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Fluorescence spectroscopy and photochemistry of phytochromes A and B in wild-type, mutant and transgenic strains of *Arabidopsis thaliana*

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Abstract

Phytochrome (P) was characterized in etiolated seedlings of wild-type, mutant and transgenic strains of *Arabidopsis* with the use of low-temperature (85 K) fluorescence spectroscopy and photochemistry. The position (λ_{max}) of the Pr emission spectrum, its intensity (F_0) proportional to [P_{tot}] and the extent of the Pr→lumi-R phototransformation at 85 K (γ_1) were shown to vary depending on the plant strains and tissues used, while the extent of the Pr→Pfr transformation at 273 K (γ_2) remained relatively constant. Depletion of phyA (*fre1-1* in Nagatani et al., Plant Physiol. 102 (1993) 269–277, and *fhy2-2* in Whitelam et al., Plant Cell 5 (1993) 757–768) resulted in a steep decrease of F_0 to ≈10%. The *phyB* mutant (*hy3-Bo64* in Reed et al., Plant Cell 5 (1993) 147–157) revealed a slight reduction (by ≈20%) of F_0 while λ_{max} and γ_1 remained practically unaffected. In *phyAphyB* mutant no P emission was observed. Overexpression of oat phyA (*13k7* and *21k15* in Boylan and Quail, Proc. Natl. Acad. Sci. USA 88 (1991) 10806–10810) brought about an increase of F_0 by two to three times, a shift of λ_{max} to 685 nm and an increase of γ_1 to 0.3–0.4. On the contrary, an increase of F_0 (up to 40%) in *Arabidopsis* and rice phyB overexpressors (*ABO* and *RBO* in Wagner et al., Plant Cell 3 (1991) 1275–1288) was followed by a decrease of γ_1 values to 0.13–0.14. These data together with the results on *phyB* (*lh*) mutant of cucumber prove the existence of the two phyA populations with high (phyA′) and low (phyA″) photochemical activity at low temperatures. PhyB emits maximally in the same region as phyA in *Arabidopsis* (≈683 nm) and at shorter wavelength (<680 nm) in rice. It is characterized by low photochemical activity at 85 K ($\gamma_1 \leq 0.05$)) and can be attributed in this respect to the same pigment type as phyA″. © 1998 Elsevier Science S.A.

Keywords: Phytochromes A and B; Fluorescence; Spectroscopy; Photochemistry; Etiolated plants; Mutant and transgenic strains; *Arabidopsis thaliana*, in situ

1. Introduction

Phytochrome is a unique photoinformation pigment consisting of a linear tetrapyrrole covalently bound to a ≈125 kDa apoprotein. It mediates a great variety of photoregulation reactions, thus allowing plants to adapt themselves to environmental light conditions. Recent success in its investigation is connected with the discovery of a number of pigment types that differ by the apoprotein encoded by a small family of genes (A through E in *Arabidopsis* and possibly more in tomato and sorghum). For the two major phytochromes, A and B (phyA and phyB), it is shown that they possess distinct functions but some of them can overlap. Less defined are the functions of the minor phytochromes (C–E), although they are not redundant (for recent reviews, see Ref. [1]).

Heterogeneity of phytochrome in the cell was also observed with the use of in situ low-temperature fluorescence spectroscopy and photochemistry (works devoted to this experimental approach are reviewed in Ref. [2]). It was found [3] that photophysical and photochemical properties of native phytochrome in its red-light-absorbing form (Pr) varied depending on the plant species and tissues used, its developmental state and environmental conditions. This was interpreted as the existence of two distinct pigment species: a longer-wavelength one with high photochemical activity at low temperatures (85 K), Pr′, and a shorter-wavelength one inactive at 85 K, Pr″. Because the *lh* mutant of cucumber lacking phyB did not practically show differences in the proportion of Pr′ and Pr″ as compared with the wild-type plants, both of them are likely to belong to phyA (phyA′ and phyA″) [4]. From the experiments [5] on transgenic potato with modified phenotypes (due to anti-sense synthesis and overexpression of phyA [6]) it was suggested that phyA′ functions in the de-etiolation process while phyA″ could be operative in green tissues. This is consistent with the observations that (i) phyA′ is light-labile while phyA″ is relatively

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light-stable [2,3], (ii) phyA remains in low quantity in green plants [7,8] and (iii) phyA is functional under constant illumination [9]. Preliminary data suggest that phyA″ could be a minor membrane (protein)-associated fraction of the pigment [10].

In the present work, we went on investigating the problem of the heterogeneous phyA population and for the first time turned to the fluorescence and photochemical characterization of phyB in its native state in the cell with the use of phytochrome mutants and overexpressors of *Arabidopsis*. The properties of *Arabidopsis* phytochrome were directly compared with those of the pigment in some other plants. Investigations of the phytochrome species from the chromophoric side acquire special interest because their functional specificity may be connected with structural differences of the chromophore-bearing N-terminal domain [11].

2. Experimental details

2.1. Plant materials and sample preparation

Wild-type (WT) mouse ear cress (*Arabidopsis thaliana* cv. Landsberg *erecta*) plants and their phytochrome-deficient mutants (*phyB* strain, *phyB-1*, previously *hy3-Bo64* and *hy3-8-36* [12,13]; *phyA* strain, *phyA-201* and *phyA-2*, previously *fre1-1* [14] and *fhy2-2* [15], respectively; *phyAphyB* strain, *phyA-2phyB-1*, obtained by Devlin and Whitelam) and phytochrome overexpressors in *Arabidopsis thaliana* (cv. Nossen) background (oat PHYA, *13k7* and *21k15* [16]; *Arabidopsis* and rice PHYB, *ABO* and *RBO*, respectively [17]) and their isogenic wild type were used. Experiments were also carried out on wild-type and *phyB* mutant (*lh*) of cucumber (*Cucumis sativus* L.) [18], wild-type and phytochrome chromophore mutant of tomato, *aurea*, *au* (*Lycopersicon esculentum* L.) containing less than 5% of the level of phytochrome in wild-type tissue [19] and wild-type garden cress (*Lepidium sativum* cv. Armada), oat (*Avena sativa* cv. unknown) and maize (*Zea mays* cv. Kubansky-13).

All the experiments were carried out on etiolated seedlings. To produce three-to-four-day-old *Arabidopsis* plants, seeds were placed on filter paper moistened with tap water for 24 h at 26°C and then kept for 1–14 days (depending on strain used) at ≈1°C in complete darkness. After such a 'cold activation' of the imbibed seeds, they were illuminated with white light (60 W incandescent lamp at a distance of 30 cm): *13k7* and *21k15* for 5 min; wild type (cv. Landsberg *erecta* and Nossen) for 10 min; *ABO* and *RBO* for 15 min; *phyA* mutant for 20 min; *phyB* mutant for 1 h; *phyAphyB* mutant for 6 h. After the light activation of germination, seeds were placed back into complete darkness at 26°C and grown for three to four days until the length of etiolated hypocotyls achieved 0.5–0.8 cm. The sizes of three-day-old hypocotyls of the wild-type plant and *phyB* mutant were almost the same (0.8 ± 0.1 and 0.7 ± 0.1 cm, respectively) while in the case of *phyA* mutant it was somewhat lower, 0.45 ± 0.05 cm. All the overexpressors were characterized by a higher rate of growth than the wild type. Already two-day-old seedlings reached a length of 0.6 ± 0.05 cm (*13k7*), 0.5 ± 0.05 cm (*21k15*), 0.6 ± 0.05 cm (*RBO*) and 0.5 ± 0.05 cm (*ABO*). Dynamics of P content in growing seedlings were followed with the use of the fluorescence technique (see below) for each strain and seedlings containing maximum P were taken for the sample.

Mutant and isogenic wild-type etiolated plants of cucumber and tomato as well as wild-type cress, maize and oat plants were also grown in complete darkness (without cold and light pre-treatment) on filter paper moistened with tap water for three to five days at 26°C.

To obtain the samples of *Arabidopsis* hypocotyls and roots, cotyledons with hook containing protochlorophyllide, which could interfere with measurements, were cut off and pieces of etiolated stems 0.4–0.5 cm long and roots were placed in a 50% water–glycerol mixture (v/v) in darkness for 5–10 min. This treatment was used to minimize the effect of freezing–thawing on the fluorescence and photochemical phytochrome parameters. In special experiments it was shown that the parameters of phytochrome in the samples were not affected by their incubation in water–glycerol mixture. From five to 15 hypocotyls and 10–20 roots were taken for the sample. An average fresh weight of the samples was about 1 mg. To obtain the samples of etiolated seedlings of cucumber and tomato, one etiolated seedling was taken (length ≈ 2–5 cm) without cotyledons, hook and roots, cut in pieces 0.5 cm long and incubated in 50% glycerol for 5–10 min at 26°C. Two or three pieces of stems were then taken for the sample. In the case of maize, the procedure was the same but root tips (3 mm growing zone) were used instead for the sample (one root tip for the sample). From three to five stems of cress were usually taken for the sample. All manipulations were carried out under safe dim green light.

2.2. Equipment and measurement procedure

Measurements of the low-temperature (85 K) fluorescence emission spectra (spectral slit width 2 nm) were carried out with the use of a laboratory-designed spectrofluorimeter based on two double-grating monochromators (conventionally used for Raman spectra measurements) described elsewhere [3]. It was modified, however, in order to allow measurements of small amounts of material in the sample with relatively low phytochrome content (in particular, in the case of etiolated *Arabidopsis* stems and roots). A He–Ne laser (LGN-207B, Russia, 1 mW) in combination with a monochromator (MDR-2, Russia) was used as a source of the exciting and actinic light. In the case of the exciting light its intensity was reduced with the use of neutral filters (NS-3 and NS-9, 2 mm thick, Russia) so that it allowed measurements of the emission spectra with reasonable precision and at the same time it was practically photochemically inactive at low temperatures within the time limits of the

spectra recording (intensities of actinic and exciting light, ≈500 and 10 W m^{-2}, respectively). The practical absence of the photochemical effect by the exciting laser beam was checked (i) by measuring the kinetics of the fluorescence intensity changes in the maximum (λ_{max}) and (ii) by obtaining the fluorescence and photochemical parameters of phytochrome in *Arabidopsis* also with the previously used technique of low-intensity monochromatic excitation [3].

The procedure of the measurements was essentially the same as described earlier [3–5] with modifications for measurements of the *Arabidopsis* seedlings. The sample (pieces of etiolated tissues) was glued with 50% glycerol on a transparent (1 mm thick) Plexiglas plate in a sample holder of the cryostat and immediately frozen in the dark at 85 K. Fluorescence emission spectra were taken (1) at this temperature (dark-adapted state with all phytochrome in its Pr form, state 0), (2) after irradiation of the same sample by full light from the He–Ne laser at 85 K to reach a photoequilibrium between Pr and lumi-R (state 1), and (3) after thawing the sample at 273 K, illumination with monochromatic red light (λ_a = 680 nm) from a laboratory-designed monochromator in combination with a cut-off red filter (KS-18, 7 mm thick) to convert Pr into the far-red-light-absorbing form, Pfr, and freezing at 85 K (state 2). Special precautions (the use of a low-intensity, ≈0.1 W m^{-2}, longer wavelength (λ_a = 680 nm) actinic band with a shorter wavelength tail reduced by strict red cut-off filters) were taken to prevent photoconversion of $Pchl^{657}$ into Chl, which could interfere with the measurements of the Pr spectra and estimations of the degree of the Pr → Pfr photoconversion (see below). Possible conversion of $Pchl^{657}$ to Chl was controlled by the absence of the decrease of the $Pchl^{657}$ emission at 657 nm in wild-type and mutant *Arabidopsis* plants and of the Chl band in the 675–685 nm region in double *phyAphyB Arabidopsis* mutant.

This was a basic scheme of the spectral measurements which provided, after correction for the background emission (see below), a number of parameters characterizing phytochrome in its native state in the cell, the major ones being: (i) the position of the fluorescence emission maximum (λ_{max}) (and from this, of the absorption maximum assuming a Stokes shift of 13–14 nm [2]); (ii) total P content, [P_{tot}], proportional to the fluorescence intensity, F_0, at λ_{max} in the dark-adapted state (state 0); (iii) extent (λ_1) of the Pr → lumi-R phototransformation to reach a photoequilibrium between the two states upon actinic illumination at λ_a = 633 nm and 85 K, measured as $\gamma_1 = (F_0 - F_1)/F_0 = \Delta F_1/F_0$; and (iv) extent of the Pr → Pfr phototransformation at 273 K and λ_a = 680 nm, $\gamma_2 = (F_0 - F_2)/F_0 = \Delta F_2/F_0$. In a number of experiments, photoreversibility of the Pr → Pfr phototransformation was checked. For this, the sample in state 2 was thawed at 273 K, illuminated with far-red light ($\lambda_a \geq 720$ nm, white light + cut-off filters KS-18 (2 mm), FS-6 (5 mm), ≈2 W m^{-2}) and frozen at 85 K and the emission spectrum was taken (state 0′). The degree of reversibility was obtained as the ratio of the fluorescence intensity at the maximum in the state 0′ to that at state 0.

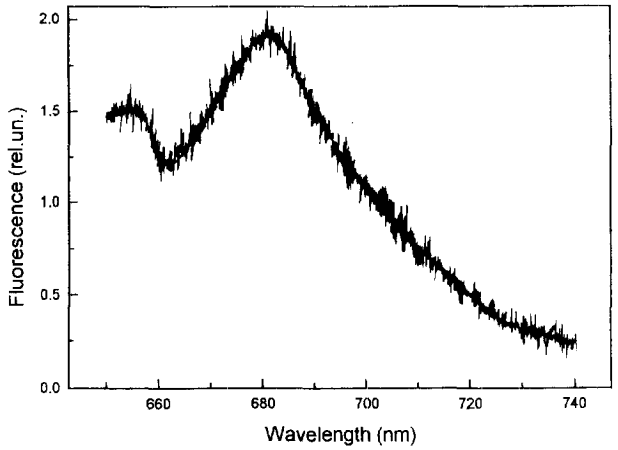

Fig. 1. Experimental recording of the low-temperature (85 K) fluorescence emission spectrum of etiolated stems of wild-type *Arabidopsis* (five hypocotyls in the sample) upon excitation with a He–Ne laser (λ_e = 633 nm) and its manual smoothing. (Here and below the spectra are not corrected for spectral sensitivity of the instrument.)

The noise level of the registration was less than 5% of the maximal signal. The typical recording of the experimental spectrum and its manual smoothing are presented in Fig. 1. Usually from seven to 11 independent experiments were carried out on different samples for each wild-type and mutant species and the precision of the estimations of the parameters λ_{max}, [P_{tot}], γ_1, γ_2 was better than ±0.5 nm, ± 15%, ±20%; and ± 10% (±SD), respectively.

3. Results

3.1. Emission spectra and phytochrome content

Experimental spectra of etiolated wild-type *Arabidopsis* plants and their respective mutant strains are presented in Fig. 2. Their analysis shows that the spectra are complex and

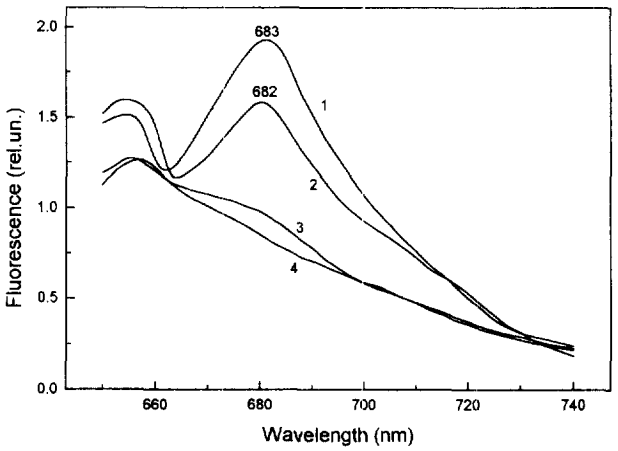

Fig. 2. Low-temperature (85 K) fluorescence emission spectra (λ_e = 633 nm) of etiolated hypocotyls (without hook and cotyledons) of wild-type *Arabidopsis thaliana* (*cv.* Landsberg *erecta*) and their phytochrome mutant strains: 1, wild type; 2, *phyB*, *hy3*; 3, *phyA*, *fre1-1*; 4, double mutant, *phyAphyB*.

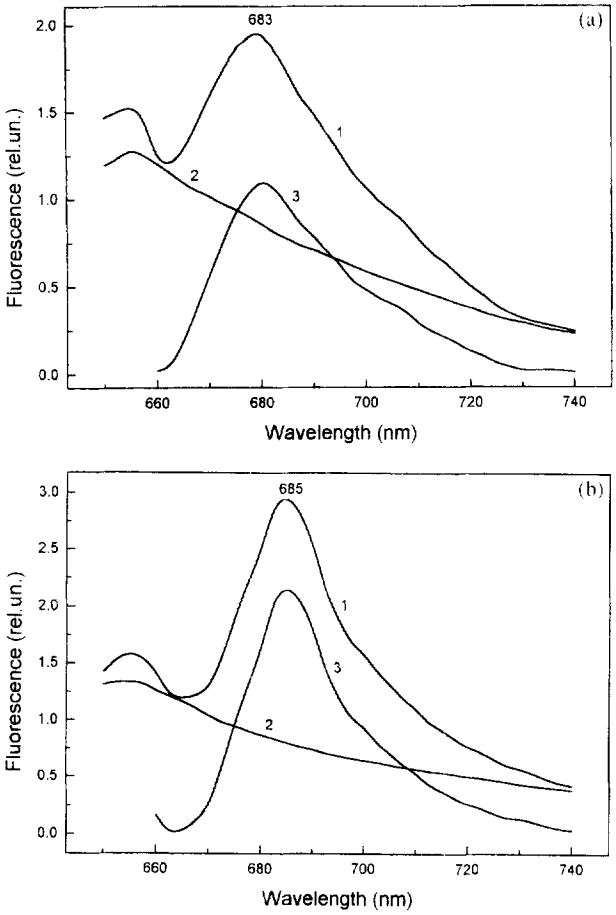

Fig. 3. Fluorescence emission spectra of etiolated hypocotyls of *Arabidopsis thaliana* (*cv.* Landsberg *erecta*) (a) and tomato (*Lycopersicon esculentum* L.) (b) measured at 85 K and $\lambda_e = 633$ nm. 1, wild-type strains; 2, their respective mutants lacking phytochrome (double mutant *phyAphyB* and phytochrome chromophore mutant, *aurea*, *au*) taken as the spectra of background emission of the sample; 3, spectrum of the red-light-absorbing phytochrome form, Pr, obtained by subtraction of spectrum 2 from spectrum 1. The spectra 1 and 2 were normalized at 660–665 nm, where the intensity of the Pr fluorescence is less than 5% of that in the maximum.

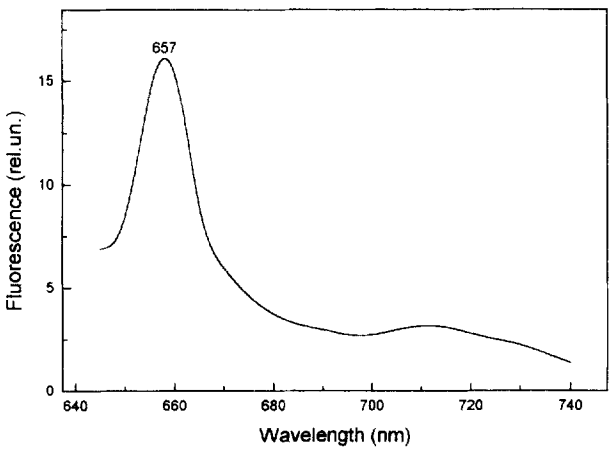

Fig. 4. Low-temperature (85 K) fluorescence emission spectrum of the cotyledons of etiolated wild-type *Arabidopsis thaliana* (*cv.* Landsberg *erecta*), $\lambda_e = 633$ nm.

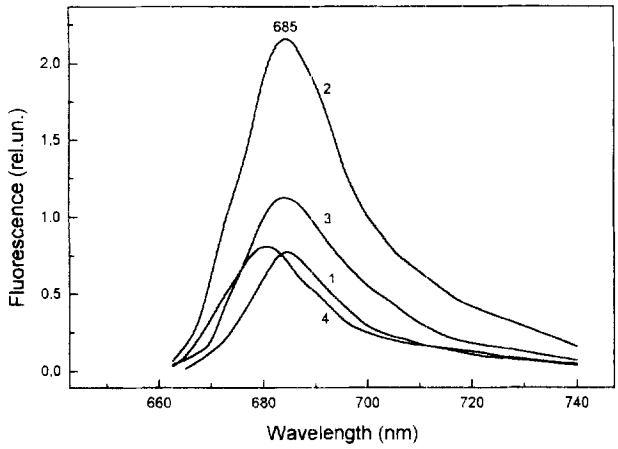

Fig. 5. Low-temperature spectra of phytochrome in etiolated stems of wild-type *Arabidopsis thaliana* (*cv.* Nossen) and respective phytochrome overexpressors: 1, wild type; 2, oat phyA overexpressor, *13k7*; 3, *Arabidopsis* phyB overexpressor, *ABO*; 4, rice phyB overexpressor, *RBO*.

comprise two major components: the main band at 680–685 nm belonging to the red-absorbing phytochrome form and unstructured spectrum of background emission, and a minor band at 657 nm of protochlorophyllide Pchl^{657}. This is suggested by the variations in the amplitude (to complete disappearance) of the 680–685 nm band in the spectra of the mutants lacking the bulk phytochrome (*Arabidopsis phyA*, *phyAphyB*, tomato *au*, *aurea*) (Fig. 3) and also by its changes in the Pr → lumi-R and Pr → Pfr photoconversions, see below. The attribution of the 655 nm band to Pchl^{657} follows, in particular, from the spectrum of *Arabidopsis* cotyledons where the content of this pigment is much higher than that of P (see Fig. 4)). The spectrum also suggests that the Pchl^{657} input in the region of the main phytochrome fluorescence (at 680–685 nm) is low and can be ignored. Thus, the spectrum of the *Arabidopsis* double mutant can be taken as a spectrum of the background fluorescence in the region of the Pr fluorescence and used to obtain, by its subtraction from the experimental spectra, the real Pr emission spectra. For this the experimental spectra were normalized at 660–665 nm where the input of the Pr fluorescence is minimal (less than 5%) and practically all the signal belongs to the background emission. The emission intensity in this region was used as an internal standard reflecting the mass of the sample under exciting light and $[P_{tot}]$ was estimated in relative units from the ratio of the Pr fluorescence in the maximum (F_0 normalized to the intensity of the background fluorescence, F_b, F_0/F_b). Using this procedure (given in more detail in Refs. [3–5,20]), we have obtained real (absolute) Pr spectra (Figs. 3, 5 and 6) and $[P_{tot}]$ and these results will be presented and discussed below.

In wild-type plants, the fluorescence emission spectrum of phytochrome in etiolated seedlings has its maximum at 683 nm, and $[P_{tot}]$ is estimated to be 0.82 ± 0.12 and 0.66 ± 0.15 rel. units (Landsberg *erecta* and Nossen, respectively) (Figs. 3, 5 and 6; Table 1). The position and relative intensity of the fluorescence was shown to depend on the part of the

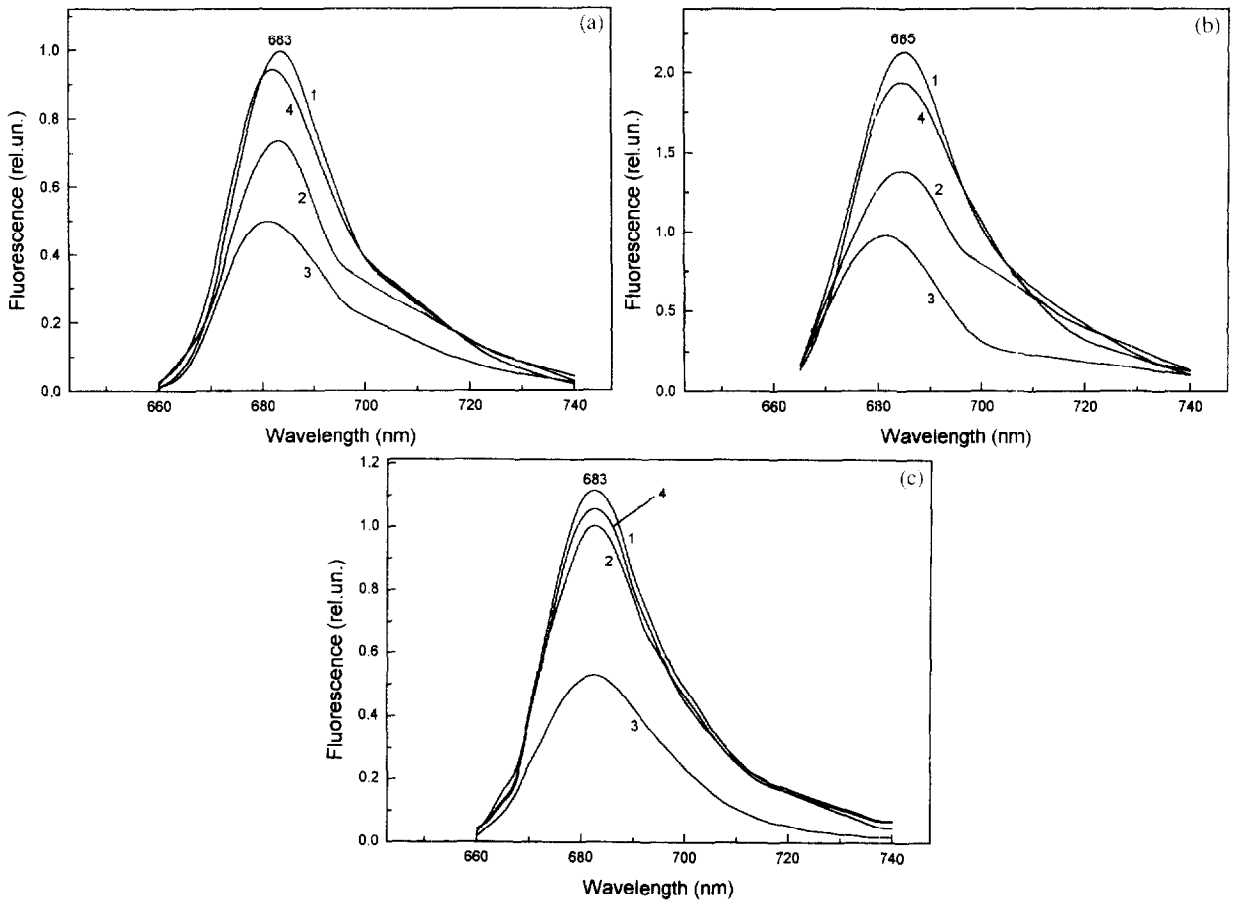

Fig. 6. Low-temperature (85 K) fluorescence emission spectra ($\lambda_e = 633$ nm) of phytochrome in *Arabidopsis* hypocotyls obtained from the experimental spectra by subtraction of the spectrum of Pchl[657] and background emission as shown in Fig. 3 and described in the text. 1, etiolated seedlings; 2, same as 1 after illumination with saturating actinic light ($\lambda_a = 633$ nm) at 85 K to convert Pr into the first photoproduct stable at low temperatures, lumi-R; 3, same as 2 after thawing at 273 K, illumination for 10 min with red actinic light ($\lambda_a = 680$ nm, ≈ 0.1 W m^{-2}) leading to a partial conversion of Pr into the far-red-absorbing form, Pfr, and freezing at 85 K; 4, same as 3 after thawing at 273 K, illumination with saturating far-red light ($\lambda_a \geq 720$ nm, ≈ 2 W m^{-2}) to convert Pfr back into Pfr and freezing at 85 K. (a) Wild type (*cv.* Nossen); (b) oat phyA overexpressor, *13k7*; (c) *Arabidopsis* phyB overexpressor, *ABO*. The fluorescence intensity scale reflects the total phytochrome content expressed as the ratio of the Pr fluorescence intensity to the intensity of the background fluorescence, F_0/Fb_{665} (see text).

seedling used for the preparation of the sample. Upper parts of stems (in Landsberg *erecta*) just below the hook region (2–3 mm sections) were characterized by $\lambda_{max} = 683$ nm and $F_0 = 0.78 \pm 0.16$, while the lower parts of the stems had $\lambda_{max} = 680$ nm and $F_0 = 0.68 \pm 0.04$; in roots $\lambda_{max} = 680$ nm and $F_0 \approx 0.2$.

Mutations bringing about the disappearance of phyB (*phyB* mutant) cause $\approx 20\%$ decrease of F_0 and the position and structure of the spectrum remain practically the same as in the wild type (Fig. 2, Table 1). Assuming that the *phyB* mutant has unchanged level of phyA (as shown in Refs. [21,22]), this reduction in F_0 could be associated with the lack of phyB and, hence, the phyB fluorescence in stems is $\leq 20\%$ of the total phytochrome emission. The fluorescence emission spectrum of phyB should be close to that of the total P fluorescence, $\lambda_{max} \approx 683$ nm, because its elimination does not practically change the position of the spectrum.

These observations on phyB content and spectrum are in line with the fluorescence measurements of *phyA Arabidopsis* mutant, *fre1-1* (Fig. 2, Table 1). A slight increment of the spectrum of the *phyA* mutant over the spectrum of the double mutant roughly fits into the estimation of the above effect of the fluorescence intensity decrease. The position of the shoulder which can be attributed to phyB on curve 3 is at 680–685 nm, i.e., within the observed band position of the total phytochrome. Double (*phyAphyB*) mutant (Figs. 2 and 3(a)), as it was discussed above, practically does not display any spectral features attributable to phytochrome.

Overexpression of oat phyA in *Arabidopsis* causes a considerable increase in the total phytochrome fluorescence, reflecting changes in the total phyA content (up to double in *13k7*, $[P_{tot}] = 1.4 \pm 0.16$ rel. units, and three times in *21k15*, $[P_{tot}] = 1.94 \pm 0.23$ rel. units) and a longer wavelength shift of the spectrum to ≈ 685 nm, i.e., 2 nm from that of the respective wild-type plants (Fig. 5, Table 1). The increase in the P_{tot} content, as judged by the fluorescence intensity changes, is, however, somewhat lower than that reported by Boylan and Quail [16] (up to four times in etiolated tissues). This may be explained by the fact that we used hypocotyls of the seedlings containing less phytochrome than the hook

Table 1
Fluorescence and photochemical characteristics of phytochrome in etiolated shoots of wild-type, mutant and overexpressor strains of *Arabidopsis* and their comparison with those of some other plant species [a]

Plant material	λ_{max} (nm)	$[P_{tot}]$ (rel. units)	γ_1	γ_{2d}^{680}	Pr', Pr'' (%)
Arabidopsis					
(cv. Landsberg) WT	683	0.82 ± 0.12	0.24 ± 0.05	0.51 ± 0.05	49, 51
phyB-1	682	0.61 ± 0.07	0.22 ± 0.04	0.49 ± 0.03	45, 55
phyA-201	680-685	≈0.1			
(cv. Nossen) WT	683	0.66 ± 0.15	0.25 ± 0.10	0.59 ± 0.01	51, 49
13k7	685	1.40 ± 0.16	0.38 ± 0.04	0.60 ± 0.04	77.5, 22.5
21k15	685	1.94 ± 0.23	0.29 ± 0.02	0.61 ± 0.03	59, 41
ABO	683	0.93 ± 0.13	0.14 ± 0.03	0.46 ± 0.04	29, 71
RBO	680	0.78 ± 0.08	0.13 ± 0.03	0.47 ± 0.05	26.5, 73.5
Cucumis					
WT	686	1.90 ± 0.20	0.33 ± 0.02	0.54 ± 0.05	67, 33
lh	686	1.60 ± 0.08	0.33 ± 0.02	0.58 ± 0.04	67, 33
Lycopersicon					
WT	686	1.40 ± 0.20	0.31 ± 0.02	0.53 ± 0.03	63, 37
Avena					
WT (coleoptile tips)	687	5.50 ± 1.04	0.48 ± 0.01		96, 4
Lepidium					
WT	683	1.53 ± 0.37	0.23 ± 0.01		47, 53
Zea mays					
WT (root tips)	685	1.13 ± 0.07		0.62 ± 0.04 0.68 ± 0.07[b]	

[a] The parameters are defined in the text.
[b] Extent of the Pr → Pfr phototransformation after reaching photoequilibrium.

area and cotyledons and in these tissues the effect of overexpression could be less pronounced. At the same time, it should be noted that the position of the emission spectrum approached that observed in oats (middle parts of coleoptiles), 685 nm [3]. This agrees with the conclusion [16] that immunological and spectral properties of oat phyA synthesized in *Arabidopsis* were similar to those of authentic oat phyA (see also photochemical parameters below).

Overexpression of phyB (*Arabidopsis*, *ABO*, and rice, *RBO* obtained by Wagner et al. [17]) also brings about changes in the phytochrome fluorescence intensity and its total content to 0.93 ± 0.13 rel. units in *ABO* and 0.78 ± 0.08 rel. units in *RBO*, although to a lower extent as compared with phyA overexpression (the increase does not exceed 50%) (Fig. 5 and Table 1).

It is interesting to note that, while overexpression of *ABO* does not practically affect the position of the emission spectrum, $\lambda_{max} = 683$ nm as it is in the wild type, the emission spectrum in *RBO* shifts by 3 nm to the blue, $\lambda_{max} = 680$ nm (Fig. 5). This suggests that *Arabidopsis* phyB is close to phyA by its spectroscopic properties and rice phyB has a shorter-wavelength position of the emission maximum ($\lambda_{max} \leq 680$ nm) (the position of the absorption maxima at 85 K can be judged by the Stokes shift of 13–14 nm; at ambient temperatures an additional 2–3 nm blue-shift is expected, see Ref. [2]).

In order to compare directly the phytochrome properties in *Arabidopsis* with those of other plant species, we have carried out measurements on dicots (cucumber, tomato, cress) and monocots (oat, maize). The wild-type tomato was characterized by $[P_{tot}] = 1.4 \pm 0.2$ rel. units and $\lambda_{max} = 686$ nm; the *aurea* mutant having less than 5% of total phytochrome did not show emission belonging to P (Fig. 3(b), Table 1). The hypocotyls of wild-type cucumber contained 1.9 ± 0.2 rel. units of P and had $\lambda_{max} = 686$ nm. Its phyB null (*lh*) mutant had only 15–20% less phytochrome fluorescence signal than the respective wild type and the same position of the spectrum, $\lambda_{max} = 686$ nm (Table 1, spectra are not presented). This agrees with the earlier observation (emission spectra of cucumber, see Ref. [4]). Wild-type oat (coleoptile tips), cress (stems) and maize (root tips) were respectively characterized by $[P_{tot}]$ of 5.50 ± 1.04, 1.53 ± 0.37 and 1.13 ± 0.07 rel. units and λ_{max} of 686, 683 and 685 nm. Thus, $[P_{tot}]$ in *Arabidopsis* is generally lower than in the other investigated plants species and λ_{max} is shifted 3–4 nm to the blue (except cress, where $[P_{tot}]$ is also not high and λ_{max} practically coincides with that of *Arabidopsis*). P content and λ_{max} in *Arabidopsis* roots are also lower than those in maize roots. On the other hand, oat phyA *Arabidopsis* overexpressors are characterized by $[P_{tot}]$ and λ_{max} similar to those of tomato and cucumber, see Table 1.

3.2. Low-temperature phototransformations of phytochrome in etiolated Arabidopsis seedlings

Parameter γ_1, which by definition is the extent of the Pr → lumi-R phototransformation, reflects the photochemical activity of Pr at low temperatures. This proved to be very sensitive and to depend on the state of phytochrome in *Ara-*

bidopsis wild-type and mutant strains and also on the parts of seedlings taken for the sample.

Fig. 6 shows a light-induced decrease of the Pr fluorescence intensity from its initial dark-adapted level F_0 (curve 1) to the level F_1 (curve 2), reflecting a decline of [Pr] to the photoequilibrium state. This transformation is followed by the appearance of the shoulder at 705 nm belonging to lumi-R. In Landsberg *erecta* γ_1 was determined to be 0.24 ± 0.05 in whole hypocotyls of wild-type seedlings (in Nossen, 0.25 ± 0.10) (Fig. 6(a), Table 1) and almost the same in the upper parts of the hypocotyl, 0.23 ± 0.02. In the lower parts of hypocotyl and roots it is much lower, 0.04 ± 0.01 and ≈ 0.05–0.1, respectively.

A very similar picture of the photoinduced fluorescence changes is observed with the *phyB* mutant (not shown) and the γ_1 value practically coincides with that of wild type, 0.22 ± 0.04 (see Table 1). Experiments with *phyA* mutant did not reveal photoinduced changes in the Pr fluorescence intensity (within the limits of sensitivity of the measurements) (spectra are not shown).

Overexpression of oat phyA in *Arabidopsis* (*13k7* and *21k15* plants), along with the increase of the total phytochrome content and longer-wavelength shift of the emission spectrum, results in an increase of the γ_1 value up to 0.29 ± 0.02 in the case of *21k15* and to 0.38 ± 0.04 in the case of *13k7* (see Fig. 6(b), Table 1). Overexpression of phyB (which causes a moderate increase in the $[P_{tot}]$ and a shorter-wavelength shift of the spectrum in the case of *RBO* and practically no effect on the position of the spectrum in *ABO*, see above) considerably decreases the γ_1 values to 0.14 ± 0.03 in *ABO* and 0.13 ± 0.03 in *RBO* (see Fig. 6(c), Table 1). This is in contrast to the above and earlier observations [3,5], when an increase of $[P_{tot}]$ is usually followed by a rise of the γ_1 parameter.

Thus, in the order of γ_1 decrease, *Arabidopsis* strains make the succession: oat phyA overexpressors, wild type, and phyB overexpressors. The γ_1 values in *13k7* and *21k15* species are comparable with those of cucumber and tomato (see Table 1). The wild-type strains (both Landsberg *erecta* and Nossen) have γ_1 values similar to those in cress, 0.23 ± 0.01 [3]. The low values of γ_1 similar to those in the phyB overexpressors were obtained earlier on plant tissues where total phytochrome content is reduced, for instance, in tissues at the base parts of stems and roots [3] and transgenic plants with inhibited, anti-sense phyA synthesis [5].

3.3. Pr → Pfr phototransformation

The extent of the phototransformation, γ_2, proved to be much more conservative, although its direct evaluation was hampered by the Pchl → Chl photoconversion at temperatures close to ambient. To exclude it, we used longer-wavelength actinic light ($\lambda_a = 680$ nm), which is weakly absorbed by photoactive $Pchl^{657}$, and also lowered its dose. The absence of the Pchl → Chl photoconversion was judged by the lack of the decrease of the Pchl fluorescence at 657 nm and of the appearance of the Chl fluorescence at 675–685 nm. The extent of the Pr → Pfr phototransformation was lower in this case than that under saturating actinic light in the maximum of absorption (≈ 667–670 nm at low temperatures) or at lower wavelength because the photoequilibrium between Pr and Pfr was shifted towards Pr and because it was not fully achieved. Under these conditions, the extent of the Pr → Pfr conversion (designated γ_{2d}^{680}, where d stands for dose dependence and 680, for $\lambda_a = 680$ nm) was ≈ 0.5–0.6 in wild-type *Arabidopsis* plants, their mutants and overexpressors (see Fig. 6 and Table 1). The same values were also obtained for cucumber and tomato (see Table 1). To evaluate the extent of the Pr → Pfr phototransformation at photoequilibrium under illumination with $\lambda_a = 680$ nm, γ_2^{680}, we have carried out experiments on maize root tips which practically do not contain Pchl and obtained $\gamma_{2d}^{680} = 0.62 \pm 0.04$. Then we determined γ_2^{680} increasing the dose (by about 10 times) to reach a full photoequilibrium. It was 0.68 ± 0.07, which is $\approx 10\%$ higher than γ_{2d}^{680}, suggesting that for *Arabidopsis* and also for cucumber and tomato γ_2^{680} could be roughly around 0.55–0.65.

The photoreversibility of Pfr into Pr under longer-wavelength illumination ($\lambda_a \geq 720$ nm) is almost complete, $\approx 95\%$ (see Fig. 6). The small deviation from full reversibility can be explained by the fact that the comparison is carried out with the initial etiolated state (state 0) where all the pigment is in its Pr form, while under the far-red illumination a photoequilibrium at $\lambda_a \geq 720$ nm between Pr and Pfr is established. We cannot, however, completely rule out a partial destruction and/or modification of the pigment by the repeated freezing–thawing of the sample.

4. Discussion

Phytochrome in wild-type *Arabidopsis* and its mutants and overexpressors was for the first time investigated with the use of low-temperature fluorescence spectroscopy and photochemistry. It was characterized from these measurements using four major parameters: relative P concentration, $[P_{tot}]$, position of the emission maximum of Pr, λ_{max}, and extent of the Pr phototransformation into lumi-R at 85 K and into Pfr at 273 K, γ_1 and γ_2, respectively.

In stems of wild-type *Arabidopsis*, $[P_{tot}] = 0.7$–0.8 rel. units, $\lambda_{max} = 683$ nm and $\gamma_1 = 0.24$–0.25. In lower parts of stems and roots, the parameters are somewhat different: $[P_{tot}] = 0.68$ and 0.23, $\lambda_{max} = 683$ and 680 nm, $\gamma_1 = 0.04$ and 0.05–0.1, respectively. In general, a dependence of λ_{max} and γ_1 on $[P_{tot}]$ in different tissues is observed such that when $[P_{tot}]$ decreases $\gamma_1 \rightarrow 0$ and $\lambda_{max} \rightarrow 680$–$681$ nm and when $[P_{tot}]$ reaches maximal values $\gamma_1 \rightarrow 0.3$ and $\lambda_{max} = 683$ nm. By these parameters phytochrome in wild-type *Arabidopsis* is close to that in stems of relative cress and also in tissues of other plants where $[P_{tot}]$ is relatively low (in particular, roots and stems near the seed in pea, cucumber, tomato, potato,

oat, maize), that is, where $[P_{tot}]$ is comparable with $[P_{tot}]$ in *Arabidopsis* [3–5].

Impairment in PHYB, PHYA and both PHYA and PHYB (double) synthesis decreases the fluorescence intensity F_0 (and $[P_{tot}]$) by ≈ 10–20%, 90% and to complete disappearance, respectively. In the case of the *phyB* mutant, where precise measurements are possible, λ_{max} and γ_l practically do not change. In the oat phyA overexpressors (*13k7* and *21k15*), a two- to three-fold increase of $[P_{tot}]$ is followed by the longer-wavelength shift of λ_{max} to 685 nm and increase of γ_l up to ≈ 0.3–0.4. On the contrary, the phyB overexpression in *Arabidopsis* (*ABO* and *RBO*) brings about a moderate (up to 40%) increase of $[P_{tot}]$ and a decrease of γ_l to ≈ 0.13–0.14. In *ABO*, λ_{max} does not change, while in *RBO* it suffers a shorter-wavelength shift to 680 nm.

Two major conclusions can be drawn from the obtained experimental results, which are essential for their interpretation. First, the 680–685 nm band in etiolated *Arabidopsis* seedlings entirely belongs to phytochrome, to its red-absorbing form, Pr. This is proved (i) by its complete disappearance in the spectra of the *phyAphyB Arabidopsis* mutant and in the phytochrome chromophore (*aurea*) mutant of tomato, which is known to have less than 5% of total phytochrome, (ii) by the decrease of the emission band in the Pr → lumi-R phototransformation and (iii) by its reversible changes in the Pr ↔ Pfr phototransformation. Secondly, the spectroscopic and photochemical parameters of P vary in different *Arabidopsis* strains and in different parts of seedlings along with the P content. These observations can be interpreted as a manifestation of the existence of at least two phenomenological emitting phytochrome species: the longer-wavelength Pr′ with relatively high γ_l and the shorter-wavelength Pr″ with low γ_l, the proportion of which changes depending on the *Arabidopsis* strain and tissue used.

The obtained data allow rough estimations of the Pr′ and Pr″ relative concentrations from their input into the total phytochrome fluorescence, more specifically, from the experimental γ_l values and individual γ_l values for Pr′ and Pr″, γ_l' and γ_l'', respectively (for procedure, see Ref. [3]). In these estimations, it is assumed that the Pr′ and Pr″ fluorescence quantum yields, φ_F, at low temperatures are the same and that γ_l' and γ_l'' are equal to 0.49 and 0, respectively, according to the earlier estimations for oat and rice [3]. The latter assumption is justified (i) by the observed limits of the variations of the experimental γ_l values in *Arabidopsis* (from 0 to 0.4), (ii) by the maximal γ_l values for oat phytochrome (0.48 ± 0.01, see Table 1) and (iii) $\gamma_l' \geq 0.4$–0.5 for cress obtained in special experiments (not shown), as described in detail for oat and rice phytochrome in Ref. [3].

In wild-type *Arabidopsis* (Landsberg *erecta* and Nossen) Pr′ and Pr″ will make up approximately equal percentages, 50 and 50%. In cress, for comparison, their relative concentrations are the same [3] and in oat coleoptile tips in this work, ≈ 95 and 5%. In *Arabidopsis* overexpressing oat phyA, the Pr′ and Pr″ ratios are ≈ 75 and 25% in *13k7* and 60 and 40% in *21k15* (see Table 1).

For the understanding of the nature of the two Pr species and their relation to phyA and phyB, it is essential to note that the *phyB Arabidopsis* mutant is pretty close by the proportion of Pr′ and Pr″ to the wild-type plant (see Table 1), that is, the elimination of phyB in the *phyB* mutant does not bring about a disappearance of Pr″. This and similar observations on the *phyB* mutant of cucumber (*lh*) in this work (see Table 1) and earlier [4] and on the *phyB* mutant of pea (*lv*) [23] (V.A. Sineshchekov, O.B. Ogorodnikova, J.L. Weller, unpublished results) firmly prove that within phyA there are two species, phyA′ and phyA″, belonging to the Pr′ and Pr″ phenomenological types. It is interesting to mention in this connection the recently found diversity of the phyA signal-transduction pathways in pea for inhibition of stem elongation in de-etiolating seedlings and promotion of stem elongation and flowering in older plants [24] and the genetic dissection of the effects of FR pulses (very low fluence response, VLFR) and continuous FR (high irradiance response, HIR) on hypocotyl growth in *Arabidopsis* mediated by phyA [25]. These observations might be interpreted in agreement with Ref. [5] in terms of the two phyA populations initiating different photoresponses.

It is essential to emphasize, on the other hand, that overexpression of phyB (both *Arabidopsis* and rice) causes a decrease in γ_l value (see Table 1). This can be explained as an indication that phyB is likely to belong to the Pr″ phenomenological type, i.e., it is characterized by the low individual γ_l value. This conclusion is supported by the direct observation obtained on the *phyA* mutant of *Arabidopsis* (*phyA-201*) in this work and on the *phyA* mutant of pea (*fun1*) [24] (V.A. Sineshchekov, O.B. Ogorodnikova, J.L. Weller, unpublished results) that phyB does not practically reveal low-temperature photochemistry ($\gamma_l \leq 0.05$). Indeed, one can evaluate that the 40% increase in $[P_{tot}]$ in the phyB overexpressor (*ABO*) would account for a decline of the experimental γ_l value from ≈ 0.25 in wild type (Nossen) to ≈ 0.17. In other words, an increase in the proportion Pr′/Pr″ from ≈ 50:50 in wild type (Nossen) to ≈ 35:65 in the overexpressor would be expected. (In these evaluations, it is assumed that (i) the individual γ_l value for phyA′ is 0.49 and 0 for phyA″ and phyB (see above); (ii) all the increase of $[P_{tot}]$ in the overexpressors is entirely due to phyB and (iii) there is no changes in the phyA′/phyA″ proportion in the overexpressor.) The experimental γ_l value is, however, somewhat lower and the proportion of Pr″ is higher, 0.14 and ≈ 70%, respectively (for *ABO*). This small discrepancy can be explained by assuming changes of the phyA′ and phyA″ relative content along with the changes in the developmental state of the overexpressors (see Section 2) because it was shown earlier [3,5] that the proportion of Pr′ and Pr″ (and, hence, of the two phyA species which make up more that 90% of $[P_{tot}]$ in etiolated seedlings) depends on the physiological state of the plant. Similarly, in *phyB* mutants one would expect a decrease in the Pr″ proportion as compared to that in wild type. This is the case with the phyB mutant of pea (*lv*) [23], where the Pr″ relative content decreases in

etiolated stems from 20% in wild type to 15% in the mutant (V.A. Sineshchekov, O.B. Ogorodnikova, J.L. Weller, unpublished results). However, in this work and earlier [4], it was observed on the *phyB* mutants of *Arabidopsis* and cucumber that [Pr″] remains the same as in wild type or even slightly increases (see Table 1). This might be explained by two reasons: first, the direct effect of phyB elimination on [Pr″] is low because of low [phyB] ($\leq 10\%$) and secondly, it is superimposed by the [phyA″] increase connected with the changes in the mutant phenotype. Finally, in oat phyA *Arabidopsis* overexpressors, a higher overexpression level (three-fold) in the *21k15* species does not bring about a higher proportion of Pr′ or phyA′ ($\approx 60\%$) as compared with the *13k7* species, which is characterized by a two-fold higher [P_{tot}] than that in wild type and [Pr′] of $\approx 75\%$. This is at variance with earlier observations [3,5] that the increase of [P_{tot}] is generally followed by an increase in the relative Pr′ content, although this dependence was not strict. Thus, the picture of the changes of the low-temperature properties of phytochrome in mutant and transgenic plants is complex and this could be a result of at least two factors: (i) direct effect of overproduction or elimination of a particular phytochrome on the relative content of the phytochrome species and (ii) changes of their concentration connected with the modification of the plant phenotype.

The data obtained suggest that spectroscopically *Arabidopsis* phyB is likely to be close to phyA, its emission λ_{max} is ≈ 683 nm, and rice phyB is blue-shifted to $\lambda_{max} \leq 680$ nm because the phyB overexpression does not change the position of the experimental λ_{max} in *ABO* and causes a blue-shift of the λ_{max} to 680 nm in *RBO*. Taking into consideration a Stokes shift of 13–14 nm between the emission and absorption spectra and the red shift of the spectra by 2–3 nm upon freezing at 85 K (see Ref. [2]), we can evaluate the positions of the absorption maximum of *Arabidopsis* and rice phyB at ambient temperature: ≈ 666 nm and below 663 nm, respectively. This agrees with the notion that in dicots the spectroscopic properties of phyB are close to those of phyA, while in monocots phyB absorption spectrum is considerably blue-shifted as compared with that of phyA (see Ref. [17] and refs. cited therein). Thus, Pr″ comprises both phyB and modified phyA″ species, while Pr′ is presented by phyA′. The heterogeneity of the fluorescence and variability of the photochemical properties at low temperatures can now be interpreted as a manifestation of the existence of at least three emitting species: two populations within phyA (phyA′ and phyA″) and phyB. Interestingly, the recently discovered putative prokariotic phytochrome from the cyanobacterium *Synechocystis* (see Ref. [26] and refs. cited therein) does not show low-temperature photochemical activity, i.e., it belongs to the Pr″ phenomenological type comprising phyB and minor phyA″ [27]. The different capacity of the phytochrome species to undergo low-temperature photochemical transformations can be associated with the existence of the activation barrier E_a for the Pr photoreaction in the excited state, which varies in the Pr′ and Pr″ pigment types (see Refs. [2,27]).

The molecular nature of the observed photochemical differences between phyA′ and phyA″ and the similarity between phyA″ and phyB remains unknown. One may speculate, however, that they could be due to post-translational modification of the pigment and/or to its different localization in the cell. It is tempting to assume also that they are connected with the functional specificity of the pigment species, which is determined, according to Ref. [11], by the structure of the chromophore-bearing N-terminal domain.

5. Abbreviations

Chl	chlorophyll
ΔF	variable fluorescence of the red-light-absorbing phytochrome form
F	fluorescence
Fb	background light
F_0, F_1, F_2	fluorescence of the red-light-absorbing phytochrome form at 85 K under different conditions of light adaptation
FR, R	far-red and red light
φ_f	fluorescence quantum yield
γ_1, γ_2	extent of the Pr phototransformation into lumi-R at 85 K and into Pfr at 273 K upon R illumination, respectively
I	intensity of actinic light
λ_a, λ_e, λ_{max}	wavelengths of the actinic and exciting light and of the maximum of phytochrome fluorescence
lumi-R	photoproduct of the phototransformation of the R-absorbing form of phytochrome
P	phytochrome
$Pchl^{657}$	protochlorophyllide emitting at 657 nm
phyA, phyB	phytochromes A and B
phyA′ and phyA″	subpopulations of phyA
PHYA and PHYB	phyA and phyB proteins
Pfr, Pr	FR- and R-light-absorbing phytochrome forms
Pr′, Pr″	different species of Pr
P_{tot}	total phytochrome

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