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Arabidopsis thaliana Expresses Three Divergent *Srp54* Genes[1]

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The *Arabidopsis thaliana Srp54* gene family was determined to consist of three genes, all of which were cloned and sequenced. In addition, cDNAs corresponding to two of the genes were obtained. To our knowledge this is the first description of multiple *Srp54* genes within an organism. In contrast to the situation in mammals, where there are only three amino acid differences between the mouse and canine sequences, there was significant amino acid sequence diversity among the genes, particularly in the methionine-rich region of the protein, which is the region responsible for binding to the 7S RNA of the signal recognition particle and to the signal sequence of newly synthesized proteins. The amino acid sequences of the GTP-binding domains of the three clones were 86% identical, whereas the methionine-rich domains were only 65% identical. RNA gel blots of various tissues and developmental stages hybridized with gene-specific probes revealed that all three genes were expressed in all the tissues investigated. There were, however, quantitative differences in expression levels.

The signal sequence hypothesis of protein targeting to the endomembrane system was proposed by Blobel and Dobberstein in 1975. Since then there have been considerable advances in understanding the mechanism of signal sequence-mediated targeting of proteins into the secretory system. In mammalian cells, the synthesis of most secreted and endomembrane proteins proceeds via SRP targeting of the nascent signal sequences to the ER, with subsequent co-translational insertion of the newly synthesized protein into the ER lumen. The mammalian SRP is an aggregate of a 7S RNA molecule and six proteins of molecular masses 72, 68, 54, 19, 14, and 9 kD (Walter and Blobel, 1980). SRP-like complexes have been isolated from *Triticum aestivum* (wheat) embryos and *Zea mays* (maize) endosperm (Prehn et al., 1987; Campos et al., 1988) and an SRP-mediated mechanism has been considered to exist for the synthesis of plant secretory proteins (Jones and Robinson, 1989; Chrispeels, 1991). Sequences for 7S RNA molecules of the maize, wheat, *Lycopersicon esculentum* (tomato), and *Arabidopsis thaliana* SRP have been reported (Haas et al., 1988; Campos et al., 1989; Marshallsay et al., 1989; Shimomura et al., 1993).

Recently, we reported the cloning from *A. thaliana* of the first plant homolog of the 54-kD protein subunit of SRP (SRP54) (Lindstrom et al., 1993). SRP54 is the protein subunit that binds to the nascent signal peptide (Krieg et al., 1986; Kurzchalia et al., 1986; Zopf et al., 1990). SRP54 homologs have been well characterized from mammals and yeasts (Bernstein et al., 1989; Hann et al., 1989; Romisch et al., 1989).

Bacteria have also been found to possess a ribonucleoprotein particle that has properties similar to eukaryotic SRP (Poritz et al., 1990; Ribes et al., 1990; Miller et al., 1994), and a bacterial protein designated Ffh has sequence homology to SRP54 (Bernstein et al., 1989; Romisch et al., 1989). *Escherichia coli* Ffh has been shown to be important in export of some proteins (Phillips and Silhavy, 1992). A chloroplast-localized homolog of SRP54 has been reported (Franklin and Hoffman, 1993) that shares more sequence similarity with the bacterial homolog than with the cytoplasmic SRP54. The chloroplast-localized homolog may target proteins to the thylakoids or chloroplast envelope (Franklin and Hoffman, 1993).

Here we report the characterization of the *A. thaliana Srp54* gene family and the expression of the genes in different tissues and developmental stages. To our knowledge this is the first description of an *Srp54* gene family in any organism. Sequences of the individual genes revealed an unexpected amino acid sequence diversity in the region of the protein that binds to the complex.

MATERIALS AND METHODS

Plant Materials

For DNA isolation, *Arabidopsis thaliana* Columbia plants were grown from seed in the greenhouse. For RNA isolation from roots and young seedlings, *A. thaliana* seeds were surface sterilized and germinated on Murashige and Skoog basal medium (Sigma) with Glc (20 g/L). Agar culture medium was used for seedlings and liquid culture medium was used for roots. The seedlings and roots were harvested after 6 and 18 d, respectively. For RNA isolation from leaves and flowers, *A. thaliana* plants were grown from seeds in the greenhouse. Leaves were harvested after 15 d. The 25-d sample was collected at the bolting stage and consisted of leaves and the developing flower spike. Flowers were harvested as either unopened or opened flowers after 30 d.

Nucleic Acid Isolation and Gel Blot Analysis

A. thaliana total DNA was isolated as described by Dellaporta et al. (1983) followed by further purification on CsCl

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Abbreviation: SRP, signal recognition particle.

gradients. Total RNA was isolated by using the guanidine-HCl method described by Cox (1968). Polyadenylated RNA was fractionated from total RNA using the PolyATract mRNA isolation system (Promega, Madison, WI).

For DNA gel blot analysis, 10 μg of restriction enzyme-digested total genomic DNA were electrophoresed in 0.8% agarose gel and transferred to Zetaprobe membranes (Bio-Rad) using the alkaline transfer method (Reed and Mann, 1985). For RNA gel blot analysis, 4 μg of poly(A)$^+$ RNA were subjected to electrophoresis in formaldehyde agarose gels and transferred to Magnagraph nylon membranes (Micron Separations, Inc., Westborough, MA) as described by Selden (1987).

Gene-specific probes were generated from the genomic clones by PCR amplification. Seventeen-base primers corresponding to the ends of the fragments were used for amplification using Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA). For use in hybridizations, the required DNA fragments, obtained either directly from a PCR product or from the appropriate clones by restriction enzyme digestions, were separated on low-melting-point agarose gels and the desired band was cut out of the gel. The fragments, in low-melting-point agarose, were labeled with [α-^{32}P]dATP by using a commercial labeling kit (Stratagene). The β-tubulin probe (provided by Dr. D.P. Snustad) was labeled by primer extension (Hu and Messing, 1982).

For both the RNA and DNA gel blots, filters were prehybridized at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 50 mM sodium phosphate, pH 6.8, 1% SDS, 100 μg/mL calf thymus DNA, and 2.5% dextran sulfate. The hybridization solution was 1×10^5 to 5×10^5 cpm/mL of ^{32}P-labeled fragment, 50% formamide, 5× SSC, 1× Denhardt's solution, 20 mM sodium phosphate, pH 6.8, 1% SDS, 100 μg/mL calf thymus DNA, and 5% dextran sulfate. The same RNA and DNA gel blots were used for all the gene-specific probes, being stripped in between hybridizations. The RNA gel blot was stripped in 5 mM Tris-Cl, pH 7.5, 0.2 mM EDTA, and 0.1% SDS at 75°C for 30 min. The DNA gel blot was stripped in 0.4 N NaOH at 42°C for 30 min. With the *Srp54* probes the RNA gel blot was exposed with an intensifying screen for 7 to 14 d. With the β-tubulin as probe the blot was exposed for 4 h.

Genomic and cDNA Library Screening and Clone Characterization

Two 104-bp PCR clones with sequence homology to mouse *Srp54* were obtained as described previously (Lindstrom et al., 1993). Radiolabeled probes were prepared from the insert fragments and were used to screen an *A. thaliana* genomic library in the λ phage vector EMBL3 SP6/T7 (Clontech Laboratories, Palo Alto, CA) as described by Huynh et al. (1985). Sixteen-base oligonucleotides, identical to one end of the fragments, were used as the primers in the labeling reactions. Positive plaques following three rounds of screening were selected for further characterization. Growth of the recombinant phage in liquid culture and λ DNA preparation were as described by Maniatis et al. (1982).

For sequencing, restriction enzyme fragments were subcloned into both M13mp18 and M13mp19 (Yanisch-Perron et al., 1985) to obtain inserts in opposite directions. Dideoxynucleotide sequencing (Sanger et al., 1977) of single-stranded templates with T7 DNA polymerase was performed by using a commercial sequencing kit (United States Biochemical) using 17-base oligonucleotides as primers.

An *A. thaliana* Columbia λ gt11 cDNA library prepared from random hexamer-primed poly(A)$^+$ RNA from 30-d-old whole seedlings was provided by Dr. E. Lam. The library was probed with the ^{32}P-labeled 674-bp *SacI/SphI* fragment of the *Srp54-1* genomic clone. In a screen of 400,000 clones, two positive plaques were identified and processed for sequencing as described above for the genomic clones.

RESULTS

Isolation of *A. thaliana Srp54* cDNA Clones

We previously reported the characterization of an *A. thaliana Srp54* genomic clone (Lindstrom et al., 1993), here designated *Srp54-1*. In a comparison of the *A. thaliana* SRP54-1 deduced amino acid sequence with SRP54 sequences from diverse organisms, the N-terminal region of the protein, which contains a conserved GTP-binding region (Dever et al., 1987) and is termed the G-domain (Bernstein et al., 1989), exhibited a high level of sequence conservation (Lindstrom et al., 1993). The 674-bp *SacI/SphI* fragment, containing the first three exons of the *Srp54-1* genomic clone, was used to probe an *A. thaliana* cDNA library. A cDNA clone corresponding to the *Srp54-1* gene was completely sequenced. The cDNA sequence confirmed the original assignment of intron positions in *Srp54-1* (Lindstrom et al., 1993), which was based on amino acid sequence homology to the mammalian sequences and on splice site consensus sequences (Breathnach and Chambon, 1981). The 5′ and 3′ ends of a second cDNA clone were sequenced and found to correspond to another gene, designated *Srp54-3* (see below). The *Srp54-1* and *Srp54-3* cDNAs contained 38 and 20 bp, respectively, of sequence 5′ to the initiator Met codon. They contained 13 and 90 bp, respectively, of 3′ noncoding sequence. Since the cDNA library was prepared by random priming, the cDNA clones did not contain the poly(A)$^+$ tail.

Isolation of Additional *A. thaliana Srp54* Genomic Clones

The *Srp54-1* genomic clone was originally cloned by using PCR to obtain a 104-bp fragment that was then used to probe a genomic library. The same PCR fragment also hybridized to another *Srp54* genomic clone, designated *Srp54-2*. In the original PCR reaction, a second 104-bp *Srp54*-like PCR fragment with a different nucleotide sequence was obtained that hybridized to a third gene designated *Srp54-3*. The *Srp54-2* and *Srp54-3* genomic clones were completely sequenced. Diagrams of the gene structures are shown in Figure 1. *Srp54-1* (Lindstrom et al., 1993) is also included for comparison. A 4-bp sequencing error in the 3′ noncoding region of the *Srp54-1* sequence, which eliminates an *SphI* site, has been corrected. From DNA gel blots using gene-specific probes (see below), it was determined that the *SacI* site at the 3′ end

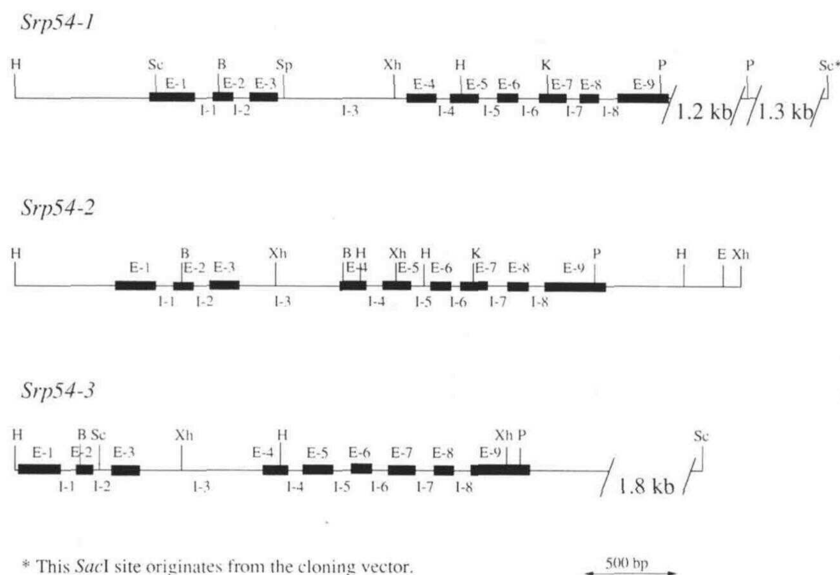

Figure 1. Structural features of the three *A. thaliana Srp54* genes. The organization of the *Srp54* genes with respect to exons (dark boxes), 5′-flanking sequence, introns, and 3′-flanking sequence (lines) is diagrammed. H, *Hin*dIII; Sc, *Sac*I; B, *Bam*HI; Sp, *Sph*I; Xh, *Xho*I; K, *Kpn*I; P, *Pst*I; E, exon; I, intron.

of the *Srp54–1* clone originated from the cloning vector and not the *A. thaliana* DNA.

The exon and intron sizes of the three genes are reported in Table I. All three genes had eight introns at identical positions in the coding sequence, and the sizes of the introns were similar. The smallest intron, intron 6 of *Srp54–2*, was 69 bp, which is 1 bp smaller than the reported minimum length for splicing in plants (Goodall and Filipowicz, 1990).

All of the exons had identical lengths, with two exceptions. The largest difference among the three genes was that exon 9 of *Srp54–1* was 45 bp smaller than exon 9 of the two other genes. Also, exon 7 of *Srp54–1* was 3 bp smaller than exon 7 of the other two genes. Overall, *Srp54–1* encoded a protein 16 amino acids smaller than the other two genes. At the nucleotide level the identities of exons 1 through 9 of the three genes ranged from 71 to 91%. As would be expected, the intron sequences were much more divergent, with identity levels ranging from 21 to 70%.

A comparison of the *A. thaliana* SRP54 deduced amino acid sequences from all three genes is shown in Figure 2. The tripartite GTP-binding motif was conserved among the three sequences. Overall, the three *A. thaliana* SRP54 amino acid sequences were 78% identical. In general, SRP54 amino acid sequences from different organisms exhibit lower sequence conservation in the carboxy-terminal Met-rich region of the protein, designated the M-domain (Bernstein et al., 1989). The M-domain is the region of the protein that binds to the signal sequence of a newly synthesized protein (Zopf et al., 1990; High and Dobberstein, 1991). Interestingly, even within a single species, *A. thaliana*, there is considerably more sequence variation in the M-domains of the proteins. The G-domains of the three amino acid sequences were 86% identical, whereas the M-domains were only 65% identical. The SRP54–2 and SRP54–3 amino acid sequences are clearly more closely related to each other than to SRP54–1. The SRP54–2 and SRP54–3 sequences are 91% identical in the G-domain and 82% identical in the M-domain. In Table II the percent amino acid identities of the *A. thaliana* sequences are compared to those of other eukaryotic SRP54 sequences.

Representation of *Srp54* Sequences in the *A. thaliana* Genome

Based on DNA gel blot analysis, we previously reported that there were at least two *Srp54* genes in *A. thaliana* (Lindstrom et al., 1993). Since we have now isolated three *Srp54*

Table I. *Exon and intron lengths of the three members of the A. thaliana Srp54 gene family*

Gene	Length in bp								
	1	2	3	4	5	6	7	8	9
Srp54-1									
Exon	231	100	154	151	149	101	155	115	281
Intron	85	96	695	81	112	115	74	77	
Srp54-2									
Exon	231	100	154	151	149	101	158	115	326
Intron	97	93	541	83	110	69	90	83	
Srp54-3									
Exon	231	100	154	151	149	101	158	115	326
Intron	76	94	648	80	113	93	88	93	

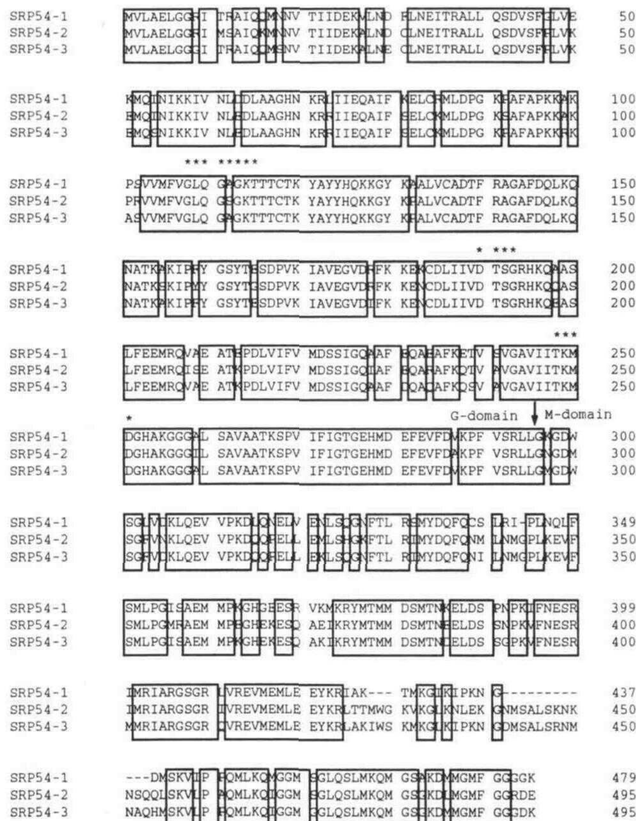

Figure 2. Amino acid sequence comparison of the three *A. thaliana* SRP54 proteins. Boxes enclose identical amino acids. The tripartite GTP-binding motif is indicated by asterisks. The separation of the amino-terminal G-domain from the carboxy-terminal M-domain (Bernstein et al., 1989) is indicated by the arrow.

genomic clones, we reinvestigated the gene copy number. In Figure 3, lane 1, a *Hin*dIII digest of *A. thaliana* DNA was probed with the *Hin*dIII fragment of *Srp54-3* cDNA, which contains exons 1, 2, 3, and part of 4, a highly conserved region of the coding sequence. In the cDNA clone the 5′ *Hin*dIII site originated from the multicloning site of the vector. Three distinct bands of the predicted sizes for the three genes were detected. In Figure 3, lane 2, a *Sac*I digest was probed with the *Bam*HI/*Kpn*I fragment of the *Srp54-1* cDNA, which consists mainly of exons 2 through 6. Again, hybridization

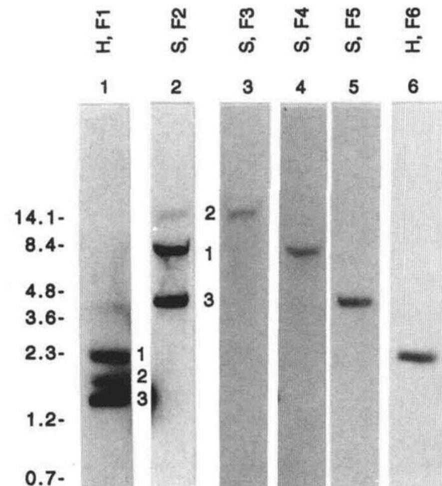

Figure 3. Genomic DNA gel blot analysis of the *A. thaliana Srp54* genes. Ten micrograms of *A. thaliana* total DNA was digested with *Hin*dIII (H) or *Sac*I (S), fractionated by electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane, and sequentially hybridized with the indicated fragments, which are described in Table III. The genes corresponding to the hybridizing bands in lanes 1 and 2 are indicated. Sizes of the DNA markers are in kb.

to three bands was observed, suggesting that there were no additional closely related *A. thaliana Srp54* genes.

Expression of the *A. thaliana Srp54* Genes

To monitor the expression of individual genes, we identified regions of the three genes that could be used as gene-specific probes. These regions are described in Table III. DNA gel blot analysis was used to confirm the gene-specific nature of the probes (Fig. 3, lanes 3–6). In each case only a single hybridizing band was observed.

The gene-specific probes, as well as a coding region fragment from the *Srp54-1* cDNA that hybridized to all three sequences, and an *A. thaliana* β-tubulin probe were used in RNA gel blot analysis of poly(A)$^+$ RNA isolated from various tissues (Fig. 4). Transcripts for all three genes were detected

Table II. *The extent of amino acid identities of SRP54 from different sources*

	A.t. 1	*A.t.* 2	*A.t.* 3	Mouse	*S.p.*	*S.c.*
A.t. 1[a]	*	81	85	54	46	42
A.t. 2		*	88	51	45	41
A.t. 3			*	54	44	43
Mouse[b]				*	53	46
S.p.[c]					*	53
S.c.[d]						*

[a] *A. thaliana* SRP54-1, 2, and 3 sequences from this study. [b] Mouse sequence from Bernstein et al. (1989). [c] *S. pombe* sequence from Hann et al. (1989). [d] *S. cerevisiae* sequence from Hann et al. (1989).

Table III. *Description of DNA fragments used as probes in DNA and RNA gel blot analysis*

Fragment Designation	Description
F1	608-bp *Hin*dIII fragment from the *Srp54-3* cDNA clone
F2	673-bp *Bam*HI/*Kpn*I fragment from the *Srp54-1* cDNA clone
F3	368-bp PCR product that consists of 230 bp upstream and 135 bp downstream of the TAG stop codon of *Srp54-2*
F4	275-bp PCR product that consists of 8 bp upstream and 264 bp downstream of the TAG stop codon of *Srp54-1*
F5	175-bp PCR product that consists of 10 bp upstream and 161 bp downstream of the TAG stop codon of *Srp54-3*
F6	Approximately 700-bp *Hin*dIII/*Sac*I fragment from the *Srp54-1* genomic clone that consists mainly of 5′ flanking sequences

in all the tissues analyzed, indicating that all three are functional genes. As assessed by film exposure time required for detection, the steady-state message levels for SRP54 were low, since the coding sequence probe (F2) required 7 d of exposure for detection, in contrast to the 4 h of exposure required for β-tubulin transcript detection.

There were some quantitative differences in expression of the individual *Srp54* genes. Because the gene-specific probes were of differing sizes, the signal intensities from the three probes could not be compared. The level of expression of the individual genes in the different tissues, however, could be compared. The most striking quantitative difference was the relatively high expression of *Srp54–1* in roots (Fig. 4, lane 4). *Srp54–2* expression was highest in the unopened flower tissue (Fig. 4, lane 5), whereas *Srp54–3* expression was more uniform in all the tissues. For all three genes the tissue with consistently the lowest transcript level was the 15-d leaf sample (Fig. 4, lane 2), a tissue that had completed most of its growth and may be synthesizing fewer proteins that enter the secretory pathway. The 25-d-old plant was at the bolting stage and consisted of leaves and the developing flower spike. The higher expression level in the 25-d-old plant (Fig. 4, lane 3) relative to the 15-d leaves (Fig. 4, lane 2) is likely due to the contribution of the rapidly growing flower spike.

DISCUSSION

In this report we have characterized three *A. thaliana Srp54* genes that appear to represent the entire gene family. From DNA gel blots probed with conserved regions of the genes, there do not appear to be any other highly homologous genes. The deduced amino acid sequences of the genes are homologous to other eukaryotic *Srp54* genes.

An *A. thaliana* nuclear-encoded, chloroplast-localized homolog of SRP54, designated 54CP, has been reported that may be involved in targeting chloroplast proteins to the thylakoids or the chloroplast envelope (Franklin and Hoffman, 1993). The protein 54CP is encoded by a single gene. The three *Srp54* clones described here do not contain N-terminal extensions suggestive of chloroplast targeting sequences, and are thus considered to code for the cytoplasm-localized SRP54. The deduced amino acid sequence of the *A. thaliana* chloroplast-localized homolog was only 25 to 26% identical to the three sequences described here.

The use of gene-specific probes revealed that all three of the *A. thaliana Srp54* genes were expressed. All three genes were expressed in all the tissues investigated. There were, however, quantitative differences in expression levels, indicating some differential regulation of expression. The presence of SRP54 would be expected in all metabolically active cells, since it is required for synthesis of most secreted and endomembrane-localized proteins. More mature tissues, such as the 15-d leaf sample, may not be synthesizing as many proteins that enter the secretory pathway and thus may require a lower level of SRP proteins. The factors that may modulate the level of expression in different tissues or developmental stages are as yet unknown.

To our knowledge this is the first description of an *Srp54* gene family in any organism. Genomic clones for the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have been reported, but these organisms contained single copies of the genes (Hann et al., 1989). The *Srp54* gene copy number in mammalian systems has not been reported. We have recently determined that in barley (*Hordeum vulgare*), SRP54 is also encoded by multiple genes (B. Chu and F.C. Belanger, unpublished data).

Within the species *A. thaliana* there is greater variability in SRP54 amino acid sequences than there is between the mouse and canine sequences, which differ by only three amino acids. It is interesting that most of the variability in the *A. thaliana* SRP54 sequences occurs in the M-domain of the protein, which is the region of SRP54 responsible for the binding of the protein to the SRP. The M-domain of SRP54 binds to the RNA component of SRP and also to the signal peptide of newly synthesized proteins (Romisch et al., 1990; Zopf et al., 1990), although the specific amino acids involved in the binding have not been determined. If the binding sites are conserved among the three *A. thaliana* proteins, the

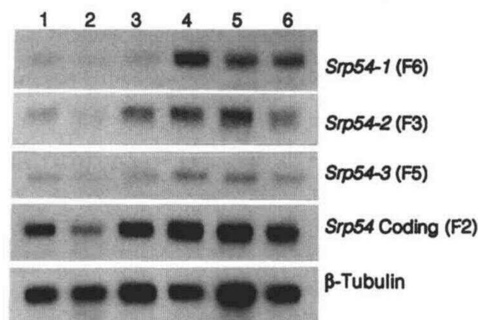

Figure 4. RNA gel blot analysis of *Srp54* transcripts. Four micrograms of poly(A)$^+$ RNA isolated from the various tissues were used for each sample. The blot was sequentially probed with the indicated fragments, which are described in Table III. Lane 1, Six-day seedling; lane 2, 15-d leaves; lane 3, 25-d plant; lane 4, 18-d roots; lane 5, unopened flowers; lane 6, opened flowers.

conserved regions of the M-domains may delimit those amino acids involved in binding. There are, however, no extended regions of amino acid identity in the M-domains of SRP54 sequences from *A. thaliana*, mouse, and yeasts (Lindstrom et al., 1993). Alternatively, the presence of variability in the region of the protein important for binding raises the question of whether the three *A. thaliana* SRP54 proteins may have different functional efficiencies.

Okita et al. (1994) discussed functional variability of SRPs as a possible mechanism for targeting particular messages to different subdomains of the ER. Multiple 7S RNAs have been recovered from the maize and wheat SRPs (Campos et al., 1989; Marshallsay et al., 1989). Subpopulations of SRP with variations in 7S RNA sequences and in SRP54 sequences may exist that may also have variability in their ability to recognize various signal sequences. Ultimately, this possibility could be investigated using in vitro binding assays.

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The GenBank accession numbers of the *Srp54-1*, *Srp54-2*, and *Srp54-3* genes are L19997, U12126, and U12127, respectively.

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