An Arabidopsis Chloroplast RNA-Binding Protein Gene Encodes Multiple mRNAs with Different 5' Ends

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An Arabidopsis cDNA (Atrbp33) encoding a nuclear-encoded chloroplast RNA-binding protein (RBP) has been isolated (A.J. DeLisle [1993] Plant Physiol 102: 313–314). ATRBP33 shares global structural homology with all known chloroplast RBPs: a chloroplast transit peptide in the amino terminus, followed by a unique acidic domain and a tandem pair of ribonucleoprotein consensus sequence-type RNA-binding domains in the carboxyl end. In vitro translation products of Atrbp33 were found to be imported into chloroplasts, suggesting that ATRBP33 is localized in chloroplasts. The expression of Atrbp33 was higher in chloroplast-containing organs than in non-chloroplast-containing organs. Furthermore, Atrbp33 was expressed in a light-dependent manner. These features are consistent with its postulated role in posttranscriptional control of chloroplast genes. Northern analyses and RNase protection assays showed that as many as nine messages are encoded by the single Atrbp33 gene. Sequence analysis of the cDNAs indicated that some of the transcripts have truncated 5' ends. Most interestingly, the multiple mRNAs potentially encode different polypeptides, one of which lacks a chloroplast transit peptide and acidic domain and contains only one intact RNA-binding domain. Unlike the chloroplast-localized ATRBP33, the truncated polypeptide may function in other cellular compartments.

Chloroplast gene expression requires coordination between plastid and nuclear genomes, because several of the structural, functional, and regulatory components of the chloroplast are encoded in the nucleus (for reviews, see Mullet, 1988; Gruissem, 1989; Taylor, 1989; Rochaix, 1992). Transcription has been demonstrated to be an important regulatory component for chloroplast genes. However, discrepancies between transcription rates and accumulation of messages during the development of chloroplasts indicate that posttranscriptional controls, including RNA processing and stability, play major roles in chloroplast gene regulation (e.g. Deng et al., 1987; Mullet and Klein, 1987; Deng and Gruissem, 1988; Salvador et al., 1993a, 1993b).

It has been shown that the inverted repeat sequences (capable of forming stem-loop structures) in the 3' untranslated region of spinach chloroplast mRNAs function as RNA processing signals in vitro (Smith and Gruissem, 1987) and stabilize upstream RNA segments in vitro (Smith et al., 1989; Adams and Stern, 1990) and in vivo (Stern et al., 1991). However, the predicted free energies of the stem-loop structures do not correlate with the stability of the cognate mRNAs in vitro (Smith and Gruissem, 1987; Stern et al., 1989). Also, the inverted repeat sequences alone cannot totally account for the developmental, organ-specific, and light-dependent changes in the accumulation and stability of specific chloroplast mRNAs. It has been hypothesized that some protein factors may function to process and stabilize plastid mRNAs (Smith and Gruissem, 1987; Stern et al., 1989).

In Chlamydomonas reinhardtii, the 5' ends of mRNAs are thought to be important for message stability (Salvador et al., 1993a): proteins that bind in vitro to the 5' region of psbA mRNA (encoding the thylakoid D1 protein of PSII) have been characterized (Danon and Mayfield, 1991). In addition, many of the factors that contribute to posttranscriptional control of chloroplast genes in C. reinhardtii are nuclear encoded (Rochaix, 1992). In several mutants of C. reinhardtii, chloroplast messages are transcribed at normal rates compared to wild type, but specific mRNAs are selectively destabilized (Kuchka et al., 1989; Sieburth et al., 1991; Monod et al., 1992). It appears that nuclear gene products in the wild type, which are absent in the mutants, stabilize specific mRNAs.

Nuclear mutants affecting chloroplast gene expression have also been isolated from higher plants. One high-Chl fluorescence mutant of maize, hcf7, is apparently defective in chloroplast rRNA processing, because 16S rRNA precursors accumulate (Barkan, 1993). In barley, nuclear genes have been identified that affect the stability of chloroplast psbD messages (Gamble and Mullet, 1989).

In efforts to isolate the protein factors that are involved in the posttranscriptional regulation of chloroplast genes, several groups have purified chloroplast RBPs and isolated the corresponding genes. In spinach, a nuclear-encoded RBP binds to chloroplast mRNA precursors and mature messages at 3' inverted repeat sequences and is apparently involved in processing the 3' end of the mRNAs in vitro (Schuster and Gruissem, 1991). Five tobacco nuclear-encoded chloroplast RBPs have also been isolated based on their ability to bind single-stranded DNA (Li and Sugiura, 1990; Ye et al., 1991). Recently, several additional nuclear-encoded chloroplast RBPs...
genes were isolated from maize (Cook and Walker, 1992), Nicotiana plumbaginifolia (Mieszczak, et al., 1992), and Arabidopsis (Bar-Zvi et al., 1992; Didier and Klee, 1992; DeLisle, 1993). All of these chloroplast RBPs share the same domain organization. The amino-terminal chloroplast transit peptide is followed by an acidic domain. The carboxyl-terminal region consists of two RNP-CS-type RNA-binding domains (Bandziulis et al., 1989). It has been demonstrated that the RNP-CS domains are the functional units for RNA-binding activity (Nietfeld et al., 1990; Scherly et al., 1990). In many eukaryotes, the RNP-CS-type RBPs are involved in splicing, polyadenylation, and ribosome biogenesis (Bandziulis et al., 1989; Mattaj, 1989). However, except for the spinach protein (Schuster and Gruissem, 1991), the functions of plant chloroplast RBPs are unknown. It has been postulated that these RBPs are involved in the posttranscriptional processing of chloroplast pre-mRNAs (Li and Sugiuara, 1990).

We previously isolated a RBP cDNA from Arabidopsis by screening an expression library for single-stranded DNA-binding proteins (DeLisle, 1993). We reported that the cDNA (Atrbp33; previously named AtrbpSl, see DeLisle, 1993) encodes a polypeptide that has the domain organization typically found in all known chloroplast RBPs (Li and Sugiuara, 1990; Schuster and Gruissem, 1991; Bar-Zvi et al., 1992; Cook and Walker, 1992; Didier and Klee, 1992; Mieszczak et al., 1992). The same Arabidopsis clones were isolated in other laboratories (Bar-Zvi et al., 1992; Didier and Klee, 1992). The composition of the first 82 amino acids is characteristic of a chloroplast transit peptide: enriched in hydroxylated amino acids (31% Ser and 7% Thr) and basic amino acids (predicted isoelectric point = 12.5). The acidic domain contains the 5-amino acid motif Ser-Glu-Gly-Asp-X repeated six times. Each of the RNA-binding domains consists of approximately 90 amino acids and contains RNP-CS 1 and RNP-CS 2 (Arg-Gly-Phe-Gly-Phe-Val-Thr-Met and Leu/Val-Phe/Tyr-Val-Gly-Asn, respectively). Although there is little sequence homology upstream of the RNA-binding domains, ATRBP33 shares 37 to 76% identity with the RNA-binding domains of chloroplast RBPs in other plants (Li and Sugiuara, 1990; Schuster and Gruissem, 1991; Cook and Walker, 1992).

To avoid confusion due to heterogeneity in the Atrbp33 mRNA 5' ends (see "Results"), base positions are designated in this report relative to the first ATG start codon (Fig. 1A) (DeLisle, 1993). There are three ATG initiation codons in the same reading frame, at positions +1, +43, and +502 (Fig. 1A). All three ATG codons are in good consensus sequences for translation initiation (Kozak, 1986). The longest ORF encodes a 329-amino acid polypeptide with a calculated molecular mass of 35.8 kD. The second ORF encodes a...
polypeptide of 314 amino acids, and its calculated molecular mass is 34.4 kD. These two polypeptides are identical except that one is 14 amino acids longer than the other at the amino terminus. The third ORF encodes a polypeptide of 167 amino acids with a predicted molecular mass of 18.3 kD. Unlike the longer polypeptides, which consist of all four domains typically found in chloroplast RBPs, the truncated polypeptide contains no transit peptide or acidic domain and has only one intact RNA-binding domain (domain II).

In this report, we present the characterization and expression of the nuclear-encoded chloroplast RBP gene \textit{Atrbp33} from \textit{Arabidopsis thaliana} (DeLisle, 1993). The expression of \textit{Atrbp33} was organ preferential, developmentally regulated, and light dependent. Most interestingly, multiple messages were encoded by \textit{Atrbp33} and one protein encoded by these messages lacked a chloroplast transit peptide. This suggests that the truncated protein may function in cellular compartments other than chloroplasts.

**MATERIALS AND METHODS**

**Cell Cultures and Plant Growth**

\textit{Arabidopsis thaliana} L. (Heyn) Columbia suspension cultures were established and maintained as previously described (Ferl and Laughner, 1989). Leaf materials were obtained from plants grown at 20°C under 10-h/14-h light/dark cycles with a light intensity of 50 to 65 \( \mu \)E m\(^{-2}\) s\(^{-1}\). Plants were grown either in potting soil or in 0.6% (w/v) agar containing Murashige and Skoog medium (Murashige and Skoog, 1963) and 3% (w/v) Suc. Roots were isolated from plants grown in agar. Inflorescences were harvested from plants grown in soil with 16-h light/10-h dark photoperiods. Tissues were harvested, immediately frozen in liquid nitrogen, and stored at \(-70°C\) until use.

**Genomic Library Screening and Sequence Analysis**

The \textit{Atrbp33} cDNA (DeLisle, 1993) was used to screen an \textit{Arabidopsis} genomic library (Clontech, Palo Alto, CA) by hybridization (Sambrook et al., 1989). A 5-kb Sac1 fragment from one of the positive clones was isolated and subcloned into pBluescript KS\((-)\) (Stratagene). DNA sequencing was performed on double-stranded templates by the dideoxy chain-termination procedure (Sanger et al., 1977) using Sequenase 2 (United States Biochemical) and following the manufacturer’s protocol. DNA sequences were analyzed using the MacVector program (International Biotechnologies, Inc., New Haven, CT).

**Chloroplast Import Assays**

The clone containing the 1.2-kb cDNA insert (as shown in Fig. 1A) was transcribed with T7 RNA polymerase in the presence of diguanosine triphosphate and was translated in a wheat germ system with \[^{3}H\]Leu as label (Cline et al., 1989). Translation was terminated by transfer to an ice bath and dilution to \(30 \mu\)M unlabeled Leu in import buffer (50 \( \mu\)M Hepes/KOH, pH 8, 0.33 M sorbitol). Intact chloroplasts were isolated from 9- to 10-d-old pea (\textit{Pisum sativum} L. cv Laxton’s Progress 9) seedlings (Cline, 1986). The plastids were suspended in import buffer and kept on ice until use. Import into intact chloroplasts was carried out in microcentrifuge tubes for 10 min at a 25°C water bath illuminated with approximately 70 \( \mu\)E m\(^{-2}\) s\(^{-1}\) (Cline et al., 1989). Assays were terminated by transfer to 0°C. Chloroplasts were then repurified on a Percoll cushion or were first treated with the protease thermolysin and then repurified. For subfractionation, recovered chloroplasts were osmotically lysed with 10 \( \mu\)M Hepes/KOH, pH 8, 10 \( \mu\)M MgCl\(_2\) and the soluble fraction was separated from the membrane fraction by centrifugation at 3,200 \( g\) for 8 min. The supernatant was further centrifuged at 40,000 \( g\). Membranes were therefore analyzed directly, washed with import buffer, and extracted with 0.1 M NaOH, or treated with thermolysin before analysis. Samples recovered from these assays were subjected to SDS-PAGE and fluorography (Cline, 1986).

**RNA Isolation and Northern Hybridization**

Total RNA from various tissues was extracted as described (Finkelstein et al., 1985). Leaf poly(A\(^+\)) RNA was isolated using oligo(dT) cellulose chromatography (type 3, Collaborative Research, Inc., Bedford, MA), size fractionated by electrophoresis in a Mops-formaldehyde 0.8% agarose gel, and transferred to a nylon membrane (Schleicher and Schuell) (Sambrook et al., 1989). A 1-kb cDNA fragment (+110 to +1139, Fig. 1A) was labeled by random priming and was used as a probe for hybridization. The membrane was hybridized in \(4 \times\) SSC (1 \( \times\) SSC equals 150 mM NaCl and 15 mM sodium citrate, pH 7), 0.1% (w/v) SDS, and 0.25% (w/v) nonfat dry milk at 50°C. Following 12 h of hybridization, the membrane was washed at room temperature in \(2 \times\) SSC, 0.1% SDS and then in \(0.2 \times\) SSC and 0.1% SDS at 65°C.

**Genomic DNA Isolation and Southern Hybridization**

\textit{Arabidopsis} genomic DNA was isolated from leaves as described (Murray and Thompson, 1980). Five micrograms of genomic DNA were digested with EcoRI. Copy number reconstruction experiments were performed with three, two, one, and one-half genomic equivalents of EcoRI-digested plasmid containing \textit{Atrbp33} cDNA. DNAs were size fractionated by electrophoresis in 0.8% agarose gels in 90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8, and blotted to a nylon membrane (Sambrook et al., 1989). DNA probes were the same as described for the northern hybridization. The filter was hybridized in 50% (w/v) formamide, \(4 \times\) SSC, 5X Denhardt’s solution, 50 mM sodium phosphate, pH 7, 0.2% SDS, and 250 \( \mu\)g mL\(^{-1}\) denatured salmon sperm DNA at 50°C. The washing conditions were the same as described for the northern hybridization.

**RPA**

Genomic DNA fragments from \textit{Atrbp33} (~524 to +490 relative to the first ATG, Fig. 1A), \textit{Aatc1} (actin gene, +1148 to +1830; Nairn et al., 1988), and \textit{Cab140} (\(Chl\) a/b binding protein gene, +56 to +854; Leutwiler et al., 1986) were cloned into pBluescript KS\((-)\). These plasmid DNAs were linearized.
with appropriate restriction enzymes. Radioactive antisense riboprobes were synthesized in vitro using T3 or T7 RNA polymerase (Promega) and purified by 5% (w/v) polyacrylamide/8 M urea gels. RPAs were carried out as described (Hod, 1992) with some modifications. Briefly, 5 μg (unless otherwise indicated) of total RNA were hybridized with the radioactive antisense RNA at 43°C for 16 h in 80% deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA. The hybridized RNA was digested with a mixture of RNase A (10 μg mL⁻¹, United States Biochemical) and RNase T1 (100 units mL⁻¹, United States Biochemical) in 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 5 mM EDTA at 37°C for 45 min. The digestion was stopped by adding an equal volume of 4 M guanidine thiocyanate. The undigested RNA was precipitated with an equal volume of isopropanol and the protected fragments were analyzed on 5% PAGE containing 8 M urea. The gels were dried and autoradiographed.

Primer Extension Analysis

Twenty micrograms each of total RNA from leaf and flower were denatured at 70°C for 10 min and hybridized to 32P-labeled primers (positions 49-110 and 268-306, see Fig. 1A) at 49°C for 5 h in 20 μL of 10 mM Tris, pH 7.5, 1 mM EDTA, 250 mM KCl. The primer extension reaction was done by adding 50 μL of reaction mixture containing 14 mM MgCl₂, 14 mM Tris-HCl, pH 8.3, 0.28 mM each of dATP, TTP, dCTP, and dGTP, 7 mM DTT, and 7.5 units of avian myeloblastosis virus reverse transcriptase (Life Science, Inc., St. Petersburg, FL) to the hybridization mix and incubating at 42°C for 30 min. The reaction was ethanol precipitated and analyzed on a 6% sequencing gel.

cDNA 5' End Amplification

RACE was employed to generate the 5' ends of the cDNAs (Frohman et al., 1988). The amplification was carried out using the 5' ampliFINDER RACE kit (Clontech) according to the manufacturer's protocol. RNA from leaf and flower was reverse transcribed using the gene-specific primer 5'-TAACTCCGAGATTTGAGCTACC-3'. The reverse-transcribed cDNAs were amplified by PCR using an internal nested primer, 5'-AATCCGAAGTCTGGGCAACGAAGG-3'. The amplified products were cloned into pBluescript KS(−) and sequenced as described for the genomic clone.

RESULTS

Gene Structure

The genomic organization of Atrbp33 was analyzed by digesting genomic DNA with restriction enzymes BglII, EcoRI, and HindIII and probing with either the entire Atrbp33 cDNA or fragments specific for the unique acidic domain or the RNA binding domains. In each case, one or two bands were detected (data not shown). Such a simple pattern suggests that Atrbp33 is encoded by a single locus. Furthermore, a copy number reconstruction experiment indicated that Atrbp33 is a single-copy gene (Fig. 1B).

A genomic clone containing introns was isolated. The sequenced region (2716 bp) includes 563 bp of 5' flanking sequence, the structural gene (1881 bp containing introns), and 274 bp of 3' flanking sequence. There are four exons and three introns of 314, 327, and 96 bp, which interrupt the coding region at positions +541, +638, and +924, respectively (Fig. 1A). The relative positions of the three introns are similar to those in tobacco and maize chloroplast RBP genes (Li et al., 1991, 1993; Cook and Walker, 1992).

In Vitro Chloroplast Import of ATRBP33 Precursor

ATRBP33 must be translocated into chloroplasts to participate in regulation of chloroplast genes. To determine whether the protein is targeted to the chloroplasts, in vitro transcription/translation products of Atrbp33 were incubated with isolated, intact pea chloroplasts and radiolabeled proteins were analyzed (Cline, 1986; Cline et al., 1989). Two in vitro translation products were found (Fig. 2, lane 1), which probably resulted from translation initiation at the two start codons at positions +1 and +43 (Fig. 1A). The predicted molecular masses of the in vitro translation products were 34.4 and 35.8 kD (Fig. 1A); however, the molecular masses were estimated to be 40 and 42 kD, respectively, by SDS-PAGE. Upon incubating the translation products with intact chloroplasts, a 32-kD band appeared that was recovered with intact chloroplasts (lane 2) and protected from exogenous protease (lane 3). The appearance of this band was dependent on ATP, time, and temperature (the 32-kD form did not accumulate at 0°C; data not shown). Upon chloroplast subfractionation, the 32-kD protein was recovered primarily in the soluble fraction. Although some of the 32-kD protein was recovered with the membrane pellet, it was only peripherally associated with intact chloroplasts. Figure 1. In Vitro Chloroplast Import of ATRBP33 Precursor. Lane 1, Translation products. Lane 2, Chloroplasts re-incubated with intact chloroplasts. Lane 3, Recovered chloroplasts were analyzed by 14% SDS-PAGE with a mixture of RNase A (10 μg mL⁻¹, United States Biochemical) and RNase T1 (100 units mL⁻¹, United States Biochemical) in 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 5 mM EDTA at 37°C for 45 min. The digestion was stopped by adding an equal volume of 4 M guanidine thiocyanate. The undigested RNA was precipitated with an equal volume of isopropanol and the protected fragments were analyzed on 5% PAGE containing 8 M urea. The gels were dried and autoradiographed.

Figure 2. Chloroplast import assay. Chloroplasts were incubated with Atrbp33 translation products as described in "Materials and Methods." Lane 1, Translation products. Lane 2, Chloroplasts re-incubated after incubation. Lane 3, Recovered chloroplasts were treated with the protease thermolysin prior to repurification. The apparent molecular masses were calculated by comparison to radiolabeled protein standards (Sigma). A picture of the fluorogram is shown.
generally bound to the membranes. The protein was largely removed upon washing and eliminated by protease treatment of the membranes or extraction with 0.1 M sodium hydroxide (data not shown). These results indicate that the precursor was imported into the chloroplast and proteolytically processed to remove the transit peptide.

**Northern Analysis**

The expression of *Atrbp33* was analyzed by a northern blot of poly(A)⁺ RNA isolated from leaves. Under relatively stringent hybridization and washing conditions (see "Materials and Methods"), the *Atrbp33* cDNA hybridized to multiple messages, with 1000- and 1200-nt bands being the most apparent (Fig. 3). Furthermore, labeled transit peptide coding region hybridized only to the 1200-nt mRNA (data not shown), indicating that the sequences at the 5' ends are different in these messages and that at least the larger mRNA could encode the RBP destined for the chloroplasts.

**Mapping 5' Ends**

Since multiple messages with different 5' ends were produced by *Atrbp33*, an RPA was performed to map the 5' ends in detail. An antisense riboprobe complementary to the region from 563 bases upstream to 451 bases downstream of the first translation initiation codon was used (Fig. 1A). There were at least nine different RNA fragments protected, ranging in length from 540 to 250 nt (Fig. 4). These RPA fragments were not artifacts of incomplete RNase digestion because the intensity of the fragments increased as the amount of RNA hybridized increased and the intensity of the fragments remained constant with increased RNase concentration (data not shown). According to their sizes, the protected fragments can be divided into two families that correspond to the 1000- and 1200-nt mRNAs detected on the northern blot (Fig. 3). The smaller family contained protected fragments of 250 to 278 nt, and the 250-nt fragment was the most prominent band. The larger family had protected fragments ranging from 427 to 540 nt, with the 490-nt band being most abundant. These results indicate that there are multiple sites of transcription initiation and/or alternative splicing for *Atrbp33*.

The various positions of the 5' ends of the transcripts were verified by primer extension analyses and 5' RACE PCR and by comparing the results to sequences of cDNAs and genomic clones. The positions of the 5' ends of the transcripts determined by RPA, 5' RACE PCR, and primer extension are summarized in Table I. Although not all of the RPA fragments have been confirmed yet by the other two techniques, in general the 5' ends mapped by the three methods were in close agreement. In particular, two 5' ends were mapped precisely at the same positions (−89 and −39) by two independent methods. The positions of the majority of the 5' ends mapped by the different techniques deviated by only a few nts (less than six). The greatest deviation was 10 nts, which occurred with the smallest transcript in the 1200-nt family. Sequence analyses of the 19 independent clones obtained from 5' RACE PCR so far indicate that some of the messages have truncated 5' ends and that there is no evidence for differential splicing. The existence of upstream 5' mini exons, however, remains a possibility.

Despite the various positions of the 5' ends, all five members of the 1200-nt family encoded a protein that possessed
Table I. Positions of transcription start sites for Atrbp33

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<th>RNA Species</th>
<th>Method of Detection</th>
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<tr>
<td>1200-nt RNAs</td>
<td>−89</td>
<td>−89</td>
<td>−89</td>
<td>−58</td>
<td>−33 (3)</td>
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<td></td>
<td>−61</td>
<td>−39</td>
<td>−22</td>
<td>−18</td>
<td>−18 to −16 (2)</td>
<td>−39</td>
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<td>+24</td>
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<td>1000-nt RNAs</td>
<td>+173</td>
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<td>+182</td>
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<td>+180</td>
<td>+201</td>
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The most abundant mRNA in the approximately 1200-nt size range. The numbers in parentheses indicate the frequency of isolation by 5’ RACE PCR. The most abundant mRNA in the flower. The most abundant mRNA in the approximately 1000-nt size range, except in flower.

the same structural framework as other chloroplast RBPs: a chloroplast transit peptide, acidic region, and two RNA-binding domains (Fig. 1A). Interestingly, the second ATG would be used as the translation start codon by the smallest transcript in the 1200-nt family, since this transcript begins at nt +24 and lacks the first ATG. The 5’ ends of the 1200-nt mRNAs begin at about 170 to 200 nt downstream from the first ATG (Fig. 1A). The only ORF found in these transcripts begins at the ATG within the first RNA-binding domain (Fig. 1A). As a result, the predicted protein encoded by the smaller transcripts is in frame with its chloroplast counterpart but is truncated and contains only part of RNA-binding domain I and all of domain II. (Fig. 1A).

Developmental Expression

To examine the expression of each of the Atrbp33 transcripts in different organs, total RNA extracted from various tissues was analyzed by RPA (Fig. 4A). Note that 3 times as much total RNA from suspension-culture cells and roots was used in RPA due to low abundance of the messages. The expression of the messages was organ preferential, being highest in leaves, followed by stems, inflorescence, suspension-culture cells, and roots. In contrast, the actin gene showed similar expression in the different organs (Fig. 4B). These results demonstrated that the relative abundance of Atrbp33 mRNAs correlates with the presence of chloroplasts. The 250- and 490-nt protected fragments represented the two predominant transcripts in all vegetative tissues examined, except that only one major Atrbp33 mRNA was observed in roots (the 490-nt fragment). Interestingly, a unique band of 275 nt was found in the inflorescence RNA sample. This fragment represented the most pronounced transcript in floral tissue; it has not yet been detected by 5’ RACE PCR but was confirmed by primer extension (Table I). Additionally, young leaves also expressed six minor transcripts, some of which were undetectable in other tissues by RPA. Moreover, all of the leaf Atrbp33 mRNAs were developmentally regulated; young leaves displayed higher expression than mature leaves (Fig. 4A). When using more sensitive techniques such as 5’ RACE PCR, however, some of these minor messages were also found in the inflorescence (Table I).

Light Induction

Many genes for nuclear-encoded, chloroplast-localized proteins, such as the Cab gene, which encodes the Chl a/b binding protein, are light regulated (Tobin and Silverthorne, 1985). To examine the effect of light on the expression of Atrbp33, light-grown plants were subjected to 4 d of darkness and then returned to the light for various lengths of time. Total RNA was isolated from the leaves of the treated plants and analyzed by RPA (Fig. 5A). In the dark the Atrbp33 messages decreased dramatically to barely detectable levels compared to those in the light. Upon return to the light, increased amounts of Atrbp33 transcripts were measured after 4 h. The messages were higher after 24 h of continuous light than before the dark treatment. Moreover, all eight Atrbp33 mRNAs were light inducible in leaves. Cab mRNA levels were similarly induced by light, whereas actin mRNA was unaffected (Fig. 5, B and C).

DISCUSSION

In higher plants, chloroplast genes are generally co-transcribed and polycistronic messages are processed into shorter RNA species. The posttranscriptional processing, including 5’ and 3’ end processing and splicing of pre-mRNAs, is thought to be a major regulatory mechanism for chloroplast gene expression (Deng et al., 1987; Mullet and Klein, 1987; Deng and Gruissem, 1988; Salvador et al., 1993a, 1993b). Chloroplast RBPs were first purified from tobacco and are postulated to be involved in the posttranscriptional processing of chloroplast RNAs (Li and Sugiura, 1990). Recently, a spinach chloroplast RBP was shown in vitro to be required for processing of the 3’ ends of chloroplast messages (Schuster and Gruissem, 1991). We have isolated a cDNA clone from Arabidopsis encoding a chloroplast RBP (DeLisle, 1993). This protein has the same structural organization as all known chloroplast RBPs and is capable of binding single-stranded DNA. Furthermore, we showed that the ATRBP33
Arabidopsis genes, the expression of the spinach 28rnp is similar in both young and mature leaves (Schuster and Gruissem, 1991). Furthermore, not all of the chloroplast RBP genes exhibit light-dependent expression. Among the five tobacco genes, only the cp28 gene is regulated by light (Li and Sugiura, 1990; Ye et al., 1991). There are two spinach 28rnp messages (1.2 and 1.4 kb) detected by northern analysis (Schuster and Gruissem, 1991), similar to what was observed for Atrbp33 (Fig. 3). However, only the 1.4-kb spinach transcript is light inducible, whereas all of the Atrbp33 mRNAs were induced by light (Fig. 5).

It appears that all of these proteins belong to a large family of nuclear-encoded chloroplast RBPs. The different patterns of expression may reflect different functions of each member, a notion supported by the fact that each member contains a highly diverged, unique acidic region. The function of these unique acidic domains is unknown, but they have been suggested to be involved in protein-protein interaction (Preugschat and Wold, 1988; Bar-Zvi et al., 1992). It has been shown that posttranscriptional processing and splicing of pre-mRNAs are complex events and require many ribonucleoproteins (e.g., the heterogeneous nuclear ribonucleoprotein splicing complex) (Bandziulis et al., 1989). Therefore, the functional specification of each chloroplast RBP may be achieved in part by interacting with other processing proteins through the acidic domains.

Didier and Klee (1992) isolated an Arabidopsis cDNA, FMV3bp, that is probably encoded by the same gene as Atrbp33. The nucleic acid sequence of FMV3bp is identical to that of Atrbp33, except for the first 21 bases, which were derived from a genomic clone (Didier and Klee, 1992). Sequence comparison of FMV3bp and the Atrbp33 genomic clone, however, revealed that the 5' 21 bases of FMV3bp were located in the first intron of the Atrbp33 genomic clone. This genomic fragment is actually located downstream of where it was placed in FMV3bp. Placement of the sequence may be the result of a cloning artifact that occurred during genomic library construction (D. Didier and H. Klee, personal communication). The genomic library used to isolate the 5' end of FMV3bp was constructed using DNA that was partially digested with EcoRI. An EcoRI site is located at the 3' end of the 21-base fragment in the intron and may have been ligated with another fragment during library construction. Therefore, FMV3bp is probably not an example of an Atrbp33 mRNA with an alternatively spliced 5' end.

Despite so much sequence identity, FMV3bp hybridized to a single RNA band of about 900 nt on a northern blot, contrasting sharply to the 1000- and 1200-nt mRNAs found in this study (Fig. 3). Furthermore, the highest expression of FMV3bp is not in leaves, as observed for Atrbp33, but in floral tissue. The reasons for such differences remain to be determined, but the patterns of expression may be influenced by the developmental stage of the tissue used. A notable difference between the two northern blots is that leaf poly(A*) RNA isolated from reproductive-stage plants was used for hybridization of FMV3bp, whereas leaf poly(A*) RNA isolated from the vegetative stage was used for Atrbp33. Perhaps the predominant 1000-nt mRNA that we observed in flowers is also most abundant in other organs of the reproductive plants.
Atrbp33 is a single-copy nuclear gene (Fig. 1B) that encodes as many as nine transcripts with different 5' ends (Fig. 4A). The heterogeneity of the messages is likely due to multiple transcription start sites. Although processing of the primary transcripts is a formal possibility, there is no precedent for 5' end processing of eukaryotic mRNAs. In spinach two messages are also encoded by the single 28rnp gene, but the mechanism by which multiple mRNAs are generated is unknown (Schuster and Gruissem, 1991). The promoter regions of both families of Atrbp33 transcripts contain no apparent TATA sequences. The absence of TATA sequences and the presence of multiple initiation sites are characteristics frequently identified in housekeeping genes (Dyman, 1986). Genes transcribed from promoters lacking TATA boxes commonly are expressed at relatively low levels, are subjected to little regulation, and show little tissue-specific expression (Dyman, 1986). Multiple transcription start sites were also observed for one of the tobacco chloroplast RBP genes (cp33) (Li et al., 1993). The cp33 message is much less abundant compared with other tobacco RBP messages (e.g. cp28) and is not light regulated, although the expression is higher in leaves than in roots (Li and Sugira, 1990). In contrast, Atrbp33 was transcribed at moderate levels and was both developmentally regulated and light regulated, and its expression showed organ preference.

In this study we showed that multiple messages are generated by the single Atrbp33 gene. Sequence analyses of cDNAs indicated that these transcripts potentially encode two distinct proteins (Fig. 1A). The larger messages encode a protein that likely functions as a RBP in chloroplasts. If the 1000-nt mRNAs are translated, the protein product is likely to function in different cellular compartments due to the absence of a chloroplast transit peptide. Different transcription start sites in the tobacco cp33 gene also result in multiple messages, but they differ only in the 5' untranslated region and thus encode the same protein (Li et al., 1993). The occurrence of the single Atrbp33 gene encoding two distinct proteins targeted to different cellular compartments is apparently unique so far among the known chloroplast RBP genes.

Little is known about proteins involved in RNA processing in other organelles in higher plants. Genes encoding proteins with only one RNA-binding domain have been isolated from maize (Ludevid et al., 1992) and recently from tobacco (Hirose et al., 1993). The predicted proteins contain a single RNP-CS-type RNA-binding domain in the amino terminus and a Gly-rich domain in the carboxyl terminus. Proteins synthesized in vitro from the maize and tobacco genes are capable of binding RNAs, indicating that they are RBPs (Ludevid et al., 1992; Hirose et al., 1993). Furthermore, it was demonstrated with the yeast poly(A)-binding protein that one of the four RNA-binding domains is sufficient for cell viability and RNA binding specificity (Sachs et al., 1987). Taken together, these data lend support for the idea that the truncated ATRBP33 protein with only one intact RNA-binding domain may also function as a RBP in Arabidopsis. Multiple messages from a single RBP gene are common in other organisms and are produced through a combination of alternative transcription start sites and differential splicing (for review, see Bandziulis et al., 1989; Smith et al., 1989). This is a mechanism by which a single gene can encode multiple proteins with specific functions in RNA processing, thereby obviating the need for a much larger number of individual genes.

All of the Atrbp33 mRNAs in leaves increased and decreased in parallel in response to light/dark treatments. This suggests that the two distinct proteins encoded by Atrbp33 have coordinated functions. One likely subcellular location of the truncated ATRBP33 is the cytoplasm, since the protein contains no apparent nuclear localization signals or other signal sequences. If so, the truncated protein may function to stabilize the 1200-nt Atrbp33 transcripts in the cytosol and thus affect the expression of chloroplast genes in an autoregulatory capacity. We have raised antibodies against the amino terminal region unique to the chloroplast form of ATRBP33 and are raising antibodies against the RNA-binding domain present in both forms of the proteins. Future immunolocalization studies will help to address the functions of the protein products of Atrbp33.

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