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Transcriptional control of plastid gene expression during development of primary foliage leaves of barley grown under a daily light-dark regime

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Abstract. Plastid DNA transcription was studied during biogenesis and maturation of chloroplasts in primary foliage leaves of barley (*Hordeum vulgare* L.) seedlings grown under a daily light-dark regime. Specific age-dependent changes in the transcript pattern of plastids have been shown by hybridization to barley plastid DNA fragments of run-on transcript probes derived from homogeneous populations of proplastids and chloroplasts of different age. In proplastids, transcription of the *rrn* operon is predominant and the increasing transcriptional activity during biogenesis of chloroplasts is mainly the consequence of additional transcription of mRNA and tRNA genes. During maturation of chloroplasts, *rrn* transcription preferentially decreases and, conversely, transcription of the *psbD* gene increases. The importance of these changes in plastid DNA transcription is discussed in relation to chloroplast ageing.

Key words: Chloroplast biogenesis and maturation – *Hordeum* (plastid gene expression) – Plastid gene expression – Transcriptional regulation (gene expression)

Introduction

In a previous paper it was demonstrated that plastid gene expression during light-dependent transformation of etioplasts into chloroplasts in 5-d-old barley seedlings apparently occurs without qualitative changes in plastid DNA transcription (Krupinska and Apel 1989). Other reports on plastid gene expression in spinach (Deng et al. 1987; Deng and Gruissem 1987; Westhoff et al. 1988) and barley (Klein and Mullet 1986; Klein et al. 1988) also demonstrated the limited role of transcriptional regulation for plastid gene expression during transformation of etioplasts to chloroplasts. Instead, the importance of post transcriptional regulation at the RNA level, i.e. changes in the stability of RNA, was emphasized (Gruissem 1989). However, such quantitative differences in the accumulation of specific RNAs in etioplasts and chloroplasts cannot explain the strict light-dependent synthesis of components for the photosynthetic apparatus (Herrmann et al. 1985). The most important control step during light-induced transformation of etioplasts to chloroplasts occurs rather at the level of translation (Klein and Mullet 1986; Laing et al. 1988). The limited role of transcriptional regulation is not restricted to light-dependent chloroplast development starting from etiolated tissue. Constitutive transcription of the plastome has also been demonstrated in non-photosynthetic root amyloplasts and in plastids from hypocotyls and cotyledons of dark-grown spinach (Deng and Gruissem 1988), and in chromoplasts of bell pepper and sunflower (Kuntz et al. 1989).

In contrast, other recent reports emphasize the importance of transcriptional control for the regulation of plastid differentiation processes (Ngernprasirtsiri 1988b; Kobayashi et al. 1990; Schrubar et al. 1990). In the monocotyledonous species *Sorghum,* light induces an enhancement in overall transcription activity and a change in the pattern of transcripts (Schrubar et al. 1990). In contrast to barley, leaf development in *Sorghum* is strictly light-dependent and chloroplast development does not proceed from differentiated etioplasts but rather from proplastid-like organelles in unfolded colourless primary leaves (Schrubar et al. 1990).

Boffey et al. (1980) and Leech (1984) emphasized that the development of functional chloroplasts during light-dependent transformation of etioplasts proceeds quite differently from chloroplast biogenesis during normal leaf development under a daily light-dark regime. The present study aimed at contributing to a clarification of the question whether the two different pathways to photosynthetically competent chloroplasts in barley are regulated differentially. For this purpose, run-on transcription was compared among proplastids and two

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Abbreviations: bp = base pairs; IR = inverted repeat; kbp = kilo base pairs; SSC = small single-copy region

chloroplast populations differing in age, isolated from seedlings grown under a daily light-dark regime. Primary foliage leaves of monocotyledonous species like barley and wheat provide an especially useful material to study chloroplast development and maturation during normal leaf development (Dean and Leech 1982). Cell division only occurs in a basal meristem resulting in a developmental sequence of cells and plastid populations from the base to the tip (Boffey and Leech 1982; Dean and Leech 1982; Baumgartner et al. 1989). Beyond a certain leaf length it is therefore possible to study plastid maturation and ageing independent of cell division and plastid division (Boffey and Leech 1982; Baumgartner et al. 1989). In the present study to investigate plastid DNA transcription during chloroplast maturation, homogeneous plastid populations were isolated from apical sections of 5-d- and 7-d-old primary foliage leaves. The transcription of plastid DNA in these normally developed chloroplasts of different ages was further compared to plastid DNA transcription in a preparation which was enriched in proplastids and was derived from a narrow colourless section at the base of 5-d-old seedlings. The results demonstrate that in contrast to chloroplast development during light-dependent greening of etiolated barley seedlings (Krupinska and Apel 1989) chloroplast biogenesis starting from proplastids in barley seedlings grown under natural light conditions is at least in part controlled at the transcriptional level. In contrast to other reports (Ngernprasirtsiri et al. 1988a, b; Kobayashi et al. 1990), but consistent with a recent paper on chromoplast formation during tomato fruit ripening (Marano and Carrillo 1991), the observed variations in plastid gene transcription are not caused by gross changes in the methylation of plastid DNA.

Material and methods

Plant material. Barley (*Hordeum vulgare* L. cv. Carina; Dr. J. Ackermann Saatzucht, Irlbach, Germany) seedlings were grown for 5 or 7 d in moist vermiculite in controlled-environment chambers at 21° C with a daily regime of 16 h light (8000 lx) and 8 h darkness.

Plastid isolation. Plastids were isolated from segments of primary foliage leaves according to the procedure of Gruissem et al. (1986). Plastids obtained from 15–30 g leaf segments were fractionated on 94%/40% Percoll step gradients. Finally, intact plastids were resuspended in a small volume of 0.33 M sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazine ethansulfonic acid (Hepes)-KOH, pH 8 (Mullet and Klein 1987). All manipulations were performed below 4° C.

Transcription in lysed plastids. Transcription in lysed plastids was carried out essentially as described before (Mullet and Klein 1987; Krupinska and Apel 1989). A 100-µl standard assay was performed with $1.5 \cdot 10^7$–$2 \cdot 10^7$ plastids (Krupinska and Apel 1989). In addition, heparin was included in some assays at a final concentration of $0.5\ mg \cdot ml^{-1}$ (Klein and Mullet 1990). Incorporation of radioactive UTP was determined with aliquots spotted onto DE81 filters as described by Hallick et al. (1976). Run-on transcripts deriving from plastids of different ages were used in hybridization experiments with Southern blots and DNA dot-blots.

Electrophoresis and blotting of plastid DNA subfragments. Recombinant pBR325 or pBluescript (Stratagene, Heidelberg, Germany) plasmids containing nine *PstI*-fragments (a-i, Krupinska and Apel 1989) of barley plastid DNA – eight of them were designated pHvC 186, 192, 203, 205, 208, 209, 222, 238 (Poulsen 1983) – were either directly digested with *EcoRI* and *BamHI* or with *PstI* to isolate the inserts. Inserts were prepared by gel-electrophoresis on 0.7–1% (w/v) agarose gels and subsequent electroelution in dialysis bags in the presence of TEA (0.04 M Tris-acetate, 1 mM EDTA, pH 8) buffer (Sambrook et al. 1989). Insert DNA was collected by ethanol precipitation and was digested with the restriction enzyme *RsaI*. So far, it has not been possible to clone a particular region of 18.4 kbp into a plasmid; however, the corresponding plastid DNA region is represented by several *EcoRI* fragments released by digest of phage DNA prepared from two recombinant λ gtWES phages of a plastid DNA library. The fragments were size-fractionated on 1% (w/v) agarose gels and were then transferred to a nylon filter (zeta probe; Biorad, München, Germany) by capillary blotting using 0.4 M NaOH as transfer medium (Reed and Mann 1985). Finally the filters were air-dried and baked for 30 min at 80° C.

Isolation and subcloning of RsaI subfragments. RsaI subfragments obtained after digestion of plastid DNA *PstI* fragments were isolated from 1% (w/v) low-melting agarose (Sigma, Deisenhofen, Germany) gels, ligated to pUC13, digested with the restriction enzyme *SmaI* and dephosphorylated by alkaline phosphatase (Boehringer, Mannheim, Germany). The recipient strain was *Escherichia coli* DH5α. Ligation, transformation and selection of recombinant clones were done according to standard procedures (Sambrook et al. 1989).

Preparation of DNA dot-blots. Various amounts of plasmid DNA (320 ng, 80 ng, 20 ng), isolated according to Birnboim and Doly (1979) and purified by CsCl-equilibrium centrifugation (Sambrook et al. 1989), were dotted onto nylon filters (zetaprobe; Biorad) using a dot-blot device according to the manufacturer's (Schleicher and Schüll, Dassel, Germany) instructions.

Identification and mapping of RsaI fragments. After subcloning into pUC13, *RsaI* fragments were sequenced from both sides up to 200 bp according to the dideoxy-chain-termination method of Sanger et al. (1977) and using a standard kit (Pharmacia, Freiburg, Germany). In addition to the M13 direct primer provided by the kit, a reverse primer (Biolabs, Schwalbach, Germany) was used. The radioactive label was α-^{35}SdATP (NEN, Dreieich, Germany). The DNA sequences of both ends of the fragments were compared with the complete sequence of the rice plastid genome (Hiratsuka et al. 1989) by using the Pharmacia DNasis software. Homologies more than 90% were found in most cases and allowed an assignment of the barley plastid DNA fragments to specific coding or noncoding parts of the rice plastome.

Hybridization experiments. Hybridization of run-on transcripts with immobilized barley DNA fragments was performed exactly as described in a previous paper (Krupinska and Apel 1989).

Preparation and endonuclease digestion of plastid DNA. Plastid DNA derived from 5-d- and 8-d-old seedlings was prepared according to Poulsen (1983). The DNA was digested with three pairs of isoschizomeric endonucleases which respond differentially to methylation (*HpaII* and *MspI, MboI* and *Sau3AI, EcoRII* and *BstNI*) according to the manufacturer's instructions (Boehringer; Gibco BRL, Eggenstein, Germany). The DNA fragments were separated electrophoretically on 1.2% (w/v) agarose gels in the presence of ethidium bromide.

Results

Survey of plastid DNA transcription in young and mature chloroplasts. In barley, the transcriptional activity of plastids derived from apical sections of primary foliage leaves of seedlings cultivated either in darkness or in

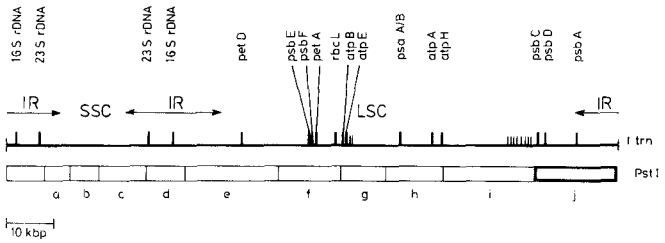

Fig. 1. Linearized physical map of barley plastid DNA showing the location of all *PstI* fragments (Poulsen 1983; Krupinska and Apel 1989). The schematic drawing starts with the left *PstI* fragment located in the middle of the inverted-repeat region (*IR*) which is identical to fragment d. The IR region is followed by the small single-copy region (*SSC*), the second IR and the large single-copy region (*LSC*). The *PstI* fragments *a–i* have been cloned into plasmids. Fragment *j* has not been cloned into a plasmid so far. The corresponding DNA region, however, is included in two recombinant λgtWES phages (j and j') of a library of plastid DNA partially digested with *EcoRI*. The positions of genes mapped on the plastid genome (Søgaard and v. Wettstein-Knowles 1987) are indicated on the uppermost line by *thick vertical lines*. *Thin vertical lines* indicate the positions of some tRNA genes (*trn*)

continuous light declines rapidly with increasing age (Mullet and Klein 1987).

In the present study the transcription of plastid DNA during normal leaf development under a light-dark regime of 16:8 h has been analyzed. First, chloroplasts derived from primary foliage leaves of 5-d-old seedlings that had almost reached the stage of maximal growth rate were compared with chloroplasts derived from primary foliage leaves of 7-d-old seedlings just after their maximal growth period at almost their final length. In both cases plastids were isolated from 2-cm leaf segments located about 1 cm below the tips of primary foliage leaves, ensuring the comparison of homogeneous chloroplast populations differing in age only. The general transcriptional activity was five- to tenfold lower in the chloroplasts of the 7-d-old seedlings compared with chloroplasts derived from the 5-d-old seedlings. Furthermore, transcription in chloroplasts of different ages was compared with transcription in a proplastid-enriched fraction, isolated from 0.5-cm basal segments of 5-d-old seedlings grown under a daily light-dark regime.

To analyze the relative transcription rates of all the different genes in the barley plastid genome during maturation of chloroplasts in primary foliage leaves developing under a light-dark regime, run-on transcripts were hybridized to plastid DNA fragments representing the complete plastid genome immobilized on nylon filters. pBluescript clones with the *PstI* inserts a–c and e–i (Fig. 1) and the insert d (Fig. 1) of the pBR325 clone pHvC205 were digested with *EcoRI* and *BamHI*, while λ-gtWES phage DNAs including the plastid DNA region j (Fig. 1) were digested with *EcoRI* only. The resulting DNA fragments were separated electrophoretically on an agarose gel (Fig. 2) and were blotted onto a nylon filter. The same filter was used in the two following experiments with run-on transcripts deriving from leaf segments of 5-d- and 7-d-old seedlings: after the first autoradiography procedure the probe was stripped off and the filter was used for the second hybridization experiment. The autoradiograms show that the intensity of the hybridization signals generally varies among the various DNA fragments (Fig. 2), indicating a non-uniform transcription of the plastid genome. Moreover, a comparison of the two autoradiograms obtained after hybridization with run-on transcripts derived from young (5-d) and older chloroplasts (7-d) reveals that the relative transcription rate in different regions of the plastid genome varies with the age of the plastids. A decrease is obvious in the hybridization intensity of bands belonging to the *PstI* fragments a(pHvC203), c(pHvC192) and d(pHvC205) with increasing age of the chloroplasts (Fig. 2). In contrast, the relative transcription rate of a 1.1-kbp *EcoRI* fragment represented by the second band in lane j of the autoradiograms in Fig. 2 seems to increase during chloroplast maturation. In other regions of the plastid genome the hybridization intensities of most fragments are similar on both autoradiograms (Fig. 2). No cross-hybridization is detectable with the linearized pBluescript (Fig. 2).

Methylation state of plastid DNA. A differential transcription of the plastid genome could be either due to changes in specificity and activity of the RNA-polymerase or could be caused by modifications of the DNA itself. Recent reports claim that transcriptional regulation of plastid gene expression during differentiation processes is based on changes in the methylation pattern of plastid DNA (Ngernprasirtsiri et al. 1988a, b; Kobayashi et al. 1990). In order to test whether a similar mechanism is responsible for the observed changes in transcript patterns during ageing of chloroplasts (Fig. 2), plastid DNA isolated from 5-d- and 8-d-old seedlings was digested with pairs of isoschizomeric restriction enzymes that can discriminate between unmethylated and methylated DNA. No differences were detected among the fragment patterns obtained after digestion of the two DNA samples with *HpaII/MspI* and *MboI/Sau3AI* and separation by agarose gel electrophoresis (Fig. 3). The same result was obtained by an analysis with the isoschizomeric enzyme pair *EcoRII* and *BstNI* (data not shown). These data indicate that transcriptional regulation of plastid gene expression during chloroplast development is not mediated by gross changes in DNA-methylation.

Relative transcription of a, c, d subfragments in proplastids and chloroplasts of different ages. Transcription in the plastid DNA regions a, c, d (Fig. 1) which decreases differentially during chloroplast maturation (Fig. 2) was further analyzed in detail. The *PstI* fragments were digested with *RsaI* to yield small subfragments, most probably containing not more than one gene. As a control the *PstI* fragment f, which showed no detectable changes in transcription between chloroplasts of 5-d- and 7-d-old seedlings (Fig. 2), was treated likewise. After electrophoresis, subfragments of about 100–1500 bp in length were blotted onto a nylon filter and were hybridized subsequently with run-on transcripts derived from proplastids and chloroplasts isolated from 5-d- and 7-d-old

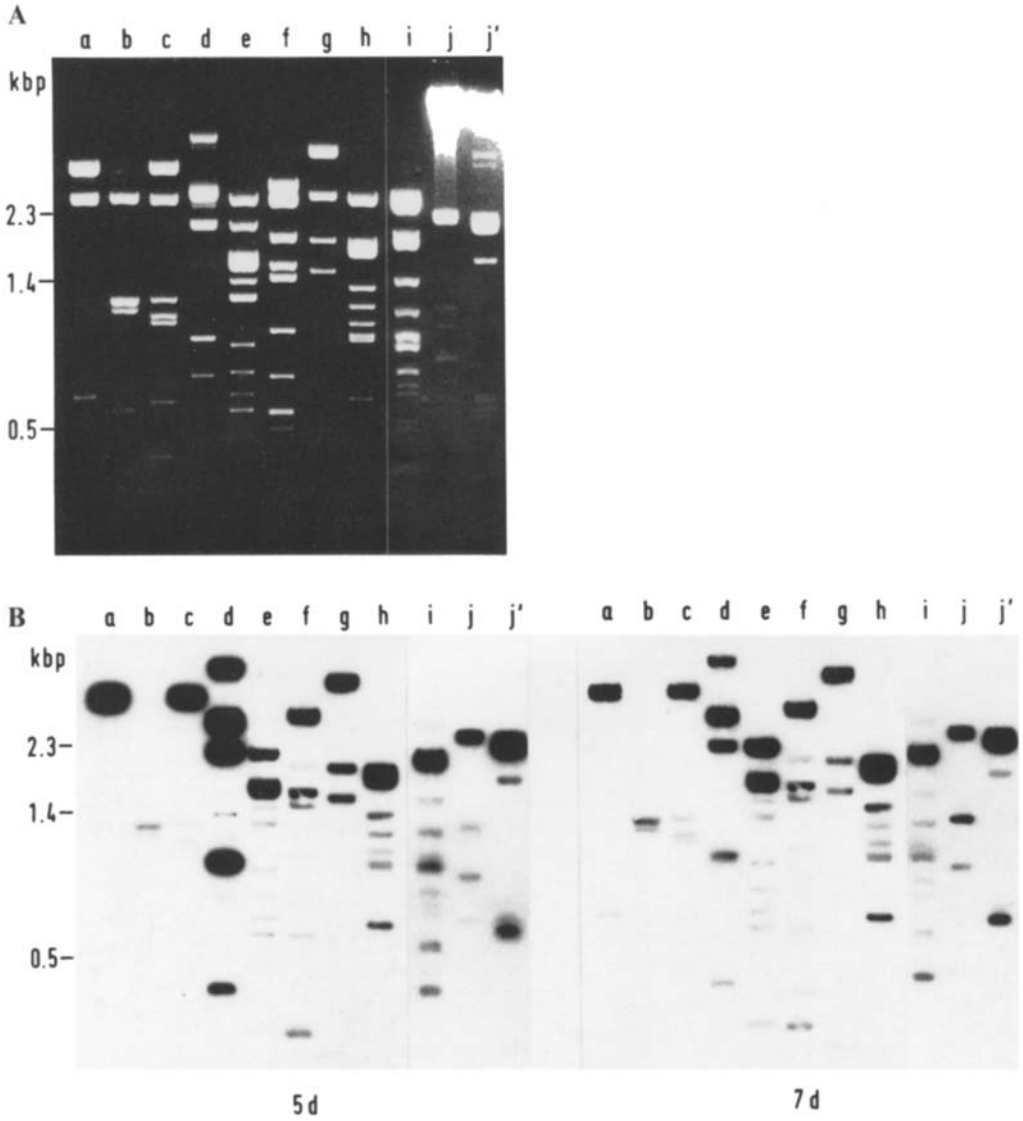

Fig. 2A, B. Analysis of barley chloroplast run-on transcripts by hybridization with barley plastid DNA fragments that in sum represent the complete barley plastome. Nine *PstI* fragments of barley plastid DNA, subcloned into pBluescript (*a–c, e–i*) or isolated from pBR325 (*d*), were digested with *BamHI* and *EcoRI*. The residue of barley plastid DNA was cloned into λgtWES and the corresponding two phages were digested with *EcoRI* (*j* and *j'*). The resulting fragments were separated electrophoretically on a 1% (w/v) agarose gel (**A**, gel stained with ethidium bromide) and were then transferred onto a nylon filter. The autoradiograms (**B**) were obtained after subsequent hybridization of the same filter to run-on transcripts derived from primary foliage leaves of barley seedlings either grown for 5-d or for 7-d under a daily light-dark regime. The probe was obtained from two standard transcription reactions (Krupinska and Apel 1989) with $3 \cdot 10^7$ plastid in total. The DNA fragment sizes (kbp) are shown on the *extreme left*

seedlings, respectively. The autoradiographic patterns obtained with this filter (Fig. 4) clearly reveal distinct changes in the relative transcription among subfragments belonging to one *PstI* fragment. This effect indicates that an endonuclease restriction digest with *RsaI* is better suited to discriminate among the various genes located on one large *PstI* fragment than a digest with *EcoRI* and *BamHI*. These development-specific changes in the relative intensities of hybridization signals are not affected by the concentration of radiolabelled run-on transcripts during hybridization (data not shown). Transcripts derived from proplastids hardly hybridize with subfragments of the *PstI* fragment f (Fig. 4, pro). Rather, in proplastids, the only transcripts which dominate are those which decline in relative intensity during development and maturation of chloroplasts. Surprisingly, subfragments of the 8.4-kbp pHvC205 insert (d), which is supposed to include almost the complete *rrn* operon (Poulsen 1983) (Fig. 1), here also show a differential age-dependent decrease in the rates of transcription (Fig. 4). Some signals in the 5-d autoradiogram and some belonging to subfragments of region d are almost undetectable in the 7-d autoradiogram while other fragments give good signals in both cases (Fig. 4). Whether these differences in the hybridization signals are due to a rapid processing of the primary *rrn* transcript or are caused by differential usage of putative internal promoters is an open question. Moreover, the exact location

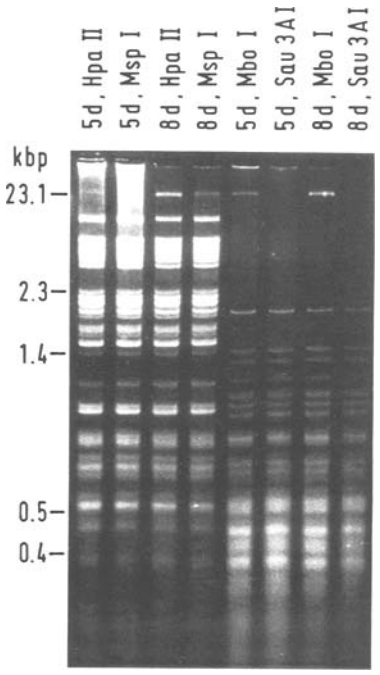

Fig. 3. Methylation analysis of barley plastid DNA isolated from 5-d and 8-d-old seedlings. The plastid DNAs were digested with the isoschizomeric restriction-enzyme pairs *HpaII/MspI* and *MboI/Sau3AI*. The resulting fragments were separated by electrophoresis on a 1.2% (w/v) agarose gel, stained with ethidium bromide. The DNA fragment sizes (kbp) are shown on the *left* of the gel

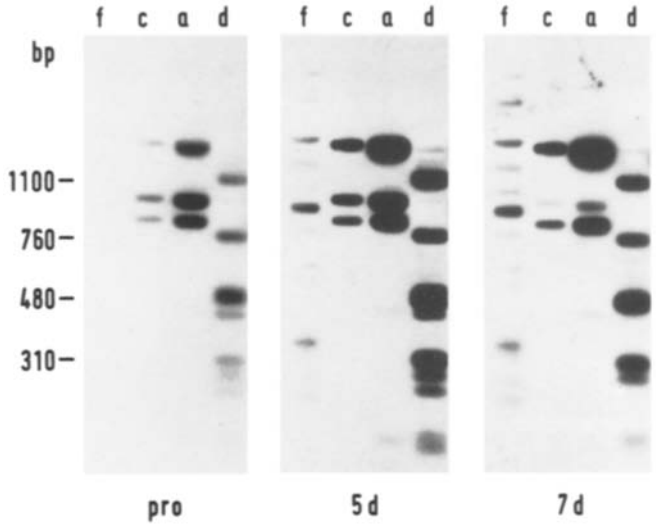

Fig. 4. Autoradiograms obtained after hybridization to barley plastid DNA fragments of barley run-on transcripts either derived from proplastids *(pro)* or from chloroplasts of different age. The probes contained run-on transcripts obtained in two standard transcription reactions, which in the case of the chloroplasts comprised $3 \cdot 10^7$ plastids in total. Chloroplasts were isolated from barley seedlings grown for 5-d or 7-d under a daily light-dark regime. *PstI* inserts of recombinant pBR325 clones pHvC186(*f*), pHvC192(*c*), pHvC203(*a*) and pHvC205 (*d*) were isolated and digested with *RsaI*. After electrophoretic separation the resulting subfragments were transferred onto a nylon filter which was subsequently used for all hybridization experiments. DNA fragment sizes (bp) are indicated on the *extreme left*

of the genes on the pHvC205 insert (d) has not yet been determined and some of the subfragments might contain genes not belonging to the *rrn* operon.

The Southern blot was also hybridized with run-on transcripts prepared in the presence of heparin. Though heparin somehow stimulated the overall incorporation of nucleotides into elongating transcripts its addition did not cause changes in the corresponding autoradiographic pattern (data not shown).

Relative transcription rates of subcloned plastid DNA fragments. Signals on a Southern blot might be mistaken if they derive from closely migrating fragments. To avoid this problem, several *RsaI* subfragments were subcloned into the *SmaI* site of pUC13 and were analyzed separately. For this purpose plasmid DNAs containing specific subfragments of the barley plastid DNA regions a, c, d were dotted onto a nylon filter. Fragments with decreasing size were named with increasing numbers due to their position on the autoradiogram (Fig. 4). Two recombinant plasmids including *EcoRI* fragments cloned into the λ gtWES (j), an 1.5-kbp *EcoRI* fragment specific for the *psbE* gene located on fragment f (Krupinska and Berry-Lowe 1988), and pUC13 DNA without insert were also dotted onto the filter. Three identical dot-blots were hybridized with run-on transcripts derived from proplastids and from chloroplasts of 5-d- and 7-d-old seedlings. In the case of the two chloroplast populations, run-on transcriptions were performed with the same amounts of plastids (2×10^7) and films were exposed for the same times. The amount of proplastids used for the preparation of run-on transcripts was about two- to threefold higher than the amount of chloroplasts. A quantitative comparison between transcription in proplastids and chloroplasts is hardly possible since the volume and the DNA content largely differ between both types of plastids (Mullet 1988). It is, however, possible to analyze the relative transcription rates of various plastid DNA regions by dot-blot analysis. Figure 5 clearly shows changes in the relative transcription during chloroplast biogenesis starting from proplastids, and demonstrates that the rate of transcription decrease during chloroplast maturation differs among various subfragments: Signals belonging to subfragments a-1 and d-1 decrease during maturation more slowly than signals belonging to other subfragments of the same regions. Moreover, the dot-blot analysis shows that transcripts specific for fragment j-2 are enhanced in the 7-d probe compared with the 5-d probe.

In case of run-on transcripts derived from chloroplasts the dot-blot analysis was repeated three times and in case of proplastids once without major changes in the relative intensities of the hybridization signals. Neither an addition of heparin during preparation of run-on transcripts nor a fivefold dilution of the hybridization probe did significantly influence the relative intensities among the dots on the autoradiograms (data not shown).

Identification of cloned fragments by partial sequencing. To elucidate whether subfragments differing in their rela-

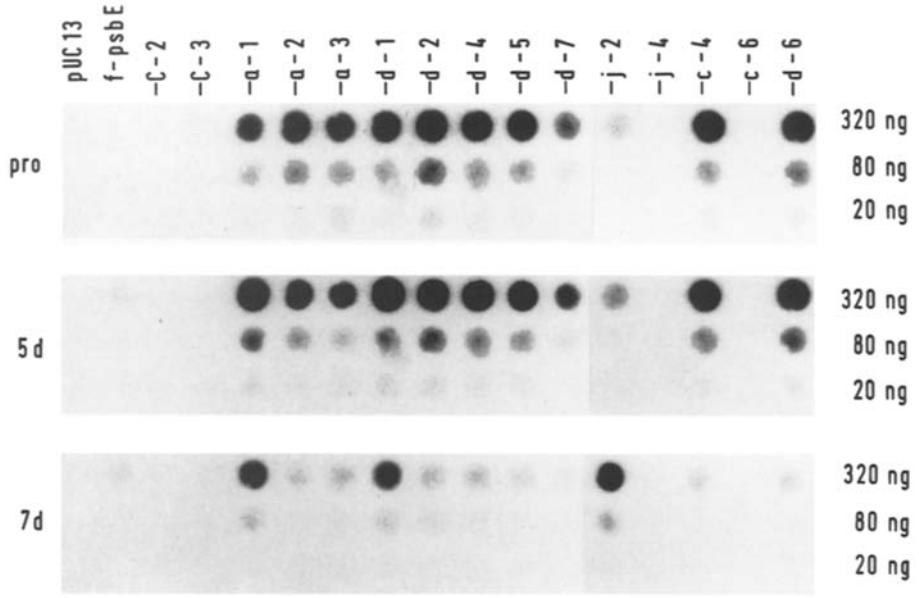

Fig. 5. Transcription analysis by hybridization of proplastid *(pro)* and chloroplast-derived run-on transcripts (*5-d* and *7-d*) to DNA dot-blots. Three identical filters carried 320-ng, 80-ng and 20-ng of each recombinant pUC13 clone containing specific barley plastid DNA fragments and of pUC13 DNA. Fragments were obtained by digestion of *PstI* fragments with *RsaI* and were named according to their positions on the autoradiogram in Fig. 4 (lower numbers belong to larger fragments and vice versa). In the case of chloroplast-derived run-on transcripts which were prepared from 5-d- and 7-d-old barley seedlings, the standard transcription assay was performed with the same number of chloroplasts ($2 \cdot 10^7$) and the autoradiograms were exposed to film for the same time. The number of proplastids was about two to three times higher

tive transcription during biogenesis and maturation of chloroplasts belong to specific groups of genes, sequence analysis was extended to more than ten different subfragments. The cloned fragments were partially sequenced from both ends and were further characterized by homology comparison with the complete sequence of the rice plastid genome (Hiratsuka et al. 1989). In the case of coding regions the nucleotide sequences of rice and barley have a homology of more than 90%. The data therefore allowed a mapping of the fragments based on their theoretical location on the rice plastid genome. In Fig. 6 the fragments are arranged on top of a map representing the small single-copy (SSC) region and parts of the inverted repeats A IR_A and IR_B on the rice plastid genome. According to the hybridization signals on the dot-blots after hybridization with 5-d run-on transcripts and 7-d run-on transcripts, the barley plastid DNA fragments were classified into three groups:

The first group (open boxes in Fig. 6) contains fragments c-2, c-6 and c-3a, which did not show a detectable hybridization signal with run-on transcripts derived from proplastids or from both chloroplast stages. On gels, c-3a co-migrates with c-3b located in the *rrn* region (Fig. 6). The c-2 and c-6 fragments are located in the SSC region at the positions of two *ndh* genes which are homologous with mitochondrial *ndh* genes coding for subunits of the NADH-ubiquinone reductase (Hiratsuka et al. 1989). The sequence of fragment c-3a shows homology with ORF393 in the rice plastid genome. Recently, it was reported that this open reading frame (ORF) is homologous to a nuclear gene encoding a 49-kDa subunit of the mitochondrial NADH-ubiquinone reductase (Fearnley et al. 1989). Though it has been suggested that these *ndh* genes are expressed for the formation of a chloroplast enzyme complex which might participate in a respiratory electron pathway (Fearnley et al. 1989), the results of the present study indicate that in chloroplasts of barley an expression of the plastid *ndh* genes is not likely.

In contrast to group-1 fragments, DNA fragments comprising the second and third group (striped and black boxes in Fig. 6) are transcribed in all stages. Compared with the third group the second group of fragments is characterized by relatively constant transcription in chloroplasts of different ages. It contains the two identical subfragments, a-1 and c-1 located in the IR region, and fragment d-1 of pHcV205. These fragments contain t-RNA genes. The t-RNA genes located on fragments a-1 and c-1 are not transcribed together with the *rrn* operon (Shinozaki et al. 1986). The d-1 fragment contains the t-RNA gene *trnV* located in front of the 16SrDNA. Strittmacher et al. (1985) demonstrated that this gene is transcribed independently of the *rrn* operon.

In contrast to fragments in group 2, the relative transcription rate of fragments in the third group is more intensive in proplastids and has a faster decline during chloroplast maturation. These fragments all represent parts of the *rrn* operon within the inverted repeats. Among fragments belonging to the *rrn* operon, c-3b, which on gels comigrates with c-3a, is identical to fragment a-2, and fragment c-4 is identical to a-3 (Fig. 6). Relative transcription of the two identical fragments a-3 and c-4, which are specific for 5SrDNA and 4.5SrDNA, seems to decline more slowly with increasing chloroplast age than transcription of the other fragments of this group.

Fragments j-2 and j-4 are not included in Fig. 6 since

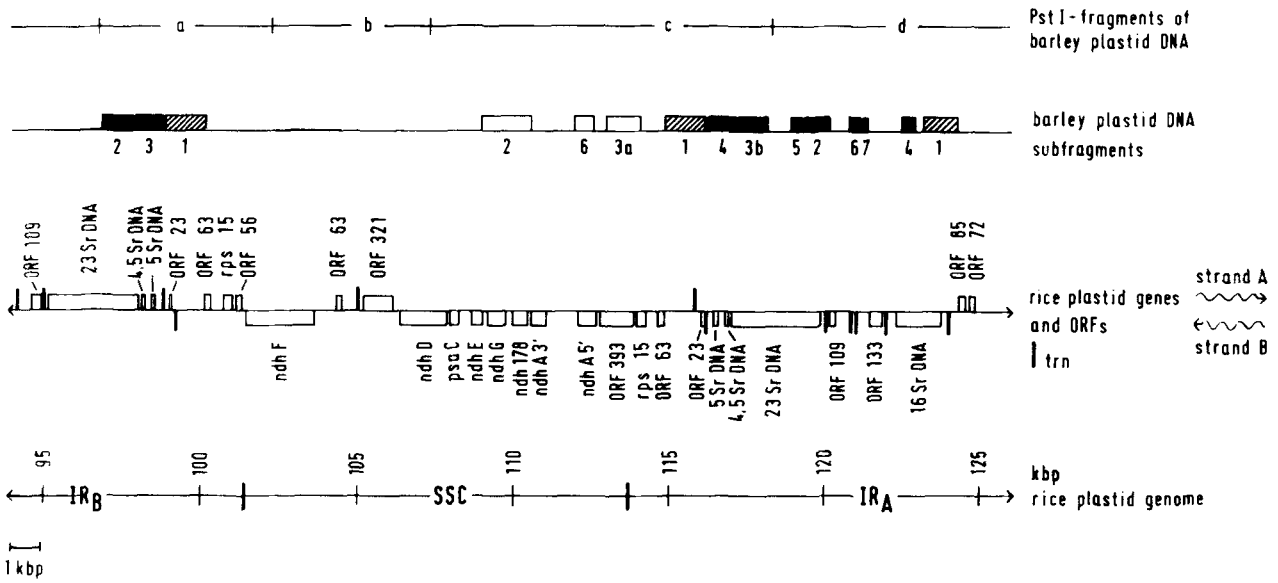

Fig. 6. Assignment of barley plastid DNA fragments, obtained after *RsaI* digestion of *PstI* fragments a, c and d of the IR and SSC regions, to specific locations on the rice plastome. The fragments in the second line are named as in Fig. 5 and are classified into three groups according to their relative transcriptional activity in chloroplasts derived from 5-d- and 7-d-old seedlings: transcription is either barely detectable *(open boxes)*; relatively constant during plastid ageing *(striped boxes)*; or decreases during plastid ageing *(black boxes)*. The third line shows a detail of the linearized physical map of the rice plastid DNA with the location of genes and open reading frames (ORFs) in the SSC and IR regions (Hiratsuka et al. 1989). *Thick vertical lines* indicate positions of tRNA genes in the third line and borders between IRs and SSC in the fourth line. The barley plastid DNA fragments were assigned to specific locations on the rice plastome after partial sequencing and homology comparison with the rice plastid DNA sequence

they belong to another region of the plastome. While j-4 shows no detectable hybridization signal, j-2 is the only fragment on the dot-blot which exhibits a dramatic increase in hybridization with run-on transcripts during biogenesis and ageing of chloroplasts (Fig. 5). Partial sequencing of this fragment allowed its assignment to the *psbD* gene coding for the D2 protein of photosystem II. Though it seems that *psbD* transcription is regulated in a unique way during chloroplast maturation, it cannot be excluded that other genes might be regulated similarily. Owing to comigration of fragments on the gels similar signals might be hidden on the Southern blots (Fig. 2, 4).

Discussion

Transcriptional activity of barley plastids in primary foliage leaves has already been studied intensively (Klein and Mullet 1987; Mullet and Klein 1987; Mullet 1988; Baumgartner et al. 1989). However, none of these investigations included primary foliage leaves grown under a daily light-dark regime as in nature. Instead, the authors studied transcription in leaves which were either grown in complete darkness or in continuous light after a certain period of darkness. In all the studies, special attention was focussed on the fifth day of growth of barley seedlings when transcriptional activity is near maximum (Mullet and Klein 1987; Klein and Mullet 1990) and RNA levels and protein synthesis are high (Klein and Mullet 1987). At this stage of development there are no great differences in the transcriptional activity and transcript patterns of etioplasts, chloroplasts obtained after 16 h illumination of etiolated seedlings (Krupinska and Apel 1989; Klein and Mullet 1990), and the 5-d-old chloroplasts used in this report. Beyond this stage of development plastid maturation may proceed quite differently under various growth conditions (Baumgartner et al. 1989; Klein and Mullet 1990). Data on gene expression in proplastids are rare (Mullet 1988). In barley, the increase in overall transcriptional activity during development of etioplasts and chloroplasts from proplastids can only in part be explained by a parallel increase in plastid number per cell and in DNA content of the plastids (Baumgartner et al. 1989), and may have a specific cause. The synthesis and accumulation of the RNA-polymerase during development (Schrubar et al. 1990) might be a candidate.

During maturation of barley chloroplasts overall transcriptional activity decreases about fivefold. A similar decrease in transcriptional activity of plastids during maturation of spinach leaves grown under greenhouse-conditions has been reported by Deng and Gruissem (1987). This age-dependent decrease in overall transcriptional activity of plastids is independent of changes in plastid DNA levels (Deng and Gruissem 1987; Baumgartner et al. 1989) and must be regulated by a more specific mechanism. Baumgartner et al. (1989) reported similar data for barley seedlings and moreover showed that during maturation the overall incorporation of nucleotides into run-on transcripts decreases faster in the light than in darkness.

In the present study, as well as variations in overall transcriptional activity of different plastid types, specific development-dependent changes in the relative transcrip-

tional activity of individual genes have been shown by hybridization of run-on transcripts derived from proplastids and chloroplasts of different ages with barley plastid DNA fragments. From this approach it transpired that the relative transcription of the *rrn* operon is predominant in proplastids and decreases during maturation of chloroplasts (Fig. 4, 5). During development of young chloroplasts the relative transcription of mRNA and tRNA genes is increasing.

While the light-induced development of chloroplasts from etioplasts during 16 h illumination of 5-d-old barley seedlings is apparently not regulated at the transcriptional level (Krupinska and Apel 1989) the normal pathway to chloroplasts starting from undifferentiated proplastids is paralleled by quantitative and qualitative changes in transcription. Taken together, the former results on plastome transcription during light-induced etioplast transformation and the present results on transcript patterns in proplastids and chloroplasts of 5-d-old barley seedlings make it evident that the two alternative pathways to functional chloroplasts (Leech 1984) are regulated differentially.

During maturation of chloroplasts the decrease in overall transcriptional activity is mainly due to a preferential decrease in *rrn* transcription (Fig. 5, 6). A decrease in *rrn* transcription was also detected during maturation of leaves of spinach (Deng and Gruissem 1987), tobacco (Wollgiehn et al. 1976) and wheat (Patterson and Smillie 1971). Mullet and Klein (1987) demonstrated that the regulation of 16SrDNA transcription was different from that of transcription of mRNA genes, e.g. *psbA* and *rbcL*, in barley seedlings, although their growth conditions were not the same as those used in the present investigation. A decline in 16SrDNA transcription occurs only in the light and hence might be a marker for chloroplast maturation (Mullet and Klein 1987). A preferential decrease in *rrn* transcription during maturation of chloroplasts might have influence on the protein-synthesis capacity of the chloroplasts. Indeed, protein synthesis is near maximum in plastids of 4.5-d-old barley seedlings (Klein and Mullet 1987) and decreases, at latest 8-d after sowing, in primary foliage leaves of seedlings grown under a 18-h daily light period (Martin et al. 1986).

Transcription rates of other genes decrease simultaneously, but at slower rates. An opposite course of transcriptional activity during chloroplast maturation has been observed for the *psbD* gene (Fig. 5). The experiments using a complex Southern blot for hybridization analysis cannot completely rule out that the transcription of further genes, e.g. *psbA*, is likewise regulated. A differential maintenance of *psbD* and *psbA* transcription rates during ageing of illuminated leaves might relate to the light-enhanced turnover of the corresponding 32-kDa proteins D2 and D1, which constitute the reaction center of photosystem II and are presumed to prevent photoinhibition by their high turnover (Schuster et al. 1988). The maintenance of *psbD* transcription during chloroplast maturation is achieved by the additional action of light-induced promoters of the *psbD–psbC* operon (Sexton et al. 1990). Accordingly, the translation capability of *psbD* is maintained in mature chloroplasts (Gamble et al. 1988) and the synthesis of at least one 32-kDa protein is maintained even during leaf senescence (Roberts et al. 1987; Bate et al. 1990). The relative transcript levels of *psbD* and *psbA* increase during senescence, while RNAs specific for other components of the photosynthetic apparatus decrease simultaneously (Bate et al. 1990). An increase in *psbD* and presumably also *psbA* transcription, may be especially important during periods of decreasing electron-transport rates and excess light energy. Though primary foliage leaves of 7-d-old barley seedlings do not senesce, and in apical sections have just reached their maximal photosynthetic capacity (data not shown), the observed changes in relative transcription of individual genes might preceed this developmental stage to enable chloroplasts to cope with the decrease in electron flow and the simultaneous risk of damage by photoinhibition during ageing. In future studies, the relationship between photosynthesis, light intensity, D1/D2 turnover, and transcription rate of *psbA* and *psbD* should be investigated.

Two possible mechanisms could have caused the observed development-specific changes in the transcription of the *rrn* operon and the *psbD* gene: modification of plastid DNA or alterations of RNA-polymerase activity. Since no remarkable differences in the methylation pattern of plastid DNA prepared from young and older chloroplasts could be detected (Fig. 3), the observed differences in transcription most probably reflect development-dependent changes in the RNA-polymerase activity. One possible explanation might be a differential regulation of two different RNA polymerases (Greenberg et al. 1984; Gruissem 1989) during chloroplast maturation. Alternatively, the activity of a single RNA polymerase might be differentially regulated by specific DNA-binding proteins or other molecules like ppGppp which controls transcription in bacteria (Lamond 1985). A biochemical comparison of RNA-polymerase fractions deriving from young and older chloroplasts could help to elucidate the mechanisms governing transcriptional regulation during chloroplast maturation. By a similar approach, Reiss and Link (1985) obtained evidence for plastid-stage-specific proteins that might be involved in the transcriptional regulation of light-dependent chloroplast formation in mustard seedlings.

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