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mRNA decay in spinach chloroplasts: *psbA* mRNA degradation is initiated by endonucleolytic cleavages within the coding region

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ABSTRACT

The expression of chloroplast genes during leaf development in higher plants is regulated on several levels as transcription, RNA processing and stability, protein stability and turnover. Differential mRNA stability is one major component which contributes to the developmentally controlled accumulation of higher plant chloroplast *psbA* mRNA, which encodes the D1 protein of photosystem II. To understand the molecular mechanisms of specific mRNA degradation an *in vitro* mRNA decay system based on lysed chloroplasts from spinach leaves was established. Employing this degradation extract the decay of *psbA* mRNA was analyzed. Half-life of the *psbA* mRNA *in vitro* is dependent on the degradation conditions as the presence of Mg^{2+}, which was found to stabilize the mRNA. Addition of tRNA stabilizes the mRNA and allows the accumulation of distinct degradation intermediates. *psbA* mRNA derived fragments of the same size were detected in degradation experiments *in vitro*, *in organello* and *in vivo*. 5′ ends of the degradation intermediates were identified by primer extension and found to be localized in the 5′ part of the coding region. The data indicate a degradation mechanism involving initiation of *psbA* mRNA decay by specific endonucleolytic cleavage and subsequent exonucleolytic degradation of the fragments. Possible models for cleavage site recognition are discussed.

INTRODUCTION

During spinach leaf development the morphology and physiology of chloroplasts drastically changes as photosynthetic thylacoid membranes are rapidly synthesized and assembled. This process is characterized by the coordinated expression of plastid encoded genes involved in gaining the full photosynthetic capacity of the plastid. Gene expression in the developing chloroplast is regulated on several levels as transcription, RNA processing and stability, translation, protein stability and turn-over (1–5).

The levels of many plastid mRNAs vary during leaf development and plastid differentiation. RNA processing and differential stability are the factors which contribute to developmental mRNA accumulation (6–9; for review see 10). Several elements of chloroplast mRNAs have been discussed as being *cis*-regulating for differential mRNA decay. Mono- and poly-cistronic protein coding transcription units in the plastid genome carry inverted repeat sequences at their 3′ non-translated regions that are capable of forming hairpin secondary structures. These structural elements are processing signals for 3′ end formation *in vitro* (11,12). They are also required for the stabilization of 5′ mRNA regions, because deletions or point mutations that alter the structure of the inverted repeats also affect the stability of the 5′ located RNA segment *in vitro* (12,13) and *in vivo* (14). It is unknown, however, whether the inverted repeats within the 3′ non-translated regions also function as *cis*-regulatory elements for differential mRNA stability or whether their function is the general stabilization of mRNAs against exonucleolytic attack. Besides in higher plants little is known about *cis*- and *trans*-regulating elements for plastid mRNA degradation. One strategy to identify those elements is the elucidation of mRNA degradation pathways, the initiating cleavage sites and the degradation direction. These informations direct at putative *cis*-regulating elements, which may be involved in the control of initiating mRNA decay.

In contrast in green algae *cis*-regulatory elements for plastid mRNA decay have already been identified. In *Chlamydomonas reinhardii* the 5′ untranslated region of several mRNAs has been assigned *cis*-regulatory functions in controlling mRNA stability. A fusion between the ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) 5′ untranslated region and the reporter gene GUS causes light-induced destabilization of the chimeric mRNA (15). The destabilization can be reversed by adding sequences of the *rbcL* coding region to the construct. Mutagenesis of the 5′ untranslated region of the cytochrome b_6/f complex subunit IV (*petD*) mRNA affects stability as well as translatability (16). The 5′ untranslated region of the mRNA encoding the D2 protein (*psbD*) acts as a target site for mRNA degradation in a mutant of *Chlamydomonas* which lacks the capability to accumulate *psbD* mRNA (17).

In bacterial systems as well as in a few mammalian and plant systems mRNA degradation pathways have been examined by analysis of degradation intermediates. In *Escherichia coli* the mRNA best elucidated is the *ompA* mRNA encoding the outer membrane protein A. The finding of degradation intermediates with their 5′ ends mapping within the 5′ untranslated region resulted in a model of the rate limiting endonucleolytic cleavage within the 5′ untranslated region being followed by rapid exonucleolytic degradation (18). Similar observations have been made for the bacterial thioredoxin mRNA (19).

Table 1. Oligonucleotides used to probe polyacrylamide RNA gel blots and for primer extension analysis

Oligonucleotide	Nucleotide no.			Oligonucleotide sequence
primer 1	–77	to	–55	5′-TCGCTAGAAATAGAAATTGAAG-3′
primer 2	18	to	37	5′-GGCTTTGCGTTTCGCGTCTC-3′
primer 3	111	to	131	5′-GCAGTCAATAAGGTAGGGATC-3′
primer 4	423	to	448	5′-AGCGGAATATGCAACAGCAATCCAA-3′
primer 5	1089	to	1110	5′-GCCCCTTACTTTCACTAACTC-3′

The nucleotide numbers of the *psbA* mRNA the primers are complementary to are given in the second column. They are numbered in respect to the translation start site which is assigned +1.

To study pathways of mRNA degradation *in vitro* degradation systems have been established as useful tools for several organisms. For mammalian cultured cells as well as in yeast a system based on polysomal fractions was used (20,21). A similar approach has been employed to examine mRNA degradation in plants. The polysome based degradation system was successfully applied in petunia, soybean and oat for mRNA degradation studies (22,23). In *Chlamydomonas reinhardii* chloroplast extracts were used to observe degradation of radiolabeled *in vitro* transcripts of *psbD* leader RNA (17).

In this work an *in vitro* degradation system for analysis of chloroplast mRNA decay in higher plants is presented. It is based on lysed chloroplasts from spinach. This kind of extract was used to ensure that all soluble factors engaged in specific mRNA decay are present. Degradation of the internal *psbA* mRNA, i.e. the mature full length transcript in complex with proteins as *in situ*, is observed. The experiments are designed to analyze the degradation intermediates that accumulate during decay of *psbA* mRNA. Usage and positions of cleavage sites are determined by high resolution Northern and primer extension analysis. The data show that *psbA* mRNA degradation is initiated by cleavages within the 5′ part of the coding region of the message and subsequent decay is facilitated by exonucleolytic activities.

The understanding of the molecular mechanisms of specific mRNA degradation will be the basis for identifying the *cis*- and *trans*-regulatory components involved in the control of mRNA stability.

MATERIALS AND METHODS

Plant material

Spinach plants (*Spinacea oleracea*) were grown on soil in the greenhouse with additional illumination during wintertime to result in 12 h light per day.

Nucleic acids

tRNA was purchased from Boehringer (Mannheim, Germany). The primers used for Northern analysis and for primer extension are summarized in Table 1. Primers were radioactively labeled according to (24).

Probes for Northern analysis. For the *psbA*-specific RNA probe, the plasmid pBluescript KS+ (Stratagene Inc.) containing the *PstI–XbaI* fragment of the *psbA* gene (25) is linearized with *XbaI* as outlined by the manufacturer. A standard transcription reaction of 50 µl contained 20 µg/ml linear DNA, 1 mM DTT, 0.5 mM ATP, GTP and CTP, 0.05 mM UTP, 50 µCi [α-^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml; Amersham), 10 U T7-polymerase (Boehringer Mannheim, Germany), and buffer supplied by the manufacturer. After incubation for 1 h at 37°C, 0.5 vol 7.5 M NH_4OAc and 3 vol ethanol were added. After pelleting the probes were used without further purification.

Preparation of chloroplasts and *in vitro* degradation extracts

Intact chloroplasts were isolated according to (26). For preparation of *in vitro* degradation extracts chloroplasts were resuspended in 20 mM *N*-2-hydroxylethylpiperazine-*N*′-2′ethane sulfonic acid (HEPES), pH 7.9, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 20% glycerol and lysed by 10–15 strokes using a potter with pestle S. For measuring mRNA decay without Mg^{2+}, $MgCl_2$ was omitted from the lysis buffer. Extracts were adjusted to ~5 mg/ml protein as determined by Bradford assay. The extracts could be stored at –70°C after freezing in liquid nitrogen.

In vitro degradation assay

In vitro degradation experiments were performed with 100 µl of degradation extract (5 mg/ml protein) per time point. The extract was thawed on ice, tRNA was added as outlined in Results and the mixture was transferred to 25°C for incubation. Reactions were stopped by adding 50 µl 6.0 M urea, 4.5 mM aurintricarboxylic acid (ATA), 1.0% SDS and 200 µl phenol/chloroform (1:1). ATA was omitted in degradation experiments which were to be analyzed by primer extension. After phenol/chloroform extraction and a subsequent chloroform extraction, nucleic acids were recovered by ethanol precipitation.

In vivo degradation assay

Experiments analyzing plastid mRNA decay *in vivo* were performed as published (8).

Preparation of crude total polysomes

To isolate polysomes from leaf tissue (27,28) 5 g leaf material was ground in liquid nitrogen using mortar and pestle. After addition of 20 ml extraction buffer (0.2 M Tris–HCl, pH 9.0, 0.2 M KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M sucrose, 1% Triton X-100, 2% polyoxyethylene-10-tridecyl ether, 0.5 mg/ml heparin, 100 mM β-mercaptoethanol, 100 µg/ml chloramphenicol) the mixture was ground until thawed. After a centrifugation of 10 000 r.p.m. for 10 min (Beckman J2–21 centrifuge, rotor: JA20.1) to sediment the debris, the supernatant was filtered through a glass wool plug to clear it from the remaining tissue pieces. Sodium-

deoxycholate was added from a 10% stock to a final concentration of 0.5%. After an incubation of 10 min on ice the solution was layered onto a two-step sucrose cushion consisting of 3 ml 1.75 M sucrose and 2 ml 0.5 M sucrose in 40 mM Tris–HCl, pH 9.0, 200 mM KCl, 30 mM $MgCl_2$, 5 mM EGTA, 0.5 mg/ml heparin, 100 µg/ml chloramphenicol. The polysomes were sedimented through the cushion 3 h at 41 000 r.p.m. using a Ti55.2 rotor (Beckman; centrifuge Beckman L8-55). The dark green supernatant was carefully removed and the pellets were dissolved in a minimal volume (usually 400 µl) polysome buffer (40 mM Tris–HCl, pH 9.0; 200 mM KCl, 30 mM $MgCl_2$, 5 mM EGTA).

Northern analysis

For Northern analysis RNAs were separated on 1.0% agarose-formaldehyde gels according to (24). The RNAs were transferred overnight to Hybond N nylon membranes (Amersham) in 5× SSC, pH 7.0 (1× SSC: 0.15 M NaCl, 0.015 M Na-citrate). The membrane was UV treated to covalently couple the RNA (120 mjoule, Stratalinker, Stratagene Inc.). All pre-hybridizations were performed for 4 h at 65°C in 6× SSC, pH 7.0, 5× Denhardt's solution, 0.5% SDS and 50 µg/ml denatured salmon sperm DNA (1× Denhardt's solution: 0.02% BSA, 0.02% ficoll 400, 0.02% polyvinylpyrolidone 40). Filters were hybridized at 68°C for ≥ 16 h using RNA probes (1×10^8 c.p.m./µg DNA-template). Filters were washed three times for 30 min at 68°C in 0.2× SSC, pH 7.0, 0.1% SDS. Filters were exposed to Kodak X-AR X-ray films.

High resolution Northern analysis

For high resolution Northern analysis RNAs were separated on denaturating 5% polyacrylamide gels containing 8 M urea, 0.5× TBE (10× TBE: 89 mM Tris, 89 mM boric acid, 1 mM EDTA). Gels were pre-run at 50 W for 20 min to allow heating and run at 40 W for 30 min. RNA samples were dissolved in 90% formamide containing 0.05% bromophenol blue, 0.05% xylene cyanole, 1 mM EDTA, heated to 85°C for 5 min and chilled on ice prior to loading onto the gel.

Gels were transferred to Hybond N nylon membrane in 0.5× TBE for 45 min by electroblotting using a Trans-Blot Semi-Dry Transfer Cell (BioRad, München, Germany). The membrane was UV treated to covalently couple the RNA (120 mjoule, Stratalinker, Stratagene Inc.). All pre-hybridizations were performed for 4 h at 50°C in 6× SSC, pH 7.0, 5× Denhardt's solution, 0.5% SDS and 50 µg/ml denatured salmon sperm DNA (1× Denhardt's solution: 0.02% BSA, 0.02% ficoll 400, 0.02% polyvinylpyrrolidone 40). Filters were hybridized at 50°C for ≥ 16 h using oligonucleotide primers (1×10^7 c.p.m./pmol DNA-primer). Filters were washed three times at 50°C in 5× SSC, pH 7.0, 0.1% SDS. Filters were exposed to Kodak X-AR X-ray films.

Primer extension

Primer extension analysis was performed as published (29). To identify positions of the 5′ ends of the primer extension products sequencing reactions using phosphorylated primers were used according to the protocol of the manufacturer (USB, Cleveland, USA).

RNA structure calculation

RNA secondary structure calculations were performed according to (30) based on a modification of the Zuker–Nussinov algorithm. The structure was calculated for a temperature of 40°C and an ionic strength of 1 M.

RESULTS

Degradation of *psbA* mRNA *in vitro*

An *in vitro* degradation extract for spinach chloroplast mRNAs was established in order to analyze the molecular mechanisms of chloroplast mRNA decay. It was used to identify stable degradation intermediates, the position of cleavages and the initial cleavage sites.

The degradation extract consists of lysed chloroplasts. This crude extract was chosen to ensure that all factors necessary for specific degradation are present. Lysed chloroplasts were preferred to experiments *in organello* to be capable of changing decay conditions as the presence of Mg^{2+} or additional tRNA.

The effect of 10 mM Mg^{2+} on mRNA decay *in vitro* was measured for three reasons. In *Chlamydomonas* it could be shown that Mg^{2+} ions are essential for RNA degradation *in vitro* (17). Secondly, polysome association of the mRNA depends on the presence of Mg^{2+} ions. Thirdly, the formation of structural elements, especially elements of tertiary structure, is influenced in stability by divalent cations.

Degradation was observed in the presence and in the absence of 0.5 mg/ml tRNA. The addition of tRNA should inhibit the activity of fast proceeding exonucleases and should therefore allow the accumulation of stable degradation intermediates.

In all experiments 500 µg protein per time point was incubated at 25°C for 1, 10, 30, 60, 120 and 180 min under the conditions outlined. Degradation kinetics of *psbA* mRNA was observed by Northern analysis using a *psbA* specific probe. The results of a series of degradation experiments performed under different conditions are shown in Figure 1. The half-life of the *psbA* mRNA in lysed chloroplasts is <30 min and therefore considerably shorter than observed *in vivo*, where it was estimated to be >8 h. (8). Half-life *in vitro*, however, is significantly dependent on the experimental conditions: in contrast to observations in *Chlamydomonas reinhardii psbA* mRNA is more stable in the presence of 10 mM Mg^{2+} indicating that either polysome assembly or structural elements of the RNA are linked to the degradation process. The addition of tRNA also increases the half-life of *psbA* mRNA *in vitro*. This is due to the inhibition of exonucleases. mRNA from isolated polysomes incubated in buffer remain stable during the whole incubation period indicating that the mRNA degrading activity is not associated with polysomes. The nucleases responsible for mRNA degradation rather have to be a component of the extract and may be either soluble or membrane bound.

Degradation intermediates can be observed in all types of degradation experiments except the polysome control. In experiments without tRNA, however, the degradation intermediates are unstable. Intermediates with a size close to full length are barely detectable. In the presence of tRNA degradation intermediates accumulate during the incubation time. The distribution of their size is shifted to longer RNA molecules indicating that those intermediates are derived by cleavages occurring early in the degradation pathway. The data show that the early steps of the

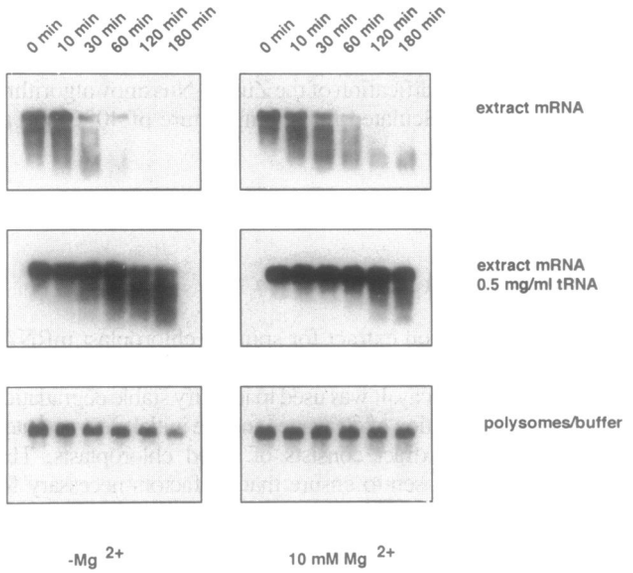

Figure 1. Degradation of the *psb*A mRNA *in vitro*. Degradation of internal *psb*A mRNA was observed in extracts of lysed chloroplasts from mature leaves. 500 µg protein per time point was incubated at 25°C in the presence and in the absence of 0.5 mg/ml tRNA, to analyze the decay of the internal RNA. Degradation was observed by Northern analysis using a *psb*A-specific riboprobe. Decay was measured in the presence and absence of Mg^{2+}.

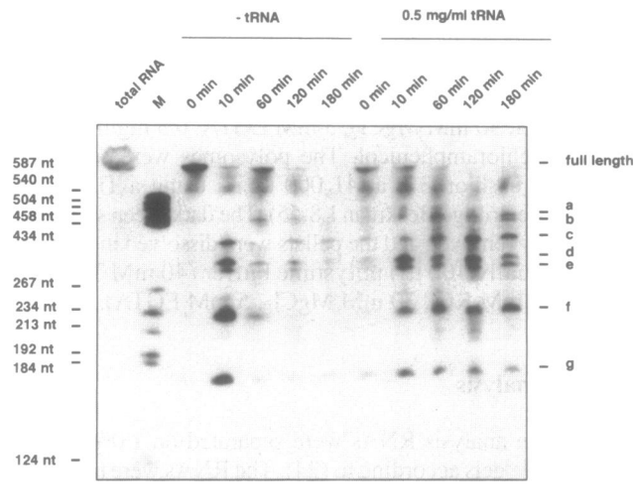

Figure 2. Characterization of stable degradation intermediates of *psb*A decay *in vitro* by high resolution Northern analysis. *In vitro* degradation of internal mRNA was performed using 500 µg protein of extracts of lysed chloroplasts from mature leaves at 25°C in the presence and in the absence of 0.5 mg/ml tRNA and in the presence of 10 mM $MgCl_2$. RNA recovered was analyzed on 5% polyacrylamide gels containing 8 M urea, 0.5× TBE, run at 55°C. The RNA was transferred to nylon membrane by electroblotting and hybridized using a ^{32}P-radiolabeled oligonucleotide complementary to position +16 to +37 of the coding region of *psb*A mRNA. Total RNA, 5 µg total spinach RNA; M, pBR322/*Hae*III.

degradation pathway are endonucleolytic followed by exonucleolytic cleavages.

Analysis of *psbA* mRNA degradation intermediates

In order to analyze *psbA* degradation intermediates in more detail, high resolution Northern analysis was performed. Degradation experiments were performed in the presence of 10 mM $MgCl_2$ and in the presence and absence of tRNA as described above. The RNA was separated on denaturating 5% polyacrylamide gels and transferred to Nylon membrane by electroblotting. Blots were probed with several primers: Figure 2 shows a hybridization experiment using a primer complementary to nucleotide +16 to +37 of the coding region of *psbA* mRNA (primer 2). The degradation intermediates observed in degradation experiments in the absence of tRNA are detectable in the 10 min timepoint, but are subsequently degraded. This result supports the model of one or several endonucleolytic cleavages, which initiate the fast exonucleolytic degradation of the mRNA. In contrast the degradation intermediates observed in the presence of tRNA are persistent during the course of the degradation kinetics. The pattern of intermediates, however, is similar for both types of degradation experiments. Seven major degradation intermediates (labeled a–g) can be detected as well as several bands of minor intensity. Calculation of the size of intermediates in respect to the position of the primer locates the 3′ ends of those degradation products within the 5′ half of the coding region.

In order to show that degradation products yielded by *in vitro* degradation are comparable to those occurring *in vivo*, total RNA from *in vivo* degradation experiments (8) was analyzed by high resolution Northern analysis in comparison to RNA derived from degradation *in vitro* and *in organello*. For *in vivo* degradation mature spinach leaves were watered with 200 µg/ml of actinomycin D to inhibit transcription. Total RNA was isolated after 0, 1, 5 and 9 h of actinomycin D treatment. mRNA decay *in organello* was performed according to degradation *in vitro* but omitting lysis of the intact chloroplasts prior to incubation at 25°C. As depicted in Figure 3, the major degradation products which can be detected *in vitro* comigrate with *psbA* mRNA fragments found *in organello* and *in vivo*. The concentrations of fragments derived *in vivo* compared to the amount of full length *psbA* mRNA are significantly lower as observed *in vitro*. No accumulation of degradation products can be observed *in vivo* during the mRNA decay kinetics. In this experiment only the steady state concentrations and no accumulation of degradation intermediates can be detected because nucleases are active in the intact cell facilitating the degradation of the intermediates. This experiment demonstrates that in respect to size of degradation intermediates *psbA* mRNA degradation *in vitro* faithfully reflects *psbA* mRNA degradation *in vivo*. Therefore it is most likely that the same cleavage sites are used *in vitro* and *in vivo*.

To gain information on the position of the early endonucleolytic cleavages which initiate mRNA decay, hybridization experiments were performed using primers complementary to a sequence located immediately at the 5′ end of the untranslated region of *psbA* mRNA (nucleotide –77 to –55, primer 1) and located within the 3′ untranslated region of the *psbA* mRNA (nucleotide 1089 to 1110, primer 5). The results shown in Figure 4 demonstrate that the 5′ end-located primer detects only the full length *psbA* transcript and one major degradation product of ~160 nt corresponding to fragment g in Figure 2 which accumulates during the course of decay (Fig. 4A). Hybridization with the 3′ end-located primer reveals a whole series of degradation products (Fig. 4B). During the early timepoints of the decay kinetics mostly intermediates of large size can be detected (10, 30, 60 min

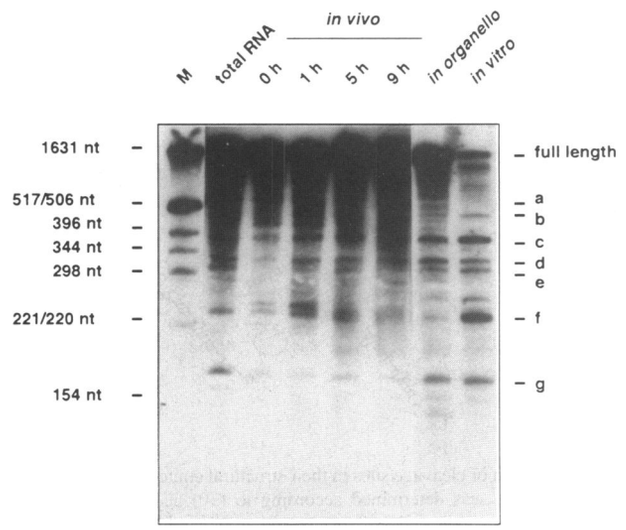

Figure 3. Comparison of degradation intermediates of mRNA decay *in vitro*, *in organello* and *in vivo*. Total spinach RNA (5 µg), RNA of mature spinach leaves treated with actinomycin D for 0, 1, 5 and 9 h (5 µg), and RNA derived after 30 min of degradation *in organello* (2 µg) and *in vitro* (2 µg) in the presence of 0.5 mg/ml tRNA and 10 mM $MgCl_2$ was separated on 5% polyacrylamide gels containing 8 M urea, 0.5× TBE, run at 55°C. The RNA was transferred to nylon membrane by electroblotting and hybridized using a ^{32}P-radiolabeled oligonucleotide complementary to position +16 to +37 of the coding region of *psb*A mRNA. M, pBR322/*Hin*fI. Degradation intermediates are labeled a–g.

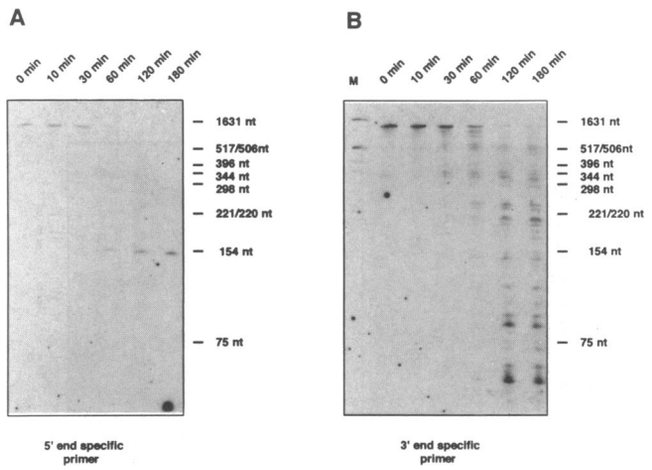

Figure 4. Characterization of stable degradation intermediates of *psb*A decay *in vitro* by high resolution Northern analysis. *In vitro* degradation of internal mRNA was performed using 500 µg protein of extracts of lysed chloroplasts from mature leaves at 25°C in the presence and in the absence of 0.5 mg/ml tRNA and in the presence of 10 mM $MgCl_2$. RNA recovered was analyzed on 5% polyacrylamide gels containing 8 M urea, 0.5× TBE, run at 55°C. The RNA was transferred to nylon membrane by electroblotting and hybridized using the ^{32}P-radiolabeled oligonucleotide 1 complementary to position –77 to –55 (**A**) and oligonucleotide 5 complementary to position +1089 to +1110 (**B**) of the *psb*A mRNA.

timepoint). During longer incubation periods also very short degradation products can be observed.

The degradation intermediates detected by primer 1 are supposed to have an intact 5′ end. Hybridization with primer 1 therefore indicates that a major cleavage site, which is located in the 5′ part of the coding region according to size, is used early in the degradation pathway of the *psbA* mRNA decay. This finding is supported by hybridization data obtained with primer 5. These degradation intermediates have an intact 3′ end. The early degradation intermediates which are of large size again direct at initial cleavage sites which are located in the 5′ part of the *psbA* mRNA. Cleavage sites in the 3′ part of the mRNA are used later during the degradation process. Hybridization with primer 2 directs at several additional cleavage sites, which are all located in the 5′ part of the mRNA, but according to the findings using primer 1 may be used with less preference. Thus, the data obtained using an *in vitro* degradation system for higher plant chloroplast *psbA* mRNA show endonucleolytic cleavage within the coding region of chloroplast *psbA* mRNA which so far has not been observed.

Identification of cleavage sites

High resolution Northern analysis allows only a rough determination of the region where endonucleolytic cleavages are located during *in vitro* degradation. The exact positions of the cleavages were determined by primer extension analysis. Figure 5A and B show the results. Two primers were used to cover that part of the mRNA in which the major cleavages are localized. The positions of the 5′ ends of the degradation intermediates correspond to the length of those identified by high resolution Northern analysis using primer 2. However, several additional cleavage sites can be detected by primer extension, which could not be resolved with the Northern technique. Only one of these is a cleavage site located in the 5′ untranslated region. In Figure 5 those cleavage sites are labeled which can be detected reproducibly in independent degradation experiments.

The positions of the cleavage sites and sequences 6 nucleotides upstream and downstream of them are summarized in Table 2. No obvious sequence motif can be found by comparison of these mRNA elements. The 3′ nucleotide at most of the cleavage sites is A. Exceptions are two intermediates beginning with G or U. The 5′ side nucleotide is preferentially an U except of two intermediates mapping to C.

The structural context of the cleavage sites was analyzed for the major intermediates. RNA secondary structure of the *psbA* mRNA was determined according to (30) at an ionic strength of 1 M and at 40°C, which corresponds to cellular salt conditions at 25°C. As shown in Figure 6 all of the cleavage sites except the one at –48G are located in single stranded regions of the mRNA considering the optimal thermodynamic structure. The finding indicates a single strand specificity of the nuclease involved. Uniform structural signals for cleavage site recognition like defined small hairpins or bulge loops cannot be detected in this structure.

DISCUSSION

In vitro mRNA degradation of chloroplast mRNAs

In this work an *in vitro* degradation system is presented which allows the analysis of spinach chloroplast mRNA degradation. Plastid *psbA* mRNA degradation is observed in extracts of lysed chloroplasts. The mRNA half-life is influenced by the presence of Mg^{2+}, which results in higher stability of the mRNA compared

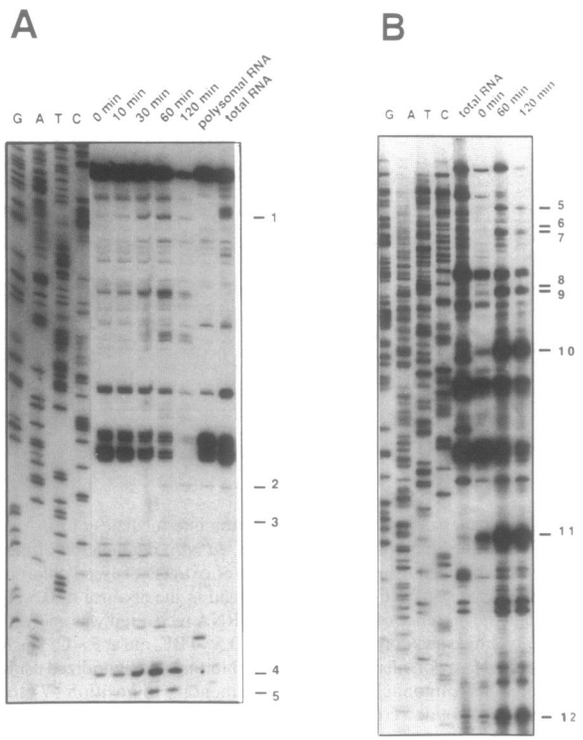

Figure 5. Determination of cleavage sites of the stable degradation intermediates of *psbA* decay *in vitro* by primer extension analysis. *In vitro* degradation of internal mRNA was performed using 500 µg protein of extracts of lysed chloroplasts from mature leaves at 25°C in the presence of 0.5 mg/ml tRNA and 10 mM $MgCl_2$. (**A**) The 5′ position of the degradation intermediates was determined by primer extension analysis using oligonucleotide 3 complementary to position +111 to +131 of the coding region of *psbA* mRNA. G, A, T, C: sequencing reaction using the phosphorylated primer: total RNA: 5 µg of total spinach RNA; polysomal RNA: 5 µg of RNA yielded from crude polysomes by phenol/chloroform extraction. (**B**) The 5′ position of the degradation intermediates was determined by primer extension analysis using oligonucleotide 4 complementary to position +423 to +448 of the coding region of *psbA* mRNA. G, A, T, C: sequencing reaction using the phosphorylated primer; total RNA: 5 µg of total spinach RNA; polysomal RNA: 5 µg of RNA yielded from crude polysomes by phenol/chloroform extraction. Degradation intermediates are labeled #1–12. It has to be noted that many of the major bands correspond to terminations of the reverse transcriptase which are unrelated to cleavage sites but presumably reflect RNA structural elements.

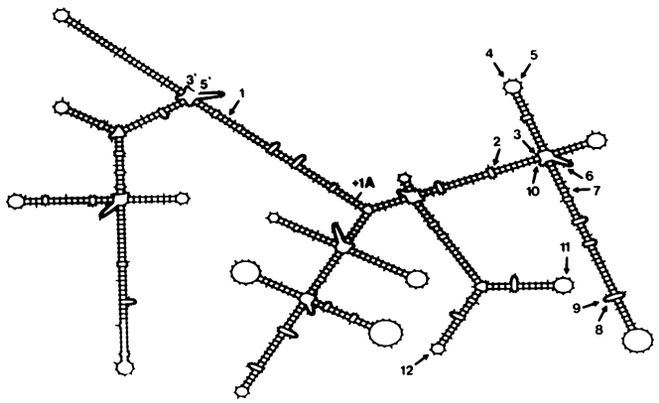

Figure 6. Position of cleavage sites in their structural context. The structure of the *psbA* mRNA was determined according to (30) at 40°C, 1 M NaCl. Cleavage sites are indicated by arrows and numbered according to their identification using primer extension analysis, the translation start site is labeled with +1A.

Table 2. Location and sequence context of 5′ ends of *psbA* mRNA degradation intermediates

Number	Nucleotide	Sequence context
1	–48G	AUUUGU GUGCUU
2	55A	UUCUGU AAUUGG
3	63A	UUCUGU AACCAG
4	86A	GUCUUU ACAUUG
5	88A	CUUUAC AUUGGA
6	145A	GUAUUU AUUAUA
7	157A	GCCUUC AUUGCU
8	229A	AAUAAU AUUAUU
9	232A	AAUAUU AUUUCG
10	280U	CACUUU UAUCCA
11	360A	CUUCUU ACUUGG
12	400A	GAACUU AGUUUC

The positions of 5′ ends of *psbA* mRNA were determined by primer extension. The positions are given in the second column. They are numbered with respect to the translation start site which is assigned +1. The sequence context (5′ to 3′) is shown in column 3. The cleavage is indicated by the gap between the bold letters.

with experiments performed with extracts prepared without Mg^{2+}. The stabilizing effect of Mg^{2+} as it is observed in these experiments is in contrast to findings in *Chlamydomonas* (17), where *in vitro* degradation rates of *psbD* mRNA are considerably diminished when Mg^{2+} is omitted. Whereas in the *Chlamydomonas* system Mg^{2+} ions may therefore act as a cofactor for the ribonuclease, it seems likely that in the spinach system Mg^{2+} ions influence the degradation substrate. Several effects of Mg^{2+} ions on mRNA degradation can be discussed. Mg^{2+} ions are known to stabilize tertiary interactions of RNA structures. If elements of tertiary structure of the *psbA* mRNA were involved in mRNA stabilization they could be destabilized in the absence of Mg^{2+} ions and this would result in a shorter mRNA half-life. On the other hand Mg^{2+} ions are involved in polysome association. If the assembly of *psbA* mRNA into polysomes is involved in mediating specific mRNA stability, disassembly by removal of Mg^{2+} ions may be responsible for the faster degradation of the mRNA.

The addition of tRNA reduces *psbA* mRNA degradation *in vitro*. High concentrations of this RNA inhibit exonucleolytic activities. The finding of longer half-lives indicates that exonucleases are responsible for the fast degradation of *psbA* mRNA. The addition of tRNA furthermore allows the accumulation of degradation intermediates. Small size intermediates also accumulate during *in vitro* degradation in the absence of tRNA, but those detected in the presence of tRNA are of larger sizes and therefore are derived by endonucleolytic cleavages occurring early in the degradation pathway. The data direct at a degradation pathway in which decay is initiated by endonucleolytic cleavages forming specific degradation products which subsequently are degraded by exonucleases.

Usage of cleavage sites

Analysis of degradation intermediates by high resolution Northern and primer extension analysis revealed major degradation products which map to cleavage sites within the coding region. The concentration of degradation fragments as detected by high resolution Northern analysis is variable. There are seven species, which can be found at larger amounts. The comparison of *psbA* mRNA-derived fragments *in vitro*, *in organello* and *in vivo* reveals degradation intermediates of the same size. The concentration of *psbA* mRNA fragments compared to the amount of full length mRNA is considerably lower *in vivo*. Therefore it can be concluded that the same cleavage sites are employed *in vivo* and *in vitro*. *In vitro* additional degradation fragments can be detected. Those are either products of cleavages not occurring *in vivo*, or those intermediates are quickly exonucleolytically degraded *in vivo* and therefore cannot be detected.

The data presented indicate that the early cleavages which may induce *psbA* mRNA degradation are located within the 5′ region of the message followed by further endonucleolytic cleavages within the downstream part of the RNA. The resulting small fragments presumably are then subject to exonucleolytic decay. The importance of exonucleases for degradation of *psbA* mRNA has been discussed above. According to these interpretations the pathway of *psbA* mRNA decay is at least biphasic consisting of the primary and secondary endonucleolytic cleavages in 5′ to 3′ direction followed by exonucleolytic degradation.

Despite the huge differences in half-lives between prokaryotes and chloroplasts similarities in mechanisms of mRNA degradation can be found in both systems. In *E.coli* 5′ non-translated regions are determined to be important for mRNA decay. In several mRNAs degradation initiates by cleavage within those regions being followed by a wave of decay in 5′ to 3′ direction (for review see 31). In *E.coli* an enzyme complex could be purified consisting of RNase E and polynucleotide phosphorylase which is involved the regulated decay of *E.coli* mRNA (32). In this model the coordinated action of both enzymes is suggested in so far as RNAse E makes the endonucleolytic cleavage followed by the exonucleolytic degradation by polynucleotide phosphorylase which attacks the newly formed 3′ end. As RNase E like enzymes are also described in organisms other than *E.coli* like human (33,34) this may be an evolutionary conserved mechanism, which could also be present in chloroplasts from higher plants.

So far degradation intermediates of chloroplast mRNA decay have only been described emphasizing the non-translated regions of the mRNAs. In *Chlamydomonas reinhardii* it could be shown by genetic analysis that the 5′ non-translated region includes a target site of *psbD* mRNA degradation (17). Employing lysed chloroplast extracts as a degradation system for *in vitro* synthesized RNA transcripts cleavage sites could be mapped within the 5′ non-translated region. However, in *Chlamydomonas reinhardii* also the relevance of the coding region as an mRNA stability determinant has already been demonstrated. In case of the *rbcL* mRNA the fusion of the 5′ untranslated region to a reporter gene destabilizes the chimeric transcript, while the addition of sequences of the 5′ part of the coding region prevent destabilization (15). Extensive work has been done on the analysis of the 3′ non-translated region of chloroplast mRNAs of higher plants. In these experiments *in vitro* synthesized transcripts of the 3′ non-translated region of *psbA* mRNA and *petD* mRNA had been used as substrates (13,35) in extracts of soluble chloroplast proteins (26). Several cleavage sites have been mapped mostly located in single stranded regions of the RNA. The location of these cleavages may be consistent with the detection of small degradation intermediates close to the 3′ end of the *psbA* mRNA during the course of degradation. However, the location of cleavage sites and the direction of mRNA decay as determined in this work indicate that the stem–loop structures located within the 3′ non-translated region of *psbA* mRNA are likely not to be directly involved in the regulation of initiation of *psbA* mRNA decay.

Endonucleolytic cleavages within the coding region which are part of the specific degradation pathway of mRNAs have been described earlier in other systems. In an *in vitro* degradation system of mammalian cells a stability determinant was identified within the coding region close to the 3′ end of the *c-myc* mRNA. This region is also subject to an endonucleolytic cleavage, which induces deadenylation-independent decay (36). In this case already a protein could be identified binding to the stability determinant and presumably regulating mRNA decay by masking the cleavage site. Stability determinants within the coding region have been extensively studied in the yeast system (37). Endonucleolytic cleavages as the rate-limiting step of mRNA degradation in the 3′ terminal part of the coding region have been reported for the phosphoglycerate kinase (*PKG1*) mRNA (38).

Cleavage products as far as they have been detected in those systems described above are derived from polysome-based degradation systems or on polysomes for nuclear encoded mRNAs (23,36). In the experiments presented here polysomes which are only incubated with buffer do not show any degradation at all; they are only degraded after addition of the chloroplast lysate. Therefore in chloroplasts the endonuclease responsible for *psbA* mRNA degradation is not polysome-associated as it has been described earlier in mammalian cells (39). Based on the results presented here a nuclease, either soluble or membrane bound, which is not present in the polysome fraction, is required.

The question has to be discussed how cleavage sites on *psbA* mRNA are recognized by endonucleases. Sequence comparison of six nucleotides upstream and downstream of the cleavage sites shows that most of the cleavages occur between a uridine at the 3′ side of the cleavage and an adenosine at the 5′ side. However, the sequences do not reveal any conserved motif which may serve as a recognition site for endonucleases. For the *E.coli* RNase E the situation is similar. The specificity of this enzyme is not defined by a fixed consensus sequence but the cleavages occur at UA-rich motives (for review see 40).

The structural analysis of *psbA* mRNA shows that in the optimal structure most of the cleavage sites are single stranded indicating the specificity of the participating nuclease. But there are no evident structural signals such as small hairpins adjacent to the cleavage sites. As the structure of the *psbA* mRNA is calculated for the deproteinized RNA, it has to be considered that *in vivo* the structure of the RNA is base-paired to a lesser extent. It could already be shown that the 5′ untranslated region of *psbA* mRNA is capable in forming a stem–loop secondary structure as demonstrated by chemical mapping (41). Therefore the structural properties of *psbA* mRNA *in vivo* will be further investigated.

The location of the majority of cleavage sites within the coding region point at the role of translation processes being involved in the recognition of cleavage sites. It has been shown earlier in barley, that ribosome stalling plays a major role in the regulation of D1 protein expression (42–44). Furthermore ribosome stalling

has been discussed in other systems to be involved in mRNA cleavage site recognition (36). Several cleavage sites detected in this work are adjacent to stalling sites at homologous codons compared to the barley sequence. The positions of the cleavage sites #4/5, #8/9 and #11 correlate with ribosome stalling. Contrary to this, cleavage sites #6/7, #10 and #12 are not in context with ribosome stalling sites as detected in barley. Therefore translational pausing may only be linked to cleavage site recognition for certain cleavage positions. Furthermore the role of RNA-binding proteins has to be considered as their binding does influence *psbA* mRNA stability.

Further studies will have to show the detailed order of usage of the specific cleavage sites. The stability determining region of the mRNA in correlation to the cleavage site usage has to be determined. The *in vitro* degradation system presented here will allow the further dissection of the steps of chloroplast mRNA degradation and thereby to understand the regulation of mRNA decay in chloroplasts of higher plants.

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