Detection of Spatial-Specific Phytochrome Responses Using Targeted Expression of Biliverdin Reductase in Arabidopsis\textsuperscript{1}[OA]

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To regulate levels of holophytochrome in a spatial-specific manner and investigate the major sites of action of phytochromes during seedling development, we constructed transgenic Arabidopsis (Arabidopsis thaliana) plant lines expressing plastid-targeted mammalian biliverdin IX\textalpha reductase (pBVR) under regulatory control of CAB3 and MERI5 promoters. Comparative photobiological and phenotypic analyses indicated that spatial-specific expression of pBVR led to the disruption of distinct subsets of phytochrome-regulated responses for different promoters: pBVR expression in photosynthetic tissues (CAB3:pBVR lines) had intermediate effects on chlorophyll accumulation, carotenoid production, anthocyanin synthesis, and leaf development responses in white-light conditions. CAB3:pBVR expression, however, resulted in distinctive phenotypes in far-red (FR) conditions. A number of FR high irradiance responses were disrupted in CAB3:pBVR lines, including FR-dependent inhibition of hypocotyl elongation and stimulation of anthocyanin accumulation. By contrast, preferential expression of pBVR in the shoot apical meristem in MERI5:pBVR lines resulted in a phytochrome-deficient, leaf development phenotype under short-day growth conditions. These results implicate leaf-localized phytochrome A as having a unique role in regulating FR-inhibition of hypocotyl elongation and meristem- and/or leaf primordia-localized phytochromes as having a novel role in phytochrome-dependent responses. Taken together, these studies demonstrate the efficacy of selectively inactivating distinct phytochrome-mediated responses by regulated expression of BVR in transgenic plants, a novel means to investigate the sites of phytochrome photoperception and to regulate specifically light-mediated plant growth and development.

Numerous growth and developmental processes of plants such as seed germination, inhibition of hypocotyl elongation, internode elongation, cotyledon and leaf expansion, plastid development and greening, and the induction of flowering are regulated by light (Franklin and Whitelam, 2004; Schepens et al., 2004; Mathews, 2006). To monitor and adapt to environmental light conditions, plants possess multiple photomorphogenic photoreceptors, those which maximally absorb UV-B, blue/UV-A, and red (R)/far-red (FR) light (Kendrick and Kronenberg, 1994; Franklin et al., 2005; Schäfer and Nagy, 2006). The most extensively studied of these photoreceptors are the R/FR reversible phytochromes. All higher plant species investigated possess multiple phytochrome isoforms, which are encoded by a small nuclear gene family. Five individual phytochrome genes, designated PHYA to PHYE, have been isolated from Arabidopsis (Arabidopsis thaliana); Sharrock and Quail, 1989; Quail, 1994). These individual phytochromes mediate overlapping, yet discrete, aspects of photomorphogenesis.

All identified higher plant phytochromes utilize a single linear tetrapyrrole chromophore precursor, phytochromobilin (Terry et al., 1993). Recent studies have shown that there are multiple genes encoding key enzymes of the plastid-localized chromophore biosynthetic pathway in Arabidopsis (Muramoto et al., 1999; Kohchi et al., 2001; Emborg et al., 2006). Phytochromobilin-deficient plants exhibit defects in light-mediated growth and development both as seedlings and adults, corresponding to a lack of multiple phytochrome-regulatory activities (for review, see Terry, 1997).

A number of recent studies have added significantly to our understanding of the in vivo functions and intracellular signaling mechanisms of phytochromes. In these studies, researchers established that phytochromes translocate in a light-dependent fashion from the cytosol into the nucleus, where phytochrome molecules interact with transcription factors to regulate gene expression (Nagy et al., 2001; Nagatani, 2004; Jiao et al., 2007; Kevei et al., 2007). In addition to nuclear functions, biochemical localization assays and injection of signaling intermediates such as cGMP, calmodulin, and calcium support additional cytosolic functions. An increased appreciation of the biochemical protein complexes and intracellular signaling pathways that are activated by phytochromes has placed phytochrome signaling on equal footing with other cellular signaling mechanisms.

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functions of phytochromes (for review, see Nagy and Schäfer, 2000). Furthermore, the analyses of mutants harboring mutations in the genes FHY1 and FHL, whose gene products have been implicated in regulating nuclear import of phyA, provide convincing evidence for cytosolic functions of phyA (Rösler et al., 2007).

Despite the progress that has been made in understanding phytochrome signaling in cells, we lack definitive molecular evidence about the distinct sites of phytochrome photoperception and cellular mechanisms of localized pools of phytochromes that regulate intercellular and organ-specific phytochrome responses in planta. Although the importance of such spatial-specific responses is beginning to be recognized in the literature, much work remains to be done to identify these responses and elucidate them fully at the molecular level (Bou-Torrent et al., 2008; Josse et al., 2008; Montgomery, 2008). Early studies, which addressed intercellular and organ-specific phytochrome responses at the physiological level, activated phytochromes in a localized region of the plant using detached plant parts or microbeam irradiation and assessed phytochrome-mediated responses at distinct sites in the plant (De Greef et al., 1971, 1973; De Greef and Verbel, 1972; De Greef and Caubergs, 1972a, 1972b; Black and Shuttleworth, 1974; Caubergs and De Greef, 1975; Mandoli and Briggs, 1982; Nick et al., 1993). Results from these experiments suggest that phytochromes exhibit cell autonomous and cell non-autonomous responses in plants. The most widely recognized of the cell nonautonomous phytochrome responses is the photoperiodic induction of flowering in photoperiod-sensitive plant species that involves the perception of light in the leaves, followed by generation and subsequent movement of an inductive substance from leaves to the shoot apex (King and Zeevaart, 1973; Parcy, 2005; Yu and Lin, 2005; Montgomery, 2006; Zeevaart, 2006; Kobayashi and Weigel, 2007; Turck et al., 2008). The perception of light in cotyledons is also responsible for intercellular signaling that results in the inhibition of hypocotyl elongation and apical hook opening (Black and Shuttleworth, 1974; Caubergs and De Greef, 1975), as well as the pattern and level of accumulation of anthocyanins (Nick et al., 1993). Results from such studies support the roles of cell- and tissue-specific phytochrome signaling pathways in controlling discrete aspects of light-dependent growth and development through intercellular and/or interorgan coordination. The implications of many of these early physiological studies were confounded, however, by light piping and light scattering, particularly in microbeam irradiation assays. These conditions potentially result in light having a direct impact on tissues or cells far from the site of application (Mandoli and Briggs, 1982; Hiltbrunner et al., 2007).

In the present investigation, we utilize a novel experimental approach to investigate the sites of photoperception for specific phytochrome-mediated responses at the molecular level. Based on previous experiments showing that constitutive expression of the gene encoding the mammalian enzyme biliverdin IXα reductase (BVR) induces a phytochrome chromophore deficiency in transgenic plants (Lagarias et al., 1997; Montgomery et al., 1999, 2001), we used cell- and tissue-specific expression of BVR to probe the phenotypic consequences of localized inactivation of phytochromes in transgenic Arabidopsis plants. We used two Arabidopsis promoters, the -400 to -9 bp region of the CAB3 promoter (Mitra et al., 1989) and the 2.4-kb MERI5 promoter (Medford et al., 1991), to direct expression of BVR to photosynthetic and meristematic tissues, respectively. These studies provide new insights into spatial-specific photoregulatory roles of phytochromes.

**Table 1.** BVR-specific activity for light-grown No-O wild-type and BVR transgenic plant extracts

<table>
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<tr>
<th>Plant Line</th>
<th>BVR-Specific Activityb</th>
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<tr>
<td></td>
<td>(I.U. mg⁻¹) x 10³</td>
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<tr>
<td><strong>No-O wild type</strong></td>
<td>0.0</td>
</tr>
<tr>
<td><strong>35S::pBVR3</strong></td>
<td>13.4</td>
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<tr>
<td>CAB3::pBVR1</td>
<td>2.3</td>
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<tr>
<td>CAB3::pBVR2</td>
<td>9.0</td>
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<tr>
<td>CAB3::pBVR3</td>
<td>2.9</td>
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<tr>
<td>MERI5::pBVR1</td>
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<td>MERI5::pBVR2</td>
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<td>MERI5::pBVR3</td>
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aSeedlings (n = 50) were grown for 7 d at 25°C under continuous cool white illumination of 60 μmol m⁻² s⁻¹ and harvested at the same time of day for each repetition. bBVR-specific activity was measured for soluble protein extracts, as described in Lagarias et al. (1997).
Spatial-Specific Expression of BVR in Transgenic Plants

We isolated three independent homozygous CAB3::pBVR and MERI5::pBVR transgenic plant lines using an approach described previously (Lagarias et al., 1997). BVR-specific activities were determined for soluble protein extracts for each line and compared with the 35S::pBVR3 line (Montgomery et al., 1999), in which BVR was constitutively expressed and targeted to plastids. As shown in Table I, one of the CAB3::pBVR lines exhibited nearly as much BVR-specific activity as the 35S::pBVR3 line. Although considerably lower, BVR activity was detectable in total soluble protein extracts of seedlings for all three MERI5::pBVR lines.

We further assessed the spatial distribution of BVR accumulation in pBVR lines to determine whether BVR expression was light and tissue specific for the CAB3 promoter and restricted to the meristem and leaf primordia as directed by the MERI5 promoter. BVR accumulation in representative CAB3::pBVR lines was observed to be light dependent by immunoblot analysis (Fig. 1A). Additionally, BVR accumulated only in the leaf tissue and not in the hypocotyl and root tissues for these CAB3::pBVR lines, whereas BVR was found in leaf and hypocotyl/root tissues of the 35S::pBVR3 line (Fig. 1B). For MERI5-driven expression, BVR accumulation was detectable only in the plastids of meristematic and recently meristematic tissues of MERI5::pBVR lines (Fig. 2).

Photomorphogenesis Is Selectively Impaired in CAB3::pBVR and MERI5::pBVR Plants

Previously, we showed that constitutive expression of BVR in Arabidopsis altered numerous aspects of light-mediated growth and development throughout

Figure 2. Whole-mount immunolocalization of BVR protein accumulation in MERI5::pBVR seedlings. A representative MERI5::pBVR line, MERI5::pBVR1, was grown at 22°C on Phytablend medium containing 1% Suc for approximately 4 d under Wc illumination of 100 μmol m⁻² s⁻¹. A, Negative control, MERI5::pBVR1 seedlings incubated without the anti-BVR primary antibody. B, DIC image of A. C, MERI5::pBVR1 seedling incubated with anti-BVR primary antibody at 1:2,500 dilution. D, DIC image of C. Each image is a representative slice from a Z-series with 0.5-μm interval size and was captured using 543-nm laser excitation with a 20× lens objective. Fluorescence images were collected using a 560- to 615-nm band pass filter. Bars = 50 μm.

Figure 3. Photomorphogenesis of Wc-grown wild-type and transgenic BVR plants. No-O wild-type (WT), 35S::pBVR3, CAB3::pBVR (CAB-1 to CAB-3), and MERI5::pBVR (MERI-1 to MERI-3) transgenic lines were grown at 20°C on Phytablend medium containing 1% Suc for 7 d under Wc light (Grolux/Grolux wide spectrum) of 100 μmol m⁻² s⁻¹ (A), Rc light of 50 μmol m⁻² s⁻¹ (B), or FRc light of 5 μmol m⁻² s⁻¹ (C). Above each seedling, cotyledons are shown that were separated and arranged to display the full cotyledon surface area.
the plant life cycle. Among the more pronounced phenotypes observed in the transgenic plants were elongated hypocotyls and petioles under all light conditions tested (Lagarias et al., 1997; Montgomery et al., 1999). Selective expression of pBVR under regulatory control of the CAB3 and MERI5 promoters affected hypocotyl growth and leaf morphology distinctively. Qualitatively similar to 35S::pBVR transgenic lines, continuous white light (Wc)-grown CAB3::pBVR lines display elongated hypocotyls compared with ecotype Nossen (No-O) wild-type seedlings, though not as severe as the 35S::pBVR3 line (Fig. 3A). The fluence rate dependence of hypocotyl growth inhibition shown in Figure 4 reflects the decreased, but not abolished, responsiveness of CAB3::pBVR plants to Wc light. By contrast with the elongated hypocotyls of Wc-grown 35S::pBVR3 and CAB3::pBVR seedlings, MERI5::pBVR plants showed wild-type, light-dependent hypocotyl growth inhibition (Figs. 3A and 4).

To more fully assess the effect of spatial-specific expression of pBVR on phytochrome-mediated hypocotyl growth inhibition, the fluence rate dependence of hypocotyl lengths for MERI5::pBVR and CAB3::pBVR lines under continuous R (Rc) and continuous FR (FRc) light was determined (Fig. 4). As was observed under Wc, MERI5::pBVR lines responded to Rc and FRc similarly to wild-type seedlings (Fig. 4, D and F). CAB3::pBVR lines were also deficient in light-mediated hypocotyl growth inhibition under Rc (Fig. 4C). Under Rc conditions, CAB3::pBVR lines exhibited limited responsiveness, but 35S::pBVR lines were impaired more significantly under these conditions. By contrast, CAB3::pBVR lines were at least as deficient in their response to FRc as the 35S::pBVR3 transgenic seedlings (Fig. 4E). Furthermore, under FRc, CAB3::pBVR lines exhibited closed cotyledons similar to a null phyA mutant (Salk_014575; Ruckle et al., 2007), whereas wild-type and 35S::pBVR3 line cotyledons were open and yellow (Fig. 3C). Notably, approximately 1.5 h of exposure to ambient white light during imaging resulted in enhanced greening of CAB3::pBVR lines, indicating that these lines are defective in the FR block to greening response similar to phyA seedlings (Fig. 3C). CAB3::pBVR lines also were the only lines to exhibit a deficiency in the FRc-mediated suppression of hypocotyl negative gravitropism (data not shown). Thus, CAB3::pBVR lines exhibit deficiencies in FR-high irra-

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**Figure 4.** Fluence response curves for hypocotyl length of wild-type and transgenic BVR seedlings. CAB3::pBVR and MERI5::pBVR lines are compared with No-O wild-type seedlings grown at 20°C on Phytablend medium containing 1% Suc for 7 d under Wc, Rc, and FRc illumination of various fluence rates. Data points represent mean (±sd) of hypocotyl lengths measured for 10 to 25 seedlings in each of three independent experiments and correspond to No-O wild-type (■), 35S::pBVR3 (●), CAB3/MERI5::pBVR1 (●, gray), CAB3/MERI5::pBVR2 (●, gray), and CAB3/MERI5::pBVR3 (●, gray) lines.
diance response (HIR) phenotypes that have been previously observed for phyA and hfr1 mutants (Barnes et al., 1996; Fairchild et al., 2000, Yanovsky et al., 2002).

A distinctive phenotype associated with meristem- and/or leaf primordia-localized phytochrome deficiencies was that the average rosette diameter of MERI5::pBVR lines was about 10% greater than wild type only when plants were grown under short-day (SD) photoperiods (Fig. 5). This phenotype was not observed in either CAB3::pBVR (Fig. 5) or 35S::pBVR lines (Montgomery et al., 1999). This increase in rosette diameter under SD photoperiod was determined to arise from an increase in the width to length ratios of MERI5::pBVR leaves as compared to wild-type and CAB3::pBVR lines (data not shown).

**Chlorophyll Accumulation Depends upon Bilin Production in Photosynthetic Tissues**

Previous findings showed that constitutive expression of BVR in both Arabidopsis and tobacco (Nicotiana tabacum) significantly reduced chlorophyll and protochlorophyll (PChl) accumulation when targeted to plastids (Montgomery et al., 1999, 2001). Plants of the 35S::pBVR lines were notably intolerant of elevated fluences of light and exhibited a striking reduction in total chlorophyll and a coincident increase in the chlorophyll a/b (chl\textsubscript{a}/chl\textsubscript{b}) ratio under these conditions. To assess the effect of site-specific BVR expression on chlorophyll accumulation, we determined the fluence rate dependence of chlorophyll accumulation in light-grown CAB3::pBVR and MERI5::pBVR seedlings. Like 35S::pBVR transgenics, CAB3::pBVR seedlings showed a general decrease in total chlorophyll levels and a measurable increase in chl\textsubscript{a}/chl\textsubscript{b} ratios (Fig. 6, A and C). By contrast to 35S::pBVR3, no dependence on light fluence rates was observed for chl\textsubscript{a}/chl\textsubscript{b} ratios for the CAB3::pBVR lines in the fluence range tested. Total chlorophyll levels for light-grown MERI5::pBVR seedlings were slightly less than for wild type at low fluences yet comparable to wild type at high fluences (Fig. 6B). Unlike the 35S::pBVR and CAB3::pBVR lines, chl\textsubscript{a}/chl\textsubscript{b} ratios for MERI5::pBVR seedlings were indistinguishable from wild-type seedlings throughout the range of fluence rates measured (Fig. 6D). We also determined the impact of

**Figure 5.** Rosette morphology of transgenic BVR plants under SD photoperiod. No-O wild-type (WT) and representative CAB3::pBVR and MERI5::pBVR lines were grown on a soil mixture for 40 d in a controlled environment growth chamber at 25°C under Wc illumination of 100 μmol m\textsuperscript{-2} s\textsuperscript{-1} with an 8-h-light/16-h-dark SD photoperiod. Shown below are average rosette diameters (±st, n = 20).

**Figure 6.** Fluence rate-dependent chlorophyll accumulation of wild-type and transgenic BVR seedlings. Transgenic CAB3::pBVR (CAB-1 to CAB-3), MERI5::pBVR (MERI-1 to MERI-3), and No-O wild-type seedlings were grown at 22°C on Phyta-blend medium containing 1% Suc for 7 d under Wc illumination of various fluence rates. Data points represent the mean obtained from three independent measurements. A and B, Total chlorophyll. C and D, chl\textsubscript{a}/chl\textsubscript{b} ratios. The symbol designations are the same as those in Figure 4.
selective phytochrome inactivation on PChl accumulation in dark-grown seedlings. PChl accumulation was largely unaffected in CAB3::pBVR and MERI5::pBVR lines (Fig. 7), with the exception of MERI5::pBVR3, which exhibited intermediate levels of PChl as compared to wild type and 35S::pBVR3.

**Anthocyanin Accumulation Is Altered in CAB3::pBVR-Expressing Seedlings**

The previous finding that constitutive BVR expression inhibits Suc-stimulated anthocyanin synthesis in transgenic Arabidopsis plants (Montgomery et al., 1999, 2001) supports the recognized regulatory role of phytochromes in anthocyanin synthesis (Kim et al., 2003; Shin et al., 2007). To determine whether the localized phytochrome deficiencies distinctly affected anthocyanin accumulation, we compared Suc-stimulated anthocyanin accumulation in Wc-grown CAB3::pBVR and MERI5::pBVR plants with that of wild type and 35S::pBVR3. Anthocyanin synthesis was reduced in all of the CAB3::pBVR lines to a level comparable to that of the constitutive BVR-expressing 35S::pBVR3 line (Fig. 8A). Two of the CAB3::pBVR lines, CAB3::pBVR2 and CAB3::pBVR3, also exhibited a reduced response to Suc, compared to that observed for No-O wild type (see fold increase, Fig. 8A). The remaining CAB3::pBVR line, CAB3::pBVR1, showed an “enhanced” response to Suc, which might reflect the insertion of the CAB3::pBVR3 transgene near a Suc regulatory locus. By contrast with the CAB3::pBVR lines, MERI5::pBVR lines did not exhibit significant changes in anthocyanin levels, nor did they show reduction in the response to Suc (Fig. 8A).

Given the marked disruption of FR-HIR responses for hypocotyl elongation and the suppression of negative gravitropism in CAB3::pBVR lines noted above, we also investigated the impact of monochromatic Rc and FRc illumination on the accumulation of anthocyanin in the presence of Suc using a representative CAB3::pBVR line, 35S::pBVR3, null phyA and phyB (Salk_022035; Mayfield et al., 2007; Ruckle et al., 2007).
T-DNA insertion mutants, and the cognate wild-type parents. Anthocyanin accumulation in Rc light was low for all lines, with the CAB3::pBVR2 line having less anthocyanin than all of the other lines, including the phyB mutant compared to its wild-type parent (Fig. 8B). Notably, CAB3::pBVR lines completely lack the FR-HIR response of accumulating anthocyanins in response to FRc, identical to the phyA null mutant (Fig. 8C).

**Carotenoid Accumulation Is Altered in CAB3::pBVR-Expressing Seedlings**

Arabidopsis phytochromes, specifically phyA and light-stable phytochromes other than phyB, have been implicated in the regulation of carotenoid biosynthetic genes (von Lintig et al., 1997). To compare the impact of constitutive and localized phytochrome deficiencies induced by BVR on carotenoid accumulation, we determined the carotenoid content of No-O wild-type, 35S::pBVR3, CAB3::pBVR, and MERI5::pBVR seedlings. These analyses indicated that constitutive (i.e. 35S-promoter driven) BVR expression yielded a significant decrease in carotenoid levels. By comparison, carotenoid accumulation was moderately reduced in all three CAB3::pBVR lines but in only one of the MERI5::pBVR lines (Fig. 9).

**DISCUSSION**

**Directed Expression of BVR Reveals Spatial-Specific Regulatory Roles for Individual Phytochrome Isoforms**

These studies support the use of spatial-specific promoters to drive BVR expression as an effective tool for generating transgenic plants with distinct subsets of phytochrome-deficient phenotypes. CAB3::pBVR expression was regulated both spatially and by light, whereas MERI5::pBVR expression was regulated spatially (Figs. 1 and 2). These diverse profiles of BVR expression led to subsets of phytochrome-deficient phenotypes that differed substantially from each other, as well as those observed for constitutive 35S::pBVR expression.

Specifically, CAB3::pBVR expression affected hypocotyl growth inhibition considerably under FRc illumination, to a lesser degree under Rc illumination, and moderately under Wc illumination. As the analysis of publicly available gene expression data using the eFP browser (http://bbc.botany.utoronto.ca/eFP/development/; Winter et al., 2007) indicated that expression of CAB3 is relatively similar in Rc, FRc, Bc, or Wc light, we ruled out the possibility that the phenotypic differences observed for different monochromatic light conditions reflect differences in the levels of BVR expression; rather, these results point to unique regulatory roles for distinct phytochromes. For example, the complete inability of CAB3::pBVR lines to inhibit hypocotyl growth (Fig. 4E) and induce anthocyanin accumulation (Fig. 8C) under FRc light suggests that CAB3::pBVR expression induces a severe phytochrome deficiency in photosynthetic tissues. Previous studies have shown that both PHYA and PHYC are expressed abundantly in Arabidopsis cotyledons (Toth et al., 2001). FRc conditions are those for which phyA has been assessed as the phytochrome responsible for the inhibition of hypocotyl elongation (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), whereas phyC has not been shown to have a highly significant role in FR-mediated inhibition of hypocotyl elongation (Monte et al., 2003; Balasubramanian et al., 2006). Thus, the elongated hypocotyls of CAB3::pBVR lines under FRc illumination, which corresponds to a phytochrome deficiency in photosynthetic tissues, suggests that leaf-localized phyA is primarily responsible for initiating a signaling cascade that results in the FR-mediated inhibition of hypocotyl elongation. This FR-dependent hypocotyl elongation phenotype is consistent with an early finding that cotyledons are the site of a phytochrome-mediated signal that controls hypocotyl growth inhibition (Black and Shuttleworth, 1974) and a more recent finding that FR perception by cotyledons impacts gene expression in the hypocotyl (Tanaka et al., 2002). Furthermore, the distinct responses of CAB3::pBVR seedlings to Rc versus FRc illumination may be related to the distinct patterns of expression of PHYA and PHYB observed in cotyledons (Toth et al., 2001).

Careful analysis of the pattern of growth of the hypocotyls of CAB3::pBVR lines under FRc illumination indicated that the hypocotyls of these lines are longer than those of the 35S::pBVR3 line (Fig. 4E). When assessing these lines relative to their individual dark controls, the 35S::pBVR3 line is virtually blind to FR light and exhibits hypocotyls that are nearly identical in length to dark-grown controls. Under these conditions, CAB3::pBVR lines perceive FR light and induce hypocotyl elongation. These observations sug-
gested that apart from the disruption of FR-mediated inhibition of hypocotyl elongation, CAB3:pBVR lines exhibit a FR-dependent induction of hypocotyl growth (Fig. 4E). As CAB3:pBVR lines do not accumulate the chromophore-degrading BVR enzyme in hypocotyl tissues and thus functional phytochromes persist in the hypocotyls of CAB3:pBVR plants, this FR-dependent induction of hypocotyl growth is likely dependent on hypocotyl-localized phytochrome signaling. This response is distinct from that observed for CAB3:pBVR seedlings grown in Rc light, which exhibit extremely limited responsiveness to Rc in regards to inhibiting hypocotyl elongation that is less than that observed for the 35S:pBVR3 line (Fig. 4C). Notably, prior results demonstrated that cotyledon-specific expression of PHYB in a phyB-deficient background was nearly as effective as expression throughout the seedling driven by the native promoter in restoring Wc-dependent inhibition of hypocotyl elongation (Endo et al., 2005). Thus, our observations of CAB3:pBVR lines, in addition to suggesting novel insight into the role of cotyledon-localized phyA, corroborate the suggestion of Endo et al. (2005) that phyB in the cotyledons is the major site of R light perception for the regulation of seedling de-etiolation.

CAB3:pBVR lines showed decreased levels of chlorophyll and carotenoid levels. As leaves are the primary site of synthesis for these pigments, reduction in the levels of these pigments as a response to inactivating phytochromes in photosynthetic tissues is anticipated. As the levels of these pigments are further reduced in 35S:pBVR lines, phytochromes in photosynthetic tissues are either not entirely responsible for this response or the reduction of phytochrome levels in CAB3:pBVR lines was not sufficient to observe a complete dampening of the accumulation of these pigments, while being sufficient for disrupting FR-mediated inhibition of hypocotyl elongation completely. MERI5:pBVR transgenic lines also exhibit leaves that have a larger surface area than either No-O wild-type or CAB3:pBVR lines under SD photoperiods (Fig. 5). These results may suggest that meristem- or leaf primordia-localized phytochromes affect primary leaf expansion.

Anthocyanin accumulation was impacted uniquely for the lines assessed. CAB3:pBVR lines had reduced anthocyanin in Rc growth conditions (Fig. 8B). This response was different from that observed for a phyB mutant and thus suggests that phyA or light-stable phytochromes other than phyB have a role in the induction of anthocyanin accumulation in R light. CAB3:pBVR completely lacked the FR-HIR response of inducing anthocyanin accumulation in response to FRc light (Fig. 8C). The response observed for CAB3:pBVR lines was identical to that of a phyA mutant. Other FR-HIR responses, including cotyledon separation and FR block to greening, were also disrupted in CAB3:pBVR lines (Fig. 3C).

In summary, our investigations indicate that spatially distinct pools of phytochrome perceive light and initiate intercellular and interorgan signaling pathways that regulate distinct aspects of de-etiolation and SD-dependent responses. Meristematic- and/or leaf primordia-specific phytochromes have a role in plant responsiveness that has not been previously reported. Our results also demonstrate a role for mesophyll-specific phyA in the regulation of numerous FR-HIR responses, including the inhibition of hypocotyl elongation, suppression of negative gravitropism, and the induction of anthocyanin accumulation. Our findings also support a potential role for hypocotyl-localized phyA in the induction of cell elongation. The definitive identification of the molecular effectors involved in these spatial-specific phytochrome responses awaits additional experimentation.

MATERIALS AND METHODS

Plasmid Constructions

The plasmid pCAB3/KS+ was obtained by subcloning a 1-kb EcoRI-PstI fragment containing the CAB3 promoter (Mitra et al., 1989) from the plasmid pUC13, a generous gift from J. Chory (Salk Institute, La Jolla, CA), into the EcoRI and PstI sites of the pBluescript II KS+ vector (Stratagene). We constructed the plant transformation vector pBIB/CAB3-pTPBVR by performing a triple ligation of a 1.2-kb EcoRV-StiI pTPBVR fragment consisting of a translational fusion between a chloroplast-targeting signal from the small subunit of Rubisco (Berry-Lowe et al., 1982) and cDNA from the plasmid pTPBVR (Lagarias et al., 1997), a HindIII-SmaI CAB3 promoter fragment from pCAB3/KS+, and the HindIII-StiI-digested vector pBIB-KAN (Becker, 1990). We obtained the plant transformation vector pMON/TpTPBVR by subcloning a 1.2-kb BamHI-SmaI TPBVR fragment into the BglII-Stud-digested vector pMON672 (Monsanto), which contained the MERI5 promoter (Medford et al., 1991).

Plant Transformation and Materials

Plasmid pBIB/CAB3-pTPBVR was mobilized into the LBA4404 strain of Agrobacterium tumefaciens and plasmid pMON/TpTPBVR into the ABI strain (Monsanto) by triparental conjugation using the helper plasmid pRK2013 (Ditta et al., 1980). Following selection of transconjugants on M9 agar medium containing 50 μg/ml kanamycin and 50 μg/ml streptomycin, we transformed Arabidopsis (Arabidopsis thaliana) No-O with each construct using a standard Agrobacterium-mediated transformation (Clough and Bent, 1998). Kanamycin selection of transformants was performed in 100-× 25-mm petri dishes on media containing 1× Murashige and Skoog salts (Gibco-BRL), 1% (w/v) Suc, 50 μg/ml kanamycin, and 0.7% agar. The transgenic 35S:pBVR3 line was previously isolated (Lagarias et al., 1997).

Plant Growth Conditions

Arabidopsis seeds were surface-sterilized for 15 min with 35% (v/v) commercial bleach containing 0.025% (v/v) SDS and rinsed at least 3 times with ultrapure water (Milli-Q, Millipore). Sterilized seeds were plated in 100-× 25-mm petri dishes containing 1× Murashige and Skoog salts, 0.9% Phytablen (Caisson Laboratories), and 0 or 1% (w/v) Suc. During imbibition, seeds were cold stratified at 4°C in darkness for 3 d. Stratified seeds were transferred to temperature- and humidity-controlled growth chambers under defined light conditions. For flowering experiments, seeds sterilized as described were germinated directly on pots containing Arabidopsis mix and grown in controlled-environment chambers at 25°C under SD (8-h-light/16-h-dark cycle).

Light Sources

Wc illumination was provided by cool-white lights (F48FT12/CW/VHO, Sylvania) or Grolux lights (F20T12/GRO and F20T12/GRO/WS) as described. Copyright © 2009 American Society of Plant Biologists. All rights reserved.
Hypocotyl Length Measurements
We determined the hypocotyl lengths of seedlings grown under defined light conditions by scanning the seeding images and quantifying them using Image J software.

Protein Extraction and Assays, BVR Enzyme Assays, and Immunochemical Analysis
Soluble protein extracts for BVR enzyme assays and immunoblot analyses were obtained as described (Lagarias et al., 1997). Biochemical fractionation and subsequent protein and enzyme analyses were performed as previously described (Lagarias et al., 1999) and quantified using equations described by Kutty and Maines (1984). Protein concentrations of soluble extracts were determined utilizing the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as a standard. SDS-PAGE and immunoblot analyses were performed as previously described (Lagarias et al., 1997; Montgomery et al., 1999) using a 1:5,000 dilution of rabbit anti-BVR antibody (QED Bioscience).

Whole-Mount Immunohistochemical Analysis
Sterilized MERIS-pBVR1 seeds were treated with a RL pulse (approximately 75 μmol m⁻² s⁻¹) for 5 min prior to imbibition. Four-day-old MERIS-pBVR1 seedlings grown at 22°C under Wc illumination of 100 μmol m⁻² s⁻¹ were subjected to whole-mount in situ protein localization as previously described with limited modifications (Sauer et al., 2006). Seedlings were treated with a paraformaldehyde-based fixative solution for 45 min. Cell walls of fixed seedlings were digested with 3% Driselase (Sigma), followed by washing with 3 × 10 min with 1× phosphate buffered saline (PBS). Fixed and permeated seedlings were incubated with rabbit anti-BVR antibody at 1:12,500 dilution in 1× PBS overnight at 4°C. Following incubation with the primary antibody and washing, seedlings were incubated with goat anti-rabbit IgG (H+L) conjugated to HiLyte Plus 555 (AnaSpec) at 0.005 mg/mL, a 543-nm laser for excitation and a 560- to 615-nm band pass filter for emission. Each optical slice of a Z series from the secondary antibody was collected using a 543-nm laser for excitation imaging. DIC imaging was performed using the 543-nm laser. Fluorescence imaging was performed with limited modifications using the LSM FCS Zeiss 510 Meta AIM imaging software.

Pigment Analyses
N,N-dimethyloformamide chlorophyll extracts were obtained from excised cotyledons of 7-d-old seedlings at 4°C (Moran, 1982) and chlorophyll concentrations determined using extinction coefficients and equations described by Inskemp and Bloom (1985). Similarly, N,N-dimethyloformamide PChl extracts were obtained under green safe light using intact 7-d-old seedlings (Moran, 1982). Fluorescence spectroscopy was utilized to determine the PChl content in the extracts as previously described (Montgomery et al., 1999). Carotenoid content was determined essentially as described (Montgomery et al., 2001). After freezing in liquid nitrogen, 7-d-old seedlings were ground to a fine powder and homogenized in 80% acetone. Extracts were then incubated at −20°C for 1 h and centrifuged at high speed in a microcentrifuge for 5 min at 4°C. Carotenoids were assayed spectrophotometrically and carotenoid concentrations determined using equations from Lichtenthaler and Wellburn (1983). Anthocyanins were extracted from intact 4- or 5-d-old seedlings and concentrations determined spectrophotometrically as previously described (Montgomery et al., 1999).

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Spatial-Specific Phytochrome Responses

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