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Photoresponses of Transgenic Arabidopsis Overexpressing the Fern *Adiantum capillus-veneris* PHY1[1]

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The phytochrome gene (*PHY1*) cDNA from the fern *Adiantum capillus-veneris* encodes an amino acid sequence that shows equal similarity (50–60%) to all five Arabidopsis phytochromes (PHYA–E). The *A. capillus-veneris* PHY1 cDNA was transformed into Arabidopsis ecotype Landsberg *erecta* to investigate its activity in angiosperms. Three of the resulting lines contained at least 8 times more spectrally active phytochrome than the wild type, indicating *that A. capillus-veneris* phytochrome can incorporate the chromophore of the host plants. Hypocotyl growth inhibition of these transgenic lines was investigated under red and far-red light. The results indicated dominant negative activity of *A. capillus-veneris* phy1 on the phytochrome A response in the host plants under continuous far-red light. However, the fern phytochrome did not interfere with the red-light repression of hypocotyl growth mediated by endogenous phytochrome B, and it failed to complement a *phyB* mutant phenotype. These observations suggest that the phy1 phytochrome molecule is too diverged from those of Arabidopsis to be fully functional.

Phytochrome is the best-characterized photoreceptor in plants and is involved in many physiological responses in diverse plant species, including algae and angiosperms (Furuya, 1993; Kendrick and Kronenberg, 1994). It has two photoreversible forms: Pr, which is the inactive form, and Pfr, which is active. Phytochrome is encoded by a small gene family in angiosperms, and five phytochrome genes (*PHYA–E*) have been identified in Arabidopsis (Sharrock and Quail, 1989; Clack et al., 1994). The phytochrome gene products are soluble apoproteins with a molecular mass of about 120 kD, and the holoprotein has a covalently attached chromophore that gives phytochrome its distinct spectral characteristics. Purified phytochrome exhibits a difference spectrum upon photoconversion from Pfr to Pr with an absorption maximum and minimum at about 665 and 730 nm, respectively.

Plants contain two physiologically distinguishable pools of Pfr, one is labile and the other is stable (Furuya, 1993). The *PHYA* gene encodes labile phytochrome (Hershey et al., 1985). A stable phytochrome was purified from green pea plants and its partial amino acid sequence (Abe et al., 1989) matches quite well with that of Arabidopsis PHYB (Sharrock and Quail, 1989). A phyB-deficient mutant has been identified (Koornneef et al., 1980; Reed et al., 1993), and phyA-deficient mutants have also been isolated and characterized in Arabidopsis (Nagatani et al., 1993b; Parks and Quail, 1993; Whitelam et al., 1993). The *phyB* mutant is deficient in the repression of hypocotyl elongation under continuous R, whereas it responds normally to continuous FR. Conversely, the *phyA* mutant has a normal response to continuous R but is deficient in its response to continuous FR.

Overexpression of monocotyledonous phyA in the dicotyledonous tobacco and tomato has shown that introduced heterologous phyA is biologically active (Boylan and Quail, 1989; Kay et al., 1989; Keller et al., 1989). Both monocot and dicot phyB were also shown to be active in Arabidopsis (Wagner et al., 1991). Studies of the photoresponses of these overexpressors have shown that the FR inhibition of Arabidopsis hypocotyl elongation is exaggerated only in phyA overexpressors, whereas R inhibition is exaggerated in both phyA and phyB overexpressors (McCormac et al., 1993). These studies suggested that overexpression of phytochrome in Arabidopsis is a suitable assay system for examining the phyA and phyB activities of introduced phytochromes. Using this approach Wagner et al. (1996a) showed that the N-terminal halves of phyA and phyB determine their respective photosensory specificities and that the C-terminal halves are interchangeable with respect to light regulation of hypocotyl elongation. It is

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Abbreviations: $\Delta\Delta A$, difference in absorbance difference; FR, far-red light; pAP1N, anti-*Adiantum* PHY1 N terminus polyclonal antibody; PHY, phytochrome apoprotein; phy, phytochrome holoprotein; R, red light.

interesting that particular fragments of phytochrome exhibit dominant negative interactions with the endogenous phyA in transgenic plants. Overexpression of mutated phyA lacking the N-terminal 52 amino acids resulted in a dominant negative phenotype under FR (Boylan et al., 1994). It has also been shown that overexpression of the C-terminal (Sakamoto and Nagatani, 1996; Wagner et al., 1996b) and chromophore-bearing N-terminal domains of phyB (Wagner et al., 1996b) cause a dominant negative phenotype under FR but not R. Thus, it was proposed that phyA and phyB may share reaction partners (Wagner et al., 1996a, 1996b).

The fern *Adiantum capillus-veneris* has a protonemal stage during the haplophase, which has allowed the investigation of many phytochrome responses such as cell elongation and phototropism in a single cell (Wada and Kadota, 1989). The phytochrome family in this fern consists of at least three members (K. Nozue, S. Fukuda, T. Kanegae, and M. Wada, unpublished data). The cDNA of one of these genes, *Adiantum PHY1*, was cloned from dark-grown diplophase leaves (Okamoto et al., 1993). The amino acid sequence showed 60 to 70% identity with phytochromes found in the fern *Selaginella martensii* (Hanelt et al., 1992), in the mosses *Physcomitrella patens* (Kolukisaoglu et al., 1993) and *Ceratodon purpureus* (Thummler et al., 1992; Hughes et al., 1996), and in the green algae *Mougeotia scalaris* (Winands and Wagner, 1996) and *Mesotaenium caldariorum* (Lagarias et al., 1996). The *Adiantum PHY1* also exhibits 50 to 60% identity to angiosperm phytochromes at the amino acid level, showing equal similarity to all five Arabidopsis sequences, *PHYA–E*. To date, whole-gene sequences are available for only a few lower plants as described above, and we do not know whether lower plants have an analogous phytochrome family to that of angiosperms. Therefore, it is impossible to predict to which angiosperm phytochrome the *Adiantum* phy1 is most closely related and whether the function of phy1 and higher plant phytochromes is conserved.

In this study we attempted to determine whether a fern phytochrome is biologically active in angiosperms. The *Adiantum PHY1* cDNA was introduced into Arabidopsis (ecotype Landsberg *erecta*) and was overexpressed under the control of the cauliflower mosaic virus 35S promoter.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of Arabidopsis (ecotype Landsberg *erecta*) were sterilized in a solution containing 5% (v/v) sodium hypochlorite and 0.02% (v/v) Triton X-100, rinsed with sterilized, distilled water, and sown aseptically on 0.8% (w/v) agar plates containing 10% (w/v) Murashige and Skoog salts. Seeds were then treated with continuous white light for 1 d to induce germination. Plants used for the transformation were grown under continuous white light for 7 d. Plants for immunoblot analyses were grown in darkness for 9 d at 25°C. Plants for experiments on hypocotyl growth were grown for 5 d under continuous FR or R or in darkness after the induction of germination. Plants used for spectroscopic analysis were grown under continuous white light at 23°C for 19 d and dark-adapted for 1 d. The biliverdin-feeding experiments were performed using agar plates containing 125 μM biliverdin (Sigma) and 7.5 mM Hepes-KOH (pH 7.4), as described by Nagatani et al. (1993b). The phytochrome chromophore mutant *hy1–100* (Chory et al., 1989; Terry, 1997) was used as a control.

Light Sources

White light used in all of the experiments was obtained from white fluorescent tubes (FL40SS.EX-N/32 h, Toshiba, Tokyo), and the fluence rate was approximately 3.7 W m^{-2}. R and FR sources used for the physiological experiments, and the green safety light used for manipulation of etiolated plants and biochemical experiments, have been described by Nagatani et al. (1993b). R and FR were attenuated with combinations of gray plastic filters (Takiron Plates S-802 and S-909, Takiron, Tokyo), as described by Nagatani et al. (1993a).

Plant Transformation

The upstream noncoding region (257 bp) of the *PHY1* cDNA (previously designated as *FP1*; Okamoto et al., 1993) of the fern *Adiantum capillus-veneris* was excised. This was achieved by PCR amplification of the cDNA between the ATG starting codon and the *Hin*dIII site, corresponding to the nucleotide positions 258 to 1136, using the 5′ primer (5′-GTCGACATGTCGAGTAC) and 3′ primer (5′-AAGCTT AACTGGCGGCA) (Fig. 1B). This fragment was inserted into the *PHY1* cDNA following digestion with *Sal*I and *Hin*dIII (Fig. 1C). The resulting *PHY1* cDNA lacking the 5′ noncoding region was excised first by digestion with *Xba*I and then blunted and digested with *Sac*I. The fragment was then inserted into the yA binary vector pBIH1-IG (Fig. 1D) that had been digested with *Xho*I, blunted, and also digested with *Sac*I (Fig. 1E). The sequence of the junction between the cauliflower mosaic virus 35S promotor and the *PHY1* coding region sequence was determined, and its sequence, especially the sequence of the PCR-amplified part of *PHY1*, was shown to be identical to that of the corresponding cDNA.

Plant transformation was performed by a callus-inducing method described by Akama et al. (1992) with *Agrobacterium tumefaciens* strain EHA 101. T_1 transgenic lines were selected for their resistance to antibiotics, kanamycin, and hygromycin. The single locus homozygote transgenic line L15 was crossed with the *phyB* (*phyB-1*) mutant, and seeds homozygous for both the *PHY1* gene and the *phyB* mutation were used for the complementation test.

Protein Analysis

Protein extracts were prepared from Arabidopsis using the extraction buffer described for *A. capillus-veneris* by Oyama et al. (1990), and proteins were concentrated with 25% (w/v) ammonium sulfate. Crude protein extracts from *A. capillus-veneris* were prepared with the same extraction buffer and 10% (w/v) PVP. Protein concentration was de-

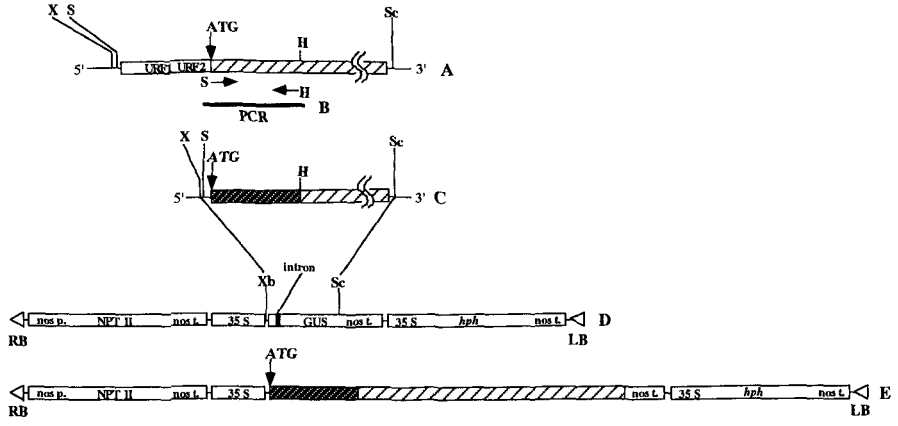

Figure 1. The construction of the binary vector used for Arabidopsis transformation. The *A. capillus-veneris* phytochrome 1 cDNA (A) was PCR-amplified between nucleotide positions 258 and 1234 (B). This fragment was inserted into the PHY1 cDNA that was excised by digestion with *Sal*I and *Hin*dIII (C). This was ligated into the binary vector pBIH1-IG (D) that was digested with *Xho*I, blunted, and digested with *Sac*I. This construct (E) was used for the transformation. NPT II, Kanamycin-resistance gene; *hph*, hygromycin-resistance gene. S, *Sal*I; Sc, *Sac*I; Xb, *Xba*I; X, *Xho*I; RB, right border; lb, left border; nos p., nopalin synthase promoter; and nos t., nopalin synthase terminator; URF1, unidentified open reading frame 1; URF2, unidentified open reading frame 2.

termined by the method of Bradford (1976) using BSA as a standard. Proteins were separated by SDS-PAGE using 7.5 and 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Western blotting was performed with anti-phytochrome antibodies and reactions were detected using the alkaline phosphatase system. A polyclonal antibody used for the detection of PHY1 polypeptide was raised in rabbits against the N terminus of the PHY1 protein (pAP1N) expressed in *Escherichia coli*. The purified N-terminal PHY1 polypeptide was obtained by making a fusion protein with an N-terminal poly-His tag (pTrcHis ABC; Invitrogen, San Diego, CA). The protein was purified using a Ni^{2+} column according to the manufacturer's instructions (Invitrogen).

Monoclonal antibodies against Arabidopsis PHYA, mAA1, PHYB, and mBA2 were described previously (Shinomura et al., 1996).

Spectroscopic Analysis

Five grams of tissue was lyophilized and ground into a powder using a mortar and pestle. Proteins were then extracted and concentrated as described by Weller et al. (1996) using 15 mL of extraction buffer containing 0.1% (w/v) polyethylenimine (Wako, Osaka, Japan). The final pellet was dissolved in 500 μL of TEGE buffer (25 mM Tris, 2 mM EDTA, 25% [v/v] ethylene glycol, 2 mM PMSF, 1 mM DTT, and 2 μg/mL leupeptin, adjusted with HCl to pH 7.8 at 5°C) and a difference spectrum between R- and FR-irradiated samples was recorded using a recording-difference spectrophotometer (model 3410, Hitachi, Tokyo, Japan), as described by Weller et al. (1996).

RESULTS

Plant Transformation

Arabidopsis (ecotype Landsberg *erecta*) was transformed with a vector containing the *A. capillus-veneris* phytochrome cDNA *PHY1* preceded by the 35S promoter (Fig. 1). As a result of the first screening, we obtained 23 T_1 transgenic lines that were resistant to the antibiotics kanamycin and hygromycin. Three lines designated as L15, L20, and L26, for which 3:1 segregation of the resistant plants was observed in the T_2 generation, were used to obtain homozygous seeds in the T_3 generation. These seeds were used for further biochemical and physiological experiments. The general phenotype of these transgenic lines grown under continuous white light was indistinguishable from that of wild-type plants.

Immunochemical and in Vitro Spectroscopic Analysis of PHY1 Transgenic Plants

The transgenic lines were examined for the presence of the PHY1 polypeptide by western-blot analysis using pAP1N. The antibody reacted with a protein of about 120 kD in extracts from etiolated tissue of the transgenic lines (Fig. 2A, lanes 3–5). The size matched well with that of the predicted PHY1 protein and was identical to that detected in *A. capillus-veneris* extracts (Fig. 2A, lane 1). As expected, no reaction was observed in the wild-type extract (Fig. 2A, lane 2). Analysis of extracts from light-grown seedlings demonstrated that the PHY1 polypeptide was also detected at significant levels under these conditions (data not shown). These observations clearly indicate that these transgenic plants accumulate *A. capillus-veneris* PHY1 polypeptide. The endogenous PHYA and PHYB proteins of Arabidopsis were then examined to determine whether the overexpression of PHY1 affected the levels of these phytochromes in the transgenic plants. The results shown in Figure 2, B and C, demonstrate that the levels of PHYA and PHYB were not significantly altered in the transgenic lines compared with the wild type.

Next we investigated whether the overexpressed *A. capillus-veneris* PHY1 could form a phytochrome holoprotein in Arabidopsis. The levels of photoactive phytochrome

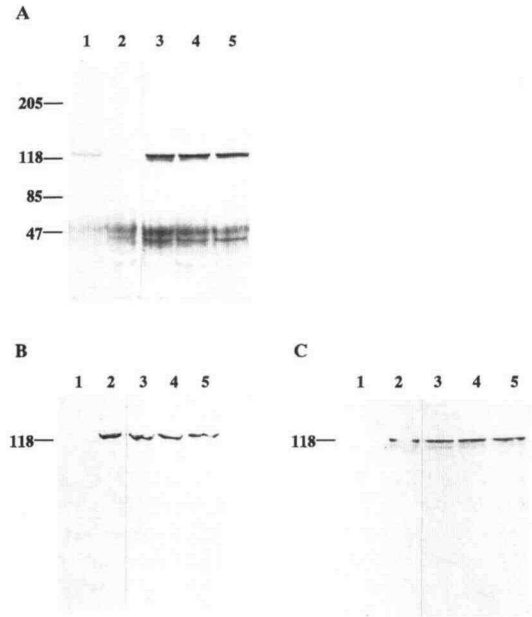

Figure 2. Immunoblot analysis of *A. capillus-veneris* PHY1 polypeptide (A) and endogenous phyA (B) and phyB (C) in PHY1 transgenic plants. For each blot: lanes 1, crude protein extract from young, dark-grown leaves of *A. capillus-veneris*; lanes 2, 3, 4, and 5, ammonium sulfate-precipitated protein from dark-grown wild-type plants and PHY1 transgenic lines L15, L20, and L26, respectively. Twenty-five micrograms of protein was loaded per lane. The polyclonal antibody pAP1N was used for the detection of the PHY1 polypeptide, and the monoclonal antibodies mAA1 and mAB2 were used for the detection of Arabidopsis phyA and phyB, respectively. The relative molecular mass of prestained markers (A) or 118 kD (B and C) is indicated on the left.

in the transgenic lines were examined by obtaining difference spectra in protein extracts from dark-adapted light-grown PHY1 transgenic and wild-type plants. Figure 3 shows that wild-type plants have only a small amount of photoactive phytochrome. In contrast to this, PHY1 transgenic plants contained at least eight times more holophytochrome than wild-type plants. The absorbance maximum and minimum of the FR-irradiated minus R-irradiated difference spectrum detected in the transgenic plants were approximately 665 and 728 nm, respectively. Although it is difficult to estimate these values exactly, they are similar to those measured in vivo in dark-grown leaves of *A. capillus-veneris* (Oyama et al., 1990) and generally consistent with those of other phytochromes. The immunochemical and spectral data therefore demonstrate that the *A. capillus-veneris* phy1 is highly expressed and spectrally active in the transgenic plants.

phy1 Is Not Involved in the R Repression of Hypocotyl Elongation

It is known that Arabidopsis hypocotyl elongation is repressed by light and that phytochrome is involved in this process; both continuous R and FR are effective. The PHY1 transgenic lines were examined to determine whether they had altered sensitivity to R or FR. Under a range of different fluence rates of R, there was no significant difference between the hypocotyl length of PHY1 transgenic lines and wild-type plants (Fig. 4A). The average level of photoactive phy1 is 0.53×10^{-3} $\Delta\Delta A$/g fresh weight, which is similar to that detected in the Arabidopsis phyB-overexpressing line ABO (Wagner et al., 1991). Even with this level of phy1 expression, we could not detect a significant difference between wild-type and PHY1 transgenic plants under any fluence rate of R. This observation suggests that *A. capillus-veneris* phy1 cannot mediate the repression of hypocotyl elongation by R in Arabidopsis. However, it is possible that the activity of phy1 is too small to detect under the background of wild-type levels of endogenous phyB. To eliminate this possibility, the biological activity of phy1 was examined in the absence of endogenous phyB. Seeds homozygous for the *PHY1* transgene and *phyB-1* were obtained by crossing the transgenic line L15 with the *phyB* mutant. When the hypocotyl length of seedlings of the double mutant were examined under 4.2 W m^{-2} of R, no recovery of the *phyB* mutant phenotype was observed (Fig. 5C). These results indicate that *A. capillus-veneris* phy1 cannot function as a phyB in Arabidopsis.

phy1 Exhibits Dominant Negative Suppression of the Action of Endogenous Arabidopsis phyA

Under continuous FR all three of the transgenic lines had longer hypocotyls than wild-type plants (Fig. 4B). This dominant negative phenotype under FR increased in a

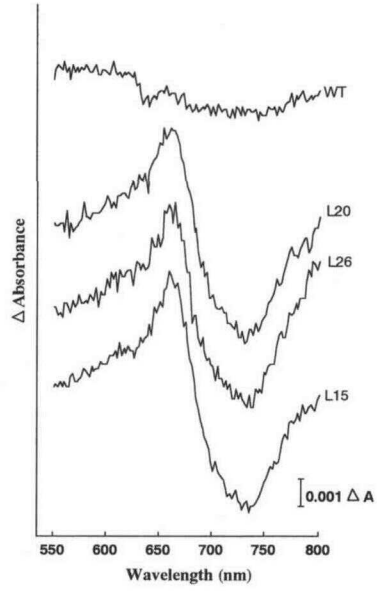

Figure 3. Spectroscopic analysis of transgenic plants overexpressing *A. capillus-veneris* PHY1 and wild-type plants grown for 19 d in continuous white light (3.7 W m^{-2}) and dark-adapted for 1 d. Protein extracts were concentrated 10-fold by ammonium sulfate precipitation, and the phytochrome content was quantitated from the FR-irradiated minus R-irradiated difference spectrum from duplicate samples. Wild type (WT; 0.6×10^{-3} $\Delta\Delta A_{666-730}$), L15 (5.3×10^{-3} $\Delta\Delta A_{666-728}$), L20 (5.6×10^{-3} $\Delta\Delta A_{666-728}$), and L26 (5.0×10^{-3} $\Delta\Delta A_{666-728}$). Representative difference spectra are shown.

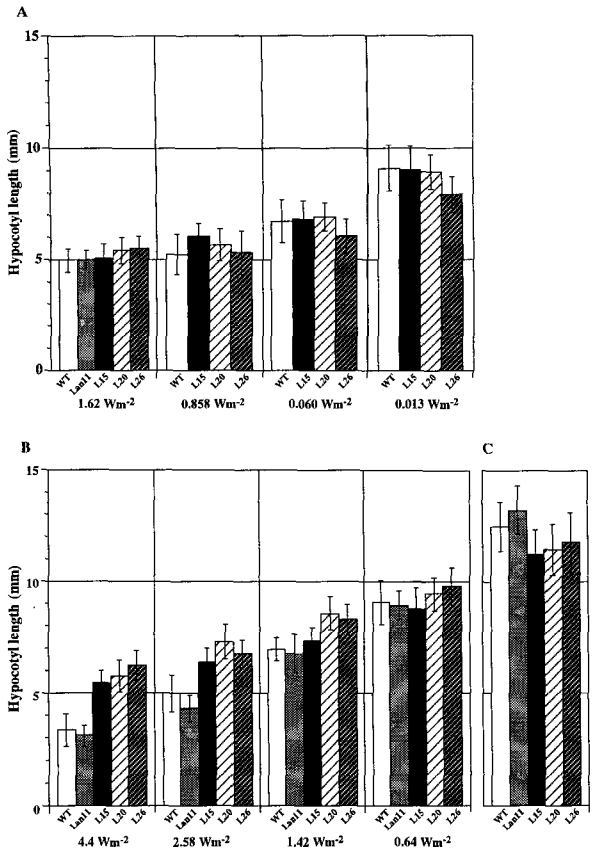

Figure 4. Photoresponses of PHY1 transgenic plants were compared with wild-type plants and plants transformed with the empty vector. The wild type (WT) and, in most experiments, the empty vector transformant Lan11 and the PHY1 transgenic lines L15, L20, and L26 were compared for their responses to different fluence rates of continuous R (A), continuous FR (B), and continuous darkness (C). Hypocotyl lengths of the plants were measured 6 d after sowing. Error bars are ± SD.

fluence-dependent manner. Under 4.4 W m^{-2} of continuous FR, all of the PHY1-overexpressing lines had hypocotyls twice the length of those of the wild-type plants (Fig. 4B). Plants transformed with an empty vector, pBI-H1IG (Lan11), were identical to wild-type plants under all of the conditions that were tested. There was no significant difference between PHY1 transgenic and wild-type plants grown in the dark (Fig. 4C).

The dominant negative phenotype of the PHY1 transgenic plant suggests that either phy1 interrupted normal FR signal transduction or directly affected the activity of the endogenous phyA. As shown in Figure 2B, the amount of Arabidopsis PHYA polypeptide was not significantly reduced in the transgenic lines compared with the wild type. However, it is possible that the high levels of PHY1 expression led to a reduction in chromophore availability for the endogenous PHYA polypeptide. To examine this possibility we grew the transgenic lines on plates containing the phytochrome chromophore precursor biliverdin. However, under these conditions the dominant negative phenotype of the PHY1 transgenic plant was still apparent (Fig. 6). Their hypocotyl lengths were not reduced by the application of biliverdin but in fact were even longer than their negative controls. In contrast, the phytochrome chromophore mutant hy1 (Koornneef et al., 1980) was reduced to a similar level to that of wild-type seedlings, as shown previously (Parks and Quail, 1991). These results indicate that the phenotype of the PHY1 transgenic plants is not a consequence of reduced chromophore availability for the endogenous PHYA in the transgenic plants.

DISCUSSION

To our knowledge, this is the first attempt to introduce an evolutionarily divergent cryptogam phytochrome (Kolukisaoglu et al., 1995; Mathews et al., 1995) into an

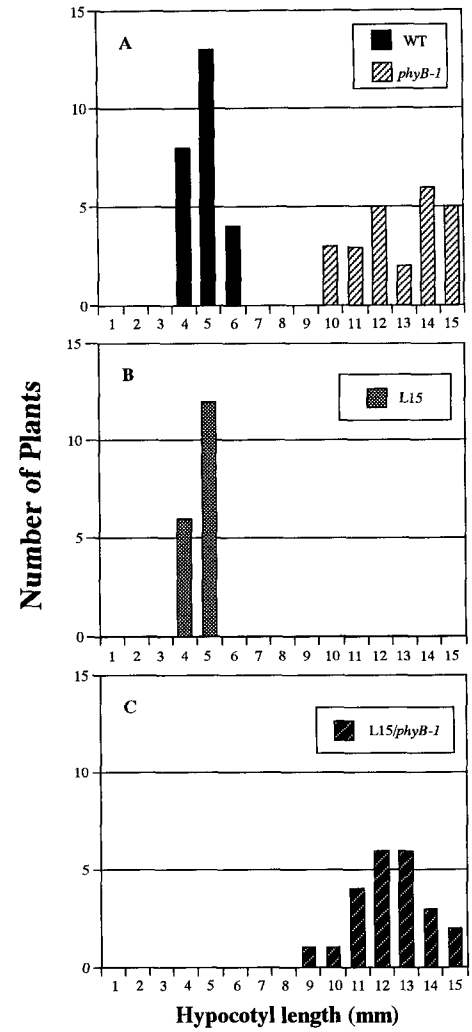

Figure 5. Frequency distribution of hypocotyl length under continuous R (4.2 W m^{-2}) of the wild type *and phyB-1* (A), transgenic PHY1 line L15 (B), and plants homozygous to *PHY1* and *phyB-1* (C). Plants homozygous for *PHY1* and *phyB-1* genes were obtained from a selfed progeny of F_1 that was obtained from a cross between transgenic PHY1 (L15) and *phyB-1*, and one of the F_2 progeny was used in this experiment. Hypocotyl lengths were measured 6 d after sowing. WT, Wild type.

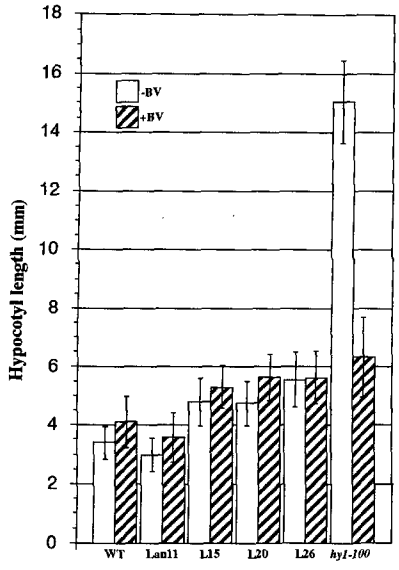

Figure 6. Transgenic PHY1 lines L15, L20, L26, wild type (WT), the empty vector transformant (Lan11), and *hy1–100* seeds were sown on agar plates with (+) or without (−) 125 μM biliverdin (BV) and grown under continuous FR (4.4 W m^{-2}). Hypocotyl lengths were measured 6 d after sowing. Error bars are ± SD, where *n* = approximately 30 seedlings for each Arabidopsis line. This is a representative result from three separate experiments.

angiosperm species. Since endogenous levels of PHYA and B polypeptides in the PHY1 transgenic lines were not altered (Fig. 2, B and C), we conclude that the excess holophytochrome is due to PHY1 that had incorporated a chromophore. The peak positions in the difference spectrum are also consistent with this interpretation. These results demonstrate that the *A. capillus-veneris* phy1 is highly expressed and spectrally active in the transgenic plants.

Even though substantial phy1 holoprotein accumulated, PHY1 transgenic plants showed only a subtle phenotype with respect to the light repression of hypocotyl elongation. This result is in contrast to those obtained with angiosperm phytochromes. Overexpression of either phyA (Boylan and Quail, 1991; Nagatani et al., 1991; Clough et al., 1995) or phyB (Wagner et al., 1991) results in a dwarf phenotype under continuous R and/or FR. Although *Adiantum* PHY1 shows equal similarity to all angiosperm phytochromes, it has two stretches of sequences, motif 1 and motif 2, that are conserved among different PHYB sequences but are not found in other angiosperm PHY sequences (Okamoto et al., 1993). However, none of the three lines overexpressing *A. capillus-veneris* PHY1 caused a dwarf phenotype in Arabidopsis under continuous R (Fig. 4A). Furthermore, crossing the PHY1 transgenic plant with a *phyB* mutant indicated that the fern phy1 failed to complement the *phyB* mutation (Fig. 5).

When the FR response was compared with PHY1 transgenic and wild-type plants (Fig. 4B), FR of higher fluence was less effective on the repression of hypocotyl elongation in PHY1 transgenic plants than in the wild type. This might be caused by either the loss of functional endogenous phyA molecules or through an effect on the phyA signal transduction pathway. A dominant negative response under FR has also been observed in overexpressors of phyA lacking an N terminus (Boylan et al., 1994; Emmler et al., 1995), full-length phyB, and a truncated phyB (Sakamoto and Nagatani, 1996; Wagner et al., 1996b). Experiments in which the N- and C-terminal domains of phyA and phyB were exchanged (Wagner et al., 1996a) suggest that these two photoreceptors may share the same reaction partners (Wagner et al., 1996a, 1996b). Our immunoblot (Fig. 2B) and biliverdin feeding (Fig. 6) results suggest there is no effect on the amount of phyA holoprotein. It is possible that phy1 forms inactive heterodimers with the endogenous phyA, which would reduce the level of functional phyA. However, in previous studies of the expression of heterologous phytochromes in Arabidopsis, no heterodimers have ever been detected (Boylan et al., 1994; Sakamoto and Nagatani, 1996; Wagner et al., 1996b), and we favor the hypothesis that phy1 is affecting the signal transduction of phyA by competing with endogenous phyA for binding to its reaction partner.

In summary, it appears that *Adiantum* phy1 is not able to transduce normal phytochrome signals in Arabidopsis. This suggests that the PHY1 protein is too divergent from angiosperm phytochromes to contain the specific structural features required for activating these signal transduction pathways. Nevertheless, the principal signal transduction mechanisms of these two divergent groups of phytochromes could be similar. The dominant negative interference suggests that at least one structural domain involved in the signal transduction is conserved to some extent in these divergent phytochromes.

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