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Editing of the chloroplast *rpo*B transcript is independent of chloroplast translation and shows different patterns in barley and maize

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Sequence analysis of amplified cDNAs derived from the maize chloroplast *rpo*B transcript which encodes the β subunit of a chloroplast specific, DNA dependent RNA polymerase reveals four C-to-U editing sites clustered within 150 nucleotides of the 5′ terminal region of the *rpo*B message. These newly identified editing sites confirm the bias of chloroplast editing for certain codon transitions and for second codon positions which both appear suggestive for an involvement of the translational apparatus in the editing process. This supposition prompted us to investigate editing of the *rpo*B transcript from ribosome deficient, and hence protein synthesis deficient, plastids of the barley mutant *albostrians*. In this mutant editing is, however, not impaired at any of the editing sites functional in the barley wild type *rpo*B transcript. This demonstrates that chloroplast editing is neither linked to nor dependent on the chloroplast translational apparatus. As a further consequence any peptide components required for chloroplast editing must be encoded in the nuclear genome. In spite of strong sequence conservation only three of the four editing sites identified in the maize *rpo*B transcript are functional in barley. This indicates that sequences surrounding an editing site alone are not sufficient as determinants for the editing process in chloroplasts, but that *trans*-acting templates carrying the editing information for each individual site may also be required.

Key words: barley/maize/ribosome deficient chloroplasts/ RNA editing/translational apparatus

Introduction

Editing has been detected for the chloroplast transcripts encoded by the genes *rpl*2, *psb*L, *ndh*A, *ndh*B and *pet*B (Hoch *et al.*, 1991; Kudla *et al.*, 1992; Maier *et al.*, 1992a,b; Freyer *et al.*, 1993) and as shown in the present work the range of plastid editing events also includes the transcripts of the maize and barley *rpo*B genes which encode the β subunit of a chloroplast specific RNA polymerase. However, nothing is known about the mechanism and the components involved in this step of chloroplast gene expression.

The 17 C-to-U editing sites now known for six different chloroplast transcripts indicate a strong preference for certain types of codon transitions, which appears suggestive for an involvement of the translational apparatus in the editing process. The major aim of the present work was therefore to examine this possibility by investigating the existence and functionality of editing sites in the barley mutant *albostrians* which in its white sectors contains ribosome deficient plastids (Hagemann and Scholz, 1962; Börner *et al.*, 1976). Editing of plastid transcripts from this mutant should be impaired either if ribosomes were directly involved in the editing process or, more indirectly, if any of the peptide components which may be essential for the editing machinery were plastome encoded and therefore dependent on translation by plastid ribosomes. Here we present evidence that the *rpo*B transcript obtained from the ribosome deficient plastids of the barley mutant *albostrians* undergoes the same three editing events as the wild type transcript. It is, therefore, concluded that chloroplast editing, despite its preference for certain codons and codon positions, is not linked to or dependent on codon recognition during translation. The deficiency of plastome encoded translation products in plastids of the *albostrians* mutant allows also the conclusion that none of the peptide components of the chloroplast editing machinery are encoded in the plastome. Surprisingly, a fourth editing site identified in the homologous region of the maize *rpo*B transcript, in spite of complete sequence conservation from −24 to +19 nucleotides around the potential editing position, does not function in the barley *rpo*B transcript. This is likely to reflect the loss in barley or gain in maize of a recognition device specific for this single editing site and lends support to a template directed recognition mechanism.

Results and Discussion

Identification of four editing sites in the rpoB transcript of maize chloroplasts

The position of the *rpo* gene cluster on the maize plastome and its constituent genes *rpo*B, *rpo*C1, *rpo*C2 and *rps*2 is shown schematically in Figure 1A and B. This arrangement, which has been observed in all species investigated so far, appears universal for the plastomes of higher plants (for review see Igloi and Kössel, 1992). Overlapping cDNAs comprising the entire *rpo*B and *rpo*C1 transcripts together with their short intergenic region were amplified using various combinations of primer pairs of which only the primers used for *rpo*B specific sequences are depicted in Figure 1. In Figure 2 the amplification products obtained with the various primer combinations are presented. All the primer combinations lead to products whose mobilities are in accordance with the expected sizes of 1029, 1197, 2148 and 1172 bp (lanes 1, 3, 5 and 7 of Figure 2, respectively; for approximate sizes see also Figure 1B).

Direct sequencing of the four amplification products, which comprise the entire *rpo*B transcript together with part

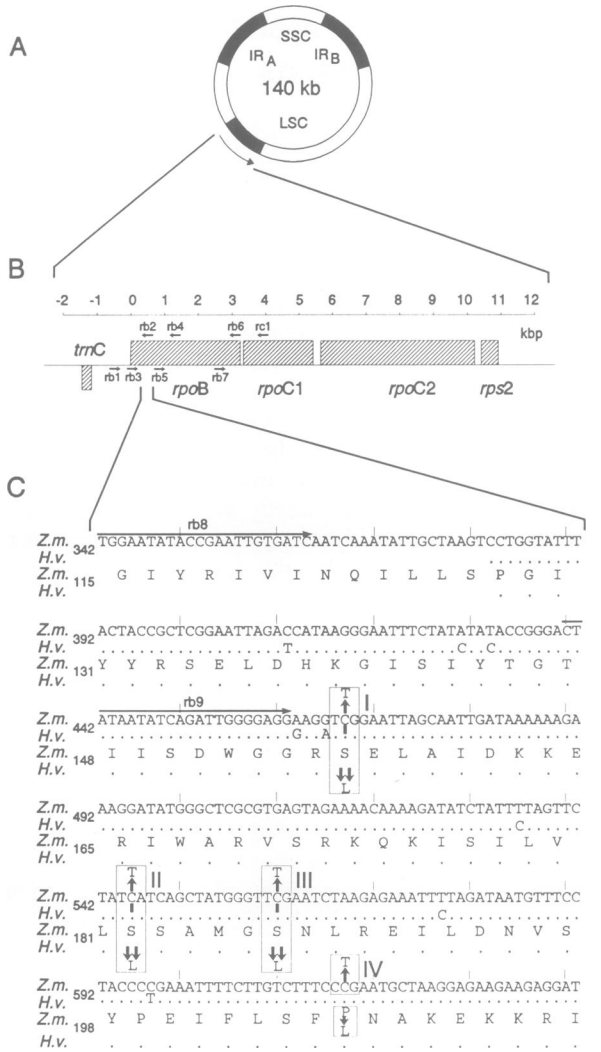

Fig. 1. Position and structure of the *rpo*B/C1/C2 operon and of the region of the *rpo*B gene encoding the four editing sites. (**A**) Location of the operon within the large single copy region (LSC), in relation to the two inverted repeat regions (IR_A and IR_B) and the small single copy region (SSC) of the maize plastome. (**B**) Enlargement of the operon showing the individual *rpo* genes together with the distal *rps*2 gene and the oppositely oriented *trn*C gene. The arrows rb1 to rb7 and rc1 designate positions and orientations of primers used for amplification and sequence analysis of the entire *rpo*B genomic and cDNA sequences. (**C**) Nucleotide and derived amino acid sequences of the *rpo*B region containing the four editing sites I–IV observed in maize (*Z.m.*). The homologous sequences from barley (*H.v.*) are also presented. Dots in the barley sequence indicate positions identical to the maize sequence. It should be noted that the determined barley nucleotide sequence starts only at the position homologous to position 382 in maize. The positions and orientations of the primers rb8 and rb9 are indicated by horizontal arrows. The C-to-T transitions are boxed and the corresponding amino acid substitutions caused by editing are indicated by upward and downward small arrows. It should be noted that editing sites I, II and III are observed in both maize and barley as indicated by the double arrows of the serine to leucine substitutions, whereas editing site IV is observed only in maize.

of the 5′ untranslated region, the *rpo*B/*rpo*C1 intergenic region and the 5′ terminal region of the *rpo*C1 transcript, with various sequencing primers (only two of which, rb8 and rb9, are indicated in Figure 1C) led us to the detection of four editing sites (I–IV), which deviate from the corresponding genomic sequences by C-to-T transitions. Sequence autoradiograms covering these editing sites are

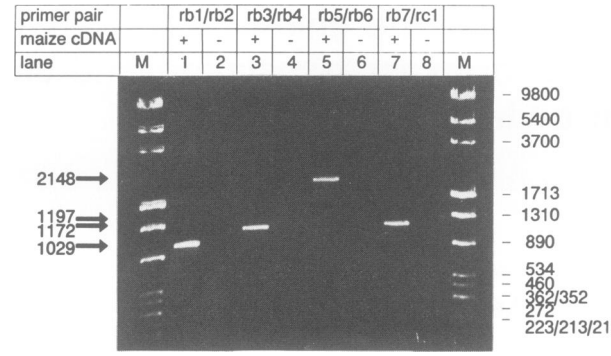

Fig. 2. Amplification products obtained by polymerase chain reactions with *rpo*B specific primer combinations and maize chloroplast cDNA. Separation of the products was achieved in all cases by gel electrophoresis in 1% agarose. The lanes M contain marker bands the size of which are indicated by the numbers (bp) to the right. The arrows to the left mark the positions of the amplification products with the numbers (bp) indicating the expected sizes of the respective products.

depicted in Figure 3 (autoradiograms 1 and 2). Interestingly, the four editing sites are clustered within a 150 bp region close to the 5′ terminus of the *rpo*B coding region (Figure 1B and C). No additional deviations between the cDNA and genomic sequences could be detected in other *rpo*B regions nor did a similar analysis of *rpo*C1 specific cDNAs (data not shown) lead to the detection of editing sites in the *rpo*C1 transcript.

The amino acid substitutions caused by the four editing events are shown in Figure 1C. The codon transitions TCG (Ser)→TTG (Leu) and TCA (Ser)→TTA (Leu) have previously been observed in the maize chloroplast *ndh*A and *ndh*B transcripts (Maier *et al.*, 1992a,b), while the transition CCG (Pro)→CTG (Leu) is observed here for the first time. An alignment of amino acid sequences encoded by the edited region of the maize *rpo*B transcript with the homologous sequences from other chloroplast species and from *Escherichia coli* is depicted in Figure 4. In accordance with the amino acid substitutions caused by other chloroplast editing sites identified previously (Kössel *et al.*, 1993), editing of the *rpo*B transcript also causes restoration of codons for highly conserved amino acid residues.

Sequence similarities between individual chloroplast and mitochondrial editing sites have been observed recently (Maier *et al.*, 1992b). In particular two closely spaced editing sites of the maize chloroplast *ndh*B transcript show a common octanucleotide preceded further upstream at slightly different spacing by common tetra- and pentanucleotides (Maier *et al.*, 1992b). A similar situation, but with the reverse orientation, is observed between editing sites III and IV of the *rpo*B transcript, which, again are in close vicinity to each other. They are flanked on their 3′ sides by the common pentanucleotide sequence CGAAT, which includes the editing position, and—with slightly different spacing—by the tetranucleotide sequence CTAA and the pentanucleotide sequence GAGAA (Figure 1C). The relevance of these sequence similarities to the specificity of the editing process remains to be determined. It is, however, clear from the results presented below that these sequence similarities alone cannot be sufficient as determinants for the editing process.

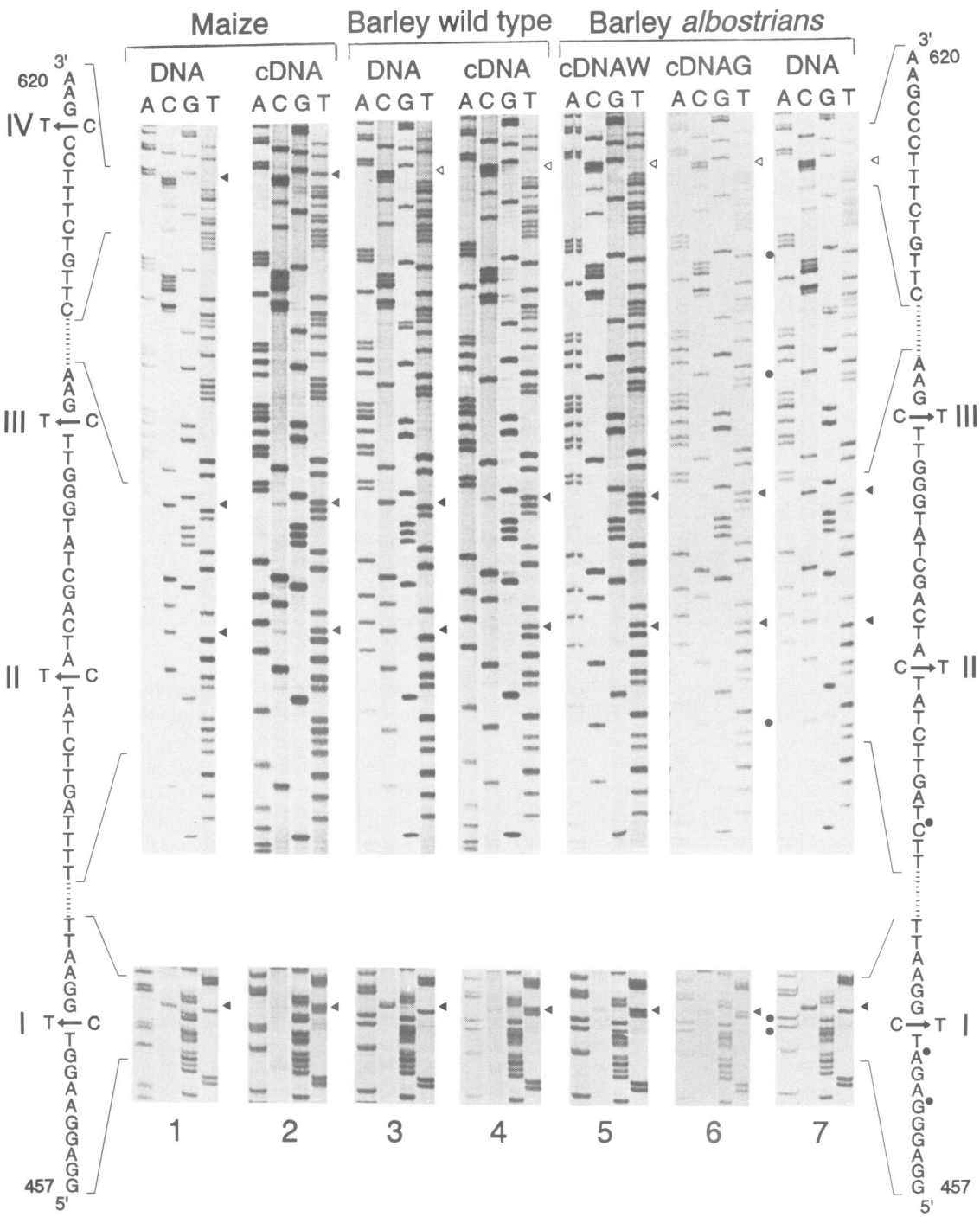

Fig. 3. Identification of editing positions in the chloroplast *rpo*B transcripts from maize, barley and the barley mutant *albostrians*. The sequences were obtained by direct sequencing of the DNAs or cDNAs amplified with the primer pair rb3/rb4 (see Figure 2) by using primers rb8 (for position I) and rb9 (for positions II−IV) as sequencing primers. All the autoradiograms represent mRNA-like sequences of the *rpo*B gene or cDNA corresponding to positions 457−620 as indicated by the partial sequences depicted for maize at the left edge and for barley at the right edge. Dots to the left of autoradiogram 7 and to the right of the partial barley sequence given to the right mark several barley specific positions, which in all the barley sequences (autoradiograms 3−7) deviate from the maize sequences (autoradiograms 1 and 2). Filled triangles to the right of all the autoradiograms mark positions where the cDNAs deviate from their respective DNA sequences by C-to-T transitions. The four and three, respectively, editing sites corresponding to these transitions are also marked in the sequences depicted on both sites. The positions of the barley sequences homologous to the editing site IV in maize are marked by open triangles. cDNA sequences of the barley *albostrians* mutant were obtained from either white (cDNAW) or green (cDNAG) seedlings of this mutant strain.

Chloroplast editing is independent of chloroplast translation

In almost all the chloroplast editing events identifed so far second codon positions are substituted (Kössel *et al.*, 1993). A single exception is observed in the maize *ndh*B transcript where a first codon position is changed (Maier *et al.*, 1992b). No editing of third codon positions or of positions in untranslated regions has yet been identified. In addition to this strong preference for second codon positions, a bias for certain codon transitions causing a preponderance of the

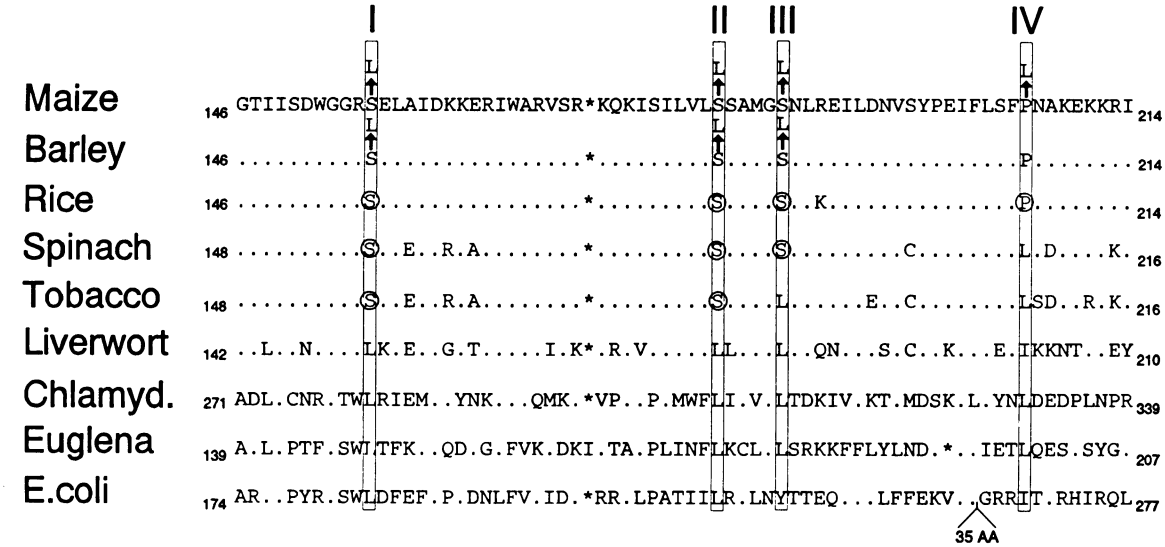

Fig. 4. Alignment of the amino acid sequences encoded by the *rpo*B regions encompassing the four editing sites identified in the maize and barley chloroplast *rpo*B transcripts. The alignment of the sequences from maize, rice, spinach, tobacco and liverwort chloroplast sequences and the *E.coli* sequence is part of an earlier alignment of the entire *rpo*B encoded amino acid sequence (Igloi *et al.*, 1990). The barley sequence was determined during this work (accession No. X73526). The *Chlamydomonas* and *Euglena* sequences are taken from the work of Fong and Surzycki (1992) and Yepiz-Plascencia *et al.* (1990), respectively. The substitutions caused by editing of the corresponding codons (see Figures 1 and 3) are marked by arrows. Circling indicates amino acid positions in the rice, spinach and tobacco sequence which are likely also to be restored to conserved amino acid residues by editing of the respective codons; this, however, remains to be determined experimentally.

amino acid substitutions serine to phenylalanine, serine to leucine and proline to leucine (but for instance not threonine to isoleucine and alanine to valine which also might be expected) can be recognized (Kössel *et al.*, 1993), although the statistical significance of altogether 17 chloroplast editing sites is still limited. Nevertheless, the preference both for second codon positions and for certain codon transitions may be taken as suggestive evidence that reading frames of chloroplast mRNAs act as determinants for the editing process and that a link exists between the chloroplast translational apparatus and the editing machinery. A more indirect dependence of the latter on chloroplast translation could also be anticipated if any peptide component of the editing machinery was plastome encoded, as the synthesis of such a component would depend on translation on chloroplast ribosomes.

The nuclear barley mutant *albostrians* (Hagemann and Scholz, 1962) appears ideally suited to test these suppositions experimentally. Eighty percent of the progeny of this mutant have a striped phenotype in which chloroplasts of the white sectors are devoid of ribosomes; 10% are completely green and 10% completely white. The latter, which can survive only up to an extended seedling state, again contain only undifferentiated, ribosomeless plastids in which no translation of plastome encoded mRNAs can take place (Börner and Hess, 1993). The complete absence of ribosomes in the plastids of white leaves and in the white sectors of striped leaves was originally observed by electron microscopy (Knoth and Hagemann, 1977). More recently this ribosome deficiency was corroborated by Northern blot analysis with a probe specific for chloroplast 16S ribosomal RNA (Hess *et al.*, 1992) as well as by Western blot analysis using an antiserum against ribosomal protein L2 (Hess *et al.*, 1993). In spite of the absence of translation in the ribosomeless plastids, high transcriptional activity for several of the plastid genes such as *rps*15, *rps*2, *rpo*A, *rpo*B, *rpo*C1 and *rpo*C2 is observed. In particular the *rpo*B/C1/C2 transcripts are produced at a high rate, apparently during all stages of the development of the mutant white leaves (Hess *et al.*, 1993) in contrast to wild type barley in which only the basal meristematic sections show higher levels of *rpo*B transcription (Baumgartner *et al.*, 1993). Therefore, in order to study the possible relationship between plastid ribosomes and editing, *rpo*B specific genomic DNA and cDNA comprising the editing sites identified in maize were isolated from white and green seedlings of the *albostrians* mutant. By using the heterologous primer pair rb3/rb4, amplification products identical in size with the 1197 bp product from maize are obtained with both wild type and mutant barley cDNA and genomic DNA (data not shown). Subsequent sequence analyses of these products (Figure 3, autoradiograms 3–7) reveal three C-to-T transitions which in all the cDNAs occur at the positions homologous to the editing sites I–III of the maize *rpo*B transcript. As evident from the cDNA sequence presented in autoradiogram 5, none of the three editing sites observed in the wild type cDNA (autoradiogram 4) or in the cDNA from green leaves of the *albostrians* mutant (autoradiogram 6) is impaired in the ribosome deficient plastids from white leaves of the *albostrians* mutant. This permits the conclusion that chloroplast editing is not linked or dependent on the chloroplast translation apparatus. This independence shows also that none of the peptide components which may be involved in the chloroplast editing process are encoded in the plastome; they must therefore be encoded in the nucleus.

One editing site of the barley rpoB transcript is not functional in spite of strong sequence conservation with the homologous maize editing site

The alignment shown in Figure 4 argues for similar editing events occurring in the chloroplast *rpo*B transcript from other chloroplast species. While this expectation could be verified in barley for editing sites I–III, no editing was observed in barley at site IV (Figure 3; positions marked by open

triangles in the upper parts of autoradiograms 3–7). This is particularly surprising as the barley sequences surrounding this site do not deviate from the maize sequences in at least 44 positions (19 positions upstream and at least 24 positions downstream; see Figure 1C). A straightforward interpretation of this observation would be that the sequences surrounding this (and other) editing site(s) do not act as determinants for the editing process. This interpretation is, however, at variance with the observation of certain sets of consensus sequences in the immediate vicinities of chloroplast editing sites which may even share sequences with mitochondria (Maier *et al.*, 1992b). A more likely interpretation is therefore that editing information is contributed by the sequences flanking individual editing sites, but that the recognition device (e.g. a guide RNA of the editing machinery) is lost or deviates when a structurally conserved editing site abandons its functionality, as is observed for the editing site IV in barley. Therefore the barley/maize comparison offers an opportunity to identify an editing template for site IV that would be expected to be functional or present only in maize chloroplasts. It should finally be pointed out that functional differences, i.e. loss of editing or partial editing, between structurally conserved editing sites have also been observed for plant mitochondrial transcripts (Covello and Gray, 1990; Wissinger *et al.*, 1990). This feature adds to the similarities already noted earlier between the editing systems of the two plant organellar systems (Maier *et al.*, 1992b).

Materials and methods

Plant material and growth conditions

Maize (*Zea mays* cv. Brummi, Inracorn) and barley wild type (*Hordeum vulgare* cv. Hassan) seedlings were cultivated with a 12 h light–12 h dark daily photoperiod, at temperatures of 28°C and 17°C, respectively and a humidity of 70%. Seeds of the *albostrians* line (*Hordeum vulgare* cv. Haisa), kindly provided by Dr G.Künzel (Gatersleben, Germany), were raised at 23°C under a 16 h light–8 h dark regime. Maize and barley wild type seedlings were harvested 4.5 days after sowing and barley *albostrians* seedlings 6 days after sowing. White and green *albostrians* seedlings were carefully checked to exclude contamination with small areas of green or white tissue, respectively. For isolation of nucleic acids from maize and barley wild type, lower leaf sections of primary leaves were taken, whereas total leaves were used from white and green barley *albostrians* plants.

Nucleic acid preparations

Wild type maize and barley nucleic acids were extracted, either from chloroplasts, isolated by Percoll gradient centrifugation as described by Robinson and Barnett (1988), or from total tissue, by guanidinium hydrochloride and CsCl gradient centrifugation (Chirgwin *et al.*, 1979). DNAs were recovered as described by Maier *et al.* (1992a). Barley *albostrians* total nucleic acids were isolated according to the procedures of Paulsen and Bogorad (1988) for RNA and Rogers and Bendich (1985) for DNA. The RNAs were then treated with DNase I, extracted with phenol–chloroform and ethanol precipitated in the presence of 0.3 M NaOAc, pH 4.8.

Reverse transcription of RNA and amplification of cDNA and DNA by polymerase chain reaction

Reverse transcription of RNA primed with hexanucleotide random primers in the presence of avian myeloblastosis virus reverse transcriptase (Angewandte Gentechnologie Systeme, Heidelberg, Germany) was performed as described by Maier *et al.* (1992a).

DNA and cDNA were amplified in the presence of 1.5 mM $MgCl_2$ using a standard protocol with 42 cycles at 93°C (for 1 min), 55°C (for 1 min) and 72°C (for 1.5 min) with a 2 min extension at 93°C of the first cycle and a 6 min extension at 72°C of the final cycle. Amplification products were separated on 1% agarose gels and visualized by ethidium bromide staining. Amplification products were purified, either directly from the reaction solutions or after agarose gel electrophoresis using SpinBind extraction units (FMC BioProducts, USA) according to the manufacturer's instructions, or as described by Maier *et al.* (1992b).

Direct sequencing of amplification products

Purified amplification products were sequenced directly by a modified chain termination method described by Bachmann *et al.* (1990) for non-fluorescent primers and by using the ΔTaq Cycle Sequencing Kit (United States Biochemicals, USA) for fluorescein labelled primers. Products of the fluorescent cycle sequencing reactions were analysed during electrophoresis by an automated laser fluorescence detection system (Ansorge *et al.*, 1986).

List of oligonucleotides

Oligonucleotides used for PCR and/or for sequencing were synthesized on a DNA synthesizer (model no. 394; Applied Biosystems, USA). The position numbers of *rpo*B specific primers are given according to the published sequence of the maize chloroplast *rpo*B/C1/C2 cluster (Igloi *et al.*, 1990). F denotes fluorescein labelling at the 5′-end of the primer. *rpo*B specific primers were as follows:

rb1: 5′-GGTGGATAACTAGATTGGCAAG-3′ (835–856)
rb2: 5′-CTAATTCCGACCTTCCTC-3′ (1856–1839)
rb3: 5′-AGATTCATATGCTCCGGAATGG-3′ (1374–1395)
rb4: 5′-CATGAACCGTTTGTGTCAATGGAT-3′ (2561–2538)
rb5: 5′-F-ATCTGTTACAAGATCAATTCGG-3′ (2337–2358)
rb6: 5′-CCCCAGATCGTAGCATTAA-3′ (4473–4455)
rb7: 5′-F-GGAGTACCCTCACGAATGAATG-3′ (3962–3983)
rc1: 5′-AAACCCCGTAATCGTAAGAAAG-3′ (5134–5113)
rb8: 5′-TGGAATATACCGAATTGTGATC-3′ (1723–1744)
rb9: 5′-CTATAATATCAGATTGGGGAGG-3′ (1821–1842).

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