Arabidopsis phytochromes C and E have different spectral characteristics from those of phytochromes A and B

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Abstract The red/far-red light absorbing phytochromes play a major role as sensor proteins in photomorphogenesis of plants. In *Arabidopsis* the phytochromes belong to a small gene family of five members, phytochrome A (phyA) to E (phyE). Knowledge of the dynamic properties of the phytochrome molecules is the basis of phytochrome signal transduction research. Beside photoconversion and destruction, dark reversion is a molecular property of some phytochromes. A possible role of dark reversion is the termination of signal transduction. Since *Arabidopsis* is a model plant for biological and genetic research, we focussed on spectroscopic characterization of *Arabidopsis* phytochromes, expressed in yeast. For the first time, we were able to determine the relative absorption maxima and minima for a phytochrome C (phyC) as 661/725 nm and for a phyE as 670/724 nm. The spectral characteristics of phyC and E are strictly different from those of phyA and B. Furthermore, we show that both phyC and phyE apoprotein chromophore adducts undergo a strong dark reversion. Difference spectra, monitored with phycocyanobilin and phytochromobilin as the apoprotein's chromophore, and in vivo dark reversion of the *Arabidopsis* phytochrome apoprotein phycocyanobilin adducts are discussed with respect to their physiological function.

Key words: Photoreceptor; Phytochrome; Chromophore; Dark reversion; Spectroscopy; Yeast

1. Introduction

Detection of light is necessary for plants to respond with differential growth, and to optimally adapt to a given light source [1]. For the detection of different light qualities and quantities in plants various types of photoreceptors exist: UV and blue light absorbing photoreceptors and red/far-red absorbing phytochromes [1,2]. In *Arabidopsis* five different phytochromes exist: phytochrome A, B, C, D and E (phyA, B, C, D, E) [3,4]. In general, phytochromes are responsible for several light mediated responses, including inhibition of hypocotyl elongation and stem growth, hook opening, expansion and positioning of the cotyledons, greening of the plant, synthesis of anthocyanins, and growth of stem hairs and side roots. Moreover, phytochromes are involved in floral induction and circadian rhythm [5]. Phytochromes are photoreversible dimers [6]. Each monomer consists of about 124 kDa [1]. After the assembly of the apoprotein with the chromophore, phytochrome exists in a red light absorbing conformation (P_r). The absorption maximum of P_r shows a peak around 660 nm. The chromophore, phytochromobilin (PΦB) is covalently linked to the apoprotein. PΦB is a linear tetrapyrrole (Fig. 1A) [7]. When red light is absorbed, PΦB changes its constitution via a $Z \rightarrow E$ isomerization, which brings the protein into a different conformation, the far-red light absorbing form (P_{fr}). The absorption maximum of P_{fr} shows a peak around 730 nm [8]. This red/far-red light dependent photoconversion is the main characteristic of all phytochrome molecules. After red light irradiation, the photoequilibrium is about 80–86% P_{fr}, after far-red light irradiation, it is 3% P_{fr} [9]. The P_{fr} form is the physiologically active conformation of the phytochromes. After transfer from red light into darkness some phytochromes undergo a conformational change back from the P_{fr} form into the P_r form. This is called dark reversion [9], and is thought to be a mechanism to inactivate the phytochrome [10]. PhyA in its P_{fr} form is rapidly degraded in plants, whereas all other phytochromes (phyB, C, D, and E) are light stable [11]. Responses of light stable phytochromes are mediated by the low fluence response (LFR), whereas phyA responses are mediated either by the very low fluence response (VLFR) or by the high irradiance response (HIR) [12].

While substantial information exists about the mode of action of phyA and phyB, less is known about phyC, D, and E [13]. Interactions between different phytochromes have been demonstrated for several physiological responses [14]. The shade avoidance syndrome is a good example of such an interaction wherein at least phyA, B, D, and E are involved [15–18]. PhyB plays a major role in the shade avoidance syndrome. However, in phyA deficient mutants, the capacity for shade avoidance is reduced, thus phyA is also needed for an optimal response. Furthermore, phyA, B, D, and E are also involved in floral induction and inhibition of hypocotyl elongation responses [18]. Since phyC deficient mutants have not been isolated up to now, it is difficult to give a

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Abbreviations: HIR, high irradiance response; LFR, low fluence response; phyA, phytochrome A; *PHYA*, cDNA of phytochrome A; PHYA, apoprotein of phytochrome A; PHYA*, phycocyanobilin adduct of phytochrome A; PHYA**, phytochromobilin adduct of phytochrome A (all abbreviations for phytochromes B, C, D, and E analogous); PCB, phycocyanobilin; PΦB, phytochromobilin; P_{fr}, far-red light absorbing form of phytochrome; P_r, red light absorbing form of phytochrome; P_{tot}, total amount of phytochrome; VLFR, very low fluence response

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statement about a specific function. From *Arabidopsis* phyC overexpressing tobacco plants it is known that phyC plays a role in expansion of cotyledons, but not in the inhibition of hypocotyl elongation [19]. The expression of phyC seems to be dependent on the expression of phyB [20]. Nothing is known about the spectroscopic properties of phyC, D, and E.

Since it was shown that phyA and phyB are expressible in yeast and assemble autocatalytically with the chromophore in vitro and in vivo, this technique was used to examine the molecular properties of phytochromes from different species [21]. In this context phycocyanobilin (PCB) was used instead of PΦB as the chromophore. The molecular difference between these two tetrapyrroles is shown in Fig. 1. It is known that PCB shifts the absorption extrema of phytochromes hypsochromatically [21]. Nevertheless, these phytochrome PCB adducts are functional in plants [22]. Moreover, PCB is supposed to be the native chromophore in the green alga *Mesotaenium* [23].

It was shown that the spectroscopically based data of the molecular properties of yeast derived phytochrome PCB adducts are comparable to the molecular properties of the native phytochromes in plants [24,25]. Given that *Arabidopsis* is a model organism in biological research [26], it was obvious to examine spectroscopically the *Arabidopsis* phytochromes in yeast to test whether spectral or kinetic differences between the different phytochromes may point to different functions.

In this work, we present spectroscopic data on yeast derived *Arabidopsis* phyA, B, C, and E. For the first time absorption maxima and minima were determined for phyC and phyE and dark reversion was detected in vivo. PhyC and E show strong spectral differences compared to phyA and B.

2. Materials and methods

2.1. Plasmid constructions and yeast strains

The yeast expression vectors were either pAA7 or pYES2 (Invitrogen, Leek, The Netherlands). Both vectors are galactose inducible and glucose repressible and bear an uracil selection. The yeast strain KN380 was used for phyA-pAA7. For all other phytochromes the strain INVSc1 (Stratagene, La Jolla, CA, USA) was used.

All cDNAs are derived from the *Arabidopsis* ecotype Columbia [3,4]. The cDNA of phyA (*PHYA*) was a gift of Peter Quail and was cloned *Bam*HI/*Xho*I into pAA7. *PHYB* was cloned *Kpn*I/*Not*I into pYES2 and was a gift of Christian Fankhauser. At the carboxy-terminus, phyB bears a streptavidin tag [10]. Carboxy-terminal tags do not hinder the spectroscopic properties of phytochromes ([10] and Lars Hennig, personal communication). *PHYC* was cloned *Kpn*I/*Bam*HI into pYES2 and was a gift of Peter Quail. *PHYE* was also cloned *Kpn*I/*Bam*HI into pYES2.

2.2. Phytochrome expression in yeast and chromophore assembly in vitro and in vivo

Yeast strains were transformed by a lithium acetate method as described previously [24]. Yeast cells were cultivated on glucose complete medium lacking uracil (CMU). For dark reversion kinetics yeast cells were grown in volumes of 100 ml galactose containing CMU up to an optical density of 1 at 600 nm. The chromophore assembly in vivo with PCB was done as described previously [27]. PCB was purified from *Spirulina* sp. as described [24]. For difference spectra yeast cells were grown from the beginning in galactose containing CMU. When a 5 l culture reached an optical density of 4 at 600 nm, two cycles in an SLM-Aminco French pressure cell at a pressure of 1250 bar followed. The crude extract was cleared by ultracentrifugation ($100\,000 \times g$). The supernatant was concentrated in a Centriprep-30® (Amicon, Beverly, MA, USA) to one third. The chromophore assembly in vitro with PCB or PΦB is described in [24]. PΦB was purified from *Porphyridium cruentum* as described in [28].

2.3. Spectroscopy

Dark reversion kinetics were measured as described previously [25,27]. The incubation temperature for all measurements was 4°C. P_{tot} and P_{fr} were detected with a dual wavelength ratio spectrophotometer [29]. Difference spectra were measured with an HP8452A diode array spectrophotometer (Hewlett Packard). The actinic light was obtained from light sources equipped with red light (664 nm) and far-red light (727 nm) interference filters from Schott (Mainz, Germany). The actual concentrations of phytochrome were calculated from the difference spectra to determine the yield of the phytochrome in yeast. We used a method with amido black to determine the total protein concentration [30].

3. Results

3.1. Difference spectra of phytochrome adducts with PCB and PΦB

The phytochromes of *Arabidopsis* were expressed in yeast. After extraction with a French pressure cell and chromophore assembly in vitro, difference spectra of the crude extracts were measured. Phytochrome concentrations, as calculated from the difference spectra, are shown in Table 1. The yield of phytochrome in yeast ranged from 0.2 to 2.7‰ of total protein. The yield of the apoprotein PCB adduct of phyD, cloned *Kpn*I/*Sma*I into pAA7, of different yeast strains was always under the spectroscopic detection limit and could therefore not be analyzed.

Fig. 2 shows the difference spectra of the apoproteins of phyA, B, C, and E adducts with PCB (PHYA*, B*, C*, and E*) as well as with PΦB (PHYA**, B**, C**, and E**). The spectroscopic properties, including the isosbestic

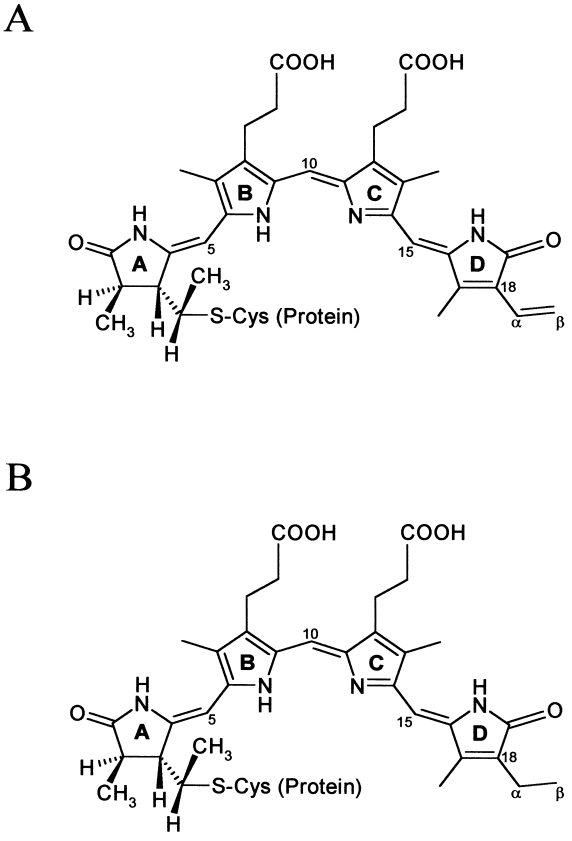

Fig. 1. Structural formula of PΦB (A) and PCB (B) [8]. At C-18 of ring D PΦB has one more double bond (vinyl group) than PCB (ethyl group). Cys = cysteine.

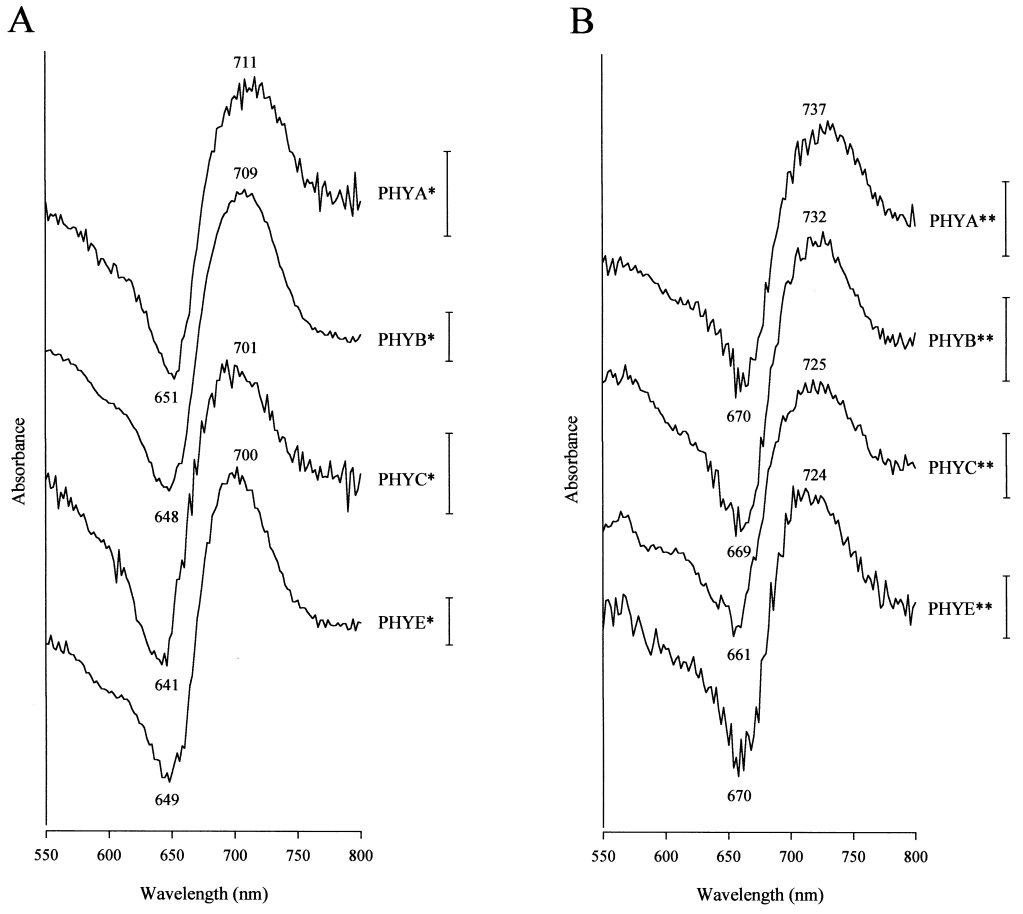

Fig. 2. Difference spectra of all *Arabidopsis* phytochrome apoprotein adducts with PCB (A) and PΦB (B). The spectra were measured using a diode array spectrophotometer after preparing crude extracts of phytochrome expressing yeast in a French pressure cell and in vitro assembly of the phytochrome apoprotein (PHY) with either PCB (PHY*) or PΦB (PHY**) [24]. The bars for PHYA*, PHYB*, and PHYE* represent an absorbance of 0.003, the bar for PHYC* represents an absorbance of 0.001 (A). The bars for PHYA**, PHYB**, and PHYE** represent an absorbance of 0.002, the bar for PHYC** represents an absorbance of 0.0005 (B). Only PHYC** was slightly electronically smoothed out ($W = 2$).

points, are summarized in Table 2. The hypsochromatic shift between the apoprotein PCB adducts and the PΦB adducts is in all cases 22–23 nm (Table 2). The relative absorption minima and maxima are essentially the same for PHYA* (651/711 nm) and PHYB* (648/709 nm), and PHYA** (670/737 nm) and PHYB** (669/737 nm), respectively, but are different for PHYC*/PHYC** and PHYE*/PHYE**. PHYC* (641/701 nm) and PHYC** (661/692) show in toto a blue shift of about 10 nm compared to PHYA*/PHYA** and PHYB*/PHYB**. The relative absorption minima of PHYE* and PHYE** (649 and 670 nm) are equal to PHYA*/PHYA** and PHYB*/PHYB**, but the relative absorption maxima (700 and 724 nm) are blue shifted by 10 nm compared to PHYA*/PHYA** and PHYB*/PHYB**. Consequently, the difference of the extrema of PHYE* (51 nm) and PHYE** (54 nm) are about 10 nm smaller than the comparable differences of the extrema of the PHYA, B, and C adducts (about 60 nm for PCB adducts and 65 nm for PΦB adducts).

3.2. Dark reversion kinetics with phytochrome apoprotein PCB adducts in vivo

Dark reversion of *Arabidopsis* phytochromes assembled in vivo in yeast was examined. Fig. 3 shows the kinetics of the dark reversion at 4°C of PHYA*, B*, C*, and E*. Table 3 summarizes the parameters of the kinetics. The dark reversion kinetics of PHYA, B, C, and E were tested for the PCB

Table 1
Expression of phytochrome constructs in yeast

Phytochrome construct in yeast	Phytochrome concentration (μg/ml)	Total protein concentration (mg/ml)	Phytochrome yield (‰ of total protein)
PHYA*	14.5	10.4	1.4
PHYB*	19.7	7.4	2.7
PHYC*	3.5	22.8	0.2
PHYE*	21.1	16.5	1.3

The phytochrome concentration was calculated from the difference spectra of the phytochrome apoprotein PCB adducts. An absorption difference ($\Delta(\Delta A)$) of 0.001 within a difference spectrum is equivalent to a phytochrome concentration of 1 μg/ml. The total protein concentration was determined with the method of Popov et al. [30].

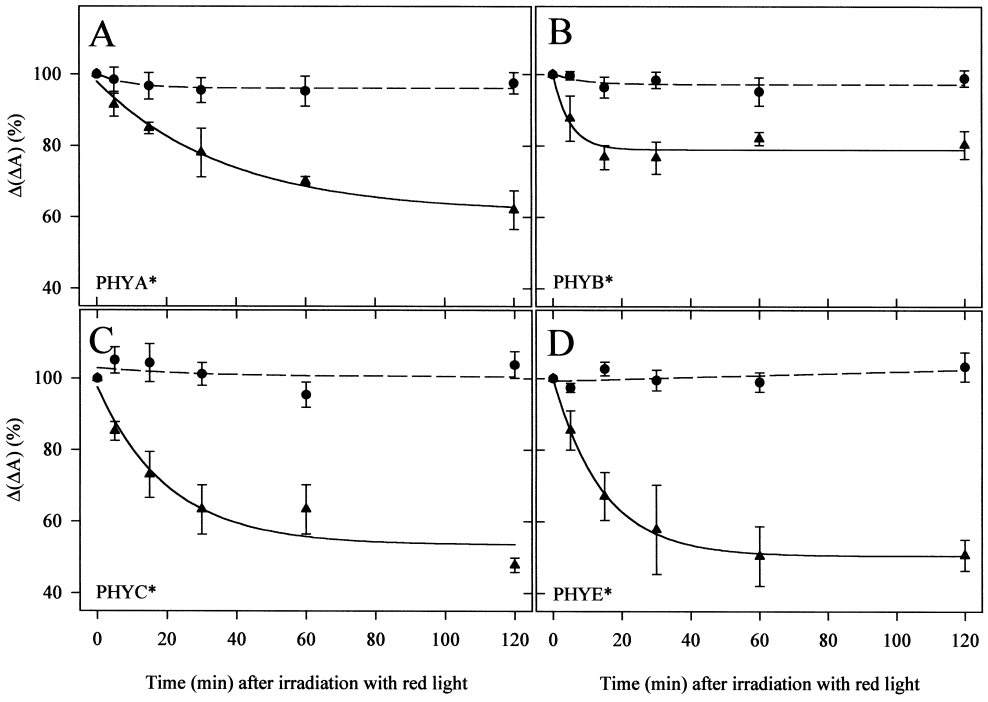

Fig. 3. Kinetics of dark reversion for different *Arabidopsis* phytochrome apoprotein chromophore adducts. The kinetics were measured in yeast after an in vivo assembly of the phytochrome apoproteins (PHY) with phycocyanobilin (PHY*) [25]. Each kinetic consists of three to five independent experiments (see Table 3) represented by the mean value. The error bars indicate the standard error. Incubation temperature was 4°C. The level of P_{fr} after red light irradiation was 80% of the total phytochrome. This photoequilibrium value was normalized to 100% P_{fr}. A = PHYA*, B = PHYB*, C = PHYC*, D = PHYE*; ●, dashed lines = P_{tot}, ▲, solid lines = P_{fr}.

adducts, because of the limited availability of PΦB (see Section 2.2).

40% of the P_{fr} molecules of PHYA* underwent dark reversion with a half-life of 30 min (Fig. 3A). Dark reversion of PHYB* exhibits a shorter half-life (estimated <6 min), but only involves 20% of the P_{fr} molecules (Fig. 3B). PHYC* and PHYE* showed the same dark reversion parameters: 50% of the P_{fr} molecules exhibited dark reversion with a half-life of 10 min (Fig. 3C,D). In all experiments, P_{tot} remained constant (dashed lines in Fig. 3).

4. Discussion

The dark reversion of *Arabidopsis* PHYA* takes place with a half-life of 30 min, which is very slow compared to reversion of PHYB*, C*, and E* (Table 3), but corresponds to PHYA* from *Nicotiana* (data not shown). Interestingly, there is no dark reversion detectable for phyA in *Arabidopsis* [31]. This observation indicates that dark reversion is a molecular property of some phytochrome molecules, including *Arabidopsis* phyA, but is modulated somehow in planta by other cellular components [31]. Because of the dark reversion of 40% of the P_{fr} molecules of PHYA*, the hypothesis of Brockmann et al. [32] that only the P_r/P_{fr} heterodimers undergo dark reversion is not valid for PHYA*, but interestingly could still hold for PHYB*. Only 20% of the P_{fr} molecules of PHYB* underwent dark reversion and, with an estimated half-life of <6 min, this dark reversion was very rapid (Fig. 3B). It is shown that phyB responses are mediated by the LFR and therefore, it is proposed that only the P_{fr}/P_{fr} homodimers act via the LFR [33]. With this background, the physiological role of dark reversion remains elusive. Nonetheless, the dark reversion kinetic of PHYB* is exactly the same as for the B-type related phytochrome, PHY2*, of the moss *Ceratodon* (data not shown). This enhances the hypothesis that the B-type phytochromes are the evolutionarily oldest ones [34].

Table 2
Properties of *Arabidopsis* phytochrome apoprotein PCB and PΦB adducts

Phytochrome adduct in yeast	$\lambda\Delta A_{min}$ (nm)	$\lambda\Delta A_{max}$ (nm)	λ Isosbestic point (nm)	Difference of extrema (nm)	Average of hypsochromatic shift (nm)	Number of difference spectra
PHYA*	651 ± 2.0	711 ± 3.3	675 ± 1.3	60	23	11
PHYB*	648 ± 1.0	709 ± 1.3	671 ± 0.7	61	23	11
PHYC*	641 ± 1.8	701 ± 2.9	670 ± 3.5	60	22	34
PHYE*	649 ± 2.3	700 ± 1.8	669 ± 1.8	51	23	34
PHYA**	670 ± 2.0	737 ± 2.8	698 ± 1.9	67	–	14
PHYB**	669 ± 2.2	732 ± 2.1	697 ± 1.9	63	–	12
PHYC**	661 ± 3.6	725 ± 3.2	692 ± 0.9	64	–	12
PHYE**	670 ± 1.8	724 ± 2.6	694 ± 0.9	54	–	12

The data were taken from Fig. 2. $\lambda\Delta A_{min}$ = minimum of the absorbance of the difference spectra (P_r), $\lambda\Delta A_{max}$ = maximum of the absorbance of the difference spectra (P_{fr}). The error represents the standard deviation.

Table 3
Summary of dark reversion kinetics

Phytochrome construct in yeast	Dark reversion half-life (min)	Dark reversion total amount (%) (within 2 h)	Number of measured kinetics
PHYA*	30	40	4
PHYB*	<6	20	3
PHYC*	10	50	5
PHYE*	10	50	4

The data were taken from Fig. 3.

It is shown here for the first time that phyC and phyE undergo dark reversion. PHYC* and PHYE* both exhibited a dark reversion in yeast of 50% of the P_{fr} molecules with a half-life of 10 min. This is the highest percentage of dark reverted molecules ever measured in yeast [27]. By using saturating red light pulses, we have excluded the possibility that the photoequilibrium has shifted to $<80\%$. At least for PHYC*, the photoequilibrium should be slightly $<80\%$, because the relative absorption minimum is 641 nm and light sources of 664 nm were used (see Section 2.3). The argument that the photoequilibrium causes a higher amount of dark reversion can be rejected by calculating the highest possible amount of P_r/P_{fr} heterodimers as 50% of P_{tot}. According to Brockmann et al. [32] the highest theoretical amount of dark reversion can be 25%, but in fact the amount is 50% for PHYC* and PHYE* (Fig. 3C,D). Therefore, we prefer to interpret our dark reversion data for PHYC* and PHYE* to indicate that, beside the P_r/P_{fr} heterodimers, a considerable portion of the P_{fr}/P_{fr} homodimers must be involved in this process.

The analysis of the difference spectra of the phytochrome apoprotein adducts of PHYC and PHYE with PCB and PΦB presented here shows clearly that these phytochromes, as well as phyA and phyB, assemble autocatalytically with their chromophore [11]. Consequently, we suggest that the autocatalytic reconstitution of phytochrome and its chromophore per se is a general property of all phytochromes. Furthermore, for the first time, we were able to determine the relative absorption extrema and isosbestic points of phyC and phyE that had been expressed in yeast. It has been shown previously that the adducts of the apoproteins with PΦB bona fide represent the native in planta situation [21].

Difference spectra of PHYA* show relative absorption extrema at 651/711 nm, those of PHYA** at 670/737 nm (Table 2). The hypsochromatic shift for all phytochrome apoprotein adducts examined in this work is an average of 22–23 nm stronger than for described phytochromes of other species [21,35,36]. Compared to native phyA, purified from *Arabidopsis* [37,38], the relative absorption extrema of the PHYA PΦB adduct (670/737 nm, Table 2) are red shifted by about 5–7 nm. In other species like *Avena*, the relative absorption extrema were shown to be identical for phyA and PHYA** [21]. The difference spectra in [37,38] exhibit a higher signal to noise ratio and hence may be less precise than the data shown here. The difference of the extrema of PHYA* is 60 nm (Table 2), which seems to be typical for phyA in general. Data from PHYA* of *Nicotiana* (58 nm; Thomas Kretsch, personal communication) and *Oryza* (62 nm) [27] support this result. Compared to the relative absorption extrema of PHYA*/PHYA**, there is no significant difference in the relative absorption extrema of PHYB*/PHYB** (Table 2). Also, the difference of the extrema is similar.

Interestingly, the difference spectra of PHYC*/PHYC** and PHYE*/PHYE** exhibit peculiarities compared to PHYA*/PHYA** and PHYB*/PHYB**. The whole difference spectra of PHYC* and PHYC** are shifted 10 nm hypsochromatically compared to either PHYA*/PHYB* or PHYA**/PHYB**. Whether this has consequences for the understanding of the physiological response of phyC remains unknown, because phyC deficient mutants have not been isolated up to now. The difference spectra of PHYE* and PHYE** are surprising. It is a distinctive feature that the relative absorption maxima are 10 nm blue shifted compared to PHYA*/PHYB* or PHYA**/PHYB**. In this sense the values are identical to PHYC* (710 nm) and PHYC** (725 nm). The relative absorption minima of PHYE* and PHYE** are, however, not shifted compared to PHYA*/PHYB* or PHYA**/PHYB**. Hence, the difference of the extrema is 10 nm smaller than for all other examined *Arabidopsis* phytochromes. Therefore, for phyE the absorption areas of red light and far-red light overlap more than for all other examined phytochromes. Along with other phytochromes, phyE is involved in the shade avoidance syndrome [18]. It is possible that phyE is more sensitive in the detection of the red/far-red ratio and thus is more readily able to detect the reflected light of the neighboring plants, whereas phyB and phyD are more important for the direct detection of the shadow of a canopy. The expression of phyE in the vascular system [39] might be a prerequisite for this sensitivity.

The strong differences in peak position and band width of the differences of phyC and E compared with phyA and B will lead to different amounts of active P_{fr} of the individual phytochromes under natural light conditions. Especially under various dense shade the different phytochromes will show different P_{fr} levels.

The expression of phytochromes in yeast is a powerful tool to examine their spectroscopic properties in vivo and in vitro. Its major advantage is to get information about the phytochrome molecule itself without disturbing or interacting cellular components. In particular, information about the molecular properties of the low abundance phytochromes, like phyC or phyE, is an important basis for further investigations in phytochrome research.

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