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Differential Patterns of Expression of the Arabidopsis *PHYB*, *PHYD*, and *PHYE* Phytochrome Genes[1]

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The *Arabidopsis thaliana* phyB, phyD, and phyE phytochrome apoproteins show higher amino acid sequence similarity to each other than to phyA or phyC, they are the most recently evolved members of this photoreceptor family, and they may interact in regulating photomorphogenesis. The expression patterns of translational fusions of the 5′ upstream regions of the *PHYB*, *PHYD*, and *PHYE* genes to the β-glucuronidase (GUS) coding sequence were compared. P_D-GUS and P_E-GUS fusions were 5- to 10-fold less active than a P_B-GUS fusion, but all three promoter regions drove expression of the reporter gene in all stages of the plant's life cycle. Over the first 10 d of seedling growth, the *PHYB* and *PHYD* promoters were more active in the dark than in the light, whereas the opposite was true of the *PHYE* promoter. Unlike the P_B-GUS construct, which was expressed in most parts of seedlings and mature plants, the P_D-GUS and P_E-GUS transgenes showed differential expression, notably in leaves, flower organs, and root tips. Tissue sections showed that the three promoters are coexpressed in at least some leaf cells. Hence, the *PHYB*, *PHYD*, and *PHYE* genes differ in expression pattern but these patterns overlap and interaction of these receptor forms within individual cells is possible.

R and FR light sensing in plants is mediated by a family of soluble photoreceptors, the phytochromes (for review, see Quail, 1994; Pratt, 1995; Quail et al., 1995). These chromoproteins undergo R/FR reversible conformational changes that trigger a large number of physiological and developmental responses to light. In *Arabidopsis thaliana*, five *PHY* genes, *PHYA*, *B*, *C*, *D*, and *E*, encode phytochrome apoproteins (Sharrock and Quail, 1989; Clack et al., 1994). Among these, the phyB and phyD polypeptides are more similar to each other (approximately 80% amino acid identity) than they are to either the phyA or phyC forms (approximately 50% identity), and the phyE form is somewhat more related to phyB and phyD than to phyA and phyC. Surveys of a large number of angiosperm plant genera indicate that *PHY* gene families similar to that of Arabidopsis are present in most, if not all, flowering plants (Mathews et al., 1995; Mathews and Sharrock, 1997).

Partial sequences of putative homologs of the *PHYA*, *PHYB*, and *PHYC* genes have been detected in diverse monocots and dicots; however, although they are present in many families of dicots, *PHYE*-like sequences have not yet been detected in monocots (Mathews and Sharrock, 1996). Phylogenetic analysis indicates that the *PHYB*/*PHYD* pair of highly related sequences in Arabidopsis is the product of a gene duplication in a relatively recent progenitor to the Cruciferae, and that similar pairs or groups of *PHYB*-like genes have evolved independently in other plant families such as the Solanacae and the Umbelliferae (Mathews et al., 1995; Pratt et al., 1995). The presence of independently evolved subgroups of closely related *PHYA*-like genes has also been observed in several dicot subclasses (Mathews et al., 1995). Hence, the *PHY* gene family exhibits significant plasticity, and recent divergence of novel subgroups of *PHY* genes appears to be characteristic of many dicotyledonous families. The Arabidopsis *PHYB*, *PHYD*, and *PHYE* genes, to our knowledge the only such subgroup that has been completely sequenced and shown to be expressed at the mRNA and protein levels (Clack et al., 1994; J. Tepperman, P. Quail, and R. Sharrock, unpublished data), represent an appropriate model for determining the extent to which evolutionary expansion of photoreceptor families in plants results in diversification of expression patterns and divergence of receptor function.

Light responses mediated by phytochromes are often localized in specific plant organs or tissues. Moreover, induction of anthocyanin synthesis in mustard cotyledons is elicited in localized groups of cells following R light microbeam irradiation (Nick et al., 1993), and microinjection of purified phytochrome into hypocotyl cells of a phytochrome-deficient tomato mutant results in cell-autonomous expression of phytochrome responses (Neuhaus et al., 1993). These observations suggest that the organ- and tissue-specific distribution of a phytochrome might spatially correlate with the responses it regulates. The functions of the Arabidopsis *PHYA* and *PHYB* genes have been identified by characterization of null mutants in these genes. Mutants lacking phyA exhibit loss of FR high-irradiance control of hypocotyl elongation, cotyledon expansion, and seed germination. Mutants lacking phyB show alteration in the photoreversible effects of R and FR light and the effects of the R/FR ratio on hypocotyl elongation, flowering time, leaf morphology, and seed germination (for review, see Smith, 1995). Recently, Shinomura et al. (1996) and Botto et al. (1996) showed that phyA also mediates the very-low-fluence seed germination response.

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Abbreviations: FR, far red; phy, phytochrome apoprotein or holoprotein; R, red.

Using fusions to the GUS reporter gene, Somers and Quail (1995a, 1995b) investigated the patterns of expression of the Arabidopsis *PHYA* and *PHYB* promoter regions. These two promoters were shown to be active very generally throughout seeds, seedlings, and mature plants, and it was concluded that there is little to suggest that differential spatial distribution of these receptors plays a role in determining their functions (Somers and Quail, 1995b). In contrast to Arabidopsis *PHYA* and *PHYB*, the expression patterns of two *PHYA* genes and one *PHYB* gene from tobacco (Adam et al., 1994, 1996), and of a *PHYA* gene from pea (Komeda et al., 1991), when assayed as promoter-GUS fusions, show distinctive organ- and tissue-specific expression. In addition, blot analyses of RNA levels from a *PHYA* and a *PHYB* gene from potato (Heyer and Gatz, 1992a, 1992b) and from five *PHY* genes of tomato (Hauser et al., 1997) indicate that these transcripts show significant variation in expression pattern in different plant organs.

Recently, a null mutation in the Arabidopsis *PHYD* gene has been identified and shown to cause changes in several of the same R/FR responses that are altered in *phyB* mutants, though phyB appears to play a more prominent role than phyD (Aukerman et al., 1997). This finding raises questions about whether these two highly related receptors might either physically interact or use common signaling pathways in the same cells. We report here a promoter fusion analysis of the expression of the Arabidopsis *PHYB*, *PHYD*, and *PHYE* genes. We describe the more specific spatial distribution of the activities of the *PHYD* and *PHYE* promoters than those of *PHYA* and *PHYB*, and the extent of overlap of expression patterns of these three genes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Nossen (No-0) was used throughout. Seeds were surface sterilized, plated on germination medium, cold treated, and grown under dark or light growth conditions as described by Wester et al. (1994).

Construction of Promoter-GUS Fusions and Plant Transformation

The P_B-GUS fusion was constructed by ligating a 2.5-kb *Sal*I fragment from a λEMBL4 genomic clone of the 5′ end of the ecotype Landsberg *PHYB* gene (Reed et al., 1993) into the *Sal*I site of pBI101.1 (Jefferson et al., 1987). This resulted in an in-frame fusion of the coding sequence for the first 64 amino acids of the phyB polypeptide plus 10 amino acids encoded by the polylinker to the GUS reading frame. The P_D-GUS fusion was constructed by subcloning a 2.8-kb *Bgl*II fragment from the λD6–1 genomic clone of the 5′ end of the Landsberg *PHYD* gene (Clack et al., 1994) into the *Bam*HI site of M13 mp18, transferring that fragment to pGEM5 as an *Sph*I/*Sma*I fragment, then excising the promoter as a 2.2-kb *Sal*I fragment and ligating it into the *Sal*I site of pBI101.1. This resulted in an in-frame fusion of the coding sequence for the first 66 amino acids of the phyD polypeptide plus 10 amino acids encoded by the polylinker to GUS. The P_E-GUS fusion was constructed by cloning a 3.0-kb *Eco*RI-*Bsr*FI fragment from the 5′ end of the λE3–2 genomic clone of the Landsberg *PHYE* gene (Clack et al., 1994) into M13 mp18, with a filled-in *Bsr*FI site ligating to a chewed-back *Pst*I site in the vector. The 2.6-kb *Hin*dIII-*Sph*I P_E fragment in this clone was moved to M13 mp19, placing a *Sal*I site downstream of the (*Bsr*FI/*Pst*I) fusion, and the 2.6-kb *Hin*dIII-*Sal*I fragment was excised and inserted into pBI101.1. This resulted in an in-frame fusion of the coding sequence for the first 42 amino acids of the phyE polypeptide plus 16 amino acids encoded by the polylinker to GUS. The P_B-GUS, P_D-GUS, and P_E-GUS genes were transferred into Arabidopsis by the root transformation protocol (Valvekens et al., 1988).

Southern-Blot Analysis

Total DNA from pooled T3 seedlings (the initial transformed plant was designated the T1 generation) of independent transgenic lines was prepared by the method of Edwards et al. (1991). Approximately 2 μg of DNA was digested with the indicated restriction enzyme, fractionated on 1% agarose gels, and transferred to Hybond filters (Amersham). For each construct, the enzyme used cuts once within the transgene and once in the flanking DNA. Hybridization and washing conditions were as described in Wester et al. (1994). Probes were random primer-labeled fragments from the promoter regions, as illustrated in Figure 1.

Fluorometric and Histochemical GUS Assays

Fluorometric assays were performed by grinding 20–30 seedlings in 300 μL of extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, and 10 mM β-mercaptoethanol) on ice, clarifying the extracts by centrifuging at 13,000*g* for 5 min at 4°C, and assaying an aliquot over a time course of 30 to 90 min, depending upon the activity level of the line being tested. The GUS reaction buffer was extraction buffer containing 1 mM 4-methyl umbelliferyl β-D-glucuronide plus 20% [v/v] methanol. Reactions were stopped with 1:10 dilution into 0.2 M sodium carbonate, and fluorescence was determined on a fluorometer (model TKO 100, Hoefer, San Fancisco, CA). Protein concentrations were determined by the method of Bradford (1976).

Histochemical assays were performed on intact seedlings or excised plant organs. Tissue samples were briefly fixed by vacuum infiltration in 100 mM sodium phosphate (pH 7.0), 0.1% formaldehyde, and 0.1% (w/v) Triton X-100 for 10 min, followed by three rinses in 50 mM sodium phosphate (pH 7.0). Samples were then incubated at 37°C for 6 to 24 h, as needed, in 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. These were rinsed, cleared of chlorophyll by a series of incubations in 25% (v/v), 50%, 75%, and 95% EtOH, and photographed under a microscope (M3C, Wild, Heerbrugg, Switzerland). Plant lines transformed with the promoterless pBI101.1 vector showed

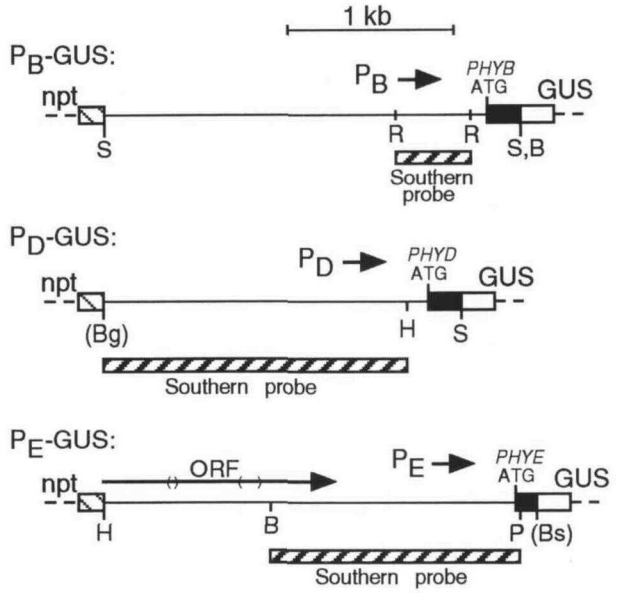

Figure 1. *PHYB*, *PHYD*, and *PHYE* promoter-GUS fusion constructs. Upstream genomic sequence (1.9–2.5 kb) from each *PHY* gene was translationally fused 100 to 200 bp downstream from the phytochrome start codon to the GUS-coding sequence of pBI101.1 (Jefferson et al., 1987). Only the promoter-containing region of the T-DNA for each construct is shown. Black areas represent phytochrome-coding sequences and open areas represent GUS-coding sequence. The restriction fragments used as probes in the Southern blots shown in Figure 2 are indicated and the upstream gene sequence, which extends into the P_E fragment, is shown with brackets representing intron regions in that gene. B, *Bam*HI; H, *Hind*III; P, *Pvu*II; R, *Eco*RI; S, *Sal*I; (Bg), disabled *Bgl*II; (Bs), disabled *Bsr*FI; ORF, open reading frame.

no detectable GUS staining under these conditions. In addition to these assay conditions, pretreatment of samples with 90% acetone at −20°C (Hemerly et al., 1994) or freeze-thawing was tested but was not found to affect the staining patterns significantly. For tissue sectioning, stained seedlings or organs were embedded in 12% gelatin. Blocks of gelatin containing the tissue samples were embedded in a mixture of one part 12% gelatin to two parts egg yolk. Egg-gel blocks were fixed in 4% formaldehyde, cut into 50-μm sections on a Vibratome (series 1000, Technical Products International, St. Louis, MO), placed on slides, and photographed under an Optiphot-2 microscope (Nikon).

RESULTS

Construction of P_B-GUS, P_D-GUS, and P_E-GUS Transgenic Lines

As shown in Figure 1, sequences including 1.9 to 2.5 kb upstream from the coding regions of the *PHYB*, *PHYD*, and *PHYE* genes and 100 to 200 bp of the phytochrome reading frames were translationally fused to the GUS-coding sequence, resulting in GUS-fusion proteins containing 64, 66, and 42 amino acids of the B, D, and E sequences as N-terminal extensions. These constructs include the multiple, small, upstream open reading frames found in the 5′ ends of these transcripts (Sharrock and Quail, 1989; Clack et al., 1994) and, in the case of the *PHYE* gene, part of an upstream transcription unit that extends to within 1100 bp of the *PHYE* start codon (Fig. 1). These fusion genes were transformed into Arabidopsis and between 15 and 40 independent transformants were isolated. The T_2-generation seedlings and plants were stained for GUS expression and representative lines were chosen for further analysis. A similar *PHYB*-GUS fusion gene, containing 155 bp less of the 5′ flanking sequence and the coding sequence for only two amino acids of the phyB N terminus, and its activity in transgenic plants have been described previously (Somers and Quail, 1995a, 1995b). Hence, only three lines carrying the P_B-GUS construct were selected for the comparative promoter analysis performed here. Eight and five representative P_D-GUS and P_E-GUS lines, respectively, were selfed to produce the T3 and T4 seeds used in these experiments. Figure 2 shows Southern-blot analysis of transgene numbers in T3 plants of these lines. By comparison with the

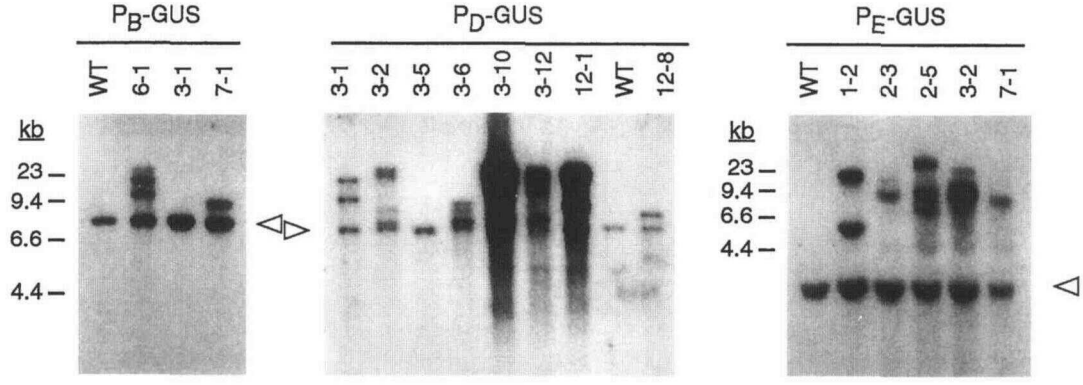

Figure 2. Southern-blot analysis of independent P_B-GUS, P_D-GUS, and P_E-GUS transgenic lines. DNA from bulked T3 seedlings of independent lines was cut with *Bam*HI (P_B-GUS lines), *Eco*RI (P_D-GUS lines), or *Hind*III (P_E-GUS lines) and hybridized to the probes for the *PHYB*, *PHYD*, and *PHYE* promoter regions shown in Figure 1. The arrowheads point to the bands resulting from hybridization to the wild-type *PHY* genes, which act as controls in each lane for a diploid DNA signal. The transgene fragments in lines P_B-GUS 3–1 and P_D-GUS 3–5 migrate close to the endogenous promoter fragments and are not resolved on these gels.

endogenous *PHYB, D,* and *E* gene hybridization signals, it is clear that the majority of lines contain 1 to 3 copies of the transgenes; however, three P_D-GUS lines, nos. 3–10, nos. 3–12, and nos. 12–1, contain higher numbers, perhaps as tandem duplications, giving rise to intense high-molecular-weight bands on the blot.

Developmental and Light-Induced Regulation of GUS Activities in the Promoter Fusion Lines

Table I gives GUS enzyme specific activities in dry seeds and in 7-d-old white-light-grown and dark-grown seedlings of the representative P_B-, P_D-, and P_E-GUS lines. These activities roughly correlate with transgene numbers (Fig. 2) and, on average, the activities of the P_D-GUS and P_E-GUS constructs in seedlings containing one or two copies of the gene are 10- to 20-fold lower than the activities of the P_B-GUS lines. As observed previously (Somers and Quail, 1995a, 1995b), the ratio of activity of P_B-GUS in the light to activity in the dark has a value of 0.6 to 0.8. A similar ratio is observed for P_D-GUS but the P_E-GUS lines show an approximately 2-fold higher activity in light compared with dark. These ratios are consistent between independent lines and show general agreement with the nearly equal mRNA levels for these gene products under these two growth conditions (Clack et al., 1994). All three promoters result in detectable GUS levels in dry seeds, indicating that the three phytochromes may be deposited during seed development. The P_E promoter is unique in having a higher expression level in dry seeds than in seedlings.

Figure 3 shows time courses for GUS specific activity in lines P_B-GUS no. 6–1, P_D-GUS no. 3–1, and P_E-GUS no. 3–2 over the first 10 d of seedling growth in darkness and continuous white light. The three promoters are continuously active throughout seedling development at levels consistent with the data in Table I. Moreover, the time courses reveal developmental control of both the *PHYB* promoter, which shows a steady increase in activity in both the light and dark, and the *PHYD* promoter, the activity of which is induced in the early seedling in both light and dark but is then reduced by d 7 of the time course. Of the four *PHY* gene promoters examined to date, *PHYA, B, D,* and *E* (Fig. 3; Somers and Quail, 1995a, 1995b), the P_E promoter is unique in being more active in the light than in the dark in mature seedlings.

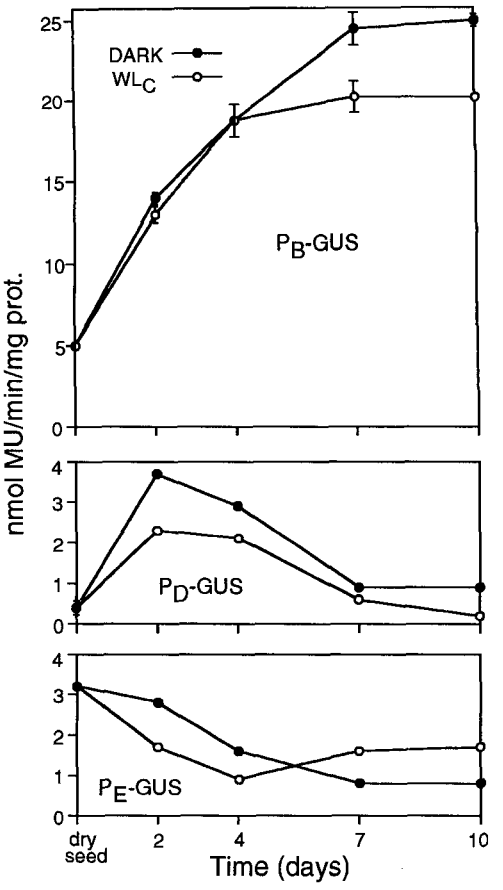

Figure 3. GUS enzyme activities in seeds and seedlings of P_B-GUS no. 6–1, P_D-GUS no. 3–1, and P_E-GUS no. 3–2 transgenic lines. GUS activities in dry seed and dark-grown and continuous white-light-grown (WL_C) seedlings (30 T3 seedlings/assay) were determined by fluorometric assay. Error bars are given for data points when the SE for three trials were greater than the size of the symbol.

Organ- and Tissue-Specific Expression of *PHY* Promoter Fusions

The observations on the histochemical localization of expression patterns of the P_B-GUS, P_D-GUS, and P_E-GUS fusion genes presented here have been confirmed in multiple independent transgenic lines shown in Table I and Figure 2, but Figure 4 shows representative results from

Table I. *GUS activities in seeds and seedlings of independent P_B-GUS, P_D-GUS, and P_E-GUS transgenic lines*

Line	P_B-GUS			P_D-GUS								P_E-GUS				
	6-1	3-1	7-1	3-1	3-2	3-5	3-6	3-10	3-12	12-1	12-8	1-2	2-3	2-5	3-2	7-1
	nmol methylumbelliferone min^{-1} mg^{-1} protein															
Seed	4	2.3	2	0.3	0.12	0.16	0.3	0.6	1.0	3	0.12	1.6	0.8	1.8	3	0.7
7d WL[a]	18	6	22	0.9	0.25	0.2	0.5	1.0	1.8	3	0.3	0.8	0.7	0.9	1.6	3
7d D[b]	23	9.5	35	2.0	0.40	0.6	2	2.5	2.4	6.5	1.2	0.7	0.3	0.6	0.7	0.7
WL:D[c]	0.8	0.6	0.6	0.45	0.6	0.3	0.25	0.4	0.75	0.45	0.25	1.1	2.3	1.5	2.3	4.3
seed:D[d]	0.2	0.2	0.1	0.2	0.3	0.3	0.2	0.2	0.4	0.4	0.1	2.3	2.7	3	4.3	1

[a] 7d WL, Seven days under continuous white light. [b] 7d D, Seven days in continuous dark. [c] WL:D, Ratio of enzyme activity in the white light to activity in the dark. [d] seed:D, Ratio of enzyme activity in dry seed to activity in the dark.

only a single line. The *PHYD* and *PHYE* mRNAs are present in plants at relatively low abundance (Clack et al., 1994), and the low-insert copy number P_D- and P_E-GUS transgenic lines required overnight incubation in the substrate to achieve significant staining. The high-copy-number P_D lines, such as P_D-GUS no. 3–10, no. 3–12, and no. 12–1 (Fig. 2), which stained more quickly (4–6 h), showed the same patterns as the low-copy-number lines.

Figure 4 shows P_B-, P_D-, and P_E-GUS expression patterns in seedlings grown for 3 or 7 d in continuous white light or continuous darkness. In general, the patterns observed at d 3 became more strongly localized and distinct by d 7. As described previously (Somers and Quail, 1995b), the P_B-GUS gene exhibited activity throughout the seedling at d 3 and 7 (Fig. 4, A-D). In contrast, at d 3, both in white light and in the dark, the P_D-GUS gene was predominantly expressed in the cotyledons, the meristem region, and the upper hypocotyl (Fig. 4, E and F). The distal end of the primary root stained in light-grown but not dark-grown P_D-GUS 3-d seedlings, with expression being limited to the root meristem and elongation zone (see Fig. 4Q). By d 7 of light growth, the cotyledons and the emerging true leaves of P_D-GUS plants showed GUS activity, as did the root tip. The meristem, hypocotyl, and proximal portion of the root stained very faintly or not at all (Fig. 4G). In 7-d-old dark-grown P_D-GUS seedlings, only the cotyledons stained, often with a sharp border between the leaf and stem tissues (Fig. 4, H and M). Three-day-old light-grown and dark-grown P_E-GUS seedlings showed expression only in the central portion of the cotyledons and the upper portion of the hypocotyl (Fig. 4, I and J), and by d 7, expression was restricted to leaf structures (Fig. 4, K and L). In light-grown seedlings, the P_E-GUS cotyledons stained evenly but emerging leaves did not stain until they expanded, and then stained predominantly in the vascular regions of the distal portion of the leaf (Fig. 4K). In dark-grown P_E-GUS seedlings, expression was restricted to a central region of the cotyledon, often with distinct boundaries separating that zone from the tip and hypocotyl (Fig. 4, L and N). Histochemical evidence for P_E-GUS activity in roots was not observed under any of these growth conditions.

The presence of GUS in roots of P_D-GUS seedlings grown in the light, and its absence in the dark (Fig. 4, E–H), suggests that this promoter region is light responsive in root tissue. Figure 4, O through Q, shows root expression in the P_B-GUS line for comparison and the results of a light-induction experiment in which P_D-GUS seedlings were grown in darkness for 7 d and then shifted to white light for 48 h. Both dark-grown (Fig. 4O) and light-grown (not shown) P_B-GUS roots stained evenly, including the root tip and cap. Dark-grown P_D-GUS roots did not stain (Fig. 4P) and 48 h of light induced GUS expression in the root meristem and elongation regions but not in the tip or cap (Fig. 4Q), the same pattern as is seen in continuous-light-grown seedlings (Fig. 4, E and G). Continuous R light induced P_D-GUS root expression, whereas FR and blue light did not (data not shown). Furthermore, excised dark-grown P_D-GUS roots are responsive to light (data not shown), indicating that the receptor for the light signal is a phytochrome located in the root itself.

Flowers from P_B-GUS lines showed a very general expression pattern, as described by Somers and Quail (1995b), including all organs except petals and with relatively weak staining in uncut pistils (Fig. 4R). In contrast, P_D-GUS and P_E-GUS flowers stained almost exclusively in sepals (Fig. 4, S and T), a pattern that was not changed by cutting open the flowers and exposing the tissues more directly to the GUS substrate (data not shown). Five of 10 independent P_E-GUS lines, including 3 of the 5 lines in Table I, also showed GUS activity in a localized region in the center of the anther, at the distal end of the filament (Fig. 4, T and U). These lines were more strongly expressing P_E-GUS transgenics, and it is possible that in the other lines, anther expression was too weak to detect. Rosette leaves of P_B-GUS and P_D-GUS lines showed GUS expression evenly over the entire leaf, with *PHYB* being expressed at a higher level than *PHYD* (Fig. 4, V and W). P_E-GUS rosette leaves continued to exhibit expression predominantly in the reticulate vasculature of distal parts of the leaf (Fig. 4X), as seen in the first emerging leaves of young seedlings (Fig. 4K).

Microscopic examination of the tissue localization of the fusion gene activities showed that, notably in leaf structures, when the *PHYB*, *D*, and *E* promoter expression patterns overlapped in macroscopic analyses (Fig. 4), they were often expressed in the same cell types. Figure 5, A through C, shows transverse sections through rosette leaves of the three lines, cut approximately one-fourth of the leaf length from the tip. The P_B- and P_D-GUS transgenes were expressed in epidermal, mesophyll, and vascular tissues, whereas the P_E-GUS promoter fusion, consistent with its macroscopic leaf expression pattern (Fig. 4X), was active primarily in vascular elements, with only faint staining of mesophyll cells. Transverse sections through the middle of stained P_B-, P_D-, and P_E-GUS flowers (Fig. 5, D–F) showed that all three promoters were highly active in vascular tissue and mesophyll cells of sepals. The P_B promoter alone was expressed strongly throughout the androecium. P_B-GUS flowers showed high GUS activity in filaments and throughout anther structures, including pollen sacs and pollen grains (Figs. 4R, and 5, D and G). P_B promoter expression was less equally distributed in the gynoecium, with the lower style showing faint expression (Figs. 4R and 5D), the upper style showing strong activity (Figs. 4R and 5G), and the stigma showing faint or no expression (Fig. 4R). These observations are consistent with those of Somers and Quail (1995b). Sections through the upper portions of the style and medial anthers of P_B-GUS flowers (Fig. 5G) illustrate the high level and very general distribution of P_B-GUS activity in these tissues, notably in the upper style (Fig. 4R). This contrasts with the localized flower expression of P_D- and P_E-GUS. P_D-GUS expression was confined to sepals (Figs. 4S and 5E). P_E-GUS activity was restricted to sepals (Figs. 4T and 5F) and to an area at the distil end of the filament, where it was enclosed by the anther (Fig. 4, T and U, and Fig. 5I).

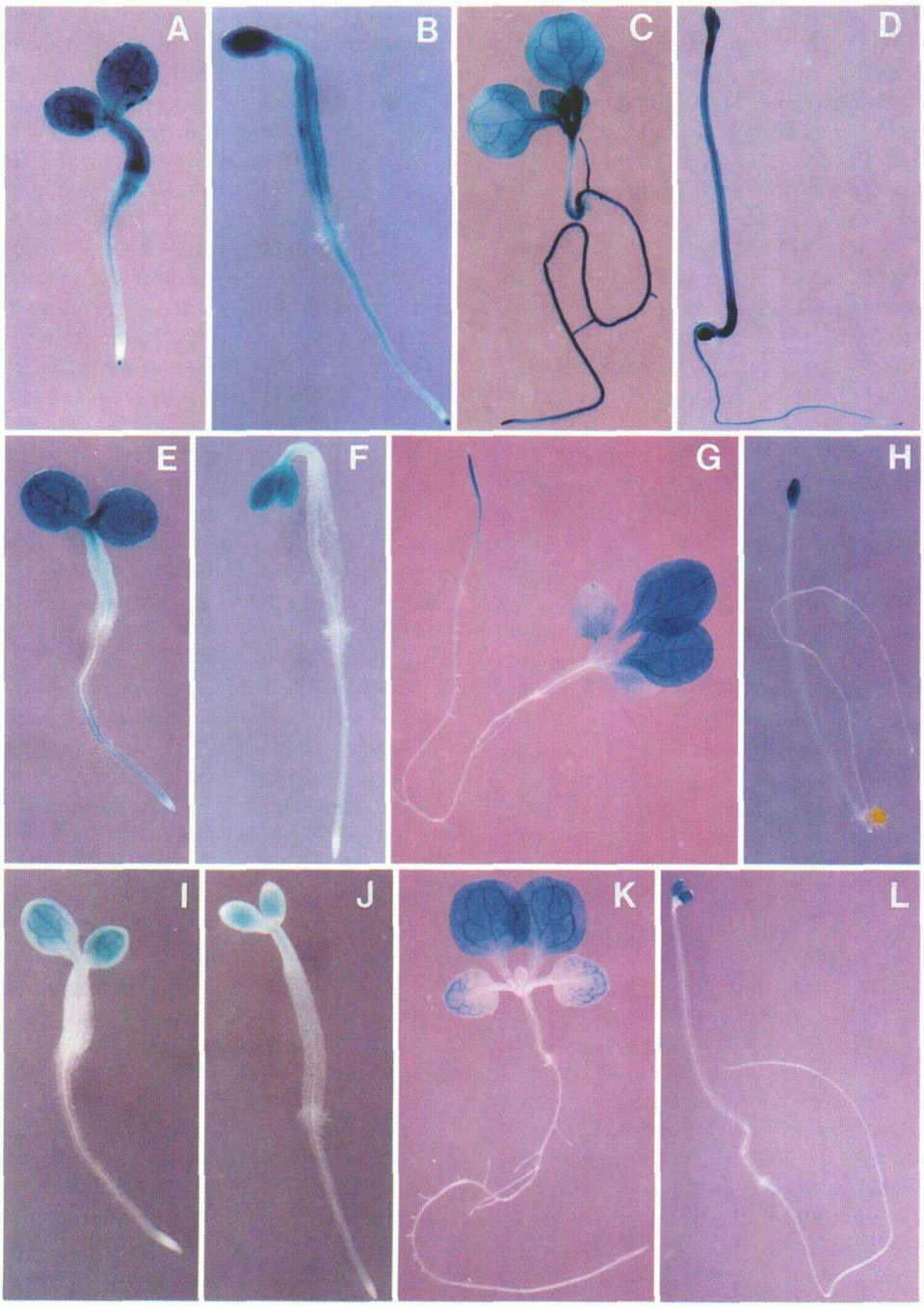

Figure 4. (Figure and legend continue on facing page.)

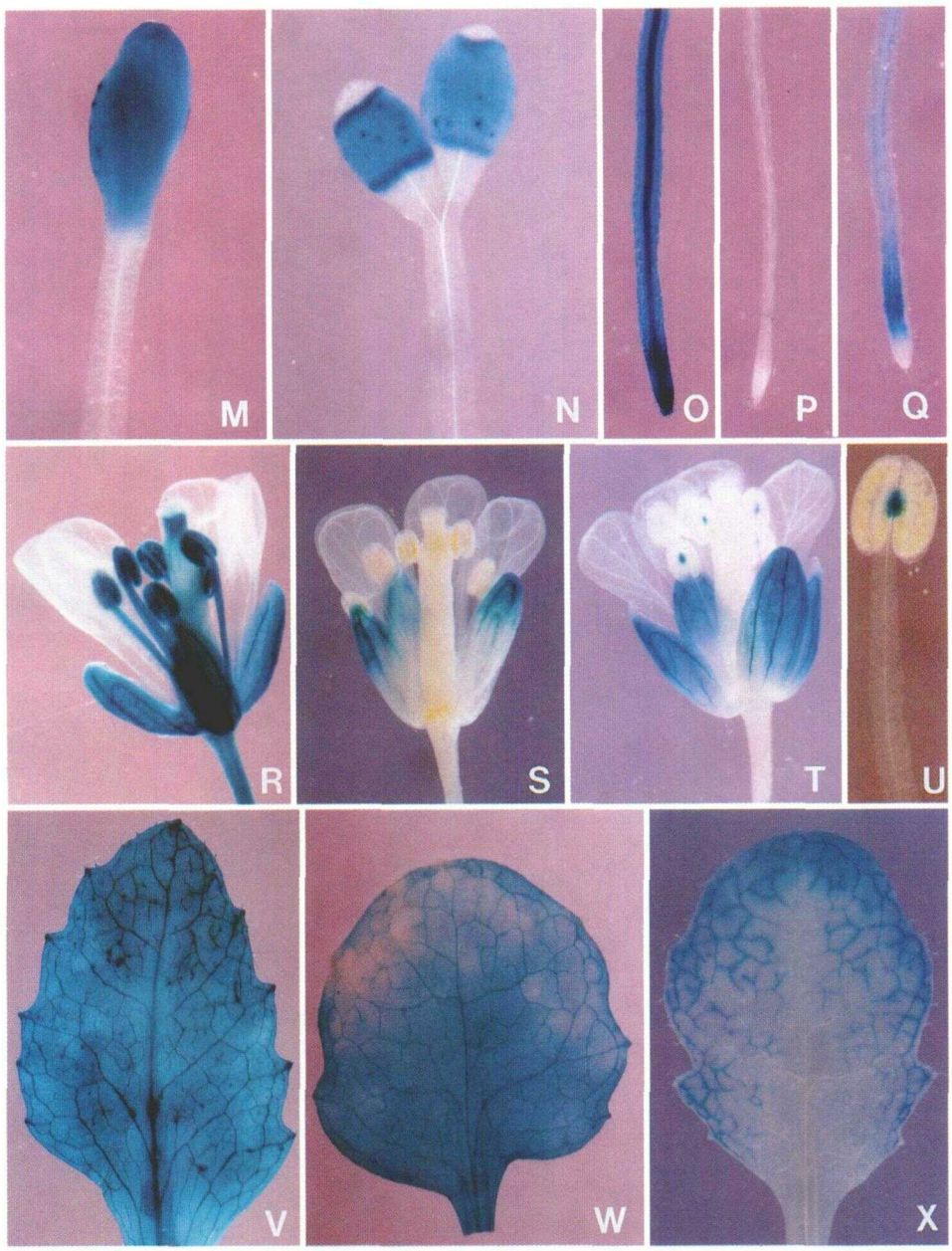

Figure 4. (Continued from facing page.) Histochemical localization of P_B-GUS, P_D-GUS, and P_E-GUS expression in *unsectioned tissues*. A through L, Each row shows, consecutively, seedlings grown for 3 d in continuous white light, 3 d in darkness, 7 d in light, and 7 d in darkness. A through D, P_B-GUS seedlings; E through H, P_D-GUS seedlings; I through L, P_E-GUS seedlings; M, cotyledons of 7-d-old dark-grown P_D-GUS seedlings; N, cotyledons of 7-d-old dark-grown P_E-GUS seedlings; O, root tip of a 9-d-old dark-grown P_B-GUS seedling; P, root tip of a 9-d-old dark-grown P_D-GUS seedling; Q, root tip of a P_D-GUS seedling grown in darkness for 7 d followed by 48 h of white light; R through T, flowers from P_B-GUS (R), P_D-GUS (s), and P_E-GUS (T) plants; U, anther from a P_E-GUS flower; V through X, young rosette leaves (approximately 1 cm) from P_B-GUS (V), P_D-GUS (W), and P_E-GUS (X) plants.

DISCUSSION

Differential spatial and temporal expression of receptor proteins in multicellular organisms can be an important determinant of their biological activities and, since light is a ubiquitous and highly penetrating environmental signal, it is possible that this is the case for the UV, blue, and R/FR photoreceptors that control plant morphogenesis. The Arabidopsis *PHY* gene family is complex in that it encodes multiple phytochrome forms, some of which are more evolutionarily, structurally, and functionally related than others (for review, see Mathews and Sharrock, 1997). We have compared the expression patterns of the upstream regulatory regions of the *PHYB*, *PHYD*, and *PHYE* genes because

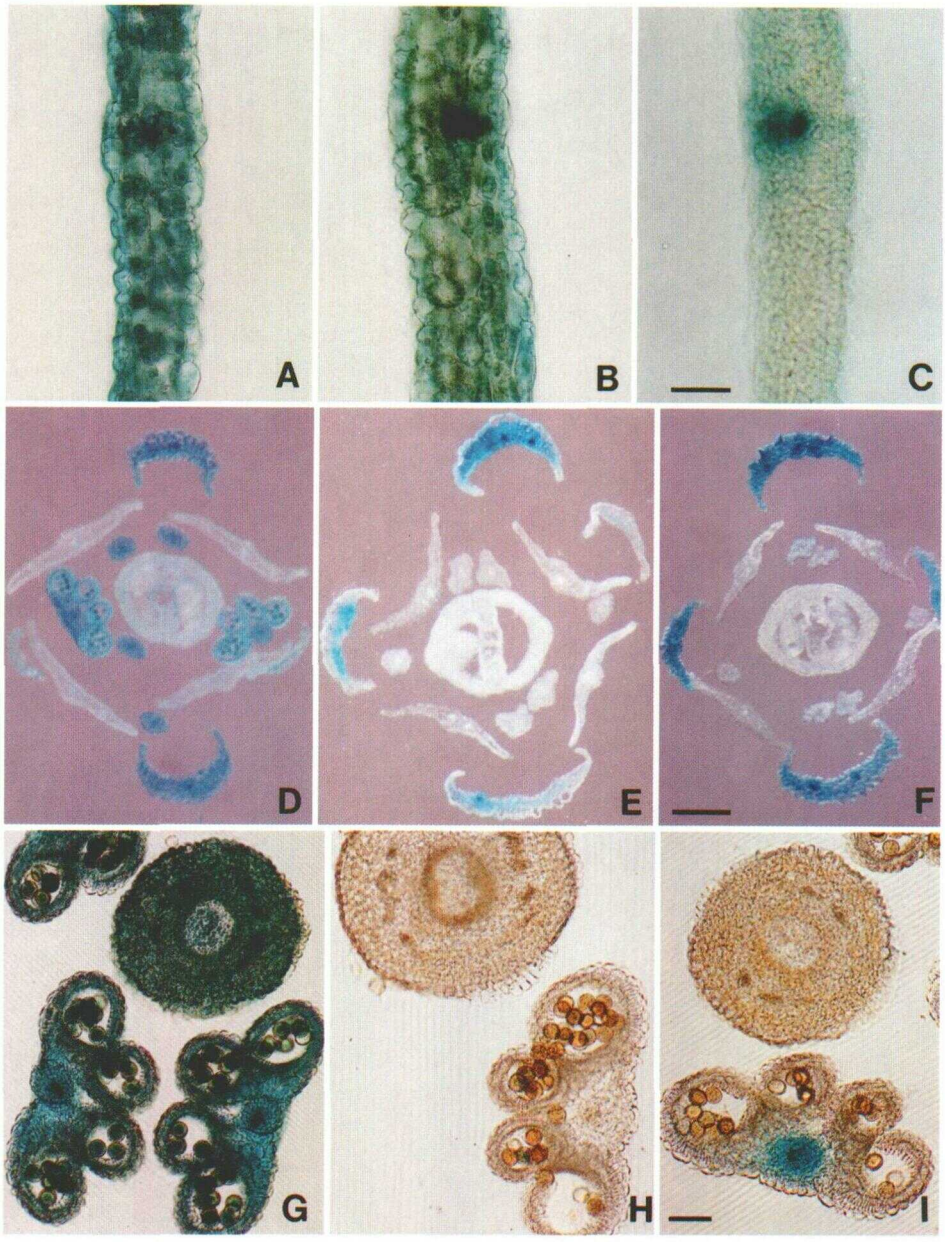

Figure 5. Tissue sections of histochemically stained P_B-GUS, P_D-GUS, and P_E-GUS leaves and flowers. A through C, Young rosette leaves (approximately 1 cm long) from P_B-GUS (A), P_D-GUS (B), and P_E-GUS (C) plants stained for GUS activity, embedded, and transverse sectioned. Scale bar = 50 μm. D through F, Open flowers from P_B-GUS (D), P_D-GUS (E), and P_E-GUS (F) plants stained for GUS activity, embedded, and sectioned through the middle of the flower. Scale bar = 200 μm. G through I, Flowers from P_B-GUS (G), P_D-GUS (H), and P_E-GUS (I) plants stained for GUS activity, embedded, and sectioned through the upper portion of the style and the medial anthers. Scale bars = 50 μm.

they constitute a subgroup of *PHY* genes that have higher sequence similarity to each other than to *PHYA* and *PHYC*, they have the most recent common ancestry among the Arabidopsis *PHY* genes, and, in the case of *PHYB* and *PHYD*, they have similar photosensory functions.

Arabidopsis *PHYB* and *PHYD* encode proteins that are approximately 80% identical in sequence and are examples of relatively recently diverged *PHY* genes. Analysis of the phenotypic effects of *phyB* and *phyD* null mutations and of the interaction of these two mutations has shown that phyB and phyD have highly overlapping functions in controlling R/FR shade-avoidance responses (Aukerman et al., 1997). The *PHYE* gene encodes a protein that is significantly more closely related to phyB and phyD (approximately 55% identity) than to phyA and phyC (approximately 47% identity), but its function is not known. *PHYE* apparently diverged from the *PHYB/D* progenitor gene early in the history of flowering plants but well after the divergence of *PHYA*, *PHYB*, and *PHYC* (Mathews and Sharrock, 1996). The *PHYB*, *PHYD*, and *PHYE* genes also differ from *PHYA*

and *PHYC* in intron number; the Arabidopsis *PHYA* gene contains an intron in its 5′ untranslated region (Dehesh et al., 1994), which *PHYB*, *C*, *D*, and *E* very likely lack (see below), and the Arabidopsis *PHYC* gene lacks a third intron in the coding region that the four other genes contain (Cowl et al., 1994). A comparative analysis of the activities of the *PHYB*, *PHYD*, and *PHYE* promoters has been performed to determine the degree to which the members of this *PHY* gene subgroup differ in expression pattern and whether a correlation can be established between those patterns and the functions of the genes.

The 1.9- to 2.5-kb upstream DNA sequences fused to GUS in these experiments clearly function to drive expression of the *PHY* reading frames, but the regulatory sequences within these promoters have not been defined. Wester et al. (1994) showed that a transgene consisting of the P_B promoter fragment used here driving the *PHYB* cDNA sequence complements a *phyB* mutation at a copy number of one per haploid genome. This indicates that the P_B promoter region contains DNA sequences adequate for expression of *PHYB* gene biological activity. The DNA sequences of the three promoters do not show extensive sequence similarities (R. Sharrock, unpublished data). Previously isolated cDNA clones of the *PHYB* mRNA (Sharrock and Quail, 1989) and 5′ rapid amplification of cDNA ends (RACE) clones of the *PHYD* and *PHYE* mRNAs (Clack et al., 1994) are colinear with genomic sequence (R. Sharrock, unpublished data). Thus, they do not indicate the presence of introns in the 5′ untranslated regions of these genes, as is seen in the *PHYA* gene (Dehesh et al., 1994). Attempts to map the 5′ ends of the *PHYD* and *PHYE* transcripts using primer extension and S1 nuclease protection have not resulted in unambiguous identification of the 5′ start sites (R. Sharrock, unpublished data), perhaps because of the presence of RNA secondary structure. The three translational fusion constructs used here contain codons for between 42 and 66 amino acids from the amino-terminal ends of the phytochromes and also contain the small, upstream reading frames found in the 5′ ends of these mRNAs (Sharrock and Quail, 1989; Clack et al., 1994), so it is expected that any posttranscriptional regulation of these genes mediated by the mRNA 5′ ends is intact. A similar but not identical P_B-GUS fusion transgene was used by Somers and Quail (1995a, 1995b) to investigate the expression and light regulation of that promoter. Results with the P_B-GUS lines presented here are, in general, consistent with their observations and are an essential component of the comparative analysis of the *PHYB*, *PHYD*, and *PHYE* promoters.

The interpretation of the results of promoter-reporter gene fusion experiments is subject to several considerations: whether patterns observed in transgenic lines are due solely to the promoter sequences being tested or also to flanking sequences and genomic interactions at the insertion site of the transgene, whether the histochemical substrate penetrates all organs and tissues being assayed, and whether all sequences important for regulation are present in the 5′ upstream region used in the fusion. With respect to these considerations, in these experiments a large number of independent transgenic lines were first screened and narrowed down to representative lines that exhibit a "consensus" pattern of staining, which is presented here. Table I shows that, although varying in specific activity, the ratios of GUS enzyme activities under different conditions are reproducible among independent lines carrying the same construct. A few primary transgenic lines showed aberrant patterns of staining, but the majority were uniform, indicating that the insertion site did not strongly affect the expression pattern. Several tissue-fixation methods were tested, but the standard method (Jefferson et al., 1987) was found to produce uniform staining of most plant parts in the P_B-GUS lines. That *PHYB* promoter activity is readily detectable in most plant parts makes it very unlikely that the distinctive patterns of expression of the *PHYD* and *PHYE* promoters are due to inaccessibility of the GUS substrate. Finally, these experiments were designed to test only the activities of the 2- to 2.5-kb upstream regions of these genes, and it cannot be excluded that downstream sequences, intron sequences, or distant regulatory elements may significantly alter the patterns in the native genes.

One further consideration is that the GUS-reporter enzyme is relatively stable in plant cells (Jefferson et al., 1987), and the results of promoter-fusion studies very likely do not reflect all posttranslational control of the native gene products. For example, there is strong light-induced proteolysis of phyA phytochrome (Vierstra, 1994) that is probably not accurately reflected in the activity of a *PHYA* promoter-GUS fusion gene (Somers and Quail, 1995b). Though phyB, D, and E are more light-stable than phyA (Somers et al., 1991; J. Tepperman, P. Quail, and R. Sharrock, unpublished data), it should be noted that the staining patterns seen here are representative of the patterns of synthesis of the phyB, D, and E apoproteins in the plant but not necessarily their ultimate distributions.

On the basis of the activity of the P_B-GUS fusion gene, *PHYB* gene expression occurs in seeds and throughout most plant tissues and organs, is induced over the first 7 to 10 d of seedling growth, and is somewhat higher in dark-grown than in light-grown seedlings. In contrast to this, expression of both the *PHYD* and *PHYE* promoters is more restricted in the plant, is not strongly induced during seedling development, and, in the case of the *PHYE* promoter, is higher in the light than in the dark. Hence, among these three *PHYB*-related genes, there is significant heterogeneity in response to both developmental signals and light regulatory mechanisms.

On the basis of the P_D and P_E fusion genes, phyD is synthesized principally in leaf structures—cotyledons, mature leaves, and sepals—and in the root tip, whereas phyE is expressed almost exclusively in cotyledons, leaves, and sepals. It is notable that in very young seedlings both the P_D-GUS and the P_E-GUS transgenes are expressed at low levels in the hook region and upper hypocotyl and as the seedlings mature in either darkness or light expression of these genes is increasingly restricted to leaves. This distribution pattern leaves open the possibility of phyD and phyE having roles in hook opening and hypocotyl elongation responses in young seedlings. Aukerman et al. (1997) have shown that phyD plays a minor but significant role in regulating hypocotyl length as well as cotyledon expansion

in response to R light. GUS activities are present in dry seeds of both P_D-GUS and P_E-GUS transgenic plants and can be histochemically detected in developing seeds (data not shown). Hence, in combination with the data of Somers and Quail (1995b), it is likely that at least four of the five Arabidopsis phytochromes (phyA, B, D, and E) are produced in seeds. However, Shinomura et al. (1996) have shown that phyA and phyB account for most or all of the seed germination response to R and FR light in Arabidopsis.

P_D-GUS and P_E-GUS fusion gene activities are not histochemically detected in dark-grown roots but P_D-GUS activity is induced by R or white light in the root meristem and elongation zone. RNA-blot data of Clack et al. (1994) showed the presence of both *PHYD* and *PHYE* mRNA in light-grown roots, which is inconsistent with the lack of P_E-GUS staining observed here. However, when P_E-GUS plants are grown under the liquid culture conditions used in the RNA experiments, the root tips stain faintly for GUS activity (data not shown). Hence, it is possible that conditions of liquid culture but not growth on solid media induce *PHYE* gene expression in roots. The functions of the apparently root-localized phyA, phyB, and phyD phytochromes are not known, though there is evidence for an effect of a *phyB* mutation on root-hair elongation (Reed et al., 1993), and the induction of expression of the *PHYD* promoter in root tips described here is itself mediated by R light, suggesting phytochrome activity.

The photosensory roles of phyA, phyB, and phyD in Arabidopsis have been defined by the isolation of null mutations in the genes coding for these apoproteins, so an attempt can be made to correlate the expression patterns of these genes with their functions. Somers and Quail (1995b) concluded that because the *PHYA* and *PHYB* promoters are expressed very generally and overlap strongly in pattern, the strikingly different photosensory activities of these receptors is likely not a function of differential localization in the plant. The expression patterns of the *PHYD* and *PHYE* promoters are more distinctive than those of *PHYA* and *PHYB* and suggest activities for these receptors, at almost all stages of plant development, predominantly in leaves. Indeed, the *phyD* mutation described by Aukerman et al. (1997) has some of its strongest effects on the size and shape of cotyledons and leaves. However, it also affects hypocotyl length and, at least in some genetic backgrounds, anthocyanin accumulation in the crown, two regions that show weak or negligible P_D-GUS activity. One variable that may account for these discrepancies is the Arabidopsis ecotype background, which has not been consistent between the mutational studies and these promoter analyses. A final implication of the promoter-fusion experiments derives from the tissue sections, which show that, in leaves in particular, all three of the phyB-related phytochromes are expressed in the same cells, providing a setting in which these receptor forms may interact, possibly by heterodimerization or through shared signal transduction pathways.

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