Plant Physiol. (1983) 73, 353–360
0032-0889/83/73/0353/08/$00.50/0

Light-Harvesting System of the Red Alga *Gracilaria tikvahiae*[1]

I. BIOCHEMICAL ANALYSES OF PIGMENT MUTATIONS

Received for publication February 8, 1983 and in revised form May 16, 1983

THOMAS A. KURSAR[2], JOHN VAN DER MEER, AND RANDALL S. ALBERTE[3]
Department of Biophysics and Theoretical Biology (T. A. K.), and Department of Biology, Barnes Laboratory (R. S. A.), The University of Chicago, Illinois 60637; and Atlantic Research Laboratory, National Research Council of Canada, Halifax, Nova Scotia, Canada (J. v. d. M.)

ABSTRACT

Wild type *Gracilaria tikvahiae*, a macrophytic red alga, and fourteen genetically characterized pigment mutants were analyzed for their biliprotein and chlorophyll contents. The same three biliproteins, phycoerythrin, phycocyanin, and allophycocyanin, which are found in the wild type are found in all the Mendelian and non-Mendelian mutants examined. Some mutants overproduce R-phycoerythrin while others possess only traces of phycobiliprotein; however, no phycoerythrin minus mutants were found. Two of the mutants are unique; one overproduces phycocyanin relative to allophycocyanin while the nuclear mutant *obr* synthesizes a phycoerythrin which is spectroscopically distinct from the R-phycoerythrin of the wild type. The phycoerythrin of *obr* lacks the typical absorption peak at 545 nanometers characteristic of R-phycoerythrin and possesses a phycoerythrobilin to phycourobilin chromophore ratio of 2.6 in contrast to a ratio of 4.2 found in the wild type. Such a lesion provides evidence for the role of nuclear genes in phycoerythrin synthesis. In addition, comparisons are made of the pigment compositions of the *Gracilaria* strains with those of *Neoagardhiella bailyei*, a macrophytic red alga which has a high phycoerythrin content, and *Anacystis nidulans*, a cyanobacterium which lacks phycoerythrin. The mutants described here should prove useful in the study of the genetic control of phycobiliprotein synthesis and phycobilisome structure and assembly.

The functional photosynthetic unit of O_2-evolving plants is composed of large arrays of light-harvesting pigment-proteins which are associated with the reaction centers and electron transport chains of PSI and PSII. While the reaction centers, electron transport chains, and dark reactions of photosynthesis are probably very similar in the major plant groups, the light-harvesting systems are quite diverse. For example, the antennae pigments fucoxanthin and Chl *c* are found in the photosynthetic unit of the diatoms and brown algae. In the red algae, Cryptomonads, and cyanobacteria, the phycobiliproteins serve this function while Chl *b* functions in the green plants (22). These pigments along with the proteins which bind them are major components of the cells and, consequently, their synthesis and assembly are major activities carried out by all photosynthetic cells. To date, it has been shown that in green plants both nuclear and chloroplast genomes function in the biogenesis of the photosynthetic apparatus (see 22); however, there is a paucity of information on this topic in the major non-green plant groups.

The red algae contain Chl *a* and the phycobilipigments as their major photosynthetic pigments. The red algal phycobiliproteins share antigenic properties and amino sequences with those of the cyanobacteria (1, 11, 17) and, to a lesser extent, with the Cryptomonads (1, 6). The blue-colored biliproteins, APC[4] and PC, are found in all cyanobacteria and red algae. The apoproteins of these bilins are composed of two related subunits, α and β, of 15 to 20 kD which can form higher aggregates (9). APC absorbs maximally at about 650 nm and fluoresces at 660 nm, while PC has an absorption maximum at 620 nm and fluoresces at 640 nm. In both pigment-proteins, the chromophores are covalently bound phycocyanobilins which are linear tetrapyrroles (9). The red biliprotein, PE, occurs in many spectral forms and is found in most red algae and in some cyanobacteria. R-PE, the spectral type found in many higher red algae, probably has an $(\alpha\beta)_6\gamma$ subunit structure (30). R-PE has absorption maxima at 498 nm, assigned to phycourobilin, and at 545 and 565 nm, assigned to phycoerythrobilin chromophores. Excitation of R-PE with 498, 545, or 565 nm light results in a major fluorescence emission peak at 575 nm (18). *In vivo*, these phycobiliproteins are organized into discrete structures called phycobilisomes. The phycobiliproteins and hence the phycobilisomes have as their primary function the absorption of light and the transfer of excitation energy to the reaction centers of PSI and PSII (7).

Van der Meer has developed the macrophyte *Gracilaria tikvahiae* as a laboratory organism for genetic studies on the Rhodophyta. Pigment mutants of *Gracilaria* have been isolated by visual screening and genetically analyzed. The wild type pigment composition of *Gracilaria* gives the thallus a brown to red-brown color. Mutations which alter the pigment composition, alter the color of the thallus and are quite readily detected by their yellow, orange, bright green, or purple color. *Gracilaria* has straightforward Mendelian transmission genetics for nuclear genes (26, 27). As it is a dioecious alga, and has both haploid and diploid vegetative phases (2), genetic parameters such as dominance, complementation, and recombination are readily determined. Several pigmentation mutants show strict maternal inheritance (25). Therefore, we examined a range of pigment phenotypes representing both Mendelian and non-Mendelian mutations in order to study the genetic regulation of phycobiliprotein synthesis and the structure of the light-harvesting system of the red algae.

[1] Supported by National Science Foundation grant PCM 78-10535, and in part by the Louis Block Foundation, The University of Chicago. National Research Council of Canada 22523.

[2] T. A. K. was supported by National Institutes of Health grant GM 23944 (to R. S. A.). Present address: Department of Biology, University of Utah, Salt Lake City, UT 84112.

[3] R. S. A. was a Mellon Foundation Fellow during a portion of the research period. To whom reprint requests should be sent: Barnes Laboratory, 5630 S. Ingleside Avenue, Chicago, IL 60637.

[4] Abbreviations: APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin; NaP_i, Na_2HPO_4/NaH_2PO_4; RC, reaction center.

MATERIALS AND METHODS

Mutagenesis and Genetic Analysis. Unialgal cultures of *Gracilaria tikvahiae* were mutagenized by treating mature, spore-bearing, fronds of tetrasporophytes with ethyl methanesulfonate as described (26). In addition to mutants isolated after chemical mutagenesis, several spontaneous mutations were also obtained, often as vegetative sectors arising on plants being maintained in culture. Genetic crosses were conducted to characterize the mutants, and most of these results have been published (27).

Representative mutants of several color classes have been examined in the present study. These include 'bright green' mutants (*vrt*^{2}, *uai*, *NMG-2*), green mutants (*vrt*, *olv*, *mos*^{2}, *vir*, *NMG-1*), brown-green mutants (*vrt*^{5}, *obr*), a purple mutant (*Pur*^{2}), an orange mutant (*ora*), and a yellow mutant (*NMY-1*). Both Mendelian and non-Mendelian mutants are represented, the latter being prefixed by the letters NM. A few double mutants obtained from green × green crosses were also examined. The mutants examined are listed in Table I.

Algal Culturing and Sample Collecting. Unialgal stocks of wild type *Gracilaria tikvahiae* originating from the Gulf of St. Lawrence, Canada, and mutants derived from these stocks were cultured in f/2 medium (12) at 24 ± 1°C with aeration. The medium was supplemented with 2 mM $NaNO_3$, 0.50 μM KBr, and 1 mM KI, and the pH was maintained at 8.2 ± 0.4 by the addition of HCl or fresh medium as needed. Incandescent lighting with a photon flux density of 80 to 160 μE m^{-2} s^{-1} of photosynthetically active radiation, and a 12:12 h photoperiod was used. The green mutants *vir*, *olv*, and *NMG-1*, and the double green mutants were cultured in Halifax as described (2) and shipped to Chicago for analysis. *Anacystis nidulans* (UTEX 625) was cultured in BG-11 and *Neoagardhiella bailyei* was collected from the field (15). *Gracilaria tikvahiae* was collected also at Waquoit Bay near Falmouth, MA. Dry weights were obtained on duplicate samples (100–200 mg fresh weight).

Pigment Content and Reaction Center Analyses. Pigment analyses were carried out on fresh samples using two methods.

Method I. All steps were carried out at 0 to 5°C unless indicated otherwise. Duplicate samples (200–500 mg) in 4.0 ml of 50 mM NaP_i buffer (pH 5.5) were French pressed at 20,000 p.s.i. (2–20°C) and then frozen at −15°C for up to 2 h. In a few cases, the

Table I. *Pigment Composition and Color of Gracilaria and Its Mutants*

The color of the algal thallus (liquid culture in the case of *Anacystis*) is given in column a. The pigment analyses were carried out by method II for *Anacystis* and *Neoagardhiella* and method I for the *Gracilaria* strains. The chromophore composition, normalized to one Chl, is expressed as the number of chromophores associated with APC, PC, PE and as the total number of bilins (APC + PC + PE): these data are presented in columns b, c, d, and e, respectively, and have a precision of ± 10%. The biliprotein composition, expressed on a weight basis, is the percentage of the total phycobiliprotein found as APC, PC, or PE; these data are presented in columns f, g, and h, respectively, and have a precision of ± 6%. The PC/APC and PE/PC ratios are tabulated in columns i and j, respectively. The Chl content is given as nmol Chl *a*/g freshly harvested tissue. (The *Gracilaria* strains are about 88% water.) These values are presented in column k and the precision of measurement for a particular culture is ± 15%. A large change in the pigment content per fresh weight can be obtained if culture conditions and growth rate are changed. For example, fast growing cultures have a significantly lower pigment content. The pigment ratios are less sensitive to changes in the culture conditions.

Species and Stock	(code)	Thallus Color (a)	Chromophore Composition				Biliprotein Composition					Chl Content (k)	Biliprotein Mass (l)
			APC/Chl (b)	PC/Chl (c)	PE/Chl (d)	Total Bilins (e)	APC (f)	PC (g)	PE (h)	PC/APC (i)	PE/PC (j)		
			bilin chromophores/Chl					%		*ratio*		*nmol Chl a/g fresh wt algae*	*D × 10^{-3} Chl*
Gracilaria tikvahiae													
wild type	(Barr)	Brown, red-brown	0.085	0.21	0.72	1.01	17	30	53	1.7	1.8	170	8.6
Mendelian mutants													
ora	(P-7-1)	Orange	0.047	0.13	1.4	1.5	7.4	15	78	2.0	5.1	150	11
Pur^2	(P-1-2)	Purple	0.32	1.07	4.9	6.3	11	27	62	2.4	2.3	42	50
vrt	(G-3-1)	Green	0.085	0.24	0.62	0.95	18	36	46	2.0	1.3	180	8.4
vrt^2	(G-3-2)	Bright green	0.15	0.38	T[a]	0.53	36	65	T	1.8	ND[b]	250	7.4
vrt^5	(G-3-5)	Brownish-green	0.086	0.25	0.79	1.1	16	33	51	2.1	1.6	170	9.6
uai	(G-13-1)	Bright green	0.11	0.31	T	0.42	33	67	T	2.0	ND	300	5.8
obr	(G-15-1)	Brownish-green	0.078	0.19	0.67	0.94	17	29	53	1.7	1.8	200	7.9
mos^2	(G-17-2)	Brownish-green	0.007[c]	0.70	0.22	0.92	1.1[c]	85	13	77[c]	0.16	175	10.3
Non-Mendelian mutants													
NMG-1		Green	0.072	0.21	0.097	0.38	28	58	14	2.0	0.24	250	4.5
NMG-2		Bright green	0.12	0.28	T	0.40	37	63	T	1.7	ND	270	5.6
NMY-1		Yellow, amber	0.045	0.053	0.038	0.48	21	17	62	0.85	3.6	200	1.7
Double mutants													
grn,mos		Blue-green	0.016[c]	0.48	T	0.50	4.4[c]	96	T	22[c]	ND	220	6.3
vir,olv		Bright green	0.099	0.24	T	0.34	37	63	T	1.7	ND	130	4.7
grt,grn		Bright green	0.072	0.19	T	0.26	35	65	T	1.9	ND	250	3.6
Anacystis nidulans		Blue-green	0.097	0.57		0.67	19.3	81		4.2		ND	8.8
Neoagardhiella bailyei		Red	0.044	0.065	1.65	1.76	6.9	7.0	86	1.0	12	165	11.4

[a] T, trace present.

[b] ND, not determined.

[c] When the PC/APC ratio is high, equation 1 is unreliable. Hence, the APC contents and the PC/APC ratios of *mos*^{2} and *grn,mos* are uncertain.

algae were frozen in liquid N_2 and homogenized with a mortar and pestle. The brei was centrifuged for 20 min at 44,000*g*. The aqueous supernatant was saved, the pellet was washed with buffer, extracted with 90% (v/v) acetone for 15 min, and centrifuged for 10 min at 12,000*g*. Chl *a* in the acetone extract was quantified spectrophotometrically (14). A second extract of the pellets using methanol yielded less than 1% of the Chl obtained in the acetone extract. The pellet was resuspended in the 50 mM NaP_i buffer (pH 5.5), and disrupted a second time in the French press. The brei was centrifuged for 20 min at 40,000*g*. This second aqueous extract yielded less than 5% of the biliprotein obtained in the first step. The absorbance of the aqueous supernatants was determined at 498.5, 614.0, and 651.0 nm and their APC, PC, and PE contents were calculated as μg/ml using the following equations:

$$\text{APC} = 181.3\ A_{651} - 22.3\ A_{614} \quad (1)$$

$$\text{PC} = 151.1\ A_{614} - 99.1\ A_{651} \quad (2)$$

$$\text{PE} = 155.8\ A_{498.5} - 40.0\ A_{614} - 10.5\ A_{651} \quad (3)$$

To convert to mols of the hexameric form, we assumed mol wt of 210, 225, and 250 kD for APC, PC, and PE $[(\alpha\beta)_6\gamma]$, respectively. To convert to chromophores, the number of chromophores per hexamer was assumed to be 12, 18, and 40 for APC, PC, and PE $[(\alpha\beta)_6\gamma]$, respectively.

Method II. The second method of pigment analysis is as described for *Anacystis* and *Neoagardhiella* (15). The sample was homogenized in 100 mM NaP_i (pH 7.0) and the Chl content of the homogenate was determined in 80% (v/v) acetone. Aliquots of the homogenate were loaded onto a Celite column and eluted with the extraction buffer. The phycobiliprotein content of the Chl-free eluate was determined spectrophotometrically as described above.

The concentration of the reaction center PSI (P_{700}) was determined from the light-induced bleaching near 700 nm in Triton-solubilized membranes (15, 20). The concentration of the reaction centers of PSII was determined from the O_2 yield per microsecond flash under steady state conditions (15).

Purification of Biliproteins. R-PE, C-PC, and APC of wild type *Gracilaria* were purified by two methods.

Method I. About 5 g fresh algae in 30 ml of 50 mM NaP_i (pH 7.0) was homogenized in a Polytron and French pressed (15). The homogenate was centrifuged for 40 min at 48,000*g*. The supernatant was fractionated with $(NH_4)_2SO_4$ followed by chromatography on Sephadex G-200 and hydroxylapatite (21). The Sephadex G-200 column (1.6 × 80 cm) was eluted with 100 mM NaP_i (pH 5.5) while the effluent was monitored at 545 nm. Upon passage through G-200, the 30 to 35% $(NH_4)_2SO_4$ cut, rich in PC, separated into two main fractions: band Ia, with a V_e/V_o value of 1.45, was primarily PC and APC while band Ib, with a V_e/V_o of 1.88 contained PC and PE. When chromatographed on G-200, the 35 to 40% $(NH_4)_2SO_4$ cut, rich in PE, separated into two components: band IIa, with a V_e/V_o value of 2.00 and was primarily PE. The PE-rich fractions (bands Ib and IIb) were combined, concentrated by ultrafiltration, and chromatographed again on Sephadex G-200. Two main factors were obtained: band IIIa, with a V_e/V_o of 1.45 (PC), and band IIIb, with a V_e/V_o of 2.20, which contained predominantly PE. Band IIIb was concentrated, equilibrated with 10 mM NaP_i (pH 6.7), 0.20 M NaCl, and loaded onto a 10-ml syringe containing 5 ml of hydroxylapatite. The bound material was eluted in a stepwise fashion with increasing concentrations of NaP_i (pH 6.7) containing 0.20 M NaCl. Spectroscopically pure PE eluted at 10 to 20 mM NaP_i. The PC- and APC-rich fractions from the G-200 columns (bands Ia and IIa) were combined and chromatographed on hydroxylapatite as described above. PC, monitored at 620 nm, eluted at 50 mM NaP_i, and APC eluted at 100 mM NaP_i. Hydroxylapatite chromatography of PC- and APC-rich fractions was repeated until spectrally pure preparations were obtained.

Method II. Purification of *Gracilaria* phycobiliproteins was achieved in less than 24 h as follows: The algae were homogenized, substituting a buffer of 100 mM NaP_i (pH 5.5), and centrifuged as described above. The supernatant was precipitated with $(NH_4)_2SO_4$ and the precipitate was dialyzed against 1.0 mM NaP_i (pH 5.5) containing 0.20 M NaCl. The preparation was fractionated on hydroxylapatite as described above except that the NaP_i buffer was pH 5.5. Under these conditions, PC elutes first at 1 to 50 mM NaP_i, PE elutes at 10 to 100 mM NaP_i, and APC, which elutes slowly at 200 mM NaP_i, can be efficiently removed from the column using a pH gradient of 5.5 to 6.0 of the 200 mM phosphate buffer.

PE purified from wild type *Gracilaria* by methods I and II have equivalent absorption and fluorescence spectra. PC and APC purified by methods I and II have identical fluorescence emission properties, though the absorption peaks of these two proteins purified by the slower procedure, method I, were shifted 2 to 3 nm to the blue. This blue shift probably indicates disaggregation resulting from dilution and the lengthy purification.

Chromophore and Protein Analyses. The chromophore composition of purified PC was determined in 8 M urea-HCl, 10 mM 2-mercaptoethanol (pH 2.0) (5, 10). The extinction coefficients were assumed to be 104,000 M^{-1} cm^{-1} at 496 nm and zero at 555 nm for phycourobilin and 43,000 M^{-1} cm^{-1} at 555 nm and 12,000 M^{-1} cm^{-1} at 496 nm for phycoerythrobilin (5, 10).

Spectroscopic Measurements. Fluorescence measurements were carried out with an Aminco SPF 500 spectrofluorometer. Absorption measurements were made with an Aminco DW-2 dual beam spectrophotometer and in a few cases with a Pye-Unicam 1800. Chl *a* concentration was determined and wavelength was calibrated using the 664.3 nm peak of Chl *a* in 90% (v/v) acetone (14).

RESULTS

Pigment Composition. To obtain a preparation which approximated the *in vivo* absorption properties of *Gracilaria*, an algal homogenate was made 0.2% (v/v) in Nonidet P-40 and clarified by centrifugation. Comparisons of the spectra of wild type, *ora* and *vrt*^{2} (Fig. 1) indicate that, when normalized to Chl *a*, *ora* has an excess of PE and *vrt*^{2} is deficient in PE. The biliproteins and Chl in these and a number of other mutants were subsequently quantified (Table I).

The pigment analyses of *Anacystis*, *Neoagardhiella*, and the *Gracilaria* strains are presented in three ways in Table I: (a) the ratio of bilin chromophores to Chl *a*, referred to as the chromophore composition; (b) the biliprotein composition; and (c) the nmol of Chl *a* per g fresh weight of algae, referred to as the Chl content. The pigment composition of the *Gracilaria* strains is compared with *Anacystis*, a cyanobacterium lacking PE, and *Neoagardhiella*, another macrophytic red alga. The pigment composition of these latter two species represents the extremes between which most of the *Gracilaria* pigment mutants are found.

The chromophore composition (Table I) describes the number of mol of bilin chromophores per mol of Chl. The APC to Chl ratio (APC/Chl) and the PC to Chl ratios (PC/Chl) of most of these mutants are within a factor of three of the wild type values (Table I, columns b and c). The chromophore ratios of the *Gracilaria* strains are comparable to those of the cyanobacterium *Anacystis*, which lacks PE, and to the red alga *Neoagardhiella* (Table I).

The absorption spectra (Figs. 2, 3, and 4) of the aqueous extracts of 11 strains demonstrate the variation in biliprotein composition in the *Gracilaria* mutants. The biliprotein composition is expressed as a weight percent of the total biliprotein

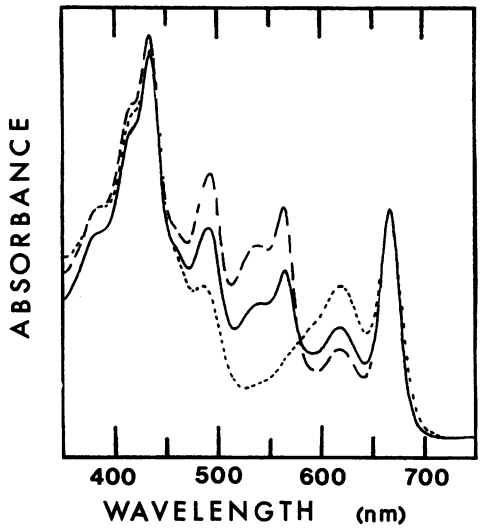

FIG. 1. Absorption spectra of total *Gracilaria* pigment extracts. The algae were disrupted in 50 mM NaP_i (pH 5.5) with a French press, made 0.2% (v/v) in Nonidet P-40, and extracted with a glass homogenizer. The detergent solution was centrifuged for 10 min at 12,000*g* and the light brown pellet was discarded. The supernatant was only slightly turbid; the absorption spectrum was measured with an opal glass and end-on photomultiplier to correct for any residual light scattering. The Chl concentration was about 10 μM and the samples were normalized to an equal absorbance at 670 nm. The samples are wild type (——), *ora* (— — —), and *vrt*^{2} (- - - -).

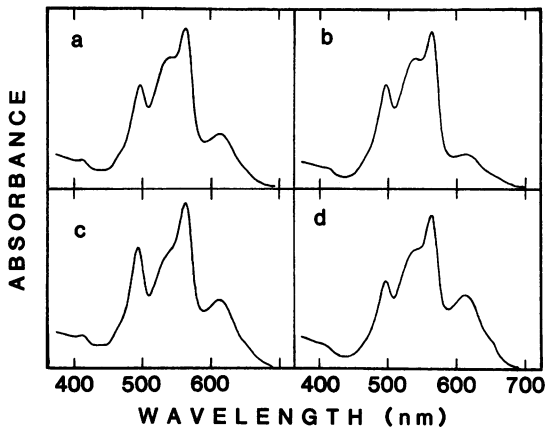

FIG. 2. Absorption spectra of total aqueous extracts of wild type and mutant *Gracilaria* having high phycoerythrin contents. The samples were prepared for pigment analysis by method I (see "Materials and Methods"). In each case, a room temperature absorption spectrum was taken of the supernatant from the first centrifugation. (a), Wild type; (b), *ora*; (c), *obr*; (d), *vrt*^{5}.

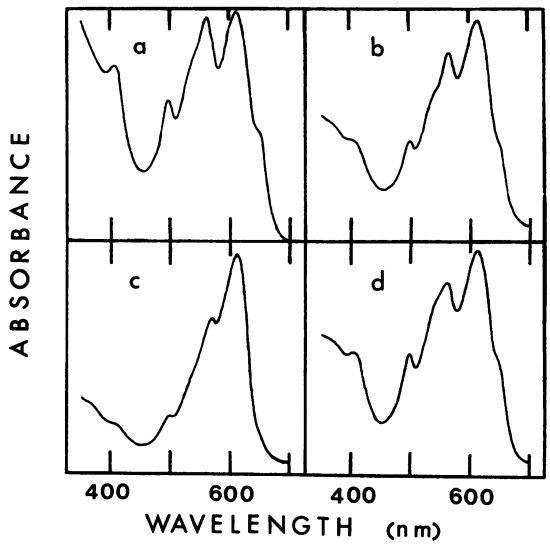

FIG. 3. Absorption spectra of total aqueous extracts from mutants of *Gracilaria* having a low phycoerythrin content. The samples were prepared as described in Figure 2 except that the samples were homogenized with a mortar and pestle. (a), *vir* (G-12-1), (31% APC, 46% PC, 23% PE); (b), *olv* (G-6-1), (28% APC, 60% PC, 12% PE); (c), *grn,mos*; (d), *NMG-1*.

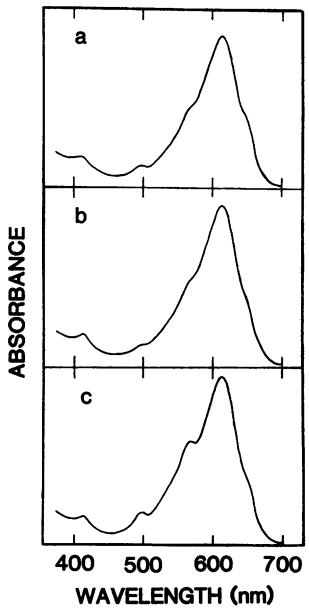

FIG. 4. Absorption spectra of total aqueous extracts from mutants of *Gracilaria* containing trace amounts of PE. The samples were prepared as described in Figure 2. (a), *vrt*^{2}; (b), *uai*; (c), *NMG-2*.

(Table I, columns f, g, and h). These values can be calculated directly from the chromophore compositions and are presented separately in order to indicate the relative abundance of the three biliproteins. The only exceptional PC to APC ratios (PC/APC; Table I, column i) of the *Gracilaria* strains are those of *NMY-1* which is 0.95, and *mos*^{2} and the double mutant *grn, mos* which are both greater than 10. In the presence of a great excess of PC, equation 1 is not reliable; hence, the APC contents of *mos*^{2} and *grn, mos* are uncertain. Otherwise the PC/APC ratios of the remaining strains are similar, 1.7 to 2.4, even though the abundance of these proteins with respect to Chl is quite variable. Possibly the synthesis of PC and APC is coupled in a manner which reflects their structural role in the phycobilisome. The PE to PC ratios (PE/PC; Table I, column j) range from 5.1 in *ora* to 1.8 in wild type, to near zero in the bright green mutants. The biliprotein composition of the mutant *ora* with 78% PE is similar to that of *Neoagardhiella* (86% PE) while the bright green mutants, which contain about 65% PC (excepting *grn, mos*), are most similar to *Anacystis* which contains 86% PC. In the wild type, the phycobiliproteins represent 10% of the total protein. The Chl content of the *Gracilaria* mutants is not altered by more than 2-fold compared to the wild type (Table I, column k). Inasmuch as Chl does not change, *Pur*^{2} has very high APC/Chl, PC/Chl, and PE/Chl values.

Reproducible pigmentation results are obtained only with material that is growing vigorously; the use of an alternate medium or culturing regime is likely to change pigmentation. The method of tissue sampling and the plant morphology will

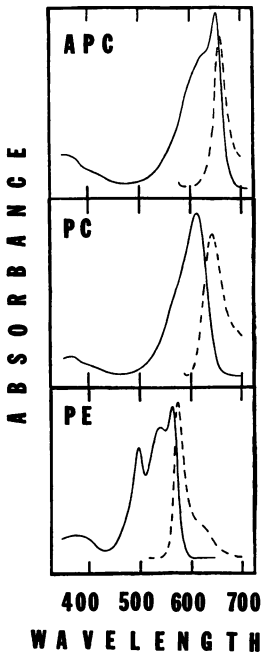

FIG. 5. Room temperature absorption and fluorescence emission spectra of the purified phycobiliproteins from wild type *Gracilaria*. The proteins were purified by the fast procedure described in "Materials and Methods" (method II) and dialyzed against 50 mM NaP_i (pH 5.5). For the absorption spectra (——) the protein concentrations were about 0.10 mg/ml. The fluorescence emission spectra (----) were corrected on a quantum basis and the maximal absorbance of the sample was less than 0.05. The excitation wavelengths were 570 nm for APC, 580 nm for PC, and 498 nm for PE.

also influence pigment analyses. In wild type and many mutants, the young tissue near the growing apex has a higher biliprotein to Chl ratio than the more mature tissue (data not shown). In this study, we did not attempt to distinguish between these different tissues and have, in all cases, analyzed the whole plant. Some strains such as *NMG-2* have many growing tips and others such as *ora* have few growing tips. The bias these morphological differences introduce into the pigment analysis results can be ignored inasmuch as the whole plant samples consist primarily of mature tissue.

Total Aqueous Extracts. Although there are a number of exceptions, a total aqueous extract of wild type or mutant *Gracilaria* has absorption peaks at 498, 545, and 565 nm assigned to R-PE, a maximum at 611 to 618 nm assigned to C-PC, and a shoulder at 650 nm assigned to APC (Fig. 2). In the bright green mutants, the absorption spectrum of R-PE is not recognizable; in two strains, the relative abundance of PC results in the loss of the 565 nm absorption maximum of PE (Fig. 4, a and b). Mixtures of wild type PE and PC in appropriate amounts mimic the spectroscopic properties of crude extracts of the mutants. Except for *obr* (discussed below), the mutants examined probably contain a PE which is spectroscopically equivalent to wild type. The total aqueous extract of *NMY-1* has an absorption maximum at 650 nm rather than a shoulder and the 611 to 618 nm peak is absent. Mos^2 and the double mutant *grn, mos* are the only mutants which lack the 650 nm shoulder assigned to APC. Mos^2 does contain APC since the PC/APC ratio of the isolated phycobilisomes of wild type and mos^2 are similar (16). Therefore, except for *obr*, the bright green mutants, mos^2, and *NMY-1*, the spectra of total aqueous extracts of the mutants examined are spectroscopically homologous to wild type *Gracilaria*.

Purified Biliproteins. The purified R-PE of wild type *Gracilaria* has absorption maxima in the visible region at 498, 545, and 565 nm and, with excitation at 498 nm, a fluorescence emission peak at 574 nm (Fig. 5, Table II). The PE of *Gracilaria* is quite similar to the R-PEs from the macrophytic red algae *Ceramium, Griffithsia*, and *Callithamnion* (8, 19, 30). The ratio of phycoerythrobilin to phycourobilin chromophores is 4.2 as determined in acid urea (see "Materials and Methods"). A second spectroscopic type of PE, similar to PE II characterized by others (29, 30), accounted for less than 10% of the total PE and was not purified. The unusually deep trough at 511 nm of an aqueous extract of *obr* (Fig. 2, panel c), a feature lacking in all other mutants, indicates that the PE of this mutant differs from that of wild type. The purified PE from *obr* has absorption peaks at 498 and 564 nm and a shoulder rather than a peak at 545 nm (Fig. 6a; Table II), and a ratio of phycoerythrobilin to phycourobilin chromophores of 2.6. However, the fluorescence emission spectrum of *obr* PE is identical to that of wild type (Figs. 5 and 6a; Table II). The purified PEs of *ora*, vrt^5, and *NMY-1* are

Table II. *Absorption and Fluorescence Characteristics of G. tikvahiae Biliproteins*

The individual biliproteins were purified in less than 24 h by method II, using hydroxylapatite chromatography, and ammonium sulfate precipitation. For spectroscopic analysis, the samples were dialyzed against a 50 mM NaP_i buffer (pH 5.5) and measured at room temperature. The protein concentration used for absorption measurements was 0.06 to 0.14 mg/ml. Fluorescence emission spectra were corrected on quantum basis and the sample absorbance was less than 0.05 at the visible absorption maximum. The PE samples were excited at 580 nm (15 nm bandpass), and the PC and APC samples were excited at 580 and 570 nm, respectively (10 nm bandpass). The emission bandpass was 1.0 nm. Samples which lacked absorption due to the presence of either of the other two biliproteins were considered to be pure.

Phycobiliprotein	Genotypes	Absorption Maxima	Principal Fluorescence Emission Maximum
		nm	
PE	Wild type, vrt^5, *ora, NMY-1*	498, 545, 565	574 ± 1
PE	*obr*	498, 545 (shoulder), 564	573 ± 1
PC	Wild type, vrt^2, vrt^5, *uai, obr, NMG-2, ora*	614 ± 1	641 ± 1
PC	*NMY-1*	613 ± 1	638 ± 1
APC	Wild type, vrt^2, vrt^5, *NMG-2, NMY-1*	651 ± 1	660 ± 1

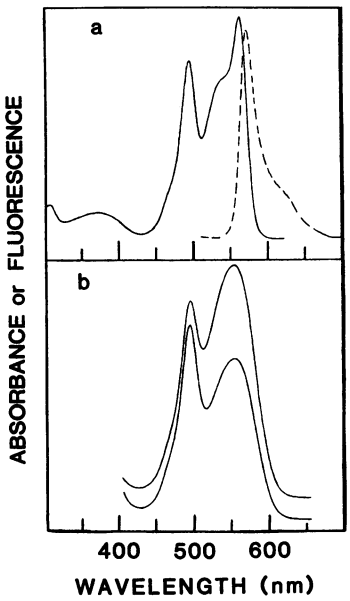

FIG. 6. (a), Absorption (——) and fluorescence emission (- - - -) spectra of the purified PE of the *obr* mutant of *Gracilaria*. Experimental conditions were as described in Figure 5 for PE. (b), Absorption spectra of *Gracilaria* wild type and *obr* PE in 50 mM P_i (pH 5.5) were diluted into 8 M urea-HCl (pH 2.0) containing 2.0 mM 2-mercaptoethanol. The final protein concentration was 0.75 μg/ml assuming equal extinction coefficients for the wild type and mutant PEs. The spectra were recorded within 5 min of sample preparation. The upper trace is wild type and the lower trace is *obr* PE.

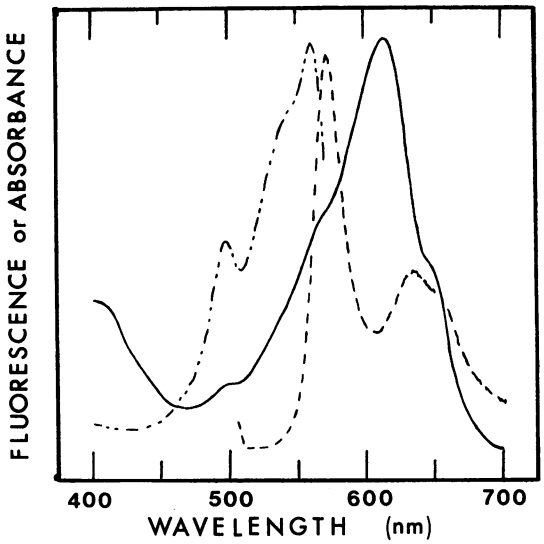

FIG. 7. Absorption spectrum and fluorescence analysis of the *Gracilaria* double green mutant *vir,olv*. The sample was prepared for pigment analysis by method I in "Materials and Methods" and the first aqueous extract was analyzed. The absorbance (——) was 0.094 at 614 nm and fluorescence was analyzed in the undiluted sample. The fluorescence spectra (- - - -) was corrected on a quantum basis. The sample was excited at 498 nm (15 nm bandpass) and the emission was scanned with a 1 nm bandpass. The fluorescence excitation spectrum (— - - —) of the 580 nm emission (15 nm bandpass) was scanned with a 2 nm bandpass and is not corrected.

spectroscopically equivalent to that of wild type (Table II). Wild type C-PC and APC have spectroscopic properties which are similar to those described from cyanobacteria and from unicellular and macrophytic red algae by other workers (Fig. 5; Table II). The PCs of *vrt*^{2}, *vrt*^{5}, *uai*, *obr*, *NMG-2*, and *ora* are spectroscopically equivalent to those of wild type (Table II). Both the absorption and fluorescence emission spectra of the purified PC of *NMY-1* are blue shifted a few nm with respect to wild type PC (Table II).

The primary criteria for the presence of wild type biliproteins in the mutants are the spectroscopic properties of the purified pigment-proteins. In many strains, the biliproteins have not been purified; if the spectral properties of the total aqueous extracts of wild type and a given mutant are similar, it is assumed that the respective biliproteins were equivalent. The total aqueous extracts of the bright green mutants, *vrt*^{2}, *uai*, *NMG-2*, and the double mutants, *grt,grn*, *vrt,olv*, and *grn,mos*, do not contain enough PE to permit reliable detection by absorption spectroscopy (Figs. 3 and 4). In these cases, fluorescence was used to demonstrate the presence of PE. A fluorescence emission peak at 574 nm characteristic of R-PE observed in crude aqueous extracts of bright green mutants (Fig. 7). This emission spectrum is nearly equivalent to that of R-PE purified from wild type *Gracilaria*. The enhanced long wavelength tail in the emission spectrum of these mutants (Fig. 7) when compared to the purified R-PE in Figure 5 is probably due to the large amount of PC present in the crude extract. By these criteria, the other five bright green mutants also contain PE (data not shown). The crude aqueous extractof *NMY-1* lacks the 615 nm peak assigned to PC, however, a PC which is spectroscopically quite similar to wild type C-PC can be purified from this mutant (Table II). Each mutant appears to be capable of making all three biliproteins which has been confirmed by protein purification, absorption spectroscopy, and by fluorescence spectroscopy.

Reaction Center Concentrations and Mutant Summary. In wild type and eight mutants of *Gracilaria*, the ratio of Chl to the RC of PSI is 160 (±30). The relative concentration of RC II as measured by the method of Emerson and Arnold (see 15) is expressed as the number of Chl per O_2 evolved in a single flash or Chl/O_2. The Chl/O_2 values are 1540 (±250) and 1450 (±250) for wild type *Gracilaria* and *ora* cultured in the lab and 1325 (± 200) for *Gracilaria* collected in the field. The absolute RC II concentration was obtained by dividing the Chl/O_2 ratio by 4 (15). Therefore, the RC I/RC II ratio is about 2.2 in wild type *Gracilaria* which is similar to the ratio in *Anacystis* and *Neoagardhiella* (15).

The properties of the individual pigment mutants can be summarized as follows: on a fresh weight basis, *Pur*^{2} has a normal complement of biliproteins and a 4-fold decrease in Chl; hence, *Pur*^{2} has a large excess of biliprotein relative to Chl; *ora* contains an excess of PE relative to Chl or PC; on a fresh weight basis, *ora* has a low PC and APC content; *vrt*, *vrt*^{5}, *vrt*^{2}, *uai*, *NMG-2*, *NMG-1*, *vir,olv*, and *grt,grn* are all low in PE to varying degrees; *mos*^{2} and *grn,mos* are low in PE and probably in APC and accumulate excess PC; *obr* contains an altered PE; and *NMY-1* is a complex mutant which is low in all three biliproteins.

DISCUSSION

The characterization of the life cycle of the red alga *G. tikvahiae* allowed for the development of a genetic system in which controlled crosses could be made, and in which stable mutations could be selected and maintained (27). This is the first known genetic system in a phycobiliprotein-containing organism among the cyanobacteria, red algae, or Cryptomonads. The genesis of this system has been described in detail by one of us (26, 27). The genetic control of the synthesis, and function of the phycobiliproteins and phycobilisomes of the red algae is being examined in *Gracilaria*. Since *Gracilaria* is eukaryotic, the cooperative interaction of the nuclear and chloroplast genomes in these processes can be examined. PE is an abundant protein in *Gracilaria* and makes up about 5% of the total protein; however, it

does not appear to be essential for photosynthesis, because mutants which possess only traces of this pigment are slow-growing but vigorous. Furthermore, none of the mutants isolated to date are PE minus; PE may serve an alternate function essential for growth.

The Chl content of the *Gracilaria* mutants varied by as much as 2-fold on a fresh weight basis. A large part of this variation appears to be related to plant morphology. Plants with slender fronds have more pigmented surface tissue per unit weight than do thicker fronds. The bright green mutants are noticeably thinner, accounting, at least in part, for their increased Chl content. Thus, the observed Chl contents (Table I, column k) cannot be interpreted in terms of the structure of the photosynthetic apparatus, unless accompanied by an anatomical study.

In most of the *Gracilaria* strains examined in the present study, the color of the thallus, or pigment phenotype, was correlated with its pigment composition. The wild type thallus appears reddish-brown in color and contains Chl *a*, carotenoids, and the phycobilipigments APC, PC, and PE. A Chl- and carotenoid-free aqueous extract of the wild type is red, due to the presence of PE. The mutants with a bright green phenotype contain little PE. Their phenotype results principally from Chl *a* and to a lesser extent from the carotenoids and the blue PC and APC. Chl free aqueous extracts of bright green mutants are blue due to the very low levels of PE. Many of the green and brownish-green mutants (*i.e. vrt*, *mos*^{2}, and *vrt*^{5}) have a higher R-PE content than the bright green mutants but a lower R-PE content than the wild type. Consequently, their aqueous extracts are purple which, when combined with Chl *a* and the carotenoids gives the frond a green-brown color. The mutant *NMY-1* is yellow in appearance for reasons which are unclear. In contrast, *ora* and *Pur*^{2} and the red alga *Neoagardhiella* have a red color which results from their high content of R-PE. The mutant *obr* possesses a brownish-green thallus color, yet has a slightly reduced level of PE compared to the wild type. This mutant synthesizes a spectroscopically altered PE resulting in a 530 nm peak in the wild type minus *obr* difference spectrum in the total pigment extract. A decrease in the *A* of 530 nm light by *obr* can account for the greenish thallus color.

The pigment mutants described above have nuclear- and non-nuclear-controlled alterations in many aspects of the assembly or synthesis of the components of the light harvesting system. As a conservative estimate, more than 30 gene products could be required to synthesize all three biliproteins (chromophores and apoproteins), enzymes which might function in chromophore attachment, and proteins required to assemble the phycobilisome. Among the mutants examined, the levels of PE and PC are dramatically influenced by nuclear mutations. If the structural genes for synthesis are not nuclear, certainly nuclear genes control the accumulation or stabilization of these phycobiliproteins.

One nuclear mutant examined, *obr*, synthesizes a PE which is spectroscopically altered though similar to naturally occurring PE from other red algal species (3, 13, 28, 30). The molar ratios of phycoerythrobilin to phycourobilin in wild type and *obr* R-PEs is 4.2 and 2.6, respectively; therefore, *obr* can synthesize phycoerythrobilin, yet its PE is deficient in this chromophore. Comparable PEs were isolated from two species of *Callithamnion* and from *Callithamnion roseum* grown under a range of light intensities (30). The PEs of wild type and *obr* chromatograph equivalently on hydroxylapatite, the PE content of *obr* is nearly the same as that of wild type, and the phycobilisomes from these two strains are also equivalent in size and structure (16). Hence, in most respects the *obr* and wild type PEs are quite similar. An interesting possibility is that *obr* is a structural gene mutation which has resulted in the replacement or loss of a cysteinyl residue, as it is unlikely (see above) that a large deletion results from the mutation. Cysteinyl residues have been shown to be responsible for chromophore attachment in both PC and APC (9, 24), and the addition of a 12 amino acid sequence containing a cysteinyl residue during evolution can account for the difference in chromophore composition between the α and β subunits of a red algal PC (24).

These findings provide the first evidence for the role of nuclear genes in the synthesis of PE. This proposal is consistent with a model in which the genes coding for the apoprotein of the major light-harvesting pigment-proteins, such as the light-harvesting Chl *a*/*b* protein of green plants, are nuclear, whereas the structural genes for reaction center pigment-proteins such as the P_{700} Chl *a* protein are encoded in the chloroplast (23).

Acknowledgments—We would like to thank Dr. R. Troxler for many helpful discussions.

LITERATURE CITED

1. BERNS DS 1967 Immunochemistry of biliproteins. Plant Physiol 42: 1569–1586
2. BIRD N, J MCLACHLAN, D GRUND 1977 Studies on *Gracilaria*. 5. In vitro life history of *Gracilaria* sp. from the Maritime Provinces. Can J Bot 55: 1282–1290
3. BONEY AD, EB WHITE 1968 Phycoerythrins from some *Acrochaetium* species. Nature 218: 1068–1069
4. BROWN AS, GD OFFNER, MM EHRHARDT, RF TROXLER 1979 Phycobilin-apoprotein linkages in the α and β subunits of phycocyanin from the unicellular rhodophyte, *Cyanidium caldarium*. Amino acid sequences of ^{35}S-chromopeptides. J Biol Chem 254: 7803–7811
5. BRYANT DA, G COHEN-BAZIRE, AN GLAZER 1981 Characterization of the biliproteins of *Gloeobacter violaceus*. Chromophore content of a cyanobacterial phycoerythrin carrying phycourobilin chromophores. Arch Microbiol 129: 190–198
6. GANTT E 1979 Phycobiliproteins of Cryptophyceae. *In* GM Levandowsky, SH Hutner, eds, Biochemistry and Physiology of the Protozoa, Ed 2, Vol 1. Academic Press, New York, pp 121–137
7. GANTT E 1981 Phycobilisomes. Annu Rev Plant Physiol 32: 327–347
8. GANTT E, CA LIPSHULTZ 1980 Structure and biliprotein composition of phycobilisomes from *Griffithsia pacifica* (Rhodophyceae). J Phycol 16: 394–398
9. GLAZER AN 1977 Structure and molecular organization of the photosynthetic accessory pigments of cyanobacteria and red algae. Mol Cell Biochem 18: 125–140
10. GLAZER AN, CS HIXSON 1975 Characterization of R-phycocyanin. Chromophore content of R-phycocyanin and C-phycoerythrin. J Biol Chem 250: 5487–5495
11. GLAZER AN, GS APELL, CS HIXSON, DA BRYANT, S RIMON, DM BROWN 1976 Biliproteins of cyanobacteria and Rhodophyta: homologous family of photosynthetic accessory pigments. Proc Natl Acad Sci USA 73: 428–431
12. GUILLARD RRL, JH RYTHER 1962 Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Deltonula confervaceae* (Cleve) Gran. Can J Microbiol 8: 229–239
13. HIROSE H, S KUMANO 1966 Spectroscopic studies on the phycoerythrins from Rhodophycean algae with special reference to their phylogenetical relations. Bot Mag Tokyo 79: 105–113
14. JEFFREY SW, GF HUMPHREY 1975 New spectrophotometric equations for the determination of chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton. Biochem Physiol Pflanzen 167: 191–194
15. KURSAR TA, RS ALBERTE 1983 Photosynthetic unit organization in a red alga. Relationships between light-harvesting pigments and reaction centers. Plant Physiol 72: 409–414
16. KURSAR TA, J VAN DER MEER, RS ALBERTE 1983 Light-harvesting system of the red alga *Gracilaria tikvahiae*. II. Phycobilisome characteristics of pigment mutants. Plant Physiol 73: 361–369
17. MACCOLL R, DS BERNS 1979 Evolution of the biliproteins. Trends Biochem Sci 4: 44–47
18. MACDOWALL FDH, T BEDNAR, A ROSENBERG 1969 Conformation dependence of intramolecular energy transfer in phycoerythrin. Proc Natl Acad Sci USA 59: 1356–1363
19. O'CARRA PO 1965 Purification and N-terminal analyses of algal biliproteins. Biochem J 94: 171–174
20. SHIOZAWA JA, RS ALBERTE, JP THORNBER 1974 The P700-chlorophyll *a* protein. Isolation and characteristics of the complex in higher plants. Arch Biochem Biophys 165: 388–397
21. SIEGELMAN HW, GA WIECZOREK, JL BAILEY 1967 Preparation of calcium phosphate for protein chromatography. Anal Biochem 13: 402–404
22. THORNBER JP, J BARBER 1979 Photosynthetic pigments and models for their organization *in vivo*. Topics Photosynth Res 3: 27–70
23. THORNBER JP, RS ALBERTE, FA HUNTER, JA SHIOZAWA, K-S KAN 1977 The organization of chlorophyll in the plant photosynthetic unit. Brookhaven

Symp Biol 28: 132–148
24. TROXLER RF, MM EHRHARDT, AS BROWN-MASON, GD OFFNER 1981 Primary structure of phycocyanin from the unicellular Rhodophyte *Cyanidium caldarium*. II. Complete amino acid sequence of the β subunit. J Biol Chem 256: 12176–12184
25. VAN DER MEER JP 1978 Genetics of *Gracilaria* sp. (Rhodophyceae, Gigartinales). III. Non-Mendelian gene transmission. Phycologia 17: 314–318
26. VAN DER MEER JP 1979 Genetics of *Gracilaria* sp. (Rhodophyceae, Gigartinales). V. Isolation and characterization of mutant strains. Phycologia 18: 47–54
27. VAN DER MEER JP 1979 Genetics of *Gracilaria tikvahiae* (Rhodophyceae). VI. Complementation and linkage analysis of pigmentation mutants. Can J Bot 57: 64–68
28. VAN DER VELDE HH 1973 The use of phycoerythrin absorption spectra in the classification of red algae. Acta Bot Néerl 22: 92–99
29. VAN DER VELDE HH 1973 The natural occurrence in red algae of two phycoerythrins with different molecular weights and spectral properties. Biochim Biophys Acta 303: 246–257
30. YU M-H, AN GLAZER, KG SPENCER, JA WEST 1981 Phycoerythrins in the red alga *Callithamnion*. Variation in phycoerythrobilin and phycourobilin content. Plant Physiol 68: 484–488