

PARTIAL PURIFICATION AND CHARACTERIZATION OF RNASE P FROM
ARABIDOPSIS THALIANA TISSUE

A Thesis

Presented in Partial Fulfillment of the Requirement for
the Degree Master of Science in the
Graduate School of The Ohio State University

By

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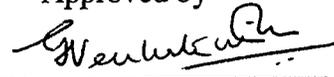
The Ohio State University
2000

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ABSTRACT

A distinct ribonuclease P (RNase P) activity that cleaves leader sequences from precursor tRNA (ptRNA) molecules to give mature 5' ends has been isolated from *Arabidopsis thaliana* tissue. The RNase P activity was isolated by ammonium sulfate precipitation of a tissue homogenate and further purified by anion exchange chromatography. The isolated activity is capable of processing tobacco ptRNA^{Gly} and cyanobacterial ptRNA^{Gln} substrates *in vitro*. The two tested substrates are processed both with and without a terminal 3'CCA sequence which, when present, increases processing by the catalytic subunit of *Escherichia coli* RNase P. The isolated activity was shown to be sensitive to micrococcal nuclease and proteinase K indicating that a ribonucleoprotein is essential for catalytic activity. Database mining of the *Arabidopsis* genome revealed a possible homolog for the Rpp38 subunit of human RNase P holoenzyme. Western blot analysis of the isolated RNase P activity revealed cross-reactivity with human Rpp38 antibody; specifically, a 19-kDa *Arabidopsis* protein is identified by the Rpp38 antibody. These results strongly suggest that *A. thaliana* RNase P is a ribonucleoprotein complex and is similar to that isolated from other eukaryotic systems.

Dedicated to the soldiers of the United States Army Chemical Corps

ACKNOWLEDGMENTS

I wish to express sincere appreciation to my advisor, Dr. Venkat Gopalan, for providing sound leadership and guidance throughout the course of this work.

I am grateful to the United States Army for funding my schooling and giving me the opportunity to learn at the highest levels.

I thank Steven Raj, Dileep Kumar, Roopa Biswas, Tim Eubank, Milan Jovanovic, Ryan Perieria and the other members of the Gopalan Lab for providing assistance on the technical aspects of performing research in Biochemistry.

I also wish to thank Dr. Randy Scholl and Mike Tilly for their generous gift of *Arabidopsis* plant tissue.

This research would not have been possible without the support and devotion of my lovely wife Marla and our two beautiful daughters, Malia and Haley. To them I express my sincerest gratitude and love.

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LIST OF ABBREVIATIONS

- BSA - bovine serum albumin
- CE - crude extract (i.e. RNase P isolated after ammonium sulfate precipitation)
- DTT - dithiothreitol
- EB - extraction buffer
- EDTA - ethylenediaminetetraacetate
- EGS - external guide sequence
- EGTA - ethylene glycol bis-(β -amino ethyl ether) tetraacetate
- fmole - femtomole
- FPLC - fast performance liquid chromatography
- GG - RNase P enzyme after glycerol gradient fractionation
- Gln - glutamine
- Gly - glycine
- GTP - guanosine triphosphate
- kb - kilo base
- M - molar
- M1 RNA - catalytic RNA subunit of *E. coli* RNase P
- mg - milligram
- ml - milliliter

mM - millimolar

MN - micrococcal nuclease

mRNA - messenger ribonucleic acid

MW - molecular weight

ng - nanogram

PCR - polymerase chain reaction

pGln - precursor glutamine transfer ribonucleic acid

pGly - precursor glycine transfer ribonucleic acid

PK - proteinase K

pmole - picomole

PMSF - phenylmethylsulfonyl flouride

ptRNA - precursor transfer ribonucleic acid

pTyr - precursor tyrosine transfer ribonucleic acid

Q - RNase P enzyme isolated by anion exchange chromatography

RNP - ribonucleoprotein

RNase - ribonuclease

Rpp - RNase P protein

RT - reverse transcriptase

TN - immunodetection buffer (Tris, NaCl)

TNT - TN + Tween 20

TrisHCl - [tris (hydroxymethyl) aminomethane] hydrochloride

tRNA - transfer ribonucleic acid

Tyr - tyrosine

μg - microgram

μl - microliter

CHAPTER 1

INTRODUCTION

1.1 RNase P in Bacteria

Ribonuclease P (RNase P) is a ubiquitous endonuclease that is uniquely responsible for the 5' maturation of all precursor tRNAs (ptRNAs) in both prokaryotes and eukaryotes (Fig. 1.1). RNase P was first discovered in *Escherichia coli* (Altman et al., 1971) and subsequent biochemical purification revealed that this enzyme consisted of one protein and one RNA subunit (Kole et al., 1980). The RNA subunit, termed M1 RNA, was found to be the catalytic subunit of the *E. coli* RNase P holoenzyme (Guerrier-Takada et al., 1983). M1 RNA is catalytically active *in vitro* in the absence of its protein cofactor (referred to as C5 protein) under high ionic strength. However, both subunits are absolutely necessary for viability. The *E. coli* RNase P holoenzyme is a well-studied prototype of all eubacterial RNase P.

1.2 RNase P in Archaeobacteria

In archaeobacteria, the RNase P holoenzyme is believed to consist of both RNA and protein in a manner similar to eubacteria. RNA subunits from the methanobacteria, thermococci, and extreme halophiles, like their bacterial homologs, are catalytically

active *in vitro* in the absence of protein at high ionic strength. Additionally, a chimeric holoenzyme consisting of the RNA subunit from *Methanobacterium thermoautotrophicum* RNase P and the protein subunit of *Bacillus subtilis* is catalytically active at low ionic strength (Pannucci et al., 1999). The RNA subunits of *E. coli*, *M. thermoautotrophicum*, and *M. jannaschii* RNase P share remarkably similar secondary structure (Fig. 1.2). These findings indicate that archaebacterial RNase P RNAs contain all of the elements necessary for substrate recognition and catalysis but require a protein cofactor for optimal catalysis.

1.3 RNase P in Non-Plant Eukarya

RNAs that co-purify with RNase P have been identified in *S. cerevisiae*, several species of *Schizosaccharomyces*, *Xenopus laevis*, and human (Krupp et al., 1986; Lee and Engleke, 1989; Baer et al., 1990; Zimmerly et al., 1990; Doria et al., 1991). Partially purified enzymes have been demonstrated to be sensitive to nuclease treatment, suggesting that nucleic acid subunits present in these preparations are essential for catalytic activity. Eukaryotic RNase P holoenzymes differ from their prokaryotic counterparts in that they possess multiple (at least seven) protein subunits and one RNA subunit. A comparison of the RNase P RNA and protein subunits identified in human and yeast is provided (Table 1.1). Thus far, the RNA subunits of eukaryotic systems have not been shown to be catalytically active in the absence of their protein subunits. The possibility exists that, through the process of evolution, the protein subunits have taken over some of the catalytic function from the RNA subunit.

1.4 RNase P in Plants

Although RNase P has been widely studied in prokaryotes and other eukaryotes, very little information is available on the subunit composition, substrate requirements, or cleavage mechanism of plant RNase P. Studies with the purified enzyme of nuclei or organelles have been hampered by the difficulties encountered during large-scale organellar preparation and the instability of the enzyme during extensive purification. All reported studies have used some type of organellar preparation or cell suspension rather than using plant tissue which is likely to be rich in nucleases. Partial studies have been reported on spinach chloroplasts (Wang et al., 1988), wheat nuclei (Arends and Schön, 1997) and mitochondria (Hanic-Joyce and Gray, 1990), potato mitochondria (Marchfelder and Brennicke, 1994), carrot (Franklin et al., 1995), and the photosynthetic organism *Cyanophora paradoxa* (Baum et al., 1996). A summary of the properties of the enzymes purified in these various studies is provided (Table 1.2). *C. paradoxa* is included in Table 1.2 because it contains a photosynthetic cyanelle and is a possible evolutionary precursor to plant chloroplasts. Whether plant RNase P contains an RNA subunit has been unclear due to conflicting results from these studies. In the studies employing spinach chloroplasts, wheat nuclei, and wheat mitochondria, RNase P was found to be insensitive to nuclease treatment, indicating the absence of a catalytically essential RNA subunit. In contrast, studies with carrot and *C. paradoxa* RNase P showed that the holoenzyme is nuclease sensitive. The notion that plant RNase P could have evolved into an enzyme devoid of a nucleic acid subunit is inconsistent with the observations that RNase P in *C. paradoxa*, a likely evolutionary precursor to plants, and higher eukaryotes (human and yeast) are RNP complexes. No attempts have been made

to isolate the protein subunits of RNase P in plants or to examine homology to the known protein subunits of human or yeast (Table 1.1). Although the RNA subunit of plant RNase P is not expected to be catalytically active in the absence of protein subunits, this hypothesis has yet to be tested.

1.5 Is Plant RNase P an RNP Complex?

Any macromolecular complex which consists of protein and RNA subunit(s) is commonly referred to as a ribonucleoprotein (RNP). Ribosomes and spliceosomes are examples of RNPs in nature. The RNase P holoenzymes of eubacteria, archaebacteria, and non-plant eukaryotes are RNPs. Therefore, it is reasonable to assume that plant RNase P would also possess protein and RNA subunit(s).

Proving the presence of an RNA subunit in RNase P has relied on two approaches: (i) treatment of the RNP complex with a nuclease to determine the contributions of an RNA to catalytic activity, and (ii) measuring the density of the RNP complex in cesium chloride/sulfate gradients. In the first method, the RNase P enzyme of interest is incubated with a nuclease, generally micrococcal nuclease (MN), in the presence of calcium (needed for MN activity) to destroy the RNA subunit of RNase P. The calcium is then chelated with EGTA to inactivate MN prior to the addition of ptRNA substrate. The inactive MN/RNase P mixture is then incubated with a ptRNA substrate and monitored for 5' processing activity characteristic of RNase P. If MN successfully degrades the RNA subunit of RNase P, 5' ptRNA substrate processing is destroyed or diminished. Successful 5' tRNA processing indicates the absence of an RNA subunit or

that the RNA subunit is not essential for catalytic activity. Prokaryotic RNase P holoenzymes are sensitive to treatment with a nuclease.

In all of the previous RNase P studies it was generally believed that an RNA component is present in the plant RNase P holoenzyme. This assumption was clouded by the results of studies involving spinach and wheat. In spinach chloroplast and two independent studies on wheat, RNase P was found to be sensitive to nuclease treatment indicating the presence of an RNA subunit; however, this result was reversible when additional poly-A RNA was introduced (Wang et al., 1988; Hanic-Joyce and Gray, 1990; Arends and Schön, 1997). The authors of all three papers attributed the initial loss of activity not to the inactivation of the RNase P RNA subunit but to substrate masking, a phenomenon where the inactive nuclease binds the ptRNA substrate and shields the cleavage site from RNase P. Addition of poly-A RNA to the inactive MN/RNase P mixture liberates the nuclease-bound ptRNA substrate making it available for 5' processing by RNase P. Conclusions from studies with carrot RNase P contradicted the results from spinach and wheat RNase P in that the 5' processing activity of carrot RNase P was sensitive to MN and the loss of 5' processing activity could be rescued by addition of RNase P enzyme to the inactive MN/RNase P mixture (Franklin et al., 1995). The restored activity was similar to that of the enzyme in the absence of nuclease and could not be attributed to substrate release by the nuclease as no competing RNA species was added. It is interesting that *C. paradoxa* RNase P was also sensitive to nuclease treatment but the activity was not restored by the addition of poly A RNA (Baum et al., 1996).

The second approach to proving the presence of an RNA subunit in RNase P relies on cesium chloride/sulfate gradients. Prokaryotic RNase P holoenzymes generally have higher densities (>1.5 g/ml) in CsCl or Cs₂SO₄ gradients than protein enzymes (1.25 g/ml) indicating the predominance of RNA in the enzyme (Darr et al., 1992). Non-plant eukaryal enzymes (human, yeast, *Xenopus laevis*), however, are light in cesium chloride/sulfate gradients and are much less dense (1.28 - 1.34 g/ml) than most RNP complexes. Wheat and spinach preparations have densities of 1.34 g/ml and 1.28 g/ml in CsCl gradients (Schön, 1996; Wang, et al. 1988) and are consistent with the densities for other eukaryal RNase P enzymes. Comparison of densities in cesium chloride/sulfate gradients to other RNase P enzymes offers a general method to confirm a high or low RNA/protein ratio.

1.6 Preface to this Study

The results of this research provide evidence that *Arabidopsis thaliana* RNase P is able to process ptRNA substrates in a comparable fashion to that observed with the catalytic RNA subunit of *E. coli* RNase P. The loss of activity observed upon treatment of *A. thaliana* RNase P with MN and PK indicates that the enzyme is an RNP complex. The same activity when probed on a western blot cross-reacts with antibody raised against the Rpp38 protein subunit of human RNase P. These results indicate that RNase P is present in *A. thaliana* and that the holoenzyme is an RNP complex with homology to human RNase P.

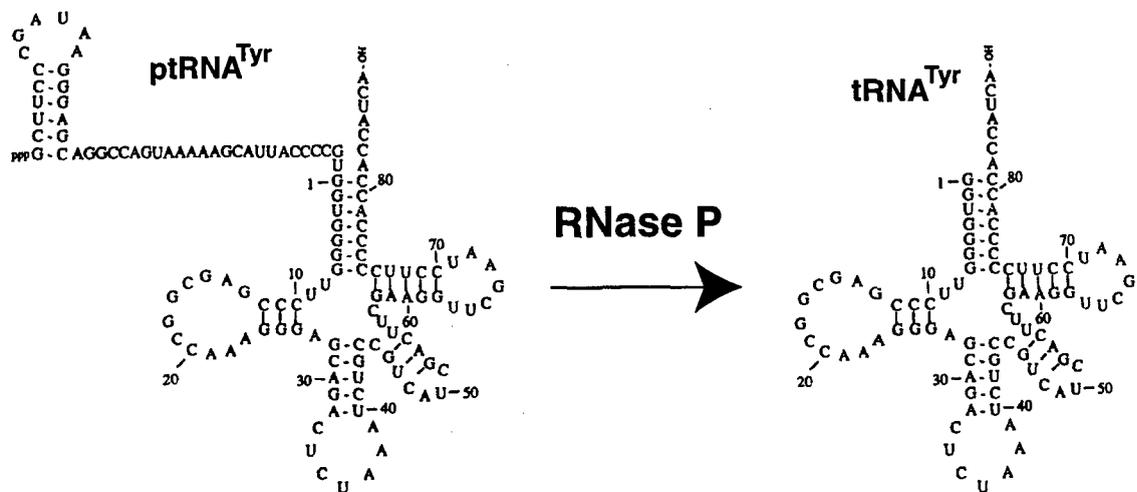


Fig. 1.1. The 5' tRNA Processing Reaction Catalyzed by RNase P

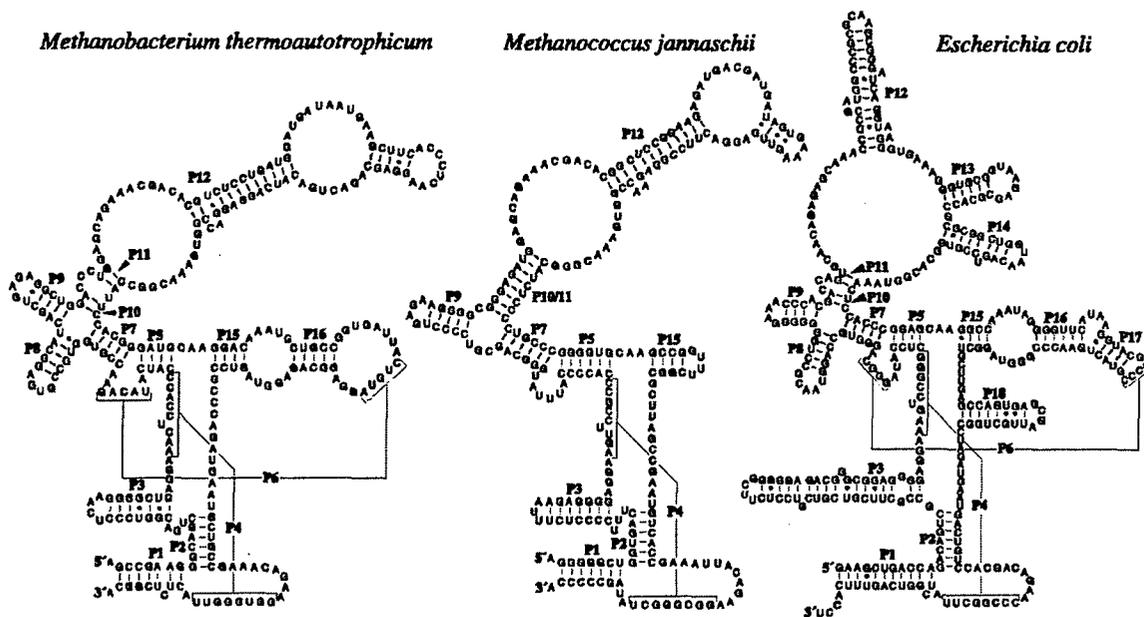


Fig. 1.2. Comparison of Secondary Structures of the RNA Subunits of Archaeal and Bacterial RNase P

<u><i>E. coli</i> RNase P</u>	<u>Human RNase P</u>	<u>Yeast RNase P</u>
<i>RNA subunit</i>	<i>RNA subunit</i>	<i>RNA subunit</i>
M1RNA, 377 nts	H1RNA, 340 nts	RPR1, 369 nts
<i>Protein subunit, kDa</i>	<i>Protein subunit, kDa</i>	<i>Protein subunit, kDa</i>
C5 protein, 13.7	Pop1	~ Pop1, 100.4
	Rpp14, 13.7	Pop3, 22.6
	Rpp20, 15.6	~ Pop7, 15.8
	Rpp29, 25.4	~ Pop4, 32.8
	Rpp30, 29.3	~ Rpp1, 32.2
	Rpp38, 31.8	Pop5, 19.6
	Rpp40, 34.5	Pop6, 18.2
		Pop8, 15.5
		Rpr2p, 16.3

Table 1.1. Subunit Composition of Representative Bacterial and Eukaryal RNase P.
Sequence homology is indicated by ~.

Source	Subcellular Locale	Density CsCl (g/ml)	Nuclease Sensitive	RNA Subunit	Protienase Sensitive
Spinach (Wang et al., 1988)	chloroplasts	1.28	-	?	+
Wheat (Arends and Schön, 1997)	nuclei	1.34	-	+(?)	no data
Wheat (Hanic-Joyce and Gray, 1990)	mitochondria	no data	-	?	no data
Potato (Marchfelder and Brennicke, 1994)	mitochondria	no data	no data	+	no data
Carrot (Franklin et al., 1995)	organellar ?	no data	+	+	+
Carrot (Franklin et al., 1995)	nuclear ?	no data	+	+	+
<i>Arabidopsis thaliana</i> (this study)	?	1.16	+	+	+
<i>Cyanophora paradoxa</i> (Baum et al., 1996)	cyanelle	no data	+	+	+

Table 1.2. Properties of RNase P in Plants and a Photosynthetic Organism.

CHAPTER 2

MATERIALS AND METHODS

2.1 *In vitro* Synthesis of M1 RNA and ptRNA Substrates

Plasmid pT7Gln used to transcribe cyanobacterial ptRNA^{Gln} was a kind gift from Dr. Agustín Vioque and was prepared as described (Pasqual and Vioque, 1999). *E. coli* ptRNA^{Tyr} was a kind gift from Dr. Sidney Altman and was prepared as described (Baer et al., 1990). The gene encoding ptRNA^{Gly} was cloned by PCR from tobacco using genomic DNA as the template and the appropriate primers. The gene encoding ptRNA^{Gly} was subcloned into pUC 19 under the control of a bacteriophage T7 RNA polymerase promoter (Stephen Raj, M.L and Gopalan, V., unpublished experiments). All of the ptRNA substrates were prepared by *in vitro* run-off transcription with T7 RNA polymerase using the respective template plasmids in the absence or presence of [α -³²P] GTP to obtain unlabeled or labeled transcripts, respectively. The ptRNA substrates described above are shown in Fig. 2.1.

A bipartite ptRNA^{Gln} (Fig. 2.2) was prepared by a combination of chemical synthesis and *in vitro* transcription. The 5' portion, referred to in this text as the Substrate (5' GGU UAAUCA AUGGGGUGUAGCCAAG 3'), is 25 nt long and was generated chemically

by Dharmacon Research, Boulder, CO. The Substrate was labeled at the 5' end with polynucleotide kinase and [γ - 32 P] ATP. The 3' portion, referred to as the External Guide Sequence (EGS), was prepared by PCR-based amplification of the ptRNA^{Gln} template. The forward primer (5' CGACGGGATCCTAATACGACTCACTATAGGTTAATCA ATGGGGTG 3') contains a *Bam* HI site, T7 promoter sequence and overlaps the template at the desired 5' end of the EGS. The reverse primer (5' GTCCCAAGCTTG GATGGACG CCTGGCTGGGGTGCTAGGATTC 3') overlaps the 3' end of the ptRNA template and contains *Fok* I, *Bst* N I, and *Hind* III sites. A PCR product of the expected size was purified, digested with *Hind* III and *Bam* HI, and ligated into pUC 18 digested with the same enzymes. The DNA sequence of the putative clone was confirmed by sequencing. EGS transcripts were prepared by T7 RNA polymerase-driven run-off transcription using template digested with *Fok* I. A *Fok* I digest generates a template without 3' CCA. The EGS and Substrate were incubated at 35° C for 15 min to allow annealing and tertiary structure formation of the bipartite ptRNA^{Gln} substrate (Fig. 2.2).

Template plasmids for *in vitro* transcription of M1 RNA were prepared as previously described (Vioque et al., 1988).

2.2 *A. thaliana* Tissue Homogenization and Protein Precipitation

Soil grown *A. thaliana* plants (CS2360 WS-2) were a kind gift from Dr. Randy Scholl, Department of Plant Biology, The Ohio State University. The aerial portion of these plants were harvested, and immediately frozen in liquid nitrogen and stored at -70° C. The plants were approximately 16 days old and harvested prior to flowering. Approximately 20 g of plant tissue were pulverized with a mortar and pestle in the

presence of liquid nitrogen to lyse cell walls. A homogenate was then prepared by adding 100 ml of extraction buffer (EB) containing 20 mM Tris-HCl (pH 8.0), 2 mM DTT, 0.05 mM PMSF, and 5 mM MgCl₂. The homogenate was centrifuged (15 min; 30,000 g) at 4° C and the supernatant was brought to 90% saturation with ammonium sulfate. The precipitated proteins of the ammonium sulfate suspension were collected by centrifugation (10 min; 10,000 g). The precipitate was resuspended and dialyzed against EB to remove salt. The dialysate was filtered with a 0.22 µm filter (Millipore, Stericup™). The filtered extract was then assayed for activity. The *Arabidopsis* RNase P enzyme preparation at this stage will be referred to as the crude extract (CE) throughout the remainder of this study.

2.3 Standard RNase P Activity Assays

A mix of [α -³²P] GTP-labeled (20 fmol) and unlabeled (1 pmole) ptRNA substrate, 20 mM Tris-HCl (pH 8.0), 2 mM DTT, 0.05 mM PMSF, 5 mM MgCl₂, 1 unit of Recombinant RNasin® Ribonuclease Inhibitor (Promega), and 5 µl enzyme preparation in a total volume of 20 µl was incubated at 35° C for 30 min. Assays were terminated by addition of 10 µl quench dye (10 M urea, 1 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, 10% phenol). To monitor product formation, ptRNA was separated from mature tRNA by subjecting the reaction/assay mixture to denaturing gel electrophoresis. For assays using ptRNA^{Gly} and ptRNA^{Tyr} substrates, 8% polyacrylamide/8 M urea gels were used. Pre- tRNA^{Gln} products were separated by 10% polyacrylamide/urea gel electrophoresis. The results of the assay were then visualized by autoradiography and quantified by using a phosphoimager (Molecular Dynamics). Initial velocity calculations

were based on cleavage assays wherein the time of incubation ranged from 1-8 min; one unit (U) of RNase P activity was described as the amount of enzyme catalyzing 1% of total substrate to product/min. Assays to determine ionic requirements were carried out according to the procedure for the standard assay with the addition of the ion of interest.

2.4 Anion Exchange Chromatography

The CE fraction (20 ml) from the ammonium sulfate precipitation was loaded onto a Hi Trap® Q sepharose column (Pharmacia Biotech, 1 ml) equilibrated with EB at 4° C. The column was washed with 20 ml EB containing 75 mM KCl. RNase P bound to the column was eluted using an FPLC (Pharmacia Biotech, LCC-501 Plus) apparatus. A 30 ml linear gradient from 75-350 mM KCl was applied and 1 ml fractions were collected. The flow rate was kept constant at 1 ml/min. The absorbance at 280 nm was measured during elution (data not shown) to obtain an estimate of the protein concentration in the eluted fractions. The collected fractions were immediately assayed for RNase P activity. The enzyme collected at this stage of purification will be referred to as the Q enzyme throughout this text.

2.5 Glycerol Gradient Fractionation

A continuous glycerol gradient was prepared by layering 2 ml volumes of 22.5%, 20%, 17.5%, 15%, and 12.5% glycerol (diluted with EB) in an ultracentrifuge tube and allowing the gradient to diffuse for three hours at 4° C. A 500 µl aliquot of the peak Q enzyme fraction was layered on the top of the gradient. The gradient was subjected to centrifugation at ~ 210,000 g for 24 hours at 4° C. The sample was collected, dropwise,

in 0.5 ml fractions from the bottom of the tube. The collected fractions were immediately assayed for RNase P activity. The enzyme collected at this stage of purification will be referred to as the GG fraction enzyme throughout this text.

2.6 Protein Concentration Estimation

Protein concentration was estimated according to the method of Bradford (1976) with BSA as standard.

2.7 Sensitivity of *A. thaliana* RNase P to Proteinase K (PK)

A 10 μ l aliquot of the Q enzyme was pre-incubated with 5 μ l PK (50 μ g/ml, Gibco BRL®) at 35° C for 15 min. As control, the Q enzyme was also pre-incubated with PK buffer to determine the effects the buffer components may have on 5' processing activity. Pre-tRNA^{Gly} substrate was then added under standard assay conditions and incubated for 30 min at 35° C. Total reaction volumes were 20 μ l. The results of the assay were analyzed by gel electrophoresis and quantified using a phosphoimager as described in section 2.3.

2.8 Sensitivity of *A. thaliana* RNase P to Micrococcal Nuclease (MN)

A 10 μ l aliquot of the Q enzyme was pre-incubated with 2 μ l MN (2 U/ μ l, Gibco BRL®) in 0.18 mM Ca⁺² and a total volume of 14 μ l at 35° C for 15 min. After the preincubation, 1 μ l EGTA (0.2 M) was added and a 5 min incubation at room temperature was carried out to chelate Ca⁺². MN loses enzymatic activity in the absence of Ca⁺². Pre-tRNA^{Gly} substrate was added under standard assay conditions and incubated

for 30 min at 35° C. To ensure that any observed loss of activity was due to RNase P inactivation by MN and not due to substrate masking, 10 µl of additional Q enzyme was added in a separate reaction mixture and incubated for 30 min at 35° C. As control, the Q enzyme was pre-incubated with Ca⁺² and subsequently chelated by EGTA in the absence of MN to determine the effects these components may have on 5' processing activity. Total reaction volumes were 20 µl and 30 µl with and without the second Q enzyme addition. The results of the assay were analyzed by gel electrophoresis and quantified using a phosphoimager as described in section 2.3.

2.9 Primer Extension Analysis

Primer extension analysis was used to identify the 5' ends of products generated by *A. thaliana* RNase P and was carried out as follows. First, a standard RNase P assay was performed as previously described using unlabeled ptRNA^{Gly} as substrate. The mature tRNA product and the uncleaved ptRNA substrate in the mixture were then precipitated with sodium acetate and 100% ethanol, washed with 70% ethanol, vacuum-dried, and resuspended in 10 µl of water. Reverse transcription (RT) of the resuspended substrate/products was then carried out at 42° C for 50 min using two units of Superscript™ II RT (Gibco BRL) and a 5' end-labeled primer (5'CCCCAAG CTTGGATGGAC GCCTGGGCGGATAGCGGGAATCGAACC3') designed against the 3' end of ptRNA^{Gly}. The RT products of the reaction were then separated on a 10% sequencing gel and were compared to a sequence ladder of ptRNA^{Gly} template DNA generated using the previously described primer. The results were visualized by autoradiography. By closely examining the sizes of the RT products with the sequence

ladder of the plasmid template, the precise cleavage location for RNase P can be determined.

2.10 Cesium Chloride Density Estimation

A continuous CsCl gradient was prepared by layering 2.5 ml volumes of 40%, 30%, 20%, and 10% CsCl (diluted with EB) in an ultracentrifuge tube and allowing the gradient to diffuse for two hours at room temperature. A 200 μ l aliquot of the Q enzyme peak fraction was layered on the top of the gradient. The sample was subjected to centrifugation at \sim 210,000 g for 24 hours at 4 $^{\circ}$ C. The sample was collected, dropwise, in 300 μ l fractions from the bottom of the tube. The refractive index of the collected fractions was determined using an ABBE Refractometer (American Optical). The density of each fraction was then computed according to the method of Bruner and Vinograd (1965) and the corresponding densities were plotted vs. fraction number. The fractions were then dialyzed individually against EB to remove CsCl which inhibits RNase P activity. The dialyzed fractions were assayed for RNase P activity to identify the most active fraction and thereby the density of *A. thaliana* RNase P.

2.11 Western Blotting and Immunodetection of Rpp38 Homolog

Samples of Q enzyme and human Rpp38 were boiled in the presence of SDS loading dye (250 mM Tris-HCl (pH 6.8), 500 mM DTT, 10% SDS, 0.5% bromophenol blue, 50% glycerol) and subjected to 15% SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane by electroblotting. Non-specific sites on the membrane were blocked with 2.5% non-fat dry milk (NFDM) dissolved in TNT buffer [10 mM Tris-HCl

(pH 8), 150 mM NaCl, 0.05% (v/v) Tween 20]. Blocking was followed by incubation with Rpp38 antibody (1:10,000 dilution). All subsequent steps were carried out using the ECL™ Western Blotting kit (Amersham Pharmacia Biotech). After washing with 1.25% NFD, the blot was incubated with secondary antibody (peroxidase-labeled anti-rabbit antibody, 1:5000 dilution). After additional washing with TNT buffer and TN buffer (TNT buffer minus Tween 20), chemiluminescence was detected by autoradiography according to manufacturer's instructions (Amersham).

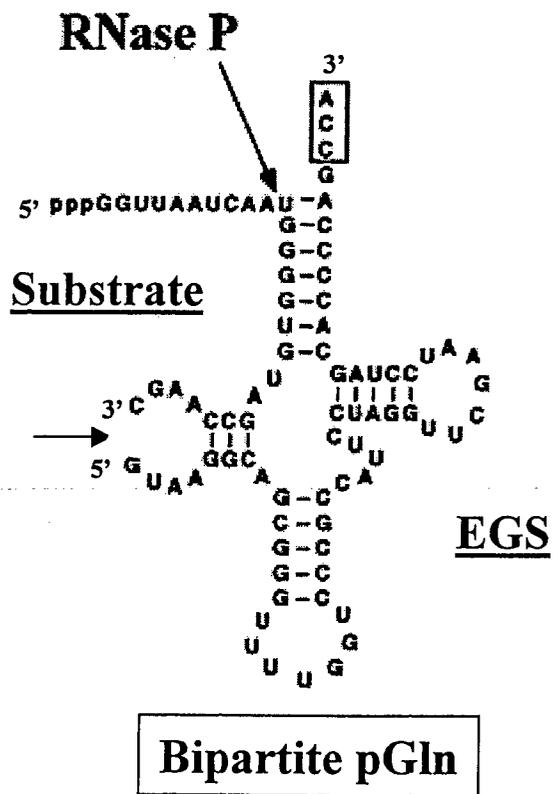


Fig. 2.2. Pre-tRNA^{Gln} Bipartite Substrate.

CHAPTER 3

RESULTS

3.1 Purification of *A. thaliana* RNase P

A. thaliana RNase P was partially purified by ammonium sulfate precipitation and anion chromatography. *A. thaliana* RNase P activity was detected in the 90% ammonium sulfate precipitate (CE) by conducting a standard RNase P assay (as described in Chapter 2.3) using ptRNA^{Gly} as substrate (Fig 3.1). The expected 5' leader sequence is not visible in Fig 3.1. The protein concentration of CE was estimated to be 2.9 mg/ml. Initial velocity calculations revealed 28.6 units of activity/mg of protein (Table 3.1).

The entire pool of CE was then subjected to anion exchange chromatography on a 1 ml Hi TrapTM Q (Amersham Pharmacia) sepharose column. Approximately 70% of the activity bound to the anion column. The remaining 30% of the activity failed to bind to the column and were present in the flow through (Fig 3.2). The activity that bound to the anion column eluted from approximately 130 mM KCl to 250 mM KCl and is referred to as the Q enzyme (Fig. 3.2). The protein concentration of Q enzyme fractions was estimated to be 0.4 mg/ml. Initial velocity experiments revealed 290 units of activity/mg of protein (Table 3.1). When considering the level of purification for *A. thaliana* RNase P in this study, it is important to note that significant purification can be attributed to the

ammonium sulfate precipitation step; however, quantifying the level of purification from the initial homogenate to CE is difficult. The high concentrations of nucleases made determining the RNase P initial velocity for the homogenate near impossible. Therefore, the purification factor of 9 was based on the level of purity from the CE fraction to the Q fraction.

Close examination of the Q fractions revealed minimal nuclease contamination, indicated by the presence of intact mature tRNA product and the 5' leader sequence in fractions 4-8 (Fig 3.2). The latter Q fractions were the source of enzyme for most of the characterization experiments performed in this study. The Q enzyme was assayed with ptRNA^{Gly} under standard conditions for 20, 40, and 60 min. (Fig 3.3). The results of this time course clearly show the presence of both the mature tRNA and the 5' leader product.

The activity found in the flow through of the anion exchange column was bound by a cation exchange column (Hi Trap SP column, 1ml) and eluted at approximately 300 mM KCl (data not shown). The enzyme isolated from the cation column was termed the S enzyme. The S enzyme was not studied in detail and therefore will not be discussed further. However, it is likely that the S enzyme activity is due to a breakdown of the Q enzyme RNA subunit (thus making it more positive) or it may be an additional isoform of RNase P that is possibly organellar (mitochondria or chloroplast) in origin.

Further purification of *A. thaliana* RNase P was attempted by glycerol gradient fractionation. A 0.5 ml fraction of the Q enzyme was loaded on a continuous 12.5-22.5% glycerol gradient (prepared as described in Chapter 2.5) and centrifuged for 24 hours at ~ 210,000 g. The activity, termed GG, was collected between 15-15.5% glycerol (Fig. 3.4). This experiment was performed three times with the same results and has been

found to be reproducible using maize and rice RNase P as well (Raj, Kumar and Gopalan, unpublished data). A time course assay (30-150 min) with the GG fraction revealed a mature tRNA^{Gly} product but did not reveal the 5' leader sequence (Fig. 3.5). The specific activity of the GG fraction was found to be unstable and was attributed to a loss of enzymatic activity over time. The GG fraction data are not included in the purification table (Table 3.1).

3.2 Primer Extension Analysis

Primer extension analysis by reverse transcription of the mature tRNA product and any uncleaved substrate in an RNase P assay mixture was carried out to prove that the Q enzyme was capable of cleaving the ptRNA^{Gly} at precisely the +1 position indicated by the arrow in Fig 1.1. Primer extension was performed by using a 5' end-labeled primer (described in Chapter 2.3) that is complementary to the 3' end of the ptRNA^{Gly} substrate. The primer is allowed to anneal to the 3' end of the mature tRNA product and any uncleaved ptRNA substrate remaining after an RNase P assay. Reverse transcriptase (RT) is then added to produce end-labeled cDNA. Primer extension analysis results are shown in Fig 3.6. The RT product of ptRNA^{Gly} assayed with the Q fraction from *A. thaliana* (Lane 3) shows the presence of ptRNA^{Gly} substrate and the presence of a second RT product corresponding to the mature tRNA^{Gly}. The mature tRNA RT product, likely synthesized using mature tRNA as the template, terminates at a G residue just prior to the last T residue in the leader sequence as also seen in Lane 2 with *E. coli* RNase P. A sequencing ladder of the plasmid used to transcribe ptRNA^{Gly} is provided in Fig. 3.6 to aid in locating the exact position of the last residue in the RT products. This experiment

proves that the activity isolated in *A. thaliana* processes the 5' end of ptRNA substrate at a position identical to *E. coli* RNase P.

3.3 Proteinase Sensitivity

A. thaliana RNase P was found to be sensitive to treatment with proteinase K (PK). The results of *A. thaliana* RNase P (Q fraction) assayed in the presence of PK are shown in Fig. 3.7. The efficiency of Q enzyme tRNA processing can be observed in each lane by comparing the intensity of the bands representing the mature tRNA and the 5' leader products. A complete loss of ptRNA^{Gly} processing activity is observed when *A. thaliana* RNase P is preincubated with PK (Lane 3). A minimal amount of the loss of activity can be attributed to inhibition of RNase P activity due to the PK buffer (Lane 4). These data suggest that *A. thaliana* RNase P contains at least one protein subunit that is essential for ptRNA processing *in vitro* under standard conditions.

3.4 Nuclease Sensitivity

A. thaliana RNase P was found to be sensitive to treatment with micrococcal nuclease (MN). The results of the assay in which *A. thaliana* RNase P (Q fraction) was preincubated with MN are shown in Fig. 3.8. The efficiency of Q enzyme tRNA processing can be observed in each lane by comparing the intensity of the bands representing the mature tRNA and the 5' leader products. When the Q enzyme is preincubated with MN and CaCl₂, ptRNA^{Gly} processing is severely diminished (Lane C). The inhibition of RNase P activity can partly be attributed to the presence of CaCl₂ and EGTA in the assay (Lane B, no MN present). The loss of activity due to inactivation of RNase P by MN

(Lane C) can be restored by adding more Q enzyme subsequent to incubation with EGTA (Lane D). The cleavage of ptRNA^{Gly} substrate in Lane D can be attributed to a combination of the processing activity seen in Lanes A (standard assay) and C. The increase in the amount of mature ptRNA product in Lane D proves that any inhibition of activity seen in Lane C is not due to MN masking the ptRNA substrate from cleavage by RNase P. If MN had masked the ptRNA substrate, then the substrate would continue to be inaccessible to processing by the Q enzyme added after the pre-incubation. Collectively, these data indicate that MN inactivates an RNA subunit in *A. thaliana* RNase P which is essential for ptRNA processing *in vitro* at standard conditions.

3.5 Density of *A. thaliana* RNase P in CsCl

The Q enzyme fraction was subjected to CsCl gradient fractionation to aid in determining if an RNA subunit was an integral part of the holoenzyme complex. A protein enzyme typically has a density of approximately 1.25 g/ml. The presence of an RNA subunit would be indicated by an increase in density to a value greater than 1.25 g/ml. In bacteria, where the RNA/protein ratio is very high (one large RNA subunit and one small protein subunit) densities are as great as 1.55 g/ml (*E.coli* RNase P; Lawrence et al., 1987).

The results of the Q enzyme on a CsCl gradient are presented as a graph of Density vs. Fractions collected (Fig. 3.9). RNase P assays of the collected fractions, after dialysis to remove CsCl, showed a peak of enzymatic activity in fraction 24 (data not shown). The density for this fraction corresponds to 1.16 g/ml (Fig. 3.9) at 4° C. This density is lower than that expected for an RNP complex. However, it does not rule out the

possibility of the presence of an RNP complex composed of one small RNA subunit and numerous large protein subunits (a low RNA/protein ratio). Additionally, data from both maize and rice RNase P give similar densities at 4° C and increase to approximately 1.22 g/ml when the gradient is centrifuged at 24° C for 24 hours at ~ 210,000 g (Raj, Kumar and Gopalan, unpublished data). Previous data (not shown) suggests that *A. thaliana* RNase P at the current level of purity loses significant enzymatic activity over long periods of time at elevated temperatures and therefore has not been subjected to CsCl gradient fractionation at temperatures greater than 4° C. Further purification and testing is needed to determine if a similar shift in density will occur with *A. thaliana* RNase P.

3.6 Identification of a Human Rpp38 Protein Homolog in *A. thaliana*

A homolog of the Rpp38 protein subunit identified in human RNase P (Jarrous et al., 1998) was identified in the *Arabidopsis* genome by database mining (Fig. 3.10). The predicted protein of *Arabidopsis* Rpp38 homolog has a molecular mass of 19-kDa. Western blot analysis, using polyclonal antisera against human Rpp38, confirmed the presence of a cross-reacting antigen in the Q enzyme fraction (Fig. 3.11) giving a band corresponding to approximately 19-kDa as expected (Lane 3). Probing a fraction devoid of *A. thaliana* RNase P enzymatic activity with polyclonal antisera against Rpp38 yielded a negative result (data not shown). These data suggest that *A. thaliana* RNase P may include a protein subunit which is similar to a protein already identified in human RNase P (Fig. 3.10).

3.7 Substrate Recognition by *A. thaliana* RNase P

Assays with $\text{ptRNA}^{\text{Gly}}$ and $\text{ptRNA}^{\text{Gln}}$ substrates with and without the 3' CCA sequence were carried out with the Q enzyme fraction. The 3' CCA sequence, when present in the ptRNA substrate, increases the rate of substrate cleavage by M1 RNA. When the *A. thaliana* Q enzyme was assayed with $\text{ptRNA}^{\text{Gly}}$ as the substrate (Fig. 3.12), processing occurred both with and without 3' CCA (Lanes 5 and 6). Pre-tRNA processing is evident in Lanes 5 and 6 and indicates the ability of the Q fraction to process both substrates regardless of the presence of 3' CCA.

When the Q enzyme was assayed with $\text{ptRNA}^{\text{Gln}}$ as the substrate (Fig. 3.13), processing occurred both with and without 3' CCA (Lanes 5 and 6). Pre-tRNA processing is evident in Lanes 5 and 6 and indicates the ability of the Q fraction to process both substrates regardless of the presence of 3' CCA.

The results of a Q fraction assay with *E. coli* $\text{ptRNA}^{\text{Tyr}}$ are provided in Fig 3.14. Although some cleavage occurs and a product forms corresponding to the proper size of the mature tRNA, it cannot be concluded that the Q fraction processes $\text{ptRNA}^{\text{Tyr}}$ correctly (Lane 3). The appearance of bands other than the expected products is an indication of aberrant processing of the $\text{ptRNA}^{\text{Tyr}}$ substrate.

3.8 Effect of KCl and CaCl_2 on tRNA Processing

The Q enzyme was subjected to the standard assay under increasing concentrations of KCl (Fig. 3.15). It was observed that $\text{ptRNA}^{\text{Gly}}$ processing was modestly inhibited by 30 mM KCl. Complete inhibition of $\text{ptRNA}^{\text{Gly}}$ processing occurred at 150 mM KCl.

The Q enzyme was also subjected to the standard assay under increasing concentrations of CaCl₂ (Fig. 3.16). It was observed that ptRNA^{Gly} processing was inhibited by even modest additions (0.5 mM) of CaCl₂ and exhibited aberrant cleavage when assayed in the presence of 1.5 mM CaCl₂.

3.9 5' Processing of a Bipartite ptRNA^{Gln} Substrate by *A. Thaliana* RNase P

The GG enzyme fraction was assayed with a bipartite ptRNA^{Gln} substrate (Fig 2.2) to determine if *A. thaliana* RNase P could serve as an effective tool for gene targeting studies in plants. Gene targeting with human RNase P has been described in detail and effectively used in disrupting viral gene expression by Liu and Altman (1995). Briefly, the rationale for gene disruption by RNase P involves targeting an mRNA of interest (Substrate) with an external guide sequence (EGS) that resembles nearly three-fourths of a tRNA structure and is complementary to the mRNA of interest. The resulting EGS/Substrate complex, although bipartite, resembles a tRNA substrate. RNase P can then act upon this tRNA-like complex and cleave the mRNA thus rendering it incapable of being translated into a functional protein.

The GG enzyme failed to properly process the bipartite substrate (data not shown). Preliminary indications are that further purification of the *A. thaliana* RNase P enzyme is necessary to show proper cleavage of a bipartite ptRNA substrate.

Protein					RNase P Activity				
Fraction	Volume (ml)	[Protein] (mg/ml)	Total (mg)	Yield (%)	Units/ml	Total Units	Specific Activity (Units/mg)	Yield (%)	Purif. Factor
Crude Extract	20	2.9	58	100	82.8	1656	28.6	100	-
Anion Fraction	5	0.4	2	3.5	96	480	240	29	9

Table 3.1. *Arabidopsis thaliana* RNase P Purification Table.

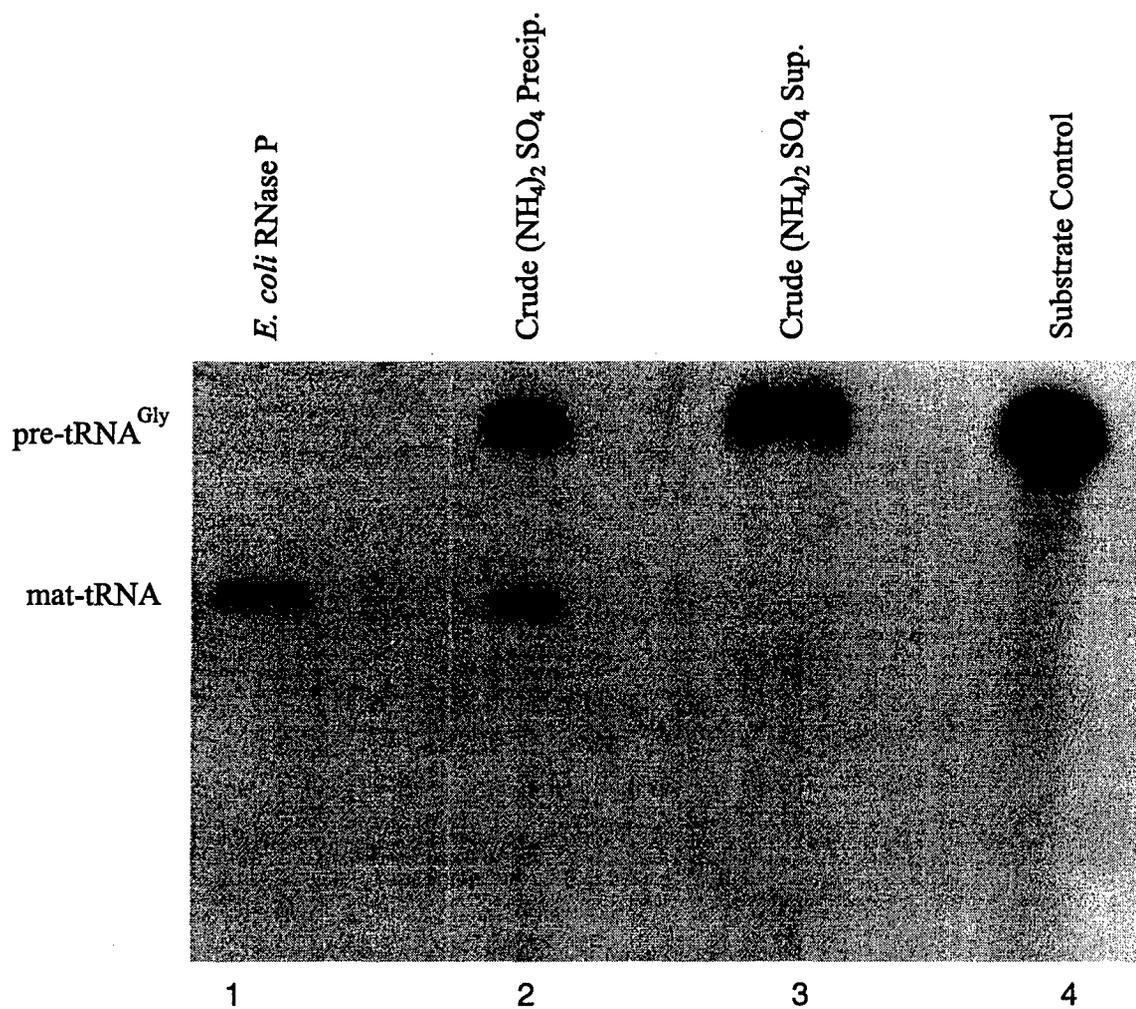


Fig. 3.1. Crude Extract Assay Results. Lane 1 shows processing of the ptRNA substrate by *E. coli* RNase P and serves as a positive control for 5' processing. Lane 2 shows 5' processing by CE resulting in a mature tRNA product. Lane 3 depicts lack of 5' processing by the supernatant of the ammonium sulfate suspension. Lane 4 is the tRNA substrate incubated in the absence of enzyme and serves as the negative control. Lane designations appear at the bottom of the figure throughout this text.

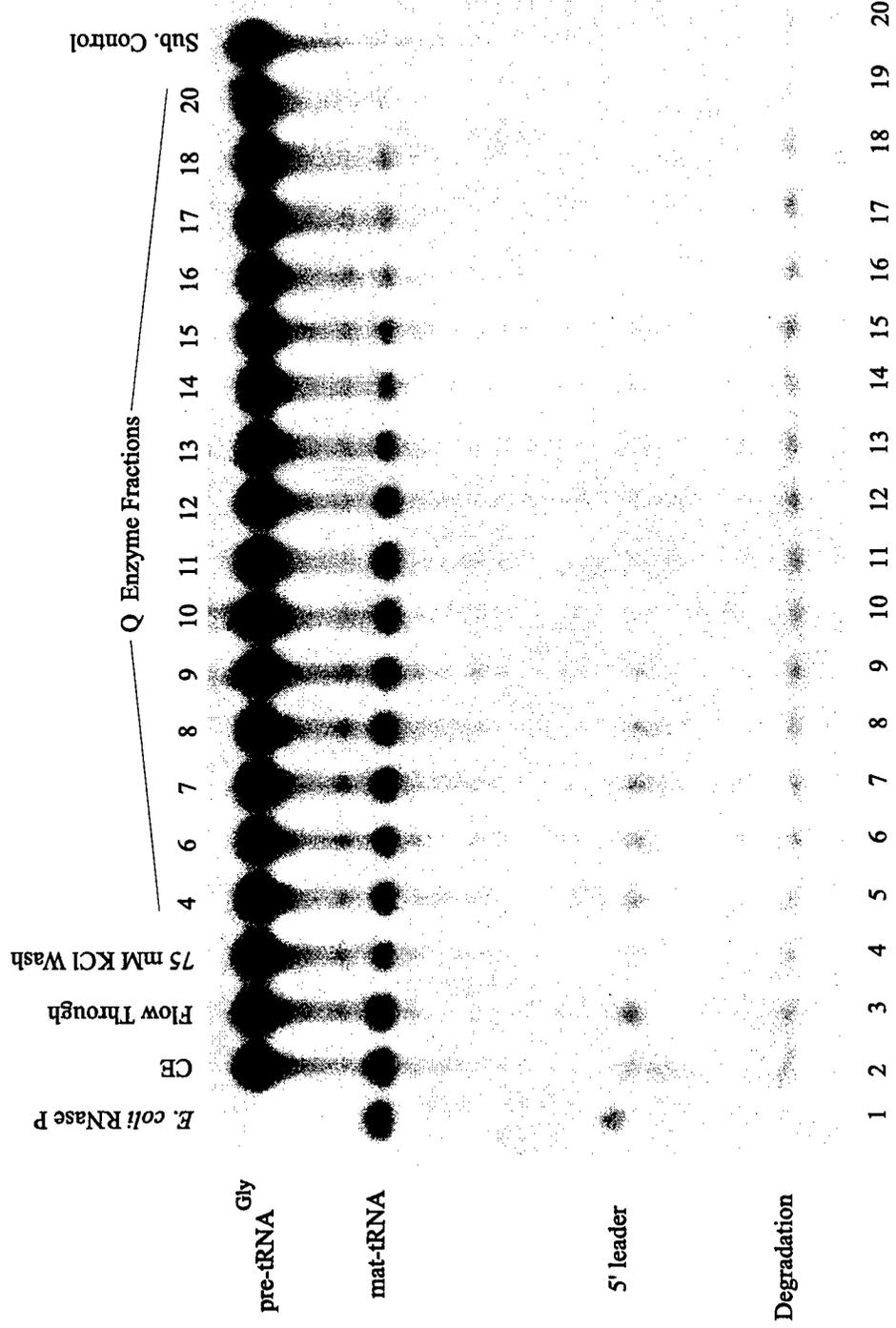


Fig. 3.2. Q Enzyme Fraction Assay Results. Lane 1 is a positive control using *E. coli* RNase P. CE is assayed in Lane 2. Lane 3 shows activity in the flow through. Lane 4 represents enzyme present in the wash. Lanes 5-19 depict ptRNA processing by aliquots from Fractions 4-20 (Q enzyme). Lane 20 is the ptRNA substrate assayed in the absence of RNase P.

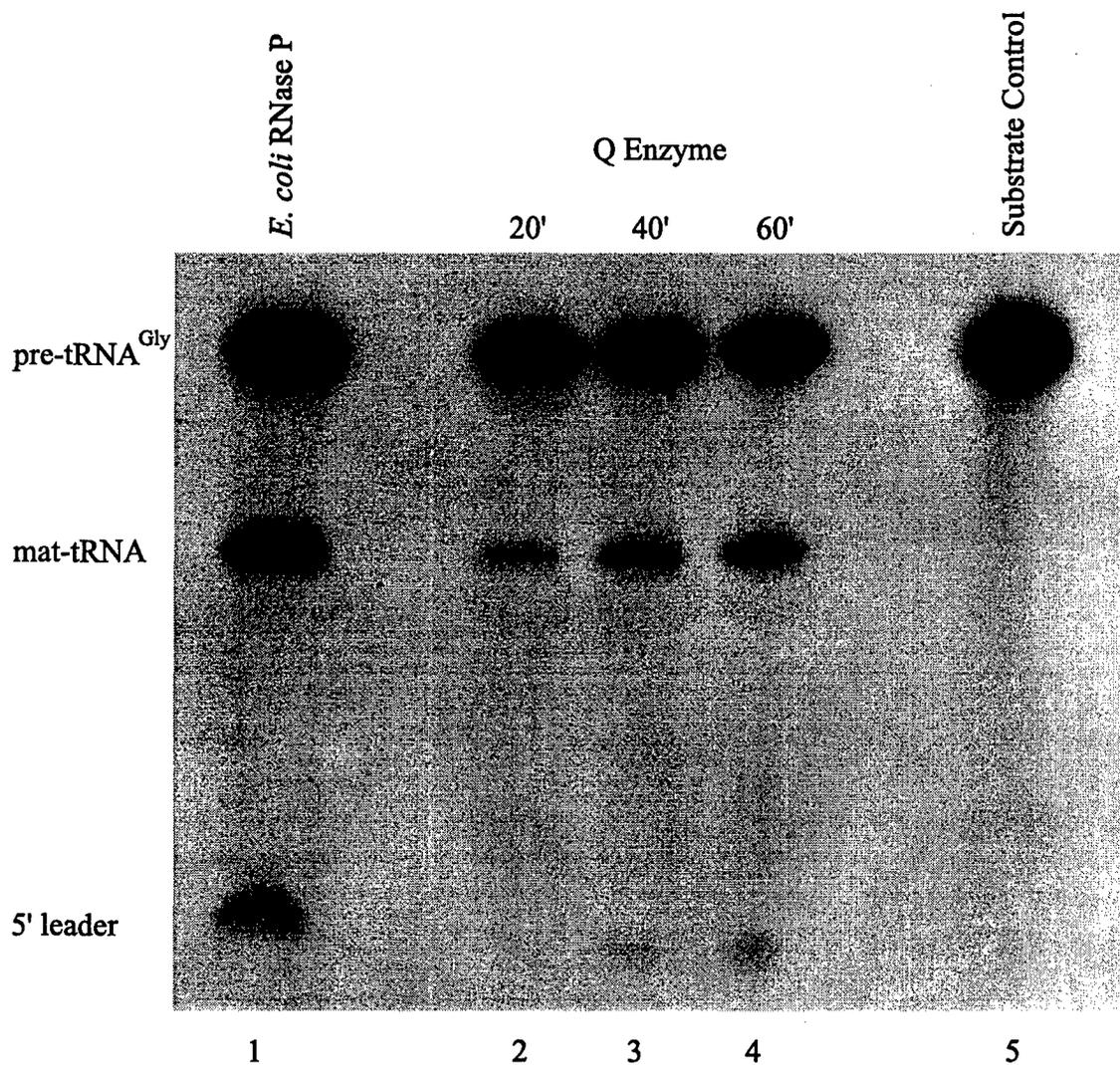


Fig 3.3. Activity of Q Peak Fraction. Lane 1 shows processing of the ptRNA substrate by *E. coli* RNase P and serves as a positive control for 5' processing. Lanes 2-4 show a time dependent increase in the presence of the mature tRNA and 5' leader products indicative of *A. thaliana* RNase P-mediated processing. Lane 5 is the ptRNA substrate incubated in the absence of enzyme and serves as the negative control.

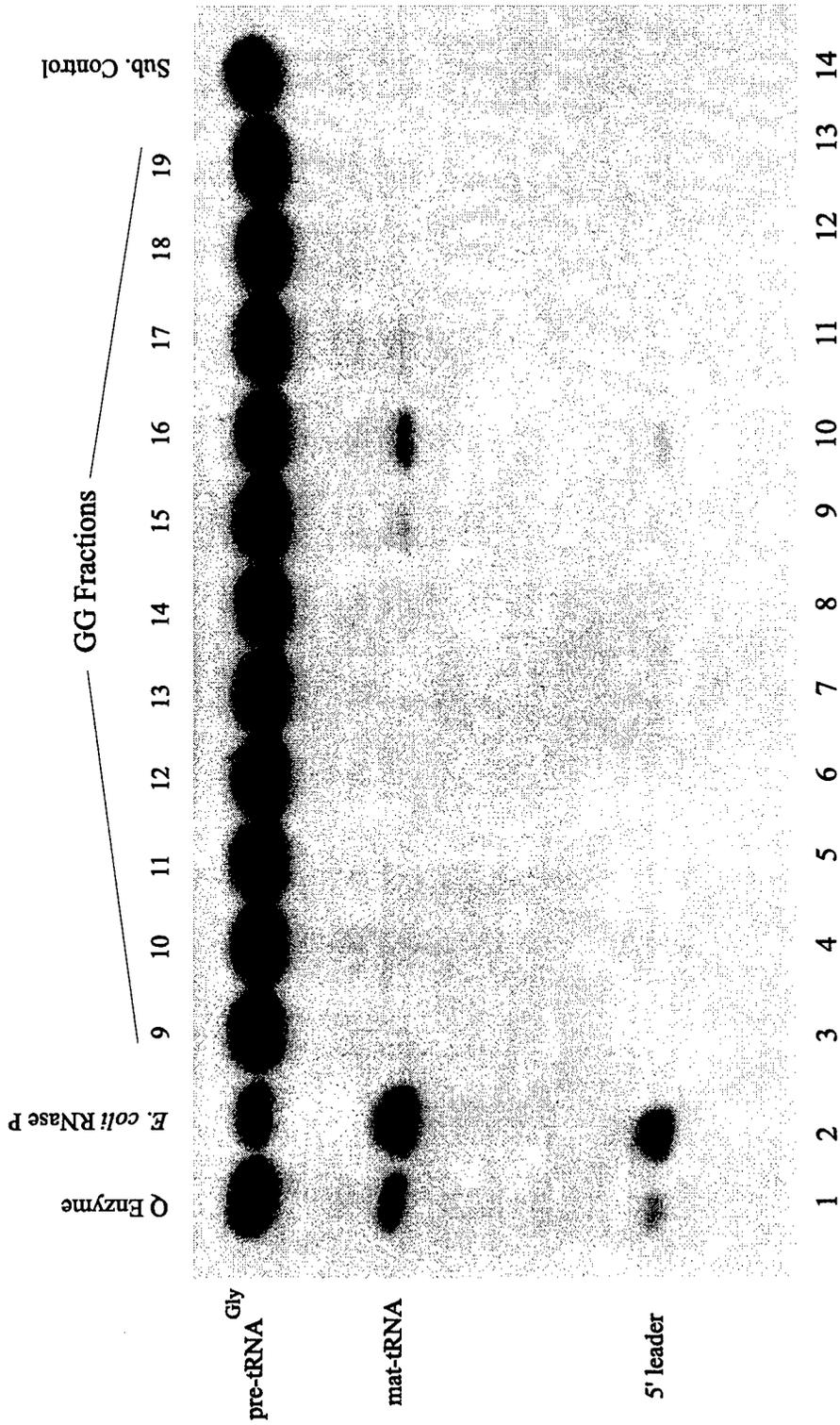


Fig. 3.4. RNase P Activity in Glycerol Gradient (GG) Fractions. Lane 1 depicts activity of the Q enzyme. Lane 2 is a positive control using *E. coli* RNase P. Lanes 3-13 depict ptRNA processing by aliquots from Fractions 9-19 (GG enzyme). Fractions 15-17 show *A. thaliana* RNase P activity. Lane 14 is the ptRNA substrate assayed in the absence of enzyme.

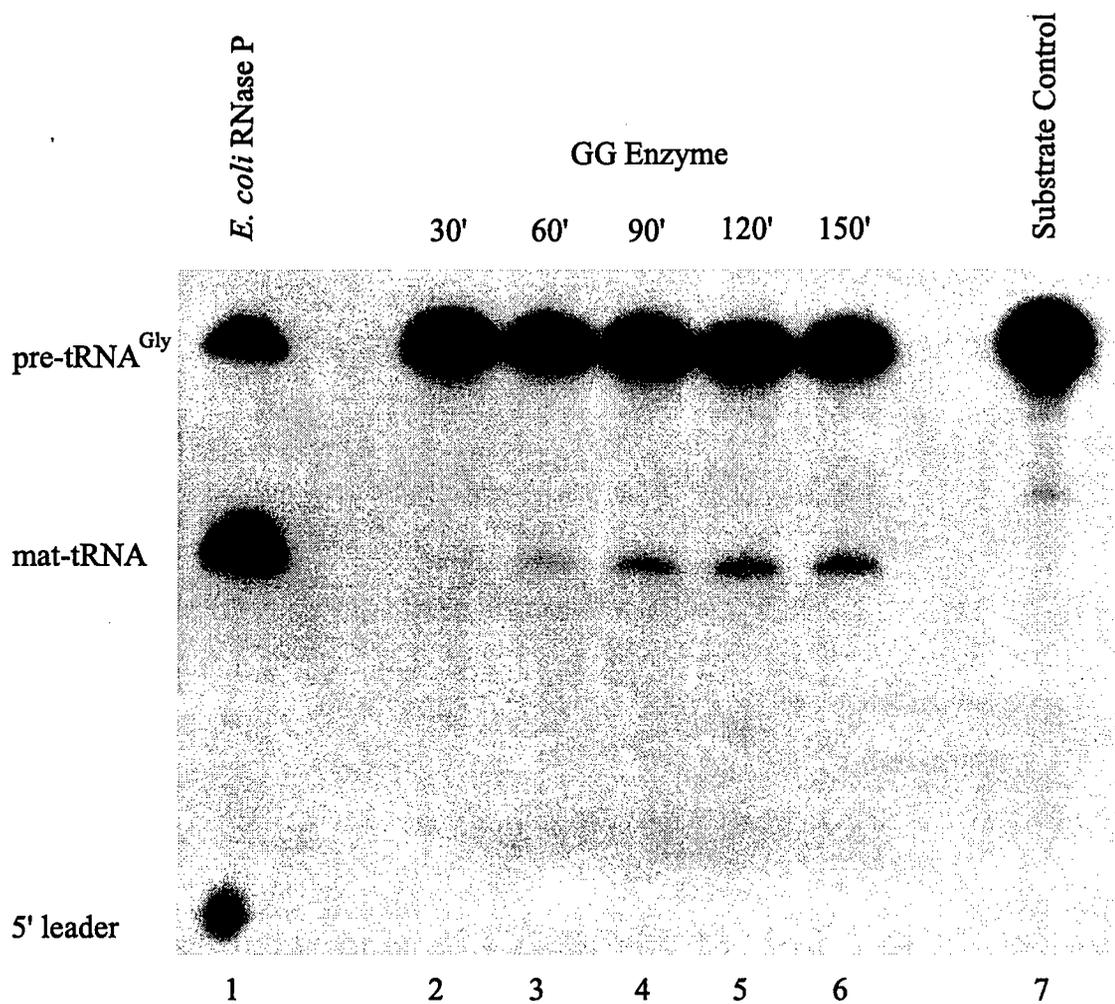


Fig 3.5. Activity of GG Peak Fraction. Lane 1 shows processing of the ptRNA substrate by *E. coli* RNase P and serves as a positive control for 5' processing. Lanes 2-6 show an increase in the presence of the mature tRNA products with time, indicating that *A. thaliana* RNase P enzymatic catalysis occurs. Lane 7 is the ptRNA substrate incubated in the absence of enzyme and serves as the negative control.

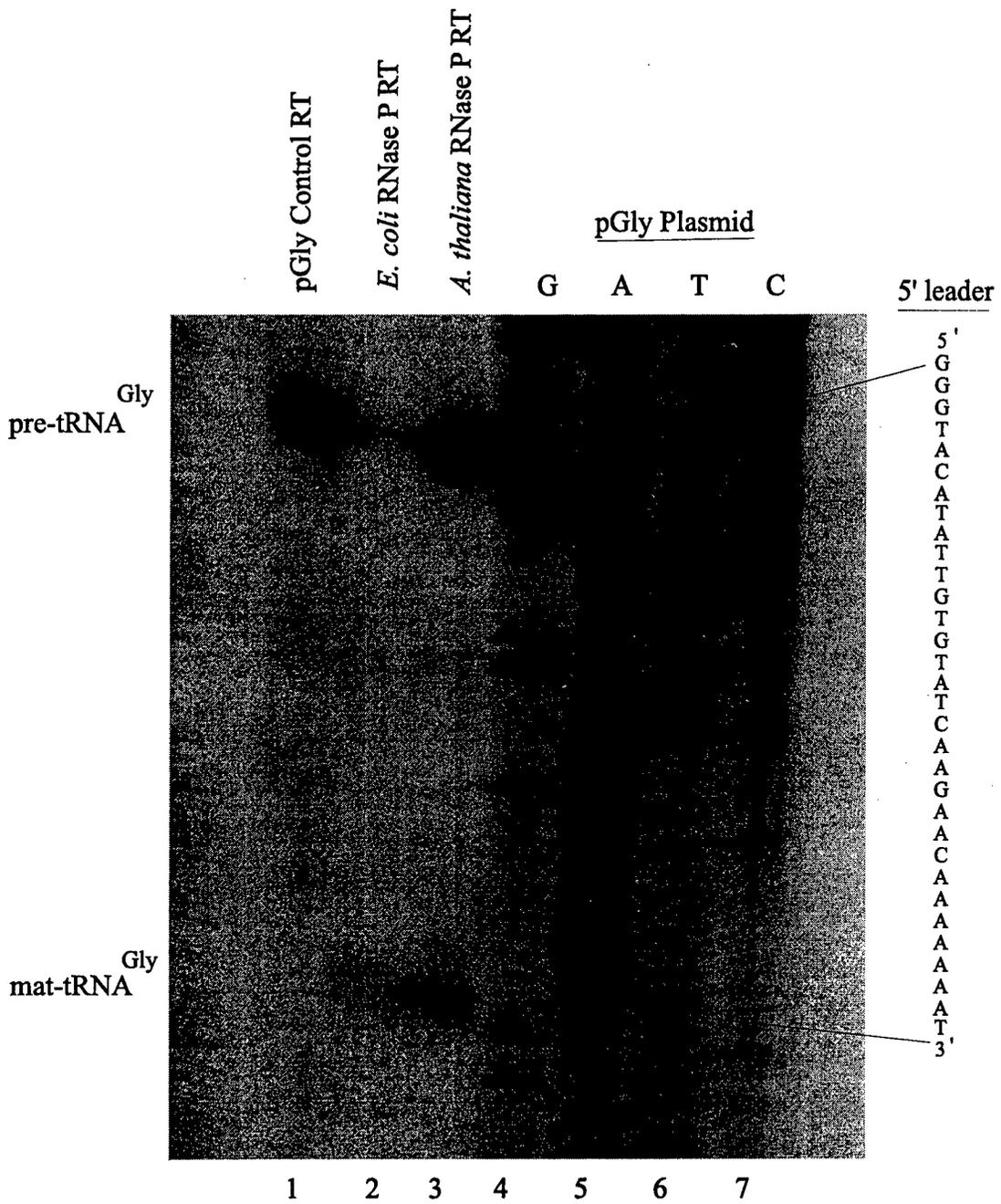


Fig 3.6 Primer Extension Analysis. Lane 1 shows the RT product of a ptRNA^{Gly} substrate control not subjected to processing by RNase P. Lane 2 shows the RT product of ptRNA^{Gly} cleaved with *E. coli* RNase P. Lane 3 shows the RT product of ptRNA^{Gly} assayed with the Q fraction from *A. thaliana*. Lanes 4-7 show a sequencing ladder of the plasmid used to transcribe ptRNA^{Gly} and is provided to aid in locating the terminating residues of the RT reactions.

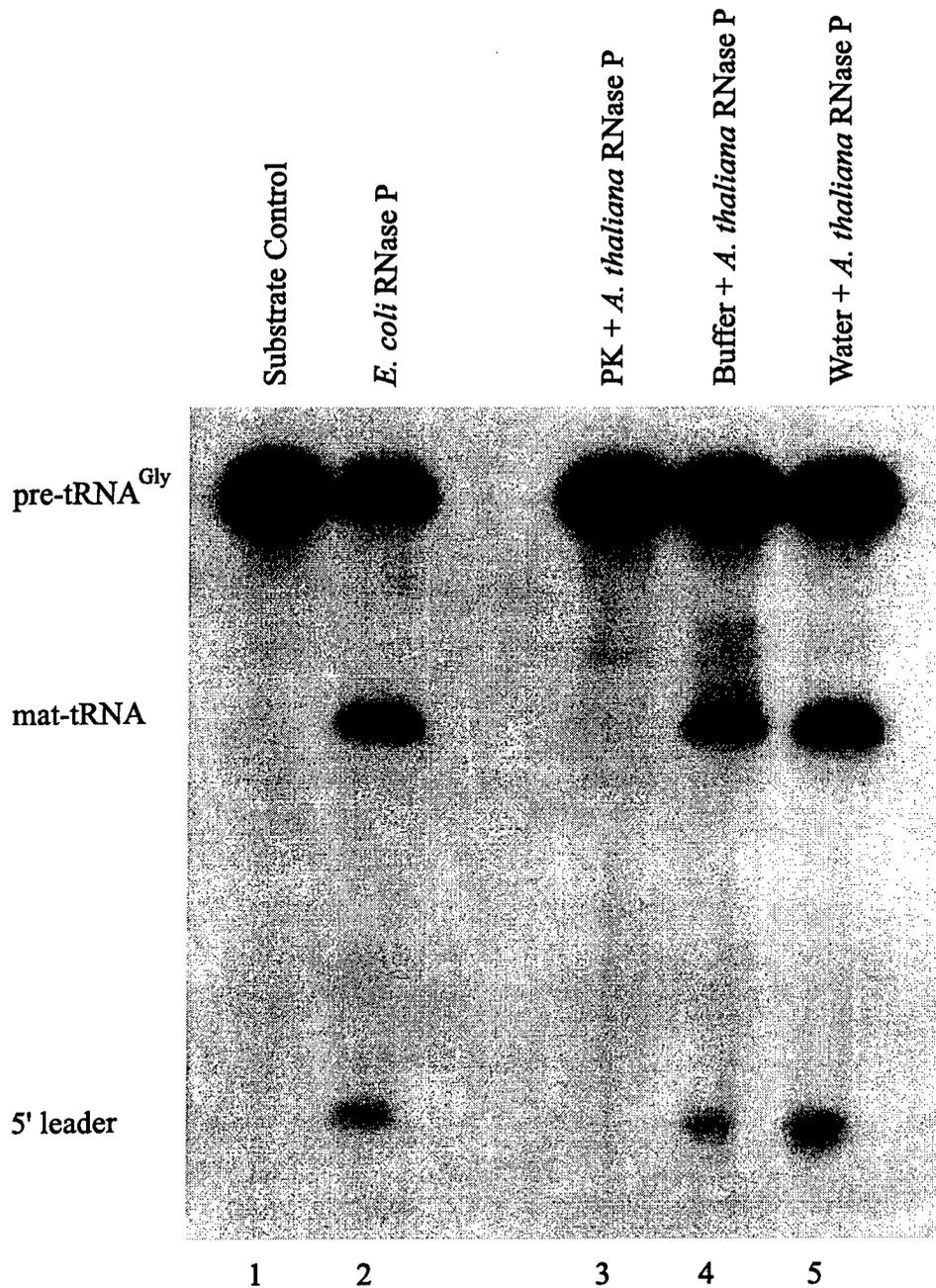


Fig 3.7. Proteinase Sensitivity of *A. thaliana* RNase P. Lane 1 shows ptRNA^{Gly} as a negative control. Lane 2 is a positive control and shows the processing of ptRNA by *E. coli* RNase P. Lane 3 shows a complete loss of *A. thaliana* RNase P ptRNA processing activity due to pre-incubation with PK. Lane 4 shows modest inhibition of RNase P activity due to the presence of PK buffer alone. Lane 5 shows the expected processing for *A. thaliana* RNase P.

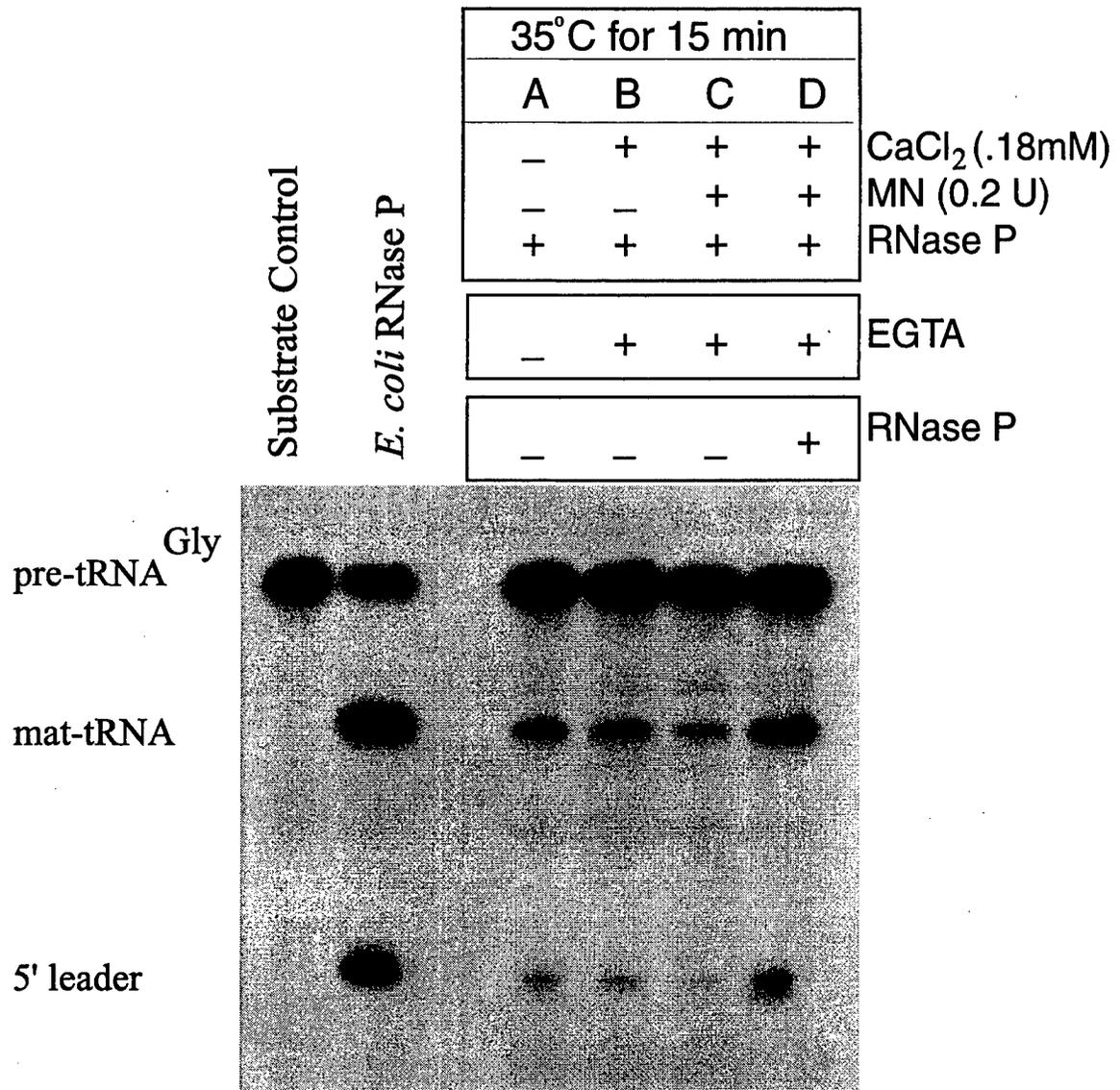


Fig. 3.8. Nuclease Sensitivity of *A. thaliana* RNase P. Lane A shows the results of the Q enzyme assayed under standard conditions with ptRNA^{Gly} substrate. Lane B shows inhibition of Q enzyme ptRNA processing activity in the presence of CaCl₂ and EGTA (no MN present). Lane C depicts the loss of ptRNA processing by the Q enzyme due to pre-incubation with MN. Lane D shows the retrieval of ptRNA processing activity by the addition of a fresh aliquot of Q enzyme to an assay mixture previously inactivated by MN. Both negative (Substrate Control) and a positive (*E. coli* RNase P) controls are included.

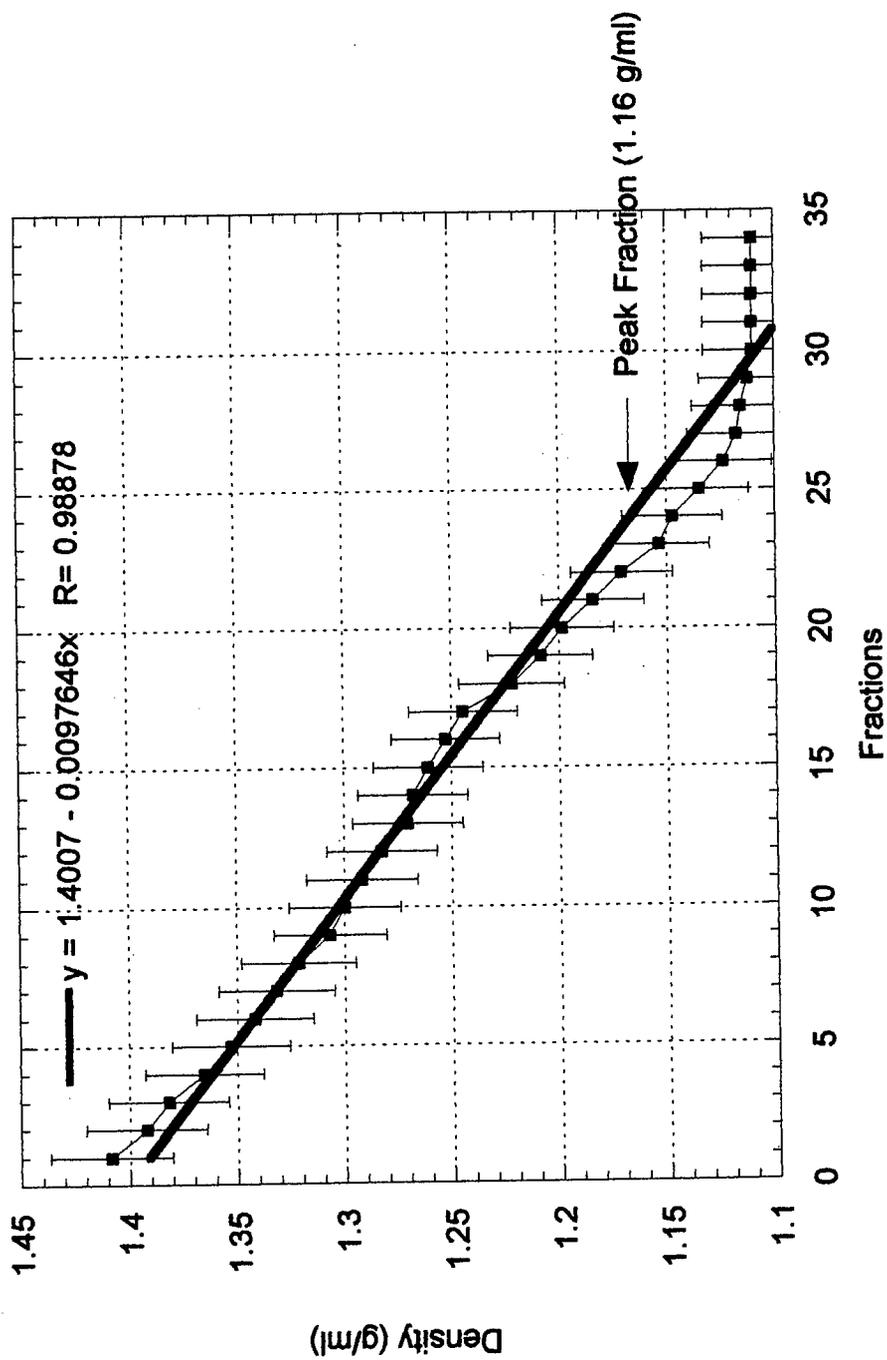


Fig. 3.9. Fractionation of *A. thaliana* RNase P on a CsCl Gradient (4° C). The density of the peak RNase P activity fraction is indicated by the arrow.

<i>Arabidopsis</i> BAC T7B11	L	K	K	Q	I	A	I	G	I	N	E	V	T	R	V	L	E	R	M	N	P	
Human Rpp38	V	R	K	Q	L	V	I	G	V	N	E	V	T	R	A	L	E	R	-	-	-	
<i>Arabidopsis</i> BAC T7B11	N	N	T	S	D	Q	Q	Q	N	P	K	G	L	Q	V	V	I	L	V	A	D	
Human Rpp38	-	-	-	-	-	-	-	-	-	-	R	E	L	L	L	V	L	V	C	K	S	
<i>Arabidopsis</i> BAC T7B11	C	K	P	R	M	L	T	K	H	I	P	N	L	A	A	S	R	N	V	P	V	
Human Rpp38	V	K	P	A	M	I	T	S	H	L	I	Q	L	S	L	S	R	S	V	P	A	
<i>Arabidopsis</i> BAC T7B11	L	Y	V	R	D	N	K	R	A	S	L	R	L	G	E	L	V	K	L	K	T	
Human Rpp38	C	Q	V	-	-	-	P	R	L	S	E	R	I	A	P	V	I	G	L	K	C	
<i>Arabidopsis</i> BAC T7B11	A	L	A	I	G	I	K	A	R	G	N	D	L	N	L	L	L	K	Q	I	L	
Human Rpp38	V	L	A	L	A	F	K	K	N	T	T	D	F	V	D	E	V	R	A	I	I	
<i>Arabidopsis</i> BAC T7B11	P	R																				
Human Rpp38	P	R																				

Fig. 3.10. Amino acid sequence alignment of human Rpp38 protein subunit and a 19-kDa homolog from *Arabidopsis*.

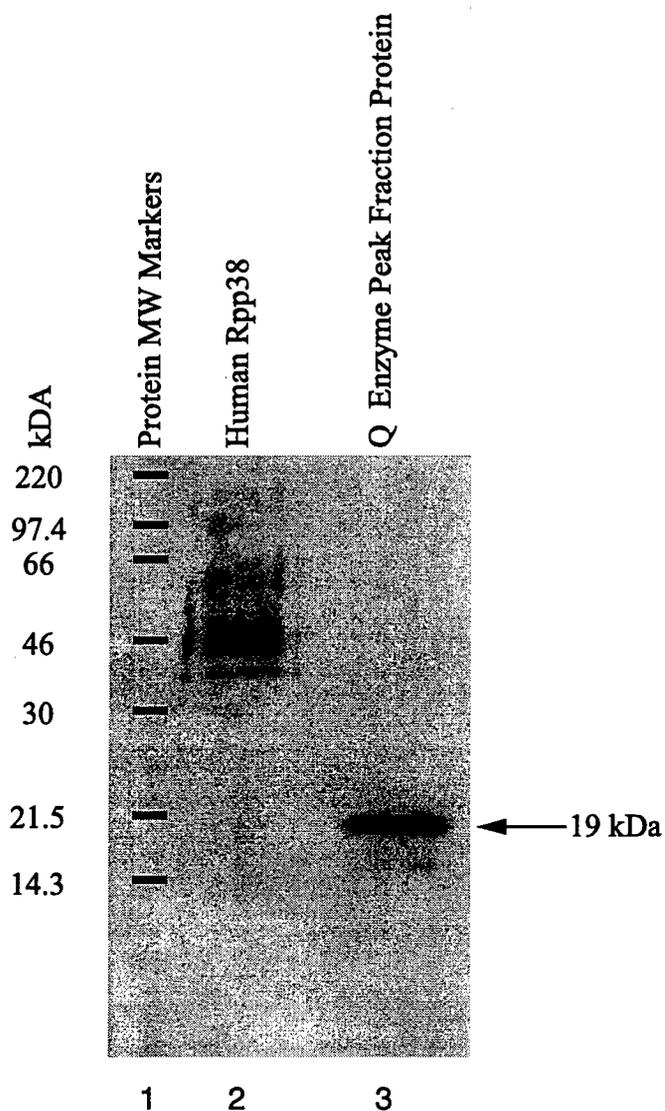


Fig. 3.11. Results of Western Blotting Analysis Using Antisera Against Human Rpp38. Lane 1 is a standard protein MW marker (Rainbow Markers, Amersham Life Science) with the appropriate mass in kDa listed on the left margin. Lane 2 shows human Rpp38 antigen as a positive control. Lane 3 contains the Q enzyme from *A. thaliana* and shows a 19-kDa band that has cross-reacted with the antibody against the Rpp38 subunit of human RNase P.

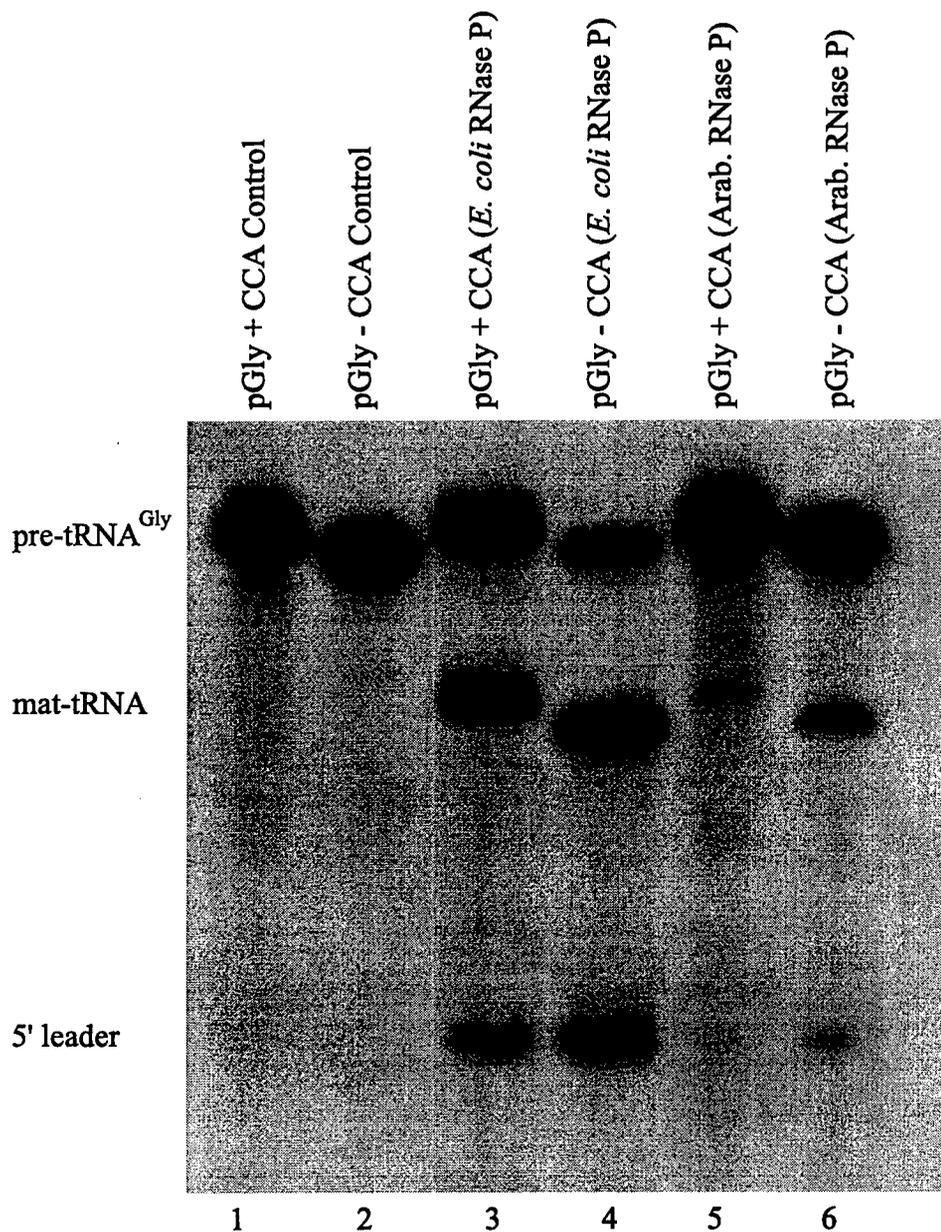


Fig. 3.12. Processing of pGly +/- 3' CCA. Lane 1 and 2 are negative controls of the +/- 3' CCA pGly substrates incubated in the absence of enzyme. Lanes 3 and 4 are positive controls of the +/- 3' CCA pGly substrates assayed with M1 RNA from *E. coli*. Lanes 5 and 6 are the +/- 3' CCA pGly substrates assayed with the Q enzyme. Pre-tRNA processing is evident in Lanes 5 and 6 indicating the ability of the Q fraction to process both substrates.

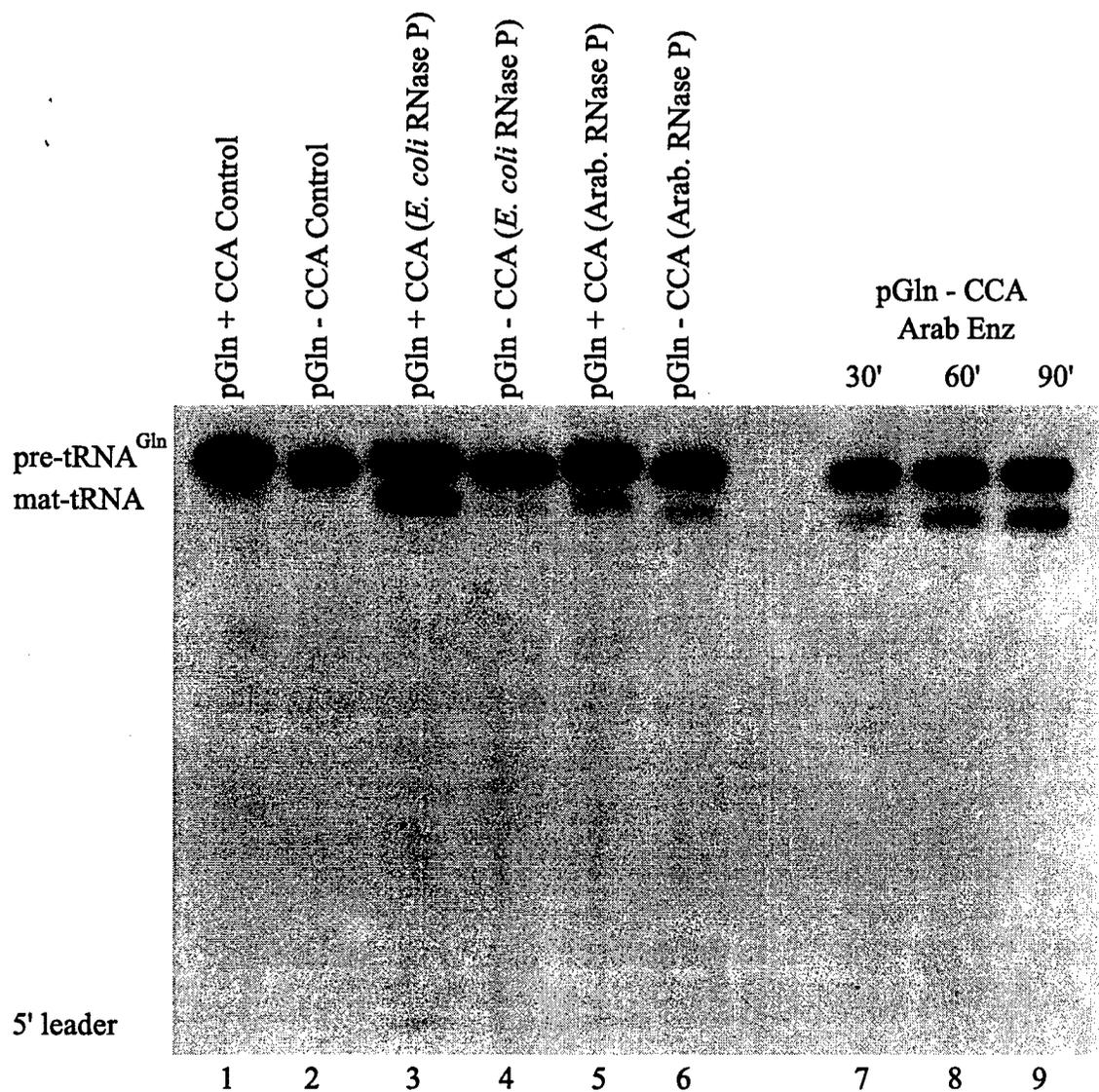


Fig. 3.13. Processing of pGln +/- 3' CCA. Lane 1 and 2 are negative controls of the +/- 3' CCA ptRNA^{Gln} substrates incubated in the absence of enzyme. Lanes 3 and 4 are positive controls of the +/- 3' CCA ptRNA^{Gln} substrates assayed with M1 RNA from *E. coli*. Lanes 5 and 6 are the +/- 3' CCA ptRNA^{Gln} substrates assayed with the Q enzyme. Pre-tRNA processing is evident in lanes 5 and 6 indicating the ability of the Q fraction to process both substrates regardless of the presence of 3' CCA. A time course assay is included in lanes 7-9 to show enzymatic processing of the ptRNA^{Gln} substrate as indicated by the increase in the amount of mature tRNA product with time.

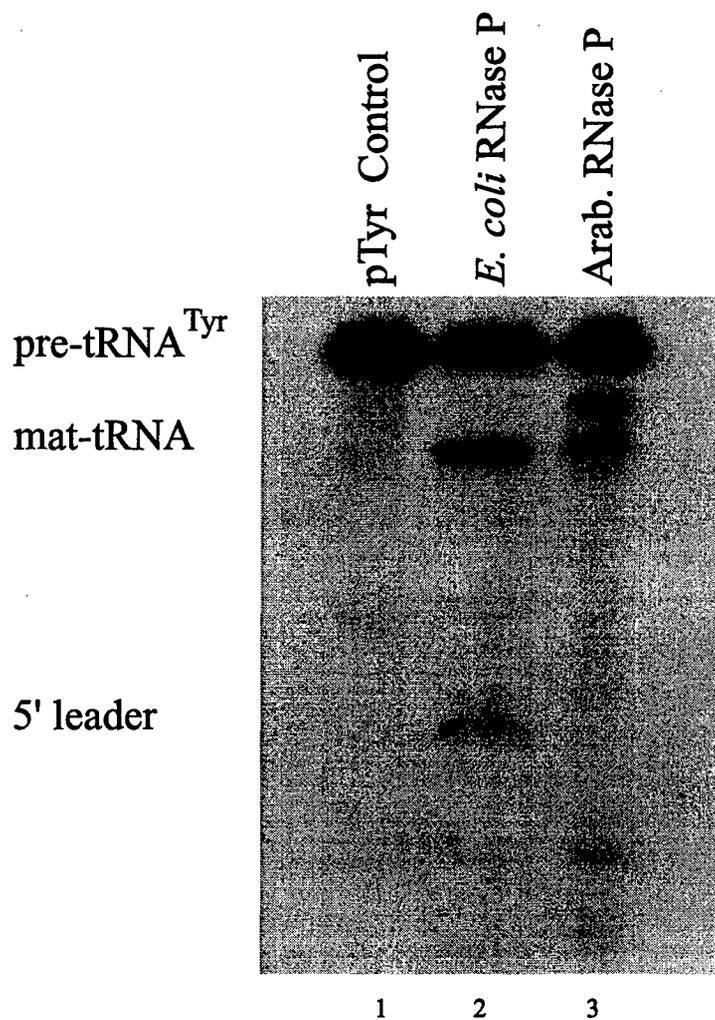


Fig. 3.14. Processing of *E. coli* ptRNA^{Tyr}. Lanes 1 and 2 are negative and positive controls as previously described. Lane 3 is the Q fraction assayed with ptRNA^{Tyr} as substrate. The Q enzyme fails to properly process ptRNA^{Tyr} substrate isolated from *E. coli* and leads to aberrant cleavage.

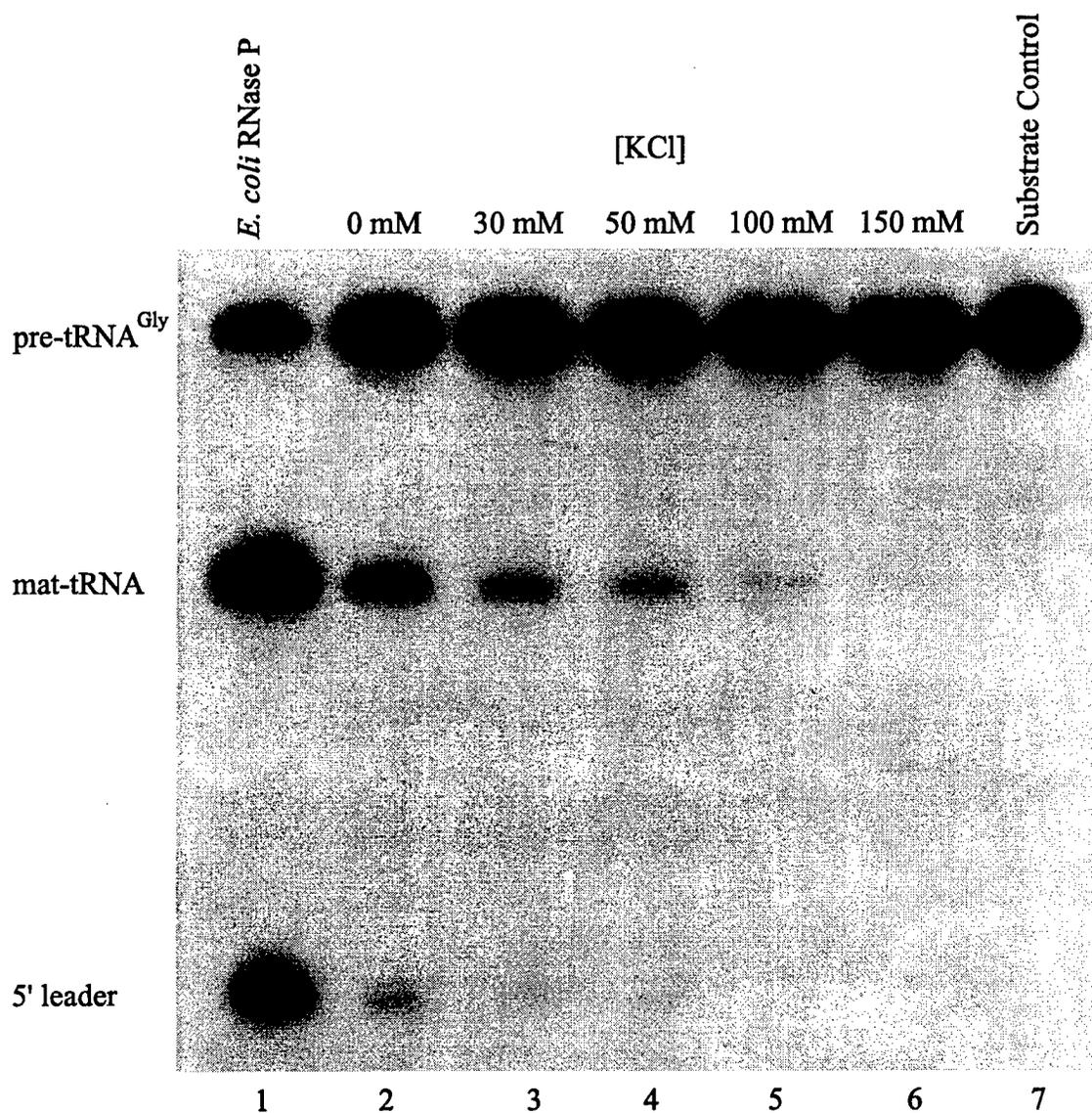


Fig. 3.15. Effect of KCl on *A. thaliana* ptRNA^{Gly} Processing. Lane 1 is a positive control using *E. coli* RNase P. Lanes 2-6 show the effect of increasing concentration of KCl on ptRNA processing by *A. thaliana* RNase P. RNase P activity is severely inhibited at 100mM. Lane 7 is a negative control.

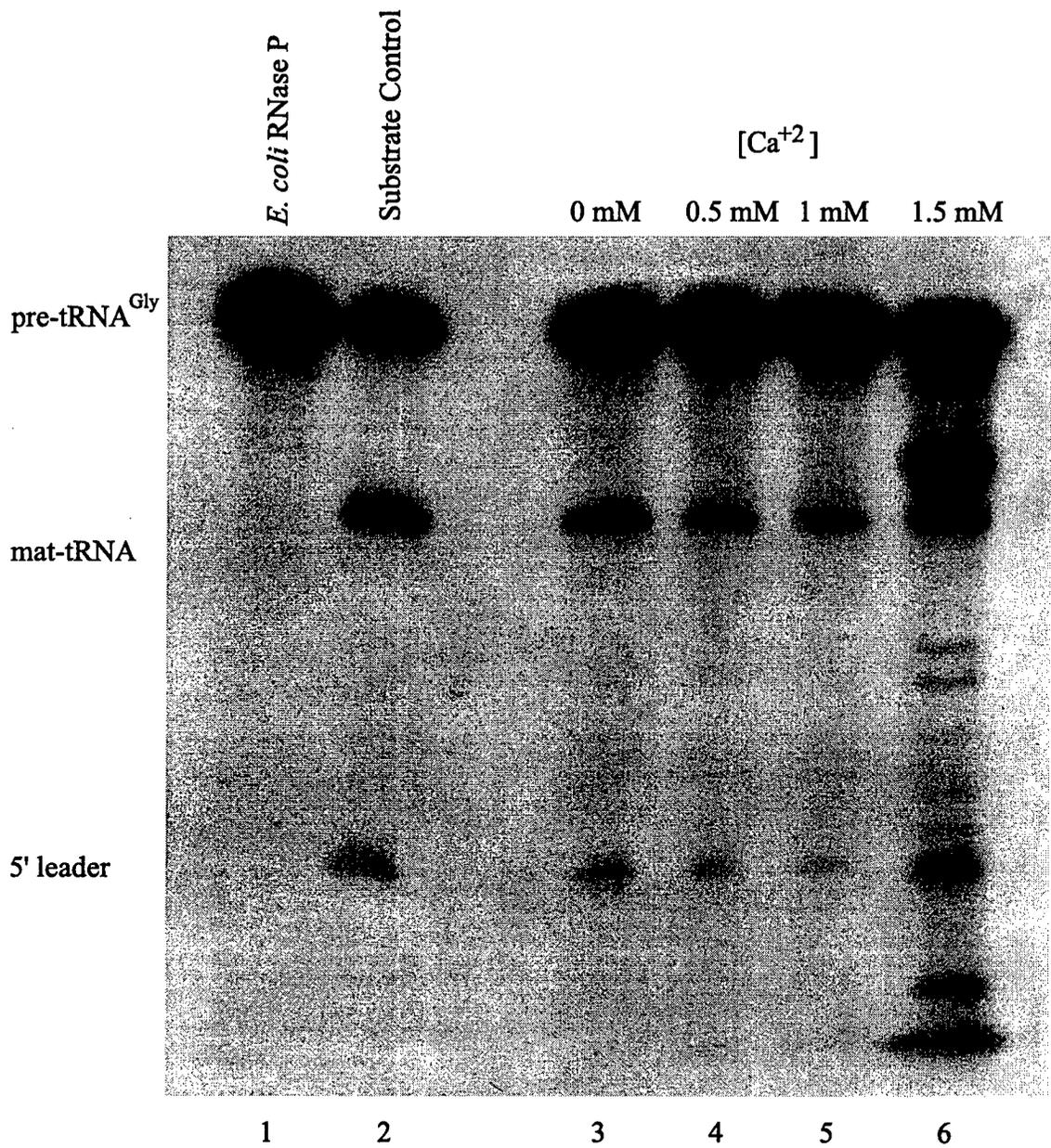


Fig. 3.16. Effect of Ca^{+2} on *A. thaliana* ptRNA^{Gly} Processing. Lane 1 and 2 are positive and negative controls as previously described. Lanes 3-6 show the effect of increasing concentrations of Ca^{+2} on ptRNA processing by *A. thaliana* RNase P. Aberrant cleavage occurs at 1.5 mM Ca^{+2} .

CHAPTER 4

DISCUSSION

4.1 Purification of *A. thaliana* RNase P

This study is the first to isolate and characterize RNase P from any *Arabidopsis* species. Purification of RNase P involved preparing a crude extract from crushed plant tissue, precipitation of proteins using ammonium sulfate and anion exchange chromatography. *A. thaliana* RNase P was purified 9-fold from the crude extract by anion exchange chromatography (Table 3.1).

A. thaliana RNase P is precipitated from a crude extract by saturation with 90% ammonium sulfate (CE) and can adequately process ptRNA substrates at this stage (Fig 3.1). Purification of the CE by anion exchange chromatography revealed the presence of two separate activities. Approximately 70% of the CE activity bound to the anion exchange column (Q enzyme) while the remaining 30% of the activity fail to bind the column and is present in the flow through.

The Q enzyme elutes from the anion exchange column between 130 to 250 mM KCl (Fig. 3.2). The leading Q fractions (4-6) eluted from the anion exchange column are generally free of nuclease contamination. A time course assay with the Q enzyme shows

the presence of mature tRNA and the 5' leader sequence expected for proper processing by RNase P (Fig 3.3). The results of the assay also proved that the Q enzyme was generally free of nuclease contamination and could be used for further characterization of *A. thaliana* RNase P.

Further purification of the Q enzyme by glycerol gradient fractionation (GG enzyme) did not improve the specific activity of RNase P (Fig 3.5). The diminished specific activity in the GG fraction may be due to RNase P breakdown during centrifugation of the glycerol gradient. Alternatively, the loss of enzymatic activity may be due to the low protein concentration (0.017 g/ml) in the collected fractions. *A. thaliana* RNase P may need the presence of other proteins in solution as stabilizing agents for enzymatic activity. Addition of nuclease-free BSA to the GG fraction might improve enzymatic activity and warrants further study.

4.2 The Isolated 5' tRNA Processing Activity is Attributable to RNase P

Various endonuclease and exonuclease activities are found in plants. Therefore, it was necessary to determine if the 5' tRNA processing activity of the Q fraction was due to RNase P and not attributable to some other nuclease. Primer extension analysis of tobacco ptRNA^{Gly} processing intermediates (Fig. 3.6) generated by the Q enzyme demonstrated that this enzyme was capable of cleaving ptRNA^{Gly} at the +1 position (Fig 1.1). Rice RNase P has also been proven to process ptRNA^{Gly} at the precise 5' position by primer extension analysis (Kumar and Gopalan, unpublished data). The results of primer extension analysis reveal that the 5' processing activity isolated from *A. thaliana* is RNase P.

4.3 *A. thaliana* RNase P is an RNP Complex

The results of this study strongly suggest that *A. thaliana* RNase P is an RNP complex. The complete loss of activity by *A. thaliana* RNase P when incubated with PK indicates that at least one protein component is essential for enzymatic activity *in vitro* at standard conditions. The sensitivity of *A. thaliana* RNase P to proteinase K (Fig. 3.7) is in agreement with previous plant studies (Wang et al., 1988; Franklin et al., 1995; Baum et al., 1996).

The partial loss of activity by *A. thaliana* RNase P when incubated with MN indicates that an RNA component is essential for enzymatic activity *in vitro*. The sensitivity of *A. thaliana* RNase P to nuclease treatment (Fig 3.8) is in agreement with the results obtained with carrot RNase P (Franklin et al., 1995). Most RNase P activities studied thus far, whether prokaryotic or eukaryotic (both nuclear and organellar), have essential RNA components. The single exception to this is in the RNase P from spinach chloroplasts (Wang et al., 1988) where it was concluded that an RNA subunit is absent. These investigators demonstrated that EGTA-inactivated MN can bind substrate ptRNAs thus, preventing its processing by RNase P. This "substrate-masking" effect can be relieved by adding a carrier polynucleotide. In the MN experiments presented here, controls were performed in which RNase P was added back to MN-treated extracts after inactivation of MN (Fig 3.8, Lane D). The ptRNA substrate was processed after introducing an additional aliquot of RNase P thus indicating that substrate masking could not account for the initial loss of activity. The results of this experiment suggest that an

RNA subunit present in the *A. thaliana* RNase P holoenzyme is essential for tRNA processing *in vitro*.

A. thaliana RNase P has a density of 1.16 g/ml in CsCl. This density is below that expected for bulk cellular protein (1.25 g/ml) and also below the range (1.27-1.61 g/ml) of previously reported RNase P enzymes (Schon, 1996). However, the low density observed can be explained by considering the effects of temperature and RNA subunit size in CsCl gradients.

RNA subunits of RNase P enzymes in the previous studies were all of approximately the same size but gave very different densities in cesium sulfate or cesium chloride gradients. Densities for RNase P holoenzymes with smaller RNA subunits have not been published. In general, it appears that RNase P enzymes with a high RNA/protein ratio, specifically those found in prokaryotes, are more dense. Eukaryotes, where the RNA/protein ratio is low, tend to be lighter in CsCl gradients and are very similar to that of bulk protein.

Data obtained from CsCl gradients are further clouded by the observation that temperature affects the density of rice RNase P (Raj, Kumar and Gopalan, unpublished data). The density of rice RNase P holoenzyme was found to increase from 1.16 g/ml to 1.22 g/ml as temperature increased from 4° to 24° C.

The density for *A. thaliana* RNase P cannot be used to confirm or deny the presence of an RNA subunit in the holoenzyme. The low density does suggest that *A. thaliana* RNase P is most likely similar to other eukaryotes and has a low RNA/protein ratio.

4.4 The Composition of *A. thaliana* RNase P is Similar to other Eukaryotes

A database search of the *Arabidopsis* genome revealed the presence of a homolog to the Rpp38 protein subunit of human RNase P. Western blot analysis, using polyclonal antisera against the human Rpp38 protein subunit, confirmed the presence of a cross-reacting antigen in partially purified preparations of *A. thaliana* RNase P (Fig 3.11).

Therefore, it is likely that *A. thaliana* RNase P might share structural homology to human RNase P. Structural similarity to human RNase P would also indicate that the *A. thaliana* RNase P is most likely a RNP complex with a low RNA/protein subunit ratio.

4.5 Can *A. thaliana* RNase P Serve as a Tool in Functional Genomics?

The results of this study fail to prove if *A. thaliana* RNase P can be used as a gene disruption tool for functional genomics studies. Since failure of partially purified *A. thaliana* RNase P to cleave a bipartite substrate (Fig. 2.2) could be easily ascribed to contaminating nucleases obscuring the cleavage by RNase P, further purification of the enzyme is needed to convincingly prove the ability to cleave a bipartite substrate. An assessment of *A. thaliana* RNase P as a functional genomics tool can be made only after establishing successful processing of a bipartite substrate.

CHAPTER 5

CONCLUSION

Arabidopsis thaliana RNase P processes ptRNA substrates in a manner similar to that of M1 RNA, the catalytic subunit of *E. coli* RNase P. The plant RNase P holoenzyme is most likely an RNP complex. It appears that both protein and RNA subunit(s) are essential for catalysis. The initial indications are that the RNP complex is very similar to other eukaryotic RNase P holoenzymes. A very low RNA/protein ratio and the presence of more than one protein subunit in the holoenzyme are expected. Confirmation of these preliminary indications will only come from additional research. Compelling evidence of an RNA subunit will come from identification of an RNase P RNA homolog in the *Arabidopsis* genome database and isolating the same RNA from purified *A. thaliana* RNase P active fractions. Additional purification of the *A. thaliana* RNase P holoenzyme is needed to determine if the enzyme will cleave a bipartite substrate and could therefore be used as a functional genomics tool.

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