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Differential transcription of plastome-encoded genes in the mesophyll and bundle-sheath chloroplasts of the monocotyledonous NADP-malic enzyme-type C_4 plants maize and *Sorghum*

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Abstract

The transcription of plastome-encoded genes in mesophyll and bundle-sheath chloroplasts of the monocotyledonous NADP-malic enzyme-type C_4 species *Zea mays* L. (maize) and *Sorghum bicolor* (L.) Moench. was investigated. RNA accumulation and transcription were assayed starting from isolated mesophyll and bundle-sheath chloroplasts and using quantitative northern and run-on transcription analysis. Determination of the mesophyll to bundle-sheath ratios of transcript abundance in maize and *Sorghum* chloroplasts showed that the mRNAs of the plastome-encoded photosystem II genes analysed (*psbA*, *psbB*, *psbD*, *psbH* and *psbE/F*) varied from 2.5- to 4.0-fold (maize) and 3.1- to 5.2-fold (*Sorghum*), respectively. The *rbcL* transcript, in contrast, was more abundant in bundle-sheath chloroplasts of both species, about 3-fold in maize and more than 10-fold in *Sorghum*. On the other hand, transcripts of genes encoding the 16S ribosomal RNA (r16) and subunits of photosystem I (*psaA*) and the cytochrome *b/f* complex (*petB*, *pet*A) accumulated to similar levels in both types of chloroplasts. Determination of absolute transcript levels for *rbcL* and *psbA* in chloroplasts from maize and *Sorghum* demonstrated that for both genes, the mesophyll to bundle-sheath differences in transcript abundance were more pronounced in *Sorghum*. Measurements of the transcriptional activities of *rbcL* and *psbA* showed that the transcription rate of *rbcL* is higher in bundle-sheath chloroplasts while *psbA* is more actively transcribed in mesophyll chloroplasts. The differences in the transcription rates between the two chloroplast types were again more pronounced in *Sorghum*, thus reflecting the differences between maize and *Sorghum* in the relative levels of the *rbcL* and *psbA* transcripts. However, although transcription rate and mRNA abundance are correlated, they did not exactly match one another. This indicates additional regulation of transcript abundance at the level of RNA stability.

Introduction

The leaves of NADP-malic enzyme (NADP-ME)-type C_4 plants are characterized by dimorphic chloroplasts. While the mesophyll chloroplasts display a high degree of grana stacking, thus resembling typical C_3 chloroplasts, the bundle-sheath chloroplasts are agranal or exhibit reduced grana [22]. In maize and *Sorghum* chloroplasts, the amount of grana stacking correlates

with both photosystem II activity [9, 43] and the abundance of photosystem II polypeptides [13, 24, 29, 35, 38]. Therefore, NADP-ME-type C_4 plants are particularly suited for studying the cell-specific regulation of plastid gene expression.

As first steps towards an understanding of the mechanisms controlling the C_4-specific expression of plastid genes, protein and mRNA levels were investigated using northern and western analysis (reviewed in [27]). For *rbcL* and the plastome-located photosystem II genes, it has been shown that the differences in the protein levels found in mesophyll and bundle-sheath chloroplasts correlate with differences in the levels of the corresponding transcripts, which implicates that the C_4-specific expression of plastid genes is regulated at the level of mRNA abundance [21, 29, 37, 38]. This view was supported by measurements of the rates of synthesis of the D1 protein of photosystem II and the large subunit of Rubisco in mesophyll and bundle-sheath chloroplasts of maize using an *in organello* translation system. It was found that the rates of translation of these proteins, like the steady-state levels, also correlate with the respective mRNA levels [25].

In general, the steady-state transcript level reflects an equilibrium between RNA synthesis and degradation. Regulation at both levels has been shown to operate in chloroplasts (reviewed in [10]). In order to determine whether transcript abundance is controlled by transcription, RNA stability or a combination of both processes, quantitative measurements of transcription rates and RNA levels are necessary. A comparison of RNA levels and RNA degradation rates should yield the same information but measurements of RNA stability in chloroplasts are technically difficult [17, 18, 28].

This study was initiated in order to clarify whether transcription or regulation of mRNA stability are responsible for the differential accumulation of plastome-encoded transcripts in mesophyll and bundle-sheath chloroplasts of monocotyledonous NADP-ME-type C_4 plants. To be able to distinguish between general and species-specific expression characteristics of plastid genes, two species of this group were examined. The species selected, maize and *Sorghum*, are particularly suited for a comparison because they differ in the extent of photosystem II reduction in the bundle-sheath chloroplasts [24, 29, 43]. Run-on transcription analysis of mesophyll and bundle-sheath chloroplasts showed that at least two genes, *psbA* and *rbcL*, are transcribed differentially in the two chloroplast types.

Materials and methods

Plants and growth conditions

Maize (*Zea mays* cv. B73; Kleinwanzlebener Saatzucht, Einbeck, Germany) and *Sorghum* (*Sorghum bicolor* cv. Tx430; Pioneer Hi-Bred, Plainview, TX) were germinated in soil. Plants were grown under greenhouse conditions with additional illumination (14 h per day from 07:00 to 21:00) provided by a combination of sodium and mercury high-pressure vapour lamps. The photon flux density was about 300 μmol m^{-2} s^{-1} at the plant height level. 8- to 10-day-old maize and 7- to 9-day-old *Sorghum* plants were used for isolation of chloroplasts. Only the upper two-thirds of the second leaf were harvested.

Isolation of intact mesophyll and bundle-sheath chloroplasts

The procedure followed essentially the protocol of Westhoff and Meierhoff [24] which was devised for the isolation of intact mesophyll and bundle-sheath chloroplasts from maize. However, here some important modifications were made to improve the final yield of intact chloroplasts. The modified protocol proved also suitable for the isolation of intact mesophyll and bundle-sheath chloroplasts from *Sorghum*. In each experiment, both mesophyll and bundle-sheath chloroplasts were isolated from the same leaf material.

The leaf material (10–20 g) was cut into small pieces (5–10 mm) with a razor blade, cooled on ice for 10 min, and then homogenized in a Waring

blender at low speed for 6–8 s in 300 ml blending medium I (0.35 M sorbitol, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-KOH pH 8.0, 10 mM EDTA, 1 mM $MgCl_2$, 0.2% (w/v) bovine serum albumin (BSA), 2 mM sodium isoascorbate and 5 mM dithiothreitol). The homogenate was filtered through two layers of Miracloth (Calbiochem, Frankfurt, Germany), centrifuged for 15 min at 1000 × *g* at 4 °C, and the resulting pellet of crude chloroplasts was resuspended in 2 ml blending medium II (0.35 M sorbitol, 25 mM Hepes-KOH pH 8.0, 10 mM EDTA, 1 mM $MgCl_2$). The suspension was layered onto two Percoll step gradients (upper layer, 10 ml of 32% (v/v) Percoll medium; lower layer, 5 ml of 90% (v/v) Percoll medium) in 30 ml Corex centrifuge tubes. The Percoll solution was prepared as described in Price *et al.* [33] using blending medium II. After centrifugation (10 min at 2500 × *g* at 4 °C) the intact chloroplasts were transferred to a fresh tube, diluted with approximately 5 volumes of blending medium II and re-centrifuged for 30 s at 6000 × *g* at 4 °C. The pellet of intact mesophyll chloroplasts was resuspended in 100–500 μl blending medium II and stored on ice until the isolation of bundle-sheath chloroplasts was finished.

The preparation of bundle-sheath chloroplasts was started during the first centrifugation step for the isolation of mesophyll chloroplasts. The crude bundle-sheath strands retained in the Miracloth gaze were ground for 30 s (maize) or 60 s (*Sorghum*), respectively, at high speed in a Waring blender in 250 ml of blending medium II. The homogenate was transferred to a second blending vessel and ground twice for 45 s at high speed. In this second blending vessel, the blades were fixed in an upside-down position. As a consequence, the strands were hit by the blunt and not by the sharp-edged side of the blades, resulting in improved yields of intact, unfragmented bundle-sheath strands. The homogenate was filtered through two layers of Miracloth and the residual bundle-sheath strands were transferred to 100 ml of digestion medium (0.35 M sorbitol, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH pH 6.0, 1 mM KH_2PO_4, 0.3% (w/v) Macerozyme R-10 and 2% (w/v) Cellulase Onozuka RS (both from Yakult Biochemicals)). The enzymatic digestion was carried out for 45 min (maize) or 75 min (*Sorghum*) at 25 °C with gentle shaking. While no bundle-sheath protoplasts were released from maize and *Sorghum* strands under these conditions – even after digestion for up to 12 h and similar treatment only low yields of bundle-sheath protoplasts were obtained (cf. [27] – the procedure rendered the bundle-sheath cells susceptible to breakage by gentle mechanical treatment. After digestion, the bundle-sheath strands were collected in a commercially available tea strainer and then transferred onto a 80 μm mesh that was submersed in 200 ml of blending medium II. Gentle stirring with a spatula broke the cells and the organelles were released into the medium. The medium containing the released bundle-sheath chloroplasts was successively filtered through 80 μm mesh and two layers of Miracloth, and centrifuged for 4 min at 2500 × *g* at 4 °C. Intact bundle-sheath chloroplasts were obtained as described for mesophyll chloroplasts, except that the Percoll gradient centrifugation was carried out at 4000 × *g*. Total chlorophyll was calculated according to Arnon [1]. Chloroplast numbers were determined by counting in a hemocytometer.

Run-on transcription reactions

Run-on transcription assays with intact mesophyll and bundle-sheath chloroplasts were essentially carried out as described by Klein and Mullet [19] with the reaction mixture modified as follows: spermidine was omitted, heparin was used at a final concentration of 0.1 mg/ml and [α-^{32}P]UTP (Amersham Buchler, Braunschweig, Germany) was added to 1.2 μCi/μl. Transcription was initiated by adding 5 × 10^6 plastids to the reaction mixture (final volume, 50 μl) and allowed to continue for 5 min at 25 °C. Transcription was terminated by adding 50 μl H_2O (treated with diethylpyrocarbonate), 100 μl of a SDS solution (100 mM Hepes pH 8.0, 400 mM LiCl, 20 mM EDTA and 2% (w/v) SDS) and 2 μl Proteinase K solution (10 mg/ml) followed by

an 30-min incubation at 37 °C. Incorporation of [α-^{32}P]UTP into transcripts was determined as described by Hallick *et al.* [11]. The transcription assays were used for filter hybridization without any further purification of the radiolabelled transcripts.

DNA and RNA probes for hybridization

Strand-specific DNA probes for hybridization analysis of plastid run-on transcripts and riboprobes for northern blot analysis of plastid transcript levels were generated from recombinant plasmids containing gene-specific fragments of spinach (*psbA*, *psbD*, *psbE/F*, *psbH*, *petA*, ORF62; [42]) and maize (*rbcL*, *psaA*, *psbB*, *petB* and r16) plastid DNA. Maize plastid DNA was isolated according to Palmer [30] and appropriate restriction fragments were cloned into pBluescript KSII$^+$ and KS$^-$ expression vectors using standard procedures [34]: a 1.8 kb *Bgl* II-*Hin*c II fragment for *rbcL*, a 0.9 kb *Apa* I fragment for *psaA*, a 0.74 kb *Bam* HI-*Hin*d III fragment for *psbB*, a 1.47 kb *Eco* RI-*Xba* I fragment for *petB* and a 3.1 kb *Bam* HI fragment for the 16S rDNA (for the exact positions of the fragments see [22] (*rbcL*), [7] (*psaA*), [33] (*psbB*, *petB*) and [36] (r16).

Riboprobes for the determination of plastid RNA levels were produced by *in vitro* transcription as described in Westhoff *et al.* [42]. Strand-specific DNA probes were generated using maize clones and the spinach *psbA* clone (for this purpose, the insert of the *psbA* clone [42] was excised and re-cloned into pBluescript KSII$^+$ and KS$^-$). Single-stranded phagemid DNA was obtained by superinfection with the f1 helper phage R408 (Stratagene Cloning Systems, San Diego, CA [6]).

Hybridization of radiolabelled run-on transcripts to DNA blots

Single-stranded DNA probes (2 and 8 pmol per dot, respectively) were immobilized onto Biodyne B nylon membranes (0.45 μm; Pall, Dreieich, Germany) using a Minifold I dot blot device (Schleicher & Schüll, Dassel, Germany). Prehybridization (12–15 h at 68 °C) and hybridization (48 h at 68 °C) were performed according to Church and Gilbert [4]. For hybridization of the ^{32}P-labelled run-on transcripts, 90–100 μl of the Proteinase K-treated reaction mixture were added to 3 ml of hybridization solution. After hybridization, blots were washed twice at 68 °C in 1 × SSC, 0.1% SDS for 30 min. Blots were then analysed by autoradiography and liquid scintillation counting of the excised spots.

Determination of relative and absolute levels of chloroplast RNA

Relative transcript levels in mesophyll and bundle-sheath chloroplasts were compared as described in Westhoff *et al.* [42]. RNA was isolated from intact mesophyll and bundle-sheath chloroplasts by the phenol/chloroform method [42] and freed from DNA by precipitation from 2 M LiCl [41]. Dilution series (0.004, 0.02 and 0.1 μg) of glyoxylated RNAs [23] were blotted onto Biodyne A nylon membranes (0.2 μm; Pall, Dreieich, Germany) using a Minifold 1 dot blot apparatus (Schleicher & Schüll, Dassel, Germany). Each blot contained mesophyll as well as bundle-sheath RNA side by side to allow a direct comparison of transcript levels. Prehybridization and hybridization with radiolabelled riboprobes was carried out at 64 °C according to Church and Gilbert [4]. The abundance of 16S rRNA was determined by hybridization with saturating amounts of a 48-mer oligonucleotide complementary to nucleotides 230 to 277 of the maize 16S rRNA [38]. The oligonucleotide was end-labelled with [γ-^{32}P]ATP according to standard procedures [34]. Hybridization was carried out for 48 h at 62 °C. After hybridization, the blots were washed 30 min each at 64 °C (riboprobes) or 62 °C (oligonucleotide), respectively, in 1 × SSC, 0.1% SDS and in 0.5 × SSC, 0.1% SDS. Radioactivity bound per dot was determined again by liquid scintillation counting.

To determine the absolute values of *rbcL* and *psbA* transcript levels, a calibration series with known amounts of *in vitro* synthesized RNAs was

co-hybridized with the mesophyll and bundle-sheath chloroplast RNA. Synthesis and purification of the *in vitro* synthesized RNAs was performed essentially as described by Rapp *et al.* [32]. The integrity of the CsCl-purified transcripts was controlled by electrophoresis on agarose gels. Hybridization of the blots and quantitation of bound radioactivity was as described above.

Enzyme measurements

Marker enzyme activities were assayed spectrophotometrically at 340 nm. Aliquots of preparations of intact chloroplasts equivalent to 2.5 μg chlorophyll were processed as follows.

1. NADP-malate dehydrogenase (NADP-MDH) activity was measured according to Kagawa and Bruno [15]. To preactivate the enzyme, aliquots were diluted to a final volume of 120 μl containing 200 mM Tris-HCl pH 8.5, 200 mM DTT and 1 mM EDTA and kept at 25 °C for 1 h. After sedimentation of membranous material, 100 μl supernatant was transferred to the assay mixture.

2. For the measurement of NADP-malic enzyme (NADP-ME) activity, chloroplasts were lysed in 50 mM Tricine-KOH pH 8.3, 1 mM EDTA, centrifuged as above and 100 μl supernatant was added to the assay mixture. Measurement of NADP-ME activity was performed according to Hatch and Mau [12]. The cross-contaminations were estimated as follows (as an example, the equation for the preparation of bundle-sheath chloroplasts is shown):

$$\% \text{ MC in BC preparation} = \frac{\text{activity of NADP-MDH in BC preparation}}{\text{activity of NADP-MDH in MC preparation}}$$

Results and discussion

Experimental design

This study aimed to investigate the mechanisms underlying the spatial expression of plastid genes in mesophyll and bundle-sheath chloroplasts of NADP-ME-type C_4 plants by comparing transcription rates and RNA levels. In a first set of experiments, methods were established for the isolation of intact and transcriptionally active chloroplasts of maize and *Sorghum*. To assay the transcriptional activities of these chloroplasts the plastid run-on transcription method was used [5, 26]. Simultaneously, with RNA isolated from intact chloroplasts a quantitative analysis of relative and absolute transcript levels was carried out.

Studies of the cell type-specific accumulation of plastid transcripts in C_4 plants have been reported repeatedly [14, 21, 35, 37, 38, 41]. Nonetheless, the quantifications presented in this study were necessary for the following reasons. Firstly, a comprehensive and quantitative analysis had not yet been carried out. Secondly, the results described so far were obtained by analysis of whole-cell RNA, while in this study plastid RNA isolated from intact chloroplasts was used. Thirdly, it was necessary to use the same plant material for the analysis of transcription and RNA levels because it is likely that the C_4-specific gene expression varies according to environmental conditions as do the anatomical and biochemical features of C_4 photosynthesis [8, 13, 39, 40].

Isolation of mesophyll and bundle-sheath chloroplasts

Intact mesophyll and bundle-sheath chloroplasts were isolated from maize and *Sorghum* seedlings by a combined mechanical and enzymatical procedure as outlined in Materials and methods. The cross-contamination of the preparations was routinely checked by assaying marker enzymes, such as the mesophyll-specific NADP-malate dehydrogenase (MDH) and the bundle-sheath-specific NADP-malic enzyme (ME). The enzyme activities and the degree of cross-contamination for a number of experiments with both maize and *Sorghum* are listed in Table 1. In general, the cross-contamination of the bundle-sheath preparations from both species was low, amounting to about 5% in maize and 3% in *Sorghum*. On the other hand, the average contamination of mesophyll

Table 1. Purities of chloroplast preparations from mesophyll and bundle-sheath cells of maize and *Sorghum*. The degree of cross-contamination was calculated by measurement of the marker enzymes NADP-MDH and NADP-ME as described in Materials and methods. Duplicate measurements were carried out.

Experiment No.	NADP-MDH activity (μmol/min per mg Chl)		% MC in BC preparation	NADP-ME activity (μmol/min per mg Chl)		% BC in MC preparation
	MC	BC		MC	BC	
A. Maize						
1	13.5	0.6	4.8	2.1	8.5	24.7
2	30.2	0.1	1.7	3.9	22.2	17.4
3	8.6	0.8	9.0	2.0	13.1	15.0
4	11.3	1.2	11.0	3.5	14.3	24.3
5	16.2	0.2	1.5	3.3	13.7	23.8
Mean ± SD	16.0 ± 7.5	0.6 ± 0.4	5.6 ± 3.8	3.0 ± 0.8	14.4 ± 4.4	21.0 ± 4.0
B. Sorghum						
1	18.9	0.4	2.0	0.6	15.8	3.7
2	13.7	0.4	2.7	0.9	24.0	3.5
3	18.8	0.6	2.9	0.7	27.5	2.4
4	20.9	0.6	2.9	1.2	35.8	3.2
5	4.8	0.1	2.7	0.3	16.6	1.7
Mean ± SD	15.4 ± 5.8	0.4 ± 0.2	2.6 ± 0.3	0.7 ± 0.3	23.9 ± 7.4	2.9 ± 0.7

chloroplasts with bundle-sheath chloroplasts was found to differ drastically between maize and *Sorghum*. While the cross-contamination was relatively high (about 20%) in maize, mesophyll chloroplasts from *Sorghum* were essentially pure and contained only about 3% bundle-sheath chloroplasts. Thus, the procedure for the isolation of intact mesophyll and bundle-sheath chloroplasts was particularly effective with *Sorghum*. This is due to the relatively rigid bundle-sheath lamella which is already established in *Sorghum* at the young seedling stage and which is largely resistant to breakage when homogenizing the leaf pieces for the release of the mesophyll chloroplasts.

Analysis of RNA accumulation in mesophyll and bundle-sheath chloroplasts

RNA dot blot experiments were performed to determine the mesophyll to bundle-sheath ratios of transcript levels in maize and *Sorghum*. In total, eleven plastid genes were analysed. *PsbA*, *psbB*, *psbD*, *psbE/F* and *psbH* transcripts were selected because they encode subunits of photosystem II which is known to be specifically reduced in the bundle-sheath chloroplasts [42]. *RbcL*, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), served as an example for a transcript whose level is reduced in the mesophyll cells [21]. The transcripts for subunits of photosystem I (*psaA/B*), the cytochrome b/f complex (*petA*, *petB/D*) and the 16S ribosomal RNA (r16) were included in the analysis because previous experiments had suggested that the levels of these transcripts do not differ significantly between mesophyll and bundle-sheath chloroplasts [42]. In addition, the transcript levels for an unidentified open reading frame ORF62, were also analysed, because this reading frame is co-transcribed with *psbD/C* (cf. [9, 42]).

In maize and *Sorghum* the levels of the *psaA* and *petA/B* mRNAs show only small differences between the two chloroplast types (Fig. 1), as is also the case for the proteins [24, 29]. The 16S rRNA accumulates to similar levels, indicating

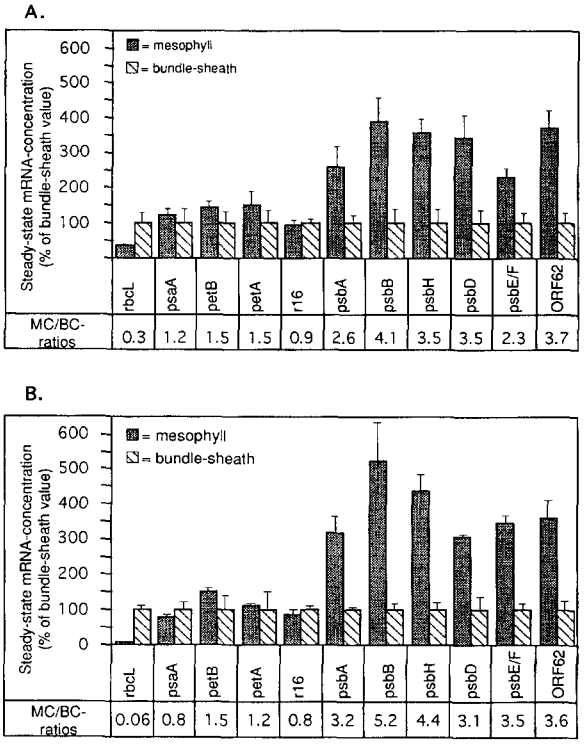

Fig. 1. Comparison of relative mRNA levels in mesophyll and bundle-sheath chloroplasts of maize and *Sorghum*. RNA from intact mesophyll and bundle-sheath chloroplasts of maize and *Sorghum* was analysed by dot-blot hybridization as described in Materials and methods. The data depicted are mean values. In the case of maize (Panel A), ten independent RNA preparations were analysed except for the 16S rRNA, for which three different RNA preparations were analysed. In the case of *Sorghum*, four preparations of bundle-sheath and two preparations of mesophyll plastid RNA were analysed. The figure shows a schematic representation of the mesophyll to bundle-sheath ratios of transcript levels. The hybridization data are expressed as percent of the bundle-sheath values which were set to 100%.

that mesophyll and bundle-sheath chloroplasts do not differ significantly in ribosome content. However, all transcripts for photosystem II subunits are less abundant in bundle-sheath chloroplasts of young seedlings of maize and *Sorghum* [9, 43]. In maize the ratios of transcript abundance range from 2.3 to 4.1 between mesophyll and bundle-sheath (Fig. 1A) while in *Sorghum* the ratios vary from 3.1 to 5.2 (Fig. 1B). The ORF62 transcript is also less abundant in bundle-sheath chloroplasts which may implicate that ORF62 encodes a protein functionally connected with photosystem II.

It has been shown repeatedly that the level of the large subunit of Rubisco is reduced in mesophyll cells of young maize leaves. However, the data describing the extent of this reduction vary [3, 37]. It was determined that the *rbcL* transcript is about 3-fold more abundant in bundle-sheath than in mesophyll chloroplasts. Thus, even taking into account a cross-contamination of up to 20%, mesophyll chloroplasts of maize must contain substantial amounts of *rbcL* mRNA. This result is in good agreement with data from *in organello* translation experiments where the large subunit of Rubisco was found to be synthesized at about 30% of the rate which was determined for bundle-sheath chloroplasts of maize [24]. In *Sorghum* the *rbcL* transcript is about 15-fold more abundant in bundle-sheath chloroplasts. At present it is not known whether this ratio is reflected at the protein level.

To determine absolute RNA levels, RNA standards for *rbcL*, *psbA* and *psaA* were co-hybridized with the chloroplast RNAs, as recently described by Rapp *et al.* [32]. The results of this analysis (Fig. 2) agree with the data from the measurements of the relative transcript levels (Fig. 1), although the mesophyll to bundle-sheath ratio of the *rbcL* mRNA estimated from the absolute

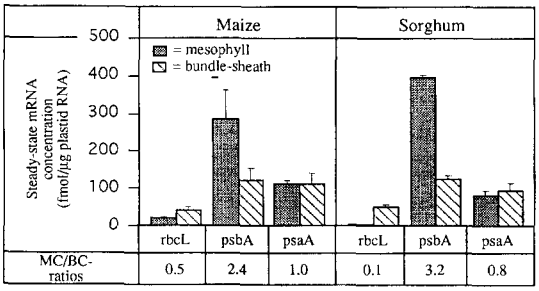

Fig. 2. Determination of absolute levels of *psbA*, *rbcL* and *psaA* mRNAs in mesophyll and bundle-sheath chloroplasts of maize and *Sorghum*. Aliquots of RNAs from mesophyll and bundle-sheath chloroplasts were co-hybridized with a dilution series of *in vitro* synthesized RNA standards for *rbcL*, *psbA* and *psaA* as described in Materials and methods. Mean values given were derived from five independent RNA preparations from maize and two (mesophyll) and four (bundle-sheath), respectively, from *Sorghum*.

RNA level data was slightly higher (0.5 compared with 0.3 for maize and 0.1 compared with 0.06 for *Sorghum*). In the two species, the differences found for *rbcL* and *psbA* are more pronounced in *Sorghum*. This was to be expected since a comparative analysis of the biogenesis of photosystem II in maize and *Sorghum* has shown that the young *Sorghum* seedlings are more advanced in developing C_4 characteristics than maize plants of the same age [24, 29].

Run-on transcription in isolated mesophyll and bundle-sheath chloroplasts of maize and Sorghum

The differences in the steady-state transcript levels of *rbcL* and the photosystem II genes that were found could be due to differences in transcription rates, RNA stability or a combination of both. To distinguish between these possibilities, the transcriptional activities of mesophyll and bundle-sheath chloroplasts were analysed in run-on transcription assays. Table 2 shows that the overall transcriptional activity of mesophyll chloroplasts from both maize and *Sorghum* (expressed as total incorporation of radioactivity per standard assay) is about 1.5- to 2-fold higher than that of bundle-sheath chloroplasts. While the average total transcriptional activity of the mesophyll chloroplasts was approximately the same in maize and *Sorghum*, bundle-sheath chloroplasts of *Sorghum* were generally somewhat more active than those of maize.

To assay the transcriptional activity of individual plastid genes, ^{32}P-labelled run-on transcripts were hybridized to dot-blots containing single-stranded antisense DNA probes. Preliminary experiments ensured that the amounts of probe immobilized onto the filters (8 pmol) were in excess. Single-stranded DNA probes were chosen in order to avoid artefacts due to bidirectional transcription of chloroplast DNA. Reproducible differences in the transcriptional activities were detectable only for the heavily transcribed *psbA* and *rbcL* genes. Therefore, the following experiments focused on assaying the transcription of these two genes. For a comparison, *psaA*, a gene whose mRNA accumulates to similar levels in the mesophyll and bundle-sheath chloroplasts of maize, was included in the hybridization

Table 2. Total transcriptional activity of mesophyll and bundle-sheath chloroplasts from maize and *Sorghum*. The activities are expressed as incorporation of [α^{32}P]UTP into transcripts after 5 min of labelling (in cpm) per 5×10^6 chloroplasts. Triplicate determinations were performed in each incorporation experiment, and the mean values are given (SD ≤ 5%).

Experiment No.	MC	BC	MC/BC ratio
A. Maize			
1	510294	272887	1.9
2	442949	224272	2.0
3	465933	93133	5.0
4	178183	198596	0.9
5	505538	252768	2.0
Mean ± SD	420579 ± 29%	208331 ± 30%	2.0
B. Sorghum			
1	454485	322061	1.4
2	364316	326161	1.1
3	184406	99204	1.7
4	548173	336896	1.6
5	671880	331390	2.0
Mean ± SD	443452 ± 37%	296372 ± 34%	1.6

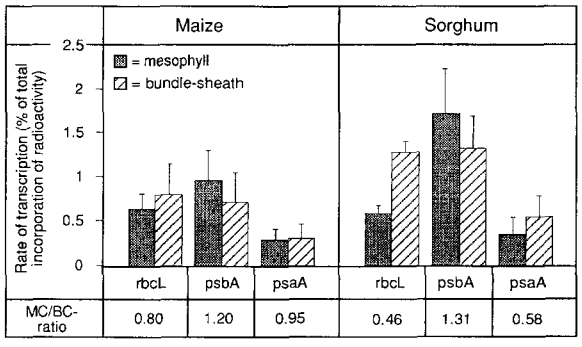

Fig. 3. Rates of transcription of *rbcL*, *psbA* and *psaA* in mesophyll and bundle-sheath chloroplasts of maize and *Sorghum*. Radiolabelled transcripts of a standard run-on transcription assay were hybridized to single-stranded DNA probes immobilized on nylon membranes, and the amounts of hybridized run-on transcripts were determined by liquid scintillation counting. Data are expressed as mean values ± S.D. of five different experiments (see Table 2).

analysis. Figure 3 depicts that both in maize and *Sorghum*, the transcription of *rbcL* is higher in bundle-sheath than in mesophyll chloroplasts. In contrast, *psbA* is more actively transcribed in mesophyll chloroplasts. The differences in the transcriptional activities of the two genes in mesophyll and bundle-sheath chloroplasts are more prominent in *Sorghum* than in maize. As was expected from the RNA measurements, the transcriptional activity of *psaA* is similar in mesophyll and bundle-sheath chloroplasts of maize. In *Sorghum*, however, this gene is transcribed somewhat stronger in the bundle-sheath chloroplasts.

Conclusion

The differences between the transcription rates of *rbcL* and *psbA* in mesophyll and bundle-sheath chloroplasts are not very pronounced (Fig. 3). However, they correlate with the transcript levels (Fig. 2). Thus, the data demonstrate that differential transcription is involved in regulating the cell type-specific expression of both genes. This view is further corroborated by comparing the differences between the two species. For example, the abundance of the *rbcL* transcript differs between mesophyll and bundle-sheath chloroplasts, viz. two-fold in maize and ten-fold in *Sorghum*, and the ratios for the transcription rates are 1.25 and 2.2 respectively.

Although transcription rate and mRNA abundance correlate, they do not exactly match one another, the differences in transcription rates of *rbcL* and *psbA* (Fig. 3) being smaller than those in the transcript levels. On one hand, this may be due to additional regulation of the accumulation of the two transcripts at the level of mRNA stability. On the other hand, it may be possible that the cell type-specific differences in the transcriptional activities are more pronounced *in vivo* and are levelled to some degree during the chloroplast isolation. Simultaneous regulation of the abundance of individual plastid transcripts at the levels of transcription and RNA stability has recently been shown in developing barley [32] and wheat [16] seedlings and is also likely to operate in mesophyll and bundle-sheath chloroplasts. However, at present it is not possible to discriminate between the two alternatives.

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