs are enriched in nucleoli (39). This is consistent with the detection of exon-junction complex (EJC) proteins in the nucleolus and the dynamic redistribution of a core EJC protein (eIF4A-III) under different growth conditions (38,50). By using isolated nucleoli and sequencing cDNA libraries generated from nucleolar RNA, we have generated full-length snoRNA and scaRNA sequences and identified novel box C/D and H/ACA snoRNA genes including U13 snoRNAs. From this and other studies, a total of 188 different snoRNA/scaRNA genes and 294 snoRNA/scaRNA gene variants are found in *Arabidopsis*. We provide direct evidence of expression for 40% of these genes/variants by cloning of cDNAs, northern analysis, *in situ* hybridization or RT-

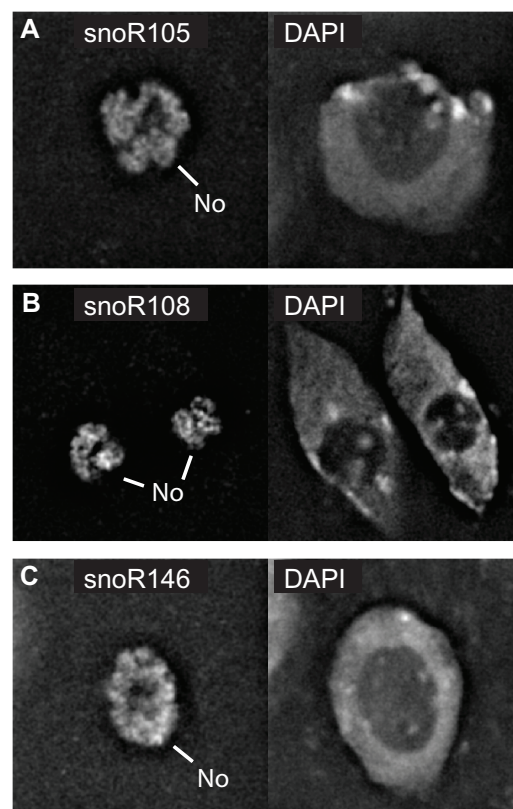


Figure 7. Nucleolar localization of U13 snoRNAs. *In situ* hybridizations with antisense probes of the U13 snoRNA variants. (A) snoR105, (B) snoR108 and (C) snoR146 labelled the nucleolus (left panel) compared to nuclear staining with DAPI (right panel). No: nucleolus.

PCR. We have also identified novel orphan snoRNAs raising the possibility of wider functions in other aspects of RNA metabolism or gene regulation.

The previous RNomic study (21) identified many *Arabidopsis* snoRNA sequences from total seedling RNA and, in particular, significantly added to our knowledge of box H/ACA snoRNAs which are difficult to detect computationally. This study also identified the first plant scaRNAs (snoR101–104) and eight orphan snoRNAs (snoR105–112) as well as many ncRNAs of unknown function. Many of the sequences from Marker *et al.* (21) were short, partial sequences and in this study we have isolated corresponding full-length clones allowing the mature snoRNA and scaRNA sequences to be defined, and to characterize 11 unknown sequences as snoRNAs (see Supplementary Table S1). Furthermore, by analysing the sequences of the orphan snoRNAs, snoR105–112 (21), snoR111 and snoR112 have putative pseudouridylation sites in 25S rRNA and U6snRNA and could be re-classified as a box H/ACA snoRNA and a new scaRNA, respectively. Similarly, *in situ* hybridization of snoR102 (scaRNA) and snoR109 strongly suggests that snoR109 is also a new scaRNA.

Three U13 snoRNAs were isolated multiple times from the capped cDNA library. Of these snoR105 and snoR108 were isolated previously as 106 and 110 bp cDNAs (21).

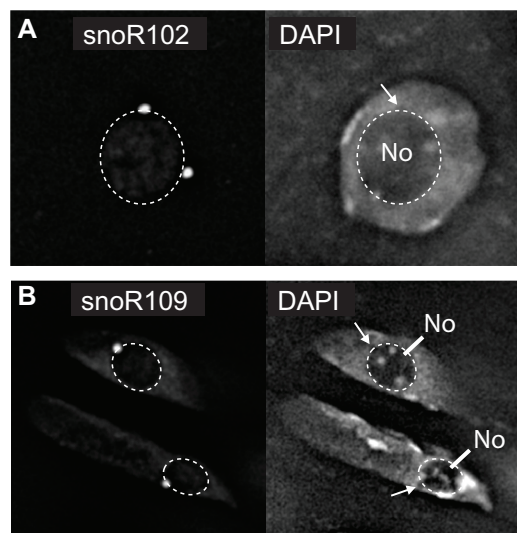


Figure 8. Subnuclear localization of the scaRNA, snoR102 and orphan snoRNA, snoR109. *In situ* hybridizations with antisense probes of (A) snoR102 and (B) snoR109 labelled bright foci on the periphery of the nucleolus (left panel) when compared to nuclear staining with DAPI (right panel). Darker regions in the DAPI stained nuclei which coincide with the labelled sites are indicated by white arrows. No: nucleolus.

The full-length sequences obtained here for snoR105, snoR108 and snoR146 showed these genes to be ~130 bp long and to be related by virtue of their mode of expression and conserved sequences—particularly in the 30 bp 5' extension from the C box (Figure 3A). The U13 snoRNAs had two regions of complementarity to the 3'-end of 18S rRNA and human U13 may function in the 3' cleavage of the 18S rRNA although its exact function remains to be elucidated (51). The three U13 genes have promoter elements (USE and TATA boxes) upstream of their coding sequences normally found in spliceosomal snRNA genes. This gene organization has only been found to date in U3 and MRP snoRNAs which are known to be capped and are required for cleavage of pre-rRNAs. Orthologues of U13 genes are present in many other plant species although there is extensive sequence variation outside of the rRNA-interacting sequences, as seen in snoR105, snoR108 and snoR146.

Orphan snoRNAs have been found in many eukaryotic organisms and recent data suggests that they may target other RNAs such as mRNAs or act as precursor molecules for the production of small regulatory RNAs (miRNAs or siRNAs) (28–31). Here, we have identified eight putative orphan snoRNAs. Our analysis of the orphan snoRNAs identified by Marker *et al.* (21) suggested that snoR109 and snoR112 are likely to be scaRNAs; and snoR111, a box H/ACA snoRNA. We were unable to find rRNA/snRNA targets for snoR106, snoR107 and snoR110 which therefore remain orphan snoRNA candidates. Some of the Arabidopsis orphan snoRNAs have orthologues in other plant species suggesting that they are *bona fide* RNA species—for example, snoR28 (18), snoR110 (21), and snoR133, snoR145,

snoR149, snoR158, snoR159, snoR163 and snoR164 (this study) (Table 2). Of the 13 currently predicted orphan snoRNAs in Arabidopsis (Table 2), snoR6 (18) and snoR157 were predicted genes due to their sequence containing features of box C/D or H/ACA snoRNAs and their position as part of polycistronic gene clusters but as yet do not have orthologues in other species or any evidence of expression. These may therefore be genes or pseudogenes which have accumulated mutations and lost their ability to interact with target RNAs or generate stable snoRNPs. Mutations resulting in the gradual loss of functional genes is reflected by the presence of gene fragments observed when comparing related polycistronic clusters and accumulation of mutations resulting in loss of complementary sequences and even the evolution of new snoRNAs has also been observed (18,34). Nevertheless, Arabidopsis and other plant species contain orphan snoRNAs which may function in different RNA metabolism pathways to affect gene regulation and ultimately plant growth and development. Orphan snoRNAs in other organisms have novel functions in mRNA alternative splicing or can be processed to snoRNA-derived small RNAs or miRNAs by RNA silencing machinery—it will be particularly interesting to elucidate the function(s) of plant orphan snoRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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