Coordination of Phytochrome Levels in phyB Mutants of Arabidopsis as Revealed by Apoprotein-Specific Monoclonal Antibodies

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Manuscript received January 26, 1998
Accepted for publication March 16, 1998

ABSTRACT

Accumulating evidence indicates that individual members of the phytochrome family of photoreceptors have differential but interactive roles in controlling plant responses to light. To investigate possible cross-regulation of these receptors, we have identified monoclonal antibodies that specifically detect each of the five Arabidopsis phytochromes, phyA to phyE (phytochrome A holoprotein; PHYA, phytochrome A apoprotein; PHYB, phytochrome B gene; phyA, mutant allele of phytochrome A gene), on immunoblots and have used them to analyze the effects of phyA and phyB null mutations on the levels of all five family members. In phyB mutants, but not in phyA mutants, a four- to six-fold reduction in the level of phyC is observed in tissues grown either in the dark or in the light. Coordinate expression of phyB and phyC is induced in the phyB mutant background by the presence of a complementing PHYB transgene. However, in transgenic lines that overexpress phyB 15- to 20-fold, phyC is not similarly overexpressed. In these overexpresor lines, the levels of phyA, phyC, and phyD are increased two- to four-fold over normal in light-grown but not dark-grown seedlings. These observations indicate that molecular mechanisms for coordination or cross-regulation of phytochrome levels are active in Arabidopsis and have implications for the interpretation of phytochrome mutants and overexpresor lines.

**RED (R) and far-red (FR) light are important environmental signals in the regulation of plant development, with major roles in seedling deetiolation, neighbor detection, and photoperiodism (Smith 1994). The plant R/FR photoreceptors, the phytochromes, are soluble chromoproteins consisting of a 120–130-kD apoprotein and a linear tetapyrrole chromophore. A small gene family encoding the phytochrome apoproteins, the PHY gene family, is found in all angiosperms that have been examined and homologous apoprotein genes have been isolated from gymnosperms, ferns, mosses, and algae (Matthews and Sharrock 1997). In the angiosperms, three major PHY gene lineages, designated the PHYA, PHYB, and PHYC genes, are found in most sampled plants from both the monocot and dicot groups. In addition to these three genes, many flowering plant families contain additional, more recently diverged PHY genes (Matthews et al. 1995).

In Arabidopsis, where an effort has been made to isolate all of the detectable phytochrome coding sequences, five PHY genes, PHYA–PHYE, have been described (Sharrock and Quail 1989; Clack et al. 1994). These genes are expressed at the level of processed mRNAs (Clack et al. 1994) and analysis of the effects of mutations in the PHYA, PHYB, and PHYD genes has demonstrated that the phyA, phyB, and phyD gene products mediate diverse R/FR light-controlled physiological responses (reviewed in Whitelam and Devlin 1997). PhyA plays a major role throughout the plant life cycle in sensing prolonged FR light (the FR high irradiance response) and in mediating very low fluence responses (Casal et al. 1997). PhyB and phyD, which are highly related in amino acid sequence and are representative of recently diverged phytochromes, have highly overlapping roles in sensing the ratio of R to FR light and in mediating what are collectively known as the shade avoidance responses (Aukerman et al. 1997; Smith and Whitelam 1997). Between the two forms, phyB has the much more prominent role. The functions of the phyC and phyE phytochromes in Arabidopsis are not currently known. Analysis of double mutant combinations of phyA, phyB, and phyD null mutations (Redd et al. 1994; Devlin et al. 1996; Aukerman et al. 1997) has revealed additive effects, synergistic interactions, and some redundancy among the functions of these receptors. This confirms that the physiological and developmental outcome of a complex natural R/FR light environment in a given plant involves integration of phytochrome photoreceptor activities and/or their cellular signaling pathways.

The distributions of the Arabidopsis PHYA–E mRNAs

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Accepted for publication March 16, 1998

Genetics 149: 525–535 (June 1998)
have been described (Clack et al. 1994) and the expression patterns of the PHYA, B, D, and E promoters have been evaluated using β-glucuronidase (GUS) fusion transgenes (Somers and Quail 1995a,b; Goosey et al. 1997). In general, these studies indicate that the phytochromes are widely distributed throughout the plant and are present in at least some of the same cells. However, because the photosensory activities of the phytochromes reside in the chromoprotein PHY gene products, a full understanding of the functions of the individual family members will require characterization of the levels, expression patterns, light stabilities, and regulation of the photoreceptors themselves. This will in turn require immunological tools that specifically detect the individual apoproteins. The isolation of monoclonal antibodies (mAbs) that recognize the Arabidopsis phytochromes was initiated by Somers et al. (1991) who generated what, at that time, were called “type-selective” mAbs to phyA, phyB, and phyC. In those studies, full-length Arabidopsis PHYA, PHYB, and PHYC apoproteins were expressed in E. coli and purified, and mAbs which selectively detected PHYB and PHYC were isolated. A mAb generated against purified oat phyA by Shanklin (1988), was shown to recognize the Arabidopsis PHYA apoprotein but not PHYB or PHYC on immunoblots (Somers et al. 1991). At the time of those studies, the PHYD and PHYE genes (Clack et al. 1994) had not been characterized and it could not be determined whether these “type-selective” antibodies cross-reacted with the phyD and phyE proteins. Recently, Aukerman et al. (1997) showed that the anti-phyB mAbs isolated by Somers et al. (1991) do in fact cross-react with phyD.

We describe here the identification of mAbs which can be used to specifically detect each of the five Arabidopsis phytochromes on immunoblots. These antibodies have been used to reevaluate the effect of phyA and phyB mutations on the levels of the other phytochromes and to examine the effects of overexpression of phyA and phyB. Assignment of discrete functions in light sensing to the individual Arabidopsis phytochromes through analysis of phenotypes conferred by mutations or by apoprotein overexpression assumes that alteration of the expression of one PHY gene does not significantly change the expression of the other PHY genes. Consistent with this, Parks and Quail (1993) reported that phyA mutants of Arabidopsis lacked phyA but had wild-type levels of phyB and phyC. Similarly, Somers et al. (1991) presented evidence that phyB mutants of Arabidopsis were deficient for phyB antigen but contained wild-type levels of phyA and phyC. Our current results partly contradict the earlier studies of Somers et al. (1991) and indicate that there is a significant degree of coordination of the levels of phyB and other phytochromes, notably phyC. The implications of these findings for interpretation of phytochrome mutants and of possible mechanisms of phytochrome interaction are discussed.

**MATERIALS AND METHODS**

**Plant materials and growth conditions:** The Arabidopsis thaliana Landsberg erecta (Ler), Nossen (No-0), and Columbia (Col) ecotypes were obtained from previously described sources (Aukerman et al. 1997). The phyB-1 (hy3-Bo64), phy-7 (hy3-1053), and phyB-8 (hy3-M4084) mutants (Koornneef et al. 1980), in the Ler genetic background, were obtained from M. Koornneef (Agricultural University, Wageningen, The Netherlands) and the phyA-201 mutant (Reed et al. 1994) was obtained from J. Chory (The Salk Institute, La Jolla, CA). The ABO phyB overexpressor transgenic line (Wagner et al. 1991), the AOX phyA overexpressor line (Boylan and Quail 1991), and the phyB-1(mPHYB) and No-0(mPHYB) lines (West et al. 1994), all in the No-0 genetic background, were as described previously.

The BOE phyB overexpression transgene, which is very similar to the transgene in the ABO line, was constructed by first substituting the GUS coding region in pBl121 (Jefferson et al. 1987), from the XbaI site of the SstI site, with the polylinker from pGEM3 (Promega, Inc., Madison, WI). Subsequently, the PHYB cDNA sequence (Sharrock and Quail 1989), from nucleotide 43 in the 5' untranslated region to the AatII site at nucleotide 3674 in the 3' untranslated region, was cloned into this vector in front of the CaMV 35S promoter. This construct was transformed into the No-0 wild type as described (West et al. 1994) and lines #2-3 and #3-2 were identified as kanamycin-resistant lines showing a phyB overexpression phenotype similar to that previously described for the ABO line (Wagner et al. 1991).

Seven-day-old seedlings were used for preparation of all protein extracts. Seeds were surface sterilized for 30 min in 15% bleach/0.2% sodium dodecyl sulfate (SDS), rinsed at least five times with sterile water, and plated on sterile filter paper overlaying germination medium (GM) agar medium in 100- x 25-mm petri dishes (West et al. 1994). Plates were kept in the dark at 4° for 2 to 3 days, treated for 2 to 3 hr with white light to induce germination, then placed either in complete darkness or under continuous light from a bank of 40 W cool-white fluorescent bulbs at room temperature.

**Cloning and expression of the PHYD and PHYE apoproteins in E. coli:** All PHYD and PHYE bacteriophage λ-clones and nucleotide positions are from Clack et al. (1994). A full-length Arabidopsis PHYD apoprotein coding sequence, from the ATG at nucleotide 242 to nucleotide 3614 in the 3' untranslated region, was assembled from polymerase chain reaction (PCR) products made from the genomic clone AD6-1, and the partial cDNA clone λ7151, with the introduction of an NdeI restriction site at the ATG and a BamHI site in the 3' untranslated region. This sequence was inserted into the pET3c vector (Rosenberg et al. 1987). A full-length PHYE apoprotein coding sequence was assembled, from the ATG at nucleotide 173 to an XbaI restriction site 25 bp downstream of the poly(A) addition site located at nucleotide 3673. The amino-terminal coding sequence, extending from the ATG to the SstI site at nucleotide 1122, and the carboxyl-terminal coding sequence, from the XhoI site at nucleotide 3324 to the XbaI site beyond the poly(A) tract, were PCR-amplified from genomic clone λE3-2, with the introduction of an NdeI site at the ATG. The central PHYE coding sequence, from the SstI site to the XhoI site, was obtained without the three intron sequences from a partial PHYE reverse transcriptase-PCR clone described in Clack et al. (1994). The 5' end, central, and 3' end PHYE sequences were assembled and inserted
into the pET3c vector. The pET3c-PHYD and pET3c-PHYE plasmids were transformed into E. coli BL-21. Overexpression of the PHYD and PHYE apoproteins was induced, inclusion bodies solubilized, and the products gel-purified as described by Somers et al. (1991). Full-length recombinant PHYA, PHYB, and PHYC apoproteins were as described (Somers et al. 1991).

**Antibody preparation:** The recombinant PHYD and PHYE apoproteins were used as antigens to produce mAbs as described previously (Somers et al. 1991; Wagner et al. 1991). Primary screens for reactive hybridoma supernatants were performed by ELISA assay against the recombinant PHYD and PHYE antigens. Following the primary screens, type-specific monoclonal supernatants were identified as follows. For the anti-phyD mAb, crude inclusion body preparations of the PHYD and the PHYB recombinant apoproteins (~30 ng/lane) were fractionated on adjacent lanes of 6% SDS-PAGE gels and transferred to nitrocellulose. The blots were cut into strips and probed with each of the ELISA-positive supernatants at a 1:50 dilution to identify mAbs that recognize PHYD but not PHYB. Candidate PHYD-specific supernatants were then tested on blots containing 25% (NH₄)₂SO₄ precipitated tissue extracts from Arabidopsis seedlings to identify mAbs exhibiting detectable reactivity with the endogenous phyD holoprotein. In this screen, only one monoclonal cell line, 2C1, was identified that produced antibody that both discriminated between the PHYD and PHYB apoproteins and recognized native phyD in plant extracts. The PHYB-specific mAbs B6-B3 and B2-A5 were identified by screening the pool of anti-phyB mAbs described by Somers et al. (1991) in an ELISA assay using the recombinant PHYB and PHYD apoproteins as antigens (10 ng/well). Anti-phyE supernatants identified in the primary ELISA screen were further screened by ELISA against recombinant PHYA, PHYB, and PHYC apoproteins (10 ng/well) and by immunoblot screen against plant extract as described above.

**Plant protein extraction and immunoblot analysis:** Direct extracts of soluble protein were prepared from 7-day-old seedlings by briefly grinding 0.25 g of frozen tissue in liquid nitrogen in a mortar and pestle, adding 0.5 ml buffer (100 mM Tris-HCl pH 8.5, 10 mM EDTA, 150 mM (NH₄)₂SO₄, 50% (v/v) ethylene glycol, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 5 µg/ml Na₂HCO₃, 1 mM di-thiothreitol) for 2 min, and centrifuging for 5 min at 13,000 rpm in a microfuge at 4°C. A sample of the supernatant was removed and the protein concentration was determined by the method of Bradford (1976). The remaining supernatant was mixed with an equal volume of 2× SDS-PAGE sample buffer (Laemmli 1970) and frozen in liquid nitrogen. For each sample, the equivalent of 50 µg of protein from the original extract was heated in a boiling water bath for 5 min and separated on a 6% SDS-polyacrylamide gel according to Laemmli (1970). The alternative tissue extraction protocol, which yields alesamone sulfate-precipitated aqueous extracts of seedlings, was performed as described in Aukerman et al. (1997). For sequential (NH₄)₂SO₄ fractionation of extracts, all steps were carried out at 4°C. Aqueous extracts of Col and Wassilewskija (Ws) wild-type dark-grown seedlings were prepared, 0.2 g/ml of dry (NH₄)₂SO₄ (w/v) was added to each, and the mixtures were stirred for 30 min. The extracts were centrifuged 20 min × 20,000 rpm, and the pellets were resuspended in 1× extraction buffer as 0–20% (w/v) (NH₄)₂SO₄ fractions. An additional 0.05 g/ml dry (NH₄)₂SO₄ was added to each supernatant and the mixtures were stirred for 30 min. These preparations were centrifuged 20 min × 20,000 rpm and the resulting pellets solubilized as 20–25% (w/v) (NH₄)₂SO₄ fractions. This procedure was repeated two more times to generate the 25–30% and 30–35% (NH₄)₂SO₄ protein fractions from each wild-type line. The protein concentration of the fractions was determined by the method of Bradford (1976) and samples of each fraction, calculated to be equivalent to 300 µg of protein in the original extract, were loaded on gels.

For Figures 1 and 2, recombinant apoproteins were electrophoresed to nitrocellulose, blocked, and developed using a secondary goat-anti-mouse antibody conjugated to alkaline phosphatase as described by Wagner et al. (1991). For Figures 3 through 9, proteins were electrophoresed to a Hybond ECL membrane (Amerham, Arlington Heights, IL), blocked in 5% nonfat dry milk in TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20), incubated with the primary mAb and horseradish peroxidase-conjugated secondary antibody, and detected with ECL reagents as recommended by the manufacturer (Amerham).

**RNA preparation and blot analysis:** RNA was isolated, enriched for poly(A) RNA by oligo(dT) chromatography, fractionated on an agarose-formaldehyde gel, blotted, hybridized with the B3' 650, C3' 600, or D3' 600 probes, and washed as described in de la Rubia et al. (1994).

**RESULTS**

Isolation of anti-phytochrome mAbs and specificity of mAbs to purified phytochrome apoproteins: The coding sequences for the PHYD and PHYE apoproteins (1991) were cloned into a T7 vector (Rosenberg et al. 1987), expressed in E. coli, and purified from polyacrylamide gels. The PHYD and PHYE proteins were used to immunize mice and hybridoma lines were generated. The anti-phyA mAb 073d previously isolated by Shanklin (1988), and the collection of anti-phyB hybridoma lines and the three anti-phyC mAbs previously isolated by Somers et al. (1991) were also used in these studies. All five full-length recombinant apoproteins, purified from E. coli, were used to screen hybridoma culture supernatants for reactivity with their purified cognate antigen and for lack of cross-reactivity to the four non-cognate phytochrome antigens, first by ELISA assays and then on immunoblots.

In Figure 1, protein blots of the five purified recombinant phytochrome apoproteins were probed with the anti-phyA mAb 073d (Shanklin 1988), the anti-phyC mAb C13 (Somers et al. 1991), and the monoclonal lines, anti-phyB mAb B6B3, anti-phyD mAb 2C1, and anti-phyE mAb 7B3. The blots indicate a lack of detectable cross-reactivity of each of these antibodies to non-cognate apophytochromes at this level of resolution. A second anti-phyB mAb, B2A5, which exhibits specificity for PHYB similar to B6B3 was also identified in this screen. The pool of all three anti-phyC mAbs used by Somers et al. (1991) was also tested on blots of the purified apoproteins and the combination of all three mAbs (C1, C11, and C13) showed no cross-reactivity to the four noncognate apoproteins (data not shown).

To obtain a more quantitative estimate of the specificity of the anti-phy mAbs, a dilution series of each of the purified apoproteins was probed with each mAb and the immunoreactivity determined on slot blots. Figure 2 shows that, at the primary antibody concentration used routinely in blot analyses, there is ~100-fold differ-
Recombinant PHY

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Figure 1.—Immunoblot analysis of recombinant Arabidopsis phytochrome apoproteins using anti-phytochrome mAbs. Crude inclusion body preparations of full-length recombinant phytochrome apoproteins PHYA-E were excised and electroeluted from 6% SDS-PAGE gels. Ten nanograms of each sample were fractionated on 6% SDS-PAGE gels and transferred to nitrocellulose. Separate replicated blots, each containing the five apoproteins, were probed with either anti-phyA mAb 073d, anti-phyB mAb B6-B3, anti-phyC mAb C13, anti-phyD mAb 2C1, or anti-phyE mAb 7B3. The bottom panel shows a silver-stained 6% gel containing the same samples used for the immunoblots. Only a small portion of the blots or the gel between approximately 115 and 125 kDa is shown.

Specificity of the anti-phytochrome mAbs in tissue extracts: Two protocols were used to prepare protein samples from plant tissues for immunoblot analysis: extraction of powdered frozen tissue into an aqueous buffer to yield “direct” extracts which were loaded on gels without further treatment or, alternatively, extraction of powdered frozen tissue into aqueous buffer as for direct extracts, followed by precipitation with 25% (w/v) ammonium sulfate (Somers et al. 1991; see materials and methods). These protocols gave similar results, but the ammonium sulfate-precipitated samples yielded immunoblots with higher signal to background and, for three of the mAbs, eliminated cross-reacting proteins recovered in the crude extracts (see below). The possibility that significant amounts of the phytochromes were pelleting in the initial centrifugation steps of these procedures was addressed by boiling the pellets from centrifugation in SDS-containing sample buffer and loading this preparation on immunoblots. Very little (<5%) antigen was detected on these blots (data not shown). Procedures involving direct extraction of lyophilized tissue into hot SDS buffer (Wang et al. 1993) or extraction of frozen tissue into cold SDS buffer followed by chloroform/methanol precipitation of total protein (Lagarias et al. 1997) were tested and yielded qualitatively similar results to the aqueous protocols but gave higher backgrounds and less clearly resolved blots.

In Figure 3A, protein blots of ammonium sulfate-precipitated tissue extracts from 7-day-old dark-grown Arabidopsis seedlings, when probed with mAbs 073d, B6-B3, C11+C13, 2C1, or 7B3, show a single immunoreactive band of relative molecular mass 110–130 kDa, consistent with the predicted molecular weights of the PHYA-E gene products (Sharrock and Quail 1989; Clack et al. 1994). Immunoblots similar to those in Figure 3A were performed with extracts from seedlings grown in the light and from older plants and no cross-reactive proteins were observed (data not shown). When Arabidopsis tissues were extracted directly and total soluble protein was loaded on gels, the anti-phyA and anti-phyC mAbs showed no crossreactive bands (not shown); however, the anti-phyB, anti-phyD, and anti-phyE mAbs detected both their respective phytochrome antigens and lower molecular weight cross-reacting antigens (Figure 3B). This result indicates that the epitope recognized by each of these three antibodies, while apparently unique to a single phytochrome type, is present on other unidentified cellular proteins.

Null mutations in the Arabidopsis PHYA, PHYB, and PHYD genes have been described previously, and extracts of these mutant lines were used to directly test for crossreactivity of the anti-phyA, anti-phyB, and anti-phyD mAbs to any of the noncognate phytochromes present in tissue extracts. The phyA-201 (Reed et al. 1994) and phyB-1 (Reed et al. 1993) alleles are transition mutations that change glutamine codons to stop codons at, respectively, amino acid 980 of the phyA apoprotein sequence and amino acid 448 of the phyB apoprotein sequence. The phyD-1 allele (Aukerman et al. 1997) is a frameshift mutation that is predicted to produce a 75-amino-acid polypeptide fragment. Immunoblots of protein extracts from dark-grown and light-grown seed-
Figure 2.—Quantitative analysis of cross-reactivity of anti-phytochrome mAbs with noncognate recombinant phytochrome apoproteins. Serial dilutions of full-length recombinant Arabidopsis phytochrome apoproteins PHYA-E, prepared as in Figure 1, were transferred to nitrocellulose by slot-blotting. The blots were probed with (A) anti-phyA mAb 073d, (B) anti-phyB mAb B6-B3, (C) anti-phyC mAb C13, (D) anti-phyD mAb 2C1, or (E) anti-phyE mAb 7B3. Video images of the blots were captured and signals quantitated in pixels using NIH Image (Wagner et al. 1996).

lings of the wild type and of phyA-201, phyB-1, or phyD-1 mutants are shown in Figure 3C. Anti-phyA mAb 073d does not detect any antigen in the size range of phytochrome in the phyA-201 mutant but, instead, detects a low level of a protein of ~110-kD apparent molecular mass. This protein corresponds in size to the predicted truncated phyA protein encoded by the phyA-201 allele (Reed et al. 1994). Anti-phyB mAbs B6B3 and B2A5 do not detect any antigen in the phytochrome size range in dark- or light-grown seedlings extracts of the phyB-1 mutant (Figure 3). It is notable that, if the cross-reactivity profiles of the purified phytochrome apoproteins in Figure 2 are applicable to the apoproteins present in tissue extracts, the high level of phyA present in dark tissue (Somers et al. 1991) might be expected to be detected by the anti-phyB B6B3 mAb and produce a significant signal. However, even when blots are loaded with high levels of dark-grown phyB-1 extract protein and exposed to film for very long times, no cross-reaction of mAb B6B3 with the phyA protein is observed. This suggests either that the affinity of the mAb B6B3 for the phyA holoprotein produced in vivo is lower than for the purified PHYA apoprotein, due to attachment of the chromophore or to another posttranslational modification, or that the large amount of carrier protein on blots of tissue extracts decreases nonspecific binding of the antibodies and improves the specificity of the mAbs as compared to blots of purified proteins.

The anti-phyD mAb 2C1 recognizes a non-phytochrome protein of ~120 kD: In contrast to the high specificity of the anti-phyA and anti-phyB mAbs on blots of tissue extracts, anti-phyD mAb 2C1 reacts with a protein of similar apparent molecular mass to phytochrome in extracts of dark-grown but not light-grown phyD-1 mutant seedlings (Figure 3C). This band is a non-phytochrome cross-reactive protein (see below), which we
Figure 3.—Immunoblot analysis of Arabidopsis seedling extracts with anti-phytochrome mAbs. (A) An aqueous extract of dark-grown Columbia wild-type seedlings was prepared and precipitated with 25% (NH₄)₂SO₄ (w/v). Samples of the solubilized protein pellet equivalent to 300 μg of original extract protein were fractionated on a 6% SDS-PAGE gel, blotted to nitrocellulose, and probed with anti-phyA mAb 073d, anti-phyB mAb B6B3, anti-phyC mAbs C11 and C13, anti-phyD mAb 2C1, or anti-phyE mAb 7B3. (B) Samples of an aqueous extract of dark-grown Columbia wild-type seedlings containing 50 μg of protein were loaded directly onto a 9% SDS-PAGE gel, fractionated, blotted to nitrocellulose, and probed with anti-phyB mAb B6B3, anti-phyD mAb 2C1, or anti-phyE mAb 7B3. (C) Aqueous extracts of dark- and light-grown Landsberg erecta (Ler) wild-type and phyA-201, phyB-1, or phyD-1 null mutant seedlings were prepared and precipitated with 25% (NH₄)₂SO₄ (w/v). Samples of the solubilized pelleted proteins equivalent to 300 μg of original extract protein for the dark-grown tissues and 400 μg extract protein for the light-grown tissues were fractionated on 6% SDS-PAGE gels, blotted to nitrocellulose, and probed with anti-phyA mAb 073d, anti-phyB mAbs B6B3 or B2A5, and anti-phyD mAb 2C1.

refer to as NPXR, and its presence complicates the use of mAb 2C1 to detect phyD. However, more than 120 hybridoma lines generated using the E. coli-expressed phyD protein as antigen were screened and mAb 2C1 was the only line which reacted strongly with phyD and did not cross-react with phyB. Hence, until new anti-phyD hybridomas are generated and screened, mAb 2C1 remains the best available antibody for the detection of phyD. The identity of NPXR is not known, but several lines of evidence indicate that it is not one of the known phytochromes. First, NPXR is present in tissue extracts of a phyA-201/phyB-1/phyD-1 triple null mutant (data not shown), demonstrating that it is not any of these three proteins. Second, though NPXR migrates in SDS gels as a slightly smaller protein than phyD (Figure 3D), it is significantly larger than phyE when analyzed in adjacent lanes of a gel (data not shown). Finally, NPXR does not cross-react with any anti-phy mAb except anti-phyD 2C1 (see below).

By using a 20% (w/v) (NH₄)₂SO₄ fraction from plant tissue extracts rather than a 25% fraction, phyD and the NPXR protein can be separated. Figure 4 shows immunoblot analysis of sequential (NH₄)₂SO₄ fractions of tissue extracts of wild-type Col seedlings, which contain both phyD and the NPXR protein, and wild-type Ws seedlings, which do not contain phyD (Aukerman et al. 1997) but do contain the NPXR protein. For phyA, phyB, phyC, and phyE, the majority of each of the proteins is precipitated by 20% (NH₄)₂SO₄ and very nearly all of the protein is precipitated by 25% (NH₄)₂SO₄, the salt concentration used in most phytochrome purifications and analyses. Trace amounts of phyC and phyE are seen in the 25–30% (NH₄)₂SO₄ fraction (Figure 4). On the immunoblot in Figure 4 probed with mAb 2C1, a strong signal is seen in the 20% (NH₄)₂SO₄ fraction of the Col extract but no signal is present in this fraction from the Ws extract. The NPXR signal, which migrates at a slightly lower molecular mass than phyD, begins to be seen in the 20–25% (NH₄)₂SO₄ fraction in both the Col and Ws extracts, is most prominent in the 25–30% (NH₄)₂SO₄ fraction, and is present in the 30–35% fraction (Figure 4). This result shows that the NPXR protein precipitates at an (NH₄)₂SO₄ concentration different from any of the five phytochromes. Figure 4 also shows that the NPXR protein is not recognized by the anti-phyA, anti-phyB, anti-phyC, or anti-phyE mAbs. Finally, a "universal" anti-phy mAb, originally isolated using rice phyB apoprotein as antigen (Wagner et al. 1991), but
Coordination of Phytochrome Levels

Figure 5.—Immunoblot analysis of direct and (NH₄)₂SO₄-precipitated extracts of Landsberg erecta (Le) wild-type and phyB null mutant seedlings. (A) Aqueous extracts of Le, phyB-1, phyB-7, and phyB-8 dark-grown seedlings were prepared and 50μg samples were loaded directly on 6% SDS-PAGE gels, fractionated, blotted to nitrocellulose, and probed with anti-phyA mAb 073d, anti-phyB mAb B6B3, anti-phyC mAbs C11 and C13, anti-phyD mAb 2C1, or anti-phyE mAb 7B3. (B) Aqueous extracts of Le, phyB-1, phyB-7, and phyB-8 dark-grown seedlings were precipitated with 25% (NH₄)₂SO₄ for the phyA, phyB, phyC, and phyE blots or with 20% (NH₄)₂SO₄ (w/v) for the phyD blot. Proteins in the pellets were solubilized and samples equivalent to 300μg of original extract protein were fractionated on 6% SDS-PAGE gels, blotted to nitrocellulose, and probed with the anti-phy mAbs as in (A) above.

which detects an epitope present on all five Arabidopsis phy apoproteins, also does not detect the NPXR protein (Figure 4). Hence, though it is striking that the NPXR protein is very similar in apparent size to the phyA-E phytochrome apoproteins and that it is present differentially in dark- and light-grown tissues (Figure 3C), there is currently no evidence that it is functionally related to phytochromes or to light regulatory pathways in cells.

Use of the anti-phytochrome mAbs on immunoblots: The cross-reactivity of anti-phyD mAb 2C1 to the NPXR protein (Figure 3C) and the cross-reactivity of the anti-phyB, anti-phyD, and anti-phyE mAbs to low molecular weight proteins in crude extracts (Figure 3B) limit the usefulness of these antibodies in applications such as immunocytochemistry and quantitative studies utilizing unfractionated crude protein preparations, such as ELISA assays. However, all available evidence indicates that the anti-phyA, B, C, and E mAb lines can be used to specifically detect and quantify these antigens on immunoblots of either crude or (NH₄)₂SO₄-fractionated Arabidopsis extracts. Moreover, until completely phyD-specific antibodies are produced, mAb 2C1 can be used to identify and quantify phyD in plant extracts if a 20% (NH₄)₂SO₄ fraction is analyzed (Figure 4). In all of the immunoblot experiments presented here, it is important to note that exposure times for detection of the chemiluminescent signal varied from blot to blot. Thus, while the chemiluminescence signals on the panels in Figure 3, 4, 5, 6, 7, and 8 accurately reflect the relative levels of each phy protein under the set of conditions being tested within the figure panel, they cannot be used to compare the levels of the different phy proteins to each other.

Phytochrome C levels are reduced in phyB mutants: Immunoblots of direct and ammonium sulfate-precipitated extracts of dark-grown Ler wild type, phyB-1, phyB-7, and phyB-8 seedlings are shown in Figure 5, A and B. As expected, all three phyB mutant lines lack the phyB antigen and contain wild-type levels of phyA, phyD, and phyE. However, the phyB lines also exhibit a reduction in the level of the protein detected by the anti-phyC mAbs relative to the wild type (Figure 5). Figure 6A shows that this reduction in phyC is on the order of 5- to 6-fold, by comparison to a dilution curve of the wild-type extract, and that the reduction is seen in lines that contain a phyB mutation, either alone or in combination with a phyA mutation, but not in lines containing only a phyA mutation. Throughout this report, quantitation
of the decreases or increases in the levels of phytochrome proteins in mutant or overexpressor lines has been performed by visual comparison of blot signals to extract dilution curves, similar to that shown in Figure 6A. However, for the sake of conciseness, these curves are not shown. The phyC blots shown in Figures 5 and 6 represent three independent experiments and each of these was repeated twice. The reduced phyC level in the phyB mutant lines was found to be reproducible in all cases. In contrast, the reductions in phyD (Figure 5A) and phyA (Figure 5B) specifically in line phyB-8 compared to the wild type, were not seen consistently on other blots, indicating that these variations are very likely blotting artifacts.

One explanation for the observed reduction of phyC in phyB mutant lines might be that the anti-phyC mAbs strongly cross-react with phyB on blots of tissue extracts, so that the absence of phyB leads to a reduction on the apparent phyC signal. We consider this to be very unlikely because of the demonstrated specificity of the antibodies (Figures 1–3) and because, in the converse experiment, 15-fold overexpression of phyB in dark-grown seedlings does not result in a significantly higher phyC signal (see Figure 8A, below). Moreover, anti-phyC mAbs C11 and C13, when used individually to probe immunoblots, show the same reduction in antigen in the phyB mutant (data not shown). Hence, we conclude that phyC is present at only 15–20% of its normal level in dark-grown plants that lack phyB and that the absence of phyA has no discernible effect on the level of any of the other phytochromes. Figure 6B shows that, in seedlings grown under continuous white light, a 3- to 4-fold reduction in phyC and a 2-fold reduction in phyD correlate with the presence of the phyB null mutation, but, again, not the phyA mutation.

**Figure 6.**—Phytochrome levels in Ler wild-type phyB-1, phyA-201, and phyA-201/phyB-1 mutant seedlings grown in the dark or light. (A) Aqueous extracts of dark-grown seedlings of the indicated genotypes were prepared and precipitated with either 25% (NH₄)₂SO₄ (w/v) for the phyA, phyB, phyC, and phyE blots or with 20% (NH₄)₂SO₄ (w/v) for the phyD blot. Proteins in the pellets were solubilized and samples equivalent to 300 µg of original extract protein were fractionated on 6% SDS-PAGE gels, blotted to nitrocellulose, and probed with anti-phyA mAb 073d, anti-phyB mAb B6B3, anti-phyC mAbs C11 and C13, anti-phyD mAb 2C1, or anti-phyE mAb 7B3. Dilution curves of the Ler wild-type protein sample are shown to illustrate the sensitivity of the assay. (B) Aqueous extracts of light-grown seedlings were prepared and precipitated with 25% (NH₄)₂SO₄ (w/v). Proteins in the pellets were solubilized and samples equivalent to 400 µg of original extract protein were fractionated on 6% SDS-PAGE gels, blotted to nitrocellulose, and probed with the anti-phyA mAbs as in (A) above.

**Coordination of phyB and phyC occurs over a physiologically significant range of phyB expression levels:** The data in Figures 5 and 6 indicate that there is dependence of the level of phyC on the level of phyB in Arabidopsis and raise the possibility that phenotypes attributed to the lack of phyB in phyB null mutants might in fact be the result of reduction in phyC or of the coordinate reduction in both photoreceptors. Small changes in the levels of specific phytochromes have been implicated as critical determinants of plant photosensory responses. For example, both phyA and phyB null mutations in Arabidopsis are incompletely recessive, showing partial mutant phenotypes in the heterozygous condition (Koornneef et al. 1980; Whitelam et al. 1993). Moreover, West et al. (1994) demonstrated that phenotypic changes caused by phyB mutations are sensitive to PHYB gene copy number and to the resulting levels of phyB phytochrome in a dosage-dependent manner, extending from the light green, elongated, early flowering phenotype of the homoygous phyB null mutant genotype to a dark green, somewhat dwarfed, late flowering phenotype caused by expression of twice the usual diploid PHYB gene complement. These results indicate that, in the range of PHYB gene activity encompassing 0, 0.5, 1, 1.5, and 2 times the intermediate diploid PHYB gene complement, each photoreceptor level yields a distinctive and characteristic photomorphogenic program.

Figures 7A and 7B show protein blot analysis of the lines from West et al. (1994), which were generated in the Arabidopsis No-0 genetic background. The phyB-1 mutation causes an absence of phyB, a strong reduction in phyC, and a small reduction in phyD both in the dark and under white light. These effects are almost
Overexpression of Arabidopsis phyB increases phyA, phyC, and phyD levels in light-grown but not dark-grown seedlings: Since a small increase in phyC was observed in lines containing twice the normal level of phyB (Figure 7, A and B), we analyzed lines in which the Arabidopsis PHYB coding sequence or the oat PHYA sequence is expressed at very high levels from transgenes driven by the CaMV 35S promoter. Such overexpression has been shown to cause a “light-exaggerated” dark green, dwarf phenotype in transgenic Arabidopsis lines (Boylan and Quail 1991; Wagner et al. 1991, 1996). Immunoblots of extracts of dark-grown and white light-grown No-0 wild-type and phyB overexpressor lines, including the ABO line of Wagner et al. (1991) and two BOE (phyB overexpressor) lines generated for these studies, are shown in Figure 8. The phyB antigen is present at ~15-fold higher levels in the transgenic lines than in the wild type when they are grown in the dark and at ~20-fold higher levels when they are grown in the light (data not shown; Wagner et al. 1991). However, there is a difference in the effect of this overexpression on the levels of the four other phytochromes under the two growth conditions. In the dark, no effect is seen on the levels of the phyA, C, D, and E antigens (Figure 8A). In the light, the levels of phyA, phyC, and phyD are all increased two- to fourfold relative to the untransformed control but phyE is unaffected (Figure 8B). The Arabidopsis transgenic line AOX overexpresses the oat phyA apoprotein from the 35S promoter (Boylan and Quail 1991). When this line is analyzed with mAb 073d, which detects both monocot and dicot phyAs, the signal from the combined oat and Arabidopsis phyA proteins is ~4-fold higher than the endogenous Arabidopsis phyA in the dark and ~10-fold higher in the light (data not shown; Boylan and Quail 1991). However, no significant effect on the level of any of the phytochromes other than phyA is observed under either growth condition (Figure 8C).

Alteration of phyC levels in phyB mutants and in phyB-overexpressing lines is post-transcriptional: Poly(A)-selected RNA samples from dark-grown wild-type and phyB-1 mutant seedlings and from light-grown wild-type and 35S-PHYB overexpressor transgenic lines were run on gels, blotted, and probed with 3'-end gene-specific hybridization probes for the PHYB, PHYC, and PHYD transcripts. Figure 9A shows that, in the phyB-1 tissues, which exhibit an absence of phyB antigen and a concomitant five- to six-fold reduction in the phyC antigen (Figures 5 and 6), there is a reduction in PHYB transcript, as was observed previously (Somers et al. 1991), but there is no effect on the PHYC transcript. In the 35S-PHYB overexpressor lines, which exhibit a ~20-fold increase in phyB antigen and a concomitant four-fold increase in phyC when grown in the light (Figure 8B), the level of PHYB transcript is 20- to 30-fold higher than that of wild type but there is no effect on the level of the PHYC transcript (Figure 9B). These data indicate

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**Figure 7.**—Phytochrome levels in the transgenically complemented phyB-1 mutant and in a wild-type transgenic line containing a two-fold increased PHYB gene dosage. (A) All lines were in the No-0 wild-type (WT) genetic background as described by Wester et al. (1994). Aqueous extracts of dark-grown seedlings were prepared and precipitated with either 25% (NH₄)₂SO₄ (w/v) for the phyA, phyB, phyC, and phyE blots or with 20% (NH₄)₂SO₄ (w/v) for the phyO blot. Proteins in the pellets were solubilized and samples equivalent to 300 μg of original extract protein were fractionated on 6% SDS-PAGE gels, blotted to nitrocellulose, and probed with anti-phyA mAb 7B3, anti-phyB mAb B6B3, anti-phyC mAbs C11 and C13, anti-phyD mAb 2C1, or anti-phyE mAb 7B3. (B) Aqueous extracts of light-grown seedlings were prepared and precipitated with 25% (NH₄)₂SO₄ (w/v). Proteins in the pellets were solubilized and samples equivalent to 400 μg of original extract protein were fractionated on 6% SDS-PAGE gels, blotted to nitrocellulose, and probed with the anti-phy mAbs as in (A) above.

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Completely reversed in the phyB-1(mPHYB) line in which the phyB-1 mutation is complemented with a mini-PHYB transgene consisting of the PHYB promoter fused to the PHYB cDNA sequence (Figure 7, A and B). However, in this cDNA-complemented line, though the level of phyB is equivalent to that in the wild type, the level of phyC is slightly reduced. In the No-0 (mPHYB) lines, the presence of a diploid copy of the mPHYB transgene in a PHYB+ background, yielding four active copies of PHYB, causes a two-fold increase in phyB, a small increase in phyC relative to the wild type, and no effect on phyD (Figure 7, A and B). The levels of phyA and phyE remain unaffected in all of the lines. These immunoblots demonstrate that parallel changes in the levels of phyB and phyC are evident over a range of PHYB gene activities and, together with the phenotypic analyses of these lines by Wester et al. (1994), indicate that each of the different phyB/phyC is associated with a strikingly different pattern of photomorphogenesis.
Figure 9.—RNA blot analysis of PHYB, PHYC, and PHYD mRNA levels in phyB-1 mutants and phyB-overexpressor transgenic lines. (A) Poly(A)-selected RNA from the Ler wild-type and the phyB-1 mutant in the Ler genetic background and from the No-0 wild-type and the phyB-1 mutant introgressed into that genetic background was fractionated on an agarose/formaldehyde gel and hybridized with 3'-end gene-specific probes for the indicated transcripts. (B) Poly(A)-selected RNA from the No-0 wild-type and three phyB overexpressor transgenic lines was fractionated on an agarose/formaldehyde gel and hybridized with 3'-end gene-specific probes for the indicated transcripts.

that the changes in phyC antigen levels which are observed coordinately with changes in phyB levels (Figures 5 through 8) are likely the result of post-transcriptional effects on phyC protein synthesis or stability.

**DISCUSSION**

**Isolation of Arabidopsis phytochrome-specific monoclonal antibodies:** Monoclonal antibodies that specifically recognize each of the five forms of phytochrome in Arabidopsis will be important tools in the analysis of light signaling in this plant. Between the antibodies identified by Somers et al. (1991) and those identified here, mAbs are now described that are, insofar as we have tested them, completely specific for phyA and phyC. However, the anti-phyB, anti-phyD, and anti-phyE mAbs, which appear to be phytochrome type-specific but which cross-react with other cellular proteins, have...
some limitations on their use. In particular, the anti-
phyD mAb 2C1, which recognizes phyD and none of the
other phytochromes, cross-reacts with an Arabidopsis
protein (NPXR) of very similar molecular mass to phyto-
chrome in unfractionated extracts of dark-grown seed-
lings. It is notable that, if a phyD null mutant (Aukerman
et al. 1997) had not become available, the NPXR protein
would not have been discovered using the procedures
widely used for immunoblot detection of the phyto-
chromes and other plant proteins. The PHYB, PHYD,
and PHYE polypeptides are the three most closely se-
quence-related PHY apoproteins in Arabidopsis (C1ack
et al. 1994) and efforts to isolate completely specific
mAbs for each of them are underway. Nonetheless, us-
ing the antibodies described here, it is now possible to
detect four of the five phytochromes in crude extracts
of Arabidopsis plants under all conditions we have in-
tested, and use of a 20% (w v) ammonium sulfate frac-
tion from the extract allows mAb 2C1 to be used to
differentially assay for phyD even in dark-grown tissues.

For analysis of in vivo phytochrome levels, several
Arabidopsis tissue extraction protocols were compared
and aqueous extraction of powdered frozen tissue was
found to yield the most consistent results. Previously,
Wang et al. (1993) investigated the efficiencies of aque-
ous versus hot SDS extraction for the recovery of phyto-
chromes from lyophilized oat seedlings. In their experi-
ments, both detergent-free and SDS-containing buffers
extracted equivalent amounts of the three oat phyto-
chromes and they found no compelling reason to use
one method rather than the other, aside from the re-
duced difficulty with proteolytic degradation in hot
SDS-containing buffers. The detection of the five Arabi-
dopsis PHY apoproteins in tissue extracts shows that all
five cloned PHY genes are expressed at the protein
level in young seedlings. The anti-phy mAbs will now
be useful in analyzing the levels and light stabilities of
these photoreceptors in the plant, their patterns of
regulation, and their tissue and cellular localizations
throughout the life cycle.

Coordination of phytochrome levels in Arabidopsis:
We have observed coordinate changes in the levels of
phyB and several of the other phytochromes in Arabi-
dopsis under conditions where phyB is either muta-
tionally eliminated or transgenically overexpressed. The
most striking coordination occurs between phyB and
phyC. In phyB null mutants of Arabidopsis, phyC is re-
duced to 15–20% of its normal level in the dark and to
25–30% of its normal level in the light. The level of
phyD is slightly (~2-fold) reduced in the phyB mutants
under these conditions but the levels of phyA and phyE
are unchanged. In contrast to this, a phyA null mutant
shows no alteration in the levels of any of the other four
phytochromes. Characterization of Arabidopsis mutants
deficient in phyA or phyB has been an important ap-
proach to defining the roles of the different members
of the phytochrome family (White et al. and Devlin
1997). In the absence of evidence to the contrary, it has
been assumed that a mutation in one PHY gene affects
only the level of that gene product. The results pre-
mitted here indicate that this is not always the case.

Somer et al. (1991) previously presented immu-
noblot data indicating that phyC levels in the phyB mu-
tants were comparable to those in the wild type. Using
the same anti-phyC mAbs used in those studies and the
same mutant lines, we have consistently observed a
strong reduction in the phyC antigen. One difference
between the earlier experiments and those presented
here is the use of chemiluminescent rather than colori-
metric detection for the immunoblots. As shown in Fig-
ure 6, chemiluminescence is sensitive to relatively small
changes in the levels of low-abundance antigens. None-
theless, we have also observed a reduction in phyC level
in phyB mutants similar to that described here using
colorimetric detection methods (R. Sharrock, unpub-
lished results).

Restoring the wild-type level of phyB in a phyB null
mutant with an intronless mini PHYB transgene (West
er et al. 1994) restores the phyC level to close to that of
the wild type both in the light and in the dark. Doubling
phyB expression by introducing the mini PHYB transgene
into a wild-type background further increases the phyC
level. These results suggest that, at levels of phyB near
the normal cellular level, there is a fairly close coordi-
ation of phyB and phyC abundance. In contrast to this,
high-level transgenic overexpression of phyB in wild-
type Arabidopsis, accomplished by driving the PHYB
coding region with the 35S viral promoter, does not
induce parallel overexpression of phyC. In the dark,
phyB overexpression has little or no effect on any of
the other phytochromes. In the light, a generalized two-
to four-fold increase in the levels of phyA, phyC, and
phyD is induced by phyB overexpression. This light-
dependent generalized increase in phytochrome levels
seen in phyB overexpressors is not observed in lines
overexpressing oat phyA. Taken together, these observa-
tions suggest that there are cellular mechanisms that
control the relative abundance of selected members
of the phytochrome family in a coordinate fashion. In
addition, these results have implications for the inter-
pretation of the effects of phyB mutations and phyB
overexpression on plant photosensory phenotypes.

Possible mechanisms for coordination of phyto-
chrome levels: The mechanism through which the
amount of phyC is reduced in phyB null mutants is
unknown and its relationship to the normal photosen-
sory function of phyB is not clear at this time. The data
presented here demonstrate that phyB is necessary to
sustain wild-type levels of phyC in Arabidopsis seedlings.
Possible mechanisms for the coordination of phyB and
phyC might act at any of several levels of cellular func-
tion. The amount of phyB in the cell may directly regu-
late the expression of the PHYC gene. The unchanged
level of the PHYC mRNA seen in phyB mutants com-

pared to wild type indicates that such an effect would have to be post-transcriptional. It is possible that the frequency or rate of translation of the phyC mRNA is responsive to phyB levels. Alternatively, the changes in the steady-state level of phyC protein that are measured on the protein blots may reflect changes in the stability rather than the synthesis rate of phyC. If phyB and phyC share a common proteolytic turnover pathway, absence of phyB in the phyB mutants could lead to increased turnover of phyC. Our results suggest that such a turnover pathway would have to be active in both the dark and the light and would be specific to phyC, phyC, and, to a small degree, phyD because phyA and phyE levels are unaffected in phyB mutants.

Finally, it is possible that there is an interaction of phyB and phyC, either a direct physical interaction via formation of phyB/phyC heterodimers, or a less direct interaction via formation of a protein complex including other proteins. Such an interaction could stabilize phyC and, when disrupted in the phyB mutants, result in phyC turnover. Qin et al. (1997) have reported that, in transgenic Arabidopsis plants containing a 35S promoter-PHYC cDNA transgene, only a three- to four-fold increase in phyC protein level is observed. This is in contrast to the 15- to 20-fold increase in phyB levels seen in 35S-PHYB transgenic lines (see Figure 8). The inability to overexpress phyC to high levels could be compatible with any of the mechanisms described above but is perhaps most consistent with a model in which phyC must bind to or interact with a molecular partner, as a heterodimer or in a complex, in order to be stabilized in the cell. Any of the mechanisms proposed here implies that a form of molecular coordination between members of the phytochrome family exists that has not been recognized previously.

The increases in the levels of phyA, phyC, and phyD in the phyB overexpressor lines are seen only in light-grown tissue and are consistent with very high phyB levels overwhelming a light-dependent proteolytic phytochrome degradation pathway. It has long been recognized that light-activated proteolysis plays a major role in determining the level of phyA in plants (Clough and Vierstra 1997). Dark-grown seedlings of many angiosperm plants contain a very high level of phyA which is rapidly degraded upon exposure to red light. The mechanism for this is postulated to involve differential ubiquitination of phyA in the Prf form as compared to the Pr form (Shanklin et al. 1987). As a result of this instability, phyA has been called a "light-labile" phytochrome. Little is known about possible cellular degradation pathways for the other phytochromes, though Somers et al. (1991) have shown that phyB and phyC are much more light stable than phyA. It is possible that the levels of the "light-stable" phytochromes are controlled to some extent by proteolytic turnover mechanisms. Nonetheless, the generalized increase in phyA, phyC, and phyD in the phyB overexpressor transgenic lines is seen only in the light, whereas the coordination of phyB and phyC levels in phyB mutants occurs in both the dark and the light. Hence, it is possible, perhaps likely, that the two mechanisms are unrelated and reflect different properties of the phytochrome receptor family.

**Implications for interpretation of phytochrome mutants and overexpressor lines:** The Arabidopsis phyA, phyB, and phyD null mutants have been used in a large number of genetic and physiological studies of phytochrome function (reviewed in Whitelam and Devlin 1997). In these studies, the phenotypes of the mutants have been interpreted to reflect the loss of the activities of those individual phytochromes. In addition, the phenotypic consequences of phy mutations in species other than Arabidopsis have been assumed to result from alteration or loss of specific phytochromes (Lopez-Juez et al. 1992; van Tuinen et al. 1995; Weller et al. 1995; Childs et al. 1997; Devlin et al. 1997). In all of these cases, the possibility that mutational alteration of one phytochrome may influence the activities of one or more of the other members of the photoreceptor family is an important consideration, though analysis of single and double mutant combinations of the Arabidopsis phyA, phyB, and phyD alleles indicates that each locus has a significant independent activity (Reed et al. 1994; Shinomura et al. 1996; Aukerman et al. 1997). The coordinate decrease in phyB and phyC in the Arabidopsis phyB mutants that we have described here indicates that is it is also necessary to consider the possibility that changes in the level of one of the phytochromes may have significant effects on the levels, and therefore the activities, of other members of the photoreceptor family. In the Arabidopsis phyB mutants, for instance, it is not currently possible to separate the phenotypic effects resulting from loss of phyB from the effects of reduced phyC.

The phenotypes of Arabidopsis transgenic 35S-PHYA, 35S-PHYB, and 35S-PHYC overexpressor lines have also been interpreted to reflect elevated activity of those individual phytochromes (Boylan and Quail 1991; Wagner et al. 1991; Qin et al. 1997). We have observed that high-level (20-fold) overexpression of Arabidopsis phyB but not oat phyA causes a small (two- to four-fold) elevation in the levels of phyA, phyC, and phyD in seedlings grown in the light. Whether these small changes play a role in any of the phyB overexpression phenotypes is not known; further experiments will be necessary to address this. Moreover, it should be noted that the predominant molecular consequences of both the phyB null mutations and transgenic phyB overexpression are on phyB levels themselves and it remains likely, therefore, that the majority of the observed phenotypic effects relate directly to phyB signaling activity. Nevertheless, since alteration of phyB levels but not phyA levels affects the amounts and therefore the activities of other phytochromes in cells, a more central role
for phyB in the coordination and activity of the plant R/FR photosensing apparatus may be indicated.

We thank the Arabidopsis Biological Resource Center, M. A. Mathews, S. Lavin, and J. C. Koornneef, and J. C. Koornneef for providing genetic stocks. We are also grateful to Dr. Alex Karu and the staff of the University of California Hybridoma Facility for assistance and guidance in the production of monoclonal antibodies. This work was supported by the National Science Foundation grant IBN-9407864 (R.A.S.), Department of Energy, Office of Basic Energy Sciences grant FG03-96ER13742 (P.H.Q.), U.S. Department of Agriculture Current Research Information System grant 5335-21000-006-000 (P.H.Q.), and Montana’s National Science Foundation Experimental Program to Stimulate Competitive Research (R.A.S.).

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Communicating editor: D. Preuss