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Modification of Photosystem I Light Harvesting of Bundle-Sheath Chloroplasts Occurred during the Evolution of NADP-Malic Enzyme C_4 Photosynthesis[1]

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Low-temperature emission spectra and excitation spectra for chlorophyll fluorescence were recorded from leaves of species of the genus *Flaveria* (Asteraceae) with C_3, C_3-C_4-intermediate, C_4-like, and C_4 photosynthesis. Among the latter two groups, high chlorophyll *b* absorption was observed in excitation spectra for photosystem I (PSI) fluorescence. By comparing leaf data with those from isolated chloroplast fractions, the high chlorophyll *b* absorption was attributed to the specific properties of the bundle-sheath chloroplasts in leaves from C_4 plants. The deconvolution of the PSI excitation spectra and the use of a model revealed that the contribution of photosystem II absorption to the functional antenna of PSI was markedly increased in leaves from three of the five C_4-like and C_4 species investigated in detail. The two other species exhibited normal, C_3-like light-harvesting properties of PSI. The former species are known for efficient carbon assimilation, the latter for decreased efficiencies of carbon assimilation. It is concluded that photosystem II becomes a substantial part of the functional PSI antenna late in the evolution of C_4 photosynthesis, and that the composite antenna optimizes the light-harvesting of PSI in bundle-sheath chloroplasts to meet the energy requirements of C_4 photosynthesis.

Photosynthetic carbon assimilation is the origin of nearly all organic matter on earth. In most land plants, atmospheric CO_2 is fixed by the enzyme Rubisco via carboxylation of a five-carbon sugar phosphate. Because the first stable products of this reaction are three-carbon compounds, this type of photosynthesis is known as C_3 photosynthesis. In light, Rubisco also exhibits oxygenase activity (Edwards and Walker, 1983). As an oxygenase, the enzyme is involved in photorespiration through which part of the oxygenated carbon is released as CO_2. Therefore, photorespiration results in carbon loss from the leaf, and when it occurs at high rates it can severely limit carbon assimilation by C_3 photosynthesis.

The oxygenase reaction of Rubisco is favored by low CO_2 concentrations in the leaf because oxygen and CO_2 compete for binding at the active site of the enzyme. Low CO_2 concentrations in the leaf prevail under dry, hot conditions. Under such conditions, plants minimize water loss from the leaves by closing their stomata, which in turn restricts CO_2 diffusion from the atmosphere into the leaves. Plants that undergo C_4 photosynthesis can effectively fix carbon at low internal CO_2 concentrations. This is achieved by CO_2 binding via specific reactions that yield four-carbon dicarboxylic acids (Hatch, 1987). This primary carbon fixation takes place in the mesophyll compartment, in which photosynthetic cells are closer to the leaf surface. In C_4 plants Rubisco is confined to the bundle-sheath compartment, in which cells surround the vascular bundles. The C_4 acids are transported from the mesophyll to the bundle-sheath compartment, where CO_2 is released for refixation by Rubisco.

In essence, C_4 biochemistry represents a biochemical CO_2 pump that increases the CO_2 concentration in the vicinity of Rubisco and thereby reduces photorespiration. In the absence of photorespiration, the quantum yield for CO_2 fixation is lower for C_4 photosynthesis than for C_3 photosynthesis because extra energy is required to fuel the reactions of the C_4 cycle (Hatch, 1987). However, when low CO_2/O_2 ratios trigger high rates of photorespiration in C_3 photosynthesis, the photosynthetic performance of C_4 plants is superior to that of C_3 plants. Therefore, a low atmospheric CO_2 concentration may represent the evolutionary pressure for the evolution of C_4 photosynthesis (Hatch, 1992). It has been suggested that C_4 plants evolved from C_3 ancestors in response to a dramatic decrease in the atmospheric CO_2/O_2 ratio that began about 100 million years ago (Moore, 1982; Ehleringer and Monson, 1993).

Among the three biochemical subtypes of C_4 photosynthesis, the biochemistry of the NADP-ME group, which also includes the crop plants maize and sugar cane, is probably the best characterized. The evolution of C_4 photosynthesis implies the compartment-specific regulation of the enzymes involved, as well as modifications in the leaf anatomy (Hatch, 1987). We have recently shown for a number of NADP-ME plants that light harvesting of PSI is also regulated in a compartment-specific manner (Pfündel et al., 1996): in bundle-sheath chloroplasts, the contribution of the PSII holocomplex to the functional antenna of PSI is much greater than in mesophyll chloroplasts.

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Abbreviations: Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; c_i, CO_2 concentration in the gas phase inside the leaf; Γ, CO_2 compensation concentration; ME, malic enzyme; Φ_T, quantum yield for PSII-to-PSI energy transfer.

In NADP-ME C_4 photosynthesis, relatively more PSI activity is required to drive CO_2 fixation than in C_3 photosynthesis (Edwards and Walker, 1983). Therefore, the peculiarity of PSI light harvesting in bundle-sheath chloroplasts may represent an adaptation to the specific energy demands of C_4 plants, and therefore may be associated with the evolution of C_4 photosynthesis. We address this question by comparing the properties of PSI light harvesting in various species of the genus *Flaveria*. This genus was chosen because it includes C_3, C_3-C_4-intermediate, and C_4 species. The intermediate *Flaveria* species fix CO_2 to varying degrees by C_4 biochemistry and most likely represent phylogenetic intermediate positions between C_3 and C_4 species (Monson and Moore, 1989; Kopriva et al., 1996). Our data demonstrate that the tight functional connection between PSII and PSI is confined to those C_4-like and C_4 species of *Flaveria* that are known for efficient carbon assimilation. We conclude that high PSII-to-PSI energy transfer indeed adapts PSI light harvesting to the energy requirements of C_4 photosynthesis.

MATERIALS AND METHODS

A listing of the species investigated and their biochemical type of photosynthesis is presented in Table I. Plants were cultivated in the greenhouse under temperatures ranging from 20 to 30°C between the fall and spring seasons. Supplementary light with an intensity of 150 to 200 μmol quanta m^{-2} s^{-1} at leaf level was given from 6 AM until 10 PM. Light sources were 400-W, high-pressure sodium lamps. During the summer plants were cultivated in a growth chamber with a 16-h light period, during which light with an intensity of 430 μmol quanta m^{-2} s^{-1} was provided by 1000-W mercury-halogen lamps. In the growth chamber the temperature was maintained at 20 and 25°C for dark and light conditions, respectively. All plants were grown on garden mulch and were propagated in greenhouse beds from root stocks (C_3 species and hybrids), in pots from cuttings (*F. brownii*, *F. chloraefolia*, *F. floridana*, and *F. pubescens*), or in pots from seeds (all other species). All measurements were performed with young, fully developed leaves that had been dark-adapted overnight. For individual species, complete data sets were acquired from plant material grown under identical conditions.

Table I. *Investigated species and F_1 hybrids of the genus Flaveria*

Species are arranged according to Γ. The classification of the photosynthetic groups is taken from the plant list compiled by Ku et al. (1991). Characters a to n identify individual species in Figures 1, 2, and 4. Reciprocal F_1 hybrids were obtained as described by Apel et al. (1989).

Character	Species	Photosynthetic Group
a	*F. cronquistii* A.M. Powell	C_3
b	*F. cronquistii* × *F. brownii*	C_3 (♀) × C_4-like (♂)
c	*F. pringlei* Gandoger	C_3
d	*F. brownii* × *F. cronquistii*	C_4-like (♀) × C_3 (♂)
e	*F. linearis* Lag.	C_3-C_4
f	*F. chloraefolia* A. Gray	C_3-C_4
g	*F. anomala* B. Robinson	C_3-C_4
h	*F. pubescens* Rydb	C_3-C_4
i	*F. floridana* J.R. Johnston	C_3-C_4
j	*F. brownii* A.M. Powell	C_4-like
k	*F. australasica* Hook	C_4 (NADP-ME)
l	*F. bidentis* (L.) Kuntze	C_4 (NADP-ME)
m	*F. palmeri* J.R. Johnston	C_4-like
n	*F. trinervia* (Spreng.) C. Mohr	C_4 (NADP-ME)

Gas-Exchange Measurements

Depending on leaf size, one to three leaves were sealed in a gas-exchange cuvette (MK-022/I, Walz, Effeltrich, Germany). The leaves were attached to whole plants or to freshly cut twigs immersed in water for pot or bed cultures, respectively. The leaves were illuminated with a 75-W quartz filament tube equipped with a cold light reflector and a heat-absorbing glass filter (KG3, Schott Glaswerke, Mainz, Germany). Light intensity was measured by a photodiode that was calibrated with a quantum sensor (model 189, Li-Cor, Lincoln, NE), and was adjusted to 750 μmol quanta m^{-2} s^{-1} by neutral-density filters. The temperature of the lower leaf surface was recorded by a NiCr-Ni thermocouple. Leaf temperature was maintained at 28°C by a peltier-cooled heat exchanger (GK 022, Walz).

Various CO_2 concentrations in the cuvette were produced by mixing pressurized CO_2-free air with pure CO_2 using CO_2-permeable silicon tubes, as described by Apel (1966), and gas-flow controllers. The moisture content of the gas entering the cuvette was adjusted to 7.5 g H_2O m^{-3} by passing the water-saturated gas through a cold trap. The moisture content in the cuvette ranged from 15 to 20 g H_2O m^{-3}.

Gas-exchange measurements were made using a setup developed by Peisker (1979). The gas was guided at constant flow rates through the cuvette and through a parallel reference channel. The CO_2 and H_2O concentrations of the gas that had passed the cuvette and the gas of the reference channel were measured alternately with an IR gas analyzer operated in the absolute mode (BINOS 1001, Rosemount, Hanau, Germany). Data were taken after steady-state conditions were attained. The CO_2 assimilation rate and c_i were calculated according to the method of von Caemmerer and Farquhar (1981). At low CO_2 concentrations (50–150 μL L^{-1} for C_3 species and 5–50 μL L^{-1} for C_4 species) the CO_2 assimilation rates correlated with c_i and, generally, correlation coefficients for the two parameter sets were $r > 0.99$. The CO_2 compensation concentration was derived from the intersection of the CO_2 assimilation rate versus the c_i regression line and the x axis.

Fluorescence Spectroscopy

To reduce artifacts caused by pigment screening and fluorescence reabsorption, we used diluted leaf powder for fluorescence spectroscopy (Weis, 1985). Diluted leaf powder was prepared under dim light by using a precooled (77 K) mortar and pestle. A leaf disc 4 mm in diameter, together with 1 mL of distilled water, was rapidly ground to a fine powder. After grinding, no intact tissue was detected by light microscopy. The chlorophyll concentration of the

powder was approximately 7 μM and matched the concentrations used by Weis (1985).

The leaf powder was placed in precooled (77 K) quartz tubes (Heralux, Hellma, Müllheim, Germany) of 1 mm i.d. Fluorescence spectra were recorded at 77 K using a fluorimeter (LS 50B, Perkin-Elmer). All fluorimeter settings were as described previously (Pfündel and Meister, 1996), except that no stray light reduction was required because of the high fluorescence intensities of the samples.

We utilized the fluorescence excitation spectra to calculate parameters of the PSII-to-PSI energy transfer (see below). In the derivation of the model applied it is assumed that the absorptance (1 minus transmission) spectra of the photosystems are proportional to their absorbance spectra (Pfündel et al., 1996), as is the case in very dilute samples. In such samples, the shape of the absorptance curves is expected to be insensitive to variations in the chlorophyll concentration. Upon increasing our chlorophyll concentration of 7 μM by a factor of 4, we observed no effect on the shape of our excitation spectra. Therefore, we were well within the concentration range required for our model calculations.

All data handling and curve fitting were done with scientific graphing software (SigmaPlot, Jandel Scientific, Erkrath, Germany). Chl *a*/Chl *b* ratios were determined fluorimetrically by the method of Meister (1992), with modifications as described by Pfündel and Meister (1996). We preferred this technique to an absorbance spectroscopic method because it is less sensitive to inaccuracies of the wavelength position of the monochromator and determines high Chl *a*/Chl *b* ratios more reliably (compare Meister, 1992).

Assessment of PSII/PSI Ratios and of the Relative Φ_T

We estimated PSII/PSI ratios and relative quantum yields of PSII to PSI from steady-state fluorescence spectra and Chl *a*/Chl *b* values for each individual species, as described previously (Pfündel et al., 1996). The PSII/PSI ratios were obtained according to the equation:

$$\frac{c_{PS2}}{c_{PS1}} = \frac{N_{Chlb,PS1}}{N_{Chlb,PS2}} \times \frac{(N_{Chla,PS1}/N_{Chlb,PS1} - c_{Chla}/c_{Chlb})}{(c_{Chla}/c_{Chlb} - N_{Chla,PS2}/N_{Chlb,PS2})} \quad (1)$$

where c_{Chla}/c_{Chlb} is the pigment ratio of Chl *a* to Chl *b* of the sample, $N_{Chla,PS1}$ represents the molecules of Chl *a* in PSI, $N_{Chla,PS2}$ represents the molecules of Chl *a* in PSII, and $N_{Chlb,PS1}$ and $N_{Chlb,PS2}$ are the molecules of Chl *b* in PSI and PSII, respectively. Using estimates of Chl *a* and Chl *b* molecules in $PSII_\alpha$ and PSI, respectively, derived from Melis (1991), we arrive at:

$$\frac{c_{PS2}}{c_{PS1}} = \frac{1}{5} \times \frac{(9 - c_{Chla}/c_{Chlb})}{(c_{Chla}/c_{Chlb} - 3/2)} \quad (2)$$

The results of Equation 2 represent PSII/PSI estimates that do not correspond to the reaction center ratios of PSII/PSI as determined by the more classical methods. As discussed previously (Pfündel et al., 1996), Equation 2 disregards PSII complexes with small antenna sizes, i.e. $PSII_\beta$. Because $PSII_\beta$ accounts for only a minor fraction of the light absorption of chloroplasts (Melis and Anderson, 1983), this characteristic is advantageous when the absorption properties of PSII are important, as is the case in this study.

Relative quantum yields of PSII-to-PSI energy transfer were estimated according to:

$$\Phi_T \times K = \frac{Fc2}{Fc1} / \frac{c_{PS2}}{c_{PS1}} \quad (3)$$

where c_{PS2}/c_{PS1} is the PSII/PSI estimate obtained by Equation 2 and the quotient $Fc2/Fc1$ represents a relative measure for the contribution of PSII to the functional PSI antenna. Quotients of $Fc2/Fc1$ were derived from the deconvolution of empirical excitation spectra for PSI fluorescence (735 nm) using Equation 4 (see also Fig. 3):

$$F_{735}(\lambda ex) = Fc1 \times e_{PS1}(\lambda ex) + Fc2 \times e_{PS2}(\lambda ex) \quad (4)$$

where $e_{PS1}(\lambda ex)$ and $e_{PS2}(\lambda ex)$ are the model spectra of pure PSI and pure PSII, respectively, and *Fc1* and *Fc2* are free parameters. $e_{PS1}(\lambda ex)$, the excitation spectrum of pure bundle-sheath chloroplasts from *C. papyrus*, was used because this spectrum exhibited almost no PSII emission at 77 K (Pfündel et al., 1996). The excitation spectrum of PSII fluorescence (685 nm) of the sample to be analyzed was $e_{PS2}(\lambda ex)$. For the definition of K, refer to Pfündel et al. (1996).

RESULTS AND DISCUSSION

Our study included 12 species of *Flaveria* and two hybrids with C_3-like behavior (Table I). We classified all plants by their Γ (Fig. 1). Our values of Γ from *Flaveria* agree with previously published data (Holaday et al., 1984; Ku et al., 1991). On this basis, we can compare the new results achieved here with the known characteristics for the different *Flaveria* species.

The value of Γ corresponds to the CO_2 concentration at which the photosynthetic carbon assimilation equals the photorespiratory and respiratory CO_2 losses from the leaf. In C_3 plants, the relatively high values of Γ are related to photorespiratory CO_2 losses. In C_4 species, the Γ is markedly lower than in C_3 species because C_4 biochemistry reduces photorespiration to trivial rates. In C_3-C_4-intermediate *Flaveria* species, an additional factor that decreases Γ is the efficient refixation of photorespired CO_2 (Rawsthorne, 1992). Therefore, Γ does not exactly reflect the degree of C_4 photosynthesis in plants, but is suitable for grouping species according to their type of photosynthesis. In our case, the group of C_4-like and C_4 species exhibited Γ values smaller than 10 μL CO_2 L^{-1}; the Γ from the intermediates ranged between 10 and 30 μL CO_2 L^{-1}; and values greater than 40 μL CO_2 L^{-1} were measured for the C_3 species and the hybrids (Fig. 1).

In bundle-sheath chloroplasts from C_4 species of *Flaveria*, light absorption by the PSII complex contributes significantly to the functional antenna of PSI despite their relatively low PSII concentrations (Pfündel et al., 1996). To investigate the expression of this phenomenon in the genus *Flaveria*, we first analyzed the PSII/PSI ratio in our species

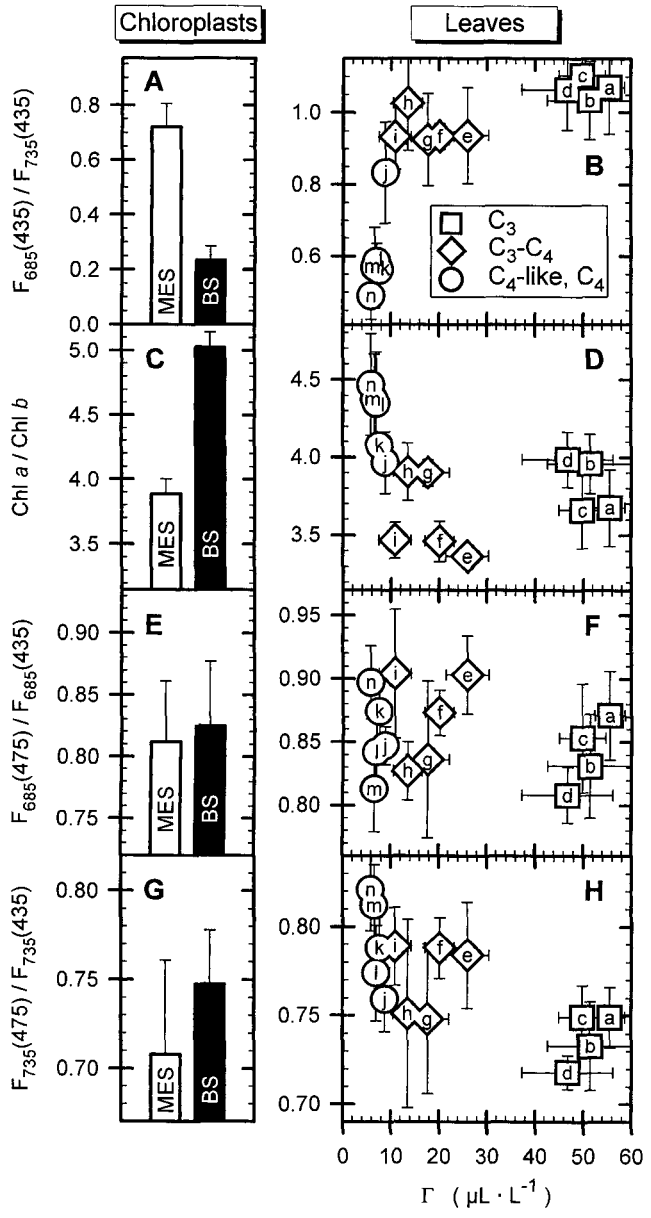

Figure 1. Fluorescence characteristics and Chl *a*/Chl *b* ratios of chloroplasts from C_4 species of *Flaveria* and of leaves from various *Flaveria* species. The figure compares mean values of pure mesophyll (MES) and bundle-sheath (BS) chloroplasts from one C_4-like and three C_4 *Flaveria* species (A, C, E, and G) with data from leaves of various *Flaveria* species and *Flaveria* hybrids (B, D, F, and H). Pure chloroplast fractions were derived from *F. australasica*, *F. bidentis*, *F. palmeri*, and *F. trinervia* (Pfündel et al., 1996), and mean values were calculated from the individual chloroplast isolations. SD values are shown as vertical bars (n = 12). The *Flaveria* species and hybrids are identified by the characters a to n, as specified in Table I. Data from leaves are plotted against the respective Γ and represent the means of 3 to 14 measurements. The SD values of the ordinate and abscissa are shown as vertical and horizontal bars, respectively. The following parameters were used as the abscissas: the value at 685 nm relative to the value at 735 nm of 77 K emission spectra (A and B); Chl *a*/Chl *b* ratios (C and D); the value at 475 nm relative to the value at 435 nm of 77 K excitation spectra for 685 nm emission (E and F); and the value at 475 nm relative to the value at 435 nm of 77 K excitation spectra for 735 nm emission (G and H).

using low-temperature emission spectra for chlorophyll fluorescence and Chl *a*/Chl *b* ratios.

Our fluorescence emission spectra (not shown) always exhibited two major peaks, one at 685 nm and the other at 735 nm. We categorized the emission spectra by the value at 685 nm relative to the value at 735 nm and plotted this ratio against the respective values of Γ in Figure 1B. This emission ratio is related to the PSII/PSI ratio because at low temperatures the 685- and 735-nm fluorescences originate from PSII and PSI, respectively (Govindjee, 1995). With the exception of the C_4-like *F. brownii*, the group of C_4-like and C_4 species exhibited emission ratios that were about 50% of those from the C_3 species and the hybrids (Fig. 1B). The emission ratios of *F. brownii* and the intermediates were slightly smaller than the C_3 values.

The Chl *a*/Chl *b* ratio is affected by the PSII/PSI ratio because the mature PSII complex binds much more Chl *b* than the PSI complex (Siefermann-Harms and Ninnemann, 1982). The highest Chl *a*/Chl *b* ratios, i.e. the lowest PSII/PSI ratios, were observed for *F. bidentis*, *F. palmeri*, and *F. trinervia*, all of which belong to the group of C_4-like and C_4 species (Fig. 1D). This agrees with the low PSII/PSI emission ratios in these species (Fig. 1B). However, the Chl *a*/Chl *b* ratios of all other species scattered considerably and disagreed with the rather homogeneous distribution of emission ratios.

Since most of the Chl *b* in the leaf is bound to the PSII complex, it is possible to explain the heterogeneity of Chl *a*/Chl *b* ratios by species-dependent variations in the PSII chlorophyll stoichiometry. The variations in PSII can be brought about by different temperatures or light regimes during growth (Anderson et al., 1995), since our growth conditions varied with season (see "Materials and Methods"). Because of the possible effects of growth conditions, we always acquired complete data sets of the individual species (Γ, fluorescence, and chlorophyll data) from plants cultivated under identical conditions.

To test if variations in PSII produce the heterogeneous behavior of the Chl *a*/Chl *b* ratios from leaves, we compared the latter data with a parameter that is related to the Chl *a*/Chl *b* ratio of PSII. This parameter was derived from excitation spectra for PSII fluorescence emission (685 nm). In the range 400 to 550 nm, the excitation spectra exhibited a major peak at around 435 and a minor peak at around 475 (not shown). As previously reported (Pfündel et al., 1996), the spectra were classified by taking the ratio of emissions obtained by exciting at 475 and 435 nm. These excitation ratios did not reveal a Γ-specific tendency (Fig. 1F). Because light absorption at 475 and at 435 nm is dominated by Chl *b* and Chl *a*, respectively (Siefermann-Harms and Ninnemann, 1982), our excitation ratios should be high for low Chl *a*/Chl *b* ratios of PSII and vice versa. Consequently, for all of the species in which the Chl *a*/Chl *b* ratio of PSII is the major determinant for the leaf Chl *a*/Chl *b* ratio, a negative correlation between the latter data and the excitation ratios of PSII should exist.

In fact, for all species with $\Gamma > 10\ \mu L\ L^{-1}$, the Chl *a*/Chl *b* ratios from leaves were strongly correlated with the PSII excitation ratios (Fig. 2). Consequently, in these plants, we

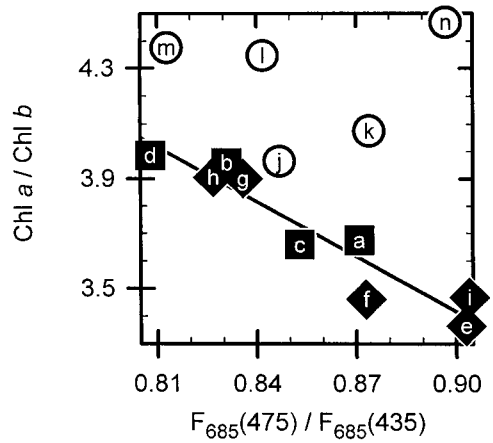

Figure 2. Relationship between Chl *a*/Chl *b* ratio values and excitation spectra for 685-nm fluorescence. Ordinate and abscissa correspond to the ordinates of Figure 1, D and F, respectively. Individual plants are identified by the characters a to n (see Table I). Data from C_4-like and C_4 species are shown as open symbols. The C_3 species, hybrids, and C_3-C_4 intermediates are shown as black symbols (squares, C_3; diamonds, C_3-C_4). The straight line was obtained by linear regression analysis of the latter data points. The correlation coefficient was $r = -0.947$ ($P = 0.0001$).

attribute the high variability in the Chl *a*/Chl *b* ratio to different chlorophyll stoichiometries in their PSII complexes, and conclude that similar PSII/PSI ratios exist. Figure 2 also illustrates that the high Chl *a*/Chl *b* ratios in leaves from our three C_4 species and the C_4-like *F. palmeri* cannot be explained by PSII effects. Instead, the Chl *a*/Chl *b* ratios in these plants indicate smaller PSII/PSI ratios than in the other species. Because the data point of *F. brownii* was close to the relationship established in Figure 2, the PSII/PSI ratio is probably similar or only slightly increased compared with that of the intermediate and C_3 species. When the species-dependent variations in PSII are taken into account, our Chl *a*/Chl *b* ratios agree well with our fluorescence emission spectra.

In pure bundle-sheath chloroplasts from our three C_4 species and *F. palmeri*, conspicuously low fluorescence emission ratios and high Chl *a*/Chl *b* ratios were measured (Fig. 1, A and C). Therefore, we are fairly certain that the decreased PSII/PSI ratio in leaves from these species is determined by the low PSII/PSI ratio in their bundle-sheath chloroplasts. This agrees with the notion that in species with NADP-ME-type C_4 photosynthesis, the redox equivalents for carbon reduction in the bundle-sheath compartment are derived from the mesophyll compartment, and that the ATP required to drive the reactions associated with carbon reduction is formed via cyclic electron transport around PSI alone (Hatch, 1987). For the intermediates, we deduced PSII/PSI ratios similar to those in C_3 species. These findings agree with those of Ku et al. (1991), who suggested that the reduced values of Γ in intermediate *Flaveria* species are predominantly brought about by efficient refixation of photorespired CO_2 and only to a minor degree by C_4 biochemistry.

After having characterized the PSII/PSI ratios, we next analyzed the contribution of PSII light absorption to the functional antenna of PSI. For this, excitation spectra for PSI fluorescence (735 nm) were recorded at 77 K. We quantified the PSI excitation spectra in an analogous fashion to the PSII excitation spectra and found that the relative 475-nm values in C_4 and C_4-like species were higher compared with C_3 species and hybrids (Fig. 1H). The relative 475-nm values in species with $\Gamma > 10$ μL L^{-1} exhibited large variations. The PSII contribution was estimated by deconvoluting the PSI excitation spectra into a PSI and a PSII model component, as described in "Materials and Methods." We chose *F. pringlei* and *F. australasica* to demonstrate typical results obtained for C_3 and C_4 species, respectively (Fig. 3). Generally, good correspondence between empirical and calculated curves was obtained in the range 400 to 500 nm. At longer wavelengths, deviations between experimental and calculated curves occurred. Because carotenoids dominate light absorption in this spectral region (Siefermann-Harms, 1985), these deviations

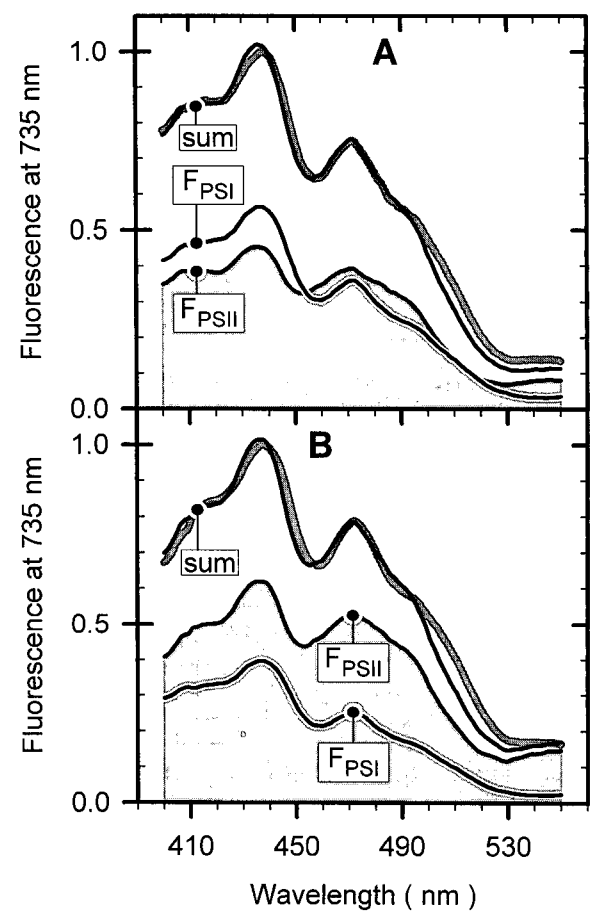

Figure 3. Deconvolution of excitation spectra of 735-nm fluorescence. Empirical 77 K excitation spectra of 735-nm fluorescence from leaves from *F. pringlei* and *F. australasica* are depicted as thick gray lines in A and B. Calculated spectra (black lines labeled sum) resulted from varying PSI and PSII model components until the best fit between the sum of both components and the measured curve was obtained (see "Materials and Methods"). The PSI and PSII components of the calculated spectra are labeled F_{PSI} and F_{PSII}, respectively, where the area below the PSII component is shaded gray. For curve fitting, the spectral range of 400 to 500 nm was considered.

were probably caused by differences in carotenoid absorption between the model spectra and the PSI and the PSII spectra in vivo.

Figure 3 clearly demonstrates that the PSII component required to simulate the empirical 735-nm excitation was substantially higher in the C_4 species *F. australasica* than in the C_3 species *F. pringlei*. For all species we quantified the PSII contribution to the empirical PSI excitation spectra by the quotient *Fc2/Fc1*, for which high values indicate high PSII contributions. The *Fc2/Fc1* values were similar for plants with $\Gamma > 10$ μL L^{-1} (Fig. 4B). This contrasts with the variations of empirical PSI excitation spectra (Fig. 1H) and can be explained by the fact that the PSII model curves, i.e. the excitation spectra for 685-nm fluorescence variations (Fig. 1F), parallel the empirical PSI excitation spectra. The fact that the *Fc2/Fc1* values in leaves from C_4 species were lower than in chloroplasts isolated from these C_4 species (Fig. 4, A and B) might be related to the different temperature and ion concentrations in the leaf and in chloroplast suspensions. These factors can affect the PSII-to-PSI energy transfer and the PSII absorption properties (Barber, 1982; Weis, 1984; Ruban et al., 1995).

In the group of C_4-like and C_4 species the *Fc2/Fc1* values from *F. bidentis* and *F. brownii* did not differ from the values of the intermediates; *F. australasica, F. palmeri,* and *F. trinervia* exhibited clearly increased values of *Fc2/Fc1*. The latter three species also showed decreased PSII/PSI ratios. Therefore, the high PSII contribution to the functional PSI antenna must be explained by increased efficiency of PSII-to-PSI energy transfer.

To calculate Φ_T, we required a parameter related to the PSII/PSI ratios (see "Materials and Methods"). Because emission ratios are sensitive to varying rates of PSII-to-PSI energy transfer, we chose ratios derived from Chl *a*/Chl *b* ratios, as described in "Materials and Methods" (Fig. 4D), for the estimation of Φ_T. We demonstrated above that the individual Chl *a*/Chl *b* ratios are influenced by varying chlorophyll stoichiometries of PSII. Therefore, the Γ-dependent course of PSII/PSI ratios was approximated by fitting an asymptotic curve to all data points. Values of the fitted curve at the Γ of the individual plants were used for the calculation of the relative Φ_T. The results show that in plants with $\Gamma > 10$ μL L^{-1} and in *F. brownii*, the relative Φ_T varied insignificantly, and that the Φ_T in *F. bidentis* was only slightly higher than the value in *F. brownii* (Fig. 4F). In *F. australasica, F. palmeri,* and *F. trinervia,* however, the relative Φ_T was up to three times higher compared with the intermediate and C_3 species.

The relative Φ_T in bundle-sheath chloroplasts from *Flaveria* C_4 species was higher than in mesophyll chloroplasts (Fig. 4A); the difference between the chloroplast fractions was significant as examined by the paired *t* test ($P = 0.0002$). A similar behavior was also observed for the *Fc2/Fc1* (Fig. 4, A and B) and the PSI excitation ratios (Fig. 1, G and H), in which significant differences between chloroplast fractions also existed ($P = 0.027$ and 0.001, respectively). The difference in PSII excitation ratios between pure mesophyll and pure bundle-sheath chloroplasts was not significant ($P = 0.204$; Fig. 1E). Consequently, the high PSII

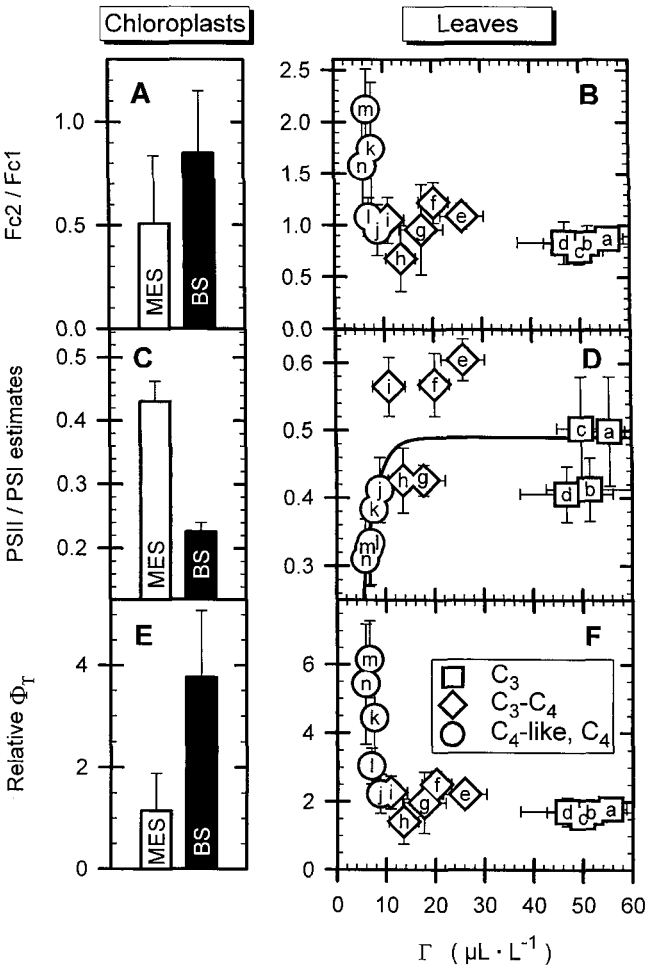

Figure 4. PSII-to-PSI energy transfer and PSII/PSI ratio estimates of chloroplasts from C_4 species of *Flaveria*, and of leaves from various *Flaveria* species. A, C, and E, and B, D, and F show data from pure chloroplast fractions from one C_4-like and three C_4 *Flaveria* species, and of leaves from various *Flaveria* species and hybrids, respectively. All leaf data are plotted against the respective Γ values. The identification of chloroplast fractions and of individual species and the statistical analysis were as described for Figure 1. The ordinate in A and B was derived from the deconvolution of empirical PSI excitation spectra as shown in Figure 3. C and D depict ratios of photosystems assessed from Chl *a*/Chl *b* values, as described in "Materials and Methods." The line in D resulted from fitting an asymptotic curve of the form $y = a + b \times r^{x}$ to all data points. Relative numbers for the yield of PSII-to-PSI energy transfer (E and F) correspond to values of *Fc2/Fc1* divided by PSII/PSI ratios (see "Materials and Methods"), where the PSII/PSI ratios were taken from the values of the asymptotic curve in D at the Γ of the individual species.

contribution to the functional antenna of PSI in leaves from *F. australasica, F. palmeri,* and *F. trinervia* may be related to the bundle-sheath chloroplasts in these species.

In summary, the evolution from C_3 plants to C_3-C_4-intermediate forms does not involve significant changes in the PSII/PSI ratio and in the light-harvesting properties of PSI. Such changes are confined to bundle-sheath chloroplasts from C_4-like and C_4 species. Efficient CO_2 fixation through the C_4 cycle has been shown for all the C_4-like and

C_4 species investigated here (Cheng et al., 1989; Moore et al., 1989). However, a different PSII/PSI ratio was not prominent in *F. brownii* (Figs. 1 and 2), and altered PSI light harvesting was not obvious in *F. brownii* and *F. bidentis* (Fig. 4)

The heterogeneity of the PSII/PSI ratio among the C_4-like and C_4 species can be related to the heterogeneous chloroplast ultrastructure in these plants. The bundle-sheath chloroplasts from *F. brownii* exhibit distinct grana stacks (Holaday et al., 1984). Grana formation can be related to the presence of PSII because the stacking is likely mediated by the major antenna complex of PSII (Barber, 1982). Granal bundle-sheath chloroplasts are also present in C_3-C_4-intermediate *Flaveria* species (Holaday et al., 1984) and are normal in C_3 species. Therefore, the C_3-like PSII/PSI ratios in *F. brownii* can be explained by the C_3-like ultrastructure of its bundle-sheath chloroplasts. By the same argument, one would expect agranal bundle-sheath chloroplasts in those species with elevated PSII/PSI ratios. Indeed, agranal bundle-sheath chloroplasts were reported for *F. bidentis*, *F. palmeri*, and *F. trinervia* (Keefe and Mets, 1983; Höfer et al., 1992; Torres-Ruiz et al., 1992). Agranal bundle-sheath chloroplasts probably also exist in *F. australasica* because the species is very closely related to *F. trinervia* (Kopriva et al., 1996).

Also, the extent of PSII-to-PSI energy transfer can be related to the chloroplast ultrastructure. In granal chloroplasts, PSII is concentrated in the grana stacks and is therefore physically separated from the PSI in the stroma lamellae (Anderson, 1986). A more homogeneous distribution of PSI and PSII is likely in agranal chloroplasts. Because the yield of PSII-to-PSI energy transfer depends on the distance between the two photosystems (Staehelin and Arntzen, 1983; Trissl and Wilhelm, 1993), it is apparent why our relative Φ_T is C_3-like in *F. brownii* and significantly increased in *F. australasica*, *F. palmeri*, and *F. trinervia* (Fig. 4F). In *F. bidentis*, however, the Φ_T and the *Fc2/Fc1* quotient were similar to those of *F. brownii*. The low Φ_T in leaves of *F. bidentis* agrees with the low Φ_T in bundle-sheath chloroplasts from *F. bidentis* compared with bundle-sheath chloroplasts from other C_4 *Flaveria* species (Pfündel et al., 1996). At present, we cannot explain why the PSII-to-PSI energy transfer of *F. bidentis* is similar to that of *F. brownii*.

Although in *F. bidentis* and *F. brownii* the reasons for the C_3-like PSII-to-PSI energy transfer may differ, the two species share photosynthetic characteristics that distinguish them from the other three species with efficient energy transfer between the photosystems. The CO_2 assimilation normalized to chlorophyll was decreased by one-third in *F. bidentis* and *F. brownii* compared with *F. australasica*, *F. palmeri*, and *F. trinervia* (Ku et al., 1991). Moreover, *F. bidentis* and *F. brownii* accumulated about twice as much inorganic carbon in the light as *F. australasica*, *F. palmeri*, and *F. trinervia*, and it was concluded that carbon refixation in the bundle-sheath compartment is less efficient in *F. bidentis* and *F. brownii* than in the other C_4-like and C_4 species (Moore et al., 1987).

In conclusion, we suggest that high PSII-to-PSI energy transfer increases the light-harvesting capacity of PSI to meet the specific requirements of NADP-ME C_4 photosynthesis in the bundle-sheath compartment, and that suboptimal light harvesting is a major factor for the reduced efficiencies of carbon reassimilation in *F. bidentis* and *F. brownii*. Compared with pure PSI, the functional association between PSII and PSI can narrow the absorption gap in the green spectral region because of the high amounts of Chl *b* in the PSII complex, and because the two major absorbance peaks of Chl *b* in the visible region are shifted toward the green spectral range compared with Chl *a*. We assume that high PSII-to-PSI energy transfer is of general importance for NADP-ME photosynthesis, since bundle-sheath chloroplasts from various other NADP-ME species also exhibit high PSII-to-PSI energy transfer (Pfündel et al., 1996).

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