

The *Azolla-Anabaena azollae* Relationship¹

XII. NITROGENASE ACTIVITY AND PHYCOBILIPROTEINS OF THE ENDOPHYTE AS A FUNCTION OF LEAF AGE AND CELL TYPE

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ABSTRACT

Nitrogenase activity was measured in leaves along the main stem axes of *Azolla pinnata* R. Br. The activity was negligible in leaves of the apical region, rapidly increased to a maximum as leaves matured, and declined in aging leaves. *In situ* absorption and fluorescence emission spectra were obtained for individual vegetative cells and heterocysts in filaments of the *A. pinnata* and *Azolla caroliniana* endophytes removed from the cavities of progressively older leaves. These spectra unequivocally demonstrate the occurrence of phycobiliproteins in the two cell types of both endophytes at the onset of heterocyst differentiation in filaments from young leaves, during the period of maximal nitrogenase activity in filaments from mature leaves, and in filaments from leaves entering senescence. Phycobiliproteins of the *A. caroliniana* endophyte were purified and extinction coefficients determined for the phycoerythrocyanin, phycocyanin, and allophycocyanin. The phycobiliprotein content and complement of sequential leaf segments from main stem axes and of vegetative cell and heterocyst preparations were measured in crude extracts. There was no obvious alteration of the phycobiliprotein complement associated with increasing heterocyst frequency of the endophyte in sequential leaf segments and the phycobiliprotein complement of heterocysts was not appreciably different from that of vegetative cells. These findings indicate that the phycobiliprotein complement of the vegetative cell precursor is retained in the heterocysts of the endophyte.

Sporophytes of the heterosporous aquatic ferns in the genus *Azolla* exhibit floating, multibranched stems bearing deeply bilobed leaves and adventitious roots (14). A symbiotic, heterocystous cyanobacterium, *Anabaena azollae* Strasb., which occurs in specialized cavities formed in the fern's aerial dorsal leaf lobes (4, 12, 14), can provide the associations with their total N requirements via N₂ fixation (17, 20). The leaves along each stem axis represent an ontogenetic sequence of leaf development. The *Anabaena* filaments in the leaf cavities undergo differentiation and development in parallel with the fern. Undifferentiated *Anabaena* filaments associated with the stem apices are partitioned into developing leaf cavities where they differentiate heterocysts (14). In the cavities of mature leaves heterocysts may account for 30% of all *Anabaena* cells (8, 9). Nitrogenase activity

increases with the increasing heterocyst frequency. Therefore, most analyses of nitrogenase activity associated with progressively older leaves, or groups of leaves, from stem axes of *Azolla caroliniana* (10, 19), *Azolla filiculoides* (9, 26), and *Azolla pinnata* (26), have revealed negligible activity in the apical segments followed by a rapid increase, a leveling off, and a decline as the leaves senesce. An exception is a report of two distinct maxima of nitrogenase activity, and a suggestion of two separate generations of heterocysts, in *A. caroliniana* and *A. pinnata* (1).

The phycobiliproteins of heterocystous cyanobacteria often are considered to be associated primarily with PSII in vegetative cells and to be absent, or greatly diminished, in the heterocysts since they exhibit only PSI activity (7). However, PBP³ occur in the heterocysts of *Anabaena variabilis* (11, 21), an *Anabaena* sp. (32), and *Azolla* endophytes (1, 29). In the *A. caroliniana* endophyte, which contains PEC, PC, and APC (28), action spectra have shown that these accessory pigments effectively harvest light energy for both O₂ evolution (24) and PSI-linked, nitrogenase-catalyzed acetylene reduction (29). PBP in *A. variabilis* heterocysts effectively promote light-dependent nitrogenase activity and sensitize P-700 oxidation (21).

In this study we determine the profile of nitrogenase activity in progressively older leaves of *A. pinnata* and compare the *in situ* PBP and Chl absorption, and PBP fluorescence, of individual vegetative cells and heterocysts in filaments of the *A. pinnata* and *A. caroliniana* endophytes removed from separate and progressively older leaf cavities. This is followed by a more detailed analysis of the *A. caroliniana* symbiosis. The total PBP and complement of PEC, PC, and APC are determined for endophyte filaments in progressively older leaves in an effort to assess any alteration that might be attributed to increasing heterocyst frequencies. This is complemented by similar analyses on vegetative cell and heterocyst fractions obtained from endophyte preparations encompassing all developmental stages.

MATERIALS AND METHODS

Plant Material and Growth Conditions. *Azolla caroliniana* population wt-v-cf and the Malaysian *Azolla pinnata* population br-m-cf from the Battelle-Kettering *Azolla* culture collection were grown on N-free medium (20) using a 16/8 h, 26 ± 1/19 ± 1°C diurnal cycle. A combination of cool-white fluorescent and incandescent lights provided a photosynthetic photon flux density of 200 μE·m⁻²·s⁻¹ as measured with a Lambda L1-1905 quantum sensor. Cultures were maintained in a manner to circumvent diminished growth associated with high plant density (20). *Anabaena cylindrica* 1403/2a was grown as described previously (23).

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³ Abbreviations: PBP, phycobiliproteins; PEC, phycoerythrocyanin; APC, allophycocyanin; PC, phycocyanin.

Phycobiliprotein Extraction and Determination of Extinction Coefficients. *Anabaena azollae* was isolated from *A. caroliniana* leaf cavities using the gentle roller method (17). Isolation and initial purification of PBP were based on previously described procedures (28, 29). Absorption spectra were obtained with a Cary 118C spectrophotometer. The absorption maxima of PEC, PC, and APC in 2.5 mM sodium phosphate buffer (pH 7.0) occurred at 572, 612, and 647 nm, respectively. Absorption at each of these wavelengths was used along with protein concentrations to determine extinction coefficients for the individual phycobiliproteins (Table I). Equations for calculating the PBP concentration in crude extracts were derived by combining the extinction coefficients with three simultaneous equations (Table I), essentially as described by Bennett and Bogorad (2). However, all of the data concerning concentrations of individual PBP in crude extracts presented in this study were obtained using a computer program developed by Dr. J. W. McDonald of this laboratory. This program was based on Cramer's Rule and incorporated a correction for residual Chl absorption.

Phycobiliprotein Content and Distribution as a Function of Leaf Age and Cell Type. To determine PBP content of the endophyte as a function of leaf age, dissected main stem axes were prepared as described previously (10). The number of main stem axes trimmed in each study was sufficient to provide a minimum of 0.2 g fresh weight for each group of segments after dissection of the axes into sequential segments and combining those of the same leaf sequence. For extraction of PBP, entire plants, main axes, and groups of segments corresponding to sequential stages in the ontogenetic sequence of leaf development were individually ground in 2.5 mM sodium phosphate containing 20 mM ascorbic acid and 1% (w/v) Polyclar AT, final pH 7.0, using a motor-driven Teflon homogenizer. Grinding buffer was used at 20 ml/g fresh weight. Homogenized material was passed twice through an Aminco French pressure cell at 20,000 p.s.i. and centrifuged at 80,000g for 60 min. Absorption spectra of the supernatants were obtained and the total PBP as well as the relative amounts of PEC, PC, and APC determined as described above.

Absorption and Fluorescence Spectroscopy of Individual Cells. Filaments of the endophytes from *A. caroliniana* and *A. pinnata* were obtained from progressively older leaves along the main stem axis of the ferns. Ferns were randomly selected and studies were duplicated using two separate cultures of each species. Immediately prior to use, an individual leaf was excised from the main stem axis and the cavity teased open under a dissecting

microscope to free filaments of the endophyte. For spectroscopy, endophyte filaments from individual leaf cavities of a known developmental age and filaments of *A. cylindrica* were placed on a microscope slide and sealed under a coverslip with dental wax. Spectroscopy was essentially as recently described for *Anabaena variabilis* (11). Room temperature absorption and fluorescence emission spectra of individual vegetative cells and heterocysts were recorded with Tracor-Northern's DARSS (diode-array rapid scanning spectrophotometer) coupled to a Leitz Ortholux II microscope and a Tracor Northern model 1710 mainframe, supplemented by modules for signal averaging, photometric processing, and wavelength calibration (11). An aperture placed in the back focal plane of the ocular limited the field of view to a single cell. Isolation of 546 nm excitation light for fluorescence measurements and procedures for measuring absorption spectra were as described (11).

PBP Complement of Vegetative Cell and Heterocyst Preparations. Filaments of *Anabaena azollae* were isolated from *A. caroliniana* by grinding fronds in N-free BG-11 medium (27) containing 1% PVP-40 (w/v), 7 ml/g fresh weight, for 60 s in a cooled, motor-driven, Teflon homogenizer. The homogenized material was sequentially passed through four and then eight layers of cheesecloth and a 100 μ m nylon mesh filter, rinsing with grinding medium. The filtrate was centrifuged at full speed in a clinical centrifuge for 20 s and the supernatant decanted. The endophyte pellets were suspended in N-free BG-11 medium without PVP and centrifuged again. After decanting the supernatant, the endophyte filaments in the pellets were suspended in 4 to 5 ml of 2.5 mM sodium phosphate buffer (pH 7.0). Due to the heterogeneity of cell size in endophyte filaments (19), the approach originally described by Fay and Walsby (5) and used in a previous study (17) was found more suitable for obtaining a heterocyst fraction from the endophyte than more recent procedures (22). The resuspended pellets were passed twice through an Aminco French pressure cell at 2000 p.s.i. and centrifuged for 30 s in a clinical centrifuge. The supernatants, comprised of disrupted vegetative cells, were decanted and centrifuged at 80,000g for 1 h. The pellets, which were estimated to contain about 85 to 90% heterocysts, 10 to 15% akinetes, and no more than 1% vegetative cells on the basis of phase contrast microscopy, were resuspended in buffer and subjected to two passes through the pressure cell at 20,000 p.s.i. and centrifugation at 80,000g for 1 h. Absorption spectra of the extracts were recorded with a Cary 118C spectrophotometer. The total PBP and the complement of PEC, PC, and APC in the vegetative cell and heterocyst enriched fractions were determined as described above.

Other Determinations. For measuring nitrogenase activity as a function of leaf age, main stem axes of *A. pinnata* were prepared as described previously for *A. caroliniana* (10). Analyses were conducted using both individual leaves and pairs of leaves sequentially dissected from the axes beginning with the apical segment. Triplicate samples of single leaves, and pooled leaf pairs of the same developmental stage, were placed in 1 ml vials containing 0.25 ml of N-free growth medium (20) and allowed to equilibrate at 25°C in the light, 200 μ E·m⁻²·s⁻¹, for 90 min. The vials were sealed with crimp-caps, evacuated, flushed and filled to a slightly positive pressure with 10% C₂H₂ in Ar. After 45 min of incubation under the conditions used for equilibration, 0.25 ml of the gas phase was removed from each vial for analysis in a Gow-Mac 750 gas chromatograph with other conditions as described previously (18). Chl was measured after extraction with 95% ethanol (30).

RESULTS

Profile of Nitrogenase Activity in *Azolla pinnata*. Analysis of light dependent nitrogenase-catalyzed acetylene reduction in in-

Table I. Extinction Coefficients of Purified *Anabaena azollae* Phycobiliproteins in 2.5 mM Sodium Phosphate Buffer (pH 7.0) and Equations for Determining Phycobiliprotein Concentrations in Crude Extracts

Phycobiliprotein	Extinction Coefficients ^a		
	$\lambda = 572$ nm	$\lambda = 612$ nm	$\lambda = 647$ nm
	ml/mg·cm		
PEC	7.052 ± 0.512	2.059 ± 0.509	0.077 ± 0.022
PC	3.940 ± 0.263	6.551 ± 0.620	1.153 ± 0.290
APC	1.650 ± 0.394	3.321 ± 0.767	4.569 ± 1.128

^a Mean of 10 determinations ± SD.

$$[\text{PEC}] = \frac{A_{572} - 0.619(A_{612}) + 0.088(A_{647})}{5.775}$$

$$[\text{PC}] = \frac{1.165(A_{612}) - 0.727(A_{647}) - 0.332(A_{572})}{5.483}$$

$$[\text{APC}] = \frac{0.049(A_{572}) - 0.205(A_{612}) + A_{647}}{4.001}$$

dividual leaves, and in pairs of leaves, sequentially dissected from main stem axes of *A. pinnata* resulted in the profiles shown in Figure 1. Whether expressed on a per leaf basis (Fig. 1A) or on the basis of fresh weight or Chl from pooled leaf pairs (Fig. 1B), the profiles concur with those reported for this (26) and two other *Azolla* species (9, 10, 19, 26). Low activity in the apical region was followed by a rapid increase, a leveling, and a decline with senescence. There was no indication of two distinct peaks of nitrogenase activity (1).

Absorption and Fluorescence Emission Spectra of Individual Vegetative Cells and Heterocysts. As with the *A. caroliniana* endophyte (29), epifluorescence microscopy demonstrated the qualitative occurrence of PBP in both vegetative cells and heterocysts of filaments of the *A. pinnata* endophyte removed from leaf cavities where N_2 fixation was maximal. *In situ* visible absorption microspectrophotometry and fluorescence emission microspectrofluorometry of individual cells provided a more quantitative assessment of their Chl and PBP content. Representative spectra obtained for the vegetative cells and heterocysts of endophyte filaments removed from progressively older leaf cavities of *A. pinnata* and *A. caroliniana* are shown in Figs. 2, A to J and 3, A to H, respectively. Spectra for *Anabaena cylindrica*, which exhibited markedly diminished PBP in heterocysts using epifluorescence microscopy (29), are presented for comparative purposes in Fig. 3, I and J. Initial studies showed that within and among filaments of the endophytes from individual leaf cavities, and from cultures of *A. cylindrica*, there was some minor variation in the spectra of individual vegetative cells and heterocysts. Therefore, in each case we randomly selected five heterocysts and five vegetative cells occupying similar positions in a filament. The spectra of the individual cells were stored in the data

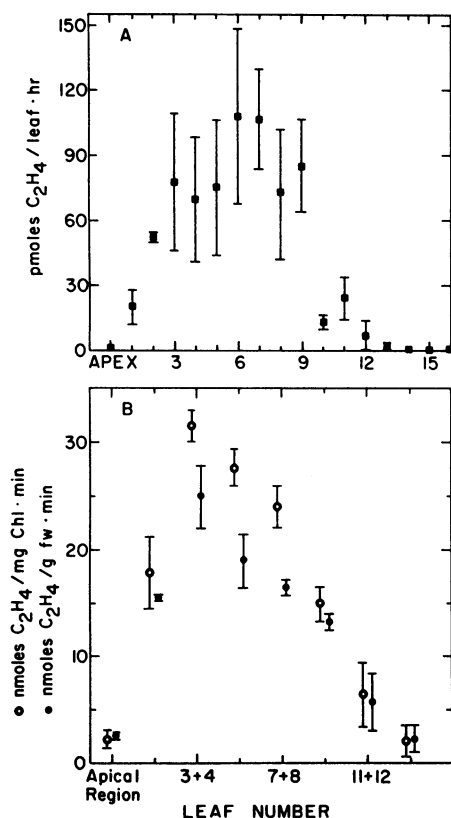


FIG. 1. Light-dependent nitrogenase-catalyzed acetylene reduction as a function of leaf age in main stem axes of *A. pinnata*. Rates are expressed on a per leaf basis (A) and, in a separate study, on the basis of fresh weight (●) and Chl (○) using pooled leaf pairs (B). Points are the mean of triplicate samples. Bars indicate SD where this exceeds size of symbol.

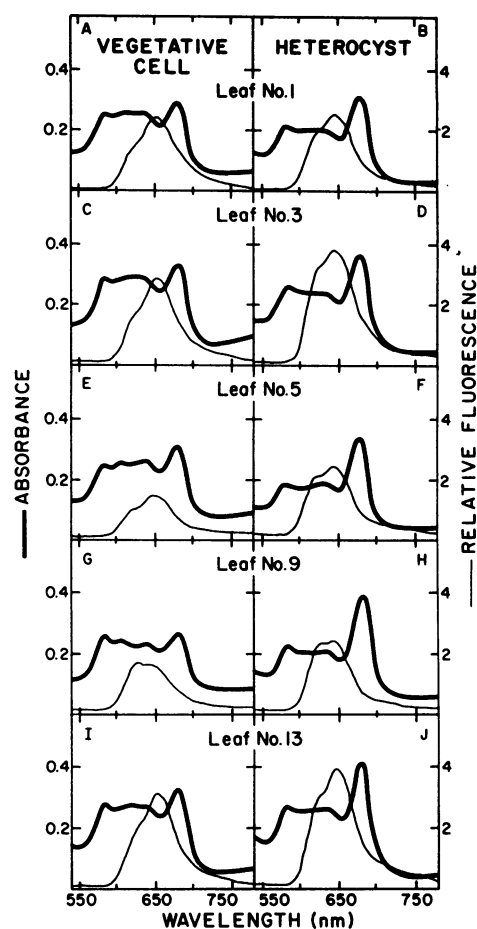


FIG. 2. Absorption and fluorescence spectra, excited at 546 nm, of individual vegetative cells and heterocysts in filaments of the *A. pinnata* endophyte from progressively older leaf cavities.

memory, and computer averaged to obtain spectra representative of an individual cell.

The Chl absorption of both cell types of the *A. caroliniana* endophyte (Fig. 3, A-H) increased with leaf age and neither cell type consistently exhibited a higher value. In the *A. pinnata* endophyte (Fig. 2, A-J) Chl absorption was consistently greater in heterocysts than in vegetative cells and only the heterocysts exhibited a trend toward increasing Chl absorption as a function of leaf age. This is clearly seen in Table II where the scatter at 750 nm has been subtracted from the absorbance of the Chl maximum and the absorbance values of the individual cell types normalized to that of leaf 1. Table II also shows the ratio of heterocyst to vegetative cell Chl absorbance for the series of determinations. Excluding the values for the *A. pinnata* endophyte from leaf number 9, the heterocyst/vegetative cell Chl ratio is reasonably constant as a function of leaf age in both species. It is important to note that an increase in Chl absorbance per cell need not reflect an increase in Chl per unit volume. The size of the endophyte vegetative cells increases in progressively older leaf cavities (9) and micrographs show that individual cell types within a filament can vary in size (1, 17, 29).

The occurrence of PBP in heterocysts as well as vegetative cells of both endophytes is unequivocally demonstrated by their absorption and fluorescence emission spectra (Figs. 2, A-J and 3, A-H). The heterocysts of both endophytes have less PBP, relative to the Chl, than the vegetative cells. The PBP absorption increases in vegetative cells of the *A. caroliniana* endophyte with leaf age (Fig. 3, A, C, E, and G), but is relatively constant in vegetative cells of the *A. pinnata* endophyte (Fig. 2, A, C, E, G, and I) and

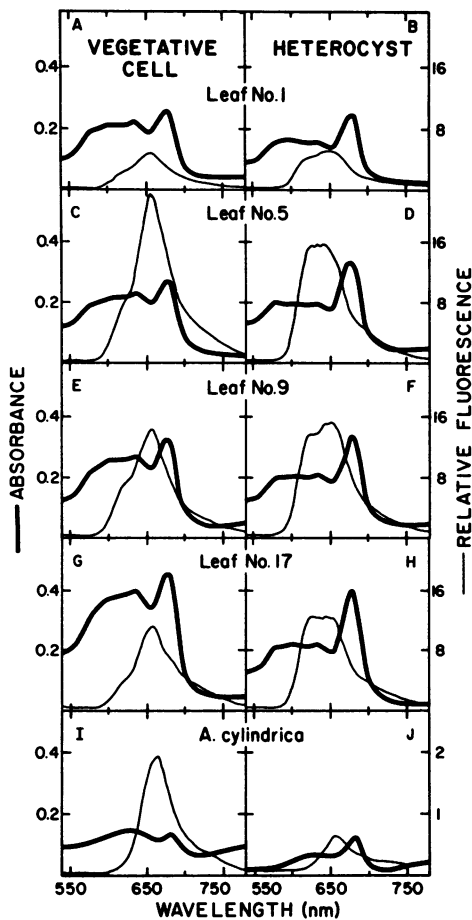


FIG. 3. Absorption and fluorescence spectra, excited at 546 nm, of individual vegetative cells and heterocyst in filaments of the *A. caroliniana* endophyte from progressively older leaf cavities (A–H) and in the free-living cyanobacterium *A. cylindrica* (I and J).

the heterocysts of both (Fig. 2, B, D, F, H, and J; Fig. 3, B, D, F, and H). In comparison to the *A. caroliniana* endophyte (Fig. 3, A–H) both vegetative cells and heterocysts of the *A. pinnata* endophyte (Fig. 2, A–J) exhibited more pronounced absorption at about 580 nm. While this suggests the occurrence of more PEC, the *A. pinnata* endophyte's PBP have not been isolated and characterized. The intensity of PBP fluorescence from 546 nm excitation was variable in both cell types of the two endophytes as a function of leaf age but more so in vegetative cells than in heterocysts (Fig. 2, A–J; Fig. 3, A–H). As indicated by the respective scales, the intensity of PBP fluorescence from cells of

the *A. caroliniana* endophyte was greater than that from cells of the *A. pinnata* endophyte. There also was some variation in the shape of the individual fluorescence emission spectra obtained for the two cell types of both endophytes.

In general, vegetative cells of the *A. pinnata* endophyte (Fig. 2, A, C, E, G, and I) fluoresced maximally at 650 nm with a prominent shoulder at 625 nm and a slight shoulder at 660 nm. In one instance (Fig. 2G), the emission at 625 nm was as intense as that at 650 nm. Its heterocysts (Fig. 2, B, D, F, H, and J) generally showed an emission maximum at about 643 nm with a prominent shoulder around 623 nm and, in some instances, a slight shoulder at 658 nm. Vegetative cells of the *A. caroliniana* endophyte (Fig. 3, A, C, E, and G) exhibited a major emission at about 656 nm, a shoulder of variable prominence around 625 nm, and indications of a shoulder at about 675 nm. Its heterocysts (Fig. 3, B, D, F, and H) showed a broader emission with apparent maxima, or pronounced shoulders, from 625 to 650 nm and indications of shoulders around 660 and 675 nm. The occurrence of strong shoulders or multiple peaks in the *in situ* PBP fluorescence emission of both cell types of the two endophytes, along with the absence of any distinct indication of room temperature PSII associated Chl fluorescence for vegetative cells, suggests the emissions from the individual cells may arise from a fraction of the cellular PBP which is uncoupled or not yet assembled into phycobilisomes. Purified PEC, PC, and APC from the *A. caroliniana* endophyte exhibit emission maxima at 630, 643, and 660 nm, respectively, using excitation at 540 nm (28).

The absence of any clearly discernible room temperature Chl fluorescence from the individual vegetative cells may be due to its being masked by the intensity of the longwave tail of the PBP fluorescence. Using a locally assembled apparatus constructed to have good sensitivity in the red region (25) and excitation at either 546 or 580 nm, the addition of 20 μM DCMU to preparations of endophyte filaments isolated from all stages of leaf development (19) resulted in an increase in room temperature fluorescence emission at 690 nm clearly attributable to PSII associated Chl (data not shown).

No attempt was made to determine the cause(s) of the variation in the intensity of the PBP fluorescence emission from the individual cells of the two endophytes. However, possible differences in the PBP complements of *A. caroliniana* vegetative cells and heterocysts suggested by the spectra were investigated further.

PBP Complement as a Function of Leaf Age. Since heterocyst frequency increases with leaf age, it was postulated that differences in the PBP complement of the two cell types might be detected in an analysis of PBP complement of the endophyte in sequential leaf segments of main stem axes. The total PBP content and the complement of PEC, PC, and APC in sequential

Table II. *Chl Absorbance of Individual Vegetative Cells (Veg) and Heterocysts (Het) of A. caroliniana and A. pinnata Endophytes as a Function of Leaf Age, their Normalization to Leaf Number 1 (in brackets) and the Het/Veg Ratio*

Chlorophyll absorption maxima of vegetative cells and heterocysts were located at 676 nm and 677 to 678 nm, respectively.

Leaf No.	<i>A. caroliniana</i>			<i>A. pinnata</i>		
	Veg	Het	Het/Veg	Veg	Het	Het/Veg
Apex	0.137 (0.54)					
1	0.255 (1.00)	0.253 (1.00)	0.99	0.226 (1.00)	0.292 (1.00)	1.29
3				0.255 (1.13)	0.329 (1.13)	1.29
5	0.258 (1.01)	0.306 (1.21)	1.19	0.227 (1.00)	0.306 (1.05)	1.35
9	0.298 (1.17)	0.305 (1.21)	1.02	0.168 (0.74)	0.346 (1.18)	2.06
13	0.393 (1.54)	0.413 (1.63)	1.05	0.275 (1.22)	0.381 (1.30)	1.39
17	0.420 (1.65)	0.389 (1.54)	0.93			

leaf segments were determined. The results of these analyses are compared to one another, and to data obtained from undissected main stem axes and entire fronds, in Figure 4. Based on prior morphological studies (3, 4, 14) and the absorption spectra shown in Figure 3, the increase in the total PBP with leaf age is attributed primarily to an increase in endophyte biomass in the first two segments and an actual increase in the PBP content of the endophyte's vegetative cells in the older two segments. When expressed as a percentage of the total PBP content, the distribution of PEC, PC, and APC in main stem axes was very similar to the combined averages of all segments and compared favorably with the distribution obtained for the entire fronds (Table III). The absence of any distinct pattern of alteration in the PBP complement of progressively older leaves on segments of the main stem axis (Table III) indicated that either there was no appreciable difference in the PBP complement of vegetative cells and heterocysts or that the increasing PBP content of the vegetative cells in progressively older leaves (Fig. 3, A, C, E, and G) was of sufficient magnitude to obscure any alteration of the PBP complement associated with an increase in heterocyst frequency. In an attempt to resolve this matter, the PBP complement in vegetative cells of the endophyte was compared with that of heterocysts.

Analysis of PBP Complement of Vegetative Cell and Heterocyst Preparations. The results from three separate determinations of the PBP complement of vegetative cell and heterocyst fractions obtained from endophytes isolated from entire fronds (17) are summarized in Table IV. The PBP concentration ($\mu\text{g}/\text{ml}$) was

Table IV. PBP Complement, Expressed as a Percentage of the Total, in Extracts of Vegetative Cells and Heterocysts from Three (a-c) Preparations of the *A. caroliniana* Endophyte

	PEC	PC	APC
Vegetative cells			
a	16	55	29
b	18	47	35
c	19	42	39
$\bar{x} \pm \text{SD}$	17.5 ± 1.5	48.0 ± 6.6	34.3 ± 5.0
Heterocysts			
a	18	65	17
b	21	50	29
c	15	52	33
$\bar{x} \pm \text{SD}$	18.0 ± 3.0	55.7 ± 8.1	26.3 ± 8.3

different in each of the extracts but the distribution of the total PBP between the vegetative cell and heterocyst fractions was quite consistent with $85.7 \pm 7.1\%$ attributed to vegetative cells and $14.3 \pm 2.1\%$ to heterocysts. In each individual determination PC accounted for a greater, and APC for a lesser, percentage of the total PBP in heterocysts than in vegetative cells (Table IV). While suggestive of a subtle alteration of the PBP complement in heterocysts relative to vegetative cells, we cannot exclude the possibility that such a modest difference is a consequence of the akinetes associated with the heterocyst preparations of the endophyte.

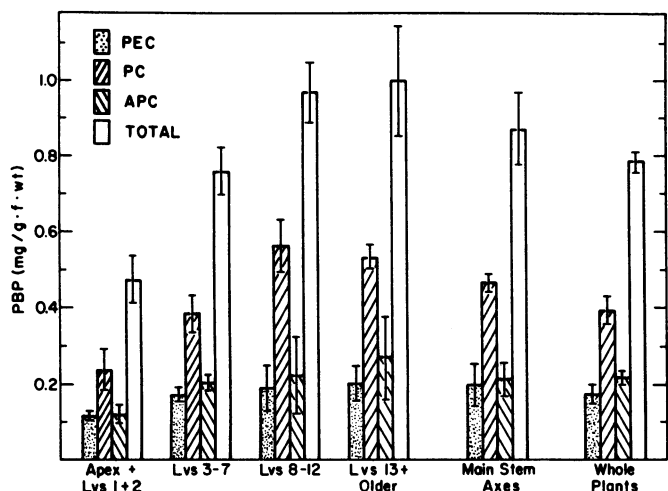


FIG. 4. The total phycobiliprotein content and complement of phycoerythrocyanin (PEC), phycocyanin (PC), and allophycocyanin (APC) in sequential leaf segments from main stem axes; intact main stem axes; and entire plants of *A. caroliniana*. Each value is based on three to five separate determinations and plotted as mean \pm SD.

Table III. PBP Complement, Expressed as Percentage of the Total PBP Content, in Sequential Leaf Segments, Main Stem Axes, and Entire Fronds

	PEC	PC	APC
	%		
Apex + leaves 1 and 2	25.4 ± 5.3	49.6 ± 5.6	25.1 ± 4.0
Leaves 3-7	22.4 ± 1.6	50.5 ± 2.7	27.1 ± 3.6
Leaves 8-12	19.6 ± 6.9	57.9 ± 4.7	22.5 ± 9.2
Leaf 13 \rightarrow older	20.0 ± 1.5	53.9 ± 8.4	26.1 ± 6.9
Combined segments	21.9 ± 2.7	53.0 ± 3.8	25.2 ± 1.9
Main stem axes	22.3 ± 4.3	53.5 ± 6.1	24.3 ± 2.8
Entire fronds	22.0 ± 3.6	50.0 ± 3.0	28.0 ± 3.0

^a Percentages are means \pm SD of at least three experiments.

DISCUSSION

Our analysis of nitrogenase activity along the ontogenetic sequence of leaf development in *A. pinnata* R. Br. yielded a profile consistent with studies (9, 10, 19, 26) in which this activity was low or negligible in the apical region, increased to a maximum in mature leaves, and declined in aging leaves. Heterocyst differentiation begins as filaments of the undifferentiated apical *Anabaena* colony are partitioned into the developing leaf cavities (14). These specialized cells are functional for a limited period with the most rapid rates of nitrogen fixation occurring shortly after their differentiation is completed. Since heterocyst frequency increases in leaves exhibiting increasing nitrogenase activity and then stabilizes, or increases only very modestly, in mature leaves (8), it follows that the heterocysts in mature cavities are of varying age and functional activity. The decline in nitrogenase activity as the leaves age reflects, at least in part, the sequential senescence of individual heterocysts in the order of their prior differentiation. This interpretation of events is consistent with the findings in a recent study of the ultrastructural ontogeny of the leaf cavity trichomes and our current understanding of this symbiosis (3, 14).

A prior study showed that the energy absorbed by PBP effectively promoted nitrogenase-catalyzed acetylene reduction in the *A. caroliniana* endophyte and demonstrated the occurrence of PBP in heterocysts of filaments removed from leaf cavities exhibiting maximal nitrogenase activity (29). In a contemporary study (1), photomicrography was used to assess the absorption and fluorescence of Chl and PBP in filaments of *Azolla* endophytes. Heterocysts of the *A. pinnata* endophyte were reported to have much less PBP and, relative to the vegetative cells, much more Chl than those of the *A. caroliniana* and *A. filiculoides* endophytes. The room temperature absorption and fluorescence emission spectra of individual cells obtained in the present study are more definitive than photomicrography of filaments. They provide quantitative rather than qualitative information on the pigment content and unequivocal evidence for the occurrence of PBP in both cell types of the *A. caroliniana* and *A. pinnata* endophytes from the onset of nitrogenase activity in leaf 1 and

continuing through all progressively older leaves. There was no indication of diminished PBP absorption by heterocysts of the *A. pinnata* endophyte relative to those of the *A. caroliniana* endophyte (1) removed from actively growing, N₂-fixing associations cultured under conditions known to yield similar growth rates and N content (20).

In contrast to free-living cyanobacteria (7, 31, 32), N starvation and PBP degradation do not appear to be associated with the transition from purely vegetative to heterocystous filaments in the *Azolla-Anabaena* symbiosis. This difference may be attributed to the interaction of host-endophyte nitrogen and carbon metabolism in this tightly coordinated and highly orchestrated symbiosis in which the endophyte is never in direct contact with the external environment and its nutritional status is controlled by the fern (14, 15). Nitrogen fixed by the endophyte in mature cavities is released and transported to the growing apices which harbor actively dividing, undifferentiated *Anabaena* filaments (10, 16). These filaments are intimately associated with specialized epidermal trichomes (4) which facilitate the partitioning of the apical colony into developing leaf cavities and, based on indirect evidence, are assumed to provide the interface through which this apical colony receives nutrients, including combined N, from the fern (3, 14). In mature leaf cavities the fern provides the endophyte with photosynthate (16) possibly via a second distinct population of epidermal trichomes (3, 4). It seems probable that the initial induction of heterocysts as filaments are partitioned into forming leaf cavities, and the high heterocyst frequencies in filaments of mature cavities, may be linked to an alteration of the endophyte C:N ratio via metabolites provided to it by the fern. However, the details of such processes are not known. It is clear from the studies presented here, however, that both the differentiation of functional heterocysts in the early stages of leaf cavity development and the subsequent differentiation leading to high heterocyst frequencies in mature leaf cavities occurs in the absence of appreciable PBP proteolysis. The absorption and fluorescence emission spectra demonstrate the occurrence of PBP in heterocysts from all stages of leaf development with nitrogenase. This, along with the absence of any distinctive alteration of the PBP complement with the increasing heterocyst frequency of the endophyte and the similarity of the PBP complement of isolated vegetative cells and heterocysts, strongly suggest that during differentiation the heterocysts retain most, if not all, of the PBP present in their vegetative cell precursor.

Yamanaka and Glazer (32) have recently discussed the differences between N starvation with heterocyst differentiation and the heterocyst differentiation which occurs in actively growing N₂-fixing cultures exhibiting biliprotein-rich vegetative cells. In the latter case, which has similarity to the situation in the *Azolla-Anabaena* symbioses, their analysis showed that the PBP found in heterocyst of *Anabaena* strain 7119 originate from the vegetative cell precursor as opposed to *de novo* synthesis during heterocyst differentiation, and exist as multimeric complexes capable of transferring absorbed light energy. Their studies also indicated the occurrence of two separate and independently regulated biliprotein degrading systems in free-living heterocystous cyanobacteria. They termed these the nitrogen starvation induced proteases and the differentiation proteases (32). The former, which are more active on phycocyanin and phycoerythrin than on allophycocyanin and require continuous protein synthesis (7, 31), would degrade entire phycobilisomes but be most active on the rod components (6). The latter would be specific for the components of the phycobilisome core (6), thereby causing a loss of allophycocyanin and allophycocyanin B during heterocyst differentiation from biliprotein-rich vegetative cell precursors (32). Since our analysis indicated that PC accounted for a slightly greater, and APC for a slightly lesser, percentage of the total PBP in heterocysts than in vegetative

cells, we cannot exclude the possibility that some differentiation protease activity occurs in the *A. caroliniana* endophyte. However, some of the akinetes, which invariably occur in older leaf cavities of *A. caroliniana* (19), were found to contain phycobillin using epifluorescence microscopy and akinetes accounted for 10 to 15% of the total cells in each of our heterocyst preparations. This precludes our attributing undue significance to the small differences in the complement of PEC, PC, and APC in the vegetative cell and heterocysts. Clearly, any alteration of the complement in the endophyte heterocysts is much less dramatic than in those free-living *Anabaena* whose heterocysts contain PC but very little or no APC (21, 32). Due to the foregoing, the possibility that differences in the fluorescence emission spectra of the *A. caroliniana* endophyte's vegetative cells and heterocysts may result from subtle alterations in their PBP complement cannot be excluded. Levels of allophycocyanin B were not determined in the present study but they could be a contributing factor to the differences in the fluorescence emission spectra of the two cell types. It is also possible that the differences reflect events associated with the turnover of PSII components and the rearrangement of the photosynthetic apparatus in heterocysts. Yamanaka and Glazer (32) have speculated that a 20 K polypeptide component of the heterocyst phycobiliprotein particle may function in the attachment of heterocyst PBP particles to the photosynthetic lamellae during such rearrangement.

The results presented here and in other recent studies (21, 32) strongly suggest that the heterocysts of at least some cyanobacteria constitute an exception to the strict structural-functional association between phycobilisomes and the PSII complex so convincingly demonstrated in the unicellular *Synechococcus* (13). The retention of a full complement of PBP in heterocysts of the endophytes is consistent with their ability to actively participate in light-harvesting for the PSI-linked process of nitrogen fixation (29). The occurrence of PBP in heterocysts of free-living *Anabaena* may have ecological importance by making more effective use of available light. Their occurrence in heterocysts of the *Azolla* endophytes is assumed to serve a similar function since *Azolla* attenuates the light which reaches *Anabaena* in the leaf cavities. It has been asserted that the occurrence of PBP in heterocysts of *Azolla* endophytes implies a less favorable environment for nitrogenase (1). We would argue, however, that PBP associated with PSI in the heterocysts of an organism whose principle function is photosynthetically driven ammonia production, but resides within the confines of a green leaf, is a reasonable adaptation which may well confer benefits to the productivity of the associations.

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