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Developmental expression of plant snRNAs

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ABSTRACT

Although the number of plant U1, U2, U4 and U5 small nuclear RNA (snRNA) variants sequenced has steadily increased over the past few years, the function of these variants in plant splicing is still not understood. In an effort to elucidate the function of plant snRNA variants, we have examined the expression of U1–U6 snRNA variants during pea seedling development. In contrast to mammalian nuclei which express a single, abundant form of each snRNA, pea nuclei express several equally abundant variants of the same snRNA. Comparison of the snRNAs in pea seeds and seedlings has revealed that four (U1, U2, U4, U5) of the five snRNAs required for pre-mRNA splicing have differentially- and developmentally-regulated forms detectable on Northerns. Only U6 snRNA, which fractionates as a single species on Northerns, appears to be constitutively expressed. Switches in the expression of the pea U1, U2 and U4 snRNAs occur at three distinct stages in development: seed maturation, seed germination and seedling maturation. Surprisingly, the snRNA profiles of mature desiccated seeds and mature leaf tissues are nearly identical and different from developing seeds and seedlings suggesting that switches in the snRNA population occur at transitions between active and inactive transcription. Sequence analysis and differential hybridization of the U1 snRNA variants has demonstrated that some of the developmentally-regulated forms represent sequence variants. We conclude that select subsets of pea snRNAs accumulate at particular stages during plant development.

INTRODUCTION

Five of the small nuclear RNAs (U1, U2, U4, U5, U6 snRNAs) found in the nucleus of eukaryotic cells mediate the excision of introns from pre-mRNAs (1). In nuclei, the U1, U2 and U5 splicesomal snRNAs exist in small ribonucleoprotein particles (snRNPs) each containing a unique snRNA and 6–10 associated snRNP proteins (2). The U4 and U6 snRNAs present in nuclei are associated via RNA–RNA interactions in a single U4/U6 snRNP particle (3, 4, 5). These particles interact with pre-mRNA to form a larger splicing complex that is capable of excising introns from precursor RNAs. In formation of this complex, the first eleven nucleotides of U1 snRNA interact directly with the 5′ splice site (6, 7), and the U2 snRNA interacts with the conserved branch site within the intron (6, 8). The U5 snRNP particle has been implicated in recognition of the 3′ splice junction via its associated snRNP protein(s) (9). The U4/U6 snRNP interacts in an undetermined manner with the splicing complex (10, 11).

In mammalian and yeast nuclei, each of the splicesomal snRNAs is usually represented by a single, abundant species (12, 13). Some vertebrate cells contain sequence variants of the U1 snRNAs, but these variants tend to be expressed in a select set of tissues and often represent a small percentage of the total snRNA population (14, 15). In other vertebrates (*Xenopus*, chicken, mouse), U1 and/or U4 snRNA variants are expressed at specific stages in development (16, 17, 18, 19). Developmental sequence variants of the U2, U5 and U6 snRNAs have not been observed in vertebrates (18).

In contrast, multiple U1, U2, U4 and U5 snRNA variants exist in plant nuclei (20, 21, 22, 23). Cloning and sequence analysis of the numerous U1, U2, U4 and U5 snRNA variants expressed in pea nuclei has demonstrated that each of these splicesomal snRNAs is represented by multiple sequence variants *in vivo* (23). The primary sequence differences in the pea U1, U2, U4 and U5 snRNA populations occur in regions implicated in the binding of small ribonucleoprotein proteins (23). Earlier studies using direct RNA sequence analysis also indicated that several U4 and U5 sequence variants exist in broad bean and pea, respectively (22, 24). In contrast, a single species of U6 snRNA is expressed in plant nuclei (20, 25). The various snRNA structures that are formed by these diverse populations potentially establish alternate snRNP particles that interact in specific subsets to promote the excision of select plant introns.

In an effort to elucidate the function of the plant snRNA variants, we have examined the expression of U1–U6 snRNA variants in pea seed and seedling development. Our experiments have demonstrated that several abundant variants of the same snRNA are expressed in pea nuclei. Comparison of the snRNAs in seeds and seedlings has revealed that four (U1, U2, U4, U5) of the five snRNAs required for pre-mRNA splicing have constitutively- and developmentally-regulated forms detectable

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on Northerns. Only U6 snRNA, which fractionates as a single species on Northerns, appears to be constitutively expressed. Cloning and sequence analysis of the U1 snRNA variants has demonstrated that the developmentally regulated forms correspond to some of the U1 snRNA sequence variants that we have previously characterized (23). We have concluded that selected subsets of the plant snRNAs are expressed at particular stages of development either due to differential transcription or post-transcriptional snRNA processing.

MATERIALS and METHODS

Reagents

The 5'-[γ-^{32}P]ATP (7000 Ci/mmol) and 5'-[α-^{35}S]dATP (1320 Ci/mmol) and Gene Screen were from New England Nuclear. Enzymes were obtained as follows: T4 polynucleotide kinase (Bethesda Research Laboratories), T7 DNA polymerase (Sequenase, U.S. Biochemicals).

Isolation of nuclear RNAs

For the isolation of 12-day-old seedling leaf RNA, pea (*Pisum sativum* var. Progress no. 9) seedlings were grown for 11 days under etiolated conditions and exposed to light for 24 h prior to harvesting of the leaf tissue. Seed RNAs were isolated from desiccated pea seeds. For intact seedling RNAs, pea seeds were germinated (24 h) and grown in vermiculite under etiolated conditions for 2−16 days and RNA was extracted from intact seedlings without prior exposure to light. For mature leaf RNAs, pea seedlings were germinated and grown under greenhouse conditions for four weeks prior to harvesting of the leaf tissue. For the isolation of the developing seed RNAs, seedlings were grown under greenhouse conditions and seeds were isolated 10 and 20 days after flowering.

Tissues were homogenized in 50 mM Tris-HCl (pH 9.0), 0.2 M NaCl, 10 mM EDTA and total RNA was extracted with phenol:chloroform (1:1) and the nucleic acids were recovered from the aqueous phase by repeated ethanol precipitations at −70°C. The snRNAs were purified from 2−5 mg total RNA by immunoprecipitation with 100 μg anti-m_3 ^{2,2,7}G IgG as described by Adams and Herrara (26) and recovered by proteinase K treatment and phenol extraction.

Electrophoresis of RNA

Immunoprecipitated snRNAs were fractionated on fully denaturing 15% acrylamide:bisacrylamide (19:1), 8.3 M urea gels (31×40×0.05 cm) containing 90 mM Tris-borate, 2 mM EDTA (1× TBE) buffer at 45 W (constant wattage) for 16 hours. Samples were diluted with an equal volume of 10 M urea, 0.1% xylene cyanol, 0.1% bromophenol blue and heated at 100°C for 1 minute prior to loading. RNAs were fractionated on partially denaturing 10% acrylamide:bisacrylamide (19:1), 4.0 M urea gels (31×38×0.05 cm) containing 1× TBE buffer at 500 V (constant voltage; 20 mA, 10 W) for 23 hours at 20°C. Samples were diluted with an equal volume of 30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue and heated for 1 min at 100°C prior to electrophoresis. Gels were blotted onto Genescreen and Northern analysis was performed essentially as described by Egeland *et al.* (20). Northerns were probed with oligodeoxyribonucleotides 5' end-labeled with [γ^{32}P]ATP and T4 polynucleotide kinase. Blots were hybridized at 25°C (U1, U2, U4, U6) or 4°C (U5) for 16 hours in 6× SSC, 5× Denhardt's solution, 0.1% SDS and washed three times for 20 minutes at room temperature in 6× SSC, 0.1% SDS. All oligodeoxyribonucleotide sequences were synthesized at the University of Illinois Biotechnology Center and were gel-purified or HPLC-purified prior to use. The oligodeoxyribonucleotide probes used in these studies are complementary to snRNAs as shown in Table I.

Table I. Oligodeoxyribonucleotide Probes

Oligonucleotide	snRNA	Complementary Nucleotides	Oligo Sequence
$U1_{1-18}$	pea U1	1−18	5'-CCCC$^{G}_{A}$TCCAGGT-AAGTAT-3'
$U1.1_{47-62}$	pea U1.1	47−62	5'-GGAGGTCACAATCCTA-3'
$U1.2_{47-62}$	pea U1.2	47−62	5'-GGAGGTCACTTGCCTA-3'
$U2_{28-42}$	pea U2	28−42	5'-CAGATACTACACTTG-3'
$U4_{54-69}$	pea U4	54−69	5'-TTTCAACCAGCAATAG-3'
$U5_{23-47}$	pea U5	23−47	5'-TAGTAAAAGGCGAAAGA-TAGTTCGC-3'
$U6_{29-42}$	bean U6	29−42	5'-TC$^{A}_{T}$TCTCTGTATTG-3'

On the right are the oligonucleotide sequences complementary to the specific snRNAs shown in the second column. The positions of the complementary nucleotides in each snRNA are designated in the third column. The snRNA sequences needed for this compilation are derived from Hanley and Schuler (23).

DNA sequence analysis

U1 snRNA clones were constructed in oligo dT-tailed pCGN1703 vector as outlined in Hanley and Schuler (23) using anti-m_3G immunoprecipitated 11-day-old pea seedling nuclear RNA. Clones were sequenced using modified T7 DNA polymerase (Sequenase, U.S. Biochemicals), 5'-[α-^{35}S]dATP and the T7 RNA polymerase promoter primer (P-L Biochemicals) as described by the manufacturer.

RESULTS

Differential expression of splicesomal snRNAs

To determine if the small nuclear RNAs (snRNAs) of pea (*Pisum sativum*) are differentially expressed during development, snRNAs from seeds and seedling leaves were immunoprecipitated from total nuclear RNA with anti-trimethylguanosine antibody (anti-m_3G) and analyzed using oligodeoxyribonucleotide probes specific for the U1, U2, U4, U5 and U6 snRNAs defined in Table I. The U1 oligonucleotide is complementary to the first 11 nucleotides that are absolutely conserved in all but two of the U1 snRNAs characterized to date (12, 27, 28) and known to interact with the 5' splice site (6, 7). The U2 oligonucleotide is complementary to nucleotides 28 to 42 in U2 snRNA, another region absolutely conserved in mammalian, yeast and plant snRNAs (12, 23, 29), which interacts with branch site sequences (6, 8). The U4 oligonucleotide probe is complementary to the conserved region in pea U4 snRNA (23) which base pairs with U6 snRNA in the U4/U6 snRNA hybrid (5). The U5 oligonucleotide was designed to complement pea U5 snRNAs in hairpin-loop I, the only region conserved in all of the pea U5 snRNA variants and in vertebrate U5 snRNAs (12, 23). The U6 snRNA probe is complementary to a single-stranded region conserved in plant, vertebrate and yeast U6 snRNAs (12).

In our initial studies, the snRNAs isolated from mature pea seeds and 12-day-old seedling leaves fractionated on 8% acrylamide denaturing gels in relatively broad bands containing U1, U2 and U4 snRNAs and multiple bands containing U5

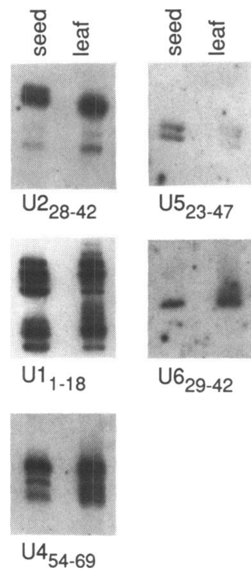

Figure 1. Northern Analysis of Seed and Leaf snRNAs. Nuclear RNAs from 12-day-old seedling leaves or mature seeds were immunoprecipitated with anti-m_3G antibody, fractionated on 15% acrylamide, 8.3 M urea denaturing gels, electrotransferred to Genescreen and sequentially hybridized with ^{32}P-labeled oligonucleotide probes. The hybridization probes are designated below each autoradiograph. The probes are complementary to snRNA sequences as outlined in Table I. In each blot: lane 1, mature seed RNA; lane 2, 12-day-old seedling leaf RNA.

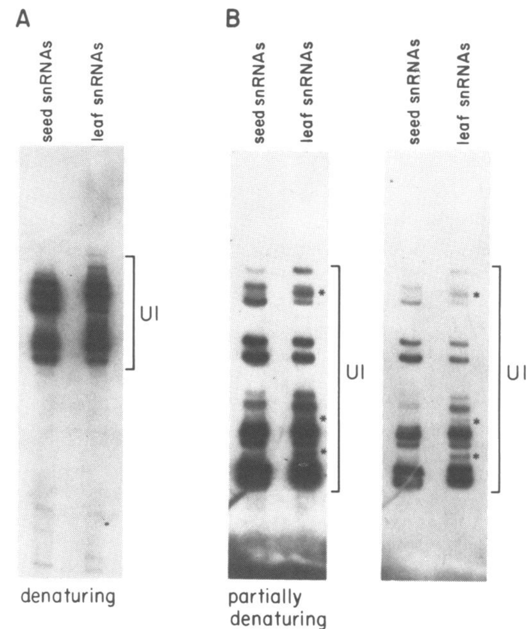

Figure 2. Northern Analysis of Seed and Leaf snRNAs on Partially Denaturing Gels. Anti-m_3G immunoprecipitated RNAs from mature seeds (lane 1) or 12-day-old seedling leaves (lane 2) were fractionated on a 15% acrylamide, 8.3 M urea denaturing gel (panel A) or a 10% acrylamide partially denaturing gel (panel B). The gels were blotted onto Genescreen and hybridized with the $U1_{1-18}$ oligonucleotide. [Panel B contains long and short exposures of the same Northern to clarify differences in abundant and nonabundant U1 snRNAs.] Leaf-specific U1 snRNA variants are designated with asterisks.

snRNAs (not shown). At this level of resolution, no obvious differences exist between the seed and seedling leaf snRNAs. Because cloning and sequence analysis of pea snRNA variants had demonstrated that multiple sequence and length variants existed in pea nuclei (23), the snRNAs were fractionated on high resolution 15% acrylamide, 8.3 M urea denaturing gels and hybridized with the snRNA-specific probes (Figure 1). Fractionation on this gel system, which separates on the basis of length and sequence variations in the snRNAs, reveals that quantitative and qualitative changes exist in the U1, U2, U4 and U5 snRNAs in seeds and seedling leaves. In contrast, only one form of U6 snRNA can be detected in these two cell types.

The most striking differences occur in the U1, U2 and U4 snRNA profiles. In addition to the seven forms of U1 snRNA expressed in mature seeds, eight additional variants of U1 snRNA appear in leaf tissue resulting in the resolution of at least fifteen forms of U1 snRNA on the high resolution gels. Like U1 snRNA, three additional forms of U4 snRNA are expressed in leaf tissue to supplement four constitutive forms expressed in mature seeds. In contrast, a new form of U2 snRNA is expressed in seed tissue in addition to the prominent, constitutive form present in leaf tissue. Multiple forms of U5 snRNA are detected in both seed and leaf tissues.

U1 snRNA variants

In order to determine if the leaf-specific forms of U1 snRNA are sequence, length, or base-modification variants, anti-m_3G precipitated snRNAs from seed and leaf tissue were fractionated on partially denaturing gels and subjected to Northern blot analysis with the $U1_{1-18}$ probe. The tissue-specific variations in U1 snRNA, first detected on high resolution denaturing gels (Figure 2A), are delineated on the partially denaturing gels (Figure 2B). Although the most abundant U1 snRNAs are constitutively expressed, at least three other minor U1 snRNA variants appear exclusively in leaf tissue (asterisks, Figure 2B).

To further define the population of U1 snRNA variants, we have cloned and sequenced seven U1 snRNAs expressed in 12-day-old seedling leaves (23). For clarity, the sequences of these variants are presented in Figure 3. The pea U1 snRNA clones represent sequence and length variants from 157 to 162 nucleotides which exhibit 85−90% sequence conservation within the U1 snRNA family. Nearly all of the length variation results from deletions of the 3′ terminal cytosine and internal insertions/deletions at nucleotides 38, 47, 53, and 131 (relative to U1.1). The pea U1 snRNA sequences vary primarily in looped, bulged or single-stranded regions. Regions of the U1 snRNA that are known to be important from phylogenetic data (circled bases; 12, 13) are conserved in the pea U1 snRNA variants.

The most variable region of pea U1 snRNA occurs in stem-loop II. To correlate the sequence variations defined in Figure 3 with the sequence variants visualized on high resolution acrylamide gels and to determine whether sequence variants in this region were differentially expressed during development, oligonucleotide probes designated $U1.1_{47-62}$ and $U1.2_{47-62}$ were designed to exactly complement the U1.1 and U1.2 snRNA variants. In addition to defining developmental variations, these probes also define the abundance of the individual U1 snRNA variants. For example, the U1.1 oligonucleotide primarily detects variants like the U1.1 and U1.15 snRNAs which contain perfect complements of the U1.1 oligonucleotide. The U1.2 oligonucleotide detects the U1.2, U1.3 and U1.36 snRNA variants. For complete analysis of the U1 snRNA variants, anti-m_3G immunoprecipitated RNAs were electrophoresed on fully- and partially-denaturing gels and sequentially probed with

Figure 3. U1 snRNA Sequence Variants Expressed in Pea Seedlings. The complete sequence for the U1.1 snRNA variant cloned from anti-m_3G immunoprecipitated nuclear RNA isolated from pea seedling tissue (23) is drawn according to the structure described for U1 snRNAs (12). Arrows followed by subscripted nucleotides (or deletions) are used to designate base variations and deletions in the U1.2, U1.3, U1.15, U1.36, U1.66 and U1.137 snRNA variants. Nucleotides phylogenetically conserved in other organisms (13) are circled. Nucleotides in the U1.1 variant which differ in phylogenetically conserved positions are designated in lower case. Boxes are drawn around the region of U1.1 complementary to the $U1.1_{47-62}$ probe and the Sm binding site (nt. 121–129).

Figure 4. Identification of Seed and Leaf Seed Variants by Northern Analysis. Anti-m_3G immunoprecipitated snRNAs from mature seeds (lane 1) or 12-day-old seedling leaves (lane 2) were fractionated on a 10% acrylamide partially denaturing gel (panel A) or a 15% acrylamide, 8.3 M urea denaturing gel (panel B), blotted onto Genescreen and sequentially analyzed using the $U1_{1-18}$, $U1.1_{47-62}$ and $U1.2_{47-62}$ probes.

oligonucleotides specific for all U1 snRNAs ($U1_{1-18}$), the U1.1 ($U1.1_{47-62}$) or the U1.2 ($U1.2_{47-62}$) snRNA variants. The abundance of variants capable of hybridizing to the $U1.1_{47-62}$ probe differs only slightly in mature seeds and seedling leaves (Figure 4B) indicating that the majority of the U1.1 variants are constitutively expressed. Several minor variants that hybridize with the U1.1 oligonucleotide are nominally more abundant in seeds and other variants are more abundant in seedling leaves.

In contrast to the U1.1 variants, several of the variants capable of hybridizing with the $U1.2_{47-62}$ oligonucleotide, designated the U1.2 variants, change significantly in seed and seedling leaf nuclei (Figure 4A). At least one novel U1.2 variant is expressed in leaf tissue in addition to the U1.2 variants constitutively expressed in both tissues. Other U1.2 variants are expressed in higher abundance in leaf nuclei or seed nuclei. Four expression patterns exist for the U1.2 snRNA variants and delineate variants that are constitutively expressed, uniquely expressed in leaf nuclei, abundantly expressed in seed nuclei and abundantly expressed in leaf nuclei. The different patterns exist because the U1.2 oligonucleotide probe hybridizes with different efficiencies with three of the seven U1 snRNA variants presented in Figure 3. As designed, the U1.2 variant hybridizes at 16/16 positions, the U1.3 variant hybridizes at 16/16 positions including one GU base pair, and the U1.36 variant hybridizes at 15/16 base pairs including one GU base pair. These small variations in the stability of the RNA:DNA hybrid can not be differentiated by Northern analysis. In contrast, snRNA variants which form less than 13/16 base pairs with the U1.1 or U1.2 oligonucleotide probes do not base pair. The absolute selectivity of these probes is evident in the nonoverlapping profiles obtained with the U1.1 and U1.2 oligonucleotide probes in Figure 4A. By comparing the Northerns analyzed with the $U1_{1-18}$, $U1.1_{47-62}$ and $U1.2_{47-62}$ probes, it is clear that the U1.1 and U1.2 probes together detect most of the major U1 snRNA variants expressed in seeds and seedling leaves.

snRNA variations during seed and seedling development

To define the period during which developmental switches in snRNA populations occur, snRNAs were isolated from maturing seeds 10 or 20 days after flowering (DAF), mature dry seeds and 12-day-old seedling leaves and analyzed by high resolution blot analysis with the $U1_{1-18}$, $U1.1_{47-62}$, $U4_{54-69}$, and $U2_{28-42}$ oligonucleotide probes (Figure 5). In this analysis, the mature seed and seedling leaf snRNAs exhibit the same developmental differences seen in Figures 1, 4 and 6. Surprisingly, the populations of U1 snRNAs in developing seeds are identical to those in the leaves of developing seedlings and distinct from the U1 snRNA populations in mature, desiccated seeds. The proportions of the abundant and most of the minor snRNA variants in the developing seed and seedling leaves hybridizing with the $U1_{1-18}$ and the $U1.1_{47-62}$ oligonucleotide probes are identical. Minor variations in the snRNAs detected with this probe in the developing seed and leaf tissues may exist but these variations are overshadowed by the more abundant variations.

As in the case of the U1 snRNAs, the U4 snRNAs detected with the $U4_{54-69}$ oligonucleotide differ significantly in mature seed and seedling leaf tissue (Figure 5). The patterns of U4 snRNA expressed in seeds 20 DAF are slightly different from those expressed 10 DAF and appear to represent a continuum of variations between those expressed 10 DAF and in mature seeds. At 20 DAF, the pattern of U4 snRNAs most closely resembles the pattern expressed in actively growing seedling leaves. From this analysis, it appears that the U1 and U4 snRNA populations in developing seeds are more similar to the populations expressed in rapidly dividing seedlings than to the populations present in quiescent, desiccated seeds.

U2 snRNA represents the third splicesomal snRNA whose population changes in development. The second U2 snRNA present in mature seeds (Figure 1) accumulates later in development; it is detectable by 20 DAF and is abundant in desiccated seeds (Figure 5, lane 5).

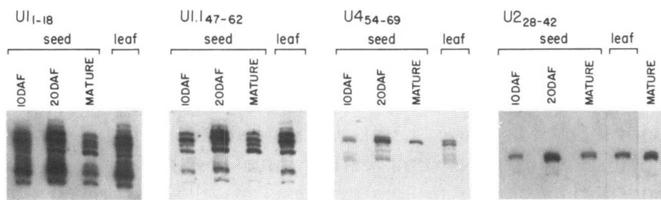

Figure 5. Northern Analysis of U1, U2 and U4 snRNAs in Leaf and Seed Tissues. Total RNAs from developing seeds and 12-day-old seedling leaves were immunoprecipitated with anti-m_3G antibody, fractionated on 15% acrylamide, 8.3 M urea denaturing gels, and subjected to gel blot analysis using the $U1_{1-18}$, $U1.1_{47-62}$ or $U2_{28-42}$ oligonucleotide probes as shown at the top of each panel. In each panel: lane 1, seed RNA isolated 10 days after flowering (DAF); lane 2, seed RNA isolated 20 days after flowering; lane 3, RNA isolated from mature dry seeds; lane 4, leaf RNA isolated from 12-day-old seedlings. In the fourth panel ($U2_{28-42}$): lane 5 is a long exposure of the Northern presented in lane 3.

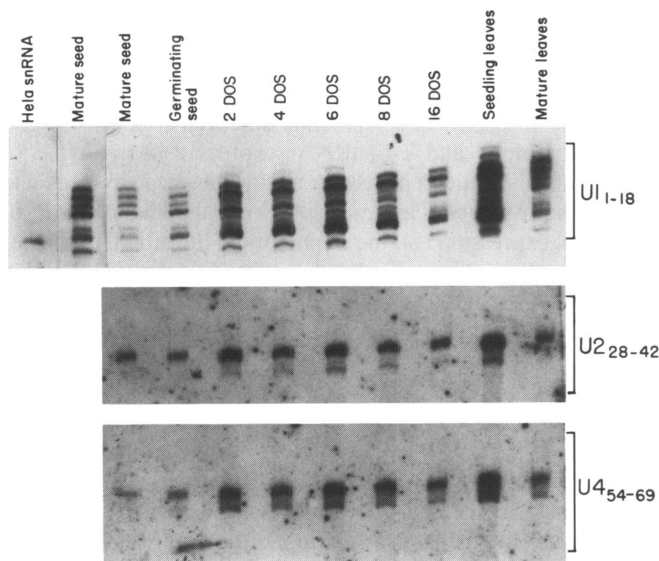

Figure 6. Developmental snRNA Variations. Total RNAs were immunoprecipitated with anti-m_3G antibody, fractionated on a 15% acrylamide, 8.3 M urea denaturing gel, and subjected to gel blot analysis using the $U1_{1-18}$, $U2_{28-42}$ or $U4_{54-69}$ oligonucleotide probes. Lane 1, HeLa cell RNA; lanes 2–3, mature seed RNA; lane 4, germinating seed RNA; lanes 5–9, RNAs isolated from 2- to 16-day-old pea seedlings (DOS); lane 10, pea seedling leaf RNA; lane 11, pea mature leaf RNA.

To determine the fate after germination of the snRNA variants present in mature seeds, snRNAs were isolated from mature and germinating seeds, 2 to 16 day old seedlings, seedling leaves and mature leaves. Northern analysis with the $U1_{1-18}$ oligonucleotide (Figure 6) indicates that the U1 snRNA populations dramatically shift as seed imbibition begins. The abundance of several more prominent U1 snRNA variants present in mature seeds decrease as germination begins and several less prominent variants increase in abundance. As germination continues and development proceeds in two day old seedlings, additional less dramatic variations in the U1 snRNA populations occur. The principal changes occurring in this developmental window are quantitative variations in the more abundant U1 snRNAs and the appearance of new, minor variants. At later stages between 2 and 16 days after germination, the U1 snRNA populations are relatively constant. Seedling leaves taken from 10 day old seedlings contain the same population of U1 snRNAs as found in 2 to 16 day old intact seedlings. Surprisingly, mature leaves contain a U1 snRNA population that is qualitatively and quantitatively identical to the U1 snRNA population expressed in mature, desiccated seeds.

The U4 snRNA population changes dramatically between germinating seeds and 2-day-old seedlings (Figure 6). After this, the U4 snRNA profile is relatively consistent throughout seedling development. As for U1 snRNAs, the U4 snRNAs expressed in 10 day old seedling leaves resemble those expressed in intact 2 to 16-day-old seedlings and developing seeds more than the U4 snRNAs expressed in mature seeds or leaves. The novel U2 snRNA expressed late in seed development, in 20 DAF and mature seed nuclei, disappears at germination and does not reappear during seedling development or in mature leaves (Figure 6). Thus, the U2 snRNA populations of mature seed and leaf tissues differ, in contrast to U1 and U4 snRNAs.

DISCUSSION

Dramatic changes in plant gene expression occur during seed and seedling development (30). In the development of pea seeds, periods of cell division and differentiation are followed by a period of seed maturation prior to desiccation. Specific subsets of genes, such as actins, tubulins and ribosomal proteins whose products are required for division and differentiation, are differentially expressed early in this process. Other subsets of genes, such as those encoding the vicilin and legumin seed storage proteins, are selectively expressed during the later period of seed maturation (30). The mature, desiccated seed which results from this development is transcriptionally quiescent. Soon after seed germination, the processes of cell division and differentiation are reinitiated and continued throughout development of the seedling into a mature plant. As individual cells and tissues differentiate, select subsets of genes are transcriptionally activated within particular developmental periods and silenced as differentiation is completed and cells mature.

Although transcription patterns change substantially throughout development, the expression of snRNAs which mediate post-transcriptional processing of the primary RNA polymerase II transcripts has not been evaluated with respect to plant development. The numerous U1, U2, U4, and U5 snRNA variants which exist in plant nuclei (20, 21, 22), represent primary sequence variants of each of these splicesomal snRNAs (23). In contrast, U6 snRNA appears to be expressed as a single species (20, 25). Cloning and sequence analysis has demonstrated that the primary sequence variations in the U1, U2, U4, and U5 snRNAs occur in regions implicated in the binding of small ribonucleoprotein proteins (snRNPs) to the snRNAs (23). As outlined in Hanley and Schuler (23), the variations in the pea U1 snRNAs occur in stem-loop I and stem II, which in mammalian cells are essential for the binding of the U1 snRNP-specific polypeptides, designated U1-A, U1-C and U1–70K (31, 32, 33, 34). U2 snRNA variations occur primarily in stems III and IV present in the 3′ half of the molecule. U4 snRNA variations occur in the central and 3′ stems which are masked with snRNP proteins in the mammalian U4/U6 snRNP complex (R.Luhrmann, communication). U5 snRNA variations are extensive and exist in both stem I and II structures that are protected by proteins in the U5 snRNP (35).

Our initial analysis of the snRNA populations in mature seeds and 12-day-old seedlings (Figure 1) demonstrated that four of the five splicesomal snRNAs, U1, U2, U4, and U5, are differentially expressed in seed and seedling tissue. Constitutive and inducible variants, which can be resolved on high resolution gels, exist for the U1, U2 and U4 snRNAs. The U5 snRNA population contains a vast number of variants (23) whose expression patterns we have not attempted to detail here. In contrast, one form of U6 snRNA is expressed at both stages in development.

The most striking differences occur in the U1, U2, and U4 snRNA populations. Analysis of the seed and seedling U1 snRNA populations on partially denaturing gels indicates that the U1 snRNA population is composed of multiple variants which assume different secondary structures rather than a unique U1 snRNA which assumes multiple conformations. Cloning and sequence analysis of the U1 snRNA variants conclusively demonstrates that multiple primary sequence variants exist.

Both the fully and partially denaturing gels indicate that novel U1 snRNA variants are expressed in seedling leaves in addition to the constitutive forms present in both seeds and seedlings. To determine if the differentially regulated forms corresponded to one select subset of the U1 snRNA variants, the snRNAs were analyzed using oligonucleotide probes complementary to the most divergent region of the pea U1 snRNAs identified by sequence analysis. These probes indicate that the majority of variants capable of hybridizing with the $U1.1_{47-62}$ probe are constitutively expressed in seeds and seedlings. Several variants which hybridize with the U1.1 oligonucleotide are expressed at different levels at these two developmental stages, but novel U1.1 snRNA variants are not readily discernible. In contrast, the expression of some variants which hybridize with the $U1.2_{47-62}$ oligonucleotide changes significantly in seeds and seedlings and can be divided into four classes: constitutive variants, novel leaf variants, abundant seed variants and abundant leaf variants. Because of the nature of the $U1.2_{47-62}$ oligonucleotide probe, all of these variants contain a stem II sequence similar to that present in U1.2 snRNA. The fact that three of the seven cloned U1 snRNAs include this stem II motif and that the U1.2 oligonucleotide probe detects the majority of U1 snRNA species visualized on partially denaturing gels (Figure 4A), indicates that a major fraction of the pea U1 snRNA population contains the stem II structure present in the U1.2 snRNA variant. Subsets of the U1.2 snRNA population are differentially expressed in development. The stem II structure present in the U1.1 snRNA exists in another prominent subset of U1 snRNAs represented by the cloned U1.1 and U1.15 variants. Together, the U1.1 and U1.2 oligonucleotide probes account for the majority of the U1 snRNA variants seen on partially denaturing gels suggesting that additional subpopulations of U1 snRNA, which might exist, contain minor U1 snRNA variants.

The expression of the different U1 and U4 snRNA variants changes dramatically at three stages in development. The first transition that we have documented occurs between 20 DAF and seed maturation as seed nuclei shift from high to low transcription rates. In *Pisum sativum*, mRNAs for the vicilin and legumin storage proteins and for lectins are essentially absent at 10 DAF (36) when cells are actively dividing. The vicilin mRNAs are then expressed at two points in seed development between 12–20 DAF and 17–27 DAF (36) and legumin mRNA accumulates between 17–25 DAF (36, 37). Thus, 20 DAF represents a period of rapid transcription and accumulation for seed storage protein and other seed-specific mRNAs. The first transition in snRNA populations, between 20 DAF and seed maturation, correlates with the transition from active to inactive transcription associated with seed desiccation. Less apparent variations in the snRNA populations expressed at earlier times, such as 10 DAF, and during storage protein synthesis at 20 DAF, potentially reflect differences in the post-transcriptional components required for processing 'housekeeping' and seed-specific RNA transcripts.

The second transition in the U1 and U4 snRNA populations occurs at seed imbibition, as seeds germinate and seedling development begins. After cell divisions and differentiations have reinitiated, the pattern of U1 and U4 snRNA expression is consistent throughout seedling development. A third transition in snRNA populations occurs between 12 days and 4 weeks after germination, as evidenced by the U1 and U4 snRNA differences between seedling and mature leaves. By high resolution Northern analysis, the snRNAs expressed in seedling leaves are essentially identical to the snRNAs expressed in intact seedlings (Figure 6) and in seedling stem or root tissue (not shown) suggesting that the individual variants are not expressed in tissue-specific manners but, rather, in specific developmental time frames. Surprisingly, the patterns of U1 and U4 snRNAs expressed in two types of transcriptionally quiescent tissue, mature desiccated seeds and mature photosynthetic leaves, are identical. The patterns of differential expression and the timing of the transitional switches suggest that the patterns of snRNA expression in pea nuclei, that we have outlined here, are more indicative of the transcriptional activity or differentiation state of a particular cell than of its cell type.

Variations in the U2 snRNA occur in time frames similar to those documented for U1 and U4 snRNAs. In contrast to U1 and U4 snRNAs which express fewer variants in mature seeds and mature leaves, a novel U2 snRNA appears in seeds 20 DAF and disappears soon after germination. The U2 variant does not reappear at leaf maturity indicating that it represents a seed-specific variant expressed exclusively during seed maturation. In contrast to these variations in the U1, U2 and U4 snRNAs, size variants of U6 snRNA have not been detected at any stage in development.

The developmental differences which typify the U1, U2, and U4 snRNA populations expressed in pea nuclei indicate that the plant RNA splicing machinery functions at a level of complexity far beyond that documented in yeast or mammalian systems. In addition to the multitude of snRNA variants constitutively expressed in pea nuclei, numerous regulated variants exist which potentially play a role in the excision of introns during periods of high transcriptional and post-transcriptional activities. In the case of U1 snRNA, the differentially expressed variants have primary sequence differences that potentially establish alternate snRNP structures at particular times in development. The differentially regulated U2 and U4 snRNA populations documented in this paper also contain sequence variations in regions implicated in the binding of small ribonucleoproteins (23). Thus, selected subsets of plant snRNAs are expressed at particular stages of development due to differential transcription and/or post-transcriptional processing of the snRNA genes. Interactions between specific subsets of the snRNAs may facilitate recognition of highly divergent plant intron sequences during periods of rapid cell growth and differentiation.

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