

Biliverdin Reductase-Induced Phytochrome Chromophore Deficiency in Transgenic Tobacco¹

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Targeted expression of mammalian biliverdin IX α reductase (BVR), an enzyme that metabolically inactivates linear tetrapyrrole precursors of the phytochrome chromophore, was used to examine the physiological functions of phytochromes in the qualitative short-day tobacco (*Nicotiana tabacum* cv Maryland Mammoth) plant. Comparative phenotypic and photobiological analyses of plastid- and cytosol-targeted BVR lines showed that multiple phytochrome-regulated processes, such as hypocotyl and internode elongation, anthocyanin synthesis, and photoperiodic regulation of flowering, were altered in all lines examined. The phytochrome-mediated processes of carotenoid and chlorophyll accumulation were strongly impaired in plastid-targeted lines, but were relatively unaffected in cytosol-targeted lines. Under certain growth conditions, plastid-targeted BVR expression was found to nearly abolish the qualitative inhibition of flowering by long-day photoperiods. The distinct phenotypes of the plastid-targeted BVR lines implicate a regulatory role for bilins in plastid development or, alternatively, reflect the consequence of altered tetrapyrrole metabolism in plastids due to bilin depletion.

Light plays an important role in growth and development throughout the life cycle of plants. It regulates a variety of processes, including seed germination, development of chloroplasts, shoot and root growth, and the induction of flowering (Kendrick and Kronenberg, 1994; Fankhauser and Chory, 1997; Jackson and Thomas, 1997). Three discrete classes of photoreceptors have been identified including UV-B, blue/UV-A, and red/far-red photomorphogenetic receptors. Phytochromes, the class of receptors primarily responsible for light absorption in the red/far red region of the light spectrum, are the most widely studied of these photoreceptors (Sage, 1992; Furuya, 1993; Quail et al., 1995; Neff et al., 2000). A family of phytochrome genes has been identified in all angiosperms examined. These include five genes (i.e. phyA-phyE) that exist in *Arabidopsis* (Clack et al., 1994), perhaps up to six genes in tomato (Alba et al., 2000) and at least three in tobacco (Adam et al., 1997). Phytochrome-deficient mutants have been widely used to determine the discrete photoregulatory activities of individual phytochromes and their roles in light-mediated plant growth and development (Koornneef and Kendrick, 1994). Extensive studies

have centered on the model species *Arabidopsis* for which multiple specific phytochrome mutants are available (for review, see Whitelam and Devlin, 1997). These studies have revealed that each phytochrome possesses distinct, and in some cases, overlapping and/or synergistic/antagonistic physiological roles (Casal, 2000).

Unlike the diversity identified for apoproteins, plants use a single linear tetrapyrrole chromophore, phytychromobilin, for the production of photoactive phytochrome (Terry et al., 1993). It is well established that phytychromobilin is synthesized from heme via the intermediacy of biliverdin IX α (BV) (Terry, 1997). Mutants in both committed steps of the chromophore biosynthesis pathway have been identified in a number of plants species (Terry, 1997). These plants exhibit photomorphogenetic defects throughout their life cycle with phenotypes consistent with deficiencies in multiple phytochrome species. Such mutants have been used to probe the global photosensory functions of phytochromes in plant development (for review, see Koornneef and Kendrick, 1994; Smith, 1995; Terry, 1997). It is interesting that all chromophore-deficient mutants retain some phytochrome responsiveness, primarily as mature plants. This observation indicates the leakiness of these mutants, suggesting the presence of alternative pathways for the synthesis of the phytochrome chromophore that likely reflect the existence of multiple, redundant genes encoding key biosynthetic enzymes.

We have previously demonstrated that constitutive expression of the mammalian enzyme biliverdin reductase (BVR) in transgenic *Arabidopsis* yields

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plants with altered photomorphogenetic development throughout their life cycle (Lagarias et al., 1997; Montgomery et al., 1999). These analyses revealed BVR-dependent phenotypes to be light dependent, pleiotropic, and dependent upon the subcellular targeting of BVR, results consistent with a loss of multiple phytochrome species. As such, these studies establish the feasibility of using BVR expression to probe the physiological roles of phytochromes in vivo. Using a similar approach, experiments described here explore the global functional roles of phytochromes in the qualitative short-day plant (SDP) tobacco (*Nicotiana tabacum* cv Maryland Mammoth) for which no phytochrome chromophore-deficient mutants have been reported. The ability to abrogate multiple phytochrome-regulated responses including the photoperiod-dependent inhibition of flowering under long days, conditions that are not usually inductive for flowering in this species, is revealed in this study.

RESULTS

Expression of BVR in Transgenic cv Maryland Mammoth Plants

Twelve homozygous, transgenic BVR lines in cv Maryland Mammoth were obtained: six each of plastid (M-pBVR) and cytosolic (M-cBVR) BVR-expressing lines. Relative BVR specific activities of total soluble protein extracts of 10-d-old light-grown seedlings were determined for all lines. As shown in Figure 1, BVR protein levels and enzyme activity were lower in M-pBVR seedlings than in M-cBVR seedlings. The doublet observed on the immunoblot is a consequence of altered processing of the expressed enzyme as previously discussed (Lagarias et al., 1997; Montgomery et al., 1999). The range of BVR expres-

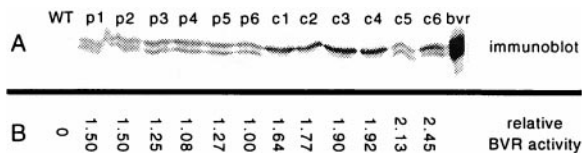


Figure 1. BVR protein and enzyme levels in soluble protein extracts from WT and transgenic M-BVR seedlings. A, Immunoblot analysis. Transgenic M-BVR (denoted p1–p6 and c1–c6) and wild-type (MMWT) seedlings were grown at 25°C on phytigel medium containing 1% (w/v) Suc for 10 d under Wc illumination of $130 \mu\text{mol m}^{-2} \text{s}^{-1}$. Whole seedling soluble protein extracts (20 μg) were used for BVR immunoblot analysis with anti-BVR antibody as described in “Materials and Methods.” A mature BVR standard (*M*, approximately 33 kDa) is shown in the far right lane. B, Relative BVR Activity. Seedlings were grown on phytigel medium containing 1% (w/v) Suc for 16 d at 25°C under continuous Gro-lux/Gro-lux Wide Spectrum illumination of $130 \mu\text{mol m}^{-2} \text{s}^{-1}$. Whole seedling soluble protein extracts were used for BVR assays as described in “Materials and Methods.” Values were calculated relative to the specific activity for M-pBVR6, which was determined to be $2.86 \times 10^{-3} \text{ IU mg}^{-1}$ protein.

sion was not large with the highest expressing M-pBVR lines, M-pBVR1 and M-pBVR2, being 1.5-fold greater than the lowest expressing line, M-pBVR6. In general, the cytosolic lines exhibited slightly greater BVR activity than the plastid-targeted lines with the highest expressing M-cBVR line, M-cBVR6, displaying a 2.5-fold greater expression than M-pBVR6. That BVR expression reduced holophytochrome levels through inducing chromophore deficiency and not by destabilization of apophytochromes was confirmed by immunoblot analyses of phyA and phyB protein levels, which showed no significant differences between BVR transgenic plants and wild type (WT, data not shown).

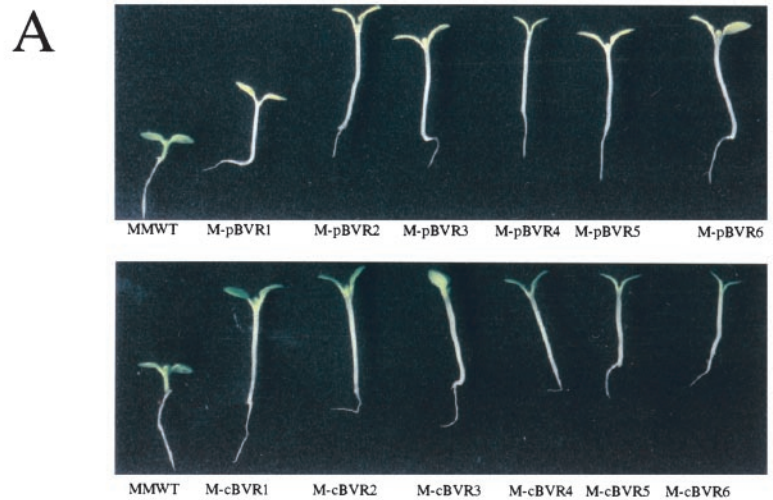
Light-Dependent Hypocotyl and Internode Lengths Are Altered in BVR Transgenic Plants

It is well established that phytochrome chromophore-deficient plants exhibit altered light-dependent growth and development throughout their life cycle. Consistent with these observations, M-pBVR and M-cBVR transgenic plants displayed aberrant phenotypes both as seedlings and as mature plants (Fig. 2). Specifically, seedlings displayed elongated hypocotyls under all fluence rates of continuous white (Wc), red (Rc), and far red (FRc) illumination examined (Figs. 2A and 3). This response was qualitatively the same for both plastid- and cytosol-targeted lines (compare Fig. 3, A, C, and E with B, D, and F) as was observed for BVR-expressing transgenic Arabidopsis (Montgomery et al., 1999). When grown in complete darkness, BVR transgenic seedlings on average had slightly longer hypocotyls than WT seedlings (Table I). This effect was more pronounced in the presence of Suc. As mature plants, M-BVR lines exhibited increased plant height with heights of all M-pBVR lines being greater than M-cBVR lines, which in turn were greater than those of WT controls (Fig. 2, B and C). This difference in height was found to be due primarily to an increase in mean internode lengths rather than an increase in total internode number (Fig. 4). This effect was more severe for M-pBVR lines than for M-cBVR lines. Leaf shape and leaf area/length ratios were also altered in M-pBVR lines that exhibited a marked decrease in area/length ratios whereas M-cBVR lines were comparable with WT plants (Fig. 2B and data not shown).

Anthocyanin Levels Are Reduced by BVR Expression

Numerous studies have shown that phytochrome plays a key role in the regulation of anthocyanin biosynthesis in a variety of plant species (Lange et al., 1970; Mancinelli et al., 1991; Kerckhoffs and Kendrick, 1997). In this regard, results from experiments with BVR-expressing Arabidopsis plants definitively established that BVR expression disrupted Suc-stimulated anthocyanin accumulation (Montgomery

Figure 2. Light-grown wild-type and BVR transgenic plants. A, cv Maryland Mammoth WT seedlings and transgenic M-BVR lines were grown at 25°C on phytigel medium containing 1% (w/v) Suc for 10 d under Wc illumination of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Mature WT and representative transgenic M-cBVR and M-pBVR plants were grown under white illumination with a 16-h light/8-h dark photoperiod of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 22°C for 4 weeks (B) and 14 weeks (C).



et al., 1999). Figure 5 shows that anthocyanin accumulation was adversely affected by BVR expression in all M-BVR transgenic seedlings tested with the

effect again being greater for M-pBVR expressing lines than for M-cBVR transgenic lines. In contrast with *Arabidopsis*, Suc did not have a significant in-

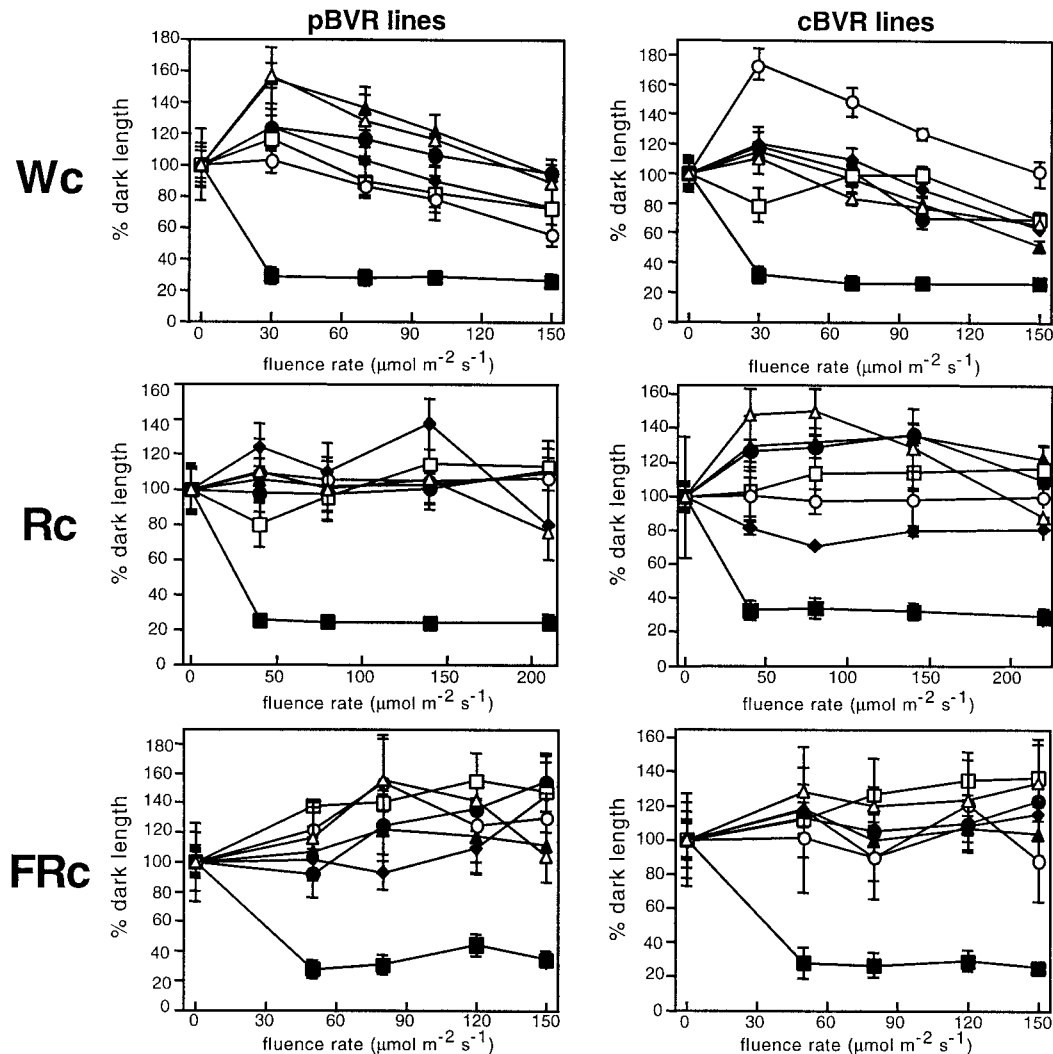


Figure 3. Hypocotyl lengths of wild-type and transgenic BVR seedlings under Wc, Rc, and FRC illumination. Plastid-targeted BVR lines (left) and cytosolic BVR lines (right) are compared with cv Maryland Mammoth WT seedlings grown at 25°C on phytagel medium containing 1% (w/v) Suc for 10 d under Wc, Rc, and FRC illumination of various fluence rates. Data points represent mean (\pm SD) of hypocotyl lengths measured for 10 to 25 seedlings and correspond to MMWT (■), p/c-BVR1 (●), p/c-BVR2 (▲), p/c-BVR3 (◆), p/c-BVR4 (□), p/c-BVR5 (○), and p/c-BVR6 (△) lines.

ductive effect on anthocyanin accumulation in WT or BVR transgenic cv Maryland Mammoth tobacco plants (data not shown).

Chlorophyll and Protochlorophyll Accumulation Are Altered by BVR Expression

Previous findings have established that chromophore-deficient plants are intolerant of elevated light fluence rates (for review, see Terry, 1997). Analysis of the fluence rate-dependent chlorophyll content of M-BVR transgenic seedlings showed that these plants exhibited fluence rate-dependent reductions of total chlorophyll levels, as well as fluence rate-dependent increases in the chlorophyll *a/b* (Chl *a/b*) ratios only when BVR was targeted to plastids (Fig. 6). Cytosolic expressing lines also displayed lower

levels of chlorophyll, but this effect was not strictly fluence rate-dependent with the notable exception of M-cBVR5. Furthermore, with the exception of the atypical M-cBVR5 line (Fig. 6D, ○), Chl *a/b* ratios were not affected by cytosolic BVR expression. In mature plants, a significant difference in chlorophyll accumulation was observed only in M-pBVR lines (Fig. 6, E and F).

Prior studies have reported a correlation between chromophore-deficiency in plastids and reduced protochlorophyll levels (Terry, 1997; Montgomery et al., 1999). Our laboratory established that in transgenic BVR Arabidopsis plants, protochlorophyll levels were affected only when BVR was expressed in plastids (Montgomery et al., 1999). When examining the effect of BVR expression on protochlorophyll content in M-BVR seedlings, we found that all lines exhibited

Table 1. Mean hypocotyl lengths for dark-grown wild-type and BVR transgenic plants on Suc-enriched or Suc-free media

Seedlings were grown for 15 d at 25°C in continuous darkness. Hypocotyl length values are means (\pm SD) measured for 10 to 25 seedlings. The average hypocotyl lengths of BVR lines grown in the absence and presence of 1% (w/v) Suc are indicated to the right of the individual values.

Plant Line	Hypocotyl Length	
	- Suc	+Suc
	<i>mm</i>	
MMWT	22.5 (\pm 2.6)	23.1 (\pm 3.8) ^b
M-pBVR1	24.6 (\pm 2.9) ^a	26.4 (\pm 3.6) ^b
M-pBVR2	25.6 (\pm 1.7) ^a	29.9 (\pm 3.1) ^b
M-pBVR3	27.0 (\pm 1.8) ^a	27.1 (\pm 3.3) ^b
M-pBVR4	25.5 (\pm 2.1) ^a	28.9 (\pm 4.0) ^b
M-pBVR5	25.0 (\pm 3.3) ^a	26.3 (\pm 2.5) ^b
M-pBVR6	27.6 (\pm 2.1) ^a	32.8 (\pm 1.4) ^b
M-cBVR1	27.3 (\pm 1.2) ^c	27.0 (\pm 0.2) ^d
M-cBVR2	26.4 (\pm 1.7) ^c	29.6 (\pm 2.7) ^d
M-cBVR3	21.2 (\pm 3.7) ^c	25.0 (\pm 3.3) ^d
M-cBVR4	26.9 (\pm 2.4) ^c	28.6 (\pm 2.3) ^d
M-cBVR5	28.9 (\pm 1.5) ^c	29.3 (\pm 1.7) ^d
M-cBVR6	24.6 (\pm 2.3) ^c	27.8 (\pm 2.4) ^d

^a Average for six lines = 25.9 (\pm 2.3). ^b Average for six lines = 28.6 (\pm 3.0). ^c Average for six lines = 25.9 (\pm 2.1). ^d Average for six lines = 27.9 (\pm 2.1).

reduced protochlorophyll levels. However, cBVR expression affected protochlorophyll accumulation to a lesser degree than pBVR expression (Fig. 7).

Carotenoid Accumulation Is Disrupted by Plastid-Targeted Expression of BVR

That phytochromes play a role in carotenoid accumulation in angiosperms particularly during the light-dependent transition from etioplast to chloroplast has long been known (Frosh and Mohr, 1980; Rau, 1983). In *Arabidopsis*, phyA and light stable phytochromes other than phyB appear to be directly involved in the regulation of specific genes in the carotenoid biosynthetic pathway (von Lintig et al., 1997). To assess the potential role of tobacco phyto-

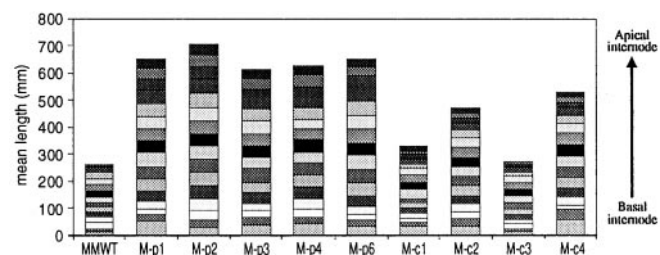


Figure 4. Mean internode lengths of wild-type and transgenic BVR plants. Transgenic M-pBVR lines, M-cBVR lines, and cv Maryland Mammoth WT plants were grown in controlled environment growth chambers as described in "Materials and Methods." Individual bars represent the mean of 10 independent measurements of the length of each internode position.

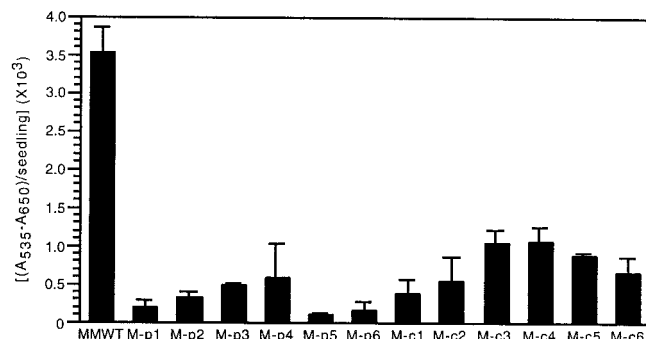


Figure 5. Anthocyanin content of wild-type and transgenic BVR seedlings. Transgenic M-pBVR lines, M-cBVR lines, and cv Maryland Mammoth WT seedlings were grown at 25°C on phytagel medium containing 1% (w/v) Suc for 10 d under Wc illumination of 100 μ mol $m^{-2} s^{-1}$. Bars represent the mean (\pm SD) of three independent measurements.

chromes in carotenoid accumulation, we determined the carotenoid content of mature, light-grown M-BVR transgenic plants. Similar to chlorophyll accumulation, a significant reduction in the total carotenoid content was only observed in transgenic plants expressing BVR in plastids (Fig. 8).

Photoperiodic Responses Are Altered in BVR-Expressing cv Maryland Mammoth Plants

It is well recognized that phytochrome mediates floral induction through the perception of photoperiod (Thomas and Vince-Prue, 1997). cv Maryland Mammoth, a qualitative SDP, is one of the most extensively studied of the photoperiod-dependent plants. The inhibitory effect of phytochrome photoactivation during inductive long nights has been well documented. We therefore investigated the effect of photoperiod on the flowering behavior of cv Maryland Mammoth M-BVR lines grown in controlled environment growth chambers at 22°C under defined photoperiods (Fig. 9A). These investigations revealed that BVR expression had no significant effect on days to flowering under inductive short, 8- to 10-h days. When the photoperiod was increased to greater than 13 h, a significant difference was observed between WT and transgenic BVR lines. Under long day conditions, M-pBVR lines again showed a more severe effect than M-cBVR expressing lines with flowering times for all M-BVR lines occurring earlier than WT plants. Two M-pBVR lines in particular, M-pBVR4 and M-pBVR6, flowered at nearly the same time under long days (16 h) as they did under inductive short-day (10.5 h) conditions. M-cBVR lines also exhibited early flowering as compared with WT plants, but all M-cBVR lines showed a photoperiod-induced delay of flowering under long days. By comparison with M-BVR plants, flowering times for WT plants were much more significantly increased when grown under long days.

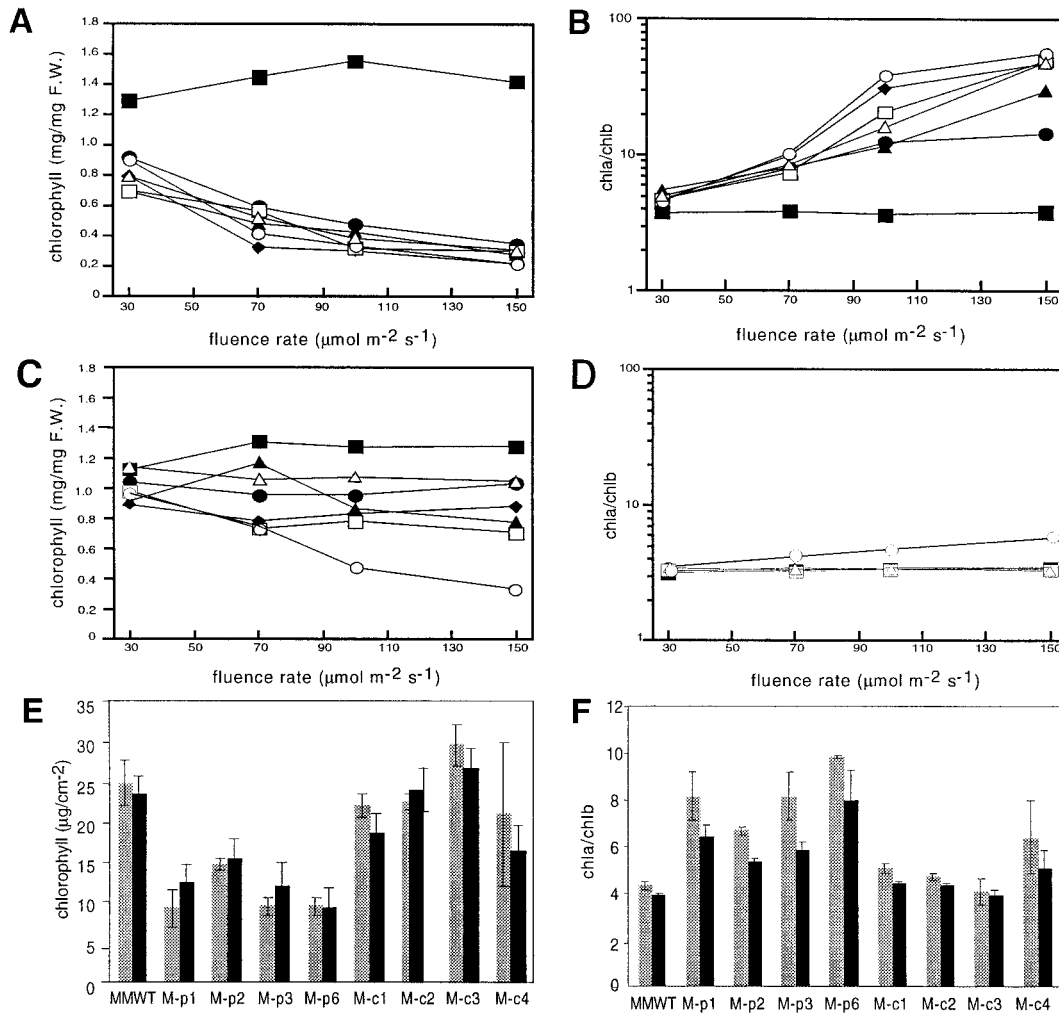


Figure 6. Fluence rate-dependent chlorophyll accumulation of wild-type and transgenic BVR seedlings and adult plants. Transgenic M-pBVR lines (A and B) and M-cBVR lines (C and D) were compared with cv Maryland Mammoth WT seedlings grown at 25°C on phytagel medium containing 1% (w/v) Suc for 10 d under Wc illumination of various fluence rates. For mature plants (E and F), chlorophyll measurements were obtained from leaves 5 and 9 of plants grown at 22°C for 40 d under white illumination with a 16-h photoperiod of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Data points represent the mean obtained from three independent measurements. A, C, and E depict total chlorophyll; B, D, and F depict Chl a/b ratios. The symbol designations are the same as those in Figure 3.

Although cv Maryland Mammoth is regarded as the “prototypical” qualitative SDP, earlier studies reported flowering under long days at reduced temperatures (Roberts, 1939). At temperatures lower than 25°C, cv Maryland Mammoth is a quantitative SDP with long-day photoperiods delaying rather than preventing floral induction. When grown under long days in the greenhouse at average temperatures of 28°C, cv Maryland Mammoth WT plants showed no evidence of flowering after 253 d, whereas all of the M-pBVR plants had flowered by 120 d (data not shown). That plastid-targeted BVR expression releases the qualitative inhibition of flowering by long-day photoperiods was also supported by flowering experiments at 28°C in long-day growth chambers (Fig. 9B). Flowering of the M-cBVR lines at 28°C under long days was considerably delayed from that

of the M-pBVR lines, but to obtain seed the M-cBVR plants were transferred to a short-day growth chamber. A quantitative comparison of the flowering behavior of M-cBVR and M-pBVR lines grown at 28°C under long days remains to be determined.

DISCUSSION

Transgenic cv Maryland Mammoth Plants Expressing Mammalian BVR Are Deficient in Multiple Phytochromes

Transgenic cv Maryland Mammoth plants expressing BVR display phenotypes consistent with phytochrome chromophore deficiency throughout their life cycle independent of subcellular localization of the enzyme to plastids or the cytosol. Phenotypes ob-

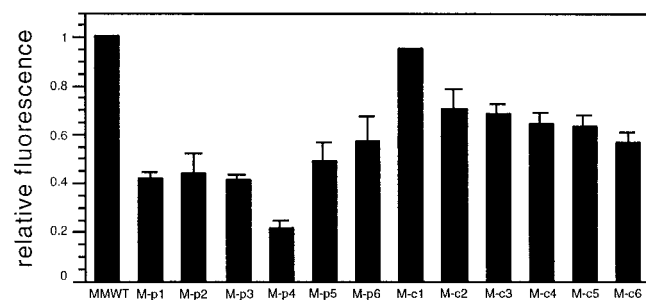


Figure 7. Protochlorophyll accumulation in wild-type and transgenic BVR seedling extracts. Transgenic M-pBVR lines and M-cBVR lines were compared with cv Maryland Mammoth WT seedlings grown at 25°C on phytigel medium containing 1% (w/v) Suc for 15 d in darkness. Bars indicate the relative protochlorophyll fluorescence values calculated by normalizing to the fluorescence value of WT seedling extracts (see "Materials and Methods" for details). Values represent the mean (\pm SD) of three independent measurements.

served were very similar to those of known chromophore-deficient mutants, e.g. elongated hypocotyls under Wc, Rc and FRc illumination, increased internode lengths, reduced protochlorophyll and chlorophyll content, reduced levels of anthocyanins and carotenoids, and altered photoperiod-dependent flowering (Terry, 1997). Many of these phenotypes are fully consistent with the loss of phyA and phyB photoregulatory activities (Whitelam and Devlin, 1997). Furthermore, the elongated internodes of BVR transgenic plants under Wc illumination are consistent with the loss of phyE photoregulatory activity (Devlin et al., 1998). Studies in *Arabidopsis* showed that such an elongated internode phenotype was only observed in a background lacking phyA and phyB, providing further support for the BVR-dependent loss of multiple phytochrome activities. In contrast to phytochrome chromophore-deficient mutants, which clearly recover phytochrome-mediated regulation of stem elongation during maturation (Weller et al., 1996; Terry, 1997), the increased internode elongation in the BVR transgenics persists in the mature plant (Fig. 4). The recovery in chromophore-deficient mutants has been attributed to the presence of additional phytochrome chromophore biosynthetic genes (Davis et al., 1999), whereas the constitutive expression of BVR in the transgenics is expected to reduce holophytochrome levels in mature plants as well as seedlings.

With the exception of hypocotyl elongation, which was qualitatively the same for both M-pBVR and M-cBVR lines, the phenotypes observed were always more severe for plastid-targeted BVR lines. This effect did not appear to be due to differences in BVR expression since quantitative levels of enzyme activity were higher for M-cBVR lines than for M-pBVR lines (Fig. 1). This suggests that either plastid-targeted BVR expression is more effective at reducing holophytochrome levels and that various photomorphogenetic responses have different phytochrome

"thresholds" or that a plastid deficiency of bilins is responsible for the more exaggerated phytochrome-deficient phenotypes.

BVR Expression Disrupts Accumulation of Chlorophylls and Carotenoids in Transgenic Plants

The accumulation of protochlorophylls, chlorophylls, and carotenoids were all affected by BVR expression. Chlorophyll levels of BVR transgenic plants were always less than WT controls even at low fluences, although the effect was more pronounced for the plastid-targeted lines (Fig. 6). As was seen for *Arabidopsis*, the reduction of chlorophyll levels and the increased Chl *a/b* ratios were fluence rate-dependent only when BVR was targeted to plastids (Fig. 6, A and B). The phenotypes observed, however, were not strictly correlated with the level of BVR activity. This suggests that factors other than the level of BVR expression in the plastid may play a role in regulating chlorophyll content. One possibility is that the initial rate of BVR synthesis during early seedling (and plastid) development may vary between lines due to positional effects of BVR transgene incorporation into the genome.

In contrast with that observed for BVR transgenic *Arabidopsis* plants (Montgomery et al., 1999), protochlorophyll accumulation was reduced independent of the subcellular localization of BVR. Nevertheless, the reduced protochlorophyll phenotypes were on average more severe when BVR was targeted to the plastid. The modest reduction of protochlorophyll levels observed for transgenic M-cBVR plants may reflect higher levels of BVR accumulation in transgenic tobacco by comparison with transgenic *Arabidopsis* plants. This suggests that the reduced levels of protochlorophyll may result from the combination of reduced global levels of phytochrome, as well as reduced levels of plastid-localized bilins.

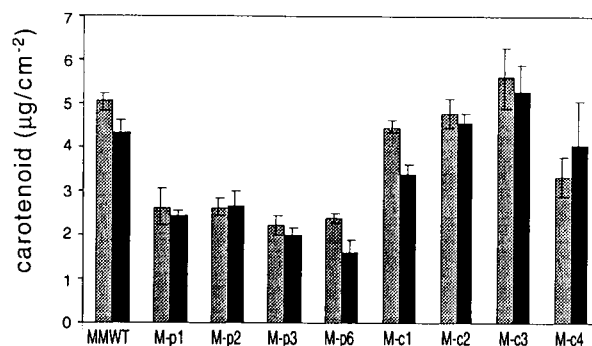


Figure 8. Carotenoid content of wild-type and transgenic BVR plants. Transgenic M-pBVR lines and M-cBVR lines were compared with cv Maryland Mammoth WT seedlings grown at 22°C for 40 d under white illumination with a 16-h photoperiod of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Bars represent the mean (\pm SE) of three independent carotenoid measurements (see "Materials and Methods" for details) from leaf 5 (gray bars) and leaf 9 (black bars).

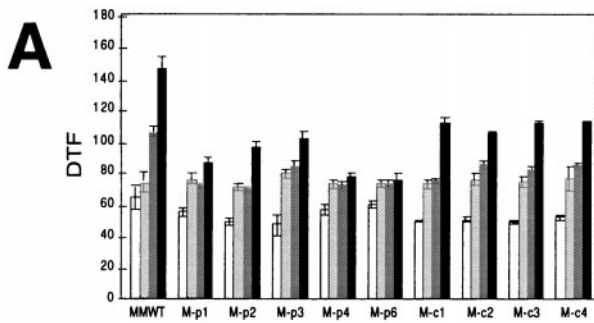


Figure 9. Flowering time analyses of wild-type *cv* Maryland Mammoth and BVR transgenic plants under various photoperiods and temperatures. A, WT *cv* Maryland Mammoth plants and representative M-pBVR and M-cBVR transgenic lines were grown on a soil mixture in controlled environment growth chambers at 22°C under white illumination of 8-h light/16-h dark (white bars), 10.5-h light/13.5-h dark (light gray bars), 13-h light/11-h dark (dark gray bars), and 16-h light/8-h dark (black bars) photoperiods. Data represent the mean of 10 plants (\pm SE). B, *cv* Maryland Mammoth WT (shown in front) and transgenic M-pBVR2 plants (in rear) were grown on soil under a long-day photoperiod of 16 h using $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light in a controlled environment growth chamber at 28°C for 18 weeks.

In response to the observed reduced levels of chlorophyll in transgenic M-BVR plants and owing to a recognized correlation between the regulation of

chlorophyll and carotenoid levels in angiosperms (Hartel and Grimm, 1998), we examined carotenoid levels in mature M-BVR plants. Our results indicate that carotenoid levels are significantly reduced only in plants expressing BVR in plastids. As the site of carotenoid biosynthesis is localized to plastids (for review, see Rau, 1983) and levels of carotenoids are not affected in lines expressing BVR in the cytosol, we propose that carotenoid levels in the plastid may be regulated by bilin metabolism within the plastid compartment.

BVR Expression Alters the Photoperiodic Flowering Response in *cv* Maryland Mammoth Plants

The flowering behavior of *cv* Maryland Mammoth is one of the most well characterized for SDPs. It has been proposed that *phyB* is responsible for the inhibition of flowering under noninductive conditions in WT *Arabidopsis* and tobacco plants and, based on transgenic experiments, that *phyC* interacts with the Mammoth gene to inhibit flowering in long days (Halliday et al., 1997; Jackson and Thomas, 1997). This suggests that a lack of phytochromes under long-day photoperiods should release the inhibition of flowering as was observed for transgenic M-BVR plants. In growth chambers maintained at 22°C, *cv* Maryland Mammoth WT plants displayed a strong delay of flowering under long days, whereas flowering of both M-pBVR and M-cBVR lines was less sensitive to extended photoperiods. Moreover, an almost complete loss of photoperiod sensitivity was observed for specific plastid-targeted BVR transgenic lines. Remarkably, under daylength-extended greenhouse conditions in which WT *cv* Maryland Mammoth showed no evidence of flowering after 8 months, we were able to observe flowering for plastid-targeted BVR transgenic plants within 4 months. Flowering of the M-cBVR lines was more strongly delayed by long-day photoperiods (than M-pBVR lines) in the greenhouse, and showed no evidence of flowering long after the M-pBVR lines had flowered. The factors contributing to the differences in flowering behavior of WT and BVR transgenic plants in the greenhouse and growth chamber (e.g. temperature, light fluence rate, nutrient/watering regimes, etc.) remain to be addressed in a future investigation. From earlier studies, it is clear that temperature plays an important role (Roberts, 1939).

Are Bilins Regulatory Molecules in the Plastid Compartment?

With the exception of the effect of BVR expression on hypocotyl elongation which was qualitatively the same for M-pBVR and M-cBVR transgenic lines, all of the measured phenotypes in *cv* Maryland Mammoth tobacco plants were more severely affected when BVR was targeted to plastids. The magnitude of these

differences can be divided into three classes. The first consists of small, or negligible, differences that were observed for hypocotyl elongation and anthocyanin accumulation. The second class includes internode elongation and the photoperiodic induction of flowering that showed more significant differences between pBVR and cBVR lines. The final class contains responses such as leaf area/length ratios and chlorophyll and carotenoid accumulation that were solely impaired in plastid-targeted lines, whereas cBVR lines showed virtually no effect of BVR expression. Several alternative hypotheses can account for these observed differences. The first relates to differential phytochrome "thresholds" for various phytochrome-dependent phenotypes. As plastids are the recognized site of chromophore biosynthesis (Terry et al., 1993), the premise that pBVR expression has a greater effect on the accumulation of linear tetrapyrroles may support the hypothesis that at least some of the differences in phenotypes may be directly attributed to different holophytochrome levels in pBVR versus cBVR lines.

A second hypothesis focuses on the fact that some phenotypes, such as chlorophyll and carotenoid accumulation, are only affected in pBVR lines. The observation that cBVR expression has no effect on these phenotypes may be explained by indirect effects on plastid development caused by manipulation of the tetrapyrrole pathway by BVR expression. In this case, the expression of BVR may have the effect of "pulling" tetrapyrroles through the pathway and thereby reducing the pool of chlorophyll precursors. The correlation between chlorophyll and carotenoid accumulation would then account for the simultaneous loss of carotenoids in BVR transgenic plants.

Alternatively, bilins, or a plastid-localized phytochrome, may have a direct signaling effect which influences plastid development. In this regard, it is presumed that in addition to exhibiting a general phytochrome deficiency, chromophore-deficient plants exhibit reduced levels of bilins in plastids. We suggest that at least some aspects of the reported phenotypes for chromophore-deficient mutants are a direct result of these reduced levels of bilins in the plastid compartment and not entirely due to secondary, or indirect, effects of lesions in the chromophore biosynthetic pathway, which would lead to phenomena such as heme feedback inhibition of tetrapyrrole biosynthesis (Terry and Kendrick, 1999). In this regard, previous studies with known chromophore-deficient mutants, with the exception of pea and tomato mutants, have established the ability of exogenously applied BV to "rescue" some aspects of the phytochrome-deficient phenotypes (Parks and Quail, 1991; Kraepiel et al., 1994; Lamparter et al., 1997; Esch and Lamparter, 1998). BV feeding should primarily increase the levels of linear tetrapyrroles in plastids, without necessarily reducing levels of heme, which would be required to overcome the feedback inhibition of tetrapyrrole bio-

synthesis. Additionally, we have been able to partially restore the defects in protochlorophyll accumulation in dark-grown chromophore-deficient plants by feeding plants a phytochrome chromophore (Montgomery and Lagarias, unpublished data), suggesting that bilin levels are at least partially responsible for regulating the levels of some pigments in plastids.

In summary, the present study has established a definitive perturbation of specific phenotypes only when BVR is expressed in the plastid compartment. These include altered leaf morphology, an intolerance to elevated light fluences, a reduction in carotenoid levels and a severe loss of photoperiodic sensitivity. In conjunction with previous results from BVR-expressing Arabidopsis plants (Lagarias et al., 1997; Montgomery et al., 1999), we believe that these findings suggest that bilins and/or phytochrome molecules have a prominent regulatory role in plastids distinct from their recognized role in the production of the phytochrome chromophore for transport to the cytosol and assembly with apophytochromes.

MATERIALS AND METHODS

Plant Material

Transgenic, plastid-targeted BVR (35S::pBVR) and cytosolic BVR (35S::cBVR) lines in tobacco (*Nicotiana tabacum* cv Maryland Mammoth) were isolated using an *Agrobacterium tumefaciens*-mediated leaf disc transformation protocol (Rogers et al., 1988). For these experiments, the previously described 35S::pBVR and 35S::cBVR constructs (Lagarias et al., 1997; Montgomery et al., 1999) in the binary transformation vector pBIB-KAN (Becker, 1990) were used. Six homozygous lines for each construct, designated M-pBVR1 to M-pBVR6 for plastid-targeted BVR lines and M-cBVR1 to M-cBVR6 for cytosolic BVR lines were used in the experiments described here.

Plant Growth Conditions

For seedling phenotype analyses, tobacco seeds were surface sterilized for 15 min with 35% (v/v) ethanol followed by 15 min with 35% (v/v) commercial bleach containing 0.025% (v/v) SDS and rinsed four times with sterile MilliQ (Millipore, Bedford, MA) water. Sterilized seeds were planted in 100 × 25 mm Petri dishes on media containing Murashige and Skoog salts (Gibco-BRL, Gaithersburg, MD), 0% or 1% (w/v) Suc, 0.3% (w/v) phytagel, and adjusted to pH 6.7 with NaOH. Imbibing seeds were cold stratified at 4°C in darkness for 4 d prior to being transferred to appropriate light conditions. Monochromatic light sources used were those previously described (Lagarias et al., 1997; Montgomery et al., 1999).

For experiments with mature plants grown in growth chambers, sterilized seeds were germinated on media containing Murashige and Skoog salts, 2% (w/v) Suc, 0.7% (w/v) agar, and adjusted to pH 5.9. Seeds were germinated at 25°C under white illumination with a 16-h photoperiod of approximately 65 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from 70-W type 84

fluorescent tubes (Philips, Eindhoven, The Netherlands). Seedlings were then transferred to pots containing a 2:1 mixture of Levington M2 bedding compost and sand, placed under white illumination with a 16-h photoperiod of $85 \mu\text{mol m}^{-2} \text{s}^{-1}$, and acclimatized for 14 d at 25°C with 70% humidity. Plants were then transferred to growth chambers with varying photoperiods at 22°C at a fluence rate of $350 \mu\text{mol m}^{-2} \text{s}^{-1}$. White light in photoperiod-controlled growth chambers was provided by Philips type 84 fluorescent tubes (Philips, Eindhoven, The Netherlands) and incandescent lights.

Long-day photoperiod greenhouse flowering experiments and comparative controlled environment growth chamber experiments were performed using seeds germinated on pots containing a 1:1 mixture of Fison's Sunshine Mix no. 1 (Bellevue, MA) and vermiculite. Plants in the greenhouse were grown under ambient sunlight conditions at an average temperature of 28°C with a daylength extended 16-h photoperiod. Daylength extension was provided by 1,000-W metal halide lamps. Plants in growth chambers for results shown in Figure 9 were grown at 28°C under a long day photoperiod (16-h light/8-h dark) using $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light provided by cool-white fluorescent tubes (F48FT12/CW/VHO, Sylvania, Danvers, MA).

BVR Enzyme and Immunochemical Analyses

Total soluble protein extracts for enzyme assays and immunoblot analyses were performed as previously described (Montgomery et al., 1999). BVR-specific enzyme activity of soluble protein fractions was measured spectrophotometrically as previously detailed (Lagarias et al., 1997) using the calculations of Kutty and Maines (1984). Protein concentrations were determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as a standard. SDS-PAGE and immunoblot analyses were performed as described previously (Lagarias et al., 1997; Montgomery et al., 1999).

Hypocotyl and Internode Length Measurements

Hypocotyl lengths of seedlings grown on Petri plates under defined light conditions were measured by scanning the plant images and quantification performed using MacBAS 2.0 software (Fuji Medical Systems, Stamford, CT). Internode lengths from the basal leaf to the plant apex were measured with a ruler.

Pigment Analyses

Chlorophyll extractions for seedling experiments were performed using *N,N*-dimethylformamide extracts (Moran, 1982) of 10-d-old seedlings. Chlorophyll concentrations were calculated using extinction coefficients and equations described (Inskeep and Bloom, 1985). For mature plants, relative chlorophyll content was measured for intact leaves using an SPAD-502 chlorophyll meter (Minolta UK, Milton Keynes, UK). Reported values represent an average of readings for 10 independent positions per leaf. Measure-

ments obtained were converted to *in vivo* chlorophyll concentrations using a calibration curve from 115 leaf tissue samples. Chlorophyll *a*, chlorophyll *b*, and carotenoid measurements were obtained for mature leaf tissue samples. Samples were frozen in liquid nitrogen and ground to a fine powder. Samples were homogenized in 80% (v/v) acetone and incubated at -20°C for 1 h. Following centrifugation at $11,000g$ for 5 min at 4°C , pigment concentrations in the supernatant were determined spectrophotometrically using equations for Chl *a* and Chl *b* from Hill et al. (1985) and for carotenoids from Lichtenthaler and Wellburn (1983).

Protochlorophyll extractions were performed under green safe light by immersion of 15-d-old seedlings in *N,N*-dimethylformamide (Moran, 1982). Protochlorophyll content in the extracts was determined from fluorescence emission curves (560–750 nm) with excitation at 438 nm. Relative fluorescence values were determined by integration of the emission spectra for each sample. Anthocyanins were extracted from intact 10-d-old seedlings using 1% (v/v) HCl in methanol as described (Feinbaum and Ausubel, 1988). Pigments were extracted overnight with agitation at 20°C (Rabino and Mancinelli, 1986). A chloroform-water partitioning was performed (Kerckhoffs et al., 1997) and anthocyanin content in the aqueous phase determined by measuring the A_{535} minus the A_{650} ($A_{535} - A_{650}$).

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