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Comparative analysis of biochemical properties of mesophyll and bundle sheath chloroplasts from various subtypes of C_4 plants grown at moderate irradiance

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The photochemical characteristics of mesophyll and bundle sheath chloroplasts isolated from the leaves of C_4 species were investigated in *Zea mays* (NADP-ME type), *Panicum miliaceum* (NAD-ME type) and *Panicum maximum* (PEP-CK type) plants. The aim of this work was to gain information about selected photochemical properties of mesophyll and bundle sheath chloroplasts isolated from C_4 plants grown in the same moderate light conditions. Enzymatic as well as mechanical methods were applied for the isolation of bundle sheath chloroplasts. In the case of *Z. mays* and *P. maximum* the enzymatic isolation resulted in the loss of some thylakoid polypeptides. It was found that the PSI and PSII activities of mesophyll and bundle sheath chloroplasts of all species studied differed significantly and the differences correlated with the composition of pigment-protein complexes, photophosphorylation efficiency and fluorescence emission characteristic of these chloroplasts. This is the first report showing differences in the photochemical activities between mesophyll chloroplasts of C_4 subtypes. Our results also demonstrate that mesophyll and bundle sheath chloroplasts of C_4 plants grown in identical light conditions differ significantly with respect to the activity of main thylakoid complexes, suggesting a role of factor(s) other than light in the development of photochemical activity in C_4 subtypes.

Keywords: bundle sheath, C_4 subtypes, mesophyll, chloroplast, chlorophyll–protein complex, PSI and PSII activity

INTRODUCTION

Leaves of C_4 plants contain two types of photosynthetic cells, mesophyll (M) and bundle sheath (BS) cells, that are quite distinctly organized, both structurally and functionally. Three C_4 subgroups are distinguished based on differences in decarboxylating mechanisms (Hatch, 1999): the NADP-ME-, NAD-ME-, and PEP-CK-type. The appearance of chloroplasts is similar in mesophyll and bundle sheath cells in plants of the NAD-ME and PEP-CK types, they contain granal thylakoids (Edwards & Walker, 1983; Hattersley & Watson, 1992). A structural dimorphism of chloroplasts is observed in plants with the NADP-ME type of C_4 photosynthesis, such as *Zea mays*. Their mesophyll chloroplasts are similar to those of C_3 higher plants, while bundle sheath chloroplasts lack grana and show extensive stroma-exposed thylakoids (Ghirardi & Melis, 1983). It is commonly accepted that in very young maize plants BS cells have granal chloroplasts but both the PSII activity and the appressed lamellae disappear during leaf differentiation (Downton & Pyliotis, 1971; Dengler & Nelson, 1999). In *Panicum miliaceum* (NAD-ME type) a size dimorphism is observed in chloroplasts of the two cell layers. Larger chloroplasts and mitochondria with a centripetal orientation are found in the BS cells. In *P. maximum* (PEP-CK type) plants there are four large BS cells with chloroplasts located uniformly (Leatsch, 1971;

Abbreviations: CFoCF1, chloroplast ATP synthase; Chl, chlorophyll; CP, chlorophyll-protein; BS, bundle sheath; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCy, ferricyanide; LDS, lithium dodecyl sulfate; LHCII, chlorophyll *a*/*b*-binding protein of photosystem II; M, mesophyl, MV, methyl viologen; NAD-ME, NAD-malic enzyme; NADP-ME, NADP-malic enzyme; OEC, oxygen evolving complex; PEP-CK, phosphoenolpyruvate carboxykinase; PSI and PSII, photosystem I and II; TMPD, tetramethyl-p-phenylenediamine.

Edwards *et al.,* 1979). There are conflicting reports regarding the activity of PSII in BS chloroplasts of the NADP-ME subtype (Andersen *et al.,* 1972; Edwards & Huber, 1981; Edwards & Walker 1983; Romanowska *et al.,* 2006). Homann and Schmid (1967) found that in 6-day-old maize seedlings BS chloroplasts were active in NADP photoreduction but this capacity was lost as the leaves maturated. Schuster *et al.* (1985) demonstrated that the agranal bundle sheath chloroplasts exhibited low PSII activity associated with reduced amounts of PSII components (traces of LHCII polypeptides at both the protein and mRNA levels) in comparison to the granal mesophyll chloroplasts. A lack of PSII activity has also been shown (Anderson *et al.,* 1971); and a lack of OEC polypeptides and ferredoxin-$NADP^+$ reductase (Bassi *et al.,* 1995) in the maize bundle sheath thylakoids. Thus, chloroplasts isolated from BS cells seem not to photoreduce NADP (Polya & Osmond, 1972) and cannot evolve oxygen similarly to the stromal lamellae of C_3 plant chloroplasts (Lavergne & Leci, 1993). This supports the view that the photosynthetic apparatus of BS chloroplasts generates ATP through a PSI-dependent cyclic pathway of electron transport. Fluorescence data obtained by Pfündel (1998) provided evidence that the two distinct photosynthetic compartments (M and BS) differed in their PSI/PSII ratio and that PSII concentration in bundle sheath cells was elevated in NAD-ME species but diminished in most of the NADP-ME plants. Therefore the question arises, what is the function of PSII if it is present in agranal BS chloroplasts? Pfündel *et al.* (1996) showed that the excitation energy of PSII was efficiently transferred to PSI in BS chloroplasts of many NADP-ME species despite their relatively low PSII concentrations, but they did not demonstrate that light energy was used for ATP synthesis. This data agree with the observation of Bassi *et al.* (1995) that light-harvesting complex II (LHCII) functions as an antenna for PSI in bundle sheath chloroplasts of maize. It was also presented that bundle sheath chloroplasts contained LHCII polypeptides but peptide composition and amount were different in both types of cells (Vainstein *et al.,* 1989). This could be due not only to transcriptional/post-transcriptional control, but also to a less efficient transport system for LHCII in the BS chloroplasts in comparison to the M ones. Our earlier studies showed that maize BS chloroplast contained a very limited electron transport activity linked with PSII, which suggests role of PSII in poising the redox state of PSI for ATP synthesis (Romanowska *et al.,* 2006). Nothing is known about the regulation of PSII biogenesis in other C_4 plants and whether similar regulatory patterns are observed in M and BS chloroplasts of all subgroups.

The reported discrepancies in regard to PSII activity in BS chloroplasts of the NADP-ME subtype may arise due to differences in the age of tissue, different light intensities during growth, methods of chloroplast isolation and degrees of chloroplast purity (Romanowska *et al.,* 2006). To date, there is no reported information on the chlorophyll-protein composition, thylakoid polypeptide profile or activities of photosystems in mesophyll and bundle sheath thylakoids of the NAD-ME and PEP-CK subtypes.

Changes in the antenna system induced by light intensity are likely to affect its interaction with photosystems resulting in altered relative absorption of light by both photosystems (Sailaja & Rama Das, 2000). In C_4 plants, where chloroplasts of mesophyll and bundle sheath cells differ structurally, light intensity may act in different manners on both types of chloroplasts.

It is known that the intensity of CO_2 assimilation in NADP-ME and NAD-ME plants is higher than in the PEP-CK type (Edwards *et al.,* 2001). It is unknown, however, whether the differences in the photosynthetic activities are related to differences in energy requirements between the subtypes or if they are due to differences in the antenna systems responsible for light absorption. Changes in the pigment composition induced by light intensity and temperature in maize leaves have been observed (Haldimann *et al.,* 1995). The content of chlorophylls and carotenoids may reflect differences in the polypeptide composition of light-harvesting complexes (LHC) resulting in changes in the light absorption and CO_2 fixation (Sarry *et al.,* 1994). In C_4 plants, there is a linear relationship between PSII activity and CO_2 fixation, where lower photosynthetic activity is accompanied by lower intensity of dark respiration (Krall *et al.,* 1991). There are no data available on ATP production in BS and M cells of C_4 subtypes in relation to activity of PSI and PSII. A number of studies provide evidence that ATP can be generated in the three different ways (cyclic, noncyclic and pseudocyclic photophosphorylation) dependent on light intensity and concentration of organic oxidants (Edwards & Huber, 1981; Sailaja & Rama Das, 2000). In NADP-ME species M and BS cells contain nearly equal amounts of total chlorophyll, but the Chl *a/b* ratio is 3.5 and 6.0, respectively (Mayne *et al.,* 1974). It is thought that the high Chl *a/b* ratio in BS chloroplasts represents a greater potential for cyclic electron transport. It is unknown whether light intensity may induce similar changes in chloroplasts in both (M and BS) cell types of PEP-CK and NAD-ME species.

In this study, we show that the mesophyll and bundle sheath chloroplasts of *Zea mays*, *Panicum miliaceum* and *Panicum maximum* plants grown in the same irradiance (moderate light) differ in chlorophyll-protein composition and photochemical

activity. These observations significantly contribute to the understanding of the significance of these differences in the photochemical activities between M and BS chloroplasts among the three subtypes of C_4 photosynthesis. We also demonstrate that enzymatic isolation of BS chloroplasts results in a variable loss of thylakoid polypeptides, which can affect the composition and function of photosystems in the examined C_4 species. Further studies are required to solve this problem.

MATERIALS AND METHODS

Plant material and growth conditions. The C_4 plants: maize (*Zea mays* L., type NADP-ME), millet (*Panicum miliaceum*, type NAD-ME) and guinea grass (*Panicum maximum*, type PEP-CK) were grown on vermiculite in a growth chamber under a 14 h photoperiod and a day/night regime at 24/19°C under medium-intensity light (approx. 180 μmol m^{-2} s^{-1}). Plants were fertilized with Knop's solution. Leaves were harvested from 3–4-week-old plants of maize and 4–5-week-old plants of millet and guinea grass.

Chloroplast isolation. The mesophyll (M) and bundle sheath (BS) chloroplasts were isolated mechanically, whereas BS chloroplasts were purified both mechanically and enzymatically, as described in details by Romanowska *et al.* (2006). The purity of each preparation was tested by determining phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) activity using the modified method described by Krömer *et al.* (1996), and also by Western blotting (Fig. 1) using antibody against PEPC according to the procedure described in Towbin *et al.* (1979).

As the enzymatic isolation of BS chloroplasts led to a loss of some thylakoid polypeptides and a lowered chlorophyll fluorescence (not shown), for all further analyses we used the chloroplasts isolated mechanically. All isolation procedures were performed at 4°C using ice-chilled media. Chlorophyll

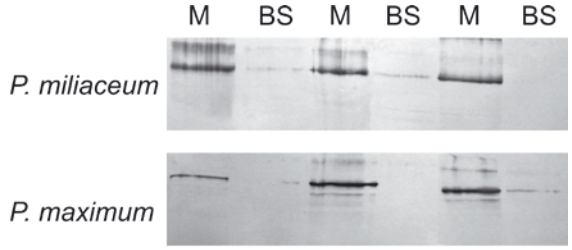

Figure 1. Analysis of purity of bundle sheath preparations (mechanical isolation) from *P. miliaceum* and *P. maximum*.
The same amount of protein (2 μg) was loaded in each gel lane and proteins were separated on SDS/PAGE. Soluble fractions of the mesophyll (M) and bundle sheath (BS) extracts from three independent experiments were analyzed by immunoblotting with antisera to PEPC.

concentration and chlorophyll *a/b* ratio were quantified after extraction with 80% acetone as described by Arnon (1979).

Protein was assayed according to Bradford (1976).

Preparation of thylakoids. For thylakoid preparations from mesophyll and bundle sheath chloroplasts, pellets of chloroplasts were washed in medium containing 50 mM Hepes buffer (pH 7.8), 4 mM $MgCl_2$ and 10 mM NaCl. After centrifugation at 6000 × *g* for 10 min the pellets were resuspended in a medium containing 330 mM mannitol, 50 mM Hepes-NaOH (pH 7.0), 4 mM $MgCl_2$ and 10 mM NaCl. Centrifugation at 6000 × *g* for 10 min was repeated and the pellets were resuspended in the above buffer and kept on ice in the dark or stored at –80°C until used.

Mildly-dissociating electrophoresis of pigment–protein complexes ("green" electrophoresis). Chlorophyll-protein (CP) complexes were separated by mild-denaturating polyacrylamide gel electrophoresis following the method of Maroc *et al.* (1987) with modifications. The electrophoresis was carried out at 4°C with an 8–16% acrylamide containing 0.1% (w/v) lithium dodecyl sulfate (LDS) and 10–15% sucrose gradient. Thylakoids (0.5 mg Chl ml^{-1}) were solubilized in a medium containing: 1.1% *n*-octyl-β-D-glucopyranoside, 0.22% LDS, 62.5 mM dithiothreitol, 20 mM Mes buffer (pH 6.6), 15 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose, using LDS:Chl ratio of 4.4:1. Relative intensities of CP bands were quantified by scans analyzed with Quantity One software (Bio Rad).

SDS/PAGE and protein immunodetection. Analytical polyacrylamide gel electrophoresis in the presence of SDS (SDS/PAGE) was performed with gradient gels (12–20% acrylamide and 10–15% sucrose) according to the method of Laemmli (1970). Chloroplasts were solubilized in 0.125 M Tris/HCl (pH 6.8), 20% glycerol, 4% SDS and 2% β-mercaptoethanol. Proteins were separated at 4°C overnight and the gels were stained with a 0.25% solution of Coomassie Brilliant Blue R-250 and destained in 10% aldehyde-free acetic acid/45% methanol.

For immunodetection proteins were separated in 15% SDS/PAGE, then transferred onto a PVDF-membrane (Millipore, Badford, MA, USA) as described by Towbin *et al.* (1979). The alkaline phosphatase color development reaction was used to visualize immunoreactive proteins. Membranes were probed with antibodies specific to the apoproteins of 33 and 23 kDa of the oxygen evolving complex; D1 and D2 proteins; the α subunit of the coupling factor and apoproteins of LHCII.

Quantity One software (Bio-Rad) was used for quantitative analysis of protein bands on the gels and the membranes.

Chlorophyll fluorescence spectroscopy. Steady-state fluorescence emission (Ex 435) spectra were measured with Shimadzu RF-5001PC spectrofluorimeter at 25°C. Scans were recorded using 0.2 nm increments with the emission optical band of 5 nm. Samples were prepared in a medium containing: 330 mM mannitol, 50 mM Hepes/NaOH (pH 7.5), 4 mM $MgCl_2$ and 10 mM NaCl, and the thylakoid suspensions were diluted to a concentration of 5 μg Chl ml^{-1}. The samples were continuously magnetically stirred to prevent chloroplast settling, and each spectrum was recorded three times.

The fluorescence emission spectra were monitored also at 77 K on a Spex FluoroMax spectrofluorimeter with 2 nm spectral resolutions for excitation and emission. Samples were thermostated using a homemade liquid nitrogen cryostat. Temperature was measured directly in the glassy solution by a diode thermometer, with an accuracy of 0.5 K. Thylakoid samples (4 μg Chl ml^{-1}) were prepared in 20 mM Hepes-NaOH buffer (pH 7.5) containing 15 mM NaCl, 4 mM $MgCl_2$ and 80% glycerol (v/v). Fluorescence emission spectra were recorded in the range of 620–850 nm and were normalized to the same area of 100 under the plot, and the F735/F685 ratio was calculated.

PSI and PSII activities. PSI and PSII activities were estimated in M and BS chloroplasts polarographically with a Clark-type oxygen electrode (TriOximatic EO200, WTW, G.M.B.H., Weilheim, Germany). After 3 min adaptation of isolated chloroplasts in the darkness, PSII and PSI activities were measured at 25°C in saturating PAR. The reaction mixture (2 ml) contained, for PSI: 330 mM sorbitol, 40 mM Tricine, pH 7.6, 2 mM EDTA, 7 mM $MgCl_2$; for PSII: 100 mM sorbitol, 50 mM Hepes/Tris, pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 2.5 mM NH_4Cl, 1 mM $MnCl_2$. PSII activity was measured as oxygen evolution with water as electron donor and 2.5 mM ferricyanide as an electron acceptor in the presence of 10 μM DBMIB as a PSI inhibitor. PSI activity was measured as oxygen uptake in a reaction using 0.2 mM TMPD reduced with 3 mM ascorbate as electron donor and 0.1 mM MV as electron acceptor; 15 μM DCMU and 5 mM NaN_3 were used as a PSII and a catalase inhibitor, respectively. For electron transport measurements chloroplast suspension containing 50 μg Chl/ml was added to the reaction mixture.

Photophosphorylation. Photophosphorylation (ATP synthesis) was measured in M and BS cell chloroplasts isolated from leaves adapted to darkness for at least 1 h. The reaction mixture (0.5 ml final volume) contained: 330 mM sorbitol, 35 mM Tricine/KOH (pH 8.0), 0.6 mM NaH_2PO_4, 6 mM $MgCl_2$, 4 mM ADP, 25 mM KCl, 0.1 mM MV, 2.5 μM diadenosine pentaphosphate. After addition of chloroplasts (20 μg of Chl) the stirred mixture was illuminated at 250 W m^{-2} for 2 min at 25°C. ATP synthesis were terminated by adding 0.5 ml 10% (v/v) $HClO_4$. ATP was determined by the firefly luciferase method (Gardeström & Wigge, 1988). Each measurement was calibrated with an ATP standard. The measurements were repeated at least 3 times in three–four separate experiments.

Measurements of photosynthesis rate. Middle segments of leaves detached from plants were introduced after dark period into a chamber with their cut ends placed in a plastic bag containing water. The photosynthesis chamber was connected to an infrared CO_2 analyzer (Beckman, 865) connected to a closed circuit system as described previously (Parys *et al.,* 1989). The photosynthetic rate was calculated from the change in CO_2 concentration in the range 380–340 μl l^{-1}. The experiments were repeated 5–7 times.

RESULTS

SDS/PAGE of M and BS thylakoids

The profiles of thylakoid polypeptides of samples isolated from mesophyll and bundle sheath cells of *Z. mays, P. maximum* and *P. miliaceum* leaves are shown in Fig. 2. CBB staining revealed a number of differences in the polypeptides in the range of 16–30 kDa between the two types of chloroplasts of the investigated C_4 species. In this range, components of the oxygen-evolving complex (OEC) and antenna

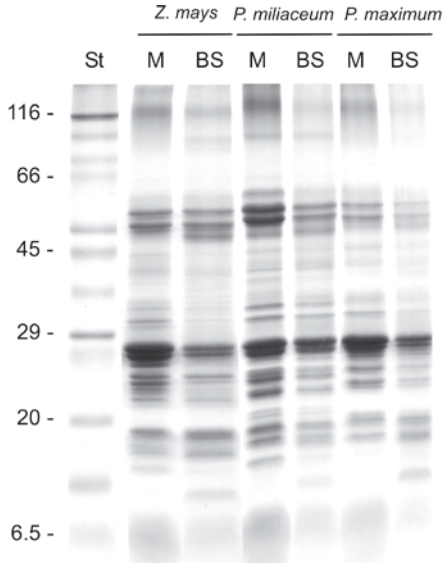

Figure 2. SDS/PAGE of mesophyll (M) and bundle sheath (BS) thylakoid polypeptides isolated from *Z. mays, P. miliaceum* and *P. maximum* leaves.
Lanes were loaded with equal amounts of protein (25 μg). St, molecular weight standards. Electrophoresis was carried out at 4°C in a 12–20% polyacrylamide gradient.

Table 1. Distribution of chlorophyll among green bands separated by LDS/PAGE of thylakoids isolated from mesophyll (M) and bundle sheath (BS) chloroplasts of *Z. mays*, *P. miliaceum* and *P. maximum*.

Values are relative intensities of CP bands to total chlorophyll as determined by densitometric scans of the gels (Fig. 3) by Quantity One software (Bio Rad). The electrophoretic patterns are representative for at last 4–6 separate experiments.

	Z. mays		*P. miliaceum*		*P. maximum*	
	M	BS	M	BS	M	BS
Complexes	% connected chlorophyll					
PSI-LHCI	12.0	20.8	32.7	20.9	26.4	20.5
PSI core complex	16.8	24.8	10.8	8.9	8.58	5.2
LHCII oligomer	11.3	4.1	22.3	10.9	25.0	13.8
CP47	4.9	4.7	4.3	3.5	4.4	3.3
CP43	4.7	5.4	3.6	4.1	2.8	3.2
PSII minor antennae + LHCII monomeric	50.3	40.2	26.3	51.7	36.0	54.0

proteins are localized. Our gel analysis showed a reduced amount of LHCII polypeptides in BS as compared to M thylakoids for all tested plants. In addition, a very small amount of LHCII was detected in BS chloroplasts of maize (Table 1, Fig. 3).

Chlorophyll–protein (CP) complexes of M and BS thylakoids

We used LDS/PAGE and densitometry to obtain information about the composition of CP complexes in the thylakoids of M and BS chloroplasts of the investigated C_4 species grown in moderate irradiance (Fig. 3). Both mesophyll and bundle sheath thylakoids were resolved into five green bands which contained the PSI–LHCI and PSI core complexes; PSII reaction center proteins (CP43 and CP47); and oligomeric and monomeric forms of antenna complexes. The identification of the CP bands was according to Maroc *et al.* (1987). The oligomers mainly corresponded to the trimeric forms of LHCII, while

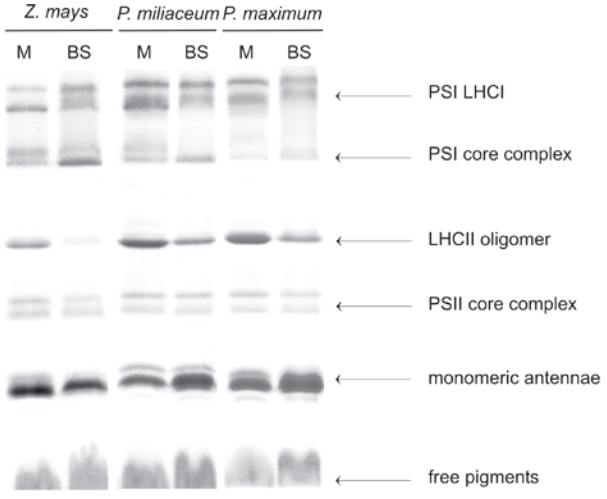

Figure 3. Analysis of chlorophyll–protein complexes with mild-denaturating green gel electrophoresis.
Native PAGE of thylakoids isolated from mesophyll (M) and bundle sheath (BS) cells from *Z. mays*, *P. miliaceum* and *P. maximum* leaves. The same amount of Chl (34.5 μg) was loaded in each lane. The electrophoretic patterns are representative for at last 4–6 independent experiments.

the monomeric forms to LHCII monomers and monomeric minor antennae. The electrophoretic patterns obtained for thylakoids isolated from a both mesophyll and bundle sheath cells differed in the relative intensity of the bands for all examined plants. A quantitative analysis of the gel bands is summarized in Table 1. The data showed that 32.7% of total Chl was associated with PSI–LHCI in *P. miliaceum* M chloroplasts and similar contents (approx. 20% of connected chlorophyll) of this complex were found in *Z. mays* and *P. miliaceum* BS chloroplasts, and also in both kinds of chloroplasts of *P. maximum*. In the bundle sheath chloroplast of *Z. mays* 24.8% of total Chl was associated with PSI core, whereas in *P. miliaceum* and *P. maximum* only about 9%. We observed that the oligomeric form of LHCII was strongly reduced in the bundle sheath chloroplasts of *Z. mays* (4.1%) with the greatest accumulation of this complex observed in mesophyll chloroplasts (11.3%). In both *Panicum* species about 22–25% and 11–14% of LHCII oligomers were in M and in BS chloroplasts, respectively. The reduced amounts of the LHCII oligomers in BS thylakoids as compared to M in *Panicum* plants were associated with a lower PSII activity in these chloroplasts (Table 2). It was found that about 50% of chlorophyll was bound to the monomeric form of the antennae in the bundle sheath chloroplasts of *P. miliaceum* and *P. maximum* and in the mesophyll chloroplasts of *Z. mays*. Moreover, we observed differences in monomeric antennae abundance in both types of thylakoids in all the plants tested. Further analyses are currently underway to investigate the LHCII and LHCI polypeptides in M and BS chloroplasts of C_4 subtypes.

Polypeptides of bundle sheath thylakoids isolated mechanically and enzymatically, and reaction with specific antibodies

We used immunoblotting to compare the polypeptide pattern of bundle sheath thylakoids isolated from *Z. mays* and *P. maximum* leaves using two distinct methods: mechanical and enzymatic (Fig. 4).

Table 2. PSI and PSII electron transport activities in isolated mesophyll and bundle sheath chloroplasts of *Z. mays*, *P. miliaceum* and *P. maximum*.

PSI: Asc/TMPD → MV, DCMU; PSII: H_2O → FeCy, DBMIB. Chloroplasts equivalent to 20 μg/ml of chlorophyll was used. ND – not detected. Experimental details are in Materials and Methods.

	Activity (μmol O_2 mg^{-1} Chl h^{-1})					
	Mesophyll chloroplasts			Bundle sheath chloroplasts		
	PSI	PSII	PSI/PSII	PSI	PSII	PSI/PSII
Z. mays	270 ± 14	57 ± 7	4.7	684 ± 94	ND	–
P. miliaceum	776 ± 51	93 ± 7	8.3	572 ± 20	63 ± 6	9.1
P. maximum	294 ± 36	106 ± 11	2.8	319 ± 47	78	4.1

We used antibodies raised against proteins localized in the lumen (the 33 kDa and 23 kDa subunit of OEC), on the stromal side of thylakoids (subunit α of CF1), and membrane proteins (LHCII and D1 and D2 proteins). Some polypeptides were lost during enzymatic isolation of thylakoids, particularly those with the apparent molecular masses of approx. 66, 45 to 29, and below 20 kDa (not presented). It is clear that the 33 and 23 kDa proteins of OEC and subunit α from maize BS chloroplasts isolated enzymatically are released during this type of isolation procedure. The D1, D2 and LHCII proteins were released only partially. Out of all the corresponding polypeptides of *P. maximum* thylakoids isolated enzymatically, only subunit α was lost. In both *Z. mays* and *P. maximum* BS chloroplasts isolated mechanically all the analyzed subunits were present. These results show clearly that the thylakoid proteins can be lost during enzymatic isolation of BS cells, and that there are differences between chloroplasts of the examined species.

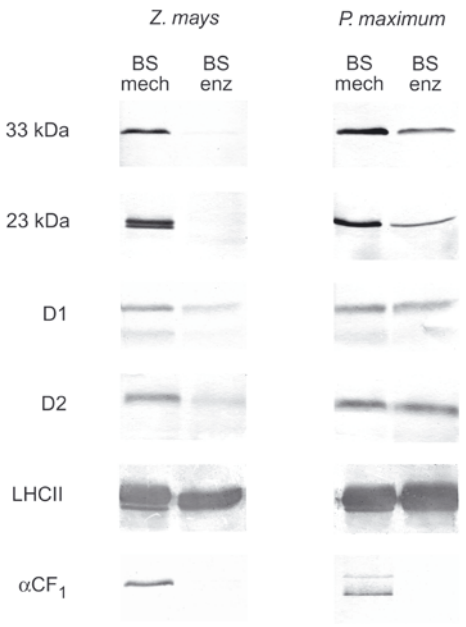

Figure 4. Immunoblot analysis of bundle sheath tylakoid proteins isolated mechanically (mech) and enzymatically (enz) from *Z. mays* and *P. maximum* leaves.
Polypeptides were probed with antibodies specific to: the α subunit of the coupling factor (CF_1); D1 and D2 proteins; 33 kDa and 23 kDa polypeptides of the oxygen-evolving complex and apoproteins to LHCII. Equal amounts of chlorophyll (2.5 μg) was loaded in each gel lane.

Fluorescence emission spectra of M and BS thylakoids

Fluorescence emission spectra were recorded at 25°C for mesophyll and bundle sheath thylakoids for the three C_4 species and then the maxima of chlorophyll *a* fluorescence at 685 nm were compared (Fig. 5). Fluorescence emission and excitation spectra at 25°C can provide information about the organization of chlorophyll–protein complexes in thylakoids of M and BS chloroplast and also about interrelations among pigments. We noticed different values of Chl *a* fluorescence for the mesophyll and bundle sheath thylakoids for all species tested. In isolated mesophyll thylakoids we observed the highest fluorescence for *Z. mays* and the lowest for *P. miliaceum*. In contrast to the M type, the BS thylakoids of maize showed little fluorescence emission at 685 nm but the highest value was observed for *P. miliaceum* chloroplasts (152% higher than in M). For all species tested, we observed (not presented)

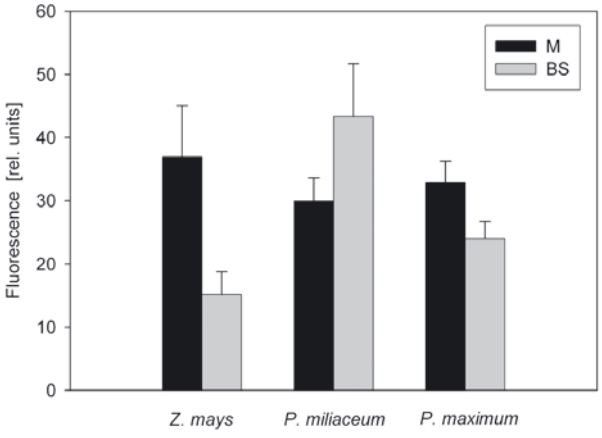

Figure 5. Comparison in maximum (at 685 nm) of chlorophyll *a* fluorescence emission spectra (Ex 435 nm) at 25°C of thylakoids isolated from mesophyll (M) and bundle sheath (BS) cells of *Z. mays*, *P. miliaceum* and *P. maximum* leaves.
Results represent mean values of three to five fluorescence measurements with S.D. values indicated by bars.

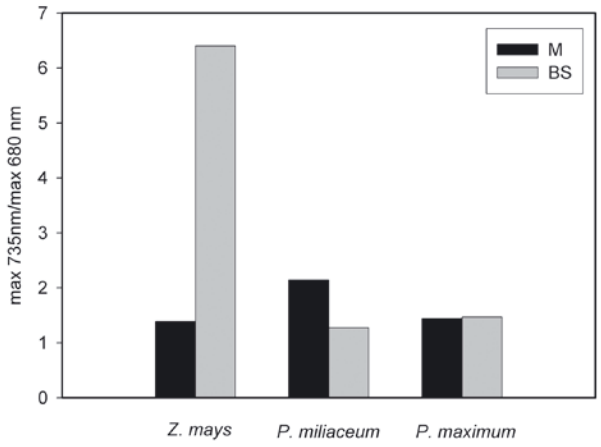

Figure 6. Ratio of the 735 nm and 680 nm maxima in fluorescence emission spectra at 77K.
The spectra were recorded in the range of 620–850 nm. Excitation wavelength was 435 nm. Results of mesophyll (M) and bundle sheath (BS) chloroplasts of *Z. mays, P. miliaceum* and *P. maximum* are given.

that chlorophyll *a* fluorescence in the BS thylakoids isolated enzymatically was reduced by 50–70% in comparison with the fluorescence from the BS chloroplasts obtained mechanically (Fig. 5).

Low-temperature (77 K) fluorescence emission spectra can show changes in the functional antennae of PSII and PSI complexes in both types of C_4 chloroplasts. At low temperatures, Chl *a* fluorescence below 700 nm is attributed to PSII, while most of the Chl fluorescence above 700 nm is emitted by PSI. The varying ratio of the short-to-long-wavelength fluorescence derived from the emission spectra of thylakoids isolated from BS and M chloroplasts may indicate varying PSII to PSI ratios (Fig. 6). Fluorescence spectroscopy at the temperature of liquid nitrogen supports our view that the BS chloroplasts of maize contain PSII. The high value (6.4) of F735/F685 in BS thylakoids of maize can be considered to be specific for PSI because of the very low concentration of PSII. These results are compatible with previous measurements of fluorescence emission spectra at 77 K, which show very low levels of PSII fluorescence (Leegood *et al.*, 1981). In M thylakoids this ratio was 1.3. On the other hand, *P. miliaceum* had a relatively higher fluorescence emission at 735 nm in the M chloroplasts (F735/F685 was 2.1), while the BS chloroplasts had fluorescence emission bands of similar heights at 735 and 685 (ratio 1.2). In *P. maximum* the fluorescence emission spectra of the M and BS thylakoids had similar emission bands at 685 and 735 nm (F735/F680 was 1.4). These observations suggest that the fluorescence characteristics of M and BS thylakoids may serve as a sensitive monitor of the contents of pigment–protein complexes responsible for regulation of the distribution of excitation energy between the photosystems.

Photosystems activity measurements

PSI and PSII activities (Table 2) were measured in M and BS chloroplasts for all tested plants. PSII function was measured as the rate of oxygen evolution using water as the electron donor and potassium ferricyanide as the electron acceptor. The oxygen evolution in M chloroplasts was significantly lower for maize than that observed for both *Panicum* species, whilst it was not detected in BS chloroplasts. The PSII activity was lower in BS chloroplasts of *P. maximum* and *P. miliaceum* than in the mesophyll ones and these differences were also reflected in the ATPase activity (Fig. 7). The rate of oxygen uptake (PSI activity) in mesophyll chloroplasts was similar for *Z. mays* and *P. maximum*, and was two-fold lower than in *P. miliaceum*. BS chloroplasts of the C_4 plants tested showed a varying PSI activity. The highest PSI activity was detected in the BS chloroplasts of maize and the lowest in *P. maximum*. The PSI activity for *P. maximum* was similar in M and BS chloroplasts, it was more than two-fold higher for maize BS chloroplasts than for M, whereas for *P. miliaceum* it was lower in BS than in M chloroplasts.

ATP synthase activity

In order to activate the membrane-bound ATPase, chloroplasts were routinely illuminated for 2 min at a light intensity of 250 W m^{-2}. The mesophyll and bundle sheath chloroplasts had different ATPase activities depending on the species (Fig. 7). Generally a higher ATP synthase activity was observed for M than for BS chloroplasts in all species tested. ATP synthase was 4.3-, 1.8-, and 1.5-fold more active in M than in BS chloroplasts isolated from *P. miliaceum*, *P. maximum* and *Z. mays*, respectively. The maximum of this activity was determined for mesophyll chloroplasts of *P. miliaceum;* compared to M chloroplasts of *Z. mays* and *P. maximum* it was higher by about 15 and 45%, respectively. As ex-

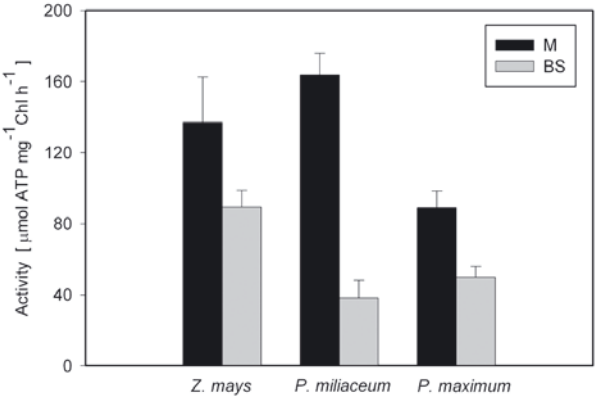

Figure 7. ATP synthase activity of chloroplasts isolated from mesophyll (M) and bundle sheath (BS) cells of *Z. mays, P. miliaceum* and *P. maximum* plants.

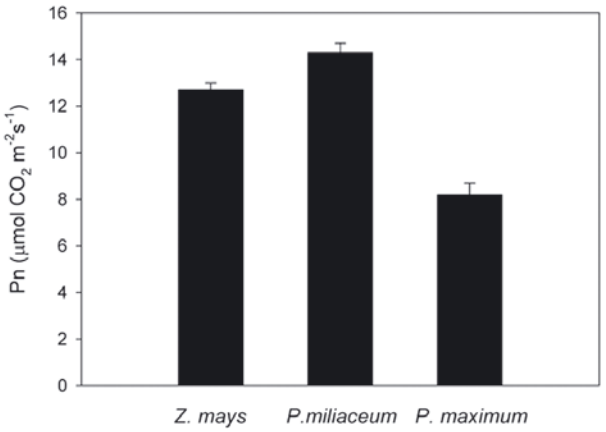

Figure 8. Photosynthesis rate (Pn) of leaves from C_4 species.
The results represent mean values ±S.E. of five to seven independent experiments.

pected, bundle sheath chloroplasts of maize showed the highest ATPase activity which was about 55 and 45% higher compared with *P. miliaceum* and *P. maximum* chloroplasts, respectively.

These results indicate that ATPase activity correlated well with the photochemical activity of photosystems and the rate of CO_2 fixation (Fig. 8).

Photosynthesis rate (Pn)

The net photosynthesis rates were measured in *Z. mays, P. miliaceum and P. maximum* leaves (Fig. 8). The highest net CO_2 fixation was observed for *P. miliaceum* and the lowest was found for *P. maximum* leaves. In *P. miliaceum* leaves Pn was higher by about 13 and 63% as compared with maize and *P. maximum*, respectively.

DISCUSSION

In the present study we compared the biochemical properties of M and BS chloroplasts isolated from various species of C_4 plants grown in moderate light, and we found that both types of chloroplasts differ in their photochemical activity among the investigated plants.

The functional and structural differences observed for PSII in BS cells of the NADP-ME type might be in many cases related to the method of isolation. It is very difficult to separate the mesophyll and bundle sheath cells and isolate intact organelles from the cells of C_4 plants. Both kinds of cells are very hard to break; moreover, there is a suberinized lamella in bundle sheath cell walls in both PEP-CK and NADP-ME types of C_4 species (Hattersley & Watson, 1992). However, bundle sheath strands isolated mechanically were used in many studies on C_4 photosynthesis. Another method applied to isolate bundle sheath cells was enzymatic digestion using a mixture of fungal cellulase-pectinase. This method was effective for obtaining protoplasts from C_4 plants, both of the NAD-ME type (Edwards *et al.*, 1979) and also from several PEP-CK ones (Ku *et al.*, 1980; Chapman & Hatch, 1983; Watanabe *et al.*, 1984). In comparative studies of chloroplasts from species representing C_4 subtypes, we used both methods to obtain pure and functional chloroplasts and thylakoids. Measurements of PEPC activity (not shown) and immunobloting analysis (Fig. 1) showed that the BS preparation obtained with the mechanical method was only about 1% contaminated with mesophyll cells. Thus, the procedure yielded pure BS chloroplasts and was suitable for subsequent investigation.

By immunodetection analyses we revealed significant differences between *Z. mays* and *P. maximum* BS thylakoids isolated by mechanical and enzymatic methods (Fig. 4). In the BS thylakoids isolated mechanically, all analysed subunits were present in both species. During enzymatic digestion of BS cells of maize we observed degradation of peripheral membrane protein (CF1 α-subunit), core protein of PSII (D1) and proteins of OEC. BS chloroplasts isolated from *P. maximum* plants were more resistant to degradation and in the enzymatic procedure only CF1 α-subunit was lost. The observed differences between species might be related to different activities/amounts of proteases present in the two types of chloroplasts. To date, no information about the mechanism of proteolysis in chloroplasts of C_4 plants is available. Proteolysis of polypeptides of PSII has been detected in C_3 plants not only at all stages of plant development but also in photoinhibited or stressed thylakoids (Andersson & Aro, 1997; Georgakopoulos *et al.*, 2002).

Our data shows that techniques used for isolation of chloroplasts may affect the composition of protein complexes in C_4 plants and thus they have influence on their structure and activity. As it is shown in Fig. 4, LHCII appears to be more resistant to degradation during isolation of BS chloroplasts than other PSII proteins. Bassi *et al.* (1995) using an enzymatic method demonstrated that PSII in BS cells was depleted in the 33 kDa polypeptide of OEC, but the amount of LHCII was unexpectedly very high. Moreover, Meierhoff and Westhoff (1993) and Wingler *et al.* (1999) found that D1 polypeptide and PEP carboxykinase protein, respectively, were also reduced in enzymatic isolation.

The observed functional and structural differences between, the photosystems in M and BS chloroplasts of C_4 plants (Figs. 2, 3, 5–7, Tables 1 and 2) are a key to the high efficiency of photosynthesis (Fig. 8). It is commonly accepted that the main

factor responsible for the adjustment of the photosystem ratio is light intensity (Anderson, 1986). As shown in Table 2, PSI/PSII ratios were different in both types of chloroplasts and there are observed differences between C_4 species grown in the identical light conditions. This suggests that different mechanisms may control the stoichiometry of photosystems in different C_4 species, which is dependent to cellular metabolic activities and independent of grana formation (not presented). The regulation of biosynthesis/assembly of proteins that brings about this effect is not known.

The fluorescence data observed for chloroplasts of C_4 species agree with their photosystems' activity. Short-wavelength fluorescence at 77 K can be attributed to PSII but fluorescence at longer wavelengths is dominated by PSI. Hence, the ratio of long/short wavelength fluorescence increases with an increasing PSI contribution to the leaf fluorescence (Fig. 6).

The composition of maize chloroplast CP and protein complexes has been investigated by genetic and biochemical approaches (Sheen & Bogorad, 1986). There are several reports on the use of native gel systems for the characterization of these complexes (Danahay *et al.,* 1984; Metz *et al.,* 1984; Maroc *et al.,* 1987; Dainese *et al.,* 1990; Peter & Thornber, 1991; Bassi *et al.,* 1995). However, there is no information in the literature about CP composition of the chloroplasts of the NAD-ME and PEP-CK subtypes.

Chloroplasts from M and BS cells of all C_4 species differ slightly in the amount of polypeptides and the composition of CP complexes as shown by both electrophoresis (Figs. 2 and 3) and fluorescence analysis (Fig. 6). PSII of maize BS chloroplasts with a small antenna size contributed insignificantly to the total light absorption. In both *Panicum* species PSII was more active in M than in BS chloroplasts, where a higher amount of an oligomeric form of light harvesting complex was observed, together with a higher fluorescence of Chl *a*. There was a close correlation between the composition of CP complexes and the activities of PSI and PSII (Table 2) in M and BS chloroplasts. A higher activity of PSI was accompanied by a larger amount of PSI–LHCI in M and BS chloroplasts of *P. miliaceum* and in BS chloroplasts of *Z. mays.* Our results show that M and BS chloroplasts of the three subgroups of C_4 plants grown under the same light conditions differ with respect to their organization and activity. Analyses of polypeptides, pigment proteins and fluorescence (Table 1 and Figs. 2–4 and 6) provide physiological evidence for the existence of a mechanism that regulates the photochemistry of photosystems by acting on their antenna components.

The effects of environmental factors on photosynthesis and the abundance of the components of the thylakoid membranes complexes and antenna systems in subtypes of C_4 plants have not been studied extensively so far. Energy requirements vary between the cell types of C_4 species because of the differences in the decarboxylation mechanism and the respective C_4 cycles. The differences in energy requirements between the cells are reflected in differences in the Chl *a/b* ratio and distribution of photochemical activities between the cells. In particular, the NADP-ME species have a relatively high proportion of non-cyclic activity in mesophyll chloroplasts and cyclic electron flow in bundle sheath cells. In NAD-ME and PEP-CK species, the main photochemical activities are localized in bundle sheath cells (Edwards *et al.,* 1976). Our data show that ATP synthase activity was higher in M than in BS chloroplasts in all examined plants (Fig. 7) and was the highest in M chloroplasts of *P. miliaceum*. The rate of ATP formation is strongly related to the rate of electron transport and to ATP consumption by the Calvin cycle. Factors influencing electron transport rates control ATP production. ATP may be provided by cyclic, noncyclic or pseudocyclic electron transport. Thus, there may be quantitative changes in ATP generated from different types of electron flow. Relatively few studies have addressed on photophosphorylation in chloroplasts of PEP-CK and NAD-ME C_4 plants, and no distinction was made between M and BS chloroplasts (Edwards & Walker, 1983). High cyclic phosphorylation capacities were observed in BS chloroplasts (Chapman *et al.,* 1980; Leegood *et al.,* 1981) of NADP-ME plants, in agreement with our data for maize. We did not observe a correlation between ATP synthase activity and electron transport rates in both types of chloroplasts of C_4 subtypes (Fig. 7). Our results suggest that differences in ATP synthase activity may play a role in regulation of photosynthetic energy conservation to allow flexibility of the ATP/NADPH ratio.

Photosynthetic activity was significantly higher in *P. miliaceum* than in *P. maximum* plants (Fig. 8), which was reflected also by higher activity of photosystems and ATP synthase.

The photosynthetic capacity (measured as the photochemical activities of PSI and PSII) was the lowest in *P. maximum* and was attributed to changes in the levels of both the CP and electron transport complexes.

In conclusion, it is clear that M and BS chloroplasts of C_4 subtypes differ in photosynthetic and ATP synthase activities, the main determinants of CO_2 assimilation in C_4 plants (Maroco *et al.,* 1998). It would appear that during growth in identical light conditions, the M and BS chloroplasts of the three C_4 subtypes develop a different photochemical pattern characteristic for each subtype, which could have important implications for the rate of photo-

synthesis. In moderate light the capacity of electron transport is reduced as compared to high light. However, the relationship between composition of polypeptides, CP complexes and PSI and PSII activities during leaf growth at higher light intensity has yet to be established.

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