In Vivo Characterization of Chimeric Phytochromes in Yeast*

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Phytochromes are plant photoreceptors that play a major role in photomorphogenesis. Two members of the phytochrome family have been characterized in some detail. Phytochrome A, which controls very low fluence and high irradiance responses, is rapidly degraded in the light, forms sequestered areas of phytochrome (SAPs), and does not exhibit dark reversion in monocotyledonous seedlings. Phytochrome B mediates red/far-red reversible responses, is stable in the light, and does not form SAPs. We report on the behavior in yeast of the phytochrome apoproteins of rice PHYA, tobacco PHYB, and chimeric PHYAB and PHYBA and on the behavior of the respective holoprotein adducts after assembly with phycocyanobilin chromophore (PHY*). SAP-like formation in yeast was not observed for PHYB, but was detectable for PHYA, PHYAB, and PHYBA. Rice PHYA did not undergo dark reversion in yeast. Surprisingly, all other tested phytochrome constructs did exhibit dark reversion, including chimeric phytochromes with a short N-terminal part of tobacco PHYB or parsley PHYA fused to rice PHYIA. Furthermore, the proportion of phytochrome undergoing dark reversion and the rate of reversion were increased for both the N terminus-swapped constructs and PHYBA*. These results are discussed with respect to structure/function analysis of phytochromes A and B.

Plants are sessile organisms that have evolved a wide spectrum of mechanisms to adapt to changes in their natural environment. Light is used not only as the energy source for photosynthesis, but also as a major environmental signal. To monitor changes in light quality and quantity and to regulate temporal and spatial patterns in photomorphogenesis, plants have evolved at least three different photoreceptor systems: UVB photoreceptors, blue UVA photoreceptors, and phytochromes (1). Of these photoreceptors, the phytochromes are the best characterized. Five genes encoding phytochrome apoproteins have been identified in Arabidopsis (2, 3). Mutants of phyA, phyB, and phytochrome D in Arabidopsis have different effects on photomorphogenesis (4–8). The dominant phytochrome of etiolated seedlings, the light-labile phyA, controls the very low fluence and the high irradiance responses, whereas the dominant phytochrome of green seedlings and mature plants, the light-stable phyB, controls responses governed by red/far-red reversibility or continuous red light (9).

Phytochromes are dimers composed of 120-kDa monomers, each of which contains two domains linked by a highly conserved hinge region. The N-terminal domain bears the chromophore, and the C-terminal domain is involved in dimerization (Fig. 1A) (10). Phytochromes are synthesized in the dark in their physiologically inactive red light-absorbing form (Pr). Upon photon absorption, Pr is converted into the physiologically active far-red light-absorbing form of phytochrome (Pfr). During this reversible process, the absorption maximum of the molecule shifts from 660 nm (red light) to 730 nm (far-red light). Under saturating red light, 80% or more of the phytochrome is in the Pfr form, whereas only 3% Pfr remains following exposure to saturating far-red light (11). In vitro spectroscopy has demonstrated that phyA undergoes dark reversion in most dicotyledonous seedlings, but not in monocotyledonous seedlings (11). Hence, some of the Pfr in dicotyledonous seedlings is converted to the physiologically inactive Pr form in total darkness.

Rapid de novo synthesis of phyA in darkness, rapid degradation of the Pfr form (half-life of 30–60 min) in light, rapid dark reversion of 20% of the Pfr molecules to Pr (half-life of 20 min) in most dicotyledonous seedlings, and light-dependent rapid aggregation (half-life of 2 s) of the protein into SAPs have been demonstrated by in vivo spectroscopy and immunological methods (1). Ubiquitin may be involved in this degradation process, which is associated with the formation of SAPs (12). In vivo characterization of phytochromes has been possible only for phyA because the less abundant phyB cannot be spectroscopically distinguished from the abundant phyA, and high levels of chlorophyll prevent measurement of phyB even if phyA is destroyed in the light. Even in mutants lacking phyA, phyB is difficult to detect because of its low concentration. In vitro spectroscopic studies of phytochrome have been performed in light-grown bleached seedlings of certain species; the results of these studies may reflect properties of phyB (13, 14). These studies indicate a very slow destruction of the light-stable phytochrome (half-life of >8 h) and a weak partial dark reversion.

A model system for spectroscopic and immunological characterization of phyB has been developed in yeast (Saccharomyces cerevisiae) (15). The yeast-derived phytochrome-chromophore adducts are functional in planta (16), and tobacco PHYB* is stable, shows a partial rapid dark reversion from Pfr to Pr, and does not form SAPs. In contrast, rice PHYA* expressed in yeast does not undergo dark reversion, but does exhibit SAPs as well as light-independent formation of SAP-like structures (15). Therefore, phytochromes expressed in yeast appear to exhibit most of the properties demonstrated in planta.
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**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Yeast Strain**—Three different classes of phytochrome constructs were prepared: (i) full-length constructs (15) of rice PHYA (21) and tobacco PHYB (22); (ii) chimeric phytochromes in which the PsiI restriction site of tobacco PHYB at nucleotide 1779 (hinge region) was used in ligation with an equivalent PsiI site in rice PHYA (created by polymerase chain reaction) to generate PHYAB and PHYBA (thus, each half of the chimeric phytochromes has nearly the same molecular mass of ~60 kDa); and (iii) phytochromes with various N-terminal domains. *BstXI* restriction sites were created in tobacco PHYAB at nucleotide 286, in parsley PHYA (23) at nucleotide 245, and in rice PHYA at nucleotide 245 to generate PHYBRA and PHYPARA. PHYBRA consists of the 10.5-kDa N terminus of tobacco PHYB fused to the remaining C terminus of rice PHYA. PHYPARA consists of the 9-kDa N terminus of parsley PHYA fused to the remaining C terminus of rice PHYA (Fig. 1B). All of the restriction sites used are located in the highly conserved domain coding for the hinge regions of tobacco phyB, rice phyA, and parsley phyA. The constructs were cloned into the *BamHI*/*SaiI* sites of the yeast vector pAA7 (24) and expressed from a galactose-inducible promoter in yeast (*S. cerevisiae*, KN380) (25).

**Expression, Chromophore Assembly, and Detection of Phytochrome in Yeast**—Analysis of the expression and detection of the apophytochromes and their in vivo assembly with phycocyanobilin (PCB) (26, 27) are described (15). PCB was purified from *Spirulina* sp. as described (28). Because the selectable marker for the pAA7 vector is the lack of uracil, the yeast cells were cultivated on glucose complete medium lacking uracil (CMU). The yeast cells were cultivated in 100 ml of CMU containing galactose until they reached an absorbance (at 600 nm) of ~1.0. To avoid phototransformation, the following steps were carried out under a green safelight (29). The cells were collected by centrifugation and then resuspended in 2 ml of non-inducing glucose CMU containing 30% dimethyl sulfoxide. Chromophore in dimethyl sulfoxide was added until a final concentration of 100–200 μM was reached. The cells were incubated for 10 min at 25 °C, after which the autacatalytic in *vivo* chromophore assembly to form the holoprotein was stopped by dilution with glucose CMU. The cells were collected again by centrifugation, and the total amount of phytochrome (Ptot) and dark reversion were measured in a dual wavelength ratio spectrophotometer (30). Because the phytochrome adduct is stable in yeast, the incubation temperature for all measurements was at the physiological level of 20 °C. In *vivo* chromophore assembly is described (28). After two cycles in an SLM-AMINCO French pressure cell at a pressure of 1250–1500 bar, the supernatant from an ultracentrifugation (100,000 × g) of the crude extract was incubated on ice for 10 min with a final concentration of 4 μM PCB. Difference spectra were measured with an HP8452A diode array spectrophotometer (Hewlett-Packard Co.). The actinic light was obtained from light sources equipped with interference filters from Schott (Mainz, Germany): red light = 664 nm and far-red light = 727 nm. To determine the phytochrome yield in yeast, we calculated the actual concentrations of phytochrome from the difference spectra. For determination of the total protein concentration, we used the method of Popov et al. (31).

The electron microscopic investigations using a Philips CM10 electron microscope are described by Kunkel et al. (15). The polyclonal antibody (pRTB) used against tobacco PHYB, PHYAB, and PHYBA recognizes the 66-kDa N terminus of tobacco PHYB (15). The polyclonal antibody (pAVK) used against rice PHYA was raised against native *Avena* phyA.

**RESULTS**

**Difference Spectra**—Difference spectra of rice PHYA* and tobacco PHYB* showed peaks at 654/716 and 658/712 nm, respectively (Fig. 2). The peaks of PHYB* agree with a previous report (28). The peak positions were blue-shifted compared with native rice phyA because PCB was used as the chromophore instead of phytochromobilin (32). The absorption maxima for PHYB* and PHYBA* did not significantly differ from those for PHYA* and PHYB*. The far-red peaks were identical to those of PHYA* and PHYB*, whereas the red peaks exhibited a blue shift of 4 nm. The far-red absorption peaks of PHYBRA* and PHYPARA* were slightly blue-shifted to 706 and 708 nm, respectively. We therefore conclude that the phytochrome chimeras obtained behaved as intact photoreversible phytochromes. Phytochrome concentrations, as calculated from the difference spectra, are shown in Table I. The yield of phytochrome in yeast ranged from 0.5 to 2% of total protein.

**Sequestering of Phytochrome**—We observed SAP-like formation in yeast cells with rice PHYA and for both chimeric constructs PHYAB and PHYBA. There were no SAP-like formation in yeast cells with tobacco PHYB (Fig. 3). The SAP-like formations have an expansion of >1 μm in diameter. To compare the SAP-like formations, the levels of phytochrome expression of the examined yeast strains were determined in all four samples. All examined samples had approximately the same concentration of phytochrome (100 μg/g [fresh weight]; data not shown).

**In Vivo Dark Reversion**—We tested the dark reversion of reconstituted rice PHYA*, tobacco PHYB*, chimeric phytochrome adducts PHYAB* and PHYBA*, and N terminus-

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2 T. Kunkel, unpublished data.
swapped phytochrome adducts PHYTBRA* and PHYYPARA* in yeast (Fig. 4 and Table II). At 20 °C, rice PHYA* did not undergo dark reversion, whereas tobacco PHYB* exhibited dark reversion of 30% with a half-life of 20 min (Fig. 4, A and B). Both PHYAB* and PHYBA* showed dark reversion. PHYAB* displayed the same kinetic as PHYB*, with 30% of the molecules undergoing dark reversion with a half-life of 20 min. For PHYBA*, the rate of dark reversion was faster (half-life of 6 min) than for both PHYB* and PHYAB*, and a higher proportion of the molecules (40%) underwent dark reversion (Fig. 4, C and D). The N terminus-swapped phytochrome adducts PHYTBRA* and PHYYPARA* exhibited the same dark reversion kinetics as PHYBA* (Fig. 4, D–F, and Table II). A decay of 20% of the protein occurred only in the yeast expressing PHYA* (Fig. 4, A, dashed line), whereas Ptot of all other constructs was stable (Fig. 4, B–F, dashed lines).

**DISCUSSION**

We investigated which domains of the phytochrome molecule are responsible for dark reversion and SAP formation. For this investigation, rice PHYA, tobacco PHYB, and various chimeric phytochrome apoproteins were expressed in yeast and reconstituted in vivo with PCB as the chromophore to enable in vivo spectroscopic examinations. Table II summarizes the results. Rice PHYA* showed no dark reversion, but showed light-independent SAP-like formation. The opposite was true for tobacco PHYB* (see also Ref. 15). Wagner et al. (19) demonstrated, by domain swap experiments in transgenic wild-type Arabidopsis seedlings, that the chromophore-bearing N-terminal domains of phyA and phyB determine the photosensory specificity and different light lability of the phytochromes. These authors used oat PHYA and rice PHYB to clone chimera for their analysis. We used a yeast expression system and rice PHYA and tobacco PHYB for our constructs. To obtain more information about the role of the N-terminal domain, we constructed chimeric PHYAB and PHYBA by swapping the 60-kDa domains, and two
additional chimera (PHYTBRA and PHYPARA) were constructed by fusing either the 10.5-kDa N terminus of tobacco PHYB or the 9-kDa N terminus of parsley PHYA, respectively, to the truncated part of rice PHYA (Fig. 1A).

Photoreversible phytochromes were obtained with all constructs, which was a prerequisite for all subsequent analyses. Interestingly, all of the PHYA* chimeras with a replaced N terminus (PHYBA*, PHYTBRA*, and PHYPARA*) showed a slightly hypsochromic shift of the far-red absorption maxima compared with PHYA* (Fig. 2).

A characteristic difference between phyA and phyB is the light-dependent SAP formation of phyA in planta and the light-independent SAP-like formation of PHYA in yeast. Light-induced SAP formation of phyA has been observed in etiolated monocotyledonous and some dicotyledonous seedlings (33). As SAP formation and pelletability (34) show parallel kinetics, temperature dependence, and light dependence, it was concluded that these two observations reflect the same process.

Therefore, SAP formation is considered as a molecular property of phyA. Although SAP formation of PHYA* and PHYA has also been found in yeast cells, it could not be obtained for PHYB* and PHYB (15). Therefore, we compared SAP-like formation of PHYAB and PHYBA chimeric phytochromes in yeast cells.

The chimeric phytochromes PHYAB and PHYBA both showed SAP-like formation. This might be explained by the fact that the N-terminal half and the C-terminal half of PHYA are each sufficient to allow SAP formation when the domains are fused to the corresponding domains of PHYB. It is possible that the surfaces of the chimeric phytochrome molecules became hydrophobic in order to aggregate more simply in a cytosolic environment. phyB is not prone to aggregate because it is possibly more hydrophilic than phyA; the addition of a hydrophobic part causes it to sequester.

Wagner et al. (19) demonstrated that phyAB shows a similar destruction as phyA and that phyBA, like phyB, shows almost...
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Table II
Summary of the results

For difference spectra and absorption maxima, see Fig. 2; for SAP-like formations, see Fig. 3; and for dark reversion including decay of the total amount of phytochrome (P_{tot} decay, see Fig. 4).

| Phytochrome constructs in yeast | Absorption maxima | SAP-like formations | Dark reversion, t_{1/2} (within 2 h) | Dark reversion, total amount | P_{tot} decay (within 2 h) |
|-------------------------------|-------------------|---------------------|-------------------------------------|-----------------------------|---------------------------|
| PHYA*                        | 654/716           | +                   | 20                                  | 20                          |
| PHYB*                        | 658/712           | −                   | 30                                  | −                           |
| PHYAB*                       | 650/716           | ++                  | 30                                  | −                           |
| PHYBA*                       | 654/712           | ++                  | 40                                  | −                           |
| PHYBRA*                      | 658/706           | ND                  | 50                                  | −                           |
| PHYPARA*                     | 652/708           | ND                  | 50                                  | −                           |

* Not done.

no destruction. The sequestering of PHYA in yeast is light-independent (15), and this was also the case with PHYB in our experiments, although no destruction occurred in planta (19). Therefore, SAP formation might be necessary, but not sufficient, for destruction (12, 35). Because of the fact that PHYBA* is stable in yeast (Fig. 4D), a specific destruction pathway like that in plants does not exist in yeast.

Spectroscopic examinations of dark reversion in vivo revealed that 20% of PHYA* decayed (Fig. 4A, dashed line), whereas P_{tot} decay for all other constructs was stable (Fig. 4, B–F, dashed lines). There was no dark reversion of PHYA*. Kunkel et al. (15) did not observe this instability of PHYA* because their measurements were performed at 4 °C, whereas we performed our analysis at 20 °C. This difference in temperature is a sufficient explanation of this result. The amount and rate of phytochrome dark reversion in yeast were independent of temperature (compare Fig. 4, A and B, with Ref. 15; data not shown for Fig. 4, C–F). In contrast to this, phyA dark reversion is strongly temperature-dependent in planta (36), indicating that this process is not a simple thermal instability of Pfr, but a catalyzed process in planta.

In most etiolated dicotyledonous seedlings, a small fraction of Pfr (~20%) undergoes dark reversion into Pr as detected by in vivo spectroscopy (36, 37), whereas in etiolated grasses, no dark reversion of Pfr can be detected in vivo (38). phyA was probably measured in these studies because etiolated plant material was used (see the Introduction). Recently, Kunkel et al. (15) demonstrated dark reversion for reconstituted tobacco PHYB* in yeast. In vitro dark reversion of extracts of potato PHYA* and PHYB* expressed in yeast was shown by Ruddat et al. (39). In vitro dark reversion of oat phyA is detectable only for the so-called large phytochrome, which lacks the 4–6-kDa N-terminal fragment (40).

Rice PHYA* did not show any dark reversion. The dark reversion rates of PHYA constructs with an N-terminal replacement (PHYB*, PHYTBR*, and PHYPARA*) were three times faster and involved a greater proportion of Pfr molecules than the dark reversion rate of PHYB* (Fig. 4, D–F). Consequently, these molecules are less stable in their physiological Pfr form, which is consistent with the previous observation that the N terminus is very important for the stability of the Pfr form of phytochrome (40). We conclude that, among other factors, the very N-terminal part of the monocotyledony phyA is necessary, but not sufficient, for the prevention of dark reversion. The prevention of dark reversion stabilizes the Pfr form of phytochrome and plays an important role in the regulation of signal transduction. This mechanism in signal transduction of phyA in monocotyledonous plants would only be attenuated by destruction of the phyA molecule.

The chimeric adducts PHYAB* and PHYBA* both exhibited dark reversion, which indicates that a special domain of phyB does not mediate dark reversion. PHYAB* showed dark reversion comparable to PHYB* and weaker than the three N-terminus-swapped phytochromes (PHYBA*, PHYTBR*, and PHYPARA*). Thus, the C terminus of phyA has a minor, but necessary, role in the prevention of dark reversion, whereas the N terminus has a major, but not sufficient, role in the prevention of dark reversion. Therefore, the very N terminus (9 kDa) and the C-terminal half of phyA are both needed to hinder dark reversion. For most phyA and phyB phytochromes, dark reversion seems to be a crucial down-regulator of signal transduction because the half-life of ~20 min is much faster than the competing destruction. The attenuation of the signal transduction of phyA in monocotyledonous and some dicotyledonous plants (e.g. Amaranthus) is obviously due to the destruction with a half-life of ~30 min, rather than due to dark reversion (41). Destruction probably replaces dark reversion as a switching-off mechanism of signal transduction. However, in all other cases, dark reversion seems to be an intrinsic molecular property of phytochromes per se and was even measured in Synechosystis phytochrome (42).

We advance the hypothesis that there exists a mechanism in phytochrome that prevents dark reversion, rather than the existence of a mechanism that promotes dark reversion. Consistent with this hypothesis is the observation that changes in the molecular structure of phytochrome result in a partial instability of Pfr, which leads to enhanced and accelerated dark reversion.

If this interpretation holds, it would also have to be taken into account for interpretations of phyA and phyB signaling mutants. The missense mutations in both phyA and phyB in Arabidopsis are all clustered in a region in the C-terminal part of phytochrome (43, 44); they lead to a loss of function without, however, changing the expression level of the photoreceptors. Therefore, this domain has been described as a signaling box (Fig. 1A). However, alterations of the extent and the velocity of dark reversion may be caused by mutations in this region, thus leading to a non-signaling mutation.

Recently, Elich and Chory (45) analyzed one of these mutations (phyB-101, a Glu-to-Lys change at amino acid 812) and, after expression of this mutated phytochrome in yeast and assembly with the chromophore in vitro, showed a 3-fold faster dark reversion with three to four times more dark-reverting Pfr obtained compared with wild-type phyB. Thus, the interpretation of these “signaling” mutants necessitates a careful in vivo spectroscopic and physiological study.

The data presented here and by Elich and Chory (45) show that the amount and velocity of dark reversion may be altered by mutations or domain swapping. Based on the observations that the dark reversion of the Pfr/Pr heterodimer is faster than destruction and that only a small percentage of the Pfr molecules undergo dark reversion, Brockmann et al. (46) hypothesized that dark reversion is only possible starting from the Pfr/Pr heterodimer state. After a light pulse shifts a phytochrome system to a 50% photoequilibrium, only 50% of the molecules can convert into the heterodimer state. This is the maximum of heterodimers in the phytochrome system. As only half of these molecules undergo dark reversion, there are only 25% of the Pfr molecules left to show such a reversion. After a saturating light pulse, the system arrives at a photoequilibrium of 80–86%. Hence, 24–32% of heterodimers exist, so 12–18% of the Pfr molecules should show dark reversion. However, as demonstrated in Fig. 4, PHYB* and PHYAB* exhibited a dark reversion of 30%, and PHYBA*, PHYTBR*, and PHYPARA* showed a dark reversion of 40–50%. Thus, the heterodimer hypothesis is not true in this context, and dark reversion must consequently also be possible from the Pfr/Pfr homodimer state.
Our data show that dark reversion is a crucial mechanism to attenuate signal transduction and that this differs for phyA and phyB. Therefore, the dynamics of the phytochrome molecule, especially dark reversion, must be considered in the analysis of the first steps in the signal transduction chain of phytochrome-dependent photomorphogenesis.

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