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Inefficient *rpl2* Splicing in Barley Mutants with Ribosome-Deficient Plastids

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Analysis of transcript accumulation and splicing in plastids of four nuclear mutants of barley revealed that the ribosomal protein L2 (*rpl2*) gene transcripts containing a group II intron remained entirely unspliced, whereas the intron of the ribosomal protein L16 (*rpl16*) gene (linked with the *rpl2* gene in the same operon) was removed in the mutant plastids. Also, the transcripts of other genes containing group II introns (ribosomal protein S16 gene, *rps16*; NADH dehydrogenase ND2 gene, *ndhB*; cytochrome *f* gene, *petD*; and intron-containing reading frame 170, *irf170*) and of the tRNA for leucine, *trnL* (UAA), possessing the only chloroplast group I intron, were found to be spliced. The mutants used in this investigation are considered to be nonallelic; this excludes the possibility that a single nuclear gene is responsible for the impaired splicing of *rpl2* transcripts. The mutants, however, have a severe deficiency in chloroplast ribosomes in common; this deficiency is evident from the lack of the essential ribosomal protein L2 and from an extremely low steady state level of plastid rRNAs. From these results, we conclude that a functioning translational apparatus of the organelle is a prerequisite for splicing of the chloroplast *rpl2* class II intron but not for splicing of at least five other group II intron-containing transcripts. This provides genetic evidence for a chloroplast DNA-encoded component (e.g., a maturase) involved in the splicing of *rpl2* pre-mRNA.

INTRODUCTION

Different types of introns have been found in a wide variety of organisms, so the question of phylogenetic relationships among them arises. Group II self-splicing introns in bacteria (Ferat and Michel, 1993) and the RNA components of the eukaryotic nuclear-splicing apparatus share significant similarities in their splicing biochemistry. In both cases, the introns are excised as branched molecules containing a 2′ to 5′ phosphodiester bond. The group II introns, which are present in eukaryotic organellar genes, could be intermediates between self-splicing bacterial group II and nuclear pre-mRNA introns (Sharp, 1985; Cech, 1986; cf. also Jacquier, 1990; Sharp, 1991). The conserved 5′ and 3′ boundary sequences of group II introns (GUGYG and AY, respectively) resemble to a certain extent those found in nuclear introns (Michel et al., 1989). However, it remains to be shown whether these similarities result from a true evolutionary relationship or simply originate from a convergence driven by mechanistic requirements (Weiner, 1993).

Maturases are thought to be involved in splicing of group I and II introns present in chloroplast and mitochondrial genes of eukaryotic cells. Their function is supposed to compensate for structural defects that have accumulated during evolution and now prevent self-splicing (cf. Saldanha et al., 1993). The exact biochemical function of maturases in splicing is, however, not yet known, despite some clues to certain enzymatic activities (e.g., Kenell et al., 1993). Evidence for a functional importance of maturase-encoding open reading frames (ORFs) found in introns has come exclusively from the analysis of fungal mitochondrial mutants (e.g., Lambowitz and Perlman, 1990).

Corresponding observations are, on the other hand, lacking for higher plant organelles, although the sequences of certain polypeptides encoded in a number of plant mitochondrial group II introns are reported to be homologous to the maturase sequences of fungal mitochondria (Wolstenholme et al., 1993). The chloroplast genome of monocots contains 17 group II introns and a single group I *cis*-intron as shown in Figure 1. Only one ORF has been detected in the chloroplasts of higher plants that potentially could encode a maturase-like polypeptide. This ORF is located in the intron of the tRNA gene for lysine (*trnK*; cf. Figure 1) and is well conserved in mustard (Neuhaus and Link, 1987), rice (Hiratsuka et al., 1989), tobacco (Shinozaki et al., 1986), barley (Sexton et al., 1990), potato (Jardin et al., 1994), liverwort (Ohyama et al., 1986), and maize (R. Maier and H. Kössel, unpublished data). Its presence in the reduced plastid genome of the parasitic, nonphotosynthetic plant *Epifagus virginiana* suggests an important function of the encoded peptide (Wolfe et al., 1992).

Mutants defective in splicing of mitochondrial or chloroplast transcripts have so far not been described in higher plants.

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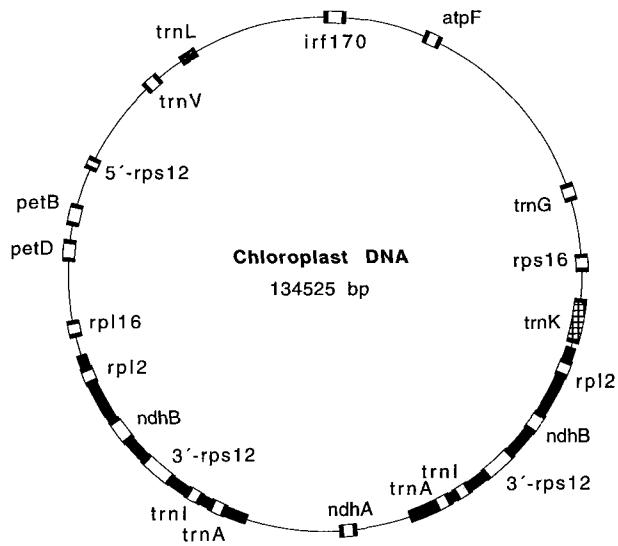

Figure 1. Location of Introns in the Chloroplast Genome of Monocots.

The 17 group II introns (white boxes) and one group I intron (grey) present in the chloroplast DNA of monocot plants are shown. The ORF with homology to fungal maturases in the *trnK* intron is indicated by the hatched segment. The data were adapted from the rice chloroplast genome map (Hiratsuka et al., 1989).

Plastids lacking their translational apparatus are obviously a powerful tool to test the possibility of chloroplast DNA–encoded peptides involved in chloroplast RNA maturation. Plastid ribosome deficiencies have been characterized in several higher plant species (cf. Börner and Sears, 1985). Plastid genes are still transcribed in such mutants as well as in heat-treated plants lacking detectable amounts of plastid ribosomes (Falk et al., 1993; Han et al., 1993; Hess et al., 1993). In the case of the barley mutant *albostrians*, the ribosome deficiency not only leads to alterations in transcription rates and transcript accumulation in plastids, but also to aberrant transcript patterns (Hess et al., 1993). These findings suggest a link between plastid translation and RNA processing.

Here, we present observations on the splicing of plastid transcripts in white and green leaves of the *albostrians* mutant. To test the possibility that there is more direct influence of this nuclear mutant allele on RNA splicing, we extended our investigation to three additional mutants of barley, which we also found to be plastid ribosome deficient. In all of these mutants, the transcript pattern of the chloroplast gene encoding the ribosomal protein L2 (*rpl2*) differed in the same way between ribosome-deficient white plastids and green chloroplasts. Even by using polymerase chain reaction (PCR), only unspliced *rpl2* transcripts could be detected in the white plastids, whereas splicing of seven other intron-containing transcripts was not affected. This provides evidence for the involvement of a chloroplast DNA–encoded gene product in the splicing of at least one, but clearly not all plastid transcripts.

RESULTS

High Level but Deviating Pattern of *rpl2* Transcripts in Mutant Plastids Are Correlated with Ribosome Deficiency

Transcripts of certain nonphotosynthetic genes were previously found to be transcribed in mutant plastids of the *albostrians* mutant and to accumulate to high abundance despite the absence of plastid ribosomes and plastid-encoded RNA polymerase subunits (Hess et al., 1993). Similarly, transcripts for *rpl2* accumulate in *albostrians* mutant plastids to an ~30-to 50-fold enhanced level in comparison to normal chloroplasts (Figure 2). In our investigations, we included RNA from three additional plastid ribosome–deficient mutants, *albina*-e16, *Saskatoon* (Figures 2A and 2B), and *albina*-d13 (Figure 2B). Mutants *albina*-d13 and -e16 were selected from 20 chlorophyll-deficient barley mutants by screening for the lack of plastid rRNA (see below). The plastid ribosome deficiency in white leaves of *Saskatoon* has been determined from previous investigations (cf. Börner and Sears, 1985). All of the ribosome-deficient mutants showed the same complex pattern of *rpl2* transcript accumulation when a 5′ exon-specific probe was used (Figure 2A). The hybridization patterns contain bands specific for RNA from either normal chloroplasts or mutant plastids. Several other bands are common to both. The longest transcript (~10 kb, labeled with arrows in Figures 2A and 2B) was present in RNA from both mutant and normal chloroplasts. It most likely represents the primary transcript that covers the entire operon, which includes in addition to *rpl2* another 12 genes plus the RNA polymerase α subunit–encoding *rpoA* gene at its distal end (Figure 2C; Ruf and Kössel, 1988). Exactly the same hybridization pattern was found for the mutants when a 528-nucleotide antisense probe, which covered the 5′ splice site (Figure 2B), was used. In the case of the RNA from green chloroplasts, differences detected by this probe, especially among transcripts of smaller size, should indicate the presence of exon-specific (mature) *rpl2* transcripts (Figure 2A; also found in Figure 2B as a result of probe specificity) and intron-specific transcripts (Figure 2B). However, both exon- and intron-specific bands were always of very low abundance and at the limit of detection by RNA gel blot hybridization (see following section).

Despite the high abundance of the putative precursor transcripts, but because of the deficiency in the translational apparatus, *albostrians* mutant plastids cannot produce the L2 protein (Hess et al., 1993). The mutant plastids of *albina*-d13, -e16, and *Saskatoon* share this feature with *albostrians* mutant plastids, as was shown by immunostaining with L2-specific antiserum (Figure 3). As a positive control, the L2 protein was easily detectable in extracts from normal chloroplasts of the *albina*-d13 line. The absence of the ribosomal protein L2 is itself indicative of an impaired translational apparatus because the L2 peptide was shown to be an essential component directly involved in the peptidyl transferase reaction in the *Escherichia coli* ribosome (Nierhaus, 1982).

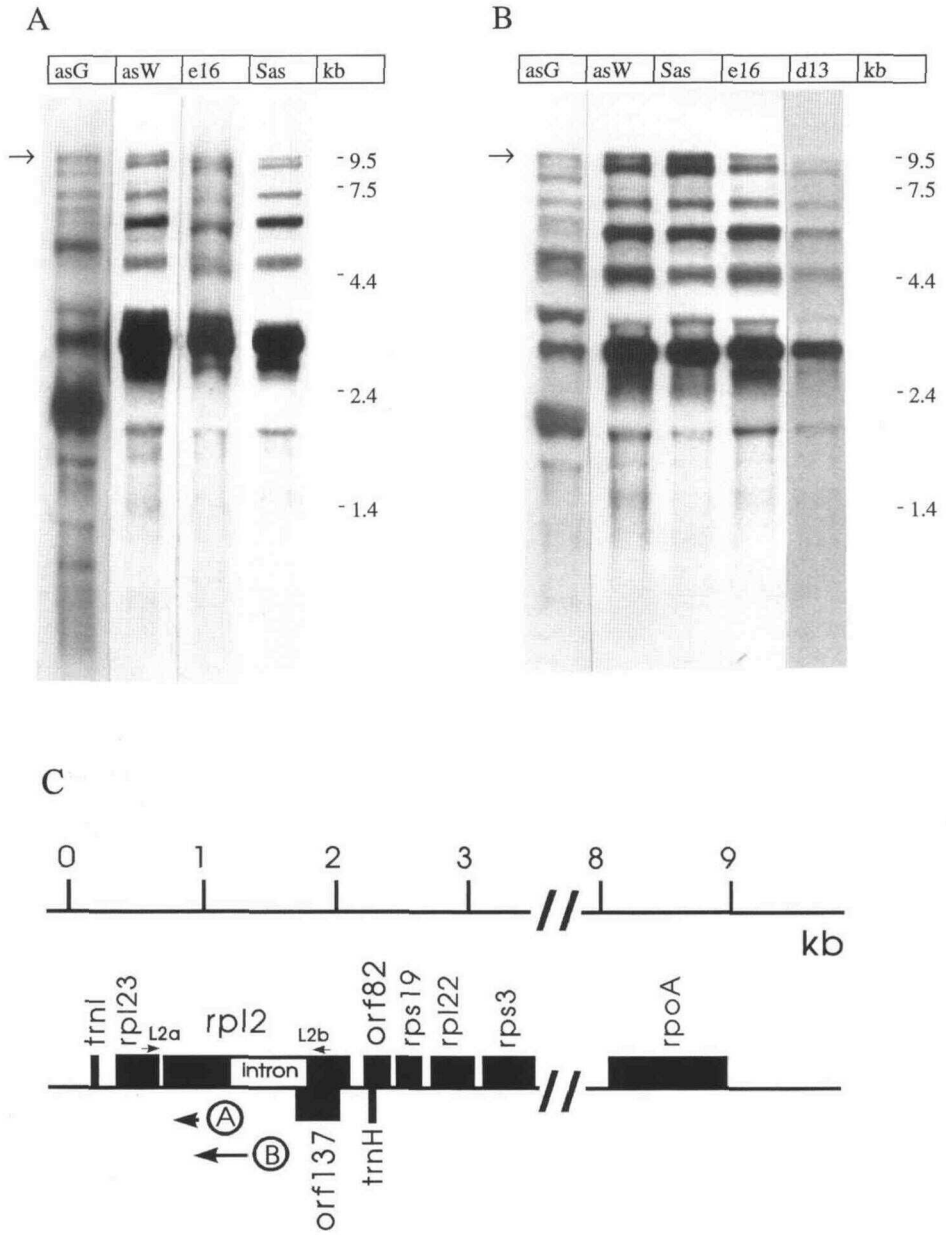

Figure 2. Comparison of Transcript Abundance for the Plastome-Encoded Ribosomal Protein L2 in Different Mutants of Barley.

(A) Exon 1–specific probe. The transcript accumulation pattern of *rpl2* differs in several bands between white mutant plastids (asW, *albostrians*; *e16*, *albina-e16*; Sas, *Saskatoon*), and phenotypically normal chloroplasts (asG) of green *albostrians* leaves used as a control. Equal amounts of RNA per lane were hybridized with a radioactively labeled 104-nucleotide antisense transcript specific for the 5′ exon of maize *rpl2*. The arrow at left indicates the longest transcript of 10 kb (also shown in **[B]**). Molecular length markers (Gibco-BRL) are shown at right in kilobases (also given in **[B]**).

(B) Junction probe. Equal amounts of RNA per lane were hybridized with a radioactively labeled probe containing part of the exon 1 and of the intron: The RNA pattern of the mutants (including d13, *albina*-d13) is analogous to the pattern obtained with the exon 1–specific probe in **(A)**. Note the differences with respect to the smaller, less abundant transcripts detected with RNA from green leaves (asG) in **(A)**. The antisense transcript probe used here was identical to that used in the RNase protection assay and comprised 241 nucleotides of the intron and 287 nucleotides of the first exon. The filters were exposed 30 times longer in the case of RNA from normal chloroplasts (asG) compared to RNA from mutant plastids to obtain bands of comparable intensity.

(C) Composition of the *rpoA* operon beginning with the *trnI* (CAU) gene in one of the two inverted repeat regions of the choroplast genome and ending with the *rpoA* gene at its distal end. The localization of the *rpl2* gene is shown (a second copy of *rpl2* is located in the corresponding incomplete operon comprising only the genes *trnI* to *rps19* in the other inverted repeat region). The genes *orf137* and *trnH* (GUG) are oppositely oriented. The arrows L2a and L2b designate positions and orientations of the primers used for amplification of cDNAs. Arrows A and B designate the positions and lengths of antisense RNA probes used in RNA gel blot hybridizations (parts **(A)** and **(B)** of this figure, respectively).

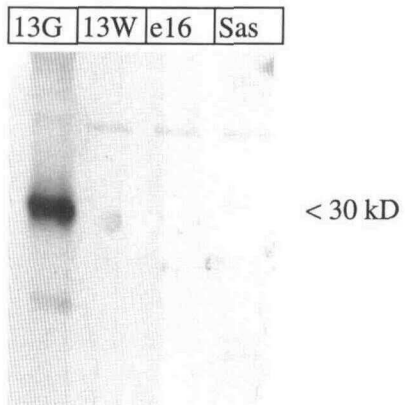

Figure 3. Immunostaining for Chloroplast Ribosomal Protein L2 Using a Specific Antiserum.

The size of the chloroplast ribosomal protein L2 (30 kD) is indicated. Protein extracts from green leaves of the *albina*-d13 mutant (13G) were taken as positive control. Equal amounts of protein per lane were probed with an L2-specific antiserum. 13 W, white leaves of *albina*-d13; e16, *albina*-e16; Sas, *Saskatoon*.

Another feature of ribosome-deficient plastids is the lack of accumulation of rRNAs. Therefore, we checked the level of rRNAs in the mutant plastids. In this experiment, a very small amount of 23S rRNA and none of the shorter rRNA species could be detected (Figure 4), which was also the case for the 16S rRNA (data not shown), hence confirming the deficiency in the plastid translational apparatus in the mutants that we analyzed. Thus, the aberrant *rpl2* transcript accumulation pattern found in these mutants could be linked to the ribosome deficiency.

rpl2 Transcript Remains Unspliced in Ribosome-Deficient Plastids

The *rpl2* gene of barley comprises 1485 bp and contains an intron of 663 bp, i.e., the gene and intron are exactly the same size as in maize chloroplasts. The barley and maize *rpl2* genes are 98.5% homologous in their exon regions and 98.9% in their intron sequences (EMBL accession number X78185 and B. Hoch, unpublished data). A 663-nucleotide unspliced intron in the *rpl2* gene could provide a possible explanation for the length difference shown in Figure 2 for the second largest transcript accumulating in mutant plastids when compared to wild-type chloroplasts. Accordingly, this band would represent a spliced and partially cleaved *rpl2* transcript in the case of normal chloroplasts, but an unspliced and partially cleaved transcript in the case of ribosome-deficient plastids. The third largest band would contain a several-times cleaved but still unspliced transcript in both types of plastids. In view of the very complex transcript pattern observed in Figure 2A when only a 104-nucleotide long and single-stranded transcript probe specific for the first exon of *rpl2* was used, we decided to test the possibility of impaired splicing by two independent and very sensitive methods, an RNase protection assay and PCR. For RNase protection, a labeled 528-nucleotide antisense transcript was generated from a homologous template obtained by PCR. After hybridization to RNA samples from green and white *albostrians* material, digestion was performed with three different concentrations of RNase (Figure 5). The observed bands show the presence of unspliced precursor RNA (522 nucleotides long) for *rpl2* containing the intron in both mutant and in green plastids. Two RNase protection assay products of shorter length were found in the case of RNA from green material. These two bands represent the protected regions of the spliced exon 1 and the free intron and, therefore, indicate

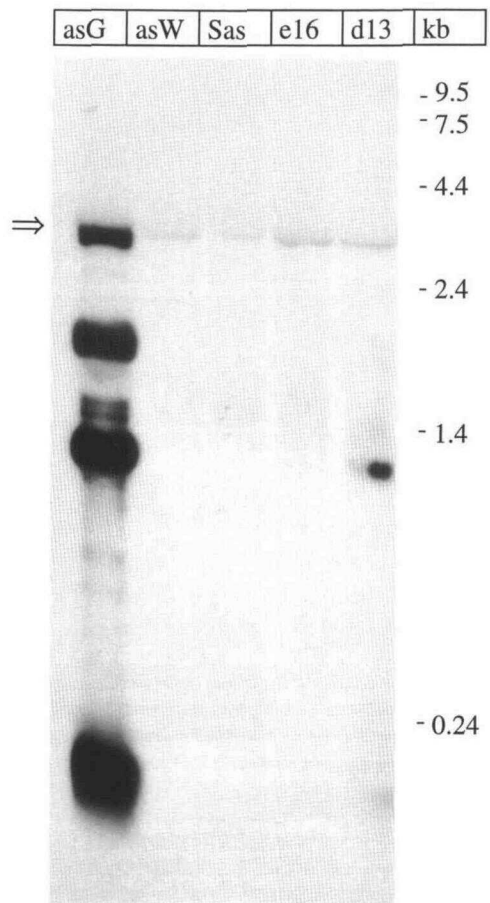

Figure 4. RNA Gel Blot Hybridization with Chloroplast rRNA.

Equal amounts of RNA per lane were hybridized with a 5.5-kb fragment of pHvC203 (Soegard and von Wettstein, 1987) containing 23S, 4.5S, and 5S rRNAs of barley chloroplasts. The full-length 23S rRNA is labeled with an arrow. RNA length standards (Gibco-BRL) are given in kilobases at right. Abbreviations are as given in the legend to Figure 2.

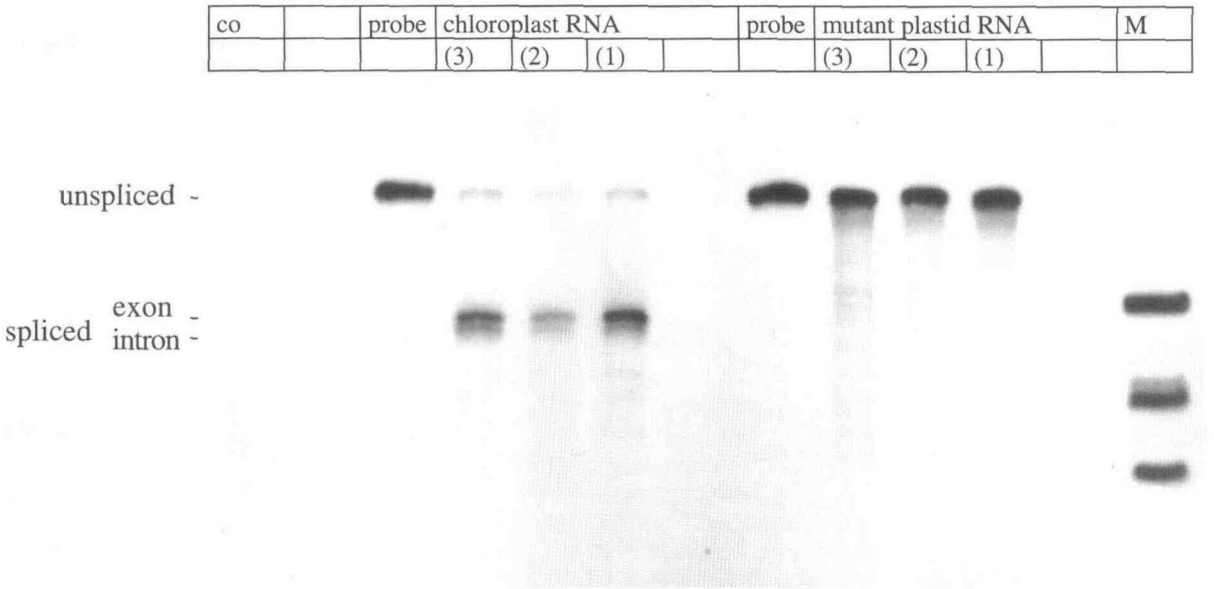

Figure 5. RNase Protection Analysis of *rpl2* Splicing in Chloroplasts and in *albostrians* Mutant Plastids.

A radiolabeled, single-stranded antisense probe spanning the 5′ splice junction (probe B in Figure 2C) was hybridized to total RNA of green and white *albostrians* mutant leaves. The hybridized RNAs were then treated each with the following concentrations of RNase: (1) 1.4 µg of RNase A and 5 units of RNase T1, (2) 3.5 µg of RNase A and 12.5 units of RNase T1, (3) 7 µg of RNase A and 25 units of RNase T1. As a control, hybridization to yeast tRNA was included (co). Single-stranded RNAs of 330, 224, and 173 bases synthesized in vitro were used as size markers (M).

splicing in the green chloroplasts. The lower abundance of the band corresponding in size to the intron directly reflects the different quantity of the respective RNA species. In addition, these bands cannot be found with RNA from white *albostrians* plastids (Figure 5). As a control, we used the sense transcript for RNase protection. No protected fragments were obtained in this case, indicating the correct conditions for RNase protection and the absence of antisense transcripts from this part of the plastid genome (data not shown).

The RNase protection assay gives a sensitive, direct reflection of the abundance of the different RNA molecules. We included PCR amplification as an even more sensitive approach to search for trace amounts of spliced *rpl2* transcripts in the mutant material. For PCR, RNA from mutant and from normal plastids was reverse transcribed and subsequently amplified using one primer located in the second exon of *rpl2* and one located 5′ to exon 1. By using this approach with cDNA derived from mutant plastids, only amplification products corresponding in size to the intron-containing *rpl2* transcripts but not to a spliced *rpl2* transcript were detectable (Figure 6). An amplified cDNA corresponding to the expected length (604 bp) of a spliced *rpl2* transcript was obtained with reverse transcribed RNA from normal chloroplasts (lane containing asG in Figure 6).

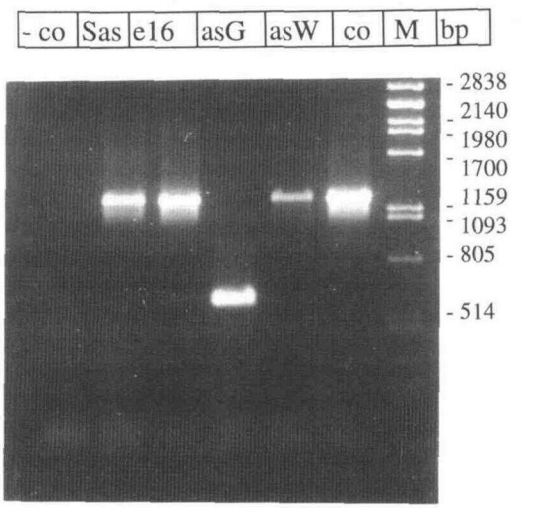

Figure 6. Amplification Products Obtained from *rpl2* Transcripts by Reverse Transcriptase-PCR.

RNA from white mutant plastids (*Saskatoon*, Sas; *albina*-e16, e16; *albostrians*, asW), *albostrians* normal green chloroplasts (asG), and DNA (co) served as templates; a buffer control reaction is labeled (−co). The primers used are designated L2a and L2b, respectively, in Figure 2C. They are identical to those described by Hoch et al. (1991). Lane M contains marker bands of PstI-digested bacteriophage λ DNA. Their lengths are indicated in base pairs at right.

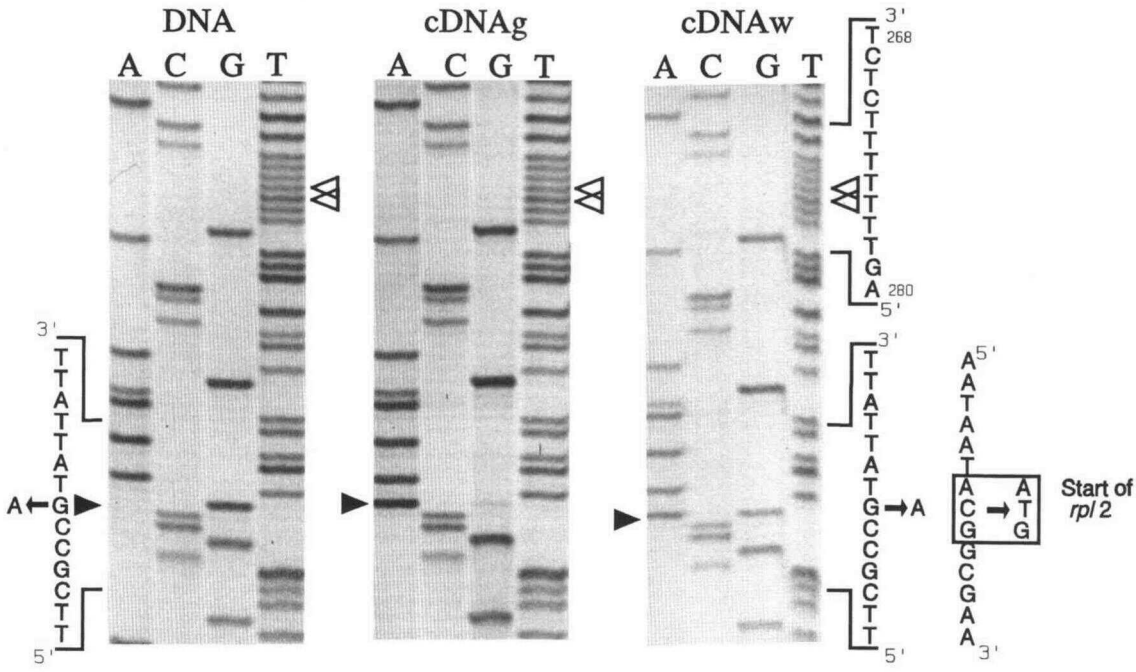

Figure 7. Sequencing Autoradiograms Representing the Barley *rpl2* Region Comprising the Edited Start Codon.

The *rpl2* start codon in barley chloroplasts was modified by RNA editing. The sequenced amplification products were obtained by PCR from *albostrians* white mutant plastids (cDNAw), phenotypically normal *albostrians* green chloroplasts (cDNAg), and barley DNA (DNA), as shown in Figure 6. The sequence complementary to the mRNA is shown because of the polarity of the primer used for sequencing. The editing position showing a G-to-A transition is marked by filled triangles; a sequence deviation between maize and barley in the region 5′ to *rpl2* is marked by open triangles. The sequence of the barley chloroplast *rpl2* gene is accessible from the EMBL data bank as accession number X78185.

Unspliced *rpl2* Transcripts of Ribosome-Deficient Plastids Are Edited

The cDNAs shown in Figure 6 for the mutant plastids amplified from unspliced RNA precursors could theoretically also be derived from contaminating plastid DNA. We used an independent approach provided by RNA editing to check if these bands were indeed amplified from unspliced RNA. The genomically encoded start codon of the maize *rpl2* gene is ACG, which is post-transcriptionally edited to an AUG codon (Hoch et al., 1991). The same situation is observed in barley (DNA in Figure 7). Thus, editing could be used to differentiate between DNA and cDNA (mRNA) as template for PCR. The editing observed by direct sequencing of the cDNA amplification product from ribosome-deficient plastids shows that an *rpl2* transcript must have served as primary template for the production of the unspliced cDNA (Figure 7). The partial editing of ~60 to 70% is in accordance with the partial editing found for the unspliced *rpl2* transcript from chloroplasts of maize (Freyer et al., 1993).

Splicing of Several Other Transcripts Is Not Impaired in Ribosome-Deficient Plastids

To check whether the splicing defect is specific to the *rpl2* transcript, we tested a series of transcripts encoded by several other intron-containing plastid genes. First, we considered the *irf170*-encoded transcript as a promising candidate, because this widely conserved ORF contains two introns and has a complex processing pattern, and its transcripts accumulate in ribosome-deficient plastids (Figure 8A). The pattern found with normal chloroplasts is very similar to the one described for maize leaves, and likewise the patterns of mutant plastids resemble in general that of the maize leaf rather than that of the maize endosperm (McCullough et al., 1992). The results in Figure 8B show the presence of spliced mRNAs lacking both introns in the ribosome-deficient plastids. The strongly accumulating transcript of 580 nucleotides in Figure 8A most probably represents the fully processed *irf170* transcript, confirming an intact splicing mechanism of *irf170* in the mutant plastids.

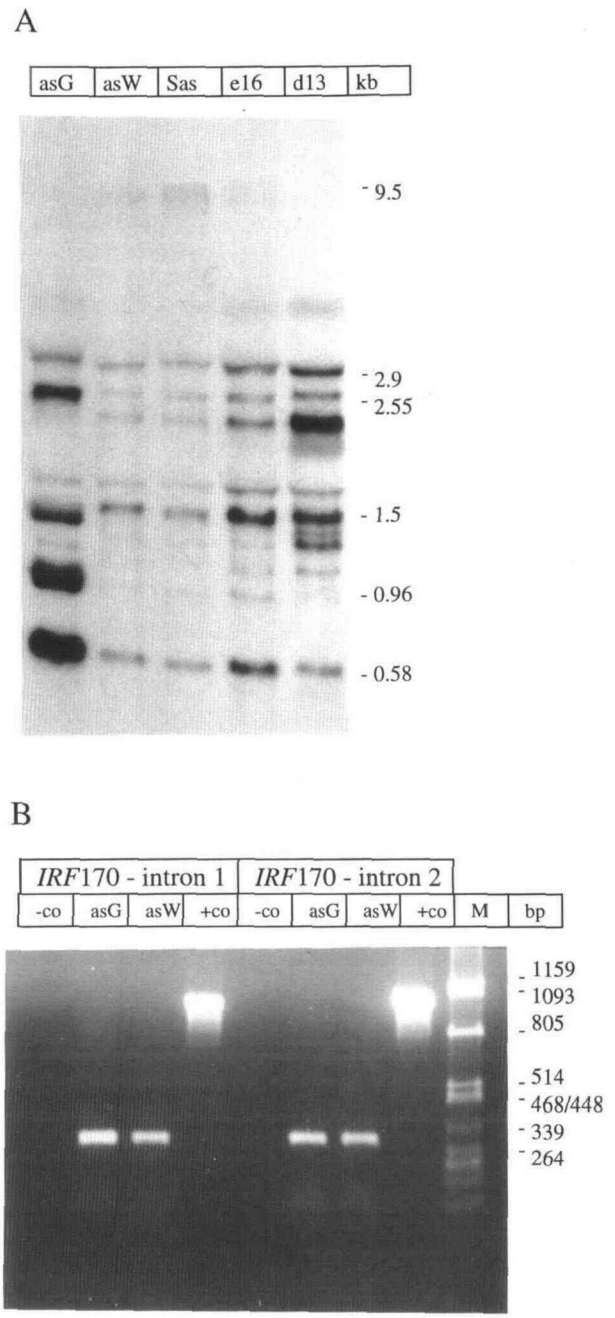

Figure 8. Transcript Accumulation Pattern of *irf170* and Products of Reverse Transcriptase-PCR.

(A) A 205-nucleotide antisense transcript probe complementary to exon 2 of barley *irf170* detected a complex RNA processing pattern, differing in presence and stoichiometry of several individual bands between RNA of normal chloroplasts (asG) and RNA of mutant plastids, but also between the individual mutants (*albostrians*, asW; *Saskatoon*, Sas; *albina*-e16, e16; *albina*-d13, d13). The lengths of the main transcripts that accumulated were determined according to an RNA length standard and are given at right in kilobases.

(B) Amplification products obtained from PCR using either primers in the first and second exon, including intron 1, or in the second and third exon, thereby including intron 2. It should be noted that absence of large amplification products is not an indication of a very low level of unspliced transcript precursors, because generation of shorter products is usually favored during PCR. Abbreviations are as given in the legend to Figure 6, (+co) is a positive control with DNA as template. Lengths of selected PstI-cleaved bacteriophage λ DNA fragments are indicated in base pairs (lane M).

The plastid operon containing *rpl2* also includes the class II intron–containing gene for ribosomal protein L16 (*rpl16*). Similar to the *rpl2* and *irf170* transcripts, we used PCR to test whether this transcript and five other transcripts containing class II introns and the only class I intron present in plastid DNA of higher plants are spliced in the *albostrians* mutant plastids. These transcripts were transcribed from the genes for the ribosomal protein S16 (*rps16*), NADH dehydrogenase subunit ND2 (*ndhB*), cytochrome *f* (*petD*), the tRNA for leucine, (*trnL* [UAA]), and from *rpl16*. At least three PCR experiments using RNAs independently isolated and reverse transcribed were performed. The results clearly indicated that none of these five additionally tested intron-containing transcripts showed impaired splicing.

Unfortunately, we failed to get reproducible amplification products from reverse-transcribed transcripts of *ndhA*, encoding NADH dehydrogenase ND1 (the only intron-containing gene in the small single-copy region), *atpF*, encoding subunit 1 of ATPase, and of transfer RNA for lysine (*trnK*). The latter contains in its intron *orf542* (rice), or *orf504* (barley), respectively, which are homologous to fungal mitochondrial maturases. These results are consistent with data from RNA gel blot hybridizations showing that these transcripts did not accumulate in ribosome-deficient plastids (data not shown).

DISCUSSION

In view of the high conservation of chloroplast genes, the 17 group II introns and the single group I intron found in the chloroplast genome of rice (Figure 1; Hiratsuka et al., 1989) and maize (R. Maier and H. Kössel, unpublished data) are expected to reside at identical positions in barley. It has been proposed previously that transcription of genes in plastids lacking ribosomes is mediated by a nuclear-encoded RNA polymerase (Falk et al., 1993; Hess et al., 1993), whereas transcriptional activity in green chloroplasts might be a result of the RNA polymerase encoded by the plastid *rpo* genes (cf. Mullet, 1993). To date, it is not known how alterations in the plastid transcript accumulation pattern observed in such systems (Han et al., 1993; Hess et al., 1993) and in nuclear mutants with defective chloroplast polysome assembly (but still containing ribosomes; Barkan, 1993) depend on the activity of nuclear or of plastid

genes and which RNA processing activities are missing or changed, respectively. If several transcripts of intron-containing genes show qualitative differences, a more general splicing defect could be involved.

Our results demonstrated that in *albostrians* ribosome-deficient plastids, the intron of at least one transcript, namely that of the *rpl2* gene, remains unspliced or is spliced with an efficiency too low even for detection by PCR or RNAse protection assays. The unspliced transcript was found to be edited, which confirms and extends our previous observation that editing in chloroplasts is independent of plastid translation (Zeltz et al., 1993) and of splicing (Freyer et al., 1993; Ruf et al., 1994).

All other investigated intron-containing transcripts were found to be spliced. Despite the unimpaired splicing, the pattern of *irf170* transcripts clearly differed between normal chloroplasts and ribosome-deficient plastids. Obviously, the mutations affect other transcript processing steps, too. Hence, such altered processing steps different from intron splicing might also contribute to the differences in the pattern of *rpl2* transcripts between chloroplasts and mutant plastids. Among the normally spliced transcripts was one containing the only group I chloroplast intron, and with *rpl16* even a gene belonging to the same operon as *rpl2*. Hence, we can rule out the possibility that the impaired splicing is specific for ribosomal protein–coding transcripts, classes of introns, or a certain primary transcript. Obviously, splicing of the *rpl2* transcript requires a factor that is lacking in the mutant plastids but is not necessary for splicing of the other seven introns studied. The product of the nuclear *albostrians* allele is not known. Thus, the defect in *rpl2* transcript splicing reported here could theoretically be caused by this allele if it would encode a peptide specifically involved in the splicing of this transcript, as was observed for several nuclear gene mutants in Chlamydomonas (Rochaix, 1992). Alternatively, the splicing deficiency could result from the ribosome deficiency resulting from a missing plastid DNA–encoded peptide component.

We identified three nonallelic mutants (*Saskatoon*, *albina*-d13, and *albina*-e16; cf. Henningsen et al., 1993) with a plastid phenotype similar to the *albostrians* mutant. It is not known whether *Saskatoon* and *albostrians* are allelic. All four mutants lack plastid ribosomes entirely or accumulate them only to an undetectable low level, and they also have the same *rpl2* splicing-deficient phenotype. The striking correlation between plastid ribosome deficiency and impaired splicing of the *rpl2* transcript may be explained in different ways. A hypothetical regulatory effect of these ribosome-deficient plastids resulting in a repression of a nuclear-encoded peptide essential for *rpl2* transcript splicing can hardly be expected, because such a plastid-derived signal chain seems to be specific for the expression of nuclear-encoded components of photosystems and Calvin cycle only (Hess et al., 1994). A direct effect of the nuclear mutations also appears very unlikely, because at least three of the four mutations are positioned at different loci. It is hard to imagine that mutations at three or four different loci would specifically lead to a defect in removal of just one intron.

Instead, the results are best explained if the removal of the *rpl2* intron, but not of seven other introns, is dependent on the presence of plastid ribosomes. Although we cannot exclude the possibility that just the presence of ribosomes (e.g., by attachment to the pre-mRNA) would be sufficient for splicing the *rpl2* transcript, we favor the idea that it is the missing translational activity in the plastids of these mutants that causes the defect in splicing. This in turn implies a role of a chloroplast gene product in *rpl2* transcript splicing. On one hand, this role could be played by an RNA. RNAs are involved in splicing of nuclear pre-mRNAs as components of the spliceosome; also, *trans*-splicing of the transcript for the photosystem I P700 apoprotein A1 (*psaA*) in Chlamydomonas requires a specific RNA transcribed from the chloroplast genome (Goldschmidt-Clermont et al., 1991). Such an RNA specifically involved in splicing of the *rpl2* transcript might not be transcribed or might not accumulate in ribosome-deficient plastids. On the other hand, a plastome-encoded protein could be essential for *rpl2* transcript splicing, e.g., the L2 protein itself. The quantitatively high level of *rpl2* transcripts in plastids lacking the encoded protein is remarkable in this context. Feedback mechanisms are known from the regulation of expression of genes encoding ribosomal proteins in bacteria (Nomura et al., 1984) and were also suggested for the nuclear *rpl2* gene of yeast (Presutti et al., 1991). However, as far as splicing is concerned, the opposite situation was reported for the eukaryotic ribosomal proteins L1 and L32 in Xenopus and yeast, respectively. These proteins seem to prevent splicing of their own pre-mRNA when present in high amounts (Bozzoni et al., 1984; Dabeva et al., 1986).

An attractive candidate for the missing splicing factor, however, is the gene product of the conserved *orf542/504*, which then would have to act in *trans*. According to sequence homologies, *orf542/504* carries the information for a protein with homology to maturases encoded by mitochondrial introns of fungi and higher plants (Neuhaus and Link, 1987). Recently, its expression at the polypeptide level has been shown (Jardin et al., 1994). Support for the idea of an important function of the *orf542/504*–encoded protein comes from the analysis of *E. virginiana* plastid DNA. In the plastome of this achlorophyllous parasitic plant, only four introns have been retained, among them that of *rpl2*. Strikingly, *orf542/504* (designated *matK* in *E. virginiana*) is also present in this reduced plastid genome, even though the two exons of *trnK* are deleted (Wolfe et al., 1992). Our attempts to analyze splicing of the *trnK* precursor transcript in ribosome-deficient plastids were not successful because of the low transcript level. However, because techniques for chloroplast transformation have now been developed (Maliga et al., 1993), inactivation of *orf542/504* and *rpl2* in vivo should be feasible and worth attempting.

METHODS

Plant Material

Seeds of the barley (*Hordeum vulgare*) mutants *Saskatoon*, *albina*-d13, and *albina*-e16 were obtained from D. von Wettstein (Carlsberg

Laboratory Copenhagen, Denmark). *albina*-d13 and *albina*-e16 are nuclear mutants expressing an albina phenotype (Henningsen et al., 1993). The lines *albostrians* and *Saskatoon* represent nuclear gene–induced plastome mutants (Arnason and Walker, 1949; Hagemann and Scholz, 1962). In both lines, a nuclear mutant allele leads to pure white and green-white striped seedlings among normal green seedlings. Only white tissues contain ribosome-deficient plastids. The material was therefore carefully checked to avoid contamination with small areas of white or green tissue, respectively. Seedlings were raised and harvested as previously described (Hess et al., 1993).

Protein Extraction and Gel Blot Analysis

Total cellular proteins were isolated by grinding 100 mg of leaf material in liquid nitrogen and extraction into 400 μL of 50 mM sodium-phosphate buffer, pH 7.0, containing 2 mM sodium bisulfite and 1 mM phenylmethylsulfonyl fluoride, supplemented with 0.1% insoluble polyvinylpyrrolidone. Samples were mixed with SDS sample buffer, and 20 μL of the samples per lane were electrophoretically separated on 12% polyacrylamide–SDS gels (Laemmli, 1970). The proteins were transferred to nylon membranes (Hybond-N, Amersham) by electrotransfer in a semidry blotter (MilliBlot-SDE, Millipore, Eschborn, Germany) with a constant current of 2.5 mA/cm^2 for 45 min. An antiserum against purified chloroplast ribosomal protein L2 of spinach (kindly provided by A. Subramanian, Max-Planck-Institute for Molecular Genetics, Berlin, Germany) was used for immunostaining of the protein gel blots (Hess et al., 1993).

Preparation and Manipulation of Nucleic Acids

Total barley DNA was isolated following the protocol of Rogers and Bendich (1985). Total RNA was isolated according to Paulsen and Bogorad (1988).

Equal amounts of RNA (20 μg) per lane were electrophoretically separated on 1.5% agarose–formaldehyde gels and transferred to nylon membranes (Hybond-N) by capillary transfer following standard protocols (Sambrook et al., 1989). The conditions of hybridization and of probe labeling were the same as described previously (Hess et al., 1992).

For reverse transcription, RNAs were precipitated twice with LiCl, treated with DNase I, extracted with phenol–chloroform, and ethanol precipitated. Polymerase chain reaction (PCR) without a previous step of reverse transcription was performed to test for possibly contaminating traces of DNA. Reverse transcription was performed according to Maier et al. (1992), and PCR was conducted as described by Zeltz et al. (1993). Products of amplification were purified by Spinbind extraction units (FMC BioProducts, Rockland, ME) and then directly analyzed in sequencing reactions using the modified chain termination method described in Bachmann et al. (1990).

Generation of RNA Probes and Ribonuclease Protection Assay

A 104-bp fragment of the first exon of the maize chloroplast gene for ribosomal protein L2 (*rpl2*), a 233-bp fragment of the second exon of barley *irf170* cloned in pBluescript KS+, and products amplified by PCR from barley chloroplast DNA by the inclusion of the bacteriophage T7 promoter in one of the primers served as templates for the generation of single-stranded RNA probes. The primers are described in the list of oligonucleotids (see below).

In vitro transcription was performed at 15°C as described in the manufacturer's protocol for the MAXIscript kit (Ambion Inc., Austin, TX). Reaction products were then separated on 4% sequencing gels, and full-length transcripts were cut out and eluted. These RNAs (cf. Figure 2C) were then used for RNase protection and as probes in RNA gel blot hybridization.

For the RNase protection assay, 30 μg of RNA was hybridized with each 1 to 3 × 10^5 cpm of labeled antisense and sense (negative control) transcripts. After digestion with RNase A and T1 and precipitation, products of RNase protection containing a comparable amount of incorporated radioactive label were separated on polyacrylamide-urea sequencing gels. The other steps were essentially as described in the protocol supplied with the RNase protection kit (Boehringer Mannheim).

Oligonucleotides Used in This Study

The following oligonucleotides were synthesized on DNA synthesizers (model no. 394, Applied Biosystems, Foster City, CA; model Oligo 1000, Beckman Inc., Fullerton, CA) and used for amplification of specific fragments by PCR. The sequences were derived from sequenced parts of the barley chloroplast genome that were available; in the other cases sequences were from rice and maize. The location of these sequences is according to the published sequence of rice chloroplast DNA (Hiratsuka et al., 1989) as follows: *rpl16*, 5′-CCAATTCGAACTATCTATGGAGTATTAGGAGTT-3′ (nucleotides 79,374 to 79,406; coding region of *rps3*); and 5′-CCCATACGTGTTTCGGTCGGTC-3′ (nucleotides 77,889 to 77,910; second exon); *rps16*, 5′-CGTTTAAAACGATGTGGTAGAAAGCAAC-3′ (5514 to 5541; first exon); and 5′-GTTGAGCACCTTTTTCAAGGAAATAGAG-3′ (4558 to 4585; second exon); *rpl2*, 5′-CCGGGTTATTCTATTCCACTTCTAGA-3′ (82,699 to 82,724; coding region of *rpl23*, primer L2a in Figure 2C); 5′-CCAAACGGACCTCCCCAGATGG-3′ (81,458 to 81,479; second exon, primer L2b in Figure 2C); *irf170*, 5′-GATAAGACCTTCTCAATTGTAGCC-3′ (43,781 to 43,804; exon 1); 5′-GCTTTGCAAAATTATTATGAAGCTACGCG-3′ (42,905 to 42,933; exon 2); 5′-TAATGACAGATCACGGCCATATTATTA-3′ (42,740 to 42,766; exon 2, antisense); and 5′-CCAGTTCTGTGCTTCAATATAATTTC-3′ (41,884 to 41,909; exon 3, antisense); *ndhB*, 5′-GGGGGAGATCGAGCTTCAAG-3′ (87,003 to 87,022; exon 1) and 5′-ATCCTGCATAATCTCGAATG-3′ (85,769 to 85,788; exon 2); cytochrome *b/f* complex subunit 4 (*petD*), 5′-TACCTCTGCTTACTGCCG-3′ (72,614 to 72,631; coding region of cytochrome B6, *petB*) and 5′-CCGTTGGCACAGAAACC-3′ (73,929 to 73,946; exon 2).

The *trnL* oligonucleotides, including the partial degeneration of the first, were chosen according to Kuhsel et al. (1990). They also fit to the corresponding gene of other, including some prokaryotic species, but not to the *trnL* (UAG) of the chloroplast genome. Sequences are as follows: *trnL* (UAA), 5′-TGG(C/T)GAAAT(C/T)GGTAGACGC(A/T)-(A/G)CGGAC-3′ (nucleotides 46,565 to 46,590; first exon) and 5′-TGGGGATAGAGGGACTTGAACCCTCACGA-3′ (nucleotides 47,154 to 47,182; second exon).

Oligonucleotides used to amplify *rpl2* templates included two of the four primers used for the amplification of barley *rpl2* templates. They contained the complete bacteriophage T7 promoter in their 5′-terminal part in addition to the sequence complementary to the target DNA. Therefore, amplification products were suitable for the direct generation of single-stranded RNA probes for ribonuclease protections assays and RNA gel blot hybridizations. These primers (nucleotides 433 to 462 and 931 to 956 in barley *rpl2*, EMBL accession number X78185) are as follows: 5′-GGATCCTAATACGACTCACTATAGGGAGGCACCGTTGTGGTAAAGGTCGTAATTCCAGA-3′ (sense, located in *rpl2* exon 1) and 5′-GGATCCTAATACGACTCACTATAGGGAGGCCGGTTCTGTGCGTGCTTCAAACAAT-3′ (antisense, located in the intron). The

corresponding primers on the opposite end that did not contain a T7 promoter are 5′-CCGGTTCTGTGCGTGCTTCAAACAAT-3′ (antisense) and 5′-CACCGTTGTGGTAAAGGTCGTAATTCCAGA-3′ (sense).

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REFERENCES

Arnason, T.J., and Walker, G.W.R. (1949). An irreversible gene-induced plastid mutation. Can. J. Res. **27C,** 172–178.

Bachmann, B., Lueke, W., and Hunsmann, G. (1990). Improvement of PCR amplified DNA sequencing with the aid of detergents. Nucl. Acids Res. **18,** 1309.

Barkan, A. (1993). Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell **5,** 389–402.

Börner, T., and Sears, B. (1985). Plastome mutants. Plant Mol. Biol. Rep. **4,** 69–92.

Bozzoni, I., Fragapane, P., Annesi, F., Pierandrei-Amaldi, P., Amaldi, F., and Beccari, E. (1984). Expression of two *Xenopus laevis* ribosomal protein genes in injected trop oocytes. J. Mol. Biol. **180,** 987–1005.

Cech, T.R. (1986). The generality of self splicing RNA: Relationship to nuclear mRNA splicing. Cell **44,** 207–210.

Dabeva, M.D., Post-Beitenmiller, M.A., and Warner, J.R. (1986). Autogenous regulation of splicing of the transcript of a yeast ribosomal protein gene. Proc. Natl. Acad. Sci. USA **83,** 5854–5857.

Falk, J., Schmidt, A., and Krupinska, K. (1993). Characterization of plastid DNA transcription in ribosome deficient plastids of heat-bleached barley leaves. J. Plant Physiol. **141,** 176–181.

Ferat, J.L., and Michel, F. (1993). Group II self-splicing introns in bacteria. Nature **364,** 358–361.

Freyer, R., Hoch, B., Neckermann, K., Maier, R.M., and Kössel, H. (1993). RNA editing in maize chloroplasts is a processing step independent of splicing and cleavage of monocistronic mRNAs. Plant J. **4,** 621–629.

Goldschmidt-Clermont, M., Choquet, Y., Girard-Bascou, J., Michel, F., Schirmer-Rahire, M., and Rochaix, R.D. (1991). A small chloroplast RNA may be required for *trans*-splicing in *Chlamydomonas reinhardtii.* Cell **65,** 135–143.

Hagemann, R., and Scholz, F. (1962). Ein Fall Gen-induzierter Mutationen des Plasmotyps bei Gerste. Der Züchter **32,** 50–59.

Han, C.D., Patrie, W., Polacco, M., and Coe, E.H. (1993). Aberrations in plastid transcripts and deficiency of plastid DNA in striped and albino mutants in maize. Planta **191,** 552–563.

Henningsen, K.W., Boynton, J.E., and von Wettstein, D. (1993). Mutants at *xantha* and *albina* loci in relation to chloroplast biogenesis in barley (*Hordeum vulgare* L.) (Copenhagen, Denmark: The Royal Danish Academy of Sciences and Letters, Munksgaard Copenhagen).

Hess, W.R., Schendel, R., Rüdiger, W., Fieder, B., and Börner, T. (1992). Components of chlorophyll biosynthesis in a barley albina mutant unable to synthesize delta-aminolevulinic acid by utilizing the transfer RNA for glutamic acid. Planta **188,** 19–27.

Hess, W.R., Prombona, A., Fieder, B., Subramanian, A.R., and Börner, T. (1993). Chloroplast *rps15* and the *rpoB/C1/C2* gene cluster are strongly transcribed in ribosome-deficient plastids: Evidence for a functioning non-chloroplast encoded RNA polymerase. EMBO J. **12,** 563–571.

Hess, W., Müller, A., Nagy, F., and Börner, T. (1994). Ribosome-deficient plastids affect transcription of light-induced nuclear genes: Genetic evidence for a plastid-derived signal. Mol. Gen. Genet. **242,** 305–312.

Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., Meng, B.Y., Li, Y.Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K., and Sugiura, M. (1989). The complete sequence of the rice (*Oryza sativa*) chloroplast genome: Intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol. Gen. Genet. **217,** 185–194.

Hoch, B., Maier, R.M., Appel, K., Igloi, G.L., and Kössel, H. (1991). Editing of a chloroplast mRNA by creation of an initiation codon. Nature **353,** 178–180.

Jacquier, A. (1990). Self-splicing group II and nuclear pre-mRNA introns: How similar are they? Trends Biochem. Sci. **15,** 351–354.

Jardin, P., Portetelle, D., Harvengt, L., Dumont, M., and Wathelet, B. (1994). Expression of intron-encoded maturase-like polypeptides in potato chloroplasts. Curr. Genet. **25,** 158–163.

Kenell, J.C, Moran, J.V., Perlman, P.S., Butow, R.A., and Lambowitz, A.M. (1993). Reverse transcriptase activity associated with maturase-encoding group II introns in yeast mitochondria. Cell **73,** 133–146.

Kuhsel, M.G., Strickland, R., and Palmer, J.D. (1990). An ancient group I intron shared by eubacteria and chloroplasts. Science **250,** 1570–1573.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lambowitz, A.M., and Perlman, P.S. (1990). Involvement of aminoacyl-tRNA synthetases and other proteins in group I and group II intron splicing. Trends Biochem. Sci. **15,** 440–444.

Maier, R.M., Hoch, B., Zeltz, P., and Kössel, H. (1992). Internal editing of the maize chloroplast *ndhA* transcript restores codons for conserved amino acids. Plant Cell **4,** 609–616.

Maliga, P., Carrer, H., Kanevski, I., Staub, J., and Svab, Z. (1993). Plastid engineering in land plants: A conservative genome is open to change. Phil. Trans. R. Soc. Lond. **342,** 203–208.

McCullough, A.J., Kangasjarvi, J., Gengenbach, B.G., and Jones, R.J. (1992). Plastid DNA in developing maize endosperm. Plant Physiol. **100,** 958–964.

Michel, F., Umesono, K., and Ozeki, H. (1989). Comparative and functional anatomy of group II catalytic introns—A review. Gene **82,** 5–30.

Mullet, J.E. (1993). Dynamic regulation of chloroplast transcription. Plant Physiol. **103,** 309–313.

Neuhaus, H., and Link, G. (1987). The chloroplast *tRNALys* (UUU) gene from mustard (*Sinapis alba*) contains a class II intron potentially coding for a maturase-related polypeptide. Curr. Genet. **11,** 251–257.

Nierhaus, K. (1982). Structure, assembly, and function of ribosomes. Curr. Top. Microbiol. Immunol. **97,** 81–155.

Nomura, M., Gourse, R., and Baugham, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. **53,** 75–118.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H., and Ozeki, H. (1986). Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322, 572–574.

Paulsen, H., and Bogorad, L. (1988). Diurnal and circadian rhythm in the accumulation and synthesis of mRNA for the light-harvesting chlorophyll *a/b* binding protein in tobacco. Plant Physiol. **88,** 1104–1109.

Presutti, C., Ciafre, S.A., and Bozzoni, I. (1991). The ribosomal protein L2 in *S. cerevisiae* controls the level of accumulation of its own mRNA. EMBO J. **10,** 2215–2221.

Rochaix, J.D. (1992). Post-transcriptional steps in the expression of chloroplast genes. Annu. Rev. Cell Biol. **8,** 1–28.

Rogers, S.O., and Bendich, A.I. (1985). Extraction of DNA from milligram amounts of fresh, herbarium, and mummified plant tissues. Plant Mol. Biol. **5,** 69–76.

Ruf, M., and Kössel, H. (1988). Structure and expression of the gene coding for the α-subunit of DNA-dependent RNA polymerase from the chloroplast genome of *Zea mays*. Nucl. Acids Res. **16,** 5741–5754.

Ruf, S., Zeltz, P., and Kössel, H. (1994). Complete RNA editing of unspliced and dicistronic transcripts of the intron-containing reading frame *irf170* from maize chloroplasts. Proc. Natl. Acad. Sci. USA **91,** 2295–2299.

Saldanha, R., Mohr, G., Belfort, M., and Lambowitz, A.M. (1993). Group I and group II introns. FASEB J. **7,** 15–24.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sexton, T.B., Jones, J.T., and Mullet, J.E. (1990). Sequence and transcriptional analysis of the barley ctDNA region upstream of *psb*D-*psb*C encoding *trn*K(UUU), *rps*16, *trn*Q(UUG), *psb*K, *psb*I, and *trn*S(GCU). Curr. Genet. **17,** 445–454.

Sharp, P.A. (1985). On the origin of RNA splicing and introns. Cell **42,** 397–400.

Sharp, P.A. (1991). "Five easy pieces." Science **254,** 663.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, H., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Shimada, A., and Sugiura, M. (1986). The complete nucleotide sequence of the tobacco chloroplast genome: Its gene organization and expression. EMBO J. **5,** 2043–2049.

Soegard, P., and von Wettstein-Knowles, P. (1987). Barley: Genes and chromosomes. Carlsberg Res. Commun. **52,** 123–196.

Weiner, A.M. (1993). mRNA splicing and autocatalytic introns: Distant cousins or the products of chemical determinism? Cell **72,** 161–164.

Wolfe, K.H., Morden, C.W., Ems, S.C., and Palmer, J.D. (1992). Rapid evolution of the plastid translational apparatus in a nonphotosynthetic plant: Loss or accelerated sequence evolution of tRNA and ribosomal protein genes. J. Mol. Evol. **35,** 304–317.

Wolstenholme, D.R., Macfarlane, C.T., Beagley, C.T., Thomson, M.C., Okada, N.A., and Fauron, C.M.R. (1993). Maize mitochondrial DNA: The *nad1* gene-mat-r gene complex, a maturase-related pseudogene linked to a *nad2* exon, and *nad* gene intron interrelationships. In Plant Mitochondria, A. Brennicke and U. Kueck, eds (Weinheim, Germany: VCH Weinheim), pp. 150–161.

Zeltz, P., Hess, W.R., Neckermann, K., Börner, T., and Kössel, H. (1993). Editing of the *rpoB* transcript is independent of chloroplast translation and shows different patterns in barley and maize. EMBO J. **12,** 4291–4296.