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

Wolfgang R. Hess, Brigitte Hoch, Patrick Zeltz, Thomas Hübschmann, Hans Kössel, and Thomas Börner

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 AV, 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 rRNA 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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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 *albosstrians*, 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 *albosstrians* 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 *albosstrians* 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 *albosstrians* 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 RNA (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, *albosstrians* mutant plastids cannot produce the L2 protein (Hess et al., 1993). The mutant plastids of *albina*-d13, -e16, and *Saskatoon* share this feature with *albosstrians* 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 trnl (CAU) gene in one of the two inverted repeat regions of the chloroplast 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 trnl 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).
rp\textsubscript{2} Transcript Remains Unspliced in Ribosome-Deficient Plastids

The rp\textsubscript{2} 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 rp\textsubscript{2} 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 rp\textsubscript{2} 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 rp\textsubscript{2} 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 rp\textsubscript{2} 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 \textit{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 \textit{rp}\textsubscript{2} 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

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 \textit{rp}\textsubscript{2} transcript accumulation pattern found in these mutants could be linked to the ribosome deficiency.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{RNA Gel Blot Hybridization with Chloroplast rRNA.}
\end{figure}

Equal amounts of RNA per lane were hybridized with a 5.5-kb fragment of p\textit{HvC203} (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 ng of RNase A and 5 units of RNase T1, (2) 3.5 ng 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).

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).
**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).

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**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.
The plastid operon containing rp2 also includes the class II intron–containing gene for ribosomal protein L16 (rp16). Similar to the rp2 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 rp16. 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 *irf770* 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 a ribosome deficiency resulting from a missing plastid DNA–encoded peptide component.

We identified three nonallelic mutants (Saskatoon, *albina-d13*, and *albina-o16*; cf. Henningson 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 *tmK* are deleted (Wolfe et al., 1992). Our attempts to analyze splicing of the *tmK* 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-o16* 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 *abrostans* and *Saskatoon* represent nuclear gene-induced plastome mutants (Arnason and Walker, 1949; Hagemann and Scholz, 1982). 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 semidy blotter (MilliBlot-SDE, Millipore, Eschborn, Germany) with a constant current of 2.5 mA/cm² for 45 min. An antiseraum 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 corresponding gene of other, including some prokaryotic species, as follows:

**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., 1993).

For reverse transcription, RNAs were precipitated twice with LiCl, treated with DNase I, extracted with phenol–chloroform, and ethanol precipitated on 12% 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’-CCAATGCAAGATCT-ATTGAGATATTTGCTAG-3’ (nucleotides 79,374 to 79,406; coding region of rpl16); and 5’-CCCATACGCTGTGCTGCGTCC-3’ (nucleotides 77,889 to 77,910; second exon); **rps16**, 5’-CGTTTAAACCATGTTGAGAGACGCAACAC-3’ (5514 to 5541; first exon); and 5’-GTGGACACATTTCCTAGAGAAGTACGC-3’ (4558 to 4585; second exon); **rpl2**, 5’-CCGGGTCATTCTGCCATCTTAGAAGAGAGC-3’ (82,699 to 82,724; coding region of rpl23, primer L2a in Figure 2C); 5’-CACACGGCCTCAGCTACAGG-3’ (81,458 to 81,479; second exon, primer L2b in Figure 2C); **ir170**, 5’-GATTTAGAAGATCTATTCTCAATTTAGACG-3’ (43,781 to 43,804; exon 1); 5’-GCTTCTTCGAAATATTATGTTGACGTCA-3’ (42,905 to 42,933; exon 2); 5’-TATATGACAGATCACGGCCATATTATTA-3’ (47,154 to 47,182; exon 3); and 5’-CCGGTGTCCGATGGATACC-3’ (41,884 to 41,909; exon 3, antisense); mvdS, 5’-GGGGGACCATTCCATCACCAGATGCTCG-3’ (87,003 to 87,022; exon 2); 5’-ATAATGACAGATCACGGCCATATTATTA-3’ (47,154 to 47,182; exon 3); and 5’-CCGGTGTCCGATGGATACC-3’ (41,884 to 41,909; exon 3, antisense).

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**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 *ir170* 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 oligonucleotides (see below).

In vitro transcription was performed at 35°C as described in the manufacturer's protocol for the MAXscript 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 x 10^6 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 incorpored 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’-CCAATGCAAGATCT-ATTGAGATATTTGCTAG-3’ (nucleotides 79,374 to 79,406; coding region of rpl16); and 5’-CCCATACGCTGTGCTGCGTCC-3’ (nucleotides 77,889 to 77,910; second exon); **rps16**, 5’-CGTTTAAACCATGTTGAGAGACGCAACAC-3’ (5514 to 5541; first exon); and 5’-GTGGACACATTTCCTAGAGAAGTACGC-3’ (4558 to 4585; second exon); **rpl2**, 5’-CCGGGTCATTCTGCCATCTTAGAAGAGAGC-3’ (82,699 to 82,724; coding region of rpl23, primer L2a in Figure 2C); 5’-CACACGGCCTCAGCTACAGG-3’ (81,458 to 81,479; second exon, primer L2b in Figure 2C); **ir170**, 5’-GATTTAGAAGATCTATTCTCAATTTAGACG-3’ (43,781 to 43,804; exon 1); 5’-GCTTCTTCGAAATATTATGTTGACGTCA-3’ (42,905 to 42,933; exon 2); 5’-TATATGACAGATCACGGCCATATTATTA-3’ (47,154 to 47,182; exon 3); and 5’-CCGGTGTCCGATGGATACC-3’ (41,884 to 41,909; exon 3, antisense); mvdS, 5’-GGGGGACCATTCCATCACCAGATGCTCG-3’ (87,003 to 87,022; exon 2); 5’-ATAATGACAGATCACGGCCATATTATTA-3’ (47,154 to 47,182; exon 3); and 5’-CCGGTGTCCGATGGATACC-3’ (41,884 to 41,909; exon 3, antisense).
corresponding primers on the opposite end and that did not contain a T7 promoter are 5'-CCCGTTCGTCGATCCTCAAACAT-3' (antisense) and 5'-CAGCGCTGATCCTCAAACAT-3' (sense).

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