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RESEARCH ARTICLE

Regulation of Photomorphogenesis by Expression of Mammalian Biliverdin Reductase in Transgenic Arabidopsis Plants

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The photoregulatory activity of the phytochrome photoreceptor requires the synthesis and covalent attachment of the linear tetrapyrrole prosthetic group phytochromobilin. Because the mammalian enzyme biliverdin IXα reductase (BVR) is able to functionally inactivate phytochromobilin in vitro, this investigation was undertaken to determine whether BVR expression in transgenic plants would prevent the synthesis of functionally active phytochrome in vivo. Here, we show that plastid-targeted, constitutive expression of BVR in Arabidopsis yields plants that display aberrant photomorphogenesis throughout their life cycle. Photobiological and biochemical analyses of three transgenic BVR lines exhibiting a 25-fold range of BVR expression established that the BVR-dependent phenotypes are light dependent, pleiotropic, and consonant with the loss of multiple phytochrome activities. Chlorophyll accumulation in BVR-expressing transgenic plants was particularly sensitive to increased light fluence rates, which is consistent with an important role for phytochrome in light tolerance. Under blue light, transgenic BVR plants displayed elongated hypocotyls but retained phototropic behavior and the ability to fully deetiolate. Directed BVR expression may prove to be useful for probing the cellular and developmental basis of phytochrome-mediated responses and for selective control of individual aspects of light-mediated plant growth and development.

INTRODUCTION

The ability of plants to adjust to changes in the light environment is critical to their survival because they depend on sunlight as an energy source and lack a neuromuscular system permitting movement toward more optimal light conditions. Plants thus possess light-sensing molecules that enable adaptation to the frequent fluctuations of intensity, direction, and spectral quality of light in their environment (Kendrick and Kronenberg, 1994). These include receptors for UV-B, blue/UV-A, and red/far-red light (R/FR) that function together to modulate genetically predetermined pathways of plant growth and development. Phytochrome is the most well characterized of these photomorphogenetic photoreceptors (Furuya, 1993; Quail et al., 1995). It mediates both inductive and adaptive responses to R and FR throughout a plant's life cycle, including seed germination, shoot and leaf development, floral initiation, fruit ripening, and ultimately senescence (Smith, 1995).

Phytochrome-mediated responses in plants can be classified into three groups: the very low fluence inductive responses (i.e., saturated by light fluences $<10^{-1}$ μmol m^{-2}), the low fluence R/FR photoreversible responses (i.e., requiring light fluences between 1 to 1000 μmol m^{-2}), and the high-irradiance responses requiring continuous irradiation. This large diversity of phytochrome-mediated responses is due to the expression of multiple phytochrome species (e.g., phyA to phyE in Arabidopsis and possibly more phy species in tomato) acting individually or in combination (Furuya, 1989; Quail, 1991; Chory, 1994; Pratt, 1995; Smith, 1995).

The molecular basis of phytochrome action is dependent on its ability to interconvert between two stable conformers—the R-absorbing Pr form and the FR-absorbing Pfr form—upon the absorption of light. This property is conferred by the linear tetrapyrrole prosthetic group phytochromobilin (PΦB), which is bound covalently to the phytochrome apoprotein via a thioether linkage (Lagarias and Rapoport, 1980). Because all phytochromes require this bilin prosthetic group for photosensory activity, plants inhibited in the synthesis of PΦB are expected to be "photomorphogenetically challenged" at multiple stages of development. That this is the case has been well established by the isolation of phytochrome chromophore-deficient long hypocotyl *hy1* and *hy2* mutants of

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Arabidopsis (Koornneef et al., 1980; Chory et al., 1989a; Parks and Quail, 1991), *aurea* (*au*) and *yellow green-2* (*yg-2*) mutants of tomato (Koornneef et al., 1985; Terry and Kendrick, 1996; Van Tuinen et al., 1996), phytochrome chromophore-deficient (*pcd1* and *pcd2*) mutants of pea (Weller et al., 1996, 1997), and partly etiolated in white light (*pew1* and *pew2*) mutants of *Nicotiana plumbaginifolia* (Kraepiel et al., 1994). Biochemical analysis of heme and linear tetrapyrrole metabolism in plastids isolated from mutant plants and/or the ability of exogenous biliverdin IXα (BV) to "rescue" these mutants (Parks and Quail, 1991; Terry and Kendrick, 1996; Weller et al., 1996, 1997) indicates that the specific lesions of these mutants are restricted to committed steps of the phytochrome chromophore biosynthetic pathway.

Extensive photobiological, physiological, and biochemical analyses have revealed that light-grown phytochrome chromophore-deficient mutants display an etiolated seedling phenotype with elongated hypocotyls and accumulate reduced levels of chlorophyll (reviewed in Koornneef and Kendrick, 1994; Terry, 1997). Phytochrome chromophore-deficient mutants also fail to deetiolate under both continuous FR illumination (FRc) and continuous R illumination (Rc)—an observation consistent with their deficiencies in both phyA and phyB (reviewed in Koornneef and Kendrick, 1994; Smith, 1995). In contrast, continuous blue light (Bc)–mediated deetiolation appears to be unaffected in these mutants. It is interesting that all phytochrome chromophore-deficient mutants characterized thus far retain some phytochrome responsivity, particularly in adult plants (e.g., end-of-day FR effects on internode elongation and flowering time). Partial reversion of the chlorophyll deficiency often occurs with age in these plants, suggesting that this phytochrome chromophore-deficient phenotype is overcome in adult mutant plants.

In view of the multiple roles for phytochromes in plant growth and development, it is not surprising that only leaky mutant alleles of the genes encoding phytochrome chromophore biosynthetic enzymes have been isolated. Null alleles of these genes might be expected to be lethal or otherwise difficult to propagate through all stages of development. The lethality of the *pew1 pew2* double mutant (Kraepiel et al., 1994) and the severely chlorotic phenotype of the *au yg-2* double mutant (Van Tuinen et al., 1996) is consistent with this hypothesis. From a metabolic perspective, lesions in the phytochrome chromophore biosynthetic pathway could also lead to an accumulation of tetrapyrrole intermediates (e.g., heme or BV), which potentially might feedback inhibit earlier steps common to both chlorophyll and heme biosynthesis (for a recent review, see Reinbothe and Reinbothe, 1996). For this reason, it is premature to conclude that the reduction of photoactive phytochrome fully accounts for the pleiotropic phenotypes of these chromophore biosynthetic mutants.

This investigation was undertaken to test the feasibility of selectively controlling photomorphogenesis in plants via expression of an enzyme that can metabolically inactivate bilin precursors of the phytochrome chromophore. A similar strategy to manipulate hormone levels in plants has led to invaluable insights into the interplay among multiple hormone-dependent pathways in plant growth and development (Klee and Romano, 1994) as well as their role in disease susceptibility (Gaffney et al., 1993). By analogy, we constructed transgenic Arabidopsis lines in which a mammalian enzyme, BV reductase (BVR), is expressed under regulatory control of a strong constitutive plant promoter. BVR is well suited for these studies for the following reasons. BVR is a soluble, monomeric enzyme of 33 kD that catalyzes the NAD(P)H-dependent reduction of BV to bilirubin (Singleton and Laster, 1965; Kutty and Maines, 1981). A cDNA for BVR has been cloned and functionally expressed in *Escherichia coli* (Fakhrai and Maines, 1992). Moreover, in addition to the reduction of BV, the known precursor of PΦB in plants (Terry et al., 1993a), BVR can efficiently metabolize PΦB to a rubinoid product that is unable to assemble with apophytochrome (Terry et al., 1993a). BVR also does not recognize corrins, porphyrins, and metalloporphyrins (i.e., chlorophylls and hemes). For this reason, the expression of BVR in transgenic plants should only affect the levels of linear tetrapyrrole intermediates committed to the synthesis of the phytochrome chromophore (i.e., BV, 3Z, and 3E isomers of PΦB). Based on biochemical and phenotypic analysis of BVR-expressing transgenic plants, our studies demonstrate the potential of this approach to regulate photomorphogenesis and in addition have provided insight into regulatory roles for phytochrome in light-mediated plant growth and development that have not been previously recognized.

RESULTS

Construction of BVR-Expressing Transgenic Plants

Because the metabolic pathway for PΦB biosynthesis is plastid localized, whereas holophytochrome assembly occurs in the plant cell cytoplasm (Terry and Lagarias, 1991; Terry et al., 1993b), we targeted the expression of BVR to plastids by translational fusion of the chloroplast transit peptide sequence of the soybean small subunit of ribulose bisphosphate carboxylase (*rbcS*) gene to a rat kidney BVR cDNA. In this way, we anticipated that both PΦB and its precursor BV would be metabolized before their release from the plastid compartment. Because our vector construction necessitated altering the primary sequence of the N terminus of BVR, we first established that the chloroplast transit peptide–BVR fusion polypeptide (TP-BVR) retained enzymatic activity after its expression in *E. coli* (data not shown). The BVR fusion protein gene construct was then placed under the control of the cauliflower mosaic viral 35S promoter in a binary plant transformation vector and used for Agrobacterium-mediated root transformation of Arabidopsis.

Two independent transformations with this construct yielded 47 kanamycin-resistant T_0 plant lines. Under continu-

ous white light (Wc), more than half of the T_0 regenerants were chlorotic, displayed elongated stems and petioles, and bolted early. These phenotypes are consistent with phytochrome-deficient plants (Koornneef and Kendrick, 1994). Such etiolated plant phenotypes were not observed in any of the 38 kanamycin-resistant transformants obtained with two control plasmids. After kanamycin selection, 22 T_1 lines were recovered from the original 47 regenerants. A high frequency of these lines (i.e., 18 of 22) produced seedlings with long hypocotyls and pale yellow cotyledons when grown under a Wc photoperiod. These mutant phenotypes always correlated with the expression of BVR enzymatic activity (data not shown). The reduced germination frequency of the resulting transgenic lines complicated genetic analysis, because very few showed the 3:1 ratio for kanamycin resistance expected in the T_1 generation for single-gene insertions.

In addition to having elongated hypocotyls, kanamycin-resistant T_1 seedlings developed extremely chlorotic leaves under Wc, especially under cool-white (CW) fluorescent illumination at light fluence rates of 100 μmol m^{-2} sec^{-1}. Most of these plants failed to develop beyond the seedling stage and ultimately died. In contrast, the combination of GroLux and wide-spectrum GroLux (GL/WS) fluorescent illumination at the same fluence rate (i.e., the light condition used serendipitously for the original regeneration experiments) was able to support more robust growth of the transgenic BVR plants. Subsequent phenotypic screens were performed using this light regime to obtain isogenic lines that were homozygous for both kanamycin resistance and the long hypocotyl phenotype. Five transgenic lines were isolated, and they were designated BVR1 to BVR5. Table 1 provides a summary of the relative BVR activities of these five lines and a comparison of their hypocotyl lengths with Wc-grown Nossen (No-0) wild-type seedlings. BVR1, BVR2, and BVR3 were chosen for detailed analysis because they represented the full range of BVR expression for all five transgenic lines.

Table 1. Relative BVR Activities and Hypocotyl Lengths for Light-Grown No-0 Wild-Type Plants and Five BVR Plant Lines

Plant Line[a]	Relative BVR Activity[b]	Hypocotyl Length (mm)[c]
No-0 WT	ND	1.83 ± 0.34
BVR1	1	6.66 ± 1.15
BVR2	8.5	8.58 ± 1.19
BVR3	15.9	7.93 ± 0.87
BVR4	5.0	7.76 ± 1.03
BVR5	14.6	8.04 ± 0.69

[a] Six-day-old seedlings for BVR and hypocotyl measurements (n = 50) were grown at 20°C under continuous GL/WS (fluence rate of 100 μmol m^{-2} sec^{-1}) and CW (fluence rate of 154 μmol m^{-2} sec^{-1}), respectively. WT, wild type.

[b] BVR assays were performed with soluble protein extracts, as described in Methods, and relative activities were calculated by normalizing to the specific activity of BVR1 seedlings. BVR activity was not detected (ND) in No-0 wild-type seedling extracts.

[c] Hypocotyl measurements reflect the average and standard deviation for 50 seedlings analyzed.

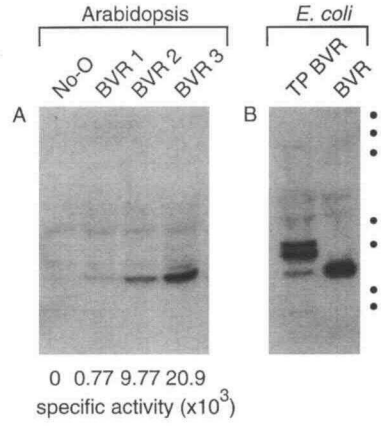

Figure 1. BVR Immunoblot Analysis of the Wild Type, Three Isogenic Transgenic BVR Lines, and *E. coli* Cells Expressing Recombinant TP-BVR and BVR.

(A) Each lane was loaded with 20 μg of soluble protein extract from 9-day-old seedlings grown under continuous GL/WS illumination of 90 μmol m^{-2} sec^{-1} at 25°C. Numbers shown for BVR-specific activity (international units per milligram of protein) have been multiplied by 1000; the actual values range from 0.00077 to 0.02090.

(B) Each lane was loaded with approximately equal amounts of BVR activity found in soluble protein extracts of *E. coli* cells expressing either mature (BVR) or precursor forms (TP-BVR) of biliverdin reductase. Filled circles at right and from top to bottom indicate the molecular masses of the protein standards (i.e., 200, 116, 97, 66, 45, 31, and 21.5 kD).

To estimate the level of BVR expression, soluble protein extracts from light-grown transgenic seedlings were analyzed for the presence of BVR protein. Figure 1A shows that the amount of immunochemically detectable BVR protein corresponded well with the measured enzyme activity in the three transgenic lines. Depending on the seedling age and light regime, we observed that BVR3 plants contain as much as 25-fold higher levels of BVR activity than the poorest expressing BVR1 line. This immunoblot also reveals that the chloroplast transit peptide was properly processed in transgenic plants because only mature-sized BVR with a molecular mass of 33 kD was detected. In this regard, the precursor form of BVR containing the transit peptide (i.e., TP-BVR) has a predicted molecular mass of 43 kD and migrated as a 43/38-kD doublet when expressed in *E. coli* cells (Figure 1B).

Transgenic BVR Plants Display Altered Photomorphogenesis

Figure 2A shows that BVR expression alters Arabidopsis seedling photomorphogenesis. Compared with wild-type

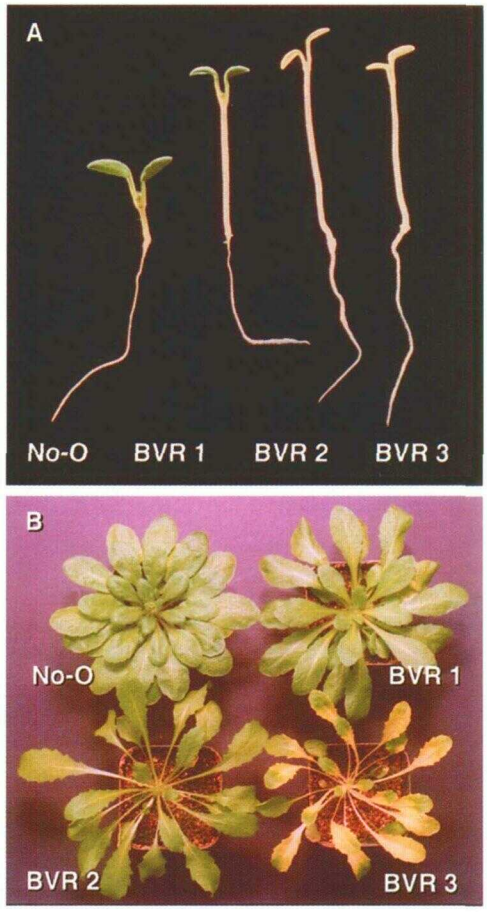

Figure 2. Light-Grown Wild-Type and Transgenic BVR Plants.

(A) No-0 wild-type seedlings and seedings from three isogenic transgenic BVR lines were grown for 4 days under continuous CW fluorescent light (175 μmol m^{-2} sec^{-1}) at 20°C.

(B) Adult No-0 wild-type plants and adult plants from three isogenic transgenic BVR lines were grown for 52 days at 20°C under continuous V/I illumination (100 μmol m^{-2} sec^{-1}) with a short-day photoperiod (8-hr light and 16-hr dark).

seedlings, light-grown transgenic plants displayed both elongated hypocotyls and smaller cotyledons. Despite the 25-fold range of BVR expression, hypocotyl lengths were similar for the three BVR lines (Figure 2 and Table 1). In contrast to hypocotyl elongation, chlorophyll accumulation in transgenic BVR plants was strikingly dependent on the level of BVR expression (Figure 3). More severe reductions in chlorophyll levels were observed in the higher expressing BVR2 and BVR3 lines, whereas total chlorophyll in BVR1 seedlings was more similar to that of wild-type seedlings (Figure 3A). Figure 3B shows that chlorophyll *a*/chlorophyll *b* (Chl*a*/Chl*b*) ratios increased with the amount of BVR expression. Interestingly, these two effects of BVR expression were more pronounced as the light fluence rate was increased from 50 to 250 μmol m^{-2} sec^{-1}. By contrast, this range of fluence rates had little effect on total chlorophyll or Chl*a*/Chl*b* ratios in wild-type seedlings. For comparative purposes, the phytochrome chromophore-deficient *hy1* and *hy2* mutants were grown under identical conditions. Although the pronounced fluence rate dependency was not observed for either mutant, *hy1* seedlings exhibited reduced chlorophyll accumulation and increased Chl*a*/Chl*b* ratios similar to those of the transgenic BVR plants (Figures 3C and 3D). By comparison, *hy2* seedlings showed no significant reduction in total chlorophyll at these light fluences; however, the Chl*a*/Chl*b* ratio was slightly elevated.

As discussed earlier, it was difficult to propagate BVR plants to adulthood under continuous CW light sources. Because BVR plants were noticeably less chlorotic when grown under light sources that produced spectral qualities more similar to sunlight, such as GL/WS and Vitalite plus incandescents (V/I; see Methods for full description of light sources), transgenic BVR plants were grown to adulthood under V/I illumination. Figure 2B illustrates the phenotypes of BVR1, BVR2, and BVR3 and wild-type adult plants grown under this light source with a short-day photoperiod. This comparison shows that the appearance of adult transgenic plants was strongly correlated with the level of BVR expression. Adult BVR plant leaves displayed decreased area/length ratios and possessed reduced levels of chlorophyll phenotypes consistent with the *hy1* and *hy2* mutants (Koornneef et al., 1980; Chory et al., 1989a). Alterations in leaf shape and reduced chlorophyll content of the higher expressing BVR2 and BVR3 plants were increasingly more pronounced by comparison with BVR1 (Figure 2B). The leaf shape of BVR2 and BVR3 plants also followed a clear trend toward more defined serrated margins when compared with leaves of wild-type and BVR1 plants.

In addition to alterations in leaf morphology and chlorophyll content, early flowering was characteristic of the transgenic BVR lines compared with No-0 wild-type plants when grown under Wc (data not shown). The flowering behavior of wild-type plants and the two higher expressing transgenic BVR lines grown under long-day photoperiods is shown in Table 2; for comparison, those of the chromophore-deficient *hy1* and *hy2* mutants are taken from the literature (Goto et al., 1991). Flowering of BVR2 and BVR3 plants was chronologically delayed under long days; however, like *hy1* and *hy2* plants, fewer rosette leaves were present at bolting. The latter result is consistent with a loss of the phytochrome-regulated delay of bolting seen for *hy1* and *hy2* mutants (Goto et al., 1991). The chronological delay in flowering under long days most likely reflects the photosynthetic deficiencies of these plants, an interpretation that is consistent with the early flowering behavior of the transgenic BVR plants under continuous light. Because of these complications, the flowering behavior of the transgenic plants will be the subject of a future investigation.

Transgenic BVR Plants Are Phytochrome Chromophore Deficient

To establish whether BVR expression reduced the level of photoactive phyA in Arabidopsis seedlings, soluble protein extracts from dark-grown wild-type and transgenic BVR plants were analyzed spectrophotometrically. PhyA was readily detected in etiolated wild-type seedlings, as expected, whereas no phyA photoactivity was observed in BVR3 extracts (data not shown). Unfortunately, when BVR3 extracts were assayed immunochemically, phytochrome A levels varied from experiment to experiment, presumably due to the instability of the PHYA polypeptide. For this reason, hot SDS was used to extract protein from freshly frozen plant tissue. This approach yielded reproducible PHYA recoveries. With this extraction protocol, PHYA levels could be quantified immunochemically. To determine whether the phytochrome A protein contained a bilin prothestic group in the transgenic BVR plants, we took advantage of the observation that (apo)PHYA has a considerably longer half-life than does phyA because of its inability to photoconvert to the Pfr form (Quail et al., 1973; Shanklin et al., 1987; Jabben et al., 1989; Parks et al., 1989).

Figure 4 shows that the light-dependent turnover of PHYA was significantly reduced in the BVR plants when compared with wild-type No-0 plants. Higher levels of BVR expression resulted in greater amounts of PHYA that were resistant to proteolysis, supporting the interpretation that the BVR1, BVR2, and BVR3 plants were increasingly more chromophore deficient. Data for wild-type Landsberg *erecta* (*Ler*)

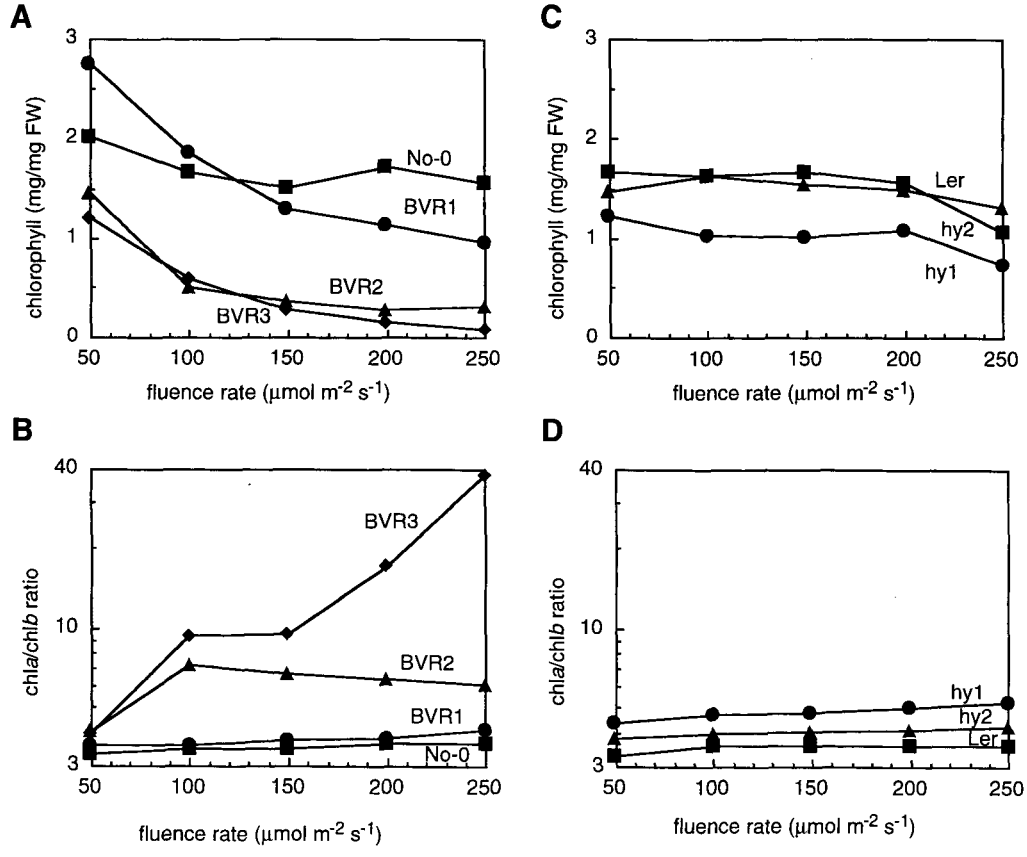

Figure 3. Fluence Rate Dependency of Chlorophyll Accumulation for the Wild Type, Transgenic BVR Plants, and Chromophore-Deficient *hy1* and *hy2* Mutants.

Transgenic BVR lines BVR1, BVR2, and BVR3, No-0 and L*er* wild-type plants, and *hy1* and *hy2* seedlings were grown at 20°C on agar medium for 7 days under continuous CW illumination of various fluence rates. Chlorophyll assays were performed as described in Methods.
FW, fresh weight; s, second.
(A) and **(C)** Total chlorophyll.
(B) and **(D)** Chl*a*/Chl*b* ratios.

Table 2. Comparative Flowering Behavior of Transgenic BVR Plants, Wild-Type No-0 and L*er* Plants, and Phytochrome Chromophore-Deficient *hy1* and *hy2* Mutants

Plant Line[a]	Days to Flowering	No. of Rosette Leaves	Reference
No-0 WT	21 ± 1	13 ± 0.7	This study
BVR2	26 ± 3	9 ± 0.8	This study
BVR3	25 ± 3	9 ± 1.0	This study
L*er* WT	18.9 ± 1.6	8.1 ± 0.2	Goto et al. (1991)
hy1	18.0 ± 2.5	6.0 ± 0.1	Goto et al. (1991)
hy2	16.9 ± 1.5	6.5 ± 0.1	Goto et al. (1991)

[a] BVR plants (n = 28) were grown at 20°C under a 16-hr-light/8-hr-dark long-day photoperiod with V/I illumination at a fluence rate of 100 μmol m^{-2} sec^{-1}. L*er* and *hy* plants (n = 70 to 80) were grown at 25°C under a 16-hr-light/8-hr-dark long-day photoperiod with high-intensity mercury discharge lamps at a fluence rate of 13 W m^{-2} (from Goto et al., 1991). WT, wild type.

and the *hy1* mutant, known to exhibit reduced light-dependent PHYA turnover (Parks et al., 1989; Parks and Quail, 1991), are also included in Figure 4 as a methodological control. Compared with that in the three BVR lines, PHYA turnover in *hy1* appeared to be more light stable. However, the relative light stability of PHYA in *hy1* versus the BVR lines reflects both the degree of PHYA chromophore attachment and differences in the inherent synthesis/turnover rates for PHYA in the L*er* and No-0 ecotypes. Until the latter issue is experimentally addressed, the precise degree of phytochrome chromophore deficiency for the three BVR plant lines cannot be estimated from this comparison.

Transgenic BVR Plants Are Deficient in Multiple Phytochrome Activities

Phytochrome mutants and phytochrome-overexpressing transgenic plants have made it possible to identify the distinct as well as overlapping biological roles that different phytochromes perform (reviewed in Whitelam and Harberd, 1994; Smith, 1995). From such studies, it has been established that phyA is responsible for FRc-dependent high-irradiance responses (e.g., seed germination) and some very low fluence responses (Furuya and Schafer, 1996), whereas phyB mediates both classic R/FR photoreversible low fluence responses, including end-of-day FR responses and Rc-dependent high-irradiance responses (Smith, 1995). The effects of both phyA and phyB deficiencies and their surfeit on light-mediated seedling development (i.e., hypocotyl extension, hook opening, cotyledon expansion, and greening) have been particularly well analyzed (reviewed in Whitelam and Harberd, 1994). These investigations indicate that phyA mediates seedling photomorphogenesis in response to FRc, whereas phyB mediates the same responses to Rc.

The ability to experimentally distinguish between phyA- and phyB-mediated seedling morphogenesis by growth under Rc or FRc has permitted us to determine whether one or both phytochrome activities are affected by BVR expression. Figure 5 shows that BVR seedlings failed to respond to either Rc or FRc, in contrast with the wild-type seedlings. For all three BVR lines, hypocotyls were elongated, hook opening was inhibited, and cotyledon expansion was severely reduced for seedlings grown under either Rc or FRc. Figure 5 also shows that dark-grown wild-type seedlings were indistinguishable from dark-grown BVR seedlings. Thus, BVR expression does not influence the development of seedlings grown in darkness.

With regard to potential deficiencies in other phytochrome activities, it is interesting that none of the *phyA*, *phyB*, or *phyA phyB* mutants of Arabidopsis displays the chlorophyll-deficient phenotype observed for the transgenic BVR plants (reviewed in Whitelam and Harberd, 1994). In this regard, the *hy1* and *hy2* mutants are similar. In itself, this observation argues that the *hy1*-, *hy2*-, and BVR-dependent chlorotic phenotypes reflect a deficiency in additional phytochromes (e.g., phyC, phyD, and phyE). Altogether, these observations strongly suggest that the phenotypic consequences of BVR expression are consonant with deficiencies in multiple phytochrome activities.

Transgenic BVR Plants Retain the Ability to Respond to Blue Light

Phenotypic analysis of wild-type and transgenic BVR seedlings grown under Bc reveals that with the exception of elongated hypocotyls, BVR expression does not significantly alter Bc-dependent deetiolation as determined by

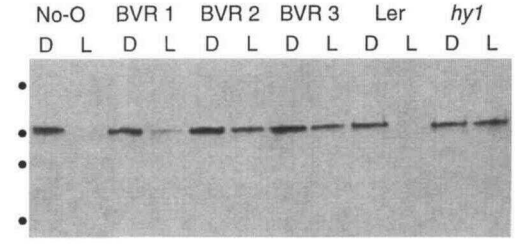

Figure 4. Immunoblot Analyses of the Phytochrome A Protein in the Wild Type, Transgenic BVR Plants, and the *hy1* Mutant.

Plants from transgenic lines BVR1 to BVR3, No-0 and L*er* wild-type plants, and *hy1* seedlings were grown on agar medium 4 days in darkness at 20°C. Half were kept in darkness for an additional 24 hr (D) at 20°C, whereas the other half were exposed to continuous GL/WS illumination (90 μmol m^{-2} sec^{-1}) at 20°C for 24 hr (L). Each lane was loaded with 25 μg of hot SDS–extracted total protein. Filled circles at left and from top to bottom indicate the molecular masses of the protein standards (i.e., 199, 120, 87, and 48 kD).

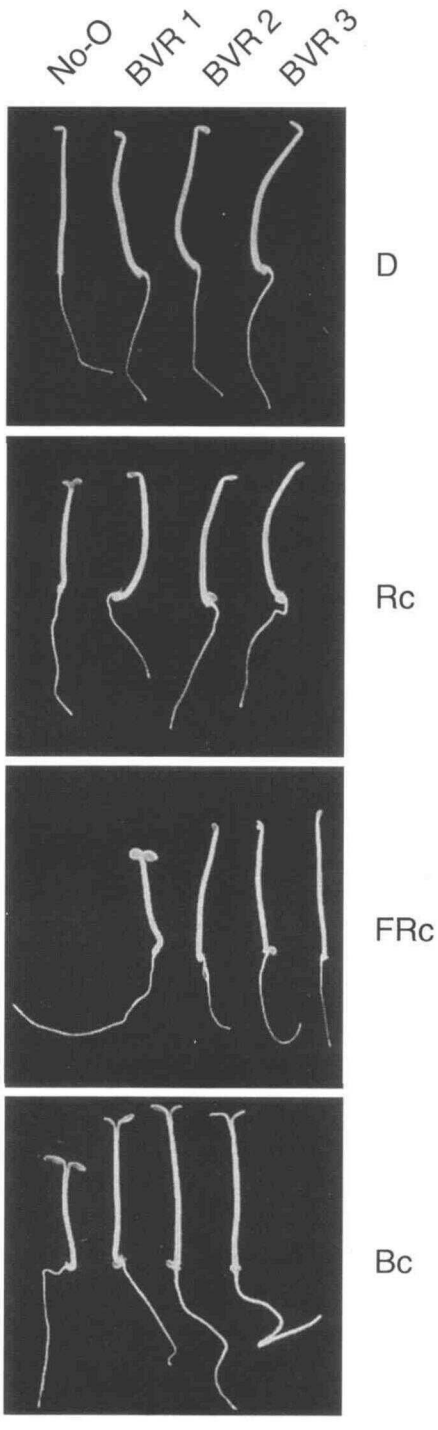

Figure 5. Phenotypes of Wild-Type and Transgenic BVR Seedlings Grown under Different Monochromatic Light Conditions.

The No-0 wild type and three isogenic transgenic BVR lines were grown for 4 days at 20°C in total darkness (D), Rc (17 μmol m^{-2} sec^{-1}), FRc (21 μmol m^{-2} sec^{-1}), or Bc (19 μmol m^{-2} sec^{-1}).

apical hook opening, cotyledon expansion, and chlorophyll accumulation (Figure 5; data not shown). Figure 6 shows that the expression of BVR also failed to alter phototropic behavior toward low fluence blue light. As controls, the phototropic sensitivities of the No-0 wild type and the nonphototropic *nph1* mutant (Liscum and Briggs, 1995) were compared with BVR3 in this experiment. Thus, it appears that many blue light responses are unaffected by BVR expression, suggesting that the altered blue light–dependent hypocotyl response of the BVR seedlings reflects the specific loss of additional phytochrome activities.

det1 Suppresses the Elongated Hypocotyl Phenotype of Transgenic BVR Plants

Because the deetiolated *det1* mutation is epistatic to phytochrome-regulated seedling development (Chory, 1992), the BVR3 *det1* double mutant was constructed to control for potential nonspecific effects of BVR expression. Figure 7 illustrates light- and dark-grown phenotypes of this double mutant, its parental lines, and the two wild-type lines. Together with phenotypic comparisons of *hy1 det1* and *hy2 det1* double mutants (Chory, 1992), this analysis shows that loss of DET1 function suppresses the elongated hypocotyl phenotype caused by BVR expression in light-grown seedlings (Figure 7A), whereas dark-grown BVR3 *det1* double mutants are indistinguishable from the *det1* parent (Figure 7B). Reduced germination of the BVR3 parental line was also suppressed in the *det1* background (data not shown). Immunoblot data and enzyme activity measurements shown in Figure 7C demonstrate that BVR protein is expressed in the double mutant. Taken together, these observations support

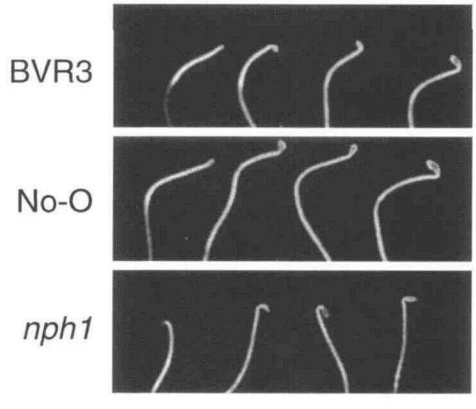

Figure 6. Blue Light–Induced Phototropic Behavior of the Wild Type, BVR3, and the Aphototropic Mutant *nph1*.

Transgenic BVR3, No-0 wild-type, and *nph1* seedlings were grown on agar medium for 3 days in darkness at 20°C and exposed to Bc illumination (0.13 μmol m^{-2} sec^{-1}) from the right. Seedlings were photographed after 10 hr of Bc.

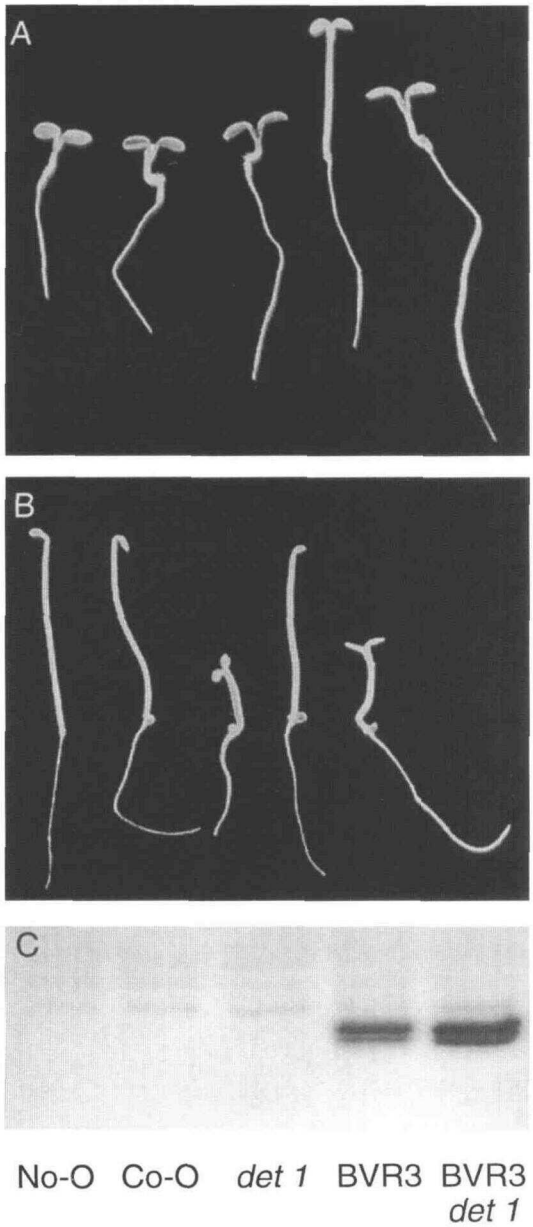

Figure 7. Comparative Phenotypic and Biochemical Analysis of the Wild-Type, Parental Strains, and BVR *det1* Double Mutants.

(A) The wild type (No-0 and Columbia [Co-0]), parental strains (*det1* and BVR3), and the BVR3 *det1* double mutants (as labeled below the gel) were grown for 4 days at 20°C under continuous CW illumination of 100 μmol m^{-2} sec^{-1}.

(B) Seedlings are as shown in **(A)**, except that they were grown for 4 days at 20°C in darkness.

(C) For BVR immunoblot analysis, each lane was loaded with 10 μg of soluble protein from 9-day-old seedlings grown under continuous GL/WS illumination (90 μmol m^{-2} sec^{-1}) at 25°C. BV-specific activities for BVR3 and BVR3 *det1* extracts were 0.018 and 0.03 international units/mg of protein, respectively.

the conclusion that the phenotypic consequence of BVR expression is phytochrome specific, as opposed to having an indirect effect on seedling growth and development (e.g., interference with plant hormone levels and/or responsiveness).

DISCUSSION

BVR Expression Phenocopies Phytochrome Chromophore-Deficient Mutations

Constitutive expression of BVR in transgenic Arabidopsis effects profound changes in light-mediated plant growth and development. Like *hy1* and *hy2* mutants, light-grown transgenic BVR plants have elongated hypocotyls, accumulate less chlorophyll, display an increased Chl*a*/Chl*b* ratio, show altered leaf morphology, exhibit increased apical dominance, flower with fewer leaves, and produce seed with greatly reduced ability to germinate. A similar combination of phenotypes has been described for other phytochrome chromophore-deficient mutants, which include the *au* and *yg-2* mutants of tomato (Koornneef et al., 1985; Terry and Kendrick, 1996; Van Tuinen et al., 1996), the *pcd1* and *pcd2* mutants of pea (Weller et al., 1996, 1997), and the *pew1* and *pew2* mutants of *N. plumbaginifolia* (Kraepiel et al., 1994).

The ability of BVR expression to phenocopy mutations in the phytochrome chromophore biosynthetic pathway suggests that the effects of BVR expression are specific to the loss of photoactive phytochrome. This conclusion is also supported by the observations that dark-grown transgenic BVR plants are indistinguishable from wild-type seedlings, are severely impaired in their responsiveness to Rc and FRc, and fully deetiolate under high fluence Bc. These phenotypes are similar to those of the phytochrome chromophore-deficient *hy1* and *hy2* mutants (Koornneef et al., 1980; Chory et al., 1989a; Liscum and Hangarter, 1991, 1994).

The observation that both light- and dark-grown BVR3 *det1* double mutant seedlings are more similar to *det1* than to the BVR3 parent also supports the conclusion that the effects of BVR expression are phytochrome specific. This result agrees with genetic data indicating that *det1* is epistatic to *hy1* and *hy2* (Chory, 1992) and is further evidence that the etiolated phenotype of light-grown transgenic seedlings is not due to nonspecific alteration of chloroplast metabolism by the presence of BVR or its rubinoid metabolites. In this regard, recent experiments in our laboratory have established that cytosolic expression of BVR produces transgenic plants that exhibit a similar range of phytochrome-deficient phenotypes (K.-C. Yeh and J.C. Lagarias, unpublished data). Taken together, these results support the conclusion that the phenotypic consequences of BVR expression mostly if not entirely result from inactivation of the linear tetrapyrrole precursors of the phytochrome chromophore.

Because heme is a potent inhibitor of the synthesis of 4-aminolevulinic acid, the first committed precursor of tetra-

pyrrole biosynthesis (reviewed in Beale, 1990; Reinbothe and Reinbothe, 1996), it has been argued that the chlorophyll-deficient phenotype of phytochrome chromophore biosynthetic mutants may be due in part to heme feedback (Terry and Kendrick, 1996). Because BVR expression would be expected to decrease the pool size of heme and other tetrapyrrole intermediates, this feedback mechanism cannot account for the chlorotic phenotype of the transgenic BVR plants and by analogy is probably not the cause of the chlorophyll deficiency in *hy1* and *hy2*.

BVR Expression Unmasks a Role for Phytochrome in Blue Light Photoperception

The observation that transgenic BVR lines grown under Bc display elongated hypocotyls is at apparent variance with the fact that *hy1* and *hy2* mutants do not (Koornneef et al., 1980; Chory et al., 1989a; Liscum and Hangarter, 1991, 1994; Ahmad and Cashmore, 1993). Because all known *hy1* and *hy2* alleles are leaky, it is conceivable that BVR is more effective at reducing the photobiological activity of phytochrome, thereby unmasking its role in Bc-mediated seedling morphogenesis. The hypothesis that phytochrome participates in Bc-dependent deetiolation has been based on the repeated observation that FR reverses the effects of blue light (reviewed in Mohr, 1994). A direct regulatory role for phytochrome in blue light–mediated seedling morphogenesis was first implicated by the evidence that hypocotyls of *hy1 hy4* double mutants were more elongated under Bc than those of their *hy4* blue light receptor mutant parent (Koornneef et al., 1980; Liscum and Hangarter, 1991). Action spectra for *phyA* and *phyB* mutants of Arabidopsis have more recently shown that phyA plays a key role in blue light–dependent seed germination (Shinomura et al., 1996). Based on these considerations, it appears likely that a deficiency in multiple phytochromes is required to manifest the long hypocotyl phenotype under Bc, as we have observed for the BVR plants, which is possibly due to the functional redundancy of different phytochrome family members for this response.

BVR Expression Reveals a Regulatory Role for Phytochrome in Light Tolerance

A novel phenotype revealed by these studies is the pronounced sensitivity of transgenic BVR plants to increased light intensity, as manifested by the marked decrease in chlorophyll content and eventual death under elevated light fluences. The asymmetric distribution of chlorosis along the BVR3 leaves suggests that this effect is a response to the localized light environment, although it could also reflect the pattern of transgenic expression. That this phenomenon is the consequence of a phytochrome deficiency is supported by the observation that a similar phenotype has been reported for the phytochrome chromophore-deficient *yg-2* and *au* mutants of tomato (Van Tuinen et al., 1996). Taken together, these results suggest that phytochrome plays an important regulatory role in light tolerance. Indeed, the severely jaundiced appearance of transgenic BVR plants grown under light fluence rates >100 μmol m^{-2} sec^{-1} indicates that these plants are unable to cope with light intensities to which wild-type plants can readily adapt. The ability to adjust to light fluences that excite the photosynthetic light-harvesting apparatus in excess of the plastid's capacity to productively utilize all of the absorbed light energy is critical to plant survival. Both rapid short-term and long-term adaptive processes are involved in the disposal of this excess light energy. The former includes light dissipation within the light-harvesting apparatus (e.g., the operation of the xanthophyll cycle), whereas the latter comprises processes required for making adjustments in the levels of light-harvesting complexes, their coupling with the two reaction centers, and the relative stoichiometry of the two photosystems (Bjorkman and Demmig-Adams, 1994; Anderson et al., 1995; Demmig-Adams and Adams, 1996). The importance of these processes to light acclimation of Arabidopsis has been well established (Walters and Horton, 1994, 1995a, 1995b), whereas a participatory role for phytochrome in light tolerance in Arabidopsis, or for that matter in any plant species, remains controversial (Anderson et al., 1995).

The precise mechanism of BVR-dependent loss of light tolerance is beyond the scope of these studies; however, we favor an active process in which reactive intermediates of photosynthetic electron transport lead to photodamage of the photosynthetic membrane rather than a passive process in which various components of the photosynthetic apparatus fail to accumulate. The former could occur via uncoordinated excitation of the two photosystems—a hypothesis supported by the observation that transgenic BVR plants are less chlorotic under light conditions enriched in FR (i.e., WS versus CW). Because FR preferentially excites photosystem I, this phenonemon suggests that changes in spectral quality may be able to compensate for the inability of the transgenic BVR plants to tolerate increased light fluences. Uncoordinated excitation of photosystems I and II could thus account for the loss of chlorophyll via the generation of reactive species in the photosynthetic membrane—an effect that would be exacerbated by the potential inability of the transgenic BVR plants to properly regulate the abundance and/or stoichiometry of the xanthophyll-cycle pigments. It is also possible that the increased survival of transgenic BVR plants under WS versus CW of the same fluence rate might simply reflect decreased activation of photosystem II. This is the photosystem most responsible for photodamage to the photosynthetic apparatus (Bjorkman and Demmig-Adams, 1994).

An alternative hypothesis for the chlorophyll deficiency of transgenic BVR plants posits a quantitative reduction in chlorophyll synthesis and/or its assembly with the apoprotein components of the photosynthetic apparatus, because it is well documented that phytochrome plays a key role in the regulation of both chlorophyll and light harvesting Chla/

Chl*b* binding protein synthesis in Arabidopsis (Karlin-Neumann et al., 1988; Lifschitz et al., 1990). However, judging by the near wild-type chlorophyll levels that accumulate in BVR plants grown under low-light fluences, transgenic BVR plants do not appear to be impaired in chlorophyll synthesis per se. A similar phenomenon has recently been reported for the phytochrome chromophore-deficient *au* mutant (Smith et al., 1993). Although it is conceivable that extreme light intensities could repress chlorophyll synthesis in phytochrome chromophore-deficient plants, the BVR-induced phytochrome deficiency is more likely to lead to an uncoordinated synthesis of chlorophyll and light-harvesting chlorophyll antennae polypeptides by potentially effecting accumulation of photodynamically active tetrapyrrole pigments. More extensive physiological analyses are clearly needed to elucidate the mechanism whereby the BVR-induced phytochrome reduction alters photosynthetic acclimation to elevated light fluences.

Transgenic BVR Plants Display Altered Leaf Morphology

In addition to altered blue light–mediated hypocotyl elongation and reduced light tolerance, transgenic BVR plants exhibit altered leaf morphology. The elongated leaves and narrow petioles of these plants are consistent with a "constitutive" shade avoidance response, which is also seen in *hy1* and *hy2* mutants (Koornneef et al., 1980; Chory et al., 1989a). In addition to these phenotypes, the leaves of transgenic BVR plants appear increasingly more serrated as the expression level of BVR increases. The dose dependency of this phenonemon suggests that the serrated leaf phenotype may also be a consequence of a phytochrome deficiency. In a recent investigation, Jones (1995) presented compelling data that the divergence between sun (i.e., more lobed) and shade (i.e., less lobed) leaf morphology in Cucurbita can be recapitulated by altering light fluence rates without significantly altering light quality (i.e., R/FR ratio). Therefore, it is possible that the serrated leaf morphology of the BVR plants is a manifestation of an alteration of a light intensity adaptation pathway rather than the classic phytochrome-mediated shade avoidance response.

Are Phytochrome Chromophore Null Mutants Viable?

In spite of the evidence for a more severe reduction in phytochrome activity in transgenic BVR plants, it is nevertheless conceivable that some photoactive phytochrome is present, which enables these plants to survive. A large number of the light-grown T_0 regenerant plants with characteristic BVR-dependent phenotypes failed to yield viable homozygous progeny lines. This suggests that higher expressing BVR lines, which effect an even more severe phytochrome chromophore deficiency, cannot survive under the conditions used for our experiments. The leakiness of all known *hy1* and *hy2* alleles, the apparent lethality of the *pew1 pew2* double mutant (Kraepiel et al., 1994), and the severely chlorotic "lethal" phenotype of the *au yg-2* double mutant (Van Tuinen et al., 1996) are consistent with this interpretation. Based on this line of reasoning, it appears that a true phytochrome chromophore null may not be viable in that it would be unable to proceed through all stages of the life cycle. Because *phyA phyB* double mutants are fully viable (Reed et al., 1994), our results imply that individual or combined photoregulatory activities of phyC, phyD, and phyE are essential for plant survival.

Future Studies: Selective Control of Photomorphogenesis by BVR Expression

BVR-dependent alterations of chlorophyll accumulation, leaf morphology, and phytochrome A turnover were all dose dependent, with the severity of the phenotypic alteration increasing with the amount of BVR activity. These results indicate that regulated expression of BVR should enable us to selectively control phytochrome levels and, potentially, the degree of the phytochrome-deficient phenotype, which may be impossible to achieve by using classical genetic approaches. Using cell-, tissue-, and organ-specific promoters to drive the expression of BVR in transgenic plants, we also hope to better define the sites of phytochrome photoperception that control individual aspects of photomorphogenesis in plants. Such information will be invaluable for tailoring desired features of light-mediated plant growth and development (e.g., onset of flowering, lateral meristem development, and internode elongation) in agronomically important crop species.

METHODS

Plasmid Constructions

Biliverdin IXα (BV) reductase (BVR) expression plasmids pRKB55/SphI and pRKB55/NcoI, containing a rat kidney BVR cDNA, were obtained by using site-directed mutagenesis of *Escherichia coli* expression plasmid pRKB55 (Fakhrai and Maines, 1992) with the degenerate primer (5′-CTCTCCAAATTTCCCGGAATTCGTAATCATGG$^{G}_{C}$CATG$^{C}_{G}$CTGTTTCCTGTGTG-3′) (Kunkel, 1985). The plasmid pTPBVR, which contains a translational fusion between a soybean small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*; Berry-Lowe et al., 1982) and rat kidney BVR, was constructed by inserting a 1.1-kb SphI-SalI BVR cDNA fragment from pRKB55/SphI into the soybean *rbcS* vector pBT22/3 that had been linearized with SphI and SalI. Plasmid pBT22/3, a kind gift of K. Archer (Trinity College, Hartford, CT), was originally constructed by inserting the 226-bp HindIII-SphI exon 1 fragment from pSRS2.1 (Berry-Lowe et al., 1982) into the SmaI and SphI sites of the pBluescript SK+ vector (Stratagene, La Jolla, CA). The HindIII site was disrupted during this process.

The plant transformation vector pBIB/35S-TPBVR was prepared in two steps. First, plasmid p35S-TPBVR was constructed by inserting the 1.3-kb EcoRV-SacI TP-BVR fragment into vector pRTL2 (Carrington et

al., 1990) at the NcoI site behind the modified cauliflower mosaic virus 35S promoter containing a dual enhancer region and the tobacco etch viral translational enhancer sequence. Before ligation with the TP-BVR fragment, the pRTL2 vector was linearized with NcoI, blunt ended with mung bean nuclease, and restricted with SacI. In the second step, plasmid pBIB/35S-TPBVR construction was accomplished by triple ligation of the HindIII-SacI–treated vector pBIB-KAN (Becker, 1990), a 1.2-kb promoter–RbcS transit peptide fragment isolated from p35S-TPBVR by restriction with HindIII and BamHI, and the 0.8-kb BVR fragment isolated by restriction of pRKB55/NcoI with BamHI and SacI.

Arabidopsis thaliana Transformation

Plasmid pBIB/35S-TPBVR was mobilized into the LBA4404 strain of *Agrobacterium tumefaciens* by triparental conjugation, using the helper plasmid pRK2013 (Ditta et al., 1980). Transconjugants were selected on M9 agar medium containing 50 μg/mL kanamycin and 50 μg/mL streptomycin. Arabidopsis (ecotype Nossen [No-0]) root explant tissue was transformed and regenerated according to the procedure of Valvekens et al. (1988). Throughout the transformation/regeneration procedure, plants were maintained at 25°C under continuous illumination with a combination of Sylvania F20T12/GRO and F20T12/GRO/WS fluorescent lamps (GL/WS) (Osram Sylvania Inc., Westfield, IN) that produce a photon fluence rate of 50 to 100 μmol m^{-2} sec^{-1}. Kanamycin selection of transformed plant lines was performed in Petri dishes consisting of Murashige and Skoog salts (Murashige and Skoog, 1962), 1% (w/v) sucrose, 50 μg/mL kanamycin, and 0.3% phytagel adjusted to pH 5.7 with KOH.

Plant Materials

The long hypocotyl mutants *hy1*(21.84N) and *hy2*(To76) and the deetiolated *det1-1* mutant (Chory et al., 1989b), which is homozygous for the closely linked morphological marker *brevipedicellus* (*bp*), were generous gifts of J. Chory (Salk Institute, La Jolla, CA). The *det1* BVR3 double mutant was obtained by pollination of emasculated *bp det1-1* flowers with pollen from a transgenic BVR plant. Kanamycin-resistant F_1 plants were selfed to obtain F_2 progeny that were homozygous for both kanamycin resistance and the *bp* phenotype. The *nph1-4* mutant was the gift of M. Liscum (University of Missouri, Columbia). Adult plants were obtained by direct seed germination in Fisons Sunshine Soil Mixture 3 (McCalif Growers Supply, Ceres, CA) and placed in a 20°C growth chamber under the appropriate light regime (see below).

Hypocotyl Length Measurements

For light-controlled hypocotyl length analyses, seeds were surface sterilized for 5 min with 70% ethanol and for 15 min with 25% (v/v) commercial bleach and 0.025% SDS, rinsed three or four times with sterile Milli-Q water (Millipore Corp., Bedford, MA), and planted in 100 × 25 mm sterile Petri dishes containing Murashige and Skoog salts, 1% (w/v) sucrose, and 0.3% phytagel adjusted to pH 5.7 with KOH. Seeds were stratified at 4°C in darkness for 4 days before placing them under the appropriate light conditions (see below). Unless otherwise indicated, all plant growth experiments were performed in E7-2 growth chambers (Conviron, Winnipeg, Canada) maintained at 20°C. Hypocotyl lengths of seedlings grown in Petri plates were determined by recording the plant images with an optical scanner and quantification, using MacBAS Version 2.0 software (Fuji Medical Systems, Stamford, CT).

Phototropism Measurements

Surface-sterilized seeds were planted one seed per well on eight-well strips (catalog number 07-200-27; Fisher Scientific, Pittsburgh, PA) containing half-strength Murashige and Skoog salts and 1% agar adjusted to pH 6.7 with KOH. Seeds were then overlayed with ~2 mm of the same agar media that had been precooled to 55°C. After a 4-day stratification period as described above, seeds were induced to germinate by irradiation for 30 min with red light and then placed in darkness at 20°C for 72 hr. Etiolated seedlings were then placed in a custom-made light chamber and irradiated unilaterally for 10 hr with continuous blue light (Bc) at a fluence rate of 0.13 μmol m^{-2} sec^{-1} (see following section for a description of the Bc light source).

Light Sources

For continuous white light (Wc) growth conditions, stratified seeds on agar media or on soil were placed under cool-white lights (CW; Sylvania F48T12/CW/VHO), wide-spectrum GroLux lights (WS; Sylvania F20T12/GRO/WS), a combination of GroLux and wide-spectrum GroLux lights (GL/WS; Sylvania F20T12/GRO and F20T12/GRO/WS), or a combination of VitaLite and incandescent lights (V/I; DuroTest 60 W F48/T12/HO; Duro-Test Lighting, Fairfield, NJ; and 60 W General Electric incandescent bulbs; GE Lighting, Cleveland, OH). Custom-built irradiation chambers used for hypocotyl measurements consisted of the following light sources: continuous red light (Rc), two Sylvania F48T12, 660 nm, VHO red fluorescent tubes filtered through 0.125-inch-thick sheets of red (No. 2423; Rohm and Haas, Philadelphia, PA) and amber (No. 2422; Rohm and Haas) Plexiglas; continuous far-red light (FRc), two Sylvania F48T12/232/VHO far-red fluorescent tubes filtered through a 0.125-inch-thick sheet of black Plexiglas (FRS 700, dye number 58015; Rohm and Haas); and Bc, two Sylvania F48T12/247/VHO blue fluorescent tubes filtered through a 0.125-inch-thick sheet of blue (No. 2424; Rohm and Haas) Plexiglas. The same light source with a mirror mounted at a 45-degree angle next to the seedlings for unilateral irradiation was used for phototropism experiments. The spectral outputs of all light sources were determined with a spectroradiometer (model 1800; Li-Cor Inc., Lincoln, NE). Peak transmittance and the half-band pass of our light sources were Rc at 658 and 20 nm, FRc at 762 and 85 nm, and Bc at 450 and 35 nm, respectively. As necessary, light fluence rates were adjusted using Rosco Lux No. 111 neutral density diffusers (Rosco, Hollywood, CA) that did not significantly alter the spectral distribution of the light impinging on the plant material. All irradiation chambers were placed in a constant temperature growth chamber and contained light-baffled fans to maintain seedlings at 20°C.

Protein Extraction Protocols

Plants were excised at the agar surface, weighed, and transferred to crucibles. After freezing in liquid nitrogen, plants were crushed to a powder by using a mortar and pestle and were homogenized in one of two ways. For BVR enzyme assays, 2 to 4 volumes of plant extraction buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM

EGTA, 142 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, and 1% dimethylsulfoxide; the latter three components were added just before use) was added to the frozen plant material (Parks and Quail, 1991), and the mixture was immediately homogenized for ~1 min, using a precooled pestle. The crude homogenate was transferred to microcentrifuge tubes on ice and then cleared of debris at high speed for 10 min in a microcentrifuge at 4°C. Supernatants were stored on ice during BVR and protein assays or stored at −70°C for later analysis. For immunoblot analysis, frozen powdered plant material was transferred to a crucible preheated in a sand bath at 100°C and containing 0.75 volume of freshly prepared 125 mM Tris-HCl, pH 6.8, 5% SDS, 5% 2-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, and 1 mM phenylmethanesulfonyl fluoride and homogenized with a hot pestle for ~30 sec. Extracts were quickly transferred to microcentrifuge tubes, boiled for 3 min, and then spun at high speed in a microcentrifuge to remove debris. An aliquot was removed for protein determination, and the remainder was frozen in liquid nitrogen and stored at −70°C until the protein assays were completed.

BVR Assays

BV for BVR assays was prepared from bilirubin (Vega Biochemicals, Tucson, AZ), as described previously (Elich et al., 1989). NADPH-dependent BVR activity was assayed in 1 mL of 0.1 M Tris-HCl buffer, pH 8.7, containing 0.1 mM $NADP^+$, 1 mM glucose 6-phosphate, 0.1 unit per mL glucose-6-phosphate dehydrogenase (type XII from torula yeast), and 10 μM BV substrate (added as a 1 mM stock solution in DMSO). Assays were performed at room temperature (25°C), and reactions were initiated by the addition of 10 to 50 μL of crude plant extract. BV reduction was monitored at 450 nm, using a UV-visible spectrophotometer (model 8450A; Hewlett Packard, Wilmington, DE). Activity calculations were performed according to Kutty and Maines (1981).

Immunoblot Analysis

SDS–polyacrylamide gels (Laemmli, 1970) were equilibrated for 10 min in cold transblot buffer (25 mM Tris, 192 mM glycine, and 20% methanol) and then transferred to a nylon membrane (Amersham) in transblot buffer at 100 V for 45 min. The membrane was transferred to Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl) and then equilibrated in TBS containing 0.1% Tween 20 (TBS–Tween). The membrane was incubated with shaking with either rat kidney BVR antiserum at a 1:5000 dilution or the phytochrome A–specific monoclonal antibody 073d (Somers et al., 1991) at a 1:1000 dilution in TBS–Tween for 45 min. After washing for 30 min with three changes of TBS–Tween, the membrane was incubated with horseradish peroxidase–conjugated goat anti–rabbit or goat anti–mouse antibody at a 1:20,000 dilution for 45 min and washed as before. Detection was performed using a chemiluminescent substrate (Du Pont–New England Nuclear).

Protein and Chlorophyll Assays

Protein in soluble extracts was measured using the Coomassie Brilliant Blue R 250 assay with BSA as the standard (Bradford, 1976). For hot SDS extracts containing phytochrome, SDS and mercaptoethanol were first removed using a modification of the MeOH/chloroform extraction method described by Wessel and Flugge (1984). To 50 μL of extract was added 200 μL of methanol with vortexing, followed by the addition of 100 μL of chloroform. After vortexing briefly, 150 μL of water was added, and the sample was vortexed for 1 min. The protein was concentrated at the interface by centrifugation at high speed in a microcentrifuge for 5 min. Liquid above and below the white pancake was discarded, and the pancake was then rinsed twice with 150 μL of methanol. After 1 min of drying under house vacuum, the pellet was redissolved in 50 μL of 125 mM Tris-HCl, pH 6.8, and 1% SDS. Sometimes it was necessary to allow the pellet to redissolve overnight at room temperature. Two to 10 μL was taken for assay by the BCA method (Smith et al., 1985), with BSA as the standard. Chlorophyll measurements were performed with excised cotyledons from 7-day-old seedlings (Moran, 1982), with extinction coefficients as described by Inskeep and Bloom (1985).

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