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Differential accumulation of the 10-, 16- and 23-kDa peripheral components of the water-splitting complex of photosystem II in mesophyll and bundle-sheath chloroplasts of the dicotyledonous C_4 plant *Flaveria trinervia* (Spreng.) C. Mohr

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Abstract. The differential expression of PSII genes was investigated in mesophyll and bundle-sheath cells of *Flaveria trinervia,* a dicotyledonous C_4 plant of the NADP-malic enzyme type. A comprehensive immunoblot analysis showed that three extrinsic proteins of the water-splitting complex (10, 16 and 23 kDa) are selectively depleted in mature bundle-sheath chloroplasts. In contrast, the reaction-centre core remained virtually unaffected as inferred from the abundance of the 47-kDa chlorophyll-a-binding protein, the D1 and D2 polypeptides, cytochrome b_{559} and the 34-kDa polypeptide. The selective depletion of the 10-, 16- and 23-kDa polypeptides in bundle-sheath chloroplasts was paralleled by a diminished PSII capacity. On the basis of oxygen evolution in the presence of the artifical electron acceptor 2,5-dimethyl-*p*-benzoquinone, bundle-sheath chloroplasts maintained up to 23% of the PSII capacity shown by mesophyll chloroplasts. However, the levels of the 10-, 16- and 23-kDa proteins and, concomitantly, PSII activity varied to some degree and appeared to be correlated with environmental factors caused by seasonal changes. The selective depletion of the three members of the water-splitting complex was not reflected at the transcript level. The corresponding mRNAs were detectable in considerable amounts in bundle-sheath cells, indicating that the depletion of these proteins is regulated by post-transcriptional events. These findings reinforce the view that the peripheral proteins of the water-splitting complex are a focal point for controlling PSII activity in bundle-sheath chloroplasts of both mono- and dicotyledonous C_4 plants of the NADP-malic enzyme subtype.

Key words: C_4 plant – *Flaveria* – Oxygen-evolving complex – Photosystem II

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Abbreviations: DMBQ = 2,5-dimethyl-*p*-benzoquinone; PEPCase = phosphoenolpyruvate carboxylase; Chl = chlorophyll; SDS = sodium dodecyl sulfate

Introduction

A characteristic of NADP-malic enzyme-type C_4 plants is the occurrence of two morphologically distinct chloroplasts. While the thylakoid membranes of mesophyll chloroplasts contain numerous appressed lamellae, those of bundle-sheath chloroplasts are almost grana-free (Laetsch and Price 1969). Concomitantly, bundle-sheath chloroplasts of mature leaves are almost devoid of PSII activity (Woo et al. 1970). However, non-cyclic electron flow has been shown to operate in the young bundle-sheath chloroplasts of *Sorghum bicolor* (Downton and Pyliotis 1971) indicating that PSII is active in undifferentiated bundle-sheath cells, but looses its activity as soon as a fully functional C_4 cycle is established.

Recently, evidence was presented that in developing bundle-sheath chloroplasts of maize, *Sorghum* and *Pennisetum* the water-oxidation complex is a prime target for initiating or stabilizing the loss of PSII activity in the bundle-sheath cells (Oswald et al. 1990). Only traces of the nuclear-encoded 10-, 16- and 23-kDa extrinsic polypeptides could be detected. It was shown for maize (Sheen et al. 1987) and *Sorghum* (Oswald et al. 1990) that the lack of these proteins was paralleled by drastically reduced levels of the corresponding mRNAs, indicating that protein abundance is controlled by RNA levels (Oswald et al. 1990). While this picture applies to all monocotyledonous NADP-malic enzyme-type C_4 species investigated so far (Schuster et al. 1985; Sheen et al. 1987; Oswald et al. 1990), nothing is known about the regulation of PSII biogenesis in dicotyledonous C_4 plants of this subgroup. This is especially interesting since C_4 plants are of polyphyletic origin (Hattersley 1987) and the question arises whether similar regulatory patterns are observed. Therefore, the present study with *Flaveria trinervia* (Asteraceae) was initiated. *Flaveria trinervia* has been classified as a NADP-malic enzyme-type C_4 species based on enzyme-activity studies and pulse-chase experiments with $^{14}CO_2$ (Apel and Maass 1981; Bauwe 1984;

Moore and Edwards 1986a). However, *F. trinervia* is also known to make use of aspartate in addition to malate as a shuttle for CO_2 (Moore and Edwards 1986a). Furthermore, owing to the low level of phosphoglycerate (Moore and Edwards 1986b) the operation of a triose-phosphate shuttle seems unlikely in this plant. This indicates the possibility that bundle-sheath chloroplasts of *F. trinervia* possess an increased capacity for non-cyclic electron transport. This in turn raises the question whether the biogenesis of PSII in bundle-sheath cells exhibits regulatory characteristics different from those observed in the monocotyledonous species. Our analysis shows that bundle-sheath chloroplasts of *F. trinervia* are severely depleted in PSII activity and that also in this C_4 plant the water-splitting complex appears to be a primary target for the deregulation of PSII.

Material and methods

Plant material. Seeds of *Flaveria trinervia* (Spreng.) C. Mohr were germinated on the surface of fine soil under conditions of high humidity. When the seedlings reached 1–2 cm in height they were transplanted into larger pots filled with peat compost (Triohum, Substrat 1; Klasmann, Geeste, Germany) and grown under greenhouse conditions with additional illumination (14 h per day from 7 a.m. to 9 p.m.) provided by a combination of sodium and mercury high-pressure vapour lamps. The photon flux density was at least 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant height. Plants were watered daily and fertilized every second week with diluted nutrient solution (Wuxal 8.8.6 super; Schering AG, Berlin, Germany). Depending on seasonal variations, the temperature varied from 24–31° C during the day and from 16–20° C at night. Under these conditions the plants developed inflorescences after 2–2.5 months. Only fully expanded leaves from different nodal regions were harvested for preparation of choroplasts and RNA.

Screening of λgt11 cDNA library with antibodies. The construction of the copy-DNA (cDNA) library of *F. trinervia* was as recently described (Börsch and Westhoff 1990). Screening of the library by antibody probes followed standard procedures (Blake et al. 1984; Huynh et al. 1985). Antibodies used included those listed previously (Oswald et al. 1990) as well as antisera to subunit 2 of PSI (Nechushtai and Nelson 1985), to the 27- to 29-kDa polypeptides of the light-harvesting complex of PSII (Mogen et al. 1990) and to plastocyanin (Haehnel et al. 1989). Further characterization of the isolated cDNA clones was as in Oswald et al. (1990).

Preparation of mesophyll and bundle-sheath protein fractions for measurement of PSII activity and immunoblot analysis. Mature leaves (100 g divided into two portions) were cut into small pieces (2–3 mm) and homogenized three times for 5 s each in 250 ml of buffer A [400 mM sorbitol, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 0.8 mM KH_2PO_4, 4 mM L-cysteine, 2 mM Na-ascorbate, 44 mM 2-(N-morpholino)ethanesulfonic acid (Mes)-KOH, pH 6.1, 0.4% (w/v) bovine serum albumin] using a Waring blendor at low-speed setting. The crude extract was passed through a 20-μm nylon mesh before being used for the preparation of mesophyll chloroplasts and soluble mesophyll contents. The retained plant material was transferred to 250 ml of buffer A (plus 0.1% (w/v) polyvinylpyrrolidone 25) and homogenized for 1 min at high speed in the Waring blendor. The suspension was filtered through a 100-μm nylon mesh and the residue washed thoroughly with buffer A. This procedure was repeated three times until most of the adhering mesophyll cells were released from the bundle-sheath strands as assessed by microscopic examination. The remaining crude bundle-sheath strands were suspended in 200 ml of buffer C [350 mM sorbitol, 1 mM KH_2PO_4, 10 mM Mes-KOH, pH 6.0, 0.5% (w/v) bovine serum albumin, 1% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Macerozyme R-10] in a 500-ml Erlenmeyer flask, and incubated for 1 h at room temperature while shaking continuously at 110 rpm. After digestion the medium was separated from the bundle-sheath strands by filtration (100-μm nylon mesh) and the residue washed with buffer A. The purifed bundle-sheath strands were then transferred to a small (120 ml) Waring blender and homogenized in buffer A by three 5-s bursts at low-speed setting. Finally the suspension was passed through a 16-μm nylon mesh and the filtrate was used for the preparation of bundle-sheath chloroplasts and soluble bundle-sheath contents.

Mesophyll and bundle-sheath chloroplasts (see above) were collected by centrifugation (RC-5B, HS-4 swing out rotor; Sorvall, Norwalk, Conn., USA; 60 s, 2300 rpm, 4° C) and resuspended in buffer B [400 mM sorbitol, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 0.8 mM KH_2PO_4, 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-NaOH, pH 6.7, 0.1% (w/v) bovine serum albumin]. The centrifugation and resuspension step was repeated twice and the final pellet resuspended in a small amount of buffer B. Soluble cytosolic mesophyll and bundle-sheath proteins (see above) were prepared as in Oswald et al. (1990).

Chlorophyll concentration was determined in 80% acetone (Arnon 1949); protein was assayed according to standard procedures (Lowry et al. 1951; Bradford 1976). Phosphoenolpyruvate-carboxylase (PEPCase) activity was determined as in Uedan and Sugiyama (1976). Sodium dodecyl sulfate (SDS) gel electrophoresis, transfer of the separated proteins to nitrocellulose (PH 79, 0.1 μm, Schleicher & Schüll, Dassel, Germany) and immunodecoration with antibodies followed the protocols outlined elsewhere (Westhoff et al. 1985; Oswald et al. 1990). Photosystem-II-mediated oxygen evolution was measured polarographically with a Clark-type electrode (Hansatech, Norfolk, UK) using 2,5-dimethyl-*p*-benzoquinone (DMBQ) as an electron acceptor (Somersalo and Krause 1990).

Isolation and analysis of mesophyll and bundle-sheath RNA. Leaf material (20–30 g) was homogenized in a Waring blendor in 250 ml of cold (2° C) medium I [600 mM sorbitol, 30 mM NaCl, 10 mM $MgCl_2$, 10 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM aurintricarboxylic acid, 10 mM dithioerythritol (DTE), 5 mM diethyldithiocarbamic acid, 200 mM Tris-HCl, pH 8.9] by mixing three times for 5 s at low speed. The crude extract was filtered through a 20-μm nylon mesh and the flow-through used for the preparation of mesophyll RNA (Westhoff et al. 1991). The residue in the cloth filter (see above) was used for the preparation of bundle-sheath RNA. The crude bundle-sheath strands were suspended in medium I (plus 0.1% diethylpyrocarbonate). Purification of the bundle-sheath strands by mechanical treatment followed the same procedure as outlined for the isolation of proteins. Diethylpyrocarbonate was omitted in the washing steps. Further isolation of RNA from purified bundle-sheath strands was as in Westhoff et al. (1991).

Ribonucleic acid was analyzed by Northern blotting (Westhoff et al. 1991) including the following modifications: cDNA probes were labelled by random priming (Feinberg and Vogelstein 1983) and hybridization was performed at 62° C in a phosphate-SDS-EDTA buffer system (Church and Gilbert 1984). When necessary, the hybridization signals were determined by liquid scintillation counting.

Miscellaneous methods. Preparation of leaf samples for light and electron microscopy followed standard procedures.

Results

Leaf anatomy. Cross sections of *Flaveria trinervia* leaf material show an anatomy typical of dicotyledonous C_4 plants. Electron micrographs were taken to examine further the structure of the mesophyll and bundle-sheath

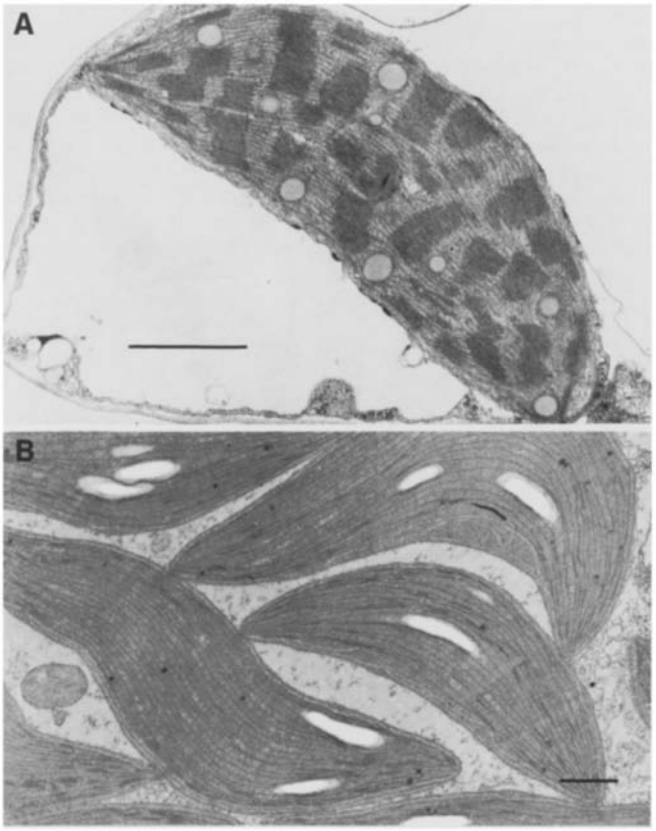

Fig. 1A, B. Ultrastructure of mesophyll (**A**) and bundle-sheath chloroplasts (**B**) of mature leaves of *Flaveria trinervia*. Scale bars represent 1 μm

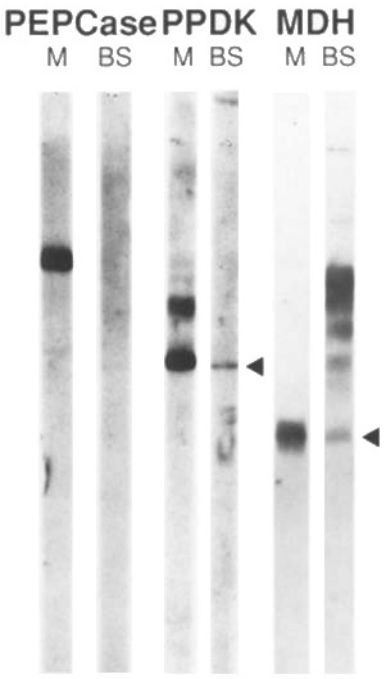

Fig. 2. Analysis of the purity of bundle-sheath preparations from *Flaveria trinervia*. Soluble fractions of the mesophyll (*M*) and bundle-sheath (*BS*) extracts were analyzed by immunoblotting with antisera to *PEPCase*, pyruvate orthophosphate dikinase (*PPDK*), and malate dehydrogenase ($NADP^+$) (*MDH*). The positions of PPDK and MDH ($NADP^+$) are labelled by *solid triangles*. The high-molecular-weight bands in the mesophyll and the bundle-sheath lanes of the PPDK and the MDH immunoreactions have not been identified

chloroplasts. Figure 1A shows that mesophyll thylakoids contain grana and non-appressed regions that are typical in the mesophyll chloroplasts of C_3 plants. In contrast, the bundle-sheath thylakoids of *F. trinervia* (Fig. 1B) largely lack grana development, but some granal stacking is apparent.

Isolation of bundle-sheath fractions for immunoblot analysis and the measurement of PSII activity. The accumulation of PSII proteins in bundle-sheath cells can only be determined when cross-contamination due to an insufficient removal of adhering mesophyll cells is avoided. Key enzymes of the C_4 biochemical pathway are strictly associated with one or the other of the two cooperating cell types. Therefore, PEPCase was chosen as a mesophyll-specific marker enzyme. Activity measurements showed that routine preparations of bundle-sheath strands exhibited less than 1% contamination of mesophyllic origin. This was confirmed by immunoblot analysis (Fig. 2) using an antiserum to the C_4 isoform of the PEPCase of *Sorghum* (Vidal et al. 1981). Thus a modified combination of a differential grinding and enzymatic procedure (Golbeck et al. 1981) yielded pure bundle-sheath strands and was suitable for this investigation. In contrast, the mesophyll fractions were severely contaminated by bundle-sheath contents (up to 30%) as assessed by immunoblot analysis with an antiserum to the maize NADP-malic enzyme (data not shown).

The 10-, 16- and 23-kDa proteins of the water-splitting apparatus are selectively deficient in bundle-sheath cells. Similar amounts of mesophyll and bundle-sheath thylakoids (on a protein basis) were separated on broad-slot SDS polyacrylamide gels and transferred to nitrocellulose filters. Care was taken to achieve comparable transfer efficiency by checking the gel after the electrotransfer by Coomassie-blue staining. Filter strips with mesophyll and bundle-sheath proteins were always carried together through the immunodetection procedure to permit direct visual comparison of the fluorographic exposures.

Similar amounts of cytochrome b_6, a representative subunit of the cytochrome b_6/f complex, and of the lumenal protein plastocyanin could be detected in mesophyll and bundle-sheath thylakoids (Fig. 3). photosystem-I reaction-centre polypeptides were slightly more abundant in the bundle-sheath fraction (Fig. 3).

Only minor differences between mesophyll and bundle-sheath cells were also apparent for subunits of the PSII core, i. e. the chlorophyll-a-binding protein CP47, the D1 and D2 polypeptides of the reaction centre, cytochrome b_{559}, and the 34-kDa protein of the water-splitting complex. The levels of these proteins were only slightly reduced in the bundle-sheath thylakoids (Figs. 4, 5).

In contrast, the levels of the 10-, 16- and 23-kDa proteins of the water-splitting complex were significantly lower in bundle-sheath thylakoids (Fig 5). However, the amounts of these proteins in the bundle-sheath chloro-

plasts are correlated with the season in which the analysis was performed (Fig. 5): bundle-sheath fractions prepared from plants in late autumn and the winter exhibited a higher content of the 10-, 16- and 23-kDa polypeptides than bundle-sheath fractions of summer-grown plants. No such seasonal variations in protein levels could be observed for the components of the PSII core polypeptides (data not shown, see Fig. 4.)

The reduction in the levels of the 10-, 16- and 23-kDa proteins correlates with PSII-mediated oxygen evolution. The relatively large amounts of PSII core polypeptides

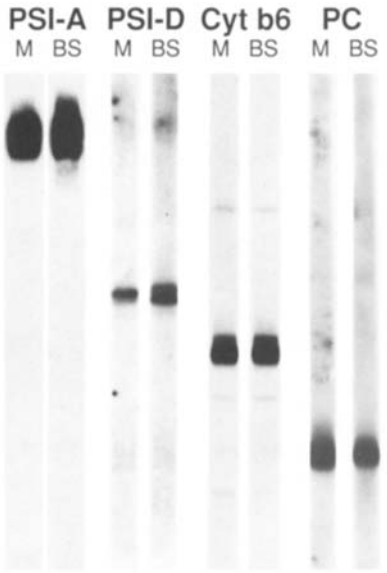

Fig. 3. Distribution of PSI subunits 1 (*PSI-A*) and 2 (*PSI-D*), cytochrome b_6 (*Cyt* b_6) and plastocyanin (*PC*) in mesophyll (*M*) and bundle-sheath (*BS*) thylakoids of *Flaveria trinervia*. The same amounts of proteins were separated on SDS-urea-polyacrylamide gels and immunodecorated as outlined in *Material and methods*. A representative fluorograph from six independent experiments (see Table 1) is shown

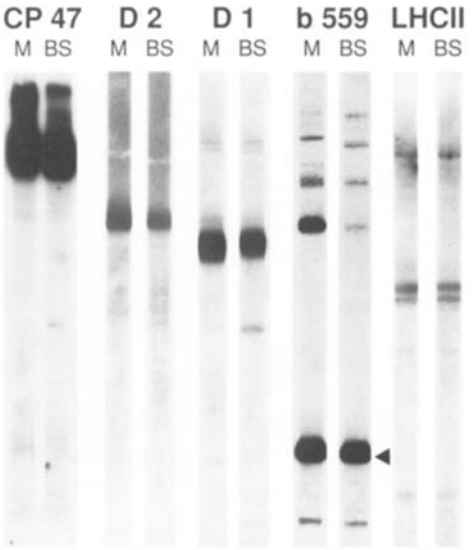

Fig. 4. Immunoblot analysis of PSII polypeptides in mesophyll (*M*) and bundle-sheath (*BS*) thylakoids of *Flaveria trinervia*. Designation of proteins: 51-kDa chlorophyll-a-binding protein (*CP47*), D2 polypeptide (*D2*), D1 polypeptide (*D1*), cytochrome b_{559}, α subunit (b_{559}) (labelled by *solid triangle*), light-harvesting chlorophyll-a/b-binding complex of PSII (*LHCII*). The higher-molecular-weight bands are the result of protein aggregation; the nature of the low-molecular-mass protein (below the indicated α subunit of cytochrome b_{559} is not known. Representative fluorographs selected from six independent experiments *(see Table 1)* are shown

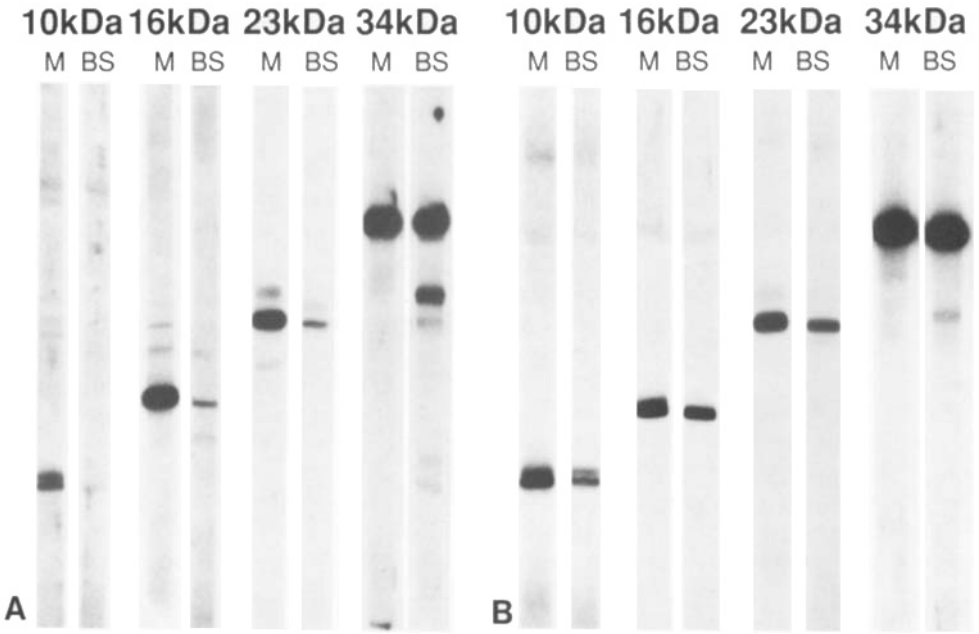

Fig. 5A, B. Immunoblot analysis of the 10-, 16-, 23- and 34-kDa proteins of the water-splitting complex of PSII in mesophyll (*M*) and bundle-sheath (*BS*) thylakoids of summer- (**A**) and winter-grown (**B**) *Flaveria trinervia*. Representative filters from three independent experiments (see Table 1) are shown

Table 1. Photosystem-II-driven oxygen evolution in mesophyll and bundle-sheath thylakoids of *Flaveria trinervia*. Numbers represent mean values of three separate measurements. Photosystem-II-dependent electron transport from water to DMBQ was measured as O_2 evolution with a Clark-type oxygen electrode in the presence of 5 mM NH_4Cl and 2 μM Gramicidin D. The PEPCase activity was assayed spectrophotometrically as NADH consumption in the presence of malate dehydrogenase ($NADH^+$). The ratio of PEPCase activity is calculated from total enzyme activity in the soluble supernatants obtained from mesophyll (M) and bundle-sheath fractions (BS) *(see Material and methods)*

Expt. No. (Date)	Ratio of PEPCase activity (%) BS/M	PSII activity ($H_2O \rightarrow DMBQ$) ($\mu mol\ O_2 \cdot (mg\ Chl)^{-1} \cdot h^{-1}$)		Ratio (%) BS/M	Ratio of Chl a to b	
		M	BS		M	BS
1 (25. 05. 1990)	0.3	204	3.4	1.6	3.8	3.7
2 (01. 06. 1990)	0.2	240	21	8.7	3.8	4.4
3 (14. 06. 1990)	0.1	296	16	5.4	4.1	4.6
4 (10. 10. 1990)	0.3	249	57	22.8	3.5	4.3
5 (12. 10. 1990)	0.1	298	70	23.4	3.7	4.4
6 (20. 12. 1990)	0.2	342	77	22.5	3.7	4.2

Table 2. Characterization of cDNA clones. Recombinant λgt11 phages were digested with EcoRI and the inserted fragments subcloned into Bluescript M13+. After partial sequence determination (see *Material and methods*) the fragments were isolated and used as hybridization probes after labelling by random priming (Feinberg and Vogelstein 1983)

Protein	Gene	Plasmid	cDNA (base pairs)	mRNA (bases)
PEPCase	*ppcA*	pcFtppc1-1	3100	3300
34-kDa protein	*psbO*	pcFtoec34-43	1200	1300
23-kDa protein	*psbP*	pcFtoec23-96	690	1200
16-kDa protein	*psbQ*	pcFtoec16-45	680	1000
10-kDa protein	*psbR*	pcFtoec10-65	560	800
Plastocyanin	*petE*	pcFtpc-71	450	700
Subunit 2, PSI (PSI-D)	*psaD*	pcFtPSI-D	580	900
Heat-shock protein 70	*hsp70*	pcFthsp-2233	840	2400
Ubiquitin	*ubi*	pcFtubi-2244	1500	1600
25S rRNA	*rrn25*	pcFtrrn25	460	–

in bundle-sheath thylakoids and the severe depletion of the 10-, 16- and 23-kDa polypeptides raised questions about the functional state of PSII in the bundle-sheath chloroplasts. Therefore, the activity of PSII was compared in mesophyll and bundle-sheath thylakoids by measuring oxygen evolution in uncoupled membranes in the presence of the electron acceptor DMBQ. Table 1 shows that the water-oxidizing activity of bundle-sheath thylakoids is significantly reduced when compared with mesophyll thylakoids, but it is, nevertheless, clearly detectable. Bundle-sheath thylakoids prepared in late autumn and winter (Expts. Nos. 4–6, Table 1) consistently exhibited higher rates of oxygen evolution than those prepared from summer-grown plants (Expt. No. 1–3). The activity of PSII thus appears to be related to the levels of the 10-, 16- and 23-kDa proteins of the water-splitting complex.

The accumulation of the 10-, 16- and 23-kDa polypeptides of the water-splitting complex is regulated at the post-transcriptional level. To determine the level of gene expression at which the accumulation of the 10-, 16- and 23-kDa proteins of the water-splitting complex is regulated in the bundle-sheath cells a detailed Northern blot analysis was performed. Hybridization probes specific for the four nuclear-encoded components of the water-splitting complex were isolated by immunoscreening from a λgt11 expression cDNA library of *F. trinervia*. For comparison, cDNAs for subunit 2 of PSI and for plastocyanin were selected as representative examples of non-PSII components of the electron-transport chain. By chance, cDNA clones encoding ubiquitin, the 70-kDa heat-shock protein and 25S rRNA were obtained during the immunoscreening procedure. (See Table 2 for characterization of cDNA clones.)

Initially isolated bundle-sheath RNA lacked a hybridization signal when filters were probed with a cDNA clone for NADP-malic enzyme (data not shown). The indicated extensive degradation of RNA was also apparent when agarose gels were stained with ethidium bromide. However, the degradation was largely observed in cytosolic, but not in chloroplastic RNAs. This indicated that during the preparation of the bundle-sheath cells the RNAses were released only in the cytosolic compartment. As outlined in *Material and methods*, intact bundle-sheath RNA could be obtained by raising the sorbitol concentration of the isolation medium to 0.6 M and by including diethylpyrocarbonate as an RNAse inhibitor during homogenization. In addition, the temperature of the isolation media was lowered to 1–4° C.

Hybridization of Northern filters with a PEPCase cDNA probe detected only traces of transcripts in the bundle-sheath RNA (Fig. 6). Quantitation of the bound probe by liquid scintillation counting revealed that the

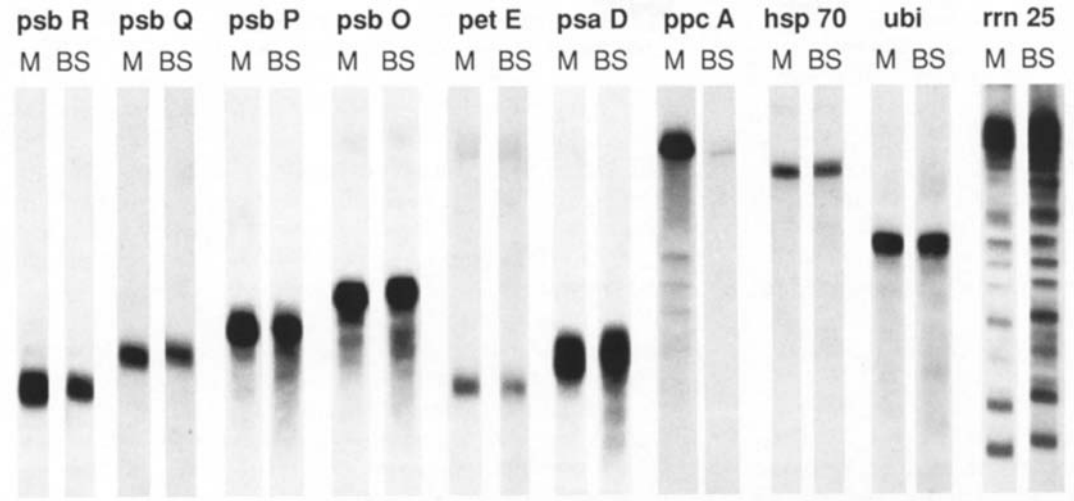

Fig. 6. Levels of cytosolic RNAs in mesophyll (*M*) and bundle-sheath (*BS*) cells of *Flaveria trinervia*. The same amounts of mesophyll and bundle-sheath RNA were denatured by glyoxylation and subjected to electrophoresis on 1.2% agarose gels. Ribonucleic acids were transferred to nylon membranes and hybridized with cDNA probes labelled by random priming as described in *Material and methods*. For designation of genes and corresponding mRNAs see Table 2. A representative out of four independent experiments is shown

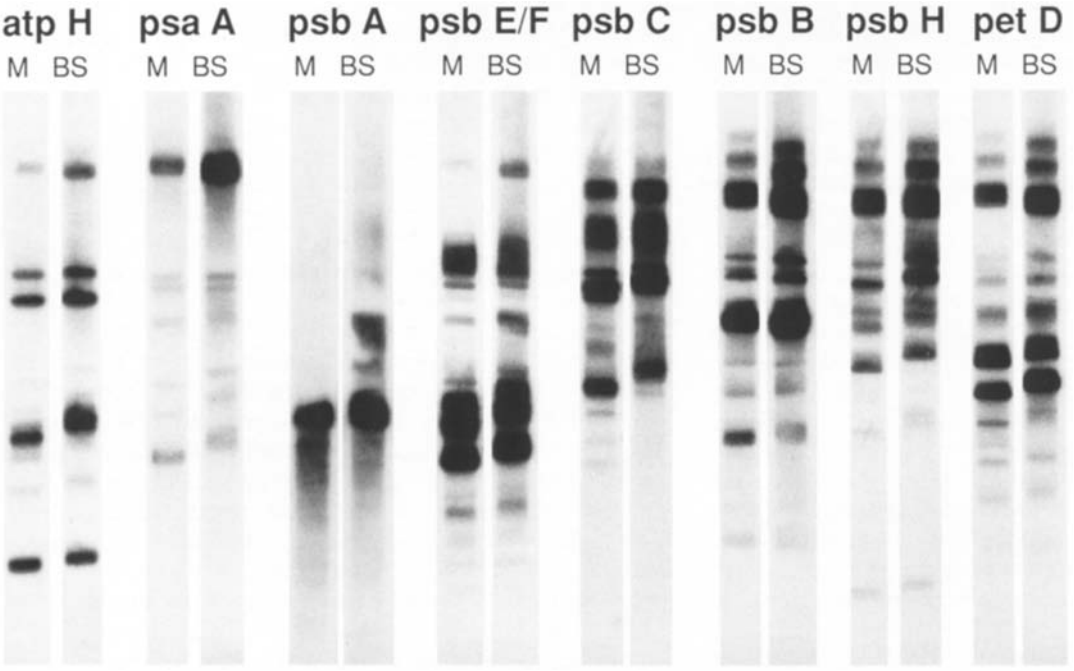

Fig. 7. Transcript profiles of plastome-encoded genes in mesophyll (*M*) and bundle-sheath (*BS*) RNA of *Flaveria trinervia*. For hybridization with antisense transcripts and probes used see Westhoff et al. (1991). Designation of genes: *atpH*, proteolipid subunit of ATP synthase; *psaA*, P700 apoprotein A of PSI; *psbA* D1 polypeptide; *psb E/F*, a (9.4 kDa) and b (4.4 kDa) subunits of cytochrome b_{559}; *psbC*, 44-kDa chlorophyll-a apoprotein (CP43); *psbB*, 51-kDa chlorophyll-a apoprotein (CP47); *psbH*, 10-kDa phosphoprotein; *petD*, subunit 4 of the cytochrome b/f complex. A representative out of four independent experiments is shown. The elevated level of the *psaA* transcript in the bundle-sheath RNA was not reproducible in the other experiments

contamination of the bundle-sheath fraction by mesophyll RNA was consistently well below 5%. This confirms the puritiy of the bundle-sheath strands. Hybridization with cDNAs encoding the 10-, 16- and 23-kDa proteins detected only slight differences (about 1.2- to 2-fold, as measured by liquid scintillation counting) in the steady-state levels of these transcripts in mesophyll and bundle-sheath RNA preparations (Fig. 6). Similar results were obtained when the blots were incubated with probes specific for the 34-kDa protein, plastocyanin, PSI-D, heat-shock protein 70 and ubiquitin (Fig. 6). In addition, no significant differences in the amounts of these RNAs in mesophyll and bundle-sheath cells could be observed when plants grown in summer and in late autumn were compared (data not shown). It follows that the severe reduction of the 10-, 16- and 23-kDa proteins

in the bundle-sheath cells cannot be controlled at the transcript level.

No major differences in transcript abundance and pattern were obvious when Northern blots of mesophyll and bundle-sheath RNA were probed by a representative set of plastid genes encoding PSII and non-PSII genes (Fig. 7). One can only conclude that the accumulation of both nuclear and plastid transcripts encoding PSII polypeptides does not appear to proceed differentially in mesophyll and bundle-sheath cells of the dicotyledonous C_4 plant *F. trinervia*.

Discussion

We are interested in understanding the regulatory mechanisms underlying the differential biogenesis of PSII in mesophyll and bundle-sheath cells of NADP-malic enzyme-type C_4 plants. In a previous paper, evidence was presented that the PSII content in developing bundle-sheath chloroplasts of monocotyledonous NADP-malic enzyme-type C_4 species varies and that the deregulation of PSII during the differentiation process is initiated at the oxidizing site (Oswald et al. 1990). In the present study the analysis has been extended to *Flaveria trinervia*, a dicotyledonous member of this subgroup.

Analysis by electron microscopy revealed only rudimentary grana development in mature bundle-sheath chloroplasts of *F. trinervia*. A strong depletion of grana formation has also been reported for *Gomphrena celesoides*, another dicotyledonous NADP-malic enzyme-type C_4 species (Repo and Hatch 1976). Thus in all cases analyzed so far the absence or reduced amounts of stacked thylakoid membrane regions in bundle-sheath chloroplasts has been correlated with a decrease in PSII activity. It has been suggested that the light-harvesting chlorophyll-a/b-protein complex (LHC II) of PSII is involved in establishing the lateral heterogeneity of thylakoid membranes (Anderson 1981). Interestingly, bundle-sheath thylakoids of *F. trinervia* contain only slightly reduced amounts of the major 27- to 29-kDa polypeptides of this antenna complex as compared to mesophyll thylakoids (Fig. 4). These data indicate that the presence of LHC II polypeptides is not sufficient for grana formation. It supports earlier conclusions derived from mutant analysis (quoted after Hiller and Goodchild 1981).

Protein fractions of both mesophyll and bundle-sheath cells were isolated and tested for the activity as well as the polypeptide organization of PSII. Only fully expanded leaves were used for these experiments. The data, therefore, represent a view of PSII organization in mesophyll and bundle-sheath cells when a functional C_4 cycle has been established (Moore et al. 1986; Moore and Edwards 1988). Bundle-sheath thylakoids isolated from summer-grown plants exhibited between 2–9% of the PSII activity measured in mesophyll thylakoids. In contrast, those from autumn- and winter-grown material showed consistently about 22% of this activity (see Table 1). Since plants in the summer were exposed to higher light intensities and temperatures as well as to a longer photoperiod than those in late autumn or winter it is tempting to conclude that the changes in environmental conditions account for the observed alterations in PSII activity in the bundle-sheath cells. It is well documented that the incident light received during growth can influence the photosynthetic properties of C_4 plants (Ludlow and Wilson 1971; Ward and Woolhouse 1986; Smith and Martin 1987). For example, high irradiance leads to an increase in the activities of several key enzymes of the C_4 cycle (Hatch et al. 1969; Fladung and Hesselbach 1989) and the C_4-like C_3–C_4 intermediate *Flaveria brownii* has been used to demonstrate that high light increases the expressed level of C_4 photosynthesis (Cheng et al. 1989). A transition from a mixed C_3/C_4 metabolism towards a fully expressed C_4 syndrome has been detected during leaf development of *F. trinervia* (Moore et al. 1986). Whether the variation in PSII activity in the bundle-sheath cells of *F. trinervia* is also paralleled by different levels of expression of the C_4 syndrome remains to be investigated.

Surprisingly, the reduction of PSII activity in bundle-sheath chloroplasts is not accompanied by a corresponding decline in the levels of the PSII core polypeptides, i.e. CP47, the D1 and D2 polypeptides, cytochrome b_{559} and the 34-kDa polypeptide. However, it is paralleled by a selective depletion of the 10-, 16- and 23-kDa polypeptides. These proteins are known to be of structural and regulatory importance for the functional integrity of the water-splitting apparatus (Homann 1988). In-vitro experiments have shown that the 16- and 23-kDa components are not an absolute prerequisite for water-oxidation provided that elevated levels of chloride and Ca^{2+} are present in the assay medium (reviewed in Miyao and Murata 1989). However, in the case of the 23-kDa protein, mutational analysis in *Chlamydomonas* has demonstrated that this polypeptide is necessary for PSII to be fully functional in vivo (Mayfield et al. 1987). Antisense experiments revealed that potato plants lacking the 10-kDa protein are not significantly affected in their growth under greenhouse conditions. Nevertheless, measurements of the relaxation of the flash-induced enhancement in the fluorescence quantum yield as determined in intact leaves showed that the elimination of the 10-kDa polypeptide affects the PSII donor site by retarding the reoxidation of Q_A^-. An analysis of the oscillation patterns of oxygen evolution in isolated thylakoid membranes indicated that the lack of the 10-kDa polypeptide introduces a greater disorder into the PSII complex (Stockhaus et al. 1990). It can be expected, therefore, that the depletion of these proteins in the bundle-sheath chloroplasts must have serious consequences for the water-oxidizing capacity of PSII, even though the intrinsic core of this photosystem does not seem to be disturbed. The data are consistent with the view that PSII activity may be deregulated by changing the amounts of the 10-, 16- and 23-kDa polypeptides attached to the PSII core. This notion is further supported by the observation that varying rates of water-oxidation activity observed in the bundle-sheath chloroplasts of summer-and autumn/winter-grown *F. trinervia* plants are associated with concomitant changes in the levels of these proteins.

Whether the regulation of the levels of the 10-, 16- and

23-kDa proteins is achieved by lowered rates of synthesis or selective degradation is not known at present. From the Northern blot analysis it has to be concluded that transcript abundance and transcription cannot account for the selective reduction of the extrinsic proteins of the water-splitting complex. Initially, preliminary experiments (see Discussion in Oswald et al. 1990) revealed low levels of the mRNAs of the 10-, 16- and 23-kDa proteins in the bundle-sheath cells, indicating a differential expression in mesophyll and bundle-sheath cells similar to monocotyledonous NADP-malic enzyme-type C_4 species (Sheen et al. 1987; Oswald et al. 1990). However, upon refining the techniques for the isolation of bundle-sheath RNA (*see Material and methods* as well as *Results*) the presence of substantial amounts of the mRNAs for these three proteins could be demonstrated in bundle-sheath cells (Fig. 6). No evidence could be obtained that the mRNAs of the 10-, 16- and 23-kDa proteins are selectively depleted in the bundle-sheath cells. Thus a post-transcriptional controlling mechanism must be responsible for regulating the abundance of these proteins in bundle-sheath chloroplasts. It is established that mRNAs encoding the 10-, 16- and 23-kDa proteins are selectively depleted in the bundle-sheath cells of the monocotyledonous C_4 plant *Sorghum bicolor* (Oswald et al. 1990). However, the data obtained with *F. trinervia* raise the question whether this reduction is indeed triggering the abundance of the corresponding protein as suggested or whether it is merely used to stabilize, but not to initiate the PSII depletion in the bundle-sheath cells.

As may be expected from the abundance of the plastome-encoded PSII subunits, i.e. CP47, D1, D2 and cytochrome b_{559}, no major differences in the levels of either the corresponding mRNAs or in the transcript profiles of PSII-gene-containing transcription units can be detected in mesophyll and bundle-sheath cells of *F. trinervia*. Thus the transcriptional activities of mesophyll and bundle-sheath chloroplasts are similar. In contrast, segmental RNAs within one single transcription unit can accumulate to different degrees in mesophyll and bundle-sheath chloroplasts of monocotyledonous NADP-malic enzyme-type C_4 species, demonstrating that in these plants the two types of chloroplasts are different with respect to their transcriptional and-or RNA-processing capacity (Westhoff et al. 1991).

It is interesting to observe that in both mono- and dicotyledonous C_4 plants of the NADP-malic enzyme subgroup the oxidizing site of PSII appears to be a focal point for regulating the functional state of this protein complex. Inactivation of the water-oxidation system is also used by the unicellular green alga *Chlamydobotrys stellata* to reduce linear electron transport when the alga is shifted from photoautotrophic to mixotrophic growth (Wiessner et al. 1981). It is tempting to speculate, therefore, that the controlling mechanism for the deregulation of PSII in the bundle-sheath cells is not a new acquisition of C_4 plants. It may be based on an evolutionary conserved, regulatory principle which is also used by C_3 plants to control the activity of photosynthetic electron flow from water to plastoquinone. The identification of genes which control the accumulation of the nuclear-encoded PSII proteins (cf. Erickson and Rochaix 1991) will, therefore, be of prime importance for understanding the differential biogenesis of this photosystem in mesophyll and bundle-sheath cells of C_4 plants.

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References

Anderson, J.M. (1981) Consequences of spatial separation of photosystem 1 and 2 in thylakoid membranes of higher plant chloroplasts. FEBS Lett. **124,** 1–10

Apel, P., Maass, I. (1981) Photosynthesis in species of *Flaveria* CO_2 compensation concentration, O_2 influence on photosynthetic gas exchange and $\delta^{13}C$ values in species of *Flaveria* (Asteraceae). Biochem. Physiol. Pflanzen **176,** 396–399

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24,** 1–13

Bauwe, H. (1984) Photosynthetic enzyme activities and immunofluorescense studies on the localisation of ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves of C_3, C_4, and C_3–C_4 intermediate species of *Flaveria* (Asteraceae). Biochem. Physiol. Pfl. **179,** 253–268

Blake, M.S., Johnston, K.H., Russel-Jones, G.J., Gotschlich, E.C. (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem. **136,** 175–179

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254

Börsch, D., Westhoff, P. (1990) Primary structure of NADP-dependent malic enzyme in the dicotyledonous C_4 plant *Flaveria trinervia*. FEBS Lett. **273,** 111–115

Cheng, S.H., Moore, B.D., Wu, J., Edwards, G.E., Ku, M.S.B. (1989) Photosynthetic plasticity in *Flaveria brownii*. Growth irradiance and the expression of C_4 photosynthesis. Plant Physiol. **89,** 1129–1135

Church, G.M., Gilbert, W. (1984) Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995

Downton, W.J.S., Pyliotis, N.A. (1971) Loss of photosystem II during ontogeny of sorghum bundle sheath chloroplasts. Can. J. Bot. **49,** 179–180

Erickson, J.M., Rochaix, J.D. (1991). The molecular biology of photosystem II. In: Topics in photosynthesis. vol. 10, The photosystems structure, function and molecular biology, Barber, J., ed. Elsevier Science Publishers, Amsterdam

Feinberg, A.P., Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13

Fladung, M., Hesselbach, J. (1989) Effect of varying environments on photosynthetic parameters of C_3, C_3–C_4 and C_4 species of *Panicum*. Oecologia **79,** 168–173

Golbeck, J.H., Martin, I.F., Velthuys, B.R., Radmer, R. (1981). A critical reassessment of the photosystem II content in bundle sheath chloroplasts of young leaves of *Zea mays*. In: Proc. Vth Int. Congr. of Photosynthesis, vol. 5, Chloroplast development, pp. 533–546, Akoyunoglou, J., ed. Balaban International Science Services, Philadelphia

Haehnel, W., Ratajczak, R., Robenek, H. (1989) Lateral distribution and diffusion of plastocyanin in chloroplast thylakoids. J. Cell Biol. **108,** 1397–1405

Hatch, M.D., Slack, C.R., Bull, T.A. (1969) Light-induced changes in the content of some enzymes of the C_4-dicarboxylic acid

pathway of photosynthesis and its effect on other characteristics of photosynthesis. Phytochemistry **8,** 697–706

Hattersley, P.W. (1987). Variations in photosynthetic pathway. In: Grass systematics and evolution, pp. 49–64, Soderstrom, T.R., Hilu, K.W., Campbell, C.S., Barkworth, M.E., eds. Smithonian Institution Press, Washington

Hiller, R.G., Goodchild, D.J. (1981). Thylakoid membrane and pigment organization. In: The biochemistry of plants, pp. 1–49, Hatch, M.D., Boardway, N.K., eds. Academic Press, London

Homann, P.H. (1988) Explorations in the "inner sanctum of the photosynthetic process", the water oxidizing system. Plant Physiol. **88,** 1–5

Huynh, T.V., Young, R.A., Davis, R.W. (1985) Constructing and screening cDNA libraries in lambda gt10 and lambda gt11. In: DNA cloning, vol. I, pp. 49–88, Glover, D.M., ed. IRL Press, Oxford-Washington

Laetsch, W.M., Price, I. (1969) Development of the dimorphic chloroplasts of sugar cane. Am. J. Bot. **56,** 77–87

Lowry, O.H., Rosebrough, N.J., Faro, A.L., Randall, R.J. (1951) Protein measurements with folin-phenol reagent. J. biol. Chem. **193,** 265–275

Ludlow, M.M., Wilson, G.L. (1971) Photosynthesis of tropical pasture plants: II. Photosynthesis and illuminance history. Aust. J. Biol. Sci. **24,** 1065–1075

Mayfield, S.P., Rahire, M., Frank, G., Zuber, H., Rochaix, J.D. (1987) Expression of the nuclear gene encoding oxygen-evolving enhancer protein 2 is required for high levels of photosynthetic oxygen evolution in *Chlamydomonas reinhardtii.* Proc. Natl. Acad. Sci. USA **84,** 749–753

Miyao, M., Murata, N. (1989). Photoinactivation of the oxygen-evolving complex of photosystem II. In: Topics in Photosynthesis, vol. 9: Photoinhibition, pp. 289–307, Kyle, D.J., Osmond, C.B., Arntzen, C.J., eds. Elsevier Science Publishers, Amsterdam-New York-Oxford

Mogen, K., Eide, J., Duysen, M., Eskins, K. (1990) Chloramphenicol stimulates the accumulation of light-harvesting chlorophyll a/b protein II by affecting posttranscriptional events in the chlorina CD3 mutant wheat. Plant Physiol. **92,** 1233–1240

Moore, B.D., Edwards, G.E. (1986a) Photosynthetic induction in a C_4 dicot, *Flaveria trinervia.* 1. Initial products of $^{14}CO_2$ assimilation and levels of whole leaf C_4 metabolites. Plant Physiol. **81,** 663–668

Moore, B.D., Edwards, G.E. (1986b) Photosynthetic induction in a C_4 dicot, *Flaveria trinervia.* 2. Metabolism of products of $^{14}CO_2$ fixation after different illumination times. Plant Physiol. **81,** 669–673

Moore, B.D., Edwards, G.E. (1988) The influence of leaf age on C_4 photosynthesis and the accumulation of inorganic carbon in *Flaveria trinervia,* a C_4 dicot. Plant Physiol. **88,** 125–130

Moore, B.D., Cheng, S.H., Edwards, G.E. (1986) The influence of leaf development on the expression of C_4 metabolism in *Flaveria trinervia,* a C_4 dicot. Plant Cell Physiol. **27,** 1159–1167

Nechushtai, R., Nelson, N. (1985) Biogenesis of photosystem I reaction center during greening of oat, bean and spinach leaves. Plant Mol. Biol. **4,** 377–384

Oswald, A., Streubel, M., Ljungberg, U., Hermans, J., Eskins, K., Westhoff, P. (1990) Differential biogenesis of photosystem II in mesophyll and bundle sheath cells of NADP malic enzyme-type C_4 plants. A comparative protein and RNA analysis. Eur. J. Biochem. **190,** 185–194

Repo, E., Hatch, M.D. (1976) Photosynthesis in *Gomphrena celosioides* and its classification amongst C_4-pathway plants. Aust. J. Plant Physiol. **3,** 863–876

Schuster, G., Ohad, I., Martineau, B., Taylor, W.C. (1985) Differentiation and development of bundle sheath and mesophyll thylakoids in maize. Thylakoid polypeptide composition, phosphorylation, and organization of photosystem II. J. Biol. Chem. **260,** 11866–11873

Sheen, J.Y., Sayre, R.T., Bogorad, L. (1987) Differential expression of oxygen-evolving polypeptide genes in maize leaf cell types. Plant Mol. Biol. **9,** 217–226

Smith, M., Martin, C.E. (1987) Field studies of photosynthetic and growth responses to irradiance in three forest understory species of the C_4 grass genus *Muhlenbergia.* Bot. Gaz **148,** 456–462

Somersalo, S., Krause, G.H. (1990) Reversible photoinhibition of unhardened and cold-acclimated spinach leaves at chilling temperatures. Planta **180,** 181–187

Stockhaus, J., Höfer, M., Renger, G., Westhoff, P., Wydrzynski, T., Willmitzer, L. (1990) Anti-sense RNA efficiently inhibits formation of the 10 kDa polypeptide of photosystem II in transgenic potato plants: Analysis of the role of the 10 kDa protein. EMBO J. **9,** 3013–3021

Uedan, K. Sugiyama, T. (1976) Purification and characterization of phosphoenolpyruvate carboxylase from maize leaves. Plant Physiol. **57,** 906–910

Vidal, J., Godbillon, G., Gadal, P. (1981) Recovery of highly purified phosphoenolpyruvate carboxylase from specific immunoadsorbent columns. FEBS Lett. **118,** 31–34

Ward, D.A., Woolhouse, H.W. (1986) Comparative effects of light during growth on the photosynthetic properties of NADP–ME type C_4 grasses from open and shaded habitats. I. Gas exchange, leaf anatomy and ultrastructure. Plant Cell Environ. **9,** 261–270

Westhoff, P., Jansson, C., Klein-Hitpaß, L., Berzborn, R., Larsson, C., Bartlett, S.G. (1985) Intracellular coding sites of polypeptides associated with photosynthetic oxygen evolution of photosystem II. Plant Mol. Biol. **4,** 137–146

Westhoff, P., Offermann-Steinhard, K., Höfer, M., Eskins, K., Oswald, A., Streubel, M. (1991) Differential accumulation of plastid transcripts encoding photosystem II components in the mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme-type C_4 plants. Planta **184,** 377–388

Wiessner, W., Dubertet, G., Henry-Hiss, Y., Mende, D., Lefort-Tran, M. (1981) Fluorescence transients as indicator for the existence of regulatory mechanisms in photosynthetic electron transport. Ber. Deutsch. Bot. Ges. **94,** 503–515

Woo, K.C., Anderson, J.M., Boardman, N.K., Downton, W.J.S., Osmond, C.B., Thorne, S.W. (1970) Deficient photosystem II in agranal bundle sheath chloroplasts of C_4 plants. Proc. Natl. Acad. Sci. USA **67,** 18–25