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### SPECTROFLUOROMETRIC CHARACTERIZATION *IN SITU* OF THE PROTOCHLOROPHYLL SPECIES IN ETIOLATED TISSUES OF HIGHER PLANTS<sup>1</sup>

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#### ABSTRACT

The fluorescence emission and excitation properties of protochlorophyll in etiolated cucumber (*Cucumis sativus* L.) cotyledons and primary bean (var. Red Kidney) leaves were characterized at 77 K. Contrary to previous studies, it appears that the short-wavelength protochlorophyll emission band consists of four fluorescent components, instead of only one nonphototransformable protochlorophyll. It was demonstrated that etiolated cucumber cotyledons synthesize and accumulate nontransformable protochlorophyll ( $E_{440}$ ,  $F_{630}$ ) as well as short-wavelength phototransformable protochlorophyll ( $E_{433}$ ,  $F_{633}$ ), ( $E_{444}$ ,  $F_{636}$ ), and ( $E_{445}$ ,  $F_{640}$ ). Long-wavelength phototransformable protochlorophyll ( $E_{450}$ ,  $F_{657}$ ) is also formed. In this context, *E* refers to the Soret excitation maxima and *F* refers to the red emission maxima of the protochlorophylls.

In etiolated bean leaves, the corresponding species were: nontransformable protochlorophyll ( $E_{440}$ ,  $F_{630}$ ), short-wavelength phototransformable protochlorophylls ( $E_{441}$ ,  $F_{633}$ ), ( $E_{442}$ ,  $F_{636}$ ), and ( $E_{443}$ ,  $F_{640}$ ), and long-wavelength phototransformable protochlorophyll ( $E_{447}$ ,  $F_{657}$ ).

It is presently acknowledged that mature etiolated tissues contain two SW<sup>3</sup> and one LW Pchl species. One of the SW species is nonphototransformable (nt) and exhibits a red excitation/absorption maximum at 628 nm and a corresponding 77 K fluorescence emission maximum at 630 to 632 nm (10). The other SW species is phototransformable (t) and exhibits a red excitation/absorption maximum at 637 nm. However, it is not considered to be highly fluorescent as it transfers most of its excitation energy to the LW Pchl species (10). Thus, the SW emission band of Pchl *in vivo* has been conventionally attributed solely to the fluorescence of nt-Pchl. On the other hand, the LW Pchl is both phototransformable and highly fluorescent and makes up the bulk of the Pchl pool in mature etiolated tissues (7, 10, 11, 16). It exhibits a red excitation/absorption maximum at 650 nm and a corresponding fluorescence emission maximum at 655 to 658 nm (9, 10).

The multiplicity of Pchl spectral forms *in situ* has been conven-

tionally attributed to the differential association of two Pchl chromophores, *i.e.* MV Pchl<sup>4</sup> and MV Pchl ester, with their lipoprotein macromolecular environment. It has recently been demonstrated, however, that, contrary to previous beliefs, the Pchl pool of etiolated plants consisted of eight different chromophores instead of the two conventional MV Pchl *a* and Pchl ester *a* components (3, 4). Four of the Pchl chromophores were identified as MV and DV Pchl *a* and MV and DV Pchl ester *a* (3, 4). The identity of the other four Pchl-like chromophores has not yet been determined, but it was proposed that two of them may represent Pchl *b* and Pchl ester *b* (3). It was subsequently shown that the MV and DV Pchl *a* and MV and DV Pchl ester *a* were convertible into MV and DV Chl(ide) *a* and MV and DV Chl *a* by a very brief light treatment (2, 5). It now appears that the multiple Chl *a* chromophoric species detectable in mature green tissues (14) originate from the MV and DV Pchl *a* and Chl(ide) *a* precursors (2, 5).

With the discovery of the aforementioned Pchl chromophoric multiplicity, it was reasonable to wonder whether this Pchl chromophoric heterogeneity was also accompanied by a concomitant multiplicity of Pchl spectral forms *in situ*. It was conjectured that a finely tuned spectrofluorometric examination of the Pchl pool of etiolated tissues *in situ* might reveal some additional Pchl spectral species that were previously overlooked. The data reported here suggest that two additional SW Pchl spectral species are detectable in etiolated tissues *in situ*.

#### MATERIALS AND METHODS

**Plant Material and Growth Conditions.** Cucumber seeds (*Cucumis sativus* L. cv. Beit Alpha MR) were purchased from the Niagara Chemical Division, FMC Corporation, Modesto, CA. Beans (*Phaseolus vulgaris* L. var. Red Kidney) were purchased from Maxwell Seed House, Champaign, IL. Seeds were germinated at 28 C in moist vermiculite in total darkness as described earlier (6), except that distilled H<sub>2</sub>O was used instead of Hoagland nutrient solution.

**Harvesting.** Etiolated cotyledons and primary bean leaves were harvested under a dim green safelight which did not phototransform Pchl to Chl(ide) *a*.

**Spectrofluorometry.** Corrected fluorescence emission and excitation spectra of Pchl in homogenates and tissue slices were recorded at 77 K in 67% glycerol with a Perkin-Elmer spectroflu-

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<sup>3</sup> Abbreviations: SW Pchl, membrane-bound, short-wavelength protochlorophyll having a broad emission maximum *in vivo* or *in situ* between 629 and 643 nm at 77 K; LW Pchl, membrane-bound, long-wavelength Pchl having an emission maximum *in vivo* or *in situ* at 657 nm at 77 K; t-Pchl, phototransformable Pchl; nt-Pchl, nonphototransformable Pchl; MV, monovinyl; DV, divinyl.

<sup>4</sup> Pchl<sup>4</sup> and Pchl ester are used here generically to designate Pchls which may differ from one another by the nature of their side-chain substituents at positions 2 and 4 of the macrocycle. MV Pchl is defined as that Pchl with a vinyl group at position 2 (or 4) of the macrocycle and an ethyl group at position 4 (or 2) of the macrocycle. DV Pchl is defined as that Pchl with vinyl groups at both positions 2 and 4 of the macrocycle.

rometer model MPF-3, equipped with a corrected spectra accessory. Emission spectra were recorded at an excitation slit width of 6 nm and an emission slit width of 3 nm; excitation spectra were recorded at an excitation slit width of 3 nm and an emission slit width of 6 nm. Spectra were recorded at the lowest scale of attenuation possible. At these settings, a resolution of  $\pm 1$  nm was achieved as evidenced by the recorded 77 K emission and excitation spectra of standard Zn-coproporphyrin tetramethyl ester dissolved in ether.

Crude homogenates of 20- to 24-h-old etiolated or illuminated cotyledons were prepared as follows. One g tissue was gently ground (25 to 30 strokes) in 2.0 ml 0.5 M sucrose, 0.2 M Tris-HCl (pH 8.0) at 0 to 4 C. The suspension was filtered through one layer of nylon mesh, and 0.1 ml of the filtrate was mixed with 0.2 ml glycerol. The mixture was transferred to the lower sample section of a cylindrical glass tube and was immediately frozen by inserting the sample tube into a long stem Dewar filled with liquid N<sub>2</sub> (7).

Tissue slices of 2- and 5-day-old etiolated or illuminated cucumber cotyledons and primary bean leaves were 10 × 1 mm. They were transferred to the sample tube in the Tris/sucrose buffer-glycerol (1:2 v/v) and immediately frozen as described above.

To measure fluorescence emission spectra, the following combination of filters was used to minimize the interference of light scattered by the frozen sample: a blue filter, Pyrex No. 5543, that was transparent in the 350- to 500-nm region, was placed between the excitation monochromator and the sample; a red sharp cut-off filter, Turner No. 25, that excluded light below 600 nm was interposed between the sample and the emission monochromator. When excitation spectra were recorded, the blue filter was removed.

**Phototransformation of Pchl.** In 20- to 24-h-old etiolated cotyledons, Pchl was phototransformed by illuminating with 3.2 w/m<sup>2</sup> white fluorescent light for 15 s.

In 5-day-old etiolated cotyledons and bean leaves, the Pchl was phototransformed by illumination with 3.2 w/m<sup>2</sup> white fluorescent light for 60 s.

**Computer Analysis of Emission/Excitation Matrices.** Deconvolution of the emission/excitation matrices of the Pchl profiles *in vivo* was performed according to Weber (18). All computations were programmed either on a Control Data Corp. computer Cyber 175 or on a Hewlett-Packard 9825S microcomputer system.

## RESULTS

**Build-up of Pchl Heterogeneity during Etiolation.** The development of the complete Pchl profile during etiolation has been described as an age-dependent, stepwise process (1, 11). SW Pchl is formed first; as tissue development proceeds in darkness, LW Pchl accumulates faster than the SW Pchl and becomes the predominant species. This process is manifested by a shift in the *in vivo* absorption maximum of the Pchl from 636 nm in juvenile etiolated tissues to 650 nm in older etiolated tissues (1, 11). A study of the 77 K fluorescence characteristics of Pchl revealed a parallel shift in the main emission maximum from about 633 to 657 nm in cucumber cotyledons grown in complete darkness (7). Data obtained during this latter investigation suggested that the SW Pchl emission band in aging etiolated tissues was complex, possibly containing some previously uncharacterized Pchl component(s). Evidence for this hypothesis is presented below.

The build-up of complexity in the SW Pchl emission band of etiolated cucumber cotyledons as a function of seedling age is depicted in Figure 1. Since the level of Pchl was low during the first 24 h (6), spectra at this developmental stage were recorded using concentrated homogenates prepared in Tris-sucrose buffer rather than tissue slices. In 2- and 5-day-old cotyledons, spectra were recorded using single cotyledonary slices. This had no influence on the results, however, since it was shown earlier that the

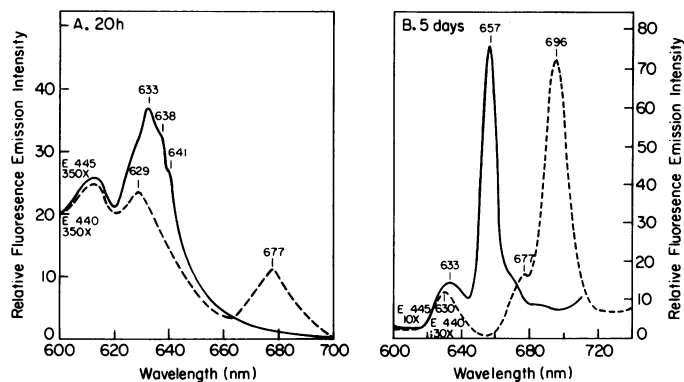


FIG. 1. Fluorescence emission spectra at 77 K of the Pchl in etiolated cucumber cotyledons, before (—) and after (---) phototransformation. A, homogenate of 20-h-old cotyledons; B, slices of a single 5-day-old cotyledon. Pchl was phototransformed with 3.2 w/m<sup>2</sup> white fluorescent light. Twenty-h-old cotyledons were illuminated for 15 s, whereas 5-day-old cotyledons were illuminated for 60 s. Pre- and postillumination spectra were normalized at 600 nm; the ordinate scale attenuation is indicated on the spectra, × 350 being the least attenuated ordinate scale.

fluorescence emission spectra of Pchl in homogenates and slices of etiolated cucumber cotyledons were similar (7). Recent spectra of cotyledonary slices prepared from 20-h-old etiolated cotyledons and recorded on a very sensitive spectrofluorometer (SLM 8000 DS) were identical to spectra recorded on concentrated homogenates prepared from the same tissue (C. A. Rebeiz, unpublished). Homogenates of cotyledons grown in darkness for 20 h exhibited a well-defined and skewed Gaussian fluorescence emission band with a maximum at 632 to 633 nm and emission shoulders at about 636 to 638 nm and 640 to 642 nm (Fig. 1A). This suggested that this band was composed of more than one Pchl species. LW Pchl was not detectable at this stage of development.

Partial deconvolution of the SW band was achieved by phototransforming the Pchl. A light treatment of 15 s converted some of the Pchl to Chl(ide) *a*, as evidenced by the appearance of a new emission band with a maximum at about 677 nm (Fig. 1A). The remaining nt-Pchl exhibited an emission band with a maximum at 628 to 629 nm; the emission shoulders at about 638 and 641 nm disappeared (Fig. 1A). These results indicated that the SW Pchl band was made up of more than one component even at this early stage of etiolation: (a) a nt-Pchl species with an emission maximum at about 629 nm, and (b) at least one or two SW t-Pchl species, apparently emitting at a longer wavelength than the nt-Pchl component.

During subsequent growth of the cotyledons in darkness, the SW Pchl band broadened considerably and exhibited a bell-shaped emission maximum between 629 and 642 nm (Fig. 1B). By 5 days, the Pchl profile became dominated by the LW t-Pchl, having an emission maximum at 657 nm (Fig. 1B). The absolute amount of SW Pchls also increased significantly. Upon illumination, the SW Pchl band underwent a decrease in amplitude and a blue shift in its emission maximum. It became narrower and assumed a more symmetrical Gaussian configuration centered around the nt-Pchl emission maximum at 630 nm (Fig. 1B). This was consistent with the results shown in Figure 1A and the notion that the SW Pchl band is made up of one or more fluorescent, t-Pchl components in addition to nt-Pchl. Further analysis of the SW Pchl emission band was undertaken as described below.

**Heterogeneity of the SW Pchl Emission Band of Etiolated Cotyledons as Revealed by Differential Excitation.** The position of the emission maximum of a given substance excited with light of different wavelengths is considered to be a constant (18). The appearance of new emission maxima and shoulders as a function of excitation wavelength is usually attributed to the presence of a



could have given rise to the multiple emission and excitation maxima that were just described. This was achieved by using Weber's matrix technique (18). Weber (18) demonstrated that, for an emission/excitation matrix ( $M$ ), whose elements ( $M_{i,j}$ ) represented the fluorescence intensity measured at wavelength  $\lambda_j$ , for an excitation  $\lambda_i$ , the number of fluorescent components in the matrix was equal to the rank of the minors for which the following inequality was obeyed.

$$\frac{\Delta}{P} \geq 3 \frac{\delta F}{\bar{F}} \quad (1)$$

where  $\Delta$  is the value of the determinant for a minor of any particular rank,  $P$  is the value of the permanent [i.e. the sum of all products in the determinant (13) for the same minor],  $\delta F$  is the noise level or minimum detectable fluorescence, and  $\bar{F}$  is the mean fluorescence intensity of the  $M_{i,j}$  elements of the minor under consideration.

Such a treatment of fluorescent data is valid only for samples which fall within the linear response range of the measuring instrument. It should also be emphasized that, although this technique will detect all the fluorescent species in a mixture, when they have reasonably well-resolved excitation bands, it fails in cases of extreme excitation-band overlap (17). It also fails to differentiate between two fluorescent species if both species possess the same excitation/absorption spectrum (18).

With these limitations in mind, the excitation/emission profile of the cucumber Pchl was submitted to matrix analysis. To this effect, 10 emission spectra were recorded at 77 K on the same sample. The emission spectra were elicited by the following excitations: 430, 435, 440, 442, 445, 447, 450, 452, 455, and 460 nm. The fluorescence emission amplitudes at 621, 624, 630, 633, 636, 640, 652, 657, 662, and 670 nm then were determined from every emission spectrum. All fluorescence emission amplitudes were normalized to the same attenuation scale. The  $10 \times 10$  matrix of the cucumber Pchl profile depicted in Table I was next assembled from the normalized emission amplitudes. The determination of all the minors of a given rank which obeyed equation 1 was achieved by means of the computer. The latter was programmed to report any minor of a given rank  $n$  with

$$\frac{\Delta}{P} > 3 \frac{\delta F}{\bar{F}}.$$

Table I. Emission/Excitation Matrix of Pchl Pool of Etiolated Cucumber Cotyledons

The  $M_{i,j}$  elements represent the fluorescence intensity measured at wavelength  $F_\lambda$  for an excitation at wavelength  $E_\lambda$ . In this matrix, a column represents an emission spectrum, whereas a row represents an excitation spectrum. The matrix was assembled as described in the text. All fluorescence amplitudes were normalized to a  $\times 100$  ordinate scale attenuation and are measured in arbitrary units.

Emission Observation Wavelengths ( $F_\lambda$ nm)	Fluorescence Emission Amplitudes at Following Excitation Wavelengths ( $E_\lambda$ nm)									
	$E_{430}$	$E_{435}$	$E_{440}$	$E_{442}$	$E_{445}$	$E_{447}$	$E_{450}$	$E_{452}$	$E_{455}$	$E_{460}$
$F_{621}$	5	8	9	6	4	3	1	0	0	0
$F_{624}$	11	16	17	13	9	7	3	1	0	0
$F_{630}$	16	23	29	25	18	14	11	8	4	2
$F_{633}$	16	22	28	26	20	16	13	11	7	2
$F_{636}$	14	19	25	24	19	16	14	12	8	2
$F_{640}$	11	15	18	19	17	14	12	11	9	3
$F_{652}$	77	79	83	80	73	70	73	77	60	50
$F_{657}$	140	140	143	147	143	140	157	153	120	97
$F_{662}$	80	70	67	70	77	63	77	73	63	53
$F_{670}$	31	37	37	37	35	41	38	38	30	26

According to Minc (13), for a  $10 \times 10$  matrix, all minors of rank  $n$  are given by:

$$\left[ \frac{10!}{n! (10-n)!} \right]^2 \quad (2)$$

All possible 63504 minors of rank 5 of the cucumber Pchl *in vivo* had  $\Delta/P$  values that were below the  $3 (\delta F/\bar{F})$  threshold. However, several thousands of the 44,100 minors of rank 4 had  $\Delta/P$  values which were higher than the  $3 (\delta F/\bar{F})$  threshold. Sixteen significant  $\Delta/P$  values of 16 minors that spanned the excitation wavelengths from 435 to 460 nm, and the emission wavelengths from 621 to 670 nm are given in Table II.

Altogether, the above data indicated that the Pchl pool of cucumber cotyledons *in vivo* was made up of at least four Pchl species. The failure of matrix analysis to detect the fifth Pchl component will be examined under "Discussion."

**Fluorescence Emission Characteristics of Pchl in Etiolated Bean Leaves.** At this point, it became desirable to determine whether the different SW Pchl species observed in etiolated cucumber cotyledons were detectable only in this particular tissue or whether their occurrence was more ubiquitous as was found to be the case for the multiple Pchl-chromophoric species of etiolated tissues (3, 4). Thus, the fluorescence profile of Pchl in etiolated bean leaves was also investigated.

As shown in Figure 3A (solid curves), the emission maximum of the SW Pchl band of 5-day-old etiolated bean leaves shifted from about 630 nm to 633, 637, and 640 nm as the excitation wavelength was varied from 435 to 455 nm at 5-nm intervals. The LW Pchl form exhibited an emission maximum at about 657 nm as was observed in cucumber cotyledons (Fig. 3A,  $E_{440}$  excitation).

A brief light treatment triggered photoconversion phenomena similar to those observed in etiolated cotyledons (Figs. 1A and 2A). The LW Pchl emitting at 657 nm was completely photoreduced; the fluorescence integral of the SW Pchl band decreased significantly, and the emission maxima at 633, 637, and 640 nm either disappeared or were replaced by much reduced and blue-shifted emissions (Fig. 3A, dashed curves). The emission maxima at 677 and 696 nm are those of Chl(ide) *a*.

These results suggested that similar SW and LW Pchl species were present in both 5-day-old etiolated bean leaves and cucumber cotyledons. The Pchls in both tissues exhibited similar phototransformability. In contrast to cucumber cotyledons, the Pchl profile in etiolated bean leaves underwent more pronounced changes during tissue development in the dark: as the proportion of the SW Pchl forms changed, the Pchl species emitting at 640 to 642 nm dominated the Pchl emission profile in 5- to 9-day-old leaves and then became less pronounced in older leaves.

**Soret Excitation Characteristics of SW and LW Pchl Species in Bean Leaves.** The Soret excitation maxima of the fluorescing Pchl species detected in etiolated bean leaves were determined as in cucumber cotyledons (see above).

Table II.  $\Delta/P$  Values for Some of  $4 \times 4$  Minors Extracted from Matrix Depicted in Table I

Observation Wavelengths ( $F_\lambda$ nm)	$\Delta/P$ at Following Excitation Wavelengths ( $E_\lambda$ nm)			
	435, 452, 455, 460	440, 452, 455, 460	442, 452, 455, 460	445, 452, 455, 460
621, 624, 636, 652	0.54	0.54	0.54	0.54
621, 624, 636, 657	0.53	0.53	0.53	0.53
621, 624, 636, 662	0.54	0.54	0.54	0.54
621, 624, 636, 670	0.55*	0.55*	0.55*	0.55*

\* The corresponding  $\delta F/\bar{F}$  values amounted to 0.02; for the other minors, the  $\delta F/\bar{F}$  values amounted to 0.01.



[i.e. Pchl ( $E_{441}$ ,  $F_{633}$ ), Pchl ( $E_{442}$ ,  $F_{636}$ ), and Pchl ( $E_{443}$ ,  $F_{640}$ )], and LW t-Pchl ( $E_{447}$ ,  $F_{657}$ ) (Table III).

The extreme Soret excitation band overlap of the four putative SW Pchl species within a wavelength range of 10 nm (Table III) may explain the failure of matrix analysis to detect the fifth Pchl. This hypothesis was tested elsewhere (3) as follows. Coproporphyrin tetramethyl ester with a Soret excitation maximum at 399 nm ( $E_{399}$ ), uroporphyrin octamethyl ester ( $E_{402}$ ), protoporphyrin IX dimethyl ester ( $E_{409}$ ), and Zn-uroporphyrin octamethyl ester ( $E_{414}$ ) were dissolved together in ether and the Soret excitation/emission spectra of the mixture were recorded at 77 K. The four Soret excitation maxima of this mixture fell within a wavelength range of 15 nm, i.e. about 5 nm more than was the case for the cucumber Pchls *in vivo*. Deconvolution of the excitation/emission matrix according to Weber (18) indicated the presence of only three fluorescent components in the mixture. However, when the wavelength range separating the Soret excitation maxima in the mixture was increased to 26 nm by substituting Zn-protoporphyrin dimethyl ester ( $E_{425}$ ) for Zn-uroporphyrin tetramethyl ester, the matrix analysis of the data detected all four fluorescent components.

The results presented here are compatible with the newly discovered Pchl chromophoric heterogeneity of etiolated tissues and suggest that the Pchl spectral multiplicity detected *in situ* may be due to the interaction of several chemically different Pchl chromophores with different macromolecular environment(s). It is noteworthy to point out that four different Pchl-binding polypeptides were recently separated electrophoretically on polyacrylamide gels (15). However, the low temperature fluorescence properties of these Pchl-protein complexes have not yet been determined. The foregoing results are also compatible with the observations of Virgin and French (19). These workers reported that the Chl(ide) *a* species apparent immediately after irradiation of etiolated barley leaves consisted of the same four major components reported to be present in all mature tissues of higher plants and algae.

Finally, the nt-SW Pchl appeared to be almost identical in both etiolated cucumber cotyledons and bean leaves, exhibiting a Soret excitation maximum at about 440 nm and an emission maximum at about 630 nm. The t-SW Pchls and t-LW Pchl exhibited emission maxima at 633, 636 to 638, 640 to 642, and 657 nm, respectively, in both tissues. The Soret excitation maxima of the phototransformable SW and LW Pchl species in etiolated bean leaves were blue-shifted, however, by 2 to 3 nm, as compared to their counterparts in etiolated cotyledons (Figs. 2B and 3B). Furthermore, although the LW Pchl in both tissues exhibited an excitation shoulder at about 463 nm, that in etiolated bean leaves lacked completely the prominent excitation shoulder at 470 nm which was observed in cucumber cotyledons. Differences were

also observed between the Soret excitation spectra of the SW and LW Pchls within the same species, notably the lack of excitation shoulders at 463 nm (and at 463 and 470 nm in cucumber) in the SW Pchl species. The reasons for these differences will be discussed elsewhere.

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