Cleavage of Bipartite Substrates by Rice and Maize Ribonuclease P. Application to Degradation of Target mRNAs in Plants[1]

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The objective of this investigation is to examine the potential for using plant ribonuclease P (RNase P) as a tool for cleaving target mRNAs and thereby disrupting gene expression in plants.

RNase P is a ubiquitous ribonucleoprotein that is uniquely responsible for the 5′ maturation of nearly sixty-odd precursor tRNAs (ptRNAs) in prokaryotes and eukaryotes (Fig. 1A; Altman and Kirsebom, 1999; Frank and Pace, 1998). Naturally occurring ribozymes, such as RNase P, have been adapted adroitly to cleave specific mRNAs by genetic engineering of either the ribozymes or their substrates (Forster and Altman, 1990; Hartmann et al., 1995; Tanner et al., 1999; Guerrier-Takada and Altman, 2000). The observation that a complex of two RNA molecules (Fig. 1B), which structurally resembles a typical ptRNA (Fig. 1A), is a good substrate for RNase P led to the idea that any cellular mRNA could be targeted for degradation by RNase P if the binding of the mRNA to an external guide sequence (EGS) forms a sequence- and structure-specific complex (Fig. 1C; Yuan et al., 1992; Yuan and Altman, 1994; Guerrier-Takada and Altman, 2000). Several recent studies have validated the use of this EGS-based approach to control gene expression (Guerrier-Takada et al., 1997; Kawa et al., 1998; Plehn-Dujowich and Altman, 1998). For instance, RNase P-mediated degradation of viral mRNAs has been used successfully to inhibit influenza virus replication in cell culture (Plehn-Dujowich and Altman, 1998).

Although the utility of the EGS approach has been demonstrated in mouse and human cells in tissue culture, similar possibilities in plant cells remain unexplored. A prerequisite for using plant RNase P in gene knockout procedures is the ability of plant RNase P to cleave bipartite substrates. This information is lacking, although partial purification and characterization of carrot (*Daucus carrota*) and wheat germ (*Triticum aestivum*) RNase P have been reported (Franklin et al., 1995; Arends and Schon, 1997). Therefore, we undertook this study with rice (*Oryza sativa*) and maize (*Zea mays*) nuclear RNase P.

By successively employing ion-exchange chromatographic and density gradient fractionation procedures, nuclear RNase P was partially purified from rice (Pusa Basmati-1) and maize (Black Mexican Sweet) calli. Details of the purification procedures will be described elsewhere (M.L. Stephen Raj, D.K. Pulukkunat, J.F. Reckard III, G. Thomas, and V. Gopalan, unpublished data). Both of these partially purified preparations exhibited accurate ptRNA processing on three different substrates (namely *Synechocystis* sp. PCC 6803 ptRNA^{Gln}, *Nicotina rustica* nuclear ptRNA^{Tyr}, and *Nicotina tabacum* chloroplast ptRNA^{Gly}. Using the ptRNA processing reaction of the well-studied *Escherichia coli* RNase P as a standard, we first confirmed the accurate cleavage of ptRNA^{Gln} by rice and maize RNase P (Fig. 2). By using two criteria, we verified that these processing events were canonical RNase P-mediated cleavages. First, by using a reverse transcriptase-based primer extension method, we have established that the ptRNA substrate is cleaved between −1 (the last nucleotide of the leader) and +1 (the first nucleotide of the mature tRNA) of the substrate (data not shown; see Fig. 2A for location of cleavage site). Second, the functional group at the 5′ end of the mature tRNA was established to be a monophosphate (data not shown).

To use plant RNase P in gene knockout experiments, it is important to determine whether a substrate for plant RNase P can be constructed from two RNA molecules. To be specific, does plant nuclear RNase P behave like its mammalian counterpart? Can EGSs (Fig. 1C) be utilized for targeted degradation of mRNAs in plants? We have adopted the following approach to address these questions. The ptRNA^{Gln} substrate was dissected into two parts which when reconstituted will generate a ptRNA-like structure (albeit with a nick in the D loop; Fig. 3A). The "EGS" part of ptRNA^{Gln} was generated by transcription in vitro, whereas the 25-nt "model target

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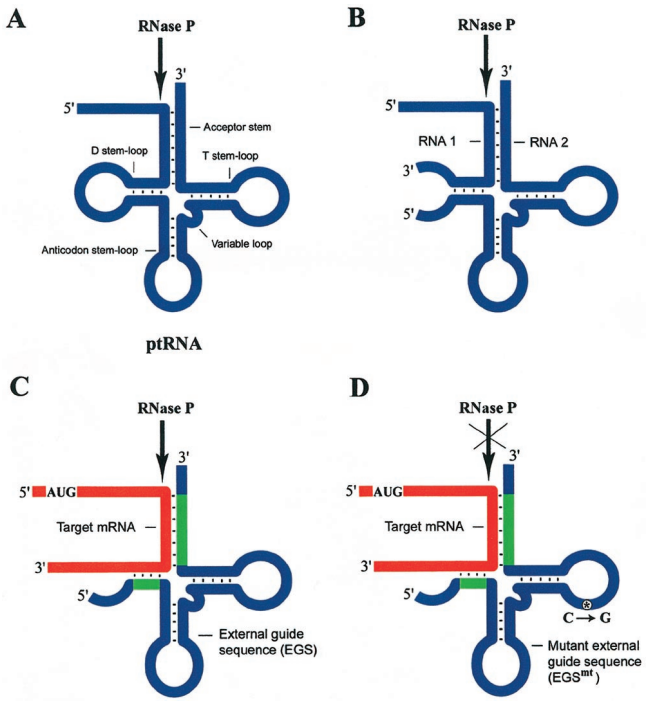

Figure 1. Rationale for cleaving target mRNAs with eukaryotic RNase P. A, The structure of a ptRNA, a typical substrate for RNase P. The arrow indicates the site of cleavage by RNase P. B, A complex of two RNAs that are non-covalently bound to form a substrate that resembles a ptRNA (albeit with a nick in the D loop). C, The concept underlying RNase P-mediated cleavage of a target mRNA in the presence of a second RNA molecule termed the external guide sequence (EGS). In addition to possessing sequences that are complementary to the target mRNA, the EGS also has the anticodon stem loop, variable loop, and T-stem loop regions of a typical ptRNA substrate. D, Inability of RNase P to cleave a ptRNA-like substrate that is formed by the interaction of the target mRNA with a mutant EGS harboring a point mutation of a conserved nucleotide in the T-loop equivalent region.

substrate" RNA was chemically synthesized. When these two molecules are mixed together, they should generate a ptRNA-like structure on account of Watson-Crick base pairing (Fig. 3A). Such a bipartite substrate is cleaved accurately and efficiently by rice and maize RNase P in vitro (Fig. 3B, lanes 7–10). Various control experiments indicate that the cleavage event is mediated by RNase P only when the target substrate and EGS RNAs are both present in the cleavage reaction. If the target substrate RNA is incubated with either rice or maize RNase P in the absence of EGS RNA, there is no cleavage (Fig. 3B, lanes 4 and 5). Moreover, in the absence of RNase P, the target substrate RNA remains unchanged when incubated in the presence of EGS RNAs (lanes 2 and 3).

As observed with human and mouse RNase P, a single nucleotide substitution in the T-loop region of the EGS RNA (Fig. 1D) renders the mRNA-EGS complex resistant to cleavage by rice and maize RNase P (Fig. 3B, lanes 11–14). By using a gel-retardation assay, we verified that the T-loop mutant EGS RNA did bind the substrate under our assay conditions (data not shown). Therefore, the failure of plant RNase P to cleave the target substrate RNA when the mutant EGS RNA was added cannot be due to lack of Watson-Crick base pairing, which is essential for generating a ptRNA-like bipartite structure. These observations, taken together with previous reports (Kawa et al., 1998; Plehn-Dujowich and Altman, 1998), indicate that substrate recognition by eukaryotic RNase P must involve some interaction with the T loop, a property already exploited in studies on RNase P-mediated cleavage of mRNAs in vivo.

Because the EGS RNAs are complementary to the target mRNA, it is conceivable that an EGS-mediated decrease in expression of the target protein in vivo is attributable to antisense effects and not due to cleavage of the target mRNA by RNase P. As mentioned above, the T-loop mutant EGS can bind the target mRNA but the resulting complex is not a substrate

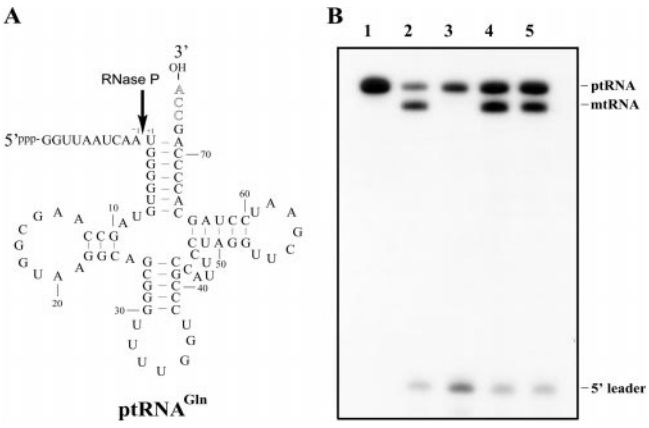

Figure 2. Cleavage of ptRNA^{Gln} by plant RNase P. A, Secondary structure of the *Synechocystis* sp. PCC 6803 ptRNA^{Gln}. B, The ptRNA^{Gln} was prepared using in vitro transcription as described by Pascual and Vioque (1999). In this study, we used the substrate lacking the 3′-terminal CCA sequence (highlighted in A). Internal labeling of the ptRNA^{Gln} was accomplished by including α-[^{32}P]GTP in the in vitro transcription reaction. For 5′-end labeling, ptRNA^{Gln} with a 5′-guanosine triphosphate was first dephosphorylated using calf intestinal alkaline phosphatase and subsequently phosphorylated using T4 polynucleotide kinase and γ-[^{32}P]ATP. The 20-μL RNase P assay mixture contained 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 7 mM dithiothreitol, 0.6 mM phenylmethylsulfonyl fluoride (PMSF), 20 units of RNasin (Promega, Madison, WI), and 50 nM ptRNA substrate (5,000 cpm). The assay was initiated by adding partially purified rice or maize RNase P. The reaction was carried out for 50 min at 37°C and terminated by adding 10 μL of stop dye solution (9 M urea, 10% [v/v] phenol, 0.1% [w/v] bromphenol blue, and 0.15% [v/v] xylene cyanol). The reference molecules for verifying the accurate processing of ptRNA^{Gln} by plant RNase P was generated by digesting ptRNA^{Gln} with *E. coli* RNase P (purified as described by Gopalan et al. [1997]). The reaction products were resolved in an 8% (w/v) polyacrylamide/8 M urea gel and visualized by autoradiography. Lane 1, ptRNA^{Gln} in the absence of enzyme; lane 2, internally labeled ptRNA^{Gln} cleaved by *E. coli* RNase P; lane 3, 5′-end-labeled ptRNA^{Gln} cleaved by *E. coli* RNase P; lanes 4 and 5, internally labeled ptRNA^{Gln} cleaved by rice and maize RNase P, respectively.

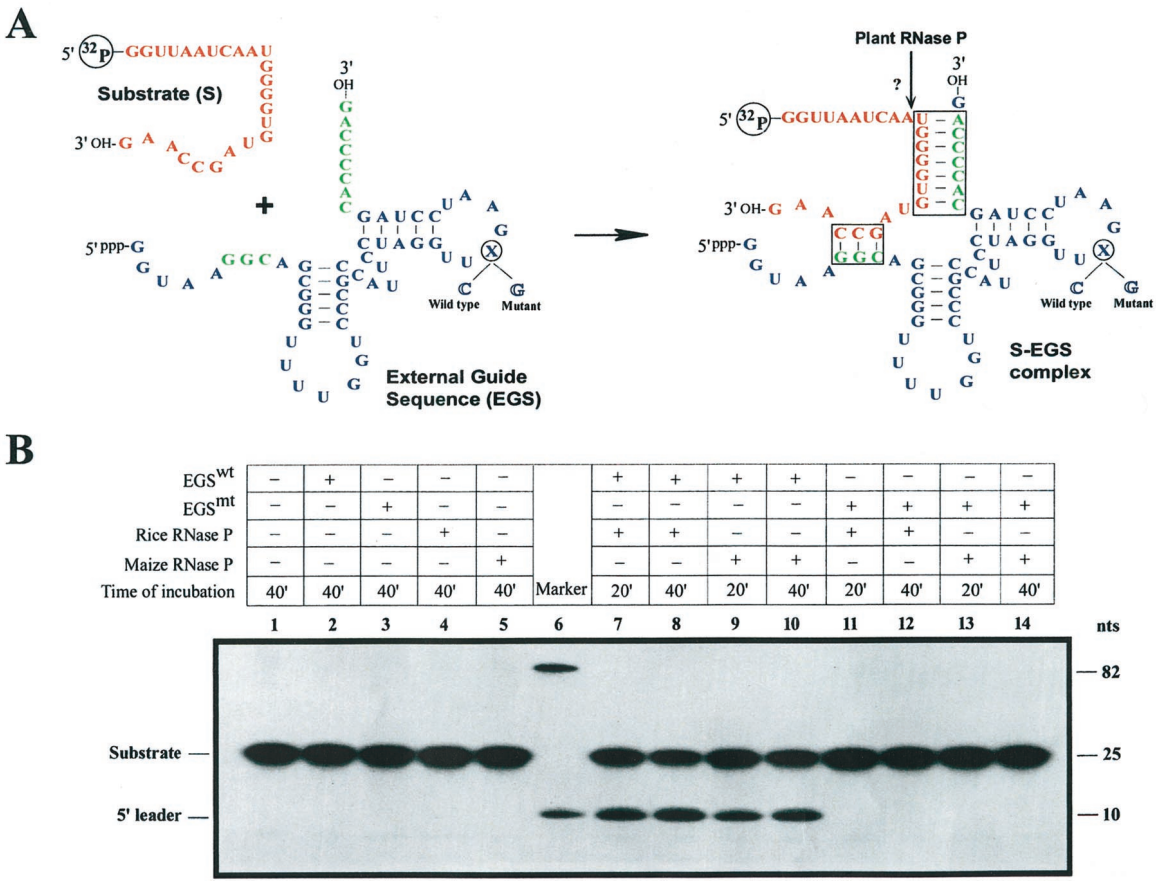

Figure 3. Cleavage of bipartite substrates by plant RNase P. A, The model target substrate and EGS RNAs were designed based on the sequence of *Synechocystis* sp. PCC 6803 ptRNA^{Gln}. The substrate RNA was chemically synthesized (Dharmacon Research, Inc., Boulder, CO) and 5′-end labeled using T4 polynucleotide kinase and γ-[^{32}P]ATP. The genes encoding the wild-type and mutant EGS RNAs (EGS^{wt} and EGS^{mt}, respectively) were cloned by PCR using the appropriate primers and pT7Gln (Pascual and Vioque, 1999) as the template. The EGS^{wt} and EGS^{mt} RNAs were generated using in vitro run-off transcription with T7 RNA polymerase. Approximately 100 nM of substrate RNA (10,000 cpm) and 200 nM of either EGS^{wt} or EGS^{mt} were mixed together in a total of 5 μL containing 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, and 0.6 mM PMSF. B, The bipartite cleavage assay was performed as follows. The substrate and EGS RNAs were first incubated at 65°C for 15 min and snap-cooled on ice. Rice or maize RNase P subsequently was added to the substrate-EGS mixture and incubated at 37°C for a specified period. The assay was performed in 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 7 mM dithiothreitol, 20 units RNasin (Promega), and 0.6 mM PMSF. Aliquots were withdrawn at 20- and 40-min intervals from the assay reaction and the activity was terminated by addition of 10 μL stop dye solution (9 M urea, 10% [v/v] phenol, 0.1% [w/v] bromphenol blue, and 0.15% [v/v] xylene cyanol). The control reactions that lack either the EGS RNA or RNase P or both also underwent similar treatment. The 5′ leader generated from the digestion of 5′-end-labeled ptRNA^{Gln} by *E. coli* RNase P was used as a reference standard for the product expected from cleavage of the bipartite substrate. The reaction products were resolved in a 12% (w/v) polyacrylamide/8 M urea gel and visualized by autoradiography. The various reactions indicated in the figure are self-explanatory. nts, Length of RNA in nucleotides.

for cleavage by RNase P. Hence, the disruption of gene expression (if any) that is observed with the mutant EGS will indicate the degree to which the disruption is due to antisense effects. In studies that employed the EGS approach in vivo, the mutant EGSs were completely ineffective relative to the wild-type EGSs in decreasing mRNA expression, thus confirming that the efficacy of the EGS-based approach is due to RNase P-mediated targeted degradation (Kawa et al., 1998; Plehn-Dujowich and Altman, 1998).

Our results suggest that a plant RNase P-based gene knockout procedure could be included in the repertoire of approaches available for delineating gene function in plants (Chuang and Meyerowitz, 2000; Wu et al., 2000). The success of the RNase P-mediated approach will depend on (a) stable expression of the EGSs (using either constitutive or regulated promoters), (b) colocalization of the target mRNA substrate and the EGS within the same subcellular compartment, and (c) accessibility of the target mRNA to the EGS. Strategies that help down-

regulate gene expression by specifically cleaving target mRNAs in a true catalytic fashion (i.e. multiple turnover) are of particular value in functional genomics because the elimination of a gene product is likely even if it is encoded by multiple copies as in polyploid plant genomes. Studies are in progress to test the potential of the RNase P-based method for targeted cleavage of mRNAs in suspension cultures of plant cells as well as in transgenic plants.

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