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CHLOROPHYLL COMPOSITION OF PHOTOSYSTEMS II_α, II_β AND I IN TOBACCO CHLOROPLASTS

A.P.G.M. THIELEN, H.J. VAN GORKOM and C.P. RIJGERSBERG

Department of Biophysics, Huygens Laboratory of the State University, P.O. Box 9504,
2300 RA Leiden (The Netherlands)

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Summary

The antenna composition of the Photosystems II_α, II_β and I was studied in tobacco chloroplasts. Absorbance spectra, recorded at 4 K, were analyzed for the wild type and the mutants Su/su and Su/su var. Aurea, containing higher concentrations of the photosystems. With chloroplasts of Su/su we measured the action spectra of the three photosystems from 625 to 690 nm. Above 675 nm absorption by Photosystem I dominated. This sytem had a maximum at 678 nm and a shoulder at 660 nm. Of the long-wavelength chlorophyll *a* forms, absorbing at 690, 697 and 705 nm at 4 K, which are generally assigned to Photosystem I, the 697 nm form occurred in an amount of four molecules per reaction center of Photosystem I in each type of chloroplast. The Photosystem II_α spectrum was characterized by maxima at 650 and 672 nm, showing clearly the participation of the chlorophyll *a* and *b* containing light-harvesting complex. In the mutants the light-harvesting complex has a chlorophyll *a* to chlorophyll *b* ratio of more than 1; the amount of the 672 nm chlorophyll *a* was normal, whereas the amount of chlorophyll *b* was markedly decreased in the mutants relative to the wild type. The Photosystem II_β spectrum mainly consisted of a band at 683 nm.

Introduction

In the preceding paper [1] we showed the existence of three efficiently functioning photosystems, PS II_α, PS II_β and PS I, in chloroplasts of tobacco. The best documented differences between the two Photosystems II are kinetic

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Abbreviations: PS, Photosystem; Chl, chlorophyll; P-700, primary donor of Photosystem I; Q, primary acceptor of Photosystem II; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

differences [2,3] and a difference in the redox potential of their primary acceptors, Q_α and Q_β [4—6]. We have shown that the difference in the rate constants of photoconversion of PS II_α and PS II_β is due to the sizes of their respective antennae [1]. In an earlier article [7] it was suggested that Chl *b* is specifically found in PS II_α. This hypothesis was based on the observation that in chloroplasts of tobacco mutants with increasing Chl *a*/*b* ratios the amount of PS II_β increases. A small difference has also been suggested between the excitation spectra of the variable fluorescence associated with the high potential and the low potential acceptor [4].

In the present study we investigated the chlorophyll composition of the two Photosystems II and Photosystem I. We used chloroplasts of tobacco and its mutants Su/su and Su/su var. Aurea, in which the ratio of PS II_α to PS II_β is decreased. After correction for the flattening effect we analyzed the absorbance spectra of the three types of chloroplasts, recorded at 4 K, into 10 bands of Chl *a* and *b* in the region from 640 to 750 nm. With chloroplasts of the Su/su mutant we made action spectra of all three photosystems, accounting for all chlorophyll absorption between 625 and 690 nm. This mutant was chosen because in Su/su Aurea the difference in antenna size between PS II_α and PS II_β is less and in the wild type the absorbance of a single chloroplast is too high to allow an acceptably homogeneous illumination with red light. Based on the action spectra and known differences between the wild type and the mutants, an attempt was made to localize the different forms of Chl *a* and *b* in the three photosystems.

Materials and Methods

Chloroplasts of tobacco, cv John William's Broad Leaf, the wild type, and the mutants Su/su and Su/su var. Aurea [8] were isolated as described in Ref. 2 and resuspended in the isolation medium, 0.4 M sucrose, 50 mM *N*-tris-(hydroxymethyl)methylglycine, 10 mM KCl and 5 mM $MgCl_2$, pH 7.8. The Chl concentrations and the Chl *a*/*b* ratios were determined according to Arnon [9]. For the measurement of the absorption spectra at 4 K we used chloroplasts of wild type tobacco with a Chl *a*/*b* ratio of 2.9, chloroplasts of the green-yellow mutant Su/su with a ratio of 4.5 and chloroplasts of the yellow mutant Su/su var. Aurea with a Chl *a*/*b* ratio of 7.5. The Su/su chloroplasts used in the measurement of the action spectra had a Chl *a*/*b* ratio of about 4, and contained 1 P-700 per 425 and 1 Q per 140 Chl molecules, determined as in the foregoing article [1]; the ratio of PS II_α to PS II_β was 0.70.

Absorbance spectra of the chloroplasts were measured at 4 K with the single-beam spectrophotometer described in [10]. Light-induced fluorescence rise and P-700 oxidation in chloroplasts of Su/su were both measured with the same apparatus [11], operated either as a fluorometer or as a split-beam absorbance difference spectrophotometer; the optical pathlength of the sample was 1.2 mm. The wavelength of actinic illumination was selected with Balzers interference filters with peak positions at 630, 638, 648, 658, 669, 678 and 686 nm, respectively. The maximum transmission was 40% and the halfwidth 10 nm. The PS I kinetics were measured at —57°C [7]. To avoid disturbance by the 686 nm actinic light, we followed the oxidation of P-700 at 707 nm. The sample contained 80 μM chlorophyll and 60% (v/v) glycerol. After averaging ten sweeps the kinetics were analyzed in a semilogarithmic plot to yield the rate constant of PS I at the intensity and wavelength of illumination used. For the fluorescence measurements 10 μM DCMU and 2 mM hydroxylamine were added to samples containing 50 μM of chlorophyll. The fluorescence was measured at 685 nm and excited with weak continuous light at 600 nm, where both PS II_α and PS II_β absorb [1,7]. The excitation light induced about 5% of the variable fluorescence in 20 s. The actinic illumination was given in the form of weak xenon flashes (1 μF, 2300 V) spaced at 0.19 s. The photomultiplier tube was shut off for 30 ms when a flash was given. The maximal fluorescence was reached in 10—15 s. The kinetics of the fluorescence rise were averaged 5-times and analyzed as described in Ref. 7 to obtain the rate constants of the primary reactions of PS II_α and PS II_β.

The amount of energy absorbed by the chloroplasts in the measurement of the PS I kinetics was measured with a Siemens BPX 65 photodiode. In the calculations we used the light-sensitive surface area and the quantum yield as specified. To measure the energy absorbed from the flashes in the experiments where the fluorescence induction was measured, we used the same photodiode connected to an integrator circuit. The sensitivity of this device was calibrated against a Laser Instrumentation energy meter. The resulting data were corrected for light losses at the cuvette walls.

Results

Upon lowering the temperature absorption bands become narrower and the maxima become higher. Due to these effects a number of absorption bands can be distinguished in the red region of low temperature spectra of chloroplasts. These bands reflect the existence of chlorophyll molecules in different environments. It seems reasonable to assume that in the three types of tobacco chloroplasts basically the same species of Chl *a* and *b* occur, but in varying amounts. Deconvolution of the absorption spectra would then show the same bands with different amplitude. However, absorption spectra of chloroplasts are distorted by scattering losses and by the flattening effect. The latter is strongest at wavelengths where absorption is highest. The proportionality constant k, relating the differential flattening factor to the absorbance at room temperature for each type of chloroplast, was determined as described in Ref. 1. We assumed that at 630 nm, where the absorption does not change much when the temperature is lowered, the flattening effect is equal at room temperature and at 4 K and that k is independent of temperature. Using the equations given by Amesz [12] we obtained the absorption spectra corrected for flattening of Fig. 1. Since we were mainly interested in differences in chlorophyll composition between the mutants and the wild type, we only corrected for scattering by subtracting the absorbance measured at 750 nm; scattering losses were small and, as shown in Ref. 1, the scattering spectra of the different types of chloroplasts are rather similar in this region.

After these corrections we analyzed the spectra. Approximate positions of the composing bands were obtained from difference spectra of mutant minus wild type. These values agreed well with results from other laboratories [13—

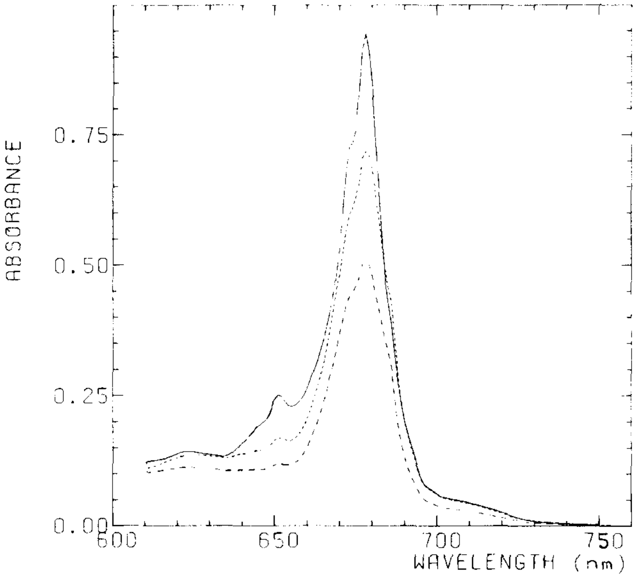

Fig. 1. Absorbance spectra of chloroplasts of wild type tobacco (———) and the mutants Su/su (· · · · · ·) and Su/su var. Aurea (- - - - - -), recorded at 4 K. The chlorophyll concentrations were different. The spectra have been corrected for flattening. Optical pathlength 1 mm, bandwidth 1.6 nm.

15]. By computer an optimal fit was determined with the same set of Gaussian-shaped bands for all three spectra of Fig. 1; no big differences in peak positions and halfwidths of the composing bands were found to occur in the three spectra. Between 620 and 750 nm at least 10 bands were required: a satellite band at 623 nm, Chl *b* bands at 644 and 651.5 nm, and Chl *a* bands at 664, 672.5, 678, 684, 690, 697 and 705 nm. In this solution the halfwidth of the band at 664 nm was almost twice the width of the neighbouring bands. A somewhat better fit was obtained with two bands, one at 660 nm and another one at 667.5 nm. Probably because they measured at 77 K, this was not seen by other investigators. Between 640 and 645 nm two bands may be present (see Refs. 14 and 16). The whole can be fitted satisfactorily, however, with only the main band at 644 nm. The results of the analysis are presented in Table I; the satellite band at 623 nm was omitted. The area under each band was calculated as percentage of the area of all bands together. In this way the contribution of each band to the spectrum of wild type and mutant chloroplasts could be compared. The Chl *a* forms at 667.5, 672.5, 684 and 697 nm were enriched in the mutants, whereas Chl *b* and the Chl *a* forms at 660 and 678 nm were present in smaller amounts than in the wild type. The amounts of 690 and 705 nm Chl *a* were increased in the Su/su mutant and about normal, as compared to the wild type, in Su/su var. Aurea. It is noteworthy that the smaller antennae of the photosystems in the mutants [1] are not characterized by the complete absence of one or more of the Chl species present in the wild type.

The shape of the fluorescence induction curve at room temperature depended on the wavelength of actinic illumination (Fig. 2). At all wavelengths

TABLE I

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The spectra of Fig. 1 were fitted by Gaussian bands at the positions and with the halfwidths given in the first two columns. The contribution of each band to the absorbance spectra of chloroplasts of wild type tobacco and the mutants Su/su and Su/su var. Aurea is listed in the third column.

Position (nm)	Width (nm)	Area (% of total)		
		wild type	Su/su	Su/su var. Aurea
644	12	6.0	5.0	4.7
651.5	8	5.5	3.8	3.5
660	11	11.1	9.2	9.2
667.5	9	12.7	14.7	15.7
672.5	6.5	11.5	11.7	12.5
678	7	28.7	25.1	24.8
684	7	11.7	15.7	16.0
690	8	5.0	5.8	5.1
697	10	0.9	1.0	1.1
705	30	7.0	7.8	7.4

the area over the induction curves could be analyzed [7] into the areas and rate constants of two kinetic phases; due to its sigmoidal shape the fast phase undoubtedly reflected the turnover of PS II_α. The initial rate constant of the sigmoidal phase (k_α) and the rate constant of the exponential phase (k_β) are proportional to the light intensity absorbed by PS II_α and PS II_β, respectively. In Fig. 3A k_α and k_β are plotted against wavelength together with the total absorbed light intensity. The ratio of k_α to k_β in Fig. 3B shows that the absorp-

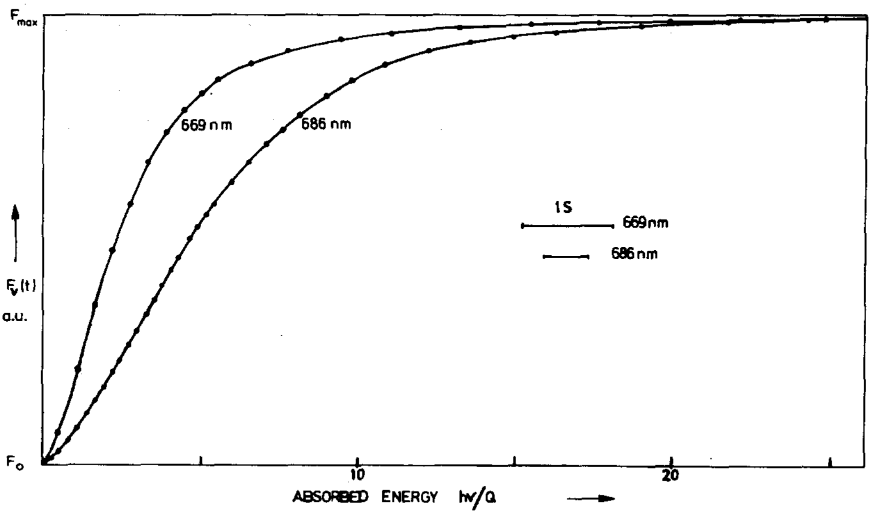

Fig. 2. Induction of fluorescence rise in chloroplasts of Su/su by flashes (1 μF, 2300 V) filtered through B-40 interference filters with maximum transmittance at 669 and 686 nm, respectively. Fluorescence was excited at 600 nm. The measured points indicate the fluorescence yield obtained after each flash; between flashes the fluorescence was constant. The smooth lines drawn through the points were used for the analysis. To facilitate comparison, the two induction curves are plotted versus absorbed quanta rather than versus time.

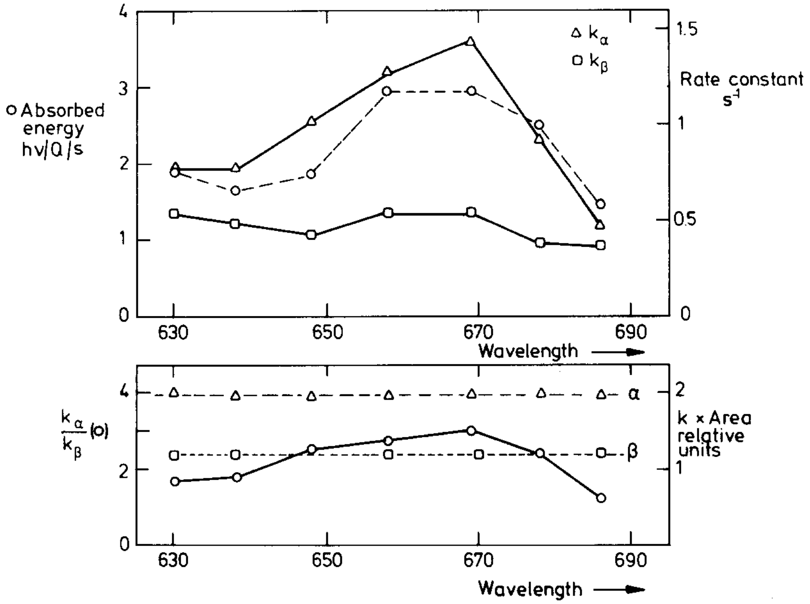

Fig. 3. Energy absorbed by chloroplasts of Su/su at the different wavelengths of the actinic flashes, expressed as number of photons per reaction center of PS II, and the rate constants of the α- and β-phases of the area growth over the fluorescence induction curve. The ratio of the rate constants of α and β is plotted in (B) together with k_α X Area$_\alpha$ and k_β X Area$_\beta$ (see text).

tions by PS II$_\alpha$ and PS II$_\beta$ have different wavelength dependencies. At all wavelengths complete reduction of Q_α and Q_β was accomplished, because the constant values of k_α times Area$_\alpha$ and k_β times Area$_\beta$, which follow from the analysis of the fluorescence induction curves, indicate that the contributions of PS II$_\alpha$ and PS II$_\beta$ to the variable fluorescence excited at 600 nm did not depend on the wavelength of the actinic flashes (Fig. 3B). One should bear in mind that, because in these experiments fluorescence exciting light and the

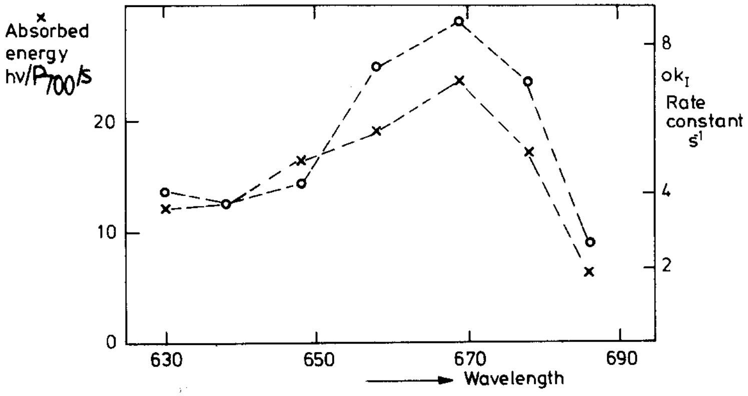

Fig. 4. Energy absorbed by chloroplasts of Su/su from the continuous light filtered through B-40 interference filters and rate constants of P-700 oxidation at the different wavelengths.

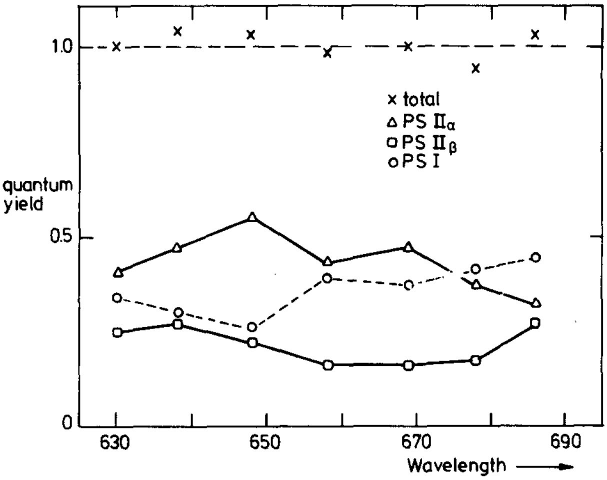

Fig. 5. Quantum yields of the primary reactions of PS I, PS II$_\alpha$ and PS II$_\beta$ at different wavelengths of the actinic light in chloroplasts of Su/su. Together they add up to an overall quantum yield of about 1.

actinic illumination were not of the same wavelength, the ratio of PS II$_\alpha$ to PS II$_\beta$ is not simply given by the ratio of the areas of the two phases in the induction curves.

For the measurement of the PS I kinetics we chose the intensity of illumination such that oxidation of P-700 was completed in less than 2 s [7]. At all actinic wavelengths PS I showed monophasic exponential kinetics. The rate constant, k_{I}, and the absorbed light intensity are plotted against wavelength in Fig. 4.

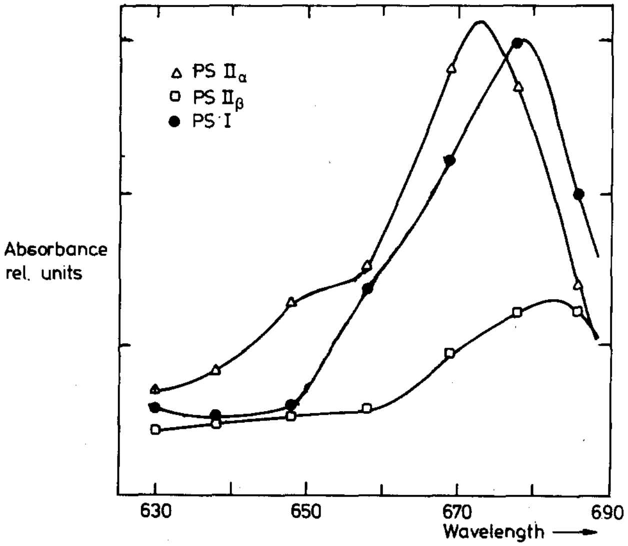

Fig. 6. The contributions of the three photosystems to the scattering-free absorbance spectrum of Su/su chloroplasts, calculated from the quantum yields at the wavelengths examined. In these chloroplasts PS I, PS II$_\alpha$ and PS II$_\beta$ were present in the ratio 1 : 1.1 : 1.6.

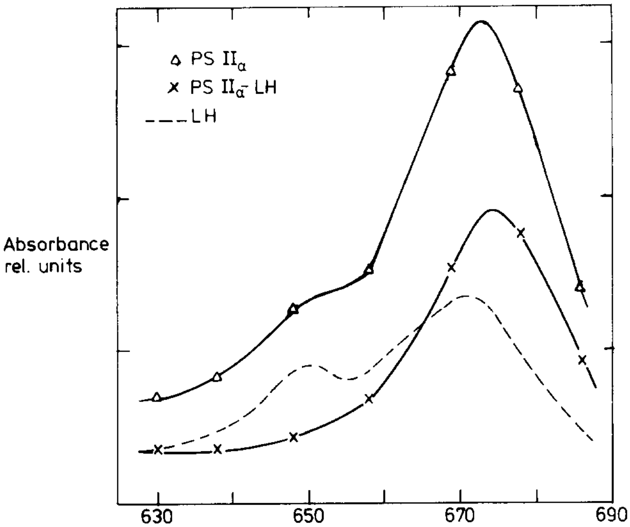

Fig. 7. Difference between the action spectrum of PS II_α (Fig. 6) and the spectrum of a light-harvesting Chl *a*/*b* complex (LH; see Ref. 25), normalized to account for all Chl *b* present in chloroplasts of Su/su. The difference spectrum still has maximal absorbance around 672 nm, and shows that essentially all Chl *b* absorbance can be accounted for by the PS II_α spectrum.

The quantum yields of PS I, PS II_α and PS II_β, calculated as described in Ref. 1, are shown in Fig. 5. In the three experiments done the partial quantum yields added up to one, with a variation of less than ±0.05 at all wavelengths. As discussed earlier [1], this is higher than expected. Besides the factors that occurred in this reference, here an additional small deviation was caused by the actinic effect of the fluorescence exciting beam. As a consequence of the high quantum efficiency of the three photosystems, the partial quantum yields at each wavelength represent the fractions of the total absorbed energy going into each of the photosystems at that wavelength. According to the partial quantum yields of Fig. 5, the contributions of the three photosystems to the scattering-free, room temperature, absorbance spectrum of Su/su chloroplasts [1] were calculated (Fig. 6).

Discussion

In the preceding article [1] a quantitative analysis is given of the photosystems present in chloroplasts of wild type and mutant tobacco, and their antenna sizes. On a chlorophyll basis the amounts of PS I and PS II_α reaction centers were somewhat higher in the mutants, whereas that of PS II_β was increased several fold. The increased amounts of reaction centers and the (partial) disappearance of chlorophyll-protein complexes from the antennae should be reflected in the chlorophyll compositions of the mutants compared to that of the wild type. In this study we analyzed the absorbance spectra of the three types of chloroplasts, recorded at 4 K and corrected for flattening, and we measured the excitation spectra of the three photosystems in chloroplasts of Su/su. With the information available we tried to determine the antenna composition of the three photosystems.

According to its spectrum in Fig. 6, PS I is not excited by quanta absorbed by Chl *b* under our experimental conditions. The maximal absorbance of this photosystem is at the position of the highest band in the low temperature absorbance spectra. Besides the 678 nm Chl *a* also the 660 nm form is found in PS I. Both forms of chlorophyll are present in smaller amounts in the mutants than in the wild type (Fig. 2). Unfortunately we could not measure the excitation spectra above 690 nm, because of too high disturbance of the P-700 measurement by the actinic light, and because too little energy was absorbed from the flashes to record reliable fluorescence rise kinetics. However, it is generally believed that the long-wavelength forms of Chl *a* at 690, 697 and 705 nm belong to the antenna of PS I [17—19]. The 690 and 705 nm forms occur in a somewhat increased amount in Su/su and may be concluded to be as abundant in the PS I antennae of Su/su as in those of the wild type. In the Aurea variety their relative amounts are diminished. The antenna size of PS I in this mutant (about 100 Chl per reaction center [1]) coincides well with the state of purification of PS I particles where the 705 nm form of Chl *a* starts to disappear [19]. The smaller amount of 705 nm Chl is probably the cause of the lower F_{735} band in the spectrum of the Aurea mutant [20,19]. The small band at 697 nm is interesting because it contained about four molecules of Chl per reaction center of PS I in each type of chloroplast (Fig. 2). Its position is close to the maximal absorbance change due to P-700 oxidation in small PS I particles [21]. Recently it was shown by Shuvalov et al. [22] that the first electron acceptor in PS I is probably a dimer of Chl *a*, having maximal absorption decrease upon reduction at about 700 nm. The four molecules in the 697 nm band possibly correspond to the two dimers of Chl *a* that act as primary donor (P-700) and intermediary acceptor.

Our action spectra indicate that absorption in both bands of Chl *b* (at room temperature they do not appear as separate bands [23]) and in the Chl *a* band at 672 nm is associated with PS II_α. Although Chl *b* is present in smaller quantities in the mutants than in the wild type, the amount of 672 nm Chl *a*, which is normally found to be coupled to Chl *b* in the light-harvesting Chl *a*/*b* protein [24,25], is not diminished. After subtraction of the spectrum of the isolated light-harvesting complex [25], normalized to account for all Chl *b* present in chloroplasts of Su/su, the maximal absorbance by PS II_α in Su/su is still found around 672 nm (see Fig. 7). It is obvious that there must be a second type of 672 nm Chl *a* which is not, or in a different stoichimetric ratio, associated with Chl *b*. From the spectra no evidence can be obtained for two spectrally different forms of 672 nm Chl *a*, although a small difference may exist in the bandwidth. Using the in vitro extinction coefficients of Chl *a* and Chl *b* we concluded that in the wild type the concentration of 672 nm Chl *a* does not exceed the Chl *b* concentration. Since it seems unlikely that in PS II_α of the mutants a completely new chlorophyll-protein complex occurs, the simplest explanation would be that in these mutants the light-harvesting complex is present, but is deficient in Chl *b*. The presence of this light-harvesting complex with a higher Chl *a*/*b* ratio may then be the cause of the broadened F_{680} band in emission spectra of chloroplasts of mutant tobacco with little Chl *b* [20].

PS II_β in chloroplasts of Su/su mainly consists of Chl *a* absorbing at 684 nm (Fig. 6). Part of this form of Chl *a*, however, may be found in PS II_α, since this photosystem still has considerable absorption above 680 nm (Fig. 7) and the cores of both Photosystems II may be basically the same. On the basis of our excitation spectra the 667.5 nm Chl *a* cannot be assigned to any of the three photosystems in particular. To make the chlorophyll antennae of PS I and PS II_α of comparable size [1] one would have to assume a function for this form of Chl *a* in the antenna of PS II_α.

The assignment of the different species of chlorophyll to PS I, PS II_α or PS II_β in chloroplasts of tobacco is mainly based on the excitation spectra that we measured for the three photosystems. In spite of the low spectral resolution and the possible uncertainty in the partial quantum yields, our results show that there are significant differences in chlorophyll composition between the photosystems. The specta measured for PS I and PS II_α are roughly in agreement with earlier data on PS I and PS II [18,26,27]. In chloroplasts of Su/su we found that Chl *b* and the 672 nm Chl *a* are primarily associated with PS II_α. The third photosystem, PS II_β, was found to consist mainly of the 684 nm form of Chl *a*. Less pronounced but qualitatively similar differences between PS II_α and PS II_β may occur in all higher plant chloroplasts.

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