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Chloroplast *rps*15 and the *rpo*B/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase

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Transcription of plastid genes and transcript accumulation were investigated in white leaves of the *albostrians* mutant of barley (*Hordeum vulgare*) and in heat-bleached leaves of rye (*Secale cereale*) as well as in normal green leaves of both species. Cells of white leaves of the mutant and cells of heat-bleached leaves bear undifferentiated plastids lacking ribosomes and, consequently, plastid translation products, among them the subunits of a putative chloroplast RNA polymerase encoded by the plastid genes *rpo*A, B, C1 and C2. The following results were obtained. (i) Plastid genes are transcribed despite the lack of chloroplast gene-encoded RNA polymerase subunits. The plastid origin of these transcripts was proven. This finding provides evidence for the existence of a plastid RNA polymerase encoded entirely by nuclear genes. (ii) Transcripts of the *rpo* genes and of *rps*15, but not of genes involved in photosynthesis and related processes (*psb*A, *rbc*L, *atp*I-H), were abundantly accumulated in ribosome-deficient plastids. In contrast, chloroplasts accumulated transcripts of photosynthetic, but not of the *rpo* genes. (iii) Differences in transcript accumulation between chloroplasts and ribosome-deficient plastids are due to different relative transcription rates and different transcript stability. (iv) The observed differences in transcription are not caused by an altered pattern of methylation of plastid DNA. Thus, the prokaryotic plastid genome of higher plants is transcribed by two RNA polymerases. The observed differences in transcription between chloroplasts and undifferentiated plastids might reflect different functions of the two enzymes.

Key words: chloroplast gene expression/*Hordeum vulgare*/plastid ribosome/plastid RNA polymerase/*Secale cereale*

Introduction

The theory that eukaryotic cell organelles are of endosymbiotic origin is now widely accepted (Schwemmler, 1984). Most genes of the original endosymbionts have been lost or presumably transferred to the plant nucleus during evolution. Even genes essential for such basic functions as replication and transcription are in general not located in mitochondrial or chloroplast genomes. The only exceptions are four genes showing homology to eubacterial RNA polymerase; these were identified in chloroplast DNA (Shinozaki *et al.*, 1986; Ohyama *et al.*, 1986; Hiratsuka *et al.*, 1989). It has been shown that these genes are expressed and that the proteins, identified immunologically and by sequencing, are involved in forming an active chloroplast RNA polymerase (Little and Hallick, 1988; Ruf and Kössel, 1988; Purton and Gray, 1989; Hu and Bogorad, 1990). The exact biochemical properties of chloroplast RNA polymerase(s), i.e. their *in vivo* subunit composition, template preference and coaction with nuclear-encoded components, are still matters of controversy (Briat *et al.*, 1987; Zaitlin *et al.*, 1989; Eisermann *et al.*, 1990; Rajasekhar *et al.*, 1991; for review see Igloi and Kössel, 1992).

It has been proposed that two different types of DNA-dependent RNA polymerase exist in maize chloroplasts due to different sensitivity to rifampicin (Bogorad and Woodcock, 1970). Separation of an RNA polymerase complexed with chloroplast DNA (called the transcriptionally active complex) from a soluble RNA polymerase, with differing biochemical properties from each other, led to the suggestion that two distinct RNA polymerases exist in *Euglena* (Greenberg *et al.*, 1984).

Indirect evidence for the existence of a nuclear-encoded chloroplast RNA polymerase has been presented based on incorporation of UTP in purified fractions of ribosome-deficient plastids (Bünger and Feierabend, 1980; Siemenroth *et al.*, 1981) or using immunodetection of proteins resulting from the translation of poly(A)$^+$ mRNA (Lerbs *et al.*, 1985).

Plants deficient in plastid ribosomes are obviously an ideal system for investigating this problem since they will be unable to translate the RNA polymerase subunits encoded in the plastid genome. Higher plants grown at non-permissive temperatures lack plastid ribosomes whereas cytoplasmic components are largely unaffected (Feierabend and Schrader-Reichhardt, 1976). Transcription of chloroplast photosynthetic genes at a very low but detectable level has been described in heat-bleached rye plants (Winter and Feierabend, 1990). The presence of minor amounts of processed 16S rRNA (Feierabend and Berberich, 1991), of tRNA^{Glu} (Hess *et al.*, 1992a) and accumulation of higher molecular weight precursors of 16S rRNA and tRNA have also been reported (Subramanian *et al.*, 1991). In contrast, neither 16S rRNA nor transcripts of the plastid *trn*E-*trn*Y-*trn*D gene cluster (Hess *et al.*, 1992b) have been found in white plants of the nuclear gene-induced plastid ribosome-deficient mutant *albostrians* (Hagemann and Scholz, 1962) of barley (*Hordeum vulgare* L. cv. Haisa).

Here we present data on the transcription of chloroplast genes for the organelle's translational and transcriptional apparatus as opposed to photosynthetic genes in heat-bleached rye and in the barley mutant *albostrians*. We show that these transcripts cannot result from promiscuous DNA in different cell organelles because they could be capped

efficiently *in vitro* and because the homologous sequences are absent from mitochondrial DNA. Absence of functional plastid ribosomes was confirmed in a very sensitive approach by using an antiserum to a functionally essential chloroplast ribosomal protein. The results strongly indicate an entirely nuclear DNA-encoded functional chloroplast RNA polymerase in higher plants.

Results

Transcripts for chloroplast-encoded ribosomal proteins and RNA polymerase subunits are present in ribosome-deficient plants

White plastids of the *albostrians* mutant are induced by the recessive nuclear *albostrians* (*as*) allele (Hagemann and Scholz, 1962). However, only part of the plastid population in homozygous *as as* plants is affected by the mutation. Thus the offspring of homozygous selfed plants are composed of ~10% green, 80% striped and 10% pure white seedlings. The pure white seedlings have no plastid ribosomes (Börner *et al.*, 1976; Knoth and Hagemann, 1977). The green plants are phenotypically wild-type and contain normal plastids, whereas striped plants possess both types of plastids. Both the green and striped seedlings were used as controls in the following experiments.

We analysed DNA fragments from different regions of the chloroplast genome (Figure 1) extensively by Northern hybridization, *in vitro* capping and run-on assays. Strong hybridization signals were obtained with the RNA from both white *albostrians* mutant and heat-bleached rye leaves when gene probes for *rpo*B, *rpo*C1 and *rps*15 were used (Figure 2).

In the case of *rps*15, the patterns for white and green barley leaves were not identical (Figure 2A). The main signals from light-grown green plants were at positions 4500, 4300, 3500, 1400 and 850 nt. Light-grown white plants gave main bands at 1250 and 850 nt. This pattern was qualitatively comparable to that obtained with RNA from the corresponding dark-grown plants. Some less prominent bands occur at 1700, 1000 and 900 nt for green and at 4500, 2700, 1700 and 1400 nt for white mutant plants. The bands of 5500, 2700 and 1250 nt (Figure 2A), which seem to appear only in the mutant RNA, perhaps represent species that are rapidly turned over in normal plants. A similar high accumulation of *rps*15 transcripts in heat-bleached rye leaves has been previously documented (Subramanian *et al.*, 1991). Dramatically high levels of transcripts for *rpo*B (Figure 2B) and *rpo*C1 (Figure 2C) genes accumulate in ribosome-deficient plastids. Interestingly, the normal green leaves of both barley and rye show extremely low levels of *rpo*B or *rpo*C1 transcripts. The major bands (*rpo*B: 3200 nt; *rpo*C1: 2700 nt) as well as several of the minor bands (e.g. 5500 nt) are of similar size in both plants. The large transcripts of ~10, 7.5, 5.5 and 4.5 kb might represent primary transcripts recognized by both probes.

Absence of chloroplast ribosomal protein L2

Lack of plastid ribosomes in the two experimental systems used here was established earlier by electron microscopical studies with the *albostrians* mutant (Knoth and Hagemann, 1977), by the absence of plastid rRNA in preparations of total RNA from white leaves (Börner *et al.*, 1976; Feierabend and Schrader-Reichhardt, 1976) and by 2-D electrophoresis of proteins from heat-bleached rye plants followed by immunostaining with antibodies raised against purified 30S and 50S ribosomal subunits (Feierabend *et al.*, 1988). In the latter case, functionality of plastid ribosomes was excluded although several ribosomal polypeptides were

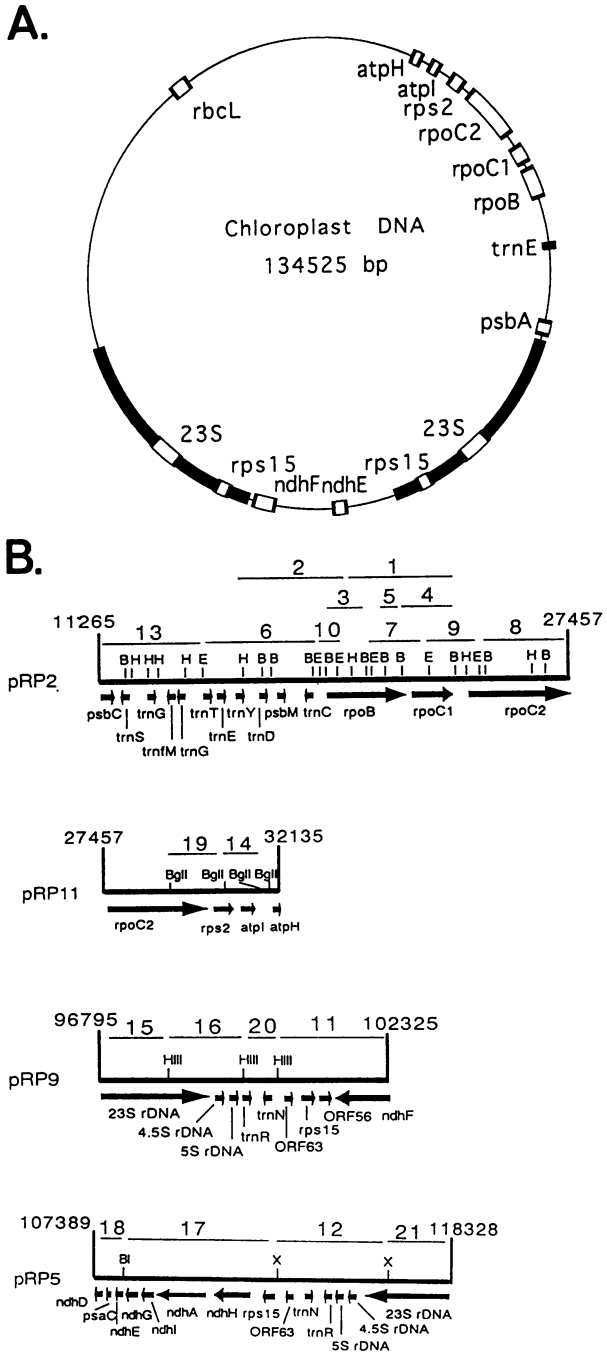

Fig. 1. (**A**) Genetic map of the chloroplast genome of monocots (e.g. rice). Only genes and operons investigated in this study are indicated. (**B**) Detailed map showing gene organization and restriction sites for *Hin*dII (H), *Bam*HI (B), *Eco*RI (E), *Bgl*II (BgII), *Hin*dIII (HIII), *Bgl*I (BI) or *Xho*I (X) on plasmids pRP2, pRP11, pRP9 and pRP5 of the rice chloroplast clone bank. Restriction fragments hybridizing in run-on assays or to *in vitro*-capped RNA are numbered 1–21 and marked by a bar. All other data were adapted from the rice chloroplast genome map (Hiratsuka *et al.*, 1989).

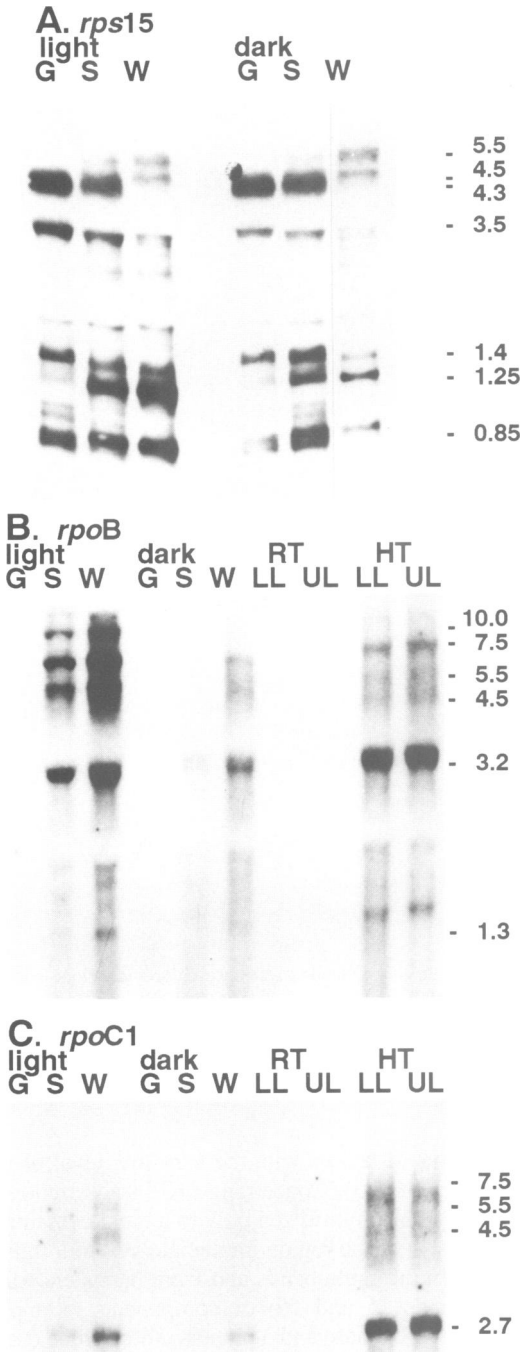

Fig. 2. Transcripts for a chloroplast-encoded ribosomal protein and for subunits of RNA polymerase accumulate in ribosome-deficient plastids. (**A**) The *rps*15 mRNA pattern for white *albostrians* (W), and for green (G) and striped (S) barley used as control. Left: RNA from light-grown seedlings. Right: similar set of RNA from dark-grown plants. A 486 bp *Eco*RI−*Cla*I fragment of rye *rps*15 was used as a probe (Prombona and Subramanian, 1989). (**B** and **C**) The *rpo*B/C1/C2 operon is strongly induced in the white plastid ribosome-deficient barley mutant (left) and in heat-bleached rye plants (right). RNA was isolated from upper (UL) and lower (LL) leaf halves of heat-bleached (HT) and control grown at room temperature (RT) rye. The *rpo*B probe was a 718 bp *Bam*HI fragment of maize (Igloi *et al.*, 1990); the *rpo*C1 probe was a 1531 bp *Mlu*I−*Eco*RV fragment of rice (Hiratsuka *et al.* 1989) chloroplast DNA. The sizes of the main RNA bands are shown on the right.

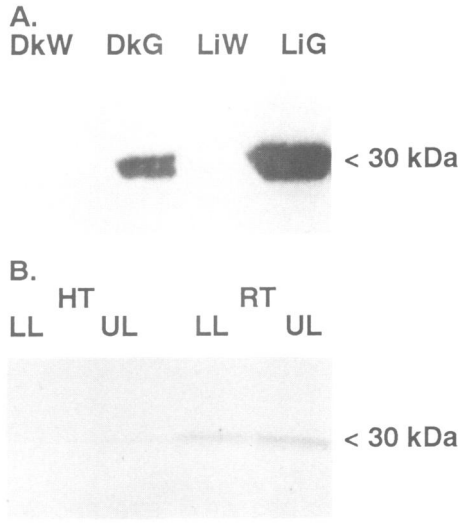

Fig. 3. The presence or absence of chloroplast r-protein L2 by immunostaining with antiserum to L2. (**A**) Barley: L2 is easily detected in the total protein extracts from green control plants grown in light (LiG) or dark (DkG) but is absent in extracts from the white *albostrians* plants (LiW, DkW). (**B**) Rye: proteins from the upper (UL) and lower leaf (LL) halves of control plants (RT) or plants grown at a non-permissive temperature (HT).

detected. It is known that ribosomes can function in the absence of certain ribosomal proteins (Dabbs, 1991). Therefore we raised an antiserum to spinach chloroplast rpL2, an essential ribosomal constituent. Absence of this protein is a very sensitive marker for absence of ribosome function since it is involved in the peptidyl-transferase centre (Nierhaus, 1982).

We carried out several Western blots using the L2 antiserum. Ribosomal protein L2 was never detected in extracts from white *albostrians* seedlings (Figure 3A). Extracts from the upper greenish leaf part of heat-grown rye plants gave a weak signal, whereas the lower pure white part was free of L2 (Figure 3B). For control plants, loading of the same amount of total protein per lane resulted in strong signals.

Transcripts of rpoB, rpoC1 and rps15 are of plastid origin

Transcripts of chloroplast genes were detected in plastid ribosome-deficient plants despite the absence of plastid translation as shown above. These transcripts need not necessarily originate from the chloroplast compartment. Theoretically they could be derived from 'promiscuous' DNA, i.e. from plastid DNA sequences localized outside the chloroplast, in either the nuclear (e.g. Pichersky *et al.*, 1991) or the mitochondrial DNA (e.g. Nugent and Palmer, 1988). We established by extensive Southern hybridizations that copies of chloroplast *rps*15, *rpo*B and *rpo*C1 are not present in the mitochondrial genome of barley (data not shown).

The RNA from the *albostrians* mutant was labelled *in vitro* by guanylyl transferase (capping reaction) and was subsequently hybridized specifically to DNA fragments from the complete rice chloroplast gene bank spanning the regions around *rps*15 and *rpo*B/C1/C2 (Figure 4). Consequently, a nuclear origin of these transcripts can be excluded.

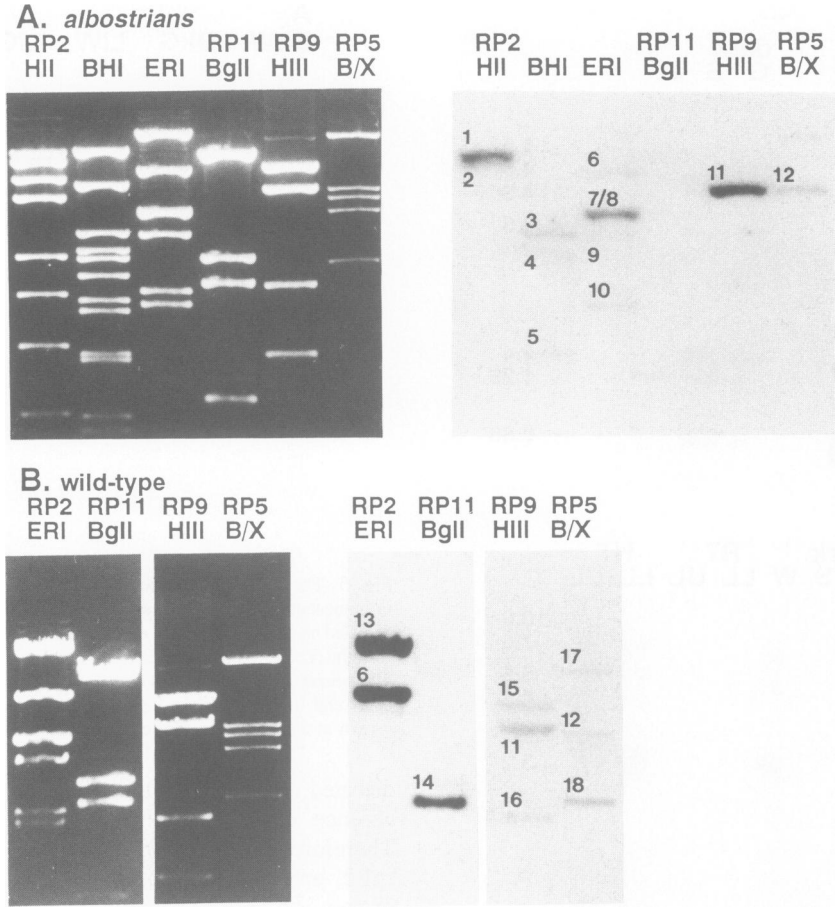

Fig. 4. *In vitro* capping experiment with (**A**) RNA from white *albostrians* or (**B**) green plants, using clones of the rice chloroplast clone bank (Hiratsuka *et al.*, 1989) that span the regions around the strongly transcribed genes *rps*15, *rpo*B and *rpo*C1. **Left:** The indicated plasmids were digested with restriction enzymes (abbreviations as in Figure 1B) and used for Southern blotting. **Right:** Its hybridization pattern with total RNA labelled by *in vitro* capping. Only the hybridizing bands are numbered. Bands hybridizing with both RNA from green and white plants are marked with the same numeral. Fragments 7 and 8 differ by only a few nucleotides in size and are labelled 7/8. The genes present in the respective hybridizing bands are given in Figure 1B.

Because only the 5′ ends of primary transcripts are labelled by this method, it is possible to define the approximate region in which the transcript was initiated. The pattern obtained with capped RNA from the white mutant upon hybridization to the rice chloroplast DNA primary clones pRP2, pRP11, pRP9 and pRP5 (Figure 1B; Hiratsuka *et al.*, 1989) was different from that of the control as shown in Figure 4. The bands 2, 3, 6 and 10 (Figure 4A) contain the spacer region 5′ to the *rpo*B gene where its transcript is most probably initiated in white plants. That bands 1, 4, 5, 7, 8 and 9 also gave a signal could be due to the length of the accumulated transcript possessing the primary 5′ end. That all fragments containing *rpo*B sequences, but none of those containing only *rpo*C2, gave signals, indicates that a 5′ *rpo*B promoter is intensely used in this operon in white plants. With RNA from green plants, however, strong signals were obtained with bands 6 and 13 (Figure 4B), representing several highly transcribed tRNA and photosynthetic genes, but no signals at all could be detected with the fragments representing the *rpo*B/C1/C2 operon. Strong signals were detected with labelled RNA from green control barley and band 14, but not with RNA from white seedlings. Fragment 14 has a spacer between *rps*2 and *atp*I; it apparently contains an intensely used promoter for the *atp* operon in green plants. The absence of signals with RNA from the white mutant in this region correlates with the very low level of mRNA for *atp*I/H (Figure 5). Concerning *rps*15 transcription, RNA from white plants hybridized only to a band containing this gene (band 11) or the region immediately 5′ to it (band 12). Signals from the same bands and from bands encoding the rRNAs (bands 15 and 16) or components of a putative NADH dehydrogenase and a photosynthesis gene (bands 17 and 18) were detected with capped RNA isolated from green plants (Figures 4B and 1B).

Transcripts of chloroplast genes encoding photosynthesis-related proteins

The abundance of certain mRNAs in plastid ribosome-deficient plants as shown above appears contradictory to earlier results describing no significant accumulation of several chloroplast transcript species (Feierabend and Berberich, 1991; Hess *et al.*, 1992b). It is therefore highly probable that transcription and/or transcript accumulation occur only for a subset of chloroplast genes under these conditions. We used gene probes for the ATPase subunits a and III (*atp*I, *atp*H), the 32 kDa protein of photosystem II (*psb*A) and the large subunit of ribulose-bisphosphate carboxylase (*rbc*L) to test this possibility.

Direct comparison of RNA from heat-grown rye plants and from the *albostrians* mutant showed that mRNA for

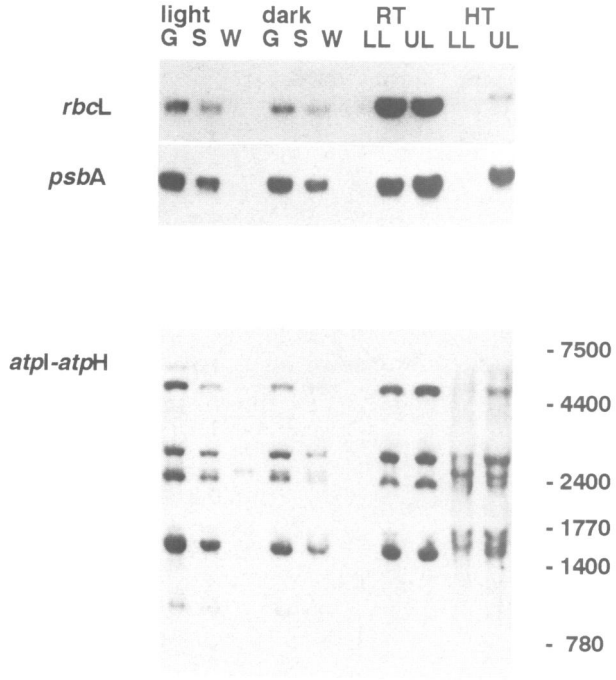

Fig. 5. Northern hybridization to show accumulation of mRNA for *rbc*L, *psb*A and *atp*I/H. These mRNA species are almost absent in plastid ribosome-deficient leaves. The size of RNA length standards (GIBCO-BRL) is indicated in the experiment with the *atp*I/H gene probe on the right margin. Hybridization probes derived from the rice chloroplast DNA clone bank were a 564 bp *Pst*I fragment of pRB1 (*rbc*L), a 1194 bp *Hpa*I−*Bgl*II fragment of pRB7 (*psb*A) and a 1519 *Sca*I−*Pst*I fragment of pRP11 (*atp*I/H; Hiratsuka *et al.*, 1989). Designations are as in Figure 2.

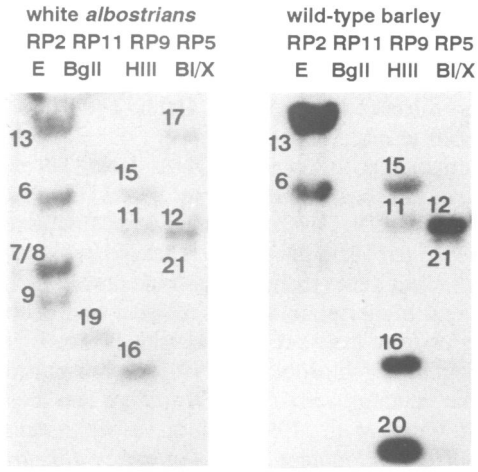

Fig. 6. Hybridization of run-on transcripts derived from plastids of white *albostrians* leaves (left) or chloroplasts of green leaves (right) to DNA fragments of the rice chloroplast clone bank (Hiratsuka *et al.*, 1989). Location of genes and arrangement of the hybridizing restriction fragments are shown in Figure 1B. The combination of plasmid and restriction enzyme, and the numbering of bands are the same as those used in Figure 4.

*psb*A and *rbc*L are reduced in the basal pure white parts of heat-grown leaves to a similar extremely low level as in the white *albostrians* plants (Figure 5). With the *atp*I-H probe two double bands were detectable in RNA from the basal leaf sections of heat-grown rye; one band in exactly the same position as a corresponding faint band in barley *albostrians*. The greenish upper halves of the heat-bleached leaves contain mRNA of all four genes, slightly less than in the controls. Interestingly, the greenish upper half contains the *atp*I-*atp*H transcripts present in the green control as well as the bands of higher size present in the white lower part (Figure 5). For control RNA the signals obtained with the *atp*I-*atp*H probe are approximately 6, 4.5, 2.9, 2.3, 1.6, 1.45 and 0.9 kb in both rye and barley. This result indicates a processing pattern similar to that described for other monocots (Westhoff *et al.*, 1991; Stahl and Subramanian, unpublished results).

Transcription in lysed plastids

It has been shown previously that relative transcript stability is very important in adjusting plastid RNA levels to different environmental and developmental conditions (e.g. Mullet and Klein, 1987; Deng and Gruissem, 1987). Only recently has it been shown that there is also regulation at the transcriptional level in plastids of barley seedlings (Klein and Mullet, 1990). We employed a plastid run-on system to investigate whether the observed pattern of transcript accumulation in the *albostrians* mutant is caused by changes at the transcriptional level or not.

The results shown in Figure 6 demonstrate that both transcriptional and post-transcriptional mechanisms contribute to the differences between transcript patterns of green and white plastids. A high rate of transcription of the *rpo*B/C1/C2 operon occurs in mutant plastids as is evident from strong hybridization of RNA to bands 7/8 and 9 and less pronounced hybridization to band 19. These fragments cover most of this operon. The same bands, after hybridization with *in vitro* transcripts from chloroplasts, gave only low signals even after very long exposure times. It is interesting to compare these results with the capping data (Figure 4). The strong signal observed after hybridization of capped RNA with fragment 10 correlates with a putative start site of *rpo*B transcripts. The weak signal of this band in run-on experiments would be due to the fact that only transcripts that had already initiated prior to plastid isolation would have been labelled under the conditions used and therefore the region near the transcript start would have been discriminated against.

The region around *rps*15 is less affected by transcriptional changes. Whereas the band 17 signal is specific for the mutant run-on assay, the fragment 11 signal was detected with transcripts of both the mutant plastid and the chloroplast run-on assay. Several other bands (12, 15, 16 and 21) also hybridized with labelled transcripts from both plastid types. This indicates that the relative transcription rate for the rRNA operon is rather high in the mutant plastids. That many more genes are transcribed in the mutant plastids than might be expected from the results of Northern experiments is further indicated by the signals with fragments 6 and 13 which are also found with transcripts from both plastid types. Only the DNA fragment in band 20 hybridized specifically with transcripts of chloroplasts. Comparable results were obtained with four independent run-on experiments.

Analysis of plastid DNA in the albostrians mutant

The large differences in transcription and mRNA accumulation observed in ribosome-deficient plastids could result from DNA modifications (for other possibilities see Discussion), e.g. methylation of certain regions of DNA or

mutations of plastid DNA. Methylation of plastid DNA has been suggested as the cause of transcriptional down-regulation in some non-photosynthetic plastids (Ngernprasirtsiri *et al.*, 1988; Gauly and Kössel, 1989; Kobayashi *et al.*, 1990).

No mutations of plastid DNA have been detected previously in heat-bleached rye plants (Herrmann and Feierabend, 1980). For the *albostrians* mutant it is not known how the white plastid phenotype is caused by the *albostrians* allele. Nuclear genes inducing plastome mutations are known for several higher plant species (Börner and Sears, 1986), but the mode of their action in plastids is mostly unknown. Association of chloroplast DNA polymorphisms to the plastome mutator activity in *Oenothera* has been shown recently (Chiu *et al.*, 1990). We have compared the DNA from normal and mutant plastids of barley *albostrians* after cleavage with the following groups of restriction endonucleases, *Msp*I/*Hpa*II/*Hap*II, *Bst*NI/*Eco*RII, *Mbo*I/*Sau*3AI/*Dpn*I, which are sensitive/insensitive to methylation of their respective recognition sequences. This should allow us to determine from one experiment if there is methylation or mutation in plastid DNA detectable at the level of restriction fragment pattern. Southern blots were prepared from total DNA and hybridized with 18 different cloned fragments representing ~80% of the barley plastid genome (Sogard and von Wettstein-Knowles, 1987). Two examples of this series of autoradiographs are shown in Figure 7. There were no detectable differences in the restriction pattern of white compared with the control plants. We conclude that (i) the *albostrians* gene does not act at the level of replication and does not lead to large deletions, inversions or transpositions of the plastid DNA, and (ii) plastid DNA is not significantly methylated in white *albostrians* mutant. Consequently modification of DNA cannot contribute to the observed differences in transcription and transcript accumulation described in this paper.

Discussion

We have used the barley *albostrians* mutant and heat-bleached rye, both of which lack plastid ribosomes (Börner *et al.*, 1976; Feierabend and Schrader-Reichhardt, 1976; Knoth and Hagemann, 1977), to investigate whether transcription of plastid-encoded genes can occur in the absence of plastid protein biosynthesis.

We confirmed the severe deficiency of the plastid translational apparatus in white plants of the *albostrians* mutant and in the lower white leaf section of heat-bleached rye by using an antiserum to the functionally important r-protein L2. A faint signal obtained with proteins extracted from the greenish upper leaf part of rye is in agreement with previous reports of the presence in this tissue of some active plastid ribosomes derived from the embryo (Feierabend and Berberich, 1991).

An extremely low level of transcript accumulation has been found in this study for photosynthesis-related chloroplast genes *rbc*L, *atp*I, *atp*H and *psb*A (Figure 5) in plastid ribosome-deficient plants. It is in principle possible that these genes were either scarcely transcribed or that the transcription rate was normal but the turnover drastically enhanced. Transcriptional down-regulation as a result of modifications of plastid DNA, however, can clearly be excluded for the white *albostrians* plastids (Figure 7).

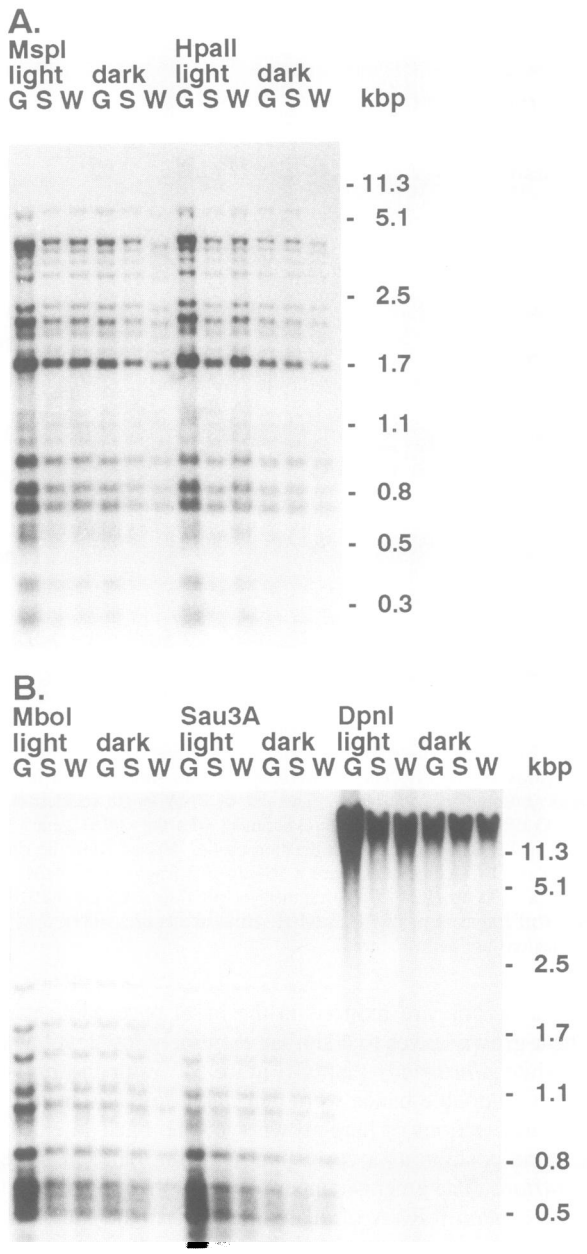

Fig. 7. Identity in plastid DNA methylation pattern in all types of seedlings of the barley *albostrians* mutant. Total DNA was isolated from white (W), striped (S) or green (G) leaves grown either in the dark (Dk) or under normal illumination (Li) and was cleaved with the isoschizomeric endonucleases *Msp*I/*Hpa*II (**A**) or *Mbo*I/*Sau*3A/*Dpn*I (**B**). The resulting fragments were separated electrophoretically, blotted onto nylon membranes and hybridized with a mixture of cloned fragments of barley plastid DNA. The probe fragments used here were the inserts of pHvC 208, 222, 238, 209, 21 and 46 (Sogard and von Wettstein-Knowles, 1987). The sizes of fragments of *Pst*I-digested lambda DNA are indicated on the right.

However, not all chloroplast transcripts are present at such a low level in ribosome-deficient plastids. Certain transcripts not only accumulated significantly but behaved as if they were specifically enhanced. This was true for genes encoding components of the translational (*rps*15) and transcriptional (*rpo*B, *rpo*C1; Figure 2) apparatus. We made similar observations with transcripts of *rps*2 and *rpo*A (data not shown).

Barley RNA preparations used in the Northern experiments could be *in vitro* labelled by guanylyl transferase (Figure 4). This result excludes a nuclear origin for the labelled transcripts because nuclear mRNAs cannot be capped *in vitro* unless they have been pretreated (Shatkin, 1976; Moss, 1981). In addition this method was employed here essentially to scan larger regions around the strongly transcribed genes. The results presented in Figure 4 agree very well with the Northern data and indicate that in ribosome-free plastids, transcripts for several photosynthetic, rRNA and tRNA genes do not accumulate whereas transcripts of genes encoding ribosomal proteins and subunits of RNA polymerase accumulate to a high level. The complex pattern in the Northern hybridizations is due to the polycistronic nature of the *rpo*B, *rpo*C1 and *rps*15 mRNAs. The particularly complex pattern of *rps*15 transcripts may originate from the two copies of this gene present in the chloroplast genomes of monocots (Hiratsuka *et al.*, 1989; Prombona and Subramanian, 1989). It is not known if both or only one copy of this gene is transcribed; differential regulation of neighbouring genes might also contribute to the complex pattern. Cotranscription of *rps*15 with the downstream *ndh*H gene has been described for tobacco (Ohto *et al.*, 1988). The 850 nt RNA species in white and green barley plastids is presumably the mature mRNA that was also present in heat-bleached and green rye plastids (Subramanian *et al.*, 1991).

The high level of transcript accumulation for *rpo*B, *rpo*C1 and *rps*15 in ribosome-deficient plastids detected in this study has some parallels to reports describing preferential expression of chloroplast r-protein genes in *Chlamydomonas* (Liu *et al.*, 1989) or overinduction of *zfp*A and *rpl*2 chloroplast genes in pea seedlings under conditions of impeded plastid translation (Sasaki *et al.*, 1989; Nagano *et al.*, 1991). These results suggest the existence of a (regulatory?) mechanism leading to an enhanced expression of these 'housekeeping' genes when chloroplast protein biosynthesis is blocked. In addition we conclude that transcript accumulation for these genes is independent of plastid protein biosynthesis.

Run-on experiments with isolated chloroplasts or ribosome-free plastids of *albostrians* barley were performed in order to analyse whether the observed pattern of mRNA accumulation was linked to altered transcription of a plastid gene.

Previous studies on the incorporation of UTP into RNA in mutant plastids indicated a lower but significant RNA synthesis capacity (Siemenroth *et al.*, 1981). Our data show that the massive accumulation of transcripts for the *rpo*B/C1/C2 operon is at least partially because the rate of transcription in white *albostrians* plastids is higher than that in normal chloroplasts. Other genes—such as the rRNA operon for which few or no transcripts were found in Northern experiments (Hess *et al.*, 1992b)—are also transcribed, but very different degradation rates contribute considerably to the observed RNA accumulation pattern. The extreme instability of rRNA in mutant plastids could well be a consequence of the absence of ribosomal proteins which normally stabilize these molecules.

Finally the question remains: what type of RNA polymerase transcribes these genes in ribosome-deficient plastids? We have demonstrated here the absence of plastid ribosomes in primary leaves by a very sensitive assay using L2 antiserum. From the leaf striping pattern and the existence of pure white seedlings it can be deduced that the induction of ribosome-deficiency in plastids occurs very early in plant development. A plastid DNA-encoded RNA polymerase synthesized before the loss of ribosomes would undergo an extreme dilution during further plant development. It is very unlikely that such a highly diluted RNA polymerase could transcribe so many plastid genes at the rate observed in our studies.

It could be argued that 'misrouting' of a nuclear coded mitochondrial polymerase into plastids occurs occasionally and generates the observed transcripts, but promoter structures, as far as they are identified, differ greatly between the two organelles (Bogorad, 1991; Brown *et al.*, 1991) and therefore the observed high transcriptional activity should not be expected. It could be speculated that the nuclear-encoded chloroplast primase, which normally does not transcribe genes (Nielsen *et al.*, 1991), becomes able to do so under extreme conditions, but the abundance and especially the lengths of the detected transcripts make this also very unlikely.

We favour the idea of an entirely nuclear-encoded RNA polymerase normally functioning in plastids. The existence of a completely nuclear-encoded RNA polymerase also gets support from recent reports describing a minimal plastid genome in the non-photosynthetic parasitic angiosperm *Epifagus virginiana*. In this plant all photosynthetic genes are deleted; also, all plastid *rpo* genes are either lost or converted to pseudogenes (Morden *et al.*, 1991); nevertheless, the remaining plastid genes are assumed to be transcribed (dePamphilis and Palmer, 1990). The differences described here between transcription rates of genes in mutant *albostrians* plastids and in chloroplasts could arise from differences in the content of transcription factors modulating the activity of the nuclear-encoded plastidic RNA polymerase. Clearly, the two types of plastid differ with respect to factors (proteins) involved in transcript stability and/or degradation. Alternatively, differential gene activity in ribosome-deficient plastids and in normal chloroplasts could be due to the existence of two plastidic RNA polymerases, one mainly plastid DNA-encoded (including nuclear-encoded σ-like factors) and the other entirely nuclear in origin. There are several reports in the literature suggesting two biochemically distinct RNA polymerases in chloroplasts (e.g. Bogorad and Woodcock, 1970; Greenberg *et al.*, 1984; see Introduction). In this case, our results suggest different functions for the two polymerases: the mainly plastid genome-encoded RNA polymerase, not active in ribosome-free plastids, would have a preference for photosynthetic and bioenergetic genes, whereas the other, i.e. the entirely nuclear-encoded RNA polymerase acting in ribosome-free plastids, would have preference for the genes of the gene expression apparatus.

Materials and methods

Plant material and growth conditions

All seedlings were grown in moist vermiculite. Control plants of rye (*Secale cereale* cv. Kustro) were cultivated at 22°C and a 12 h light/12 h dark daily photoperiod. Heat-treated, plastid ribosome-free seedlings were grown in an environmental chamber set to 37°C (adequate to 34°C ground temperature) with constant illumination of 50 W/m^2 (Feierabend, 1982). Seeds of *albostrians* (*Hordeum vulgare* cv. *Haisa*) were kindly provided by Dr G.Künzel, Gatersleben, Germany. Barley seedlings were raised at

23°C in a dark room or under a 16 h light/8 h dark regime with an illumination of 32 W/m^2 (normal illumination).

Plants were harvested 6 days after sowing. Primary leaves of heat-treated rye seedlings were cut into a greenish upper half and a lower pure white half. White *albostrians* plants were carefully checked to exclude contamination with small areas of green tissue. Green and striped *albostrians* plants were used as controls. Seedlings grown in constant darkness were harvested under green safety light into liquid nitrogen and sorted under normal illumination into etiolated yellow control and white mutant plantlets.

Protein extraction and Western blots

Total proteins were isolated as described previously (Hess *et al.*, 1992b). Samples were mixed with SDS-sample buffer and electrophoresed on 12% polyacrylamide–SDS gels (Laemmli, 1970). The proteins were transferred to nylon membranes (Hybond-N, Amersham) by electrotransfer in a semi-dry blotter (MilliBlot-SDE, Millipore) with a constant current of 2.5 mA/cm^2 for 45 min. Antiserum to purified spinach chloroplast r-protein L2 was raised in rabbits according to the procedure described previously (Smooker *et al.*, 1990).

Isolation and blot analysis of nucleic acids

Total RNA was isolated according to Paulsen and Bogorad (1988), separated on 1.5% agarose–formaldehyde gels, transferred to nylon membrane by capillary blotting and hybridized as described by Hess *et al.* (1992b).

Total DNA was isolated from 1–2 g of seedlings with cetyltrimethyl-ammonium bromide (CTAB), according to Rogers and Bendich (1985). DNA was cleaved with restriction enzymes, electrophoresed and transferred to nylon membrane by standard methods (Sambrook *et al.*, 1989).

Preparation and labelling of hybridization probes

Plasmids were cleaved with the appropriate restriction enzymes. Fragments were separated by agarose gel electrophoresis and isolated via 'spinbind DNA extraction units' (FMC Bioproducts). Probes were labelled to a high specific activity by random priming (using an Amersham megaprime kit) according to the procedure recommended by the manufacturer.

In vitro capping

30 μg of total RNA were *in vitro* capped in a total volume of 50 μl in 50 mM Tris–HCl pH 7.8, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 0.1 mM EDTA with 4.625 MBq of [α-^{32}P]GTP by the action of 12.5 U guanylyl transferase (BRL) and in the presence of 90 U of RNasin (Promega). After 30 min incubation at 37°C a further 12.5 U of guanylyl transferase were added and incubation was continued for another 30 min. Labelled RNA was precipitated by adding 1 vol 5 M ammonium acetate and 2 vol ethanol, washed twice with 70% ethanol and dried. RNA was then dissolved directly in prewarmed hybridization buffer (Hess *et al.*, 1992b) and hybridized to the filter-bound fragments of a rice chloroplast library (Hiratsuka *et al.*, 1989) in a total volume of 0.8 ml using 10 ml screw-capped glass centrifuge tubes. We avoided an RNase treatment after hybridization in order to prevent removal of the label from imperfectly protected primary transcripts because homology between barley and rice chloroplast DNA is incomplete, especially in the non-coding regions.

Isolation of plastids and run-on transcription

The upper two-thirds of primary leaves of 6 day old plants were homogenized in a Waring blender into 380 mM sorbitol, 25 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, filtered through cheesecloth and steel sieves of declining pore size, reaching finally 20 μm. Mutant plastids were obtained following the procedure of Siemenroth *et al.* (1981). Chloroplasts were fractionated on 80–35% Percoll step gradients, adapted from Klein and Mullet (1986).

Pulse–chase labelling of plastid transcripts was done as described by Mullet and Klein (1987) with the following modifications. Reactions were performed in a volume of 200 μl containing [α-^{32}P]UTP (>15 TBq/mM, Amersham) 3.7 MBq for chloroplasts or 7.4 MBq for mutant plastids. The buffer was supplemented with 40 U RNase inhibitor (Ambion) and the reaction was started by adding 30 μl plastid suspension (~$3-5\times10^7$ plastids). Incubation was terminated after 5 min and RNA was purified with extraction buffer and phenol (Mullet and Klein, 1987). The labelled RNA was then used for hybridization as described for *in vitro* capping.

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