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CSP41, a Sequence-Specific Chloroplast mRNA Binding Protein, Is an Endoribonuclease

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Correct 3′ processing of chloroplast precursor mRNAs (pre-mRNAs) requires a stem-loop structure within the 3′ untranslated region. In spinach, a stable 3′ stem-loop–protein complex has been shown to form in vitro between *petD* pre-mRNA, encoding subunit IV of the cytochrome b_6/f complex, and chloroplast proteins. This complex contains three chloroplast stem-loop binding proteins (CSPs), namely, CSP29, CSP41, and CSP55. Here, we report the purification of CSP41 and cloning of the *csp41* gene and show that CSP41 is encoded by a single nuclear gene. Characterization of bacterially expressed CSP41 demonstrates that this protein binds specifically to the 3′ stem-loop structure and a downstream AU-rich element of *petD* pre-mRNA and that its binding affinity is enhanced by associating with CSP55. Our data also show that CSP41 has substantial nonspecific endoribonuclease activity. These data suggest that CSP41 could be involved in 3′ processing of *petD* pre-mRNA and/or in RNA degradation. The fact that different reaction conditions favor RNA binding over ribonuclease activities suggests a possible mode of in vivo regulation.

INTRODUCTION

Chloroplasts are specialized organelles in plant cells that perform photosynthesis, and their structural proteins and enzymes are encoded by both the nuclear and the chloroplast genomes. During chloroplast development under illumination, accumulation of chloroplast-encoded mRNAs depends mainly on post-transcriptional events, including the regulation of mRNA stability (reviewed in Mullet, 1988; Gruissem, 1989). Like bacterial mRNAs, most chloroplast precursor mRNAs (pre-mRNAs) and mature mRNAs contain an inverted repeat (IR) sequence in their 3′ untranslated region (UTR) that can fold into a stable stem–loop structure. The functions of these 3′ stem–loop structures have been studied in vitro by using RNA processing and degradation assays and in vivo by using chloroplast transformation to alter the 3′ UTR. The results obtained by these approaches have demonstrated that these structures are required for correct 3′ processing of pre-mRNAs and for stabilization of mature mRNAs but that the IRs do not efficiently terminate transcription (Stern and Gruissem, 1987; Blowers et al., 1993; Stern and Kindle, 1993). The mechanism of chloroplast pre-mRNA 3′ end processing involves both processive 3′ to 5′ exoribonuclease and site-specific endoribonuclease activities, with exonucleolytic degradation being impeded by the 3′ stem–loop structure, thus generating stable and mature mRNAs. In the absence of stable secondary structure, or by readthrough of the stem–loop structure, mRNA becomes heterogeneous and generally unstable (Stern and Gruissem, 1987, 1989; Stern et al., 1991; Blowers et al., 1993). Therefore, the 3′ IR has a primary role in controlling chloroplast mRNA accumulation through pre-mRNA processing and protection of upstream sequences.

rpsO mRNA encoding *Escherichia coli* ribosomal protein S15 and mRNAs encoding animal cell cycle–regulated histones also contain stem–loop structures in their 3′ UTRs, which determine correct 3′ formation during pre-mRNA processing. Extensive studies have shown that both exonucleases and endonucleases participate in 3′ processing by interacting with the 3′ stem–loop structures and flanking RNA sequences (reviewed in Marzluff, 1992; Hajnsdorf et al., 1994). In addition, a 3′ stem–loop binding protein is involved in histone pre-mRNA 3′ processing (Vasserot et al., 1989). In chloroplasts, several RNA binding proteins (RNPs) or their genes have been purified or cloned from spinach, tobacco, pea, mustard, and Arabidopsis (Li and Sugiura, 1990; Li et al., 1991; Schuster and Gruissem, 1991; Ye et al., 1991; Mieszczak et al., 1992; DeLisle, 1993; Nickelsen and Link, 1993; Subbaiah and Tewari, 1993; Hirose et al., 1994; Liere and Link, 1995; Ohta et al., 1995). Although most bind to RNA nonspecifically and are of unknown function, 28- and 100-kD spinach RNPs have been implicated in the 3′ end maturation of chloroplast pre-mRNAs (Schuster and Gruissem, 1991; Hayes et al., 1996). Although the precise role of the 28-kD RNP is not known, the 100-kD RNP gene has strong homology with the polynucleotide phosphorylase (PNPase, a 3′ to 5′ exoribonuclease).

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Spinach chloroplast *petD* mRNA encodes subunit IV of the cytochrome b_6/f complex, and both mRNA and protein levels increase modestly during light-induced chloroplast development. *petD* pre-mRNA contains a 3′ stem–loop structure with the functions described above, as well as two identical eight-nucleotide AU-rich elements upstream and downstream of the stem–loop, which are conserved in *petD* pre-mRNAs of other plants (Chen and Stern, 1991). In our laboratory, an effort has been made to identify and functionally characterize chloroplast proteins that interact with this region. Proteins of 55 and 29 kD were shown previously to bind to the stem–loop structure based on UV cross-linking experiments (Stern et al., 1989; Chen and Stern, 1991). A stable 3′ stem–loop–protein complex also can be detected by gel mobility shift (GMS) assays, and we have recently shown that this complex contains not only 55- and 29-kD proteins but also a novel 41-kD protein (Chen et al., 1995). Site-directed mutagenesis showed that complex formation is mediated by both the stem–loop structure and the downstream AU-rich element that is removed during pre-mRNA processing, suggesting that this complex can form with *petD* pre-mRNA but not with mature *petD* RNA (Chen et al., 1995).

To understand whether and how the 3′ stem–loop–protein complex functions in *petD* mRNA processing and stabilization, we decided to characterize the protein components of the complex, which we have termed chloroplast stem–loop binding protein (CSP) 29, CSP41, and CSP55. In this report, we describe the purification and characterization of CSP41. Our results show that CSP41 is a light-induced nuclear-encoded chloroplast endoribonuclease with sequence-specific stem–loop binding activity. The possible roles of CSP41 in *petD* mRNA processing and stabilization are discussed.

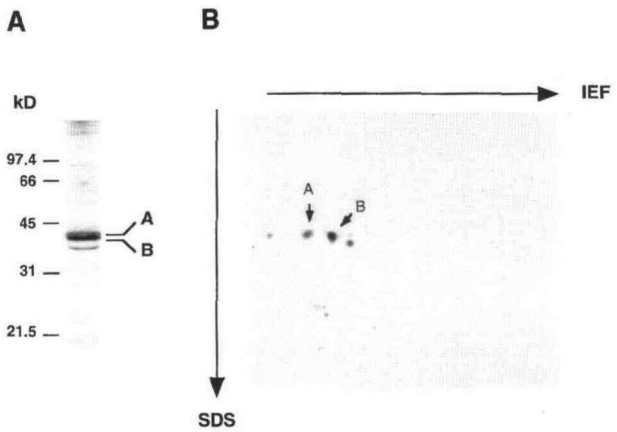

Figure 1. *CSP41 Copurifies with GAPDH.*

(A) SDS-PAGE analysis of the CSP41-enriched protein fraction. One microgram of protein of the CSP41-enriched fraction obtained from heparin–FPLC was electrophoresed on a 12% SDS–polyacrylamide gel and visualized by silver staining. Protein molecular mass markers in kilodaltons are indicated at left. A and B indicate the two 41-kD proteins. **(B)** Separation of the 41-kD proteins by two-dimensional gel electrophoresis. Thirty-five micrograms of the CSP41-enriched fraction was electrophoresed in an isoelectric focusing (IEF)–SDS two-dimensional gel system and visualized by Coomassie blue staining. N-terminal amino acid sequences for proteins A and B are shown below the gel. X denotes an unidentified amino acid.

RESULTS

Purification of CSP41 and Gene Cloning

Previously, we described the identification of CSP29, CSP41, and CSP55, which interact with the 3′ stem–loop structure of *petD* pre-mRNA. To purify CSP41, a spinach chloroplast protein extract containing the stem–loop binding activity was subjected to heparin–fast protein liquid chromatography (FPLC). Proteins that bind to the heparin column were eluted with a linear gradient of 0 to 500 mM KCl, and stem–loop–specific binding activity was monitored by GMS using ^{32}P-labeled petDΔ50 RNA, a 200-nucleotide transcript containing 84 nucleotides of the subunit IV coding region and 107 nucleotides of 3′ UTR sequences, including the stem–loop structure and the upstream and the downstream AU-rich elements (box I and box II; Chen et al., 1995). The GMS assay indicated that the stem–loop binding activity eluted after the major protein peak, and SDS–polyacrylamide gel examination revealed that a doublet of 41-kD proteins copurified with the binding activity (data not shown). The fractions most highly enriched in these proteins were combined as the 41-kD–enriched protein fraction. Separately, proteins were eluted in bulk from the heparin column with 1 M KCl instead of a gradient, yielding a crude binding fraction that contained the stem–loop binding activity, which was used for several experiments presented below.

Figure 1A shows the profile of the 41-kD–enriched protein fraction in an SDS–polyacrylamide gel, with a doublet of 41-kD proteins (proteins A and B) and a major 36-kD protein contaminant. By running a parallel SDS–polyacrylamide gel loaded with the stem–loop–protein complex, which was formed with petDΔ50 RNA and the binding fraction and excised from a native gel (Chen et al., 1995), we were able to see that the migration of CSP41 in the complex matched that of protein B in the 41-kD–enriched fraction (data not shown). Proteins A and B of the 41-kD doublet were separated further by isoelectric focusing in two-dimensional polyacrylamide gels (Figure 1B) and transferred to a polyvinylidene difluoride membrane for N-terminal sequencing. A comparison of the sequences shown at the bottom of Figure 1B with those in the data base suggests that protein A is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an abundant chloroplast enzyme, and that protein B is a novel protein. Because it was reported previously that GAPDH has RNA binding activity (Singh and Green, 1993), we tested its properties using a commercial preparation (Sigma) and our substrates and reaction conditions.

We found its binding to be weak and nonspecific relative to the activity of the 41-kD–enriched fraction (data not shown), and we therefore identified protein B as CSP41. To obtain additional sequence information, several peptides were generated from protein B by tryptic digestion and subjected to sequence analysis. The three internal peptide sequences obtained in this way are underscored in Figure 2B.

Complete sequencing of chloroplast genomes from several plant species has revealed that most chloroplast genes encode components of the photosynthesis apparatus and the transcription/translation machinery (reviewed in Umesono and Ozeki, 1987). Furthermore, none of the unidentified open reading frames (ORFs) encoded in chloroplast DNA has obvious homology with the RNP or with the peptide sequences of CSP41. Therefore, it is likely that CSP41 is encoded in the nuclear genome. To clone the *csp41* gene, a degenerate primer JYBIN(+) and primer T7BS were used to amplify a gene-specific DNA fragment from a spinach leaf λZapII cDNA library by polymerase chain reaction (PCR), yielding a 585-bp DNA fragment (see Methods). DNA sequence analysis indicated that this fragment contains a partial cDNA with deduced amino acid sequences identical to two of the trypsin-generated peptides from CSP41. This DNA fragment was then used as a probe to isolate additional *csp41* cDNA clones. The longest *csp41* cDNA, as shown in Figure 2A, contains a 1248-bp ORF encoding an incomplete N-terminal putative chloroplast transit peptide and the entire mature CSP41 protein. The messenger also contains 155 bp of 3′ UTR and a poly(A) tail. The structural features of the *csp41* messenger, including the putative transit peptide and poly(A) tail, indicate that CSP41 is a typical nuclear-encoded chloroplast protein.

Within the ORF, mature CSP41 begins where the deduced amino acid sequence is identical to the N-terminal amino acid sequence of purified CSP41. Figure 2B shows the deduced amino acid sequence for CSP41; the mature part contains 332 amino acids with a predicted molecular mass of 36 kD. We found several short regions of similarity to existing protein sequences; a 50–amino acid region with 42% similarity to nuclease P1 was of particular interest (see Discussion). However, no known functional motifs, such as RNA recognition motifs, were located in CSP41. To determine gene copy number, total spinach DNA was digested with restriction enzymes and analyzed by DNA filter hybridization. For DNA samples digested with BamHI or EcoRI, a single band was detected with a DNA probe made from the *csp41* cDNA clone (Figure 2C). Therefore, it is likely that CSP41 is encoded by a single nuclear gene.

The *csp41* Gene Is Differentially Expressed

To obtain a large amount of homogeneous CSP41 protein for in vitro studies, we cloned the mature *csp41* coding region immediately downstream of a sequence encoding a six-histidine peptide in the *E. coli* expression vector pQE30. After induction with isopropyl β-D-thiogalactopyranoside, the recombinant

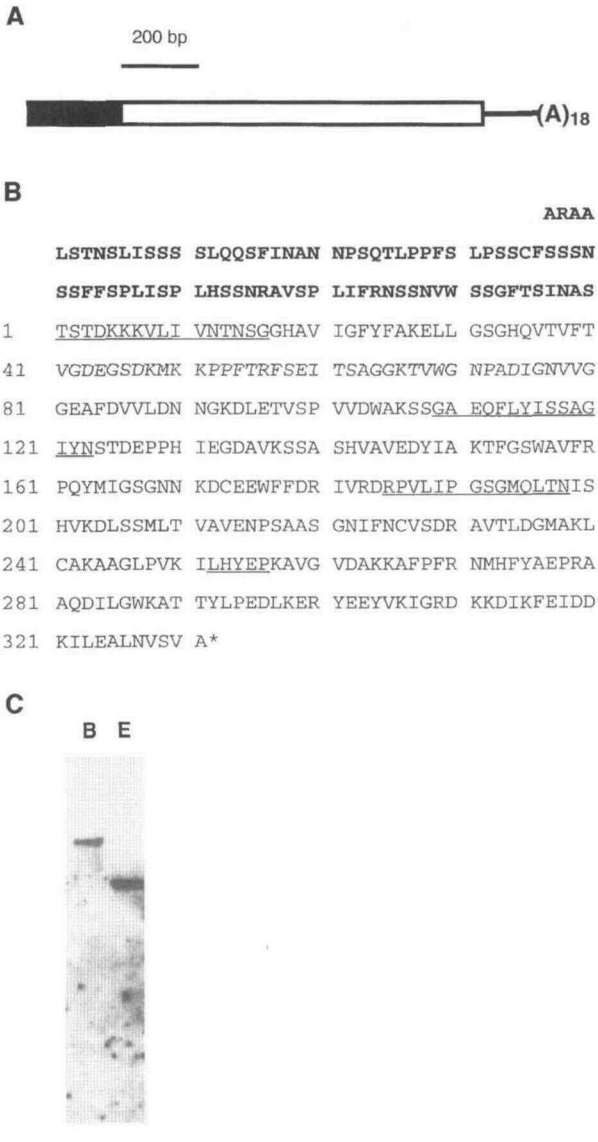

Figure 2. CSP41 Is Encoded by a Single Nuclear Gene.

(A) Structure of the longest spinach *csp41* cDNA. A 996-bp ORF encoding mature CSP41 (open box) is followed by a 155-bp 3′ UTR (solid line) and a poly(A) tail ($(A)_{18}$). The ORF is preceded by an incomplete chloroplast transit peptide (filled box).

(B) The predicted amino acid sequence of CSP41. The N-terminal amino acid sequence and three internal peptide sequences of mature CSP41 are underlined, and the putative transit peptide is indicated by boldface letters that are not numbered. The sequence has GenBank accession number U49442.

(C) DNA filter hybridization analysis of the *csp41* gene. Ten micrograms of spinach total DNA digested with BamHI (B) or EcoRI (E) was separated in a 0.7% agarose gel, transferred to a nylon membrane, and probed with *csp41* cDNA.

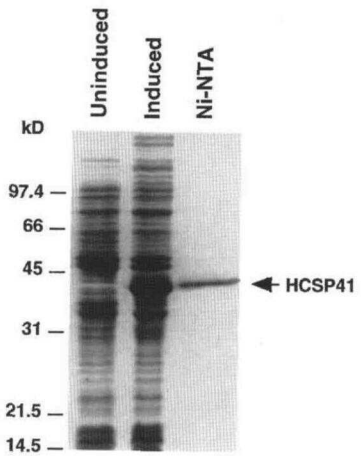

Figure 3. Expression of HCSP41 in *E. coli*.

HCSP41 was expressed in *E. coli*, as described in Methods. Protein extracts from 100 μL of uninduced and induced SG13009 cell cultures and 1 μg of HCSP41 protein purified through a Ni–nitrilotriacetate (NTA) column were analyzed on a 12% SDS–polyacrylamide gel. Protein profiles were visualized by Coomassie blue staining, and the migration of HCSP41 is shown at right. Protein molecular mass markers in kilodaltons are indicated at left.

plasmid produced an N-terminal histidine-tagged CSP41 protein (HCSP41) in insoluble form. This fusion protein has a relative molecular mass of ~41 kD in an SDS–polyacrylamide gel. Using the histidine tag, HCSP41 could be obtained at a purity of ≥98% under denaturing conditions on a nickel column, directly from a lysate of the induced host strain (Figure 3). After refolding in buffer E containing 2 mM DTT (see Methods), HCSP41 was used to develop anti-CSP41 polyclonal antibodies in rabbits. For biochemical activity assays, homogeneous HCSP41 was obtained by heparin–FPLC chromatographic purification of the protein enriched on the Ni–nitrilotriacetate column.

We followed CSP41 accumulation in spinach plants by immunoblot analysis, using CSP41 antiserum. Total protein extracts from young leaves, mature leaves, roots, etiolated cotyledons, and cotyledons from etiolated plants that had been illuminated for 24 hr were analyzed. Figure 4 shows that CSP41 accumulates in photosynthetic tissues but not in roots of a light-grown spinach plant or in etiolated cotyledons. After 24 hr of illumination, the cotyledons showed a significant accumulation of CSP41. This accumulation pattern clearly indicates that CSP41 accumulates preferentially in photosynthetic tissues and can be induced by illumination. This result is in contrast with data obtained for other spinach chloroplast RNPs. For example, 33RNP and 28RNP, which may play a role in RNA processing (L. Gabay, I. Lisitsky, A. Kotler, and G. Schuster, unpublished data; Schuster and Gruissem, 1991), accumulate in etiolated tissues and are not affected by 24 hr of illumination (Figure 4; also see Schuster and Gruissem, 1991).

CSP41 Is Essential for Formation of the Stem-Loop–Protein Complex

We elected to use the anti-CSP41 polyclonal antibody to prove unambiguously that the gene we cloned indeed encodes the 41-kD component of the *petD* pre-mRNA stem–loop–protein complex. For this purpose, a gel slice containing the complex formed between the ^{32}P-labeled petDΔ50 transcript and the binding fraction (Figure 5, lane G2 at left) was excised from a native polyacrylamide gel and electrophoresed onto a 12% SDS–polyacrylamide gel. Protein components of the complex were separated in this gel and then transferred to nitrocellulose and challenged with CSP41 antiserum. Of the three protein components of the complex (CSP29, CSP41, and CSP55; Chen et al., 1995), only CSP41 was recognized by the antibody (Figure 5, lane G2 at right). In a control reaction, a GMS assay without RNA added was subjected to native gel electrophoresis, and a gel slice was excised from it at a position parallel to slice G2 (Figure 5, lane G1 at left). This slice did not contain any proteins that could be recognized by the antiserum in the subsequent immunoblot analysis (Figure 5, lane G1 at right). Thus, we conclude that the gene we have cloned indeed encodes the 41-kD component of the stem–loop–protein complex.

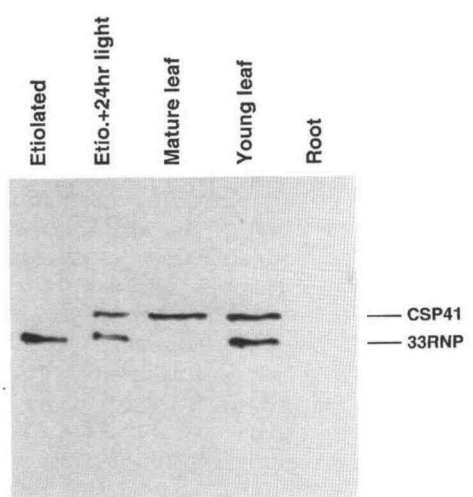

Figure 4. CSP41 Accumulates after Illumination.

Immunoblot analysis of CSP41 in spinach plants was conducted. Six micrograms of total protein extracted from the indicated organs and developmental stages was fractionated on a 12% SDS–polyacrylamide gel. Immunodetection was performed with 1:2000 dilutions of CSP41 antiserum and 33RNP antiserum (L. Gabay, I. Lisitsky, A. Kotler, and G. Schuster, unpublished data). Reactive proteins were visualized by enhanced chemiluminescence. Etio., etiolated.

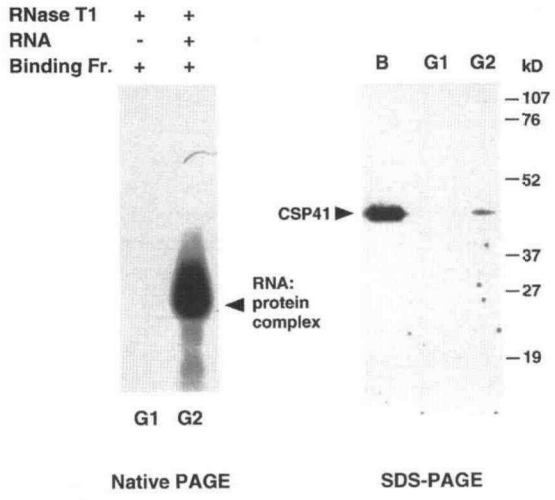

Figure 5. CSP41 Antiserum Detects a 41-kD Species in the Stem–Loop–Protein Complex.

Immunodetection of CSP41 in the stem–loop–protein complex was conducted. A large-scale GMS assay (at left) was performed as described in Methods. Following electrophoresis in a 5% Tris–glycine native polyacrylamide gel, the gel slice containing the complex in lane G2 and that in lane G1, with a position corresponding to the complex, were excised and loaded on a 12% SDS–polyacrylamide gel. One microgram of the binding fraction (lane B at right) was also loaded. Proteins were transferred to nitrocellulose and immunodetected by anti-CSP41 antibodies and ^{125}I-labeled protein A (as shown at right). Protein molecular mass markers in kilodaltons are indicated at right. Fr., fraction; (+), presence; (–), absence.

Although CSP41 is present in the complex, it was of interest to determine whether it is an essential component. To address this issue, we depleted CSP41 from the binding fraction by using anti-CSP41 IgG bound to protein A. The degree of depletion, as monitored by immunoblot analysis, was essentially complete, whereas the majority of CSP41 remained in the preimmune IgG-depleted binding fraction, as shown in Figure 6 in the lower immunoblot. The depleted binding fractions and controls were examined for their stem–loop binding activities by GMS. The results shown at the top in Figure 6 indicate that the depletion of CSP41 dramatically reduced the stem–loop binding activity of the binding fraction (lane A) relative to a control fraction depleted with preimmune IgG (lane P). It is significant that the reduced stem–loop binding activity in the anti-CSP41 IgG-depleted binding fraction can be largely restored by HCSP41 (lane C), although HCSP41 alone interacts with the RNA substrate very weakly in this condition (lane H; also see below). These results strongly suggest that CSP41 is essential for formation of the stem–loop–protein complex on *petD* pre-mRNA 3′ UTR but that other proteins are also involved.

CSP41 Alone Can Bind to the *petD* mRNA 3′ UTR

Of the three protein components of the stem–loop–protein complex formed with *petD* pre-mRNA 3′ UTR, CSP29 and CSP55 have been identified previously as RNPs by UV cross-linking (Stern et al., 1989; Chen and Stern, 1991). However, CSP41 cannot be labeled by UV cross-linking, which raises the question of whether CSP41 is itself an RNP or whether it participates in the complex solely through protein–protein interactions. In the experiment shown in Figure 6 (lane H), we noted that HCSP41 alone formed a complex, but the signal could be seen only by prolonged exposure to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) screen (data not shown). This experiment was therefore repeated using HCSP41 purified by heparin–FPLC and at a higher protein concentration. Two RNA substrates were used: the wild-type sequence and an RNA containing mutations in box II, which is the sequence motif immediately downstream of the stem–loop that is required for complex formation with the binding fraction (Chen et al., 1995). Figure 7 shows that HCSP41 alone forms a complex with the wild-type RNA, although it migrates more slowly than does a complex formed with the

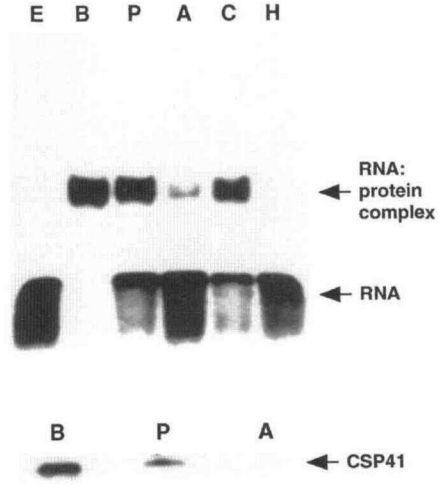

Figure 6. Immunodepletion of Stem–Loop Binding Activity with CSP41 Antiserum.

(Top) Binding fraction proteins (0.8 μg) were treated as follows and examined for stem–loop binding activity by a GMS assay: lane E, no added protein; lane B, 0.8 μg of the binding fraction protein; lane P, depletion by preimmune IgG-insoluble protein A particles; lane A, depletion by anti-CSP41 IgG-insoluble protein A particles; lane C, as given for lane A with the addition of 0.5 μg of bacterially expressed HCSP41; lane H, 0.5 μg of bacterially expressed HCSP41.

(Bottom) Immunoblot detection of CSP41 in samples contained in lanes B, P, and A by using CSP41 antiserum and horseradish peroxidase–linked secondary antibodies. Relative to the GMS assay, three times as much of each sample was used.

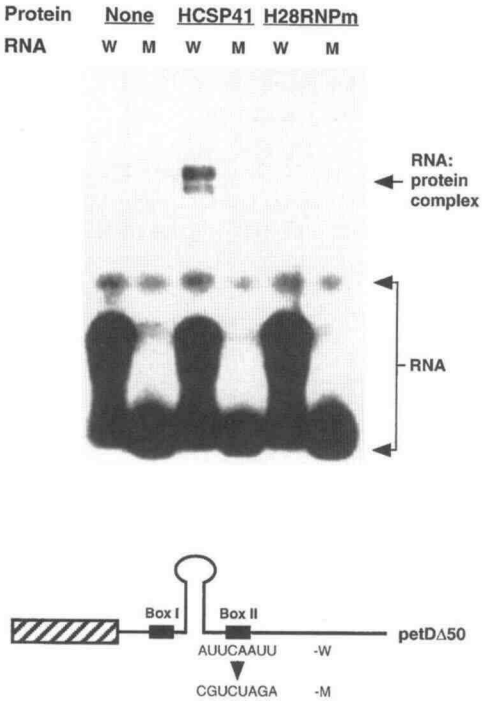

Figure 7. CSP41 Binds Specifically to the *petD* 3′ UTR.

(Top) A GMS assay was performed with 1 μg of bacterially expressed HCSP41 and ^{32}P-labeled wild-type (W) or BIIa mutant (M) petDΔ50 RNA. Assays were also performed without protein and with 1 μg of bacterially expressed H28RNPm. Both HCSP41 and H28RNPm were purified in the final step by heparin–FPLC.

(Bottom) Box II sequences in wild-type (-W) and BIIa (-M) petDΔ50 RNAs. Because two guanosines were introduced into the mutant, unbound BIIa mutant RNAs were cleaved into fragments smaller than those of unbound wild-type RNAs by RNase T1 in the GMS assay. The diagonally striped box indicates the C terminus of the *petD* coding region included in petDΔ50 RNA.

binding fraction (see below). Complex formation did not occur when RNA with a mutated box II was used, suggesting that this binding has the same sequence specificity as native chloroplast proteins. The acidic domain of spinach chloroplast 28RNP with an N-terminal histidine-tag (H28RNPm), which does not bind RNA (Lisitsky et al., 1994), was expressed and purified at the same time as HCSP41. This protein did not bind to either transcript, demonstrating that the binding activity of HCSP41 did not result from the histidine tag or from bacterial contamination. Because complex formation requires the downstream AU-rich element, whether it contains only CSP41 or all three components, we believe that CSP41 alone can bind to the *petD* 3′ UTR by interacting with the stem–loop structure and the downstream AU-rich motif.

To better understand the difference between the slower migrating complex containing only CSP41 and the faster migrating complex containing the three CSPs, a reconstitution experiment was performed using the homogeneous HCSP41 preparation and a CSP55-enriched protein fraction. The CSP55-enriched fraction was one of the fractions obtained by heparin–FPLC during preparation of the 41-kD–enriched fraction. This fraction contains abundant CSP55 but a very limited amount of CSP41 (data not shown). Figure 8 shows that the CSP55-enriched fraction alone forms a complex migrating at the same position as the complex containing all three proteins (lane B), but the signal is very weak, presumably due to the low level of CSP41. In contrast, HCSP41 alone forms a slower migrating complex (Figure 8, fourth lane from the left). However, when the CSP55-enriched fraction and HCSP41 were combined, the signal for the faster migrating complex became much stronger and that of the slower migrating complex nearly disappeared. The H28RNPm protein was used as a negative control and did not possess or enhance binding activities.

We conclude that although CSP41 alone can bind to the *petD* mRNA 3′ UTR, its interaction with other proteins, such as CSP55, results in the formation of a different and more stable complex. Whether the slower migrating complexes contain one or more copies of CSP41 is not clear, although the doublet seen in Figures 7 and 8 is consistent with complexes containing a different number of CSP41 subunits.

CSP41 Is an Endoribonuclease

While purifying CSP41 protein as a stem–loop binding protein, we also started identifying and purifying chloroplast

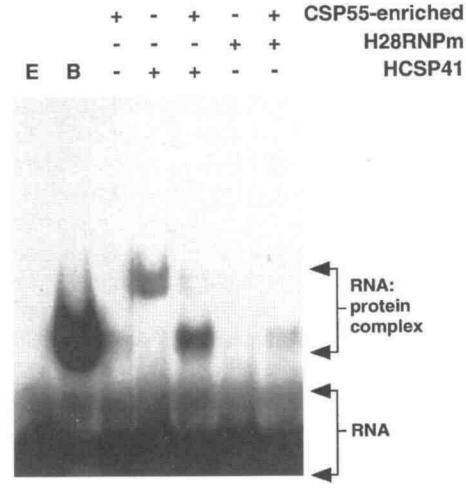

Figure 8. CSP41 Binding Is Stimulated by CSP55.

One microgram of bacterially expressed HCSP41 or H28RNPm was combined with 0.5 μg of the CSP55-enriched fraction as indicated, and RNA binding activity was examined by a GMS assay. Buffer E containing no protein (lane E) or 2.0 μg of the binding fraction (lane B) was used as a control. Both HCSP41 and H28RNPm proteins were purified as given in the legend to Figure 7. (+), presence; (–), absence.

ribonucleases, which may be involved in processing and/or degradation of chloroplast RNA. In this approach, a protein fraction highly enriched in ribonuclease activity was obtained by using a purification procedure similar to that for CSP41. Indeed, the staining patterns of two-dimensional gels of the most highly purified fractions were similar, containing two major proteins of ~41 kD, although the RNase fraction was much reduced for the 36-kD protein and other contaminants (see Figure 1). The two 41-kD proteins of the RNase-enriched fraction were subjected to N-terminal amino acid sequencing, which revealed that these two proteins were apparently identical to the 41-kD GAPDH and CSP41. This result suggested either that one of these proteins comigrated with a third protein with RNase activity and a blocked N terminus or that CSP41 itself was the ribonuclease (GAPDH does not possess ribonuclease activity).

To address the question of whether CSP41 is a ribonuclease, we relied on bacterially expressed and highly purified CSP41 to avoid possible contamination from minor chloroplast proteins. Heparin–FPLC–purified HCSP41 was renatured in buffer E containing 0.02 mM oxidized and 2 mM reduced glutathione, instead of buffer E containing 2 mM DTT. H28RNPm protein was purified and renatured under the same conditions as a control. As shown in Figure 9A, when incubated with a ^{32}P-labeled petDΔ50 transcript, HCSP41 degraded most of the RNA into a range of smaller fragments within 15 min. In contrast, the H28RNPm preparation did not display any RNA degradation activity. The accumulation of multiple degradation products suggested that CSP41 is an endoribonuclease, because a continuous ladder of products is expected from an exonuclease. To confirm this hypothesis, we employed polyethylenimine cellulose thin-layer chromatography to analyze the RNA degradation products generated by HCSP41. Figure 9B (gel at left) shows that these products do not migrate on the polyethylenimine–cellulose sheet, indicating that HCSP41 releases only truncated RNA fragments or oligomers but not free nucleotides. In contrast, PNPase, a well-documented 3′ exonuclease, generates ^{32}P-UDP, which migrates to a higher position (Figure 9B, gel at right). We conclude that CSP41 is an endoribonuclease that is highly active and nonspecific under our reaction conditions.

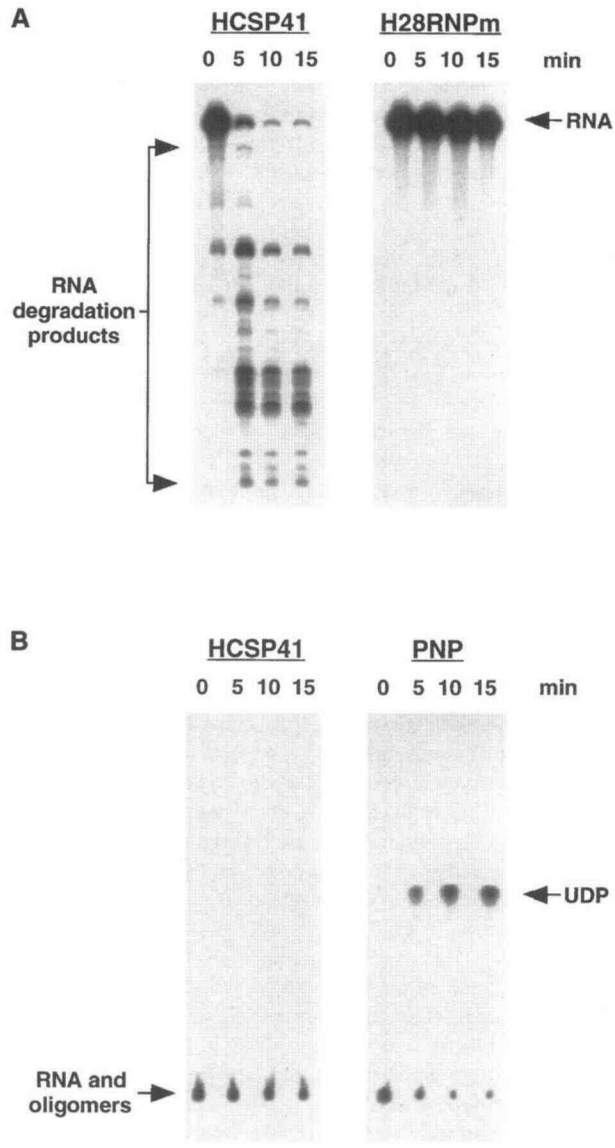

Figure 9. CSP41 Is an Endoribonuclease.

(A) RNA degradation activity of HCSP41. Bacterially expressed HCSP41 (at left) or H28RNPm (at right) (0.1 μg) was reacted with 20 fmol of ^{32}P-labeled petDΔ50 RNA, as described in Methods. At the times indicated, one-sixth of each reaction was stopped and analyzed in a 6% sequencing gel. For this assay, purified bacterially expressed proteins were denatured and refolded in buffer E containing glutathione.
(B) Identification of RNA degradation products by polyethylenimine–cellulose thin-layer chromatography. An RNase assay was performed as given in **(A)**. Five percent of this reaction was stopped at the times indicated and analyzed by thin-layer chromatography, as described in Methods. Bacterial polynucleotide phosphorylase (1.5 units) was used in a similar assay to yield the products of exonucleolytic degradation.

DISCUSSION

Previously, we described a stable stem–loop–protein complex formed in vitro between the 3′ UTR of spinach chloroplast *petD* pre-mRNA and spinach chloroplast proteins, and we have shown that the complex contains CSP55, CSP41, and CSP29 (Chen et al., 1995). Because the 3′ stem–loop structure plays an important role in *petD* pre-mRNA processing and stabilization, we have speculated that these proteins could regulate or be essential for the functions of the 3′ stem–loop structure. Here, we report the cloning of the nuclear *csp41* gene, which encodes the chloroplast CSP41 protein. We demonstrate, by

using bacterially expressed CSP41, that this protein binds in a sequence-specific manner to the stem–loop region and use immunodepletion to show that CSP41 is essential for complex formation. In addition, we find unexpectedly that CSP41 has a strong endonuclease activity. These apparently antagonistic activities raise interesting possibilities for a CSP41 regulatory function.

A Sequence-Specific Chloroplast RNP

Chloroplast RNPs have been purified from several plant species, and some of their cognate genes have been cloned using classical or PCR-based approaches. Although most of these proteins contain canonical RNA recognition motifs and prefer G- or U-rich sequences over A- or C-rich ones, their binding activities are largely nonspecific (Li and Sugiura, 1991; Ye and Sugiura, 1992; Subbaiah and Tewari, 1993; Hirose et al., 1994; Lisitsky et al., 1995). One exception is a 54-kD mustard chloroplast protein that preferentially binds to a U-rich sequence downstream of the *trnK* gene (Nickelsen and Link, 1989, 1993). In contrast, CSP41 shows no significant similarity to known RNPs and does not contain RNA recognition motifs. In addition, it cannot be labeled by UV cross-linking and is normally one component of a three-component complex. Although these features might suggest that CSP41 interacts indirectly with *petD* pre-mRNA via protein–protein interactions, we show that CSP41 alone is capable of binding the *petD* stem–loop region and indeed that this binding requires the wild-type sequence in the AU-rich box II element. This specificity is the same as that of the three-component complex and suggests that direct interactions between CSP41 and *petD* RNA are required for complex formation to occur. The presence of CSP55, however, appears to stabilize greatly this interaction.

The sequence-specific binding of CSP41 is consistent with a regulatory function. For example, the sequence-specific binding of the iron-responsive element binding protein to ferritin and transferrin receptor mRNAs regulates their translation and stability, respectively (reviewed in Klausner et al., 1993). Similar interactions occur during cell cycle–regulated histone mRNA processing (reviewed in Marzluff, 1992). In vitro, we have found that *petD* pre-mRNA containing mutations in box II is processed less efficiently than is its wild-type counterpart (Q. Chen, J. Yang, and D.B. Stern, unpublished data). This implies a role for the stem–loop–protein complex, and hence CSP41, in *petD* pre-mRNA processing.

Nonspecific Endonuclease Activity of CSP41

We found unexpectedly that CSP41 has RNase in addition to RNA binding activity. This was most likely overlooked during our RNA binding assays because of differences in cation optima (see below) and because of the presence of yeast tRNA as a nonspecific competitor. Under conditions optimized for its RNase activity, CSP41 had little substrate specificity. CSP41 degraded wild-type and box II mutant *petD* pre-mRNAs with similar kinetics and also rapidly degraded other chloroplast mRNAs (data not shown). Although CSP41 does not have overall similarity to any known nuclease, a region from positions 127 to 176 shows 42% similarity to a fragment of *Penicillium citrinum* nuclease P1, an endonuclease cleaving both RNA and single-stranded DNA. This region includes the proposed active site, which is Pro-Pro-His in CSP41 and Pro-Leu-His in nuclease P1 (Maekawa et al., 1991).

We found that in vitro, the RNA binding and endoribonuclease activities of CSP41 have different reaction optima. First, we observed that ATP enhances formation of the stem–loop–protein complex but not RNase activity (J. Yang and D.B. Stern, unpublished data). Second, we noted during purification of CSP41 that RNA binding activity could be monitored in up to 50 mM $MgCl_2$ or 200 mM KCl, whereas RNase activity could not be detected under these conditions. Third, bacterially expressed HCSP41, if refolded in buffer E containing 2 mM DTT, has RNA binding activity but not RNase activity (Figure 7 and data not shown). Strong endoribonuclease activity could be recovered only when the HCSP41 protein was refolded in buffer E containing a weaker reducing agent, glutathione (Figure 9). Together, these observations imply that the two biochemical activities of CSP41 may be regulated by several mechanisms, including phosphorylation status, cation concentration, and redox potential. These factors may regulate CSP41 activities directly, or activities may be regulated indirectly by its recruitment into or release from the multiprotein complex.

Because ribonucleases necessarily interact with RNA to perform their enzymatic functions, a nonspecific RNA binding activity would be expected of CSP41. However, protein fractions containing CSP41, such as our chloroplast soluble protein extract, contain highly specific RNA binding and processing rather than nonspecific degradative activities (Stern and Gruissem, 1989; Chen and Stern, 1991; Chen et al., 1995). This combination of nonspecific RNase and specific RNA binding can be compared with that of a complex of *E. coli* proteins that includes RNase E and PNPase, which binds stably to stem–loop containing RNAs (Py et al., 1994), as well as that of the 54-kD mustard chloroplast protein mentioned above, which was reported to have nonspecific endonuclease activity (Nickelsen and Link, 1993).

Possible Functions of CSP41

A number of sequence-specific RNPs are involved in various steps of RNA metabolism, especially RNA processing and stabilization (reviewed in Burd and Dreyfuss, 1994). Recently, additional proteins with enzymatic activities have been shown to function at specific RNA sequences by associating with the RNPs. For example, yeast poly(A) binding protein–dependent poly(A) ribonuclease has been found to interact with poly(A) binding protein and function in poly(A) tail shortening (Boeck

et al., 1996). Another example is *E. coli* RNase E and PNPase, which form a complex with other as yet unidentified proteins (Py et al., 1994).

Spinach CSP41 is unusual because its RNA binding activity is sequence specific but its endonuclease activity has little substrate specificity. These and other data suggest three major possibilities for CSP41 function. A conservative model for CSP41 function is that only the 3′ stem–loop region of *petD* pre-mRNA is a target of CSP41 RNase, due to its highly specific RNA binding activity in association with CSP55 and CSP29. This suggests a role in *petD* pre-mRNA processing, because box II is removed during *petD* mRNA maturation. This might be analogous to RNase E, which controls 3′ processing of *rpsO* pre-mRNA by cleaving at an RNA sequence downstream of its 3′ stem–loop structure (Regnier and Hajnsdorf, 1991), which leads to maturation by RNase II or PNPase (Hajnsdorf et al., 1994). Similar two-step processing models have been suggested for spinach chloroplast *petD* pre-mRNA (Hayes et al., 1996) and Chlamydomonas chloroplast *atpB* mRNA (Stern and Kindle, 1993).

A second possibility is that in addition to a specific role in *petD* pre-mRNA processing, CSP41 has a nonspecific degradation activity. For example, CSP41 could participate in net 5′ to 3′ endonucleolytic degradation of mRNA in chloroplast in a pathway similar to that demonstrated for *ompA*, *lacZ*, and other bacterial mRNAs (reviewed in Petersen, 1992). In support of this hypothesis are the facts that CSP41 RNase activity is nonspecific in vitro (Figure 9) and that a majority of CSP41 purifies on gel filtration columns as an apparent monomer (data not shown). Although the degradation pathways of chloroplast mRNAs are largely obscure, molecular genetic evidence has shown that in the case of Chlamydomonas chloroplast *psbD* mRNA, critical RNA stability determinants lie in the 5′ UTR (Nickelsen et al., 1994).

Finally, it is possible that complex formation itself regulates CSP41 function. In this model, association with CSP55 and CSP29 would inactivate CSP41 as an RNase, and this complex would effectively protect *petD* pre-mRNA from degradation by processing enzymes, perhaps the 67- and 100-kD spinach chloroplast proteins proposed to have these functions (Hayes et al., 1996). When not engaged in complex function, CSP41 might be involved in bulk RNA degradation, as described above. This process would repress CSP41 RNase activity by sequestration in a multiprotein complex in a manner analogous to that of, for example, plant hormones, which can be stored as inactive conjugates (Brzobohaty et al., 1993), or transcription factors, such as c-Jun, which can function as activators or repressors in different types of heterodimers (De Cesare et al., 1995). Placed in developmental context of CSP41 expression (Figure 4), this third scenario is perhaps the most appealing. Both chloroplast RNA synthesis and degradation occur at low levels in dark-grown plants. When illuminated, there is a burst of transcription (Deng and Gruissem, 1987), upon which both *petD* mRNA processing factors (CSP41 binding activity) and general enzymes in RNA metabolism (CSP41 RNase activity) would be required.

METHODS

Protein Purification and Sequencing

Chloroplast protein extracts were prepared from leaves of hydroponically grown spinach, as described by Gruissem et al. (1986). To prepare the chloroplast stem–loop binding protein 41 (CSP41)–enriched fraction, 10 mg of the extract was loaded onto a 1-mL HiTrap heparin–fast protein liquid chromatography (FPLC) cartridge (Pharmacia Biotechnology) at 2 mL/min. After being washed with buffer E (buffer E is 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 17% glycerol, 20 mM Hepes), proteins were eluted with a 30-mL linear gradient of buffer E containing 0 to 500 mM KCl at the same rate and collected into 1-mL fractions. Stem–loop binding activity was monitored by gel mobility shift (GMS) assays, and the protein profile was examined by SDS-PAGE. The half-peak stem–loop binding fractions eluting in higher salt were combined as a CSP41-enriched protein fraction and dialyzed against buffer E. To prepare the binding fraction, the same procedure was performed, except proteins loaded onto the heparin cartridge were eluted with buffer E containing 1 M KCl, and all the peak protein fractions were combined.

Proteins of the CSP41-enriched fraction were further separated by mini two-dimensional PAGE (Bio-Rad), according to the manufacturer's instructions. Briefly, isoelectric-focusing gel rods contained 2% ampholytes (20% Bio-Lyte 3/10 and 80% Bio-Lyte 5/7; Bio-Rad), and CSP41-enriched proteins were fractionated for 5 hr at 750 V. The second dimension was in 12% SDS-PAGE for 1.5 hr at 120 V. The separated proteins were transferred to an Immobilon-P (polyvinylidene difluoride) membrane (Millipore Corp., Bedford, MA) in CAPS buffer (CAPS buffer is 10 mM CAPS [3-cycloherylamino-1-propanesulfonic acid, pH 11], 10% methanol) for 30 min at 90 V and visualized by staining in 0.25% Coomassie Brilliant Blue R in 40% methanol and destaining in 50% methanol. Proteins A and B shown in Figure 1B were excised from the membrane, and their N-terminal sequences were determined by using an ABI 476A sequencer (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) at the Center for Protein Analysis at Technion University (Haifa, Israel). To obtain internal peptide sequences of protein B, the separated proteins were directly visualized in the gel with 0.2% Coomassie blue in 50% methanol/10% acetic acid. Protein B was subjected to in-gel tryptic digestion, and peptides were separated by HPLC and sequenced (Rosenfeld et al., 1992).

Ribonuclease activity was monitored by measuring the degradation rate (disappearance of precursor) of *psbA* pre-mRNA (Stern and Gruissem, 1987), with one unit defined as the activity required to degrade 0.125 fmol RNA in 60 min at 25°C. The activity was 30 units/mg chloroplast protein in the total soluble protein extract and 5000 units/mg protein in the heparin column peak fractions. The enzyme had a pH optimum of 7.0 and a temperature optimum of 37°C. Other properties of the RNase activity are reported in the Discussion.

Gene Cloning and DNA Analysis

Two oligonucleotide primers were synthesized. Primer JYBIN(+), CCHGGIWSHGGIATGCA (where H stands for A, C, and T; I for inosine; W for A and T; S for C and G), was based on the peptide sequence PGSGMQ. Primer T7BS has the sequence GTAATACGACTCACTATAGGGC and is complementary to the T7 promoter region on the right arm of the phage vector λ Zap II (Stratagene). A *csp41* gene-specific fragment was amplified from a spinach leaf λ Zap II cDNA

library (3.5×10^8 plaque-forming units per mL; Schuster and Gruissem, 1991) by polymerase chain reaction (PCR). The 25-μL reaction mixture contained 2 μM primer JYBIN(+), 0.2 μM primer T7BS, 2 μL of the cDNA library suspension, 2 mM $MgCl_2$, 2.5 μM each of the deoxynucleotide triphosphates, and 2.5 μL of 10 × Taq DNA polymerase buffer provided by the manufacturer (Promega) and was denatured for 5 min at 98°C before adding one unit of Taq DNA polymerase (Promega). Amplification was performed for 40 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. The PCR products were ligated into the TA cloning vector (Invitrogen, San Diego, CA) and sequenced by dideoxynucleotide chain termination.

A 585-bp cDNA fragment was identified. It contained nucleotide sequences for primer JYBIN(+) and the internal peptide LHYEP of CSP41. This fragment was labeled by random priming (Feinberg and Vogelstein, 1983) and used to rescreen the cDNA library with a final wash at 65°C in 0.1 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% SDS. Fifteen positive phage plaques were isolated from the library and excised into pBluescript SK− plasmids harboring the cDNA inserts in *Escherichia coli* strain XL1-Blue, according to the protocol provided by Stratagene. The plasmid containing the largest cDNA insert was completely sequenced on both strands by a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA) in the DNA analysis facility of Cornell University (Ithaca, NY). Homology searches were performed using BLAST service (National Center for Biotechnology Information, Bethesda, MD) and the MOTIFS program of the Genetics Computer Group (Madison, WI) software package.

For DNA filter hybridizations, the labeled 585-bp cDNA fragment was also used as probe. Spinach total DNA was prepared by the cetyltrimethylammonium bromide method (Murray and Thompson, 1980) and digested with BamHI or EcoRI. The digested DNA samples were subjected to filter hybridization, as described by Stern et al. (1991).

Fusion Protein Expression and Purification

The *csp41* cDNA in pBluescript SK− was digested with ScaII and XhoI, liberating a 1152-bp fragment precisely, including the entire mature CSP41 coding region and the 3′ untranslated region (UTR). This fragment was then subcloned into the SmaI and XhoI sites of pBluescript SK+ to generate suitable restriction sites. The *csp41* fragment was liberated from pBluescript SK+ with BamHI and KpnI and inserted into the same sites of the pQE30 expression vector (QIAGEN, Chatsworth, CA). The resulting pQE30-*csp41* plasmid was expected to express a CSP41 fusion protein histidine-tagged CSP41(HCSP41) with six histidines and three other amino acid residues at the N terminus. *E. coli* SG13009[pREP4] was transformed by the pQE30-*csp41* plasmid and grown to an OD_{600} of 1.0 in 2 × YT broth (1 L contains 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl) at 37°C. Expression of HCSP41 was induced by 2 mM isopropyl β-D-thiogalactopyranoside for 3 hr at 37°C. HCSP41 was purified on a Ni–nitrilotriacetate column by using the denatured purification method provided by QIAGEN, renatured, and refolded initially against buffer E containing 8 M urea. During the next 72 hr, the urea concentration was progressively reduced to zero. The resulting HCSP41 preparation was further purified on a HiTrap heparin–FPLC cartridge (Pharmacia Biotechnology) by the same method as that used for the binding fraction described above. Homogeneous HCSP41 was dialyzed against buffer E and used for RNA binding assays. To recover its RNase activity, homogeneous HCSP41 was denatured by adding urea to a final concentration of 8 M and renatured as described above, except that 0.02 mM oxidized and 2 mM reduced types of glutathione were substituted for 2 M DTT.

Antibody Preparation and Protein Analysis

To raise antibodies, 150 μg of bacterially expressed HCSP41 protein purified on the Ni–nitrilotriacetate column was injected into a rabbit, followed by two boosters of 150 μg each. The crude antiserum specifically recognized CSP41 in spinach leaf total protein extracts in immunoblot analysis. Total protein extracts were prepared by grinding 0.1 g of plant material in liquid nitrogen, resuspending it in 0.5 mL buffer E, and removing debris by microcentrifugation. For immunoblot analysis, protein samples were fractionated in 12% SDS–polyacrylamide gels and electroblotted to nitrocellulose membranes. The membranes were incubated overnight with a 1:2000 dilution of antiserum at 4°C and then reacted with horseradish peroxidase–linked secondary antibodies for 3 hr at 4°C. The signals were visualized by chemiluminescence (ECL kit; Amersham Corp.). To identify CSP41 in the stem–loop–protein complex, ^{125}I-labeled protein A (Du Pont–New England Nuclear, Boston, MA) was used as a secondary antibody (Figure 5). For immunodepletion of CSP41 from the binding fraction, 200 μL of insoluble protein A (Sigma) was incubated with 100 μL of 50 μg/μL BSA for 30 min, then with 100 μL of antiserum for 30 min at room temperature, and washed with 5 × 300 μL of buffer E. Two hundred microliters of the diluted binding fraction (80 ng/μL) was incubated with the IgG-bound protein A particles for 30 min on ice and clarified by microcentrifugation. As a control, the same experiment was performed using preimmune serum instead of antiserum.

Biochemical Assays

The petDΔ50 transcript includes 84 nucleotides of the 3′ end of the *petD* coding region and 107 nucleotides of the 3′ UTR, encompassing the stem–loop structure and its upstream and downstream AU-rich elements (box I and box II). Wild-type and box II mutant (BIIa) of petDΔ50 transcripts were synthesized and labeled with α-^{32}P-UTP, as described by Chen et al. (1995).

For a standard GMS assay, the 20-μL reaction mixture contained the protein sample, 40 ng yeast tRNA, 5 fmol ^{32}P-labeled RNA, 40 mM KCl, 10 mM $MgCl_2$, 0.05 mM EDTA, 3 mM DTT, 8.5% glycerol, and 10 mM Hepes, pH 7.9. The reaction was incubated for 10 min at room temperature, treated with 40 units of RNase T1 for 15 min at 37°C, and electrophoresed in a 5% Tris–glycine native polyacrylamide gel for 2 hr. To identify CSP41 in the stem–loop–protein complex, a large-scale reaction was performed (Figure 5). For this purpose, 1 pmol of trace-labeled petDΔ50 RNA and 125 μg protein from the binding fraction were used in a 20-μL reaction. The reaction was treated with 1000 units of RNase T1 before loading in the gel. The wet gel was exposed to x-ray film to locate the complex.

To measure ribonuclease activity, the 30-μL reaction contained the same components as the GMS reaction, except that 20 fmol of ^{32}P-labeled RNA was used and yeast tRNA was omitted. Reactions were performed at 25°C and analyzed in 6% sequencing gels, as described by Stern and Gruissem (1987). For thin-layer chromatography assays, 1.5-μL aliquots of the RNase assay were stopped in equal volumes of 20 μg/μL proteinase K and spotted on a polyethylenimine–cellulose sheet (Sigma) and chromatographed in 0.25 M KH_2PO_4 (Carpousis et al., 1994).

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