elongated mesocotyl1, a Phytochrome-Deficient Mutant of Maize[1]

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To begin the functional dissection of light signal transduction pathways of maize (*Zea mays*), we have identified and characterized the light-sensing mutant *elm1* (*elongated mesocotyl1*). Seedlings homozygous for *elm1* are pale green, show pronounced elongation of the mesocotyl, and fail to de-etiolate under red or far-red light. Etiolated *elm1* mutants contain no spectrally active phytochrome and do not deplete levels of phytochrome A after red-light treatment. High-performance liquid chromatography analyses show that *elm1* mutants are unable to convert biliverdin IXα to 3*Z*-phytochromobilin, preventing synthesis of the phytochrome chromophore. Despite the impairment of the phytochrome photoreceptors, *elm1* mutants can be grown to maturity in the field. Mature plants retain aspects of the seedling phenotype and flower earlier than wild-type plants under long days. Thus, the *elm1* mutant of maize provides the first direct evidence for phytochrome-mediated modulation of flowering time in this agronomically important species.

The phytochrome family of photoreceptors mediates many of the responses that plants display to changes in their light environment (Smith, 2000). The basis of phytochrome action is a reversible photoconversion between a red light (R)-absorbing form (Pr) and a far-red light (FR)-absorbing form (Pfr; Quail, 2002). In lower plants, the family is represented by a small number of nuclear genes (Schneider-Poetsch et al., 1998). However, gene duplication and evolutionary divergence have resulted in the formation of functionally diverse multigene families in flowering plants. In Arabidopsis, the phytochrome family consists of five genes: *PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE* (Clack et al., 1994), whereas the grasses have three phytochromes: *PhyA*, *PhyB*, and *PhyC* (Mathews and Sharrock, 1996). In maize (*Zea mays*), an ancestral genomic duplication has increased the total family size to at least six: *PhyA1*, *PhyA2*, *PhyB1*, *PhyB2*, *PhyC1*, *PhyC2*, and possibly *PhyC3* (Christensen and Quail, 1989; Childs et al., 1997; Basu et al., 2000). Although loss-of-function *phy* mutants have been characterized in a broad range of plants, including Arabidopsis (for review, see Whitelam et al., 1998), sorghum (*Sorghum bicolor*; Childs et al., 1997), barley (*Hordeum vulgare*; Hanumappa et al., 1999), and rice (*Oryza sativa*; Takano et al., 2001), no phytochrome gene mutants have been characterized in maize. Gene duplication within the maize phytochrome family and the accompanying potential for functional redundancy may have obscured genetic screens for *phy* mutants.

The photoactive holoprotein (phy) consists of a *PHY* apoprotein (PHY) covalently attached to a linear tetrapyrrole (bilin) chromophore, 3*E*-phytochromobilin (PΦB; Terry, 1997). The first committed step in the synthesis of PΦB is the conversion of heme to biliverdin (BV) IXα by the enzyme heme oxygenase (Weller et al., 1996). BV IXα is then reduced to 3*Z*-PΦB by PΦB synthase and subsequently isomerized to 3*E*-PΦB (Terry et al., 1995). Of these three activities, genes encoding the first two have now been cloned (Davis et al., 1999; Muramoto et al., 1999; Kohchi et al., 2001). The *HO1* (*HY1*) gene encodes heme oxygenase, which is targeted to the plastid (Muramoto et al., 1999). The *HY2* gene encodes PΦB synthase, a ferredoxin-dependent BV reductase, which is also plastid localized (Kohchi et al., 2001). It is not yet known whether the isomerization of 3*Z*-PΦB to 3*E*-PΦB is enzyme mediated or whether it occurs spontaneously.

Although phytochrome apoproteins are encoded by a multigene family, it is likely that all plant apophytochromes bind the same chromophore. Therefore, genetic disruption of linear tetrapyrrole synthesis offers a way to specifically inactivate the entire phytochrome system. There are a number of known mutants in which linear tetrapyrrole synthesis is disrupted. These include the *hy1* and *hy2* mutants of Arabidopsis (Koornneef et al., 1980; Muramoto et al.,

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1999; Davis et al., 1999; Kohchi et al., 2001), the *pew1* (*partially etiolated-in-white-light1*) and *pew2* mutants of *Nicotiana plumbaginifolia* (Kraepiel et al., 1994), the *pcd1* (*phytochrome chromophore-deficient1*) and *pcd2* mutants of pea (*Pisum sativum*; Weller et al., 1996, 1997), the *au* (*aurea*) and *yg-2* (*yellow-green2*) mutants of tomato (*Lycopersicon esculentum*; Koornneef et al., 1985; Terry and Kendrick, 1996), and the *se5* (*photoperiodic sensitive5*) mutant of rice (Yokoo and Okuno, 1993; Izawa et al., 2000). All these mutants have lesions in either heme oxygenase or PΦB synthase and show a reduction in light responsiveness. However, a common characteristic of these mutants is that they show a partial recovery of light sensitivity during development (López-Juez et al., 1990; Weller et al., 1996), suggesting that other enzymes are present that can partially complement these mutations. Support for such an idea has recently come from Davis et al. (2001), who have shown that heme oxygenase is encoded by a small gene family that may be functionally redundant.

Although many molecular characterizations of phytochrome signaling have focused on seedling responses, a number of studies have demonstrated the importance of phytochrome in mature, field-grown plants (Robson et al., 1996; Schmitt, 1997; Shlumukov et al., 2001). In several cases, the mutation of a single *phy* gene has dramatically changed the mature plant phenotype. Two such examples are the early flowering ma_3^R line of sorghum (Childs et al., 1997) and the photoperiod-insensitive BMDR1 line of barley (Hanumappa et al., 1999). Overexpression of an oat (*Avena sativa*) *PHYA* in tobacco (*Nicotiana tabacum*) resulted in dramatic morphological changes that increased harvest index (Robson et al., 1996). Characterization of the *se5* heme-oxygenase mutant of rice has also demonstrated a significant contribution of the phytochrome system to the regulation of flowering time in this crop species (Izawa et al., 2000).

In this study, the isolation and initial characterization of the *elongated mesocotyl1* (*elm1*) mutant of maize is presented. We show that the *elm1* mutant has a reduced accumulation of active phytochrome. Under long-day (LD) growth conditions, *elm1* mutants flower earlier than near-isogenic wild-type plants, indicating that phytochrome signaling can modulate flowering time in maize.

RESULTS

Isolation of the *elm1* Mutant

The *elm1* mutation was identified in seedling screens of an *Ac*-mutagenized population. All lines in this population were maintained in a standard W22 inbred, enabling near-isogenic comparisons between any alleles recovered. To identify light-signaling mutants, *Ac* transpositions were selected from several donor elements located throughout the maize genome. F_1 plants were grown and self-pollinated to generate approximately 100 F_2 families. Approximately 20 kernels from each ear were then screened in greenhouse sandbenches to identify elongated pale-green seedlings. A similar screen was previously used to identify long-hypocotyl mutants of Arabidopsis (Koornneef et al., 1980) and tomato (Koornneef et al., 1985). A single line was identified that segregated pale-green seedlings with elongated mesocotyls as a simple recessive trait. The mutation was designated *elm1*. Southern-blot analysis has failed to detect linkage of the transposable element *Ac* to the *elm1* locus (data not shown); thus, it is unlikely that the *elm1* allele contains an *Ac* insertion.

Mature, field-grown *elm1* plants have elongated internodes (Fig. 1), pale-green leaves, and display a tendency to lodge (fall down). Under LD growth conditions in the field (14–16 h of light, Ithaca, NY), *elm1* plants flowered approximately 5 d earlier than wild type (*Elm1*, $n = 60$, mean = 79.1 d after planting [dap], SE = 0.22; *elm1*, $n = 63$, mean = 74.4 dap, SE = 0.38; Wilcoxon two-sample, non-paired, rank test, $U = 3{,}639.5$, $P < 0.01$). The similarity of *elm1* seedling and mature plant phenotypes to previously characterized mutants of Arabidopsis and tomato

Figure 1. Mature plant phenotypes of wild-type (*Elm1*) and mutant (*elm1*) plants. Wild-type (left) and *elm1* mutant (right) plants grown at summer field site (LD conditions). *elm1* mutants are taller, have pale internodes, and flower earlier than near-isogenic siblings.

suggested that the *elm1* mutant is impaired in light perception or signal transduction.

elm1 Mutant Seedlings Show a Disruption of the De-Etiolation Response

The phenotype of *elm1* seedlings grown under white light (W) is shown in Figure 2. Under these conditions, *elm1* showed a moderately elongated phenotype (Fig. 2), lower levels of chlorophyll (Chl), and an increased Chl *a*:*b* ratio (Table I). Carotenoid levels were also reduced in *elm1* compared with wild-type seedlings (Table I). Detailed genetic analyses in Arabidopsis have indicated that phyB is the primary photoreceptor mediating de-etiolation in response to red (R), whereas phyA is the primary photoreceptor mediating responses to far-red (FR) (Quail, 2002). To further examine the light signal transduction pathway in *elm1* mutants, we examined the inhibitory effect of R and FR on mesocotyl elongation. The mesocotyl can be considered functionally analogous to the dicot hypocotyl and, in wild-type seedlings, is greatly elongated in the absence of light stimuli (see Fig. 2). Wild-type and *elm1* plants were grown in dark (D), R (3 μmol m^{-2} s^{-1}), or FR (3 μmol m^{-2} s^{-1}) and mesocotyl lengths measured 10 dap. As shown in Figure 2, etiolated (D) wild-type and *elm1* seedlings showed similar elongation of the mesocotyl. In wild-type plants, elongation was strongly inhibited by both R and FR. In contrast, mesocotyl length was similar in *elm1* seedlings under all growth conditions tested (Fig. 3). This morphology is indicative of a lack of responsiveness to either R or FR.

Table I. *Pigment measurements*

Pigment	*Elm1*	*elm1*
Total Chl (mg g^{-1} fresh wt)[a]	1.16 ± 0.16	0.48 ± 0.07
Chl *a*:*b* ratio	3.73 ± 0.17	5.55 ± 1.03
Carotenoids (mg g^{-1} fresh wt)	0.27 ± 0.04	0.15 ± 0.01

[a]Chl and carotenoids were measured in acetone extracts from 2-week-old W-grown seedlings (values shown are the mean ± SE).

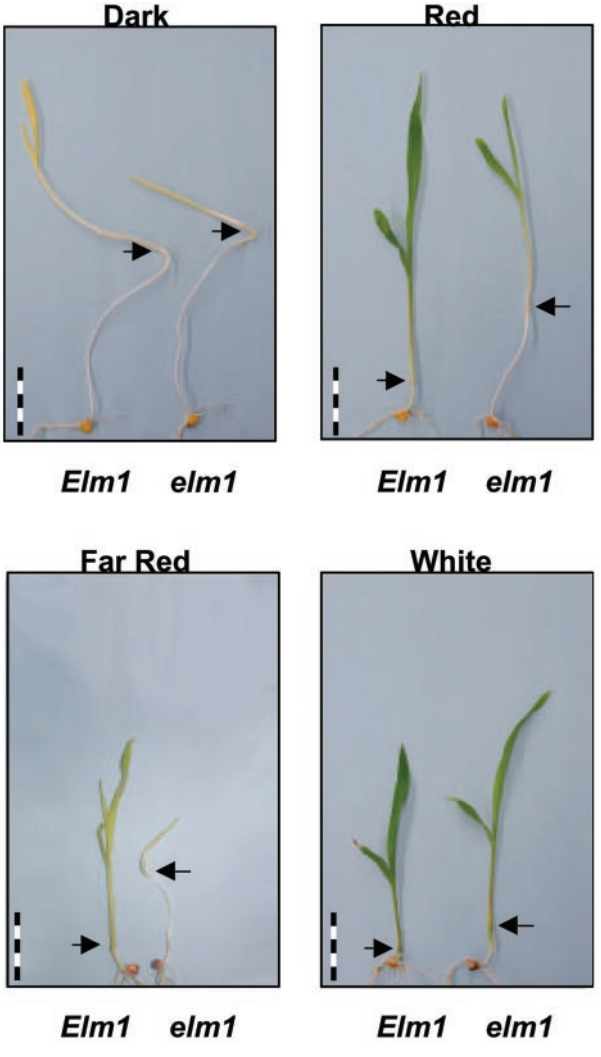

Figure 2. Seedling phenotypes of wild-type (*Elm1*) and mutant (*elm1*) plants. Representative seedlings were photographed after 10 d of growth in D, R (3.0 μmol m^{-2} s^{-1}), FR (1.2 μmol m^{-2} s^{-1}), or W (100 μmol m^{-2} s^{-1}). Arrows indicate boundary between mesocotyl and first internode. Scale bar divisions are in centimeters.

elm1 Seedlings Do Not Contain Spectrally Active Phytochrome Pools

The nonresponsiveness of *elm1* seedlings to both R and FR suggests disruption of both phyA- and phyB-mediated responses. To further investigate the activity of phytochrome in *elm1* mutants, spectrophotometrically active pools of phytochrome were directly measured in etiolated *elm1* and wild-type seedlings. Using in vivo spectrophotometry, the signal from *elm1* seedlings was below the level of detection, whereas the signal from etiolated wild-type seedlings was 4.9 ± 0.1 (n = 2) units (1 unit is 1×10^{-3} $\Delta\Delta A_{730-800\ nm}$). The level of detection is <0.3 units; therefore, *elm1* seedlings contain <6% of the spectrally active phytochrome present in wild-type plants.

As an additional assay of phytochrome activity, PHYA accumulation was examined in *elm1* seedlings. phyA is the most abundant phytochrome in etiolated tissue, but is rapidly degraded upon illumination.

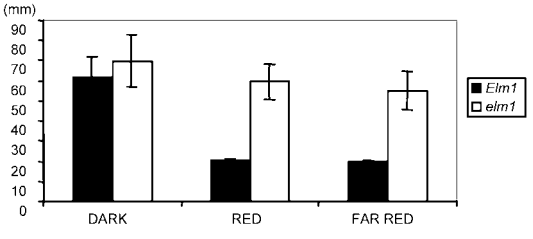

Figure 3. De-etiolation responses of wild-type (*Elm1*) and mutant (*elm1*) plants. Mean (±SE) mesocotyl length measurements in wild-type (*Elm1*) and mutant (*elm1*) seedlings grown for 10 d in D, R (3.0 μmol m^{-2} s^{-1}), or FR (2.0 μmol m^{-2} s^{-1}) growth conditions. Sample size is 15 to 20 seedlings per treatment/genotype.

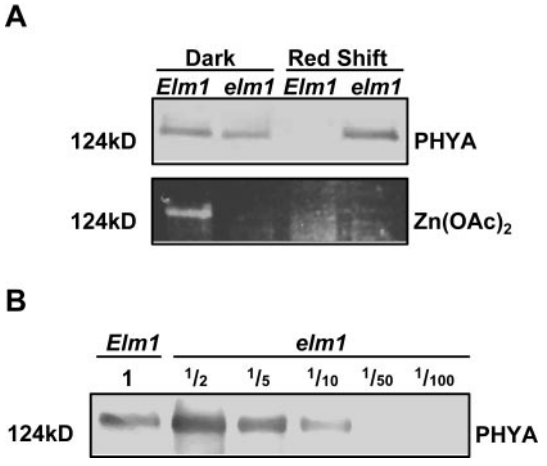

Figure 4. Immunoblot analysis of PHYA stability in wild-type (*Elm1*) and mutant (*elm1*) plants. A, Top, Immunodetection of PHYA apoprotein after SDS-PAGE and western blotting of crude protein extracts from wild-type (*Elm1*) and mutant (*elm1*) seedlings grown in dark (D) or given a 4-h R treatment (Red Shift; 10.0 μmol m^{-2} s^{-1}). Lanes were loaded on an equivalent fresh weight basis and loading confirmed by Coomassie Blue stain (data not shown). Molecular masses (kD) were determined using prestained markers (see "Materials and Methods"). Bottom, Detection of bound PΦB by zinc-induced fluorescence after gel staining with $Zn(OAc)_2$. Lanes as above. B, Immunodetection of PHYA apoprotein in crude protein extracts of R shift (4 h, 10.0 μmol m^{-2} s^{-1}) wild-type (*Elm1*) and mutant *elm1* seedlings. Extracts of *elm1* seedlings were diluted from 2-fold (1/2) to 100-fold (1/100) to allow semiquantitative determination of relative PHYA levels.

Because this degradation requires Pfr formation, the change in phyA stability after a light treatment can be used to assay the degree of photoconversion (Parks et al., 1989; Weller et al., 1996, 1997). As shown in Figure 4A, wild-type and *elm1* seedlings accumulate PHYA in D. After 4 h of R (10 μmol m^{-2} s^{-1}), phyA pools are rapidly depleted in wild-type but not in *elm1* seedlings. Figure 4B shows that levels of PHYA were approximately 5- to 10-fold higher in *elm1* seedlings relative to wild-type plants after a 4-h R treatment. This suggests that although PHYA accumulates in *elm1* seedlings, it is not bound to PΦB.

To confirm that *elm1* is deficient in holophytochrome, zinc-induced fluorescence was used to visualize the covalently bound chromophore (Berkelman and Lagarias, 1986). In wild-type plants, the presence of bound chromophore was detected as a fluorescent band in zinc-stained SDS-PAGE gels that comigrated with PHYA (Fig. 4A). However, although *elm1* plants accumulate high levels of PHYA, no bound chromophore could be detected in extracts of either etiolated or red-shifted seedlings. The absence of chromophore associated with PHYA in etiolated *elm1* seedlings suggests that there is either a disruption in holoenzyme assembly or PΦB synthesis in *elm1* mutants.

elm1 Mutants Are Unable to Convert BV IXα to 3*Z*-PΦB

All phytochrome chromophore-deficient mutants characterized to date are blocked in one of two steps: heme to BV IXα or BV IXα to 3*Z*-PΦB (Terry, 1997). To examine the accumulation of heme oxygenase in *elm1*, we used an antibody raised to the HO1 (HY1) protein of Arabidopsis (Muramoto et al., 1999). Etiolated *elm1* seedlings had an identical level of immunodetectable HO1 protein as wild-type seedlings (data not shown). This result suggests that heme oxygenase is unaffected in *elm1* seedlings and that *elm1* may be deficient in PΦB synthase. To directly assay PΦB synthase activity in *elm1*, we isolated plastids from dark-grown wild-type and *elm1* seedlings, incubated these with BV IXα and heme, and analyzed the products by HPLC. Figure 5 shows that incubation of wild-type plastids with BV IXα results in the synthesis of two products, identified by their absorbance maxima and co-injection of authentic standards isolated from pea (data not shown), as 3*Z*-PΦB (peak 2, trace B) and 3*E*-PΦB (peak 3, trace B), respectively. In contrast, incubation of *elm1* plastids with BV IXα produced no PΦB peaks (trace C). The major peak under these conditions was confirmed as

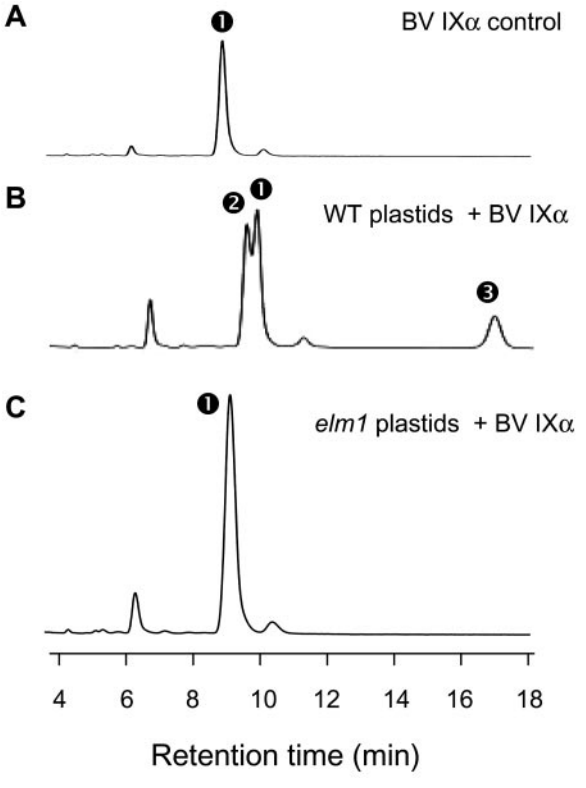

Figure 5. HPLC analysis of BV metabolism by isolated plastids from wild-type (WT) and mutant (*elm*) seedlings. HPLC analysis of BV metabolism by isolated plastids from wild-type (WT) and mutant (*elm*) seedlings. A, Control incubation with BV IXα and all reaction components except plastids. B and C, Bilin products obtained after incubation of WT or *elm1* mutant plastids with BV IXα. Peaks identified as 1 BV IXα, 2 3*Z*-PΦB, and 3 3*E*-PΦB are indicated.

the substrate BV IXα by its absorbance maximum and co-injection of authentic BV IXα (data not shown). To confirm that the *elm1* mutation specifically affects PΦB synthase, we also assayed the same etioplast samples for heme oxygenase activity. Wild-type etioplasts were capable of converting heme to both BV IXα and 3*Z*-PΦB. In contrast, incubation of heme with *elm1* etioplasts resulted in a small and reproducible increase in BV IXα synthesis, but no synthesis of PΦB (data not shown). These data strongly suggest that *elm1* is specifically deficient in the enzyme PΦB synthase.

Photosynthetic Transcripts Accumulate to Reduced Levels in *elm1* Seedlings

To investigate the requirement of phytochrome for photosynthetic development, the levels of transcripts encoding several plastidic proteins were examined in *elm1* seedlings. Wild-type and *elm1* seedlings were grown in D, under two fluences of R and in W. RNA gel-blot analysis was used to assay the accumulation of transcripts encoded by *Cab*, *RbcS*, *rbcL*, and *psbA* genes (Fig. 6). *Cab* and *RbcS* are nuclear transcripts encoding the light-harvesting Chl *a*/*b* protein (LHC-PII) and the small subunit of Rubisco, respectively. The transcription of *Cab* and *RbcS* genes is regulated by both phytochrome and blue light-mediated signaling (Fluhr et al., 1986; Tobin and Silverthorne, 1986). *rbcL* and *psbA* are chloroplast genes encoding the large subunit of Rubisco and the D1 peptide of photosystem II, respectively. As observed with *Cab* and *RbcS*, transcripts encoded by *rbcL* and *psbA* accumulate to higher levels after illumination (Bedbrook et al., 1978; Crossland et al., 1984). Under D growth conditions, wild-type and *elm1* mutants showed similar low-level accumulation of photosynthetic transcripts. As expected, all photosynthetic transcripts examined in wild-type seedlings accumulated to much higher levels under R relative to D growth conditions. In contrast, there was a relatively modest accumulation of all photosynthetic transcripts examined in *elm1* seedlings grown under low- or high-R conditions. Under W, *rbcL* and *psbA* transcripts accumulated to similar levels in wild-type and *elm1* mutant seedlings. *RbcS* transcripts accumulated to slightly lower levels in W-grown *elm1* relative to wild type, but *Cab* transcripts were significantly lower in *elm1* plants relative to W-grown wild-type seedlings. These data show that the R-mediated nuclear (*Cab* and *RbcS*) and plastid (*rbcL* and *psbA*) transcript accumulation is impaired in *elm1* seedlings.

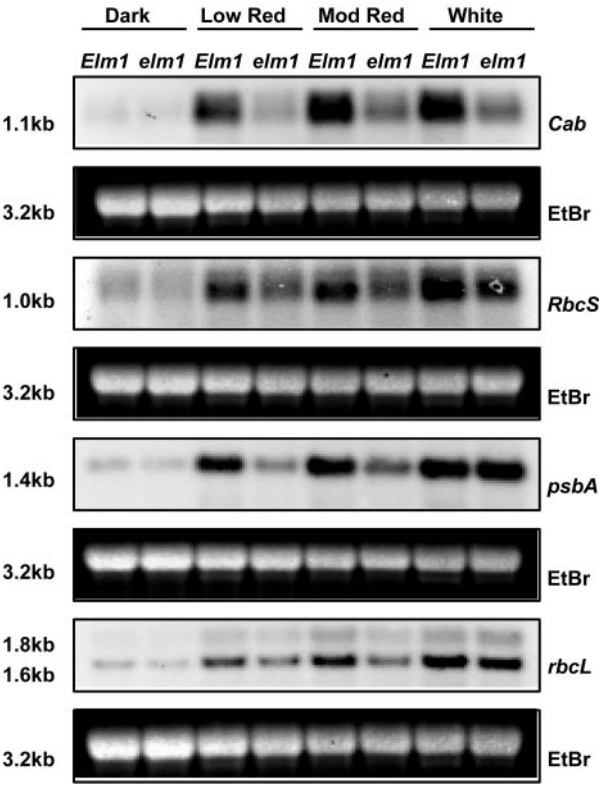

Figure 6. Northern-blot analysis of photosynthetic transcript accumulation in wild-type and *elm1* seedlings. Total RNA was extracted from wild-type and *elm1* mutant seedlings grown under D, low R (3 μmol m^{-2} s^{-1}), high R (30 μmol m^{-2} s^{-1}), or W (100 μmol m^{-2} s^{-1}) light conditions. Filters were hybridized to gene-specific fragments for the *Cab*, *RbcS*, *psbA*, and *rbcL* genes as described in "Materials and Methods." Approximately 5 μg of total RNA was loaded per lane. The 26S ribosomal band was visualized by ethidium bromide staining and was used as a loading control. Approximate transcript sizes (kb) are shown on the left.

DISCUSSION

The data presented here show that the *elm1* mutant of maize is severely deficient in photoreversible phytochrome and responds only weakly to both R and FR irradiation. These results indicate that *elm1* lacks multiple phytochromes and is consistent with a deficiency in phytochrome chromophore synthesis or assembly. Mutants disrupted in the synthesis of the phytochrome chromophore have been characterized in a number of species (Terry, 1997) and are disrupted at one of two loci encoding heme oxygenase or PΦB synthase. To date, no locus has been implicated in the regulation of chromophore synthetic enzymes, post-synthetic chromophore processing or holophytochrome assembly. Measurement of PΦB synthesis in isolated etioplasts demonstrated that *elm1* was unable to synthesize 3*Z*-PΦB from BV IXα. This result, together with the retention of heme oxygenase protein and activity, suggest that *elm1* is specifically deficient in PΦB synthase. Therefore, the *elm1* mutant is similar to the *hy2* mutant of Arabidopsis (Koornneef et al., 1980; Kohchi et al., 2001), *pcd2* of pea (Weller et al., 1997), and the *au* mutant of tomato (Koornneef et al., 1985; Terry and Kendrick, 1996), but is not equivalent to any known mutants from monocot species. In Arabidopsis, the *HY2* gene

has recently been cloned and shown to encode PΦB synthase (Kohchi et al., 2001). Unfortunately, searches of public maize expressed sequence tag collections have failed to identify putative maize orthologs of *HY2*. This may be due to the relatively low abundance of *HY2*-like transcripts in maize or may reflect a high degree of sequence divergence between the maize and Arabidopsis gene sequences. The rapid progress being made in the sequencing of the rice genome could soon help to provide a bridge to the isolation of a maize *HY2* ortholog and possibly to the cloning of *Elm1* in maize.

elm1 mutants are pale green both as seedlings and as mature field-grown plants, a phenotypic trait observed in most chromophore-deficient mutants (Terry, 1997). Investigation of greening in *elm1* seedlings has demonstrated a reduction in the accumulation of transcripts encoding a number of chloroplast components. Under R, *Cab*, *RbcS*, *rbcL*, and *psbA* transcripts accumulated to reduced levels in *elm1* seedlings. Under W, *rbcL* and *psbA* transcripts accumulated to similar levels in *elm1* and wild-type seedlings, suggesting that R and possibly blue light-signaling systems function redundantly to promote the accumulation of chloroplast-encoded transcripts. In contrast to *rbcL* and *psbA*, *Cab* and *RbcS* transcripts did not accumulate to similar levels in wild-type and *elm1* seedlings grown in W. This result suggests that the blue-light signal transduction pathway is unable to compensate for impaired phytochrome signaling in *elm1* mutants. However, it does not exclude the possibility that the reduced levels of *Cab* and *RbcS* transcripts reflect an altered physiology directly or indirectly responsible for lower Chl and carotenoid levels in *elm1* plants.

Although non-plastidic photoreceptor systems may signal directly to chloroplasts, the probable targets of light regulation are nuclear factors required for the accumulation of plastid-encoded transcripts. The identification of nuclear-encoded plastid-localized RNA polymerase (Greenberg et al., 1984; Lerbs-Mache, 1993; Young et al., 1998) and plastid-targeted sigma factors (Tiller et al., 1991; Lahiri et al., 1999) has revealed the importance of transcriptional control in the regulation in plastid gene expression. The accumulation of both nuclear-encoded plastid-localized RNA polymerase and plastid-localized sigma factors is normally light regulated (Chang et al., 1999; Lahiri et al., 1999) and, therefore, may be disrupted in *elm1* plants. Furthermore, genetic analyses have revealed a number of nuclear loci required for the processing of chloroplast mRNAs and highlight the importance of posttranscriptional regulation of transcript abundance (Stern et al., 1997). Thus, although we observed dramatic decreases in levels of *psbA* and *rbcL* transcripts in *elm1* mutants, it is unclear if this reflects a decreased rate of transcription, an increased rate of transcript degradation, or a combination of both.

Although it is clear from the above discussion that deficiencies in light signaling are likely to be important in determining the pale-green phenotype of *elm1*, the analysis of chromophore-deficient mutants from other species has indicated that other factors may play a role (Terry, 1997). Inconsistencies between the degree of Chl deficiency and the level of functional phytochrome have led to the proposal that feedback inhibition of Chl synthesis results from the block in plastidic heme degradation (Terry and Kendrick, 1999). Consistent with this hypothesis, dark-grown *au* and *yg-2* mutants have a reduced level of the Chl precursor protochlorophyllide (Terry and Kendrick, 1999) and this phenomenon has also been observed in chromophore-deficient mutants of pea and Arabidopsis (Terry et al., 2001). The pale-green phenotype and reduced Chl accumulation in *elm1* plants, therefore, may result in part from a similar negative feedback of Chl biosynthesis. Examination of protochlorophyllide levels in D-grown *elm1* seedlings should resolve this issue.

The *elm1* mutant represents the first light-signaling lesion to be characterized in maize and presents evidence that phytochrome influences flowering time in this species. Flowering time in many species is modulated by the relative duration of light and darkness during a daily cycle (the photoperiod; Thomas and Vince-Prue, 1997). In some plants, flowering is promoted by short days (SD), whereas in others, flowering is promoted by LD. Genetic analyses, notably of Arabidopsis (an LD plant), have indicated that both phytochrome and blue light-signaling pathways interact in the perception of photoperiod and in the regulation of flowering. As a generalization, phyA acts to promote flowering (Johnson et al., 1994), whereas phyB is required to inhibit flowering (Goto et al., 1991; Reed et al., 1993). The blue light-sensing cryptochromes act to promote flowering, both in a phytochrome-independent manner and by antagonism of phyB signaling (Koornneef et al., 1991; Bagnall et al., 1996; Guo et al., 1998; Mockler et al., 1999).

Although accessions of maize grown in the United States are generally considered day neutral, the early flowering phenotype of *elm1* shows that flowering is repressed under LD in the W22 inbred. The response is similar to that seen in the *se5* mutant of rice (an SD plant), although the magnitude of the effect in rice is greater (under LD wild-type rice flowered after 100.8 ± 0.8 d, *se5* flowered after 46.6 ± 0.6 d; Izawa et al., 2000). The expansion of cultivated maize beyond semitropical regions of early domestication has required the selection of day-neutral lines from ancestrally SD stocks. Nevertheless, the *elm1* mutant suggests that standard U.S. inbreds retain a weak response to photoperiod. The *elm1* mutant, the *se5* mutant of rice, the BMDR-1 mutant of barley, and the ma_3^R line of sorghum collectively show that the establishment of early flowering under nonpermissive

photoperiods can be achieved by selection for reduced phytochrome signaling. The utility of loss-of-function alleles in components of the phytochrome pathway has made this an efficient target for past selection and an attractive candidate for future genetic modification.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Homozygous *elm1* mutants and near-isogenic wild-type seed stocks were maintained in a standard maize (*Zea mays*) W22 inbred line. Seeds were surface sterilized for 15 min in a 10% (v/v) commercial bleach solution containing 0.1% (v/v) Tween 20, rinsed five times in deionized water, and imbibed overnight with shaking at room temperature. For mesocotyl measurements, seeds were grown in Rootrainers (http://www.hummert.com) containing vermiculite. On d 10, mesocotyl length was measured to the nearest millimeter using digital calipers. All seedlings were grown under continuous light or in constant darkness at 28°C in Percival Scientific (Boone, IA) model E-30LED light chambers with the exception of white-light treatments. LED light modules provided red and far-red light sources with narrow wavebands and peak emissions at 664 and 736 nm, respectively. White-light growth used a combination of incandescent and cool-white fluorescent lighting. Plants were grown for 10 d under light treatments before photography, RNA isolation, and mesocotyl measurements.

Measurement of Photosynthetic Pigments

Wild-type and *elm1* plants were grown for 2 weeks for 16 h in 180 μmol m^{-2} s^{-1} W and 8 h in D at 23°C. Three leaf discs were taken from the third leaf of four different plants and Chl and carotenoids were extracted into 80% (v/v) acetone and quantified according to Lichtenthaler (1987).

Protein Gel-Blot Analysis

Seedlings were frozen in liquid nitrogen, ground to a fine powder, and suspended at 2 mL g^{-1} in extraction buffer (37.5% [v/v] ethylene glycol, 75 mM Tris-HCl [pH 8.3], 7.5 mM Na_4EDTA, 15 mM NaS_2O_5, 0.11% [v/v] polyethylenimine, and 1.5 mM phenylmethylsulfonyl fluoride; Davis et al., 2001). Extracts were clarified by centrifugation at 3,000*g* for 30 min at 4°C, fractionated by SDS-PAGE (7.5% [w/v] acrylamide gel) and transferred to nitrocellulose membrane (Schleicher & Schull, Keene, NH). Gels were loaded by mass of starting tissue and equal loading confirmed by Coomassie Blue staining. Samples were equivalent to approximately 50 μg of total protein as determined using the Bio-Rad DC protein assay antibody (Bio-Rad, Hercules, CA). PHYA protein was detected using the monoclonal antibody O73D (Boylan and Quail, 1991), horseradish peroxidase-conjugated goat-anti-mouse secondary antibody and the Bio-Rad Opti-4CN substrate kit. To detect PΦB, acrylamide gels were incubated for 2 h in 1 M zinc acetate and visualized under UV light (Berkelman and Lagarias, 1986). Molecular masses (kD) were determined using prestained markers (SeeBlue Plus 2, Invitrogen, Carlsbad, CA).

Spectrophotometric Assay for Phytochrome

Phytochrome levels in wild-type and *elm1* seedlings were assayed by in vivo spectroscopy as described previously (Weller et al., 1996). Seedlings were grown for 7 d in D at 25°C and the top 1.5 cm of eight seedlings were used for each sample.

Assays for PΦB Synthesis

Maize seeds were sown in wet vermiculite (washed to remove fine particles before use), cold treated for 24h at 4°C, and then grown in the dark at 23°C for a further 8 to 10 d before etioplast isolation. Maize etioplasts were isolated as described previously (Weller et al., 1996) and PΦB synthesis from BV IXα was assayed essentially as described before for pea (*Pisum sativum*; Weller et al., 1996; Terry, 2001), but with the following modifications; PΦB assays were performed in 1 mL of reaction buffer, 20 mM TES, 10 mM HEPES-NaOH (pH 7.7) containing 500 mM sorbitol, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 0.5 mM dithiothreitol, and an NADPH regenerating system (1.2 mM $NADP^+$, 10 mM Glc-6-phosphate, and 2.5 units mL^{-1} Glc-6-phosphate dehydrogenase), 3,000 U mL^{-1} catalase, 1 mM desferrioxamine, and 5 mM ascorbate. The reaction was initiated by the addition of 10 μL of BV IXα (Porphyrin Products Inc., Logan, UT) to give a final substrate concentration of 10 μM. Bilins were recovered and concentrated using a C18 cartridge (SepPak Plus, Waters Corporation, Milford, MA) as described previously (Terry et al., 1995; Terry, 2001). HPLC analysis was performed using an LC-10 system (Shimadzu Corp., Kyoto) running VP-5 software and using an SPD-M10A photodiode array detector. An LC-18 column (5 μm; 250 × 4.6 mm; Supelco UK, Poole, UK) was used with a mobile phase of acetone:ethanol:100 mM formic acid (25:65:10 [v/v]) at an isocratic flow rate of 1 mL min^{-1} (Weller et al., 1996; Terry, 2001). The photodiode array detector was used to monitor spectra between 300 and 800 nm over 60 min.

RNA Gel-Blot Analysis

Seedling tissue was harvested at the base of the coleoptile and flash frozen in liquid nitrogen. Total RNA was extracted from approximately 1 g of tissue as previously described (Van Tunen et al., 1988). Approximately 5 μg of total RNA was fractionated on 1.5% (w/v) agarose gels containing 6.8% (v/v) formaldehyde and photographs taken of ethidium bromide-stained gels to visualize ribosomal bands. RNA was transferred to GeneScreen Plus nylon membrane (NEN, Boston, MA) through capillary transfer in 20× SSC. Digoxygenin (DIG)-labeled DNA probes were synthesized using the PCR DIG Probe Synthesis kit (Roche, Indianapolis, IN) according to the manufacturer's recommendations, using T7 and T3 primers. Gene-specific fragments for *RbcS* (pJL12), *rbcL* (pJL12), *psbA* (pSD7), and *Cab* (LHCP1020) were described previously (Roth et al., 1996; Hall et al., 1998). Hybridizations were performed using the Roche DIG Easy Hyb solution. Hybridization buffers and conditions were according to the manufacturer's recommendation (DIG Easy Hyb, Roche). In brief, membranes were prehybridized in 15 to 25 mL of DIG Easy Hyb buffer (Roche) for 30 min to 1 h. The prehybridization buffer was removed and 5 mL of hybridization buffer was added to 3 μL of labeled probe. Hybridization was performed overnight at 43°C. Membranes were washed twice in 2× SSC and 0.1% (w/v) SDS at room temperature for 5 min and twice in 0.1× SSC and 0.1% (w/v) SDS at 68°C for 15 min each. Membranes were then washed in 1× maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl, pH 7.5) for 3 min followed by 1 to 2 h of shaking in blocking solution (10% [w/v] casein, 0.1 M maleic, and 0.15 NaCl) acid before addition of 5 μL of Anti-DIG-AP Fab fragments (Roche). Blots were incubated for 30 min at room temperature with gentle shaking, washed for 15 min twice in 1× Washing buffer (3% [v/v] Tween 20, 0.1 M maleic acid, and 0.15 M NaCl). Blots were incubated with detection buffer (0.1 M Tris and 0.1 M NaCl, pH 9.5) for 3 min and placed in plastic sheet protectors. Excess liquid was removed and 2 mL of CDP-Star solution (20 μL of CDP-Star reagent + 2 mL of detection buffer) were applied directly to membranes. Blots were incubated for 5 min and exposed on an image station 440 CF (Eastman-Kodak, Rochester, NY). Eastman-Kodak 1D 3.5.4 Image Analysis software was used to determine relative transcript abundance.

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