Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8596–8600, September 1995
Biochemistry

Mutational analysis of phytochrome B identifies a small COOH-terminal-domain region critical for regulatory activity

(regulatory photoreceptor/site-directed mutagenesis/structure–function analysis/regulatory domain)

DORIS WAGNER AND PETER H. QUAIL*

Department of Plant Biology, University of California, Berkeley, Berkeley, CA 94720; and U.S. Department of Agriculture, Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710

Communicated by Eldon H. Newcomb, University of Wisconsin, Madison, WI, June 2, 1995 (received for review April 18, 1995)

ABSTRACT **Overexpression of phytochrome B (phyB) in transgenic *Arabidopsis* results in enhanced deetiolation in red light. To define domains of phyB functionally important for its regulatory activity, we performed chemical mutagenesis of a phyB-overexpressing line and screened for phenotypic revertants in red light. Four phyB-transgene-linked revertants that retain parental levels of full-length, dimeric, and spectrally normal overexpressed phyB were identified among 101 red-light-specific revertants. All carry single amino acid substitutions in the transgene-encoded phyB that reduce activity by 40- to 1000-fold compared to the nonmutagenized parent. The data indicate that the mutant molecules are fully active in photosignal perception but defective in the regulatory activity responsible for signal transfer to downstream components. All four mutations fall within a 62-residue region in the COOH-terminal domain of phyB, with two independent mutations occurring in a single amino acid, Gly-767. Accumulating evidence indicates that the identified region is a critical determinant in the regulatory function of both phyB and phyA.**

Phytochrome is the best characterized of the photoreceptors controlling light-dependent plant development (1–8). Of the five members of the phytochrome family in *Arabidopsis*, phytochrome A to E (phyA to phyE, respectively) (9), two have been well characterized: phyA, which is most abundant in dark-grown tissue and is light-labile, and phyB, which is more abundant in light-grown tissue and is light-stable (10, 11). phyA and phyB have different photosensory specificity in young seedlings. phyA controls deetiolation (hypocotyl elongation, hook opening, cotyledon separation, and expansion) in continuous far-red light (FRc), whereas phyB controls deetiolation in continuous red light (Rc) (12–14).

The phytochromes are dimeric chromoproteins with a covalently attached linear tetrapyrrole chromophore on the NH_2-terminal domain of each subunit (15). Two putative dimerization sites reside on the COOH-terminal domain (16). The biological function of the different phytochrome domains is poorly understood. Overexpression of *in vitro*-generated deletion derivatives of phyA in transgenic plants has shown that the NH_2-terminal domain is necessary and sufficient for chromophore attachment and photoconversion and that regions at the extreme NH_2 and COOH termini are important for normal biological function (17–19). A Ser repeat at the phyA NH_2 terminus is not specifically required for activity as replacement of those residues with Ala resulted in increased activity in transgenic *Arabidopsis* (20). To our knowledge, no similar studies for phyB have yet been reported.

Phytochrome mutations that lead to loss of biological activity without reducing either the levels or spectral activity of the molecules are indicative that the resulting molecules retain normal photoperception function but are defective in the regulatory function that facilitates signal transmission to downstream transduction components. Two recently identified missense mutations in endogenous phyA are candidates for this type of defect (7, 14, 21). One missense mutation has been reported for endogenous phyB (13). The resulting protein is present at parental levels, but spectral activity was not determined, because the low levels of endogenous phyB make spectrophotometric analysis difficult (11). Thus it is not clear which phytochrome function is affected in this case.

We have designed a screen to specifically identify residues important to the regulatory function of phyB that overcomes this limitation. phyB overexpression in transgenic *Arabidopsis* results in strong enhancement of Rc-mediated deetiolation (11). We have mutagenized a homozygous, single-locus, and single-insert transgenic line overexpressing phyB at levels that are readily spectrophotometrically assayable and have screened for tall revertants that maintain parental levels of full-length spectrally active phyB. This screen is anticipated to target changes in the transgene-encoded phyB sequence that cause substantial reduction in activity because phyB levels in this line are at least 20-fold more than the level needed for full inhibition of hypocotyl elongation (11). Although a large ratio of nonsense to missense mutations is expected, the screen should enhance recovery of missense mutations in critical residues. Loss of endogenous phyB activity will not be detected as the phenotypic consequences of phyB overexpression in the wild-type (wt) background are indistinguishable from those in a *phyB* null background (data not shown).

MATERIALS AND METHODS

Mutagenesis. *Arabidopsis* seeds of a homozygous single-locus single-insert *Arabidopsis* phyB-overexpressing line, ABO (11), were mutagenized in 0.3% ethyl methanesulfonate. Approximately 2000 seeds were sown directly onto soil in individual flats. Each flat was considered a M_1 family, averaged 500–1000 plants, and was harvested collectively.

Plant Growth and Revertant Screen. Eighty to 160 mg of M_2 seed was screened per M_1 family as described in ref. 22. Seedlings were grown essentially as described in ref. 17. The plates were screened visually for tall plants after 5 days of growth in Rc (22 μmol per m^2 per s). Six hundred and seventeen tall revertants were isolated in 79 M_1 families. A subset of revertants were screened again in Rc and in FRc (19 μmol per m^2 per s) in the M_3 generation.

Linkage to Transgene. Rc-specific revertants were backcrossed to the wt (ecotype Nössen). The F_1 and F_2 generations were assayed both for inhibition of hypocotyl elongation in Rc and for kanamycin resistance conveyed by the transferred DNA (T-DNA) carrying the phyB transgene. The mutation was considered linked to the transgene when 100% of the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Rc, continuous red light; FRc, continuous far-red light; phyB, phytochrome B; phyA, phytochrome A; wt, wild-type.

*To whom reprint requests should be addressed.

progeny in the F_1 generation and all kanamycin-resistant progeny in the F_2 generation exhibited the revertant phenotype.

Protein Extraction, Immunoblot, and Spectrophotometric Analysis. Crude extracts and native and denaturing gel electrophoresis were as described (11), except centrifugation was at 400,000 × *g* before native electrophoresis. All extracts were loaded on an equal crude protein basis. phyB was visualized with a monoclonal antibody directed to an epitope between amino acid 93 and amino acid 100 on phyB (unpublished data). Twentyfold-concentrated ammonium sulfate extracts were prepared as described in ref. 11. Spectrophotometric analyses ($\Delta\Delta A$ and difference spectra measurements) were performed with the instrumentation described in ref. 11. For $\Delta\Delta A$ measurements, 600 μl of concentrated extract was added to 450 mg of $CaCO_3$. Native gel electrophoresis was performed at 4°C on 4–30% gradient gels (pH 8.0) with 4% stacking gels (pH 8.0). The running buffer was 0.025 M Tris/0.19 M glycine (pH 8.6). Molecular mass was calculated based on migration of native size markers (electrophoresis calibration kit, Pharmacia).

Sequence Analysis of Mutants. Crude plant DNA was prepared as described in ref. 23. The phyB transgene was amplified specifically by using long PCR (24) with primers complementary to the 35S promoter and NOS 3′ terminator regions. The 4-kb PCR product was separated from the primers using QIAquick-spin columns (Qiagen, Chatsworth, CA). One hundred nanograms of the PCR product was sequenced by using the thermocycler and the fmol sequencing kit (Promega).

RESULTS

Isolation of Rc-Specific Tall Revertants. To permit isolation of mutations in the transgene-encoded phyB (cis mutations), we identified and mutagenized a single-locus single-insert phyB-overexpressing *Arabidopsis* line (ABO) (data not shown). We isolated tall revertants in Rc (reduction of the enhanced deetiolation response caused by phyB overexpression). In the M_3 generation, revertants were screened for inhibition of hypocotyl elongation in FRc. Sixty-one percent of all revertants analyzed were Rc- specific (and thus presumably phyB specific). One hundred and one Rc-specific revertants were tested by immunoblot for the presence of full-length overexpressed phyB protein at parental levels (data not shown). Only 6 of 101 Rc revertants were found to have this characteristic (protein$^+$). Backcrosses to the wt and subsequent segregation analysis in the F_2 generation revealed that four of these mutants were linked to the transgene (cis mutations). Two additional protein$^+$ revertants were unlinked and carry recessive second-site (trans) mutations (unpublished data). Of the remaining Rc-specific revertants, 75 were null for the transgene-encoded phyB, 14 resulted in phyB truncations, and 6 had reduced levels of transgenic phyB. These revertants were linked to phyB where tested (data not shown).

Homozygous F_3 progeny of the backcross to wt of the transgene-linked protein$^+$ revertants were examined further for hypocotyl elongation in Rc, FRc, and darkness (Fig. 1). In FRc and darkness, all revertants are not significantly different from ABO and wt, confirming that the phenotype is Rc-specific. Revertants 35-4 and 38-5 exhibit the strongest loss of activity in Rc compared to ABO. Revertant 60-2 also displays significant loss of activity, whereas 37-3 is less drastically affected.

Transgene-Linked Revertants Have at Least Parental Levels of Spectrally Active phyB. Immunoblots of extracts derived from Rc-grown seedlings show the high levels of phyB in the transgene-linked protein$^+$ revertants (Fig. 2*A*). Endogenous phyB levels are undetectable under these conditions (lane 1). The level of phyB in the 35-4 and 38-5 revertants (lanes 4 and 5) is similar to that in the parental ABO line (lane 2), whereas

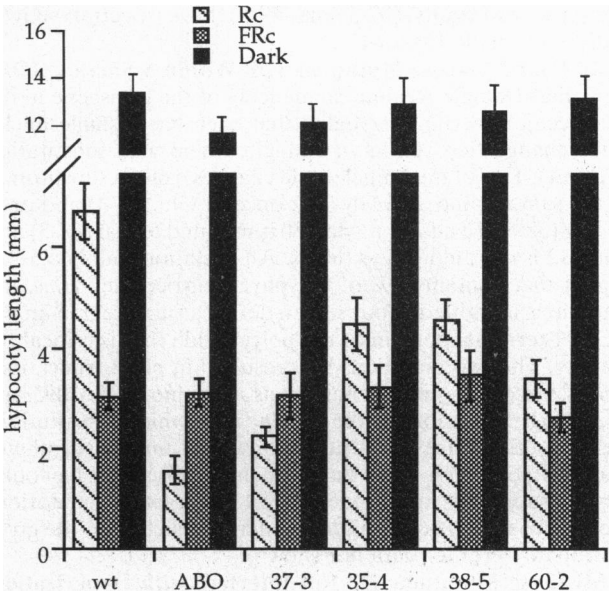

FIG. 1. Revertant phenotype is red-light-specific. Hypocotyl elongation in Rc (22 μmol per m^2 per s), FRc (19 μmol per m^2 per s), and dark was analyzed in four transgene-linked revertants (37-3, 35-4, 38-5, and 60-2), the unmutagenized parental phyB-overexpressing line (ABO), and wt. Mean hypocotyl length was determined for 20 seedlings. Each error bar denotes one standard deviation.

both the 37-3 and 60-2 revertants (lane 3 and 6) show increased levels of phyB compared to ABO. For comparison, two mutations that result in truncated phyB (mutants 37-5 and 49-8) also exhibit a reduction in protein levels (lanes 7 and 8). The amount of spectrally active protein ($\Delta\Delta A$) was determined in concentrated extracts from Rc-grown seedlings (Fig. 2*B*). In Rc the $\Delta\Delta A$ value reflects the amount of overexpressed phyB, as the levels of the endogenous phytochromes drop below the limit of detection under these assay conditions. All putative missense revertants are fully spectrally active. Higher levels of spectrally active protein compared to the parental ABO line are found in the extracts from lines 37-3 and 60-2, in accordance with the immunoblot results (Fig. 2*A*). The putative

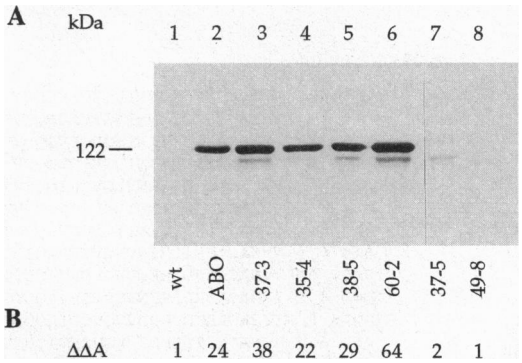

FIG. 2. Transgene-linked revertants contain parental or higher levels of full-length spectrally active transgenic phyB. (*A*) After denaturing electrophoresis, phyB was visualized on immunoblots with a monoclonal antibody that recognizes an epitope near amino acid 100 of phyB (D.W., unpublished data). Sixty micrograms of total protein from crude extracts of Rc-grown seedlings was loaded per lane. Molecular mass of full-length 122-kDa phyB is indicated. (*B*) Spectrally active phytochrome was determined by $\Delta\Delta A$ analysis of concentrated extracts from the Rc-grown seedlings used in *A*. Spectral activity is given in arbitrary units/μg of crude protein. Lane wt represents the untransformed wt (lane 1), and lane ABO is the parental phyB-overexpressing line (lane 2). Revertants (37-3, 35-4, 38-5, 60-2, 37-5, and 49-8) were analyzed in lanes 3–8.

nonsense revertants (37-5 and 49-8) have spectral activity indistinguishable from wt.

All Four Missense Mutations Fall Within a Small COOH-Terminal-Domain Region. Sequencing of the transgene in the Rc-specific revertants revealed that each has a single nucleotide change that results in a single amino acid substitution (Fig. 3*A*). Two of these nucleotide changes cause a substitution in the same amino acid Gly-767, once to Glu (35-4) and once to Arg (38-5). In addition Ala-750 is mutated to Val (37-3) and Glu-812 is mutated to Lys (60-2). All mutations fall in a 62-aa region that contains 5% of the phyB polypeptide. Thus, the mutations identified in our screen define a small region on the COOH-terminal domain of the polypeptide that is critical for activity. The two mutations that resulted in phyB truncations (Fig. 2*A*) carry nonsense mutations at amino acids 1095 and 1136 (Fig. 3*A*). Most of the residues for which substitutions were identified are not strictly conserved among all phytochromes (Fig. 3*B*), even though conservation can be found among subgroups of phytochromes. None of the mutations identified is predicted to alter secondary structure or the gross hydropathy profile (data not shown).

Missense Mutations Do Not Interfere with Dimerization. To assess whether any of the missense mutations in phyB interfere with dimerization, crude extracts from Rc-grown wt, ABO, and the revertants were subjected to native and denaturing gel electrophoresis followed by immunoblot analysis (Fig. 4). Under native conditions, all mutant polypeptides form a single band at 317 kDa indistinguishable from full-length transgenic phyB (Fig. 4 *A* and *B*, lanes 2) and endogenous phyB (Fig. 4*B*, lane 1). The amount of protein detected within a line is comparable for both types of electrophoresis (Fig. 4 *A* and *C*), indicating that the mutant phyB molecules dimerize as efficiently as ABO.

Spectral Properties Are Unaltered in the Mutant Phytochromes. To analyze the spectral properties of the mutant phyB molecules, difference spectra were recorded for wt, ABO, and the missense revertants (Fig. 5). Difference spectra are a sensitive indicator of structural integrity of all domains involved in interaction with the chromophore. The spectra obtained for all phyB-overexpressing seedlings show the same absorption maxima (664 nm) and minima (729 nm) as described (11). Thus none of the single amino acid changes alters the spectral properties of phyB.

Missense Mutations Result in 40- to 1000-Fold Loss of phyB Activity. As none of the phyB mutations cause complete loss of activity compared to wt (Fig. 1), we determined the extent of loss in biological activity of each. All revertants were grown under a range of Rc light fluence rates and compared to wt, the parental ABO line, and other lines overexpressing unmutagenized phyB at different levels (Fig. 6). The weakest revertant (37-3) requires a 40-fold greater fluence rate to obtain the same response (hypocotyl length of 4.2 mm) as in ABO, despite the fact that the phyB levels of this line are 50% greater than those in ABO (Fig. 2*B*). Both 35-4 and 38-5 have phyB activities that are reduced by at least three orders of magnitude by the same criteria (Fig. 6). Revertant 60-2 requires a 150-fold higher fluence rate for equivalent response even though a 2-fold increase in phyB levels is observed in this line relative to ABO. Thus, as predicted, the screen identified few missense mutations, all of which strongly reduce phyB

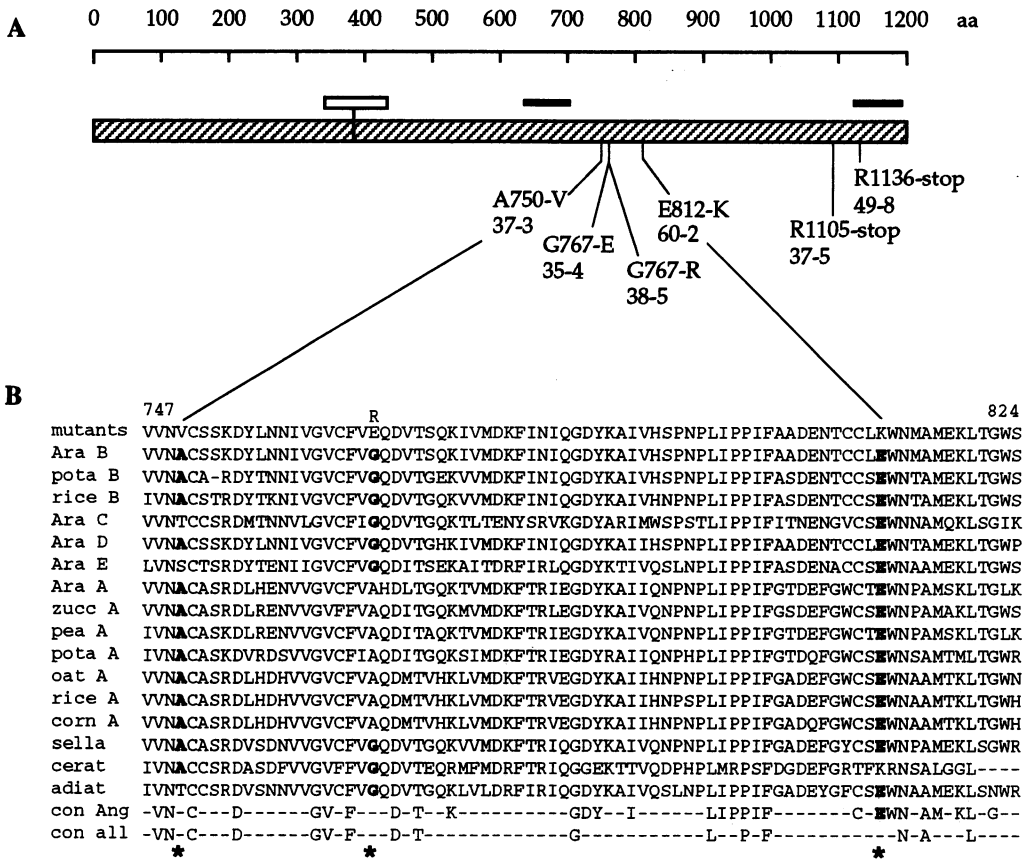

FIG. 3. Location of missense and nonsense mutations in transgenic phyB. (*A*) Schematic map of phyB (hatched box). The positions of the single amino acid changes caused by missense or nonsense mutations and the corresponding revertant line designations are indicated below phyB. The open box at amino acid 380 denotes the linear tetrapyrrole chromophore, and the two solid boxes near amino acids 650 and 1150 denote putative dimerization sites. (*B*) Sequence comparison of 13 angiosperm and 3 lower plant phytochromes in the region where the amino acid substitutions occur. Alignment and nomenclature are according to ref. 28. The missense mutations are indicated in the first line (mutants) and amino acid conservation with phyB at those positions is shown by boldface type. Asterisks indicate the positions of amino acid substitutions.

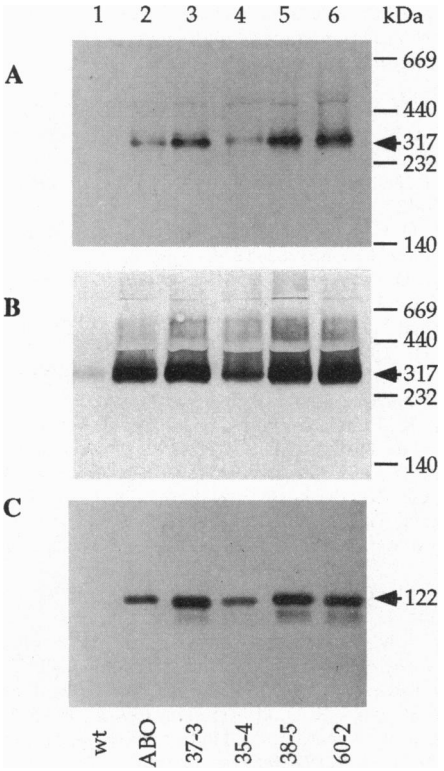

FIG. 4. Missense mutations do not interfere with dimerization. Apparent molecular mass of phyB in each crude extract was determined under native (*A* and *B*) and denaturing (*C*) gel-electrophoresis conditions. Thirty micrograms of crude protein was loaded per lane, except for *B* where 60 μg of crude protein was loaded and the blot was deliberately overdeveloped to visualize endogenous phyB. After transfer to nitrocellulose, phyB was stained with a monoclonal antibody specific to the NH_2 terminus of phyB. No additional bands besides those shown were detected. The untransformed wt and the parental ABO line are shown in lanes 1 and 2, respectively. Lanes 3–6 show the Rc-specific revertants carrying missense mutations (37-3, 35-4, 38-5, and 60-2). Molecular masses of native size markers (tick marks) and calculated molecular mass of phyB (arrows) are indicated.

activity. Revertants exhibiting no residual Rc-enhanced deetiolation were all thus far found to be null for transgenic phyB (data not shown).

DISCUSSION

Mutagenesis of transgenic phyB-overexpressing *Arabidopsis* has resulted in the isolation of four missense mutations that define a small COOH-terminal domain region critical for phyB activity. None of the substitutions identified alter the gross structural characteristics of phyB: the resulting molecules are expressed at or greater than parental levels, form dimers, and show full levels of normal spectral activity. These results indicate that these molecules are normal in photosignal perception, but defective in the regulatory function responsible for activation of downstream signal-transduction pathway components.

Several lines of evidence indicate that the regulatory function of the COOH-terminal domain is conserved between phyB and phyA: (*i*) In recent domain swap experiments, we have shown that the COOH-terminal domains of phyA and phyB, which are necessary for the activity of each photoreceptor (ref. 17; unpublished data), are reciprocally interchangeable and, thus, carry functionally equivalent determinants (D.W., R. M. Kuhn, and P.H.Q., unpublished data). (*ii*) Two missense mutations in phyA (14, 21), which like the phyB mutations studied here result in loss of biological but not

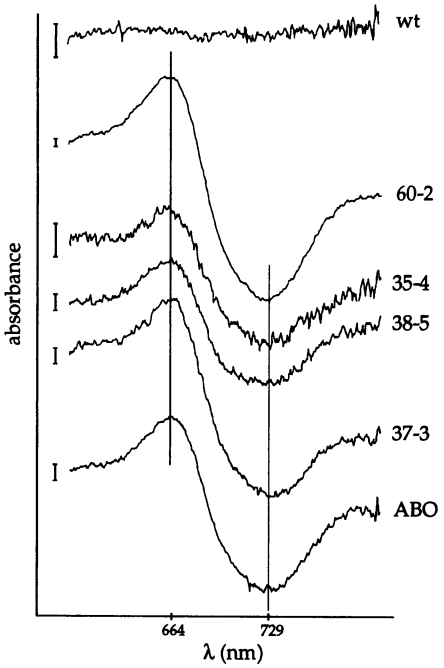

FIG. 5. Missense mutations do not alter the spectral properties of phyB. Difference spectra were recorded for extracts of Rc-grown wt and the unmutagenized parental line (ABO), and the revertants 37-3, 38-5, 35-4, and 60-2. The wavelengths of the maxima and minima are indicated (λ). The bar to the left of each spectrum represents 10^{-3} absorbance units. The spectra were derived from equal volumes of concentrated extract and were not adjusted for equal protein.

spectral activity (7, 14), occur in or near the region identified here (at the equivalent of residues 669 and 762 in phyB). Moreover, 66% of the additional regulatory missense mutations identified recently in phyA (ref. 29; M. Boylan and P.H.Q., unpublished data) also fall in a region that overlaps with the region identified herein. A total of 43% of all

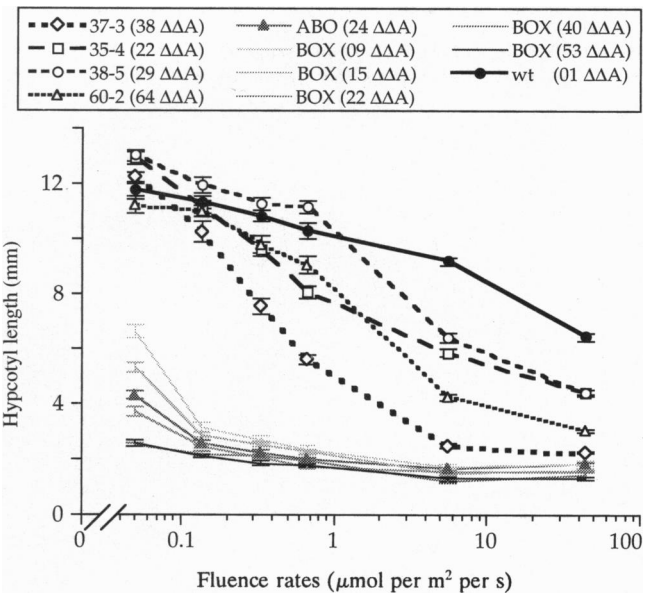

FIG. 6. Biological activity of mutant phyB molecules is reduced by 40- to 1000-fold. Hypocotyl elongation was analyzed for various red light (Rc) fluence rates. The revertants (37-3, 35-4, 38-5, and 60-2) were compared to the parental ABO line, five additional lines overexpressing unmutagenized phyB at different levels (BOX), and the wt. The amount of spectrally active phyB in each line analyzed is indicated (Δ*A*). Each error bar denotes one SEM.

mutations identified for phyB and phyA localize to the 17-aa region defined by 37-3, 35-4, and 38-5, and all residues in this region are mutated multiple times. (*iii*) Replacement of Ala-750 with Val (37-3) was identified in mutants of both phyB (Fig. 3) and phyA (M. Boylan and P.H.Q., unpublished data). Thus, despite the differential photosensory activity and light lability of phyA and phyB, common determinants necessary for regulatory activity may reside within a small region of the COOH-terminal domain of both photoreceptors. This finding suggests that the primary interaction partner of phyA and phyB may be similar in nature. As we were unable to find significant homology between motifs in other proteins in the databases and the critical region identified herein (data not shown), the regulatory activity of phytochrome may involve a distinct biochemical mechanism.

The COOH-terminal domain of phytochrome has previously been implicated in regulatory activity (3, 7, 8) in part based on limited homology of this domain with the transmitting domain of bacterial two-component system receptors (25). However, directed mutagenesis of residues conserved between phytochromes and two-component receptor kinases has no effect on phyA activity (3). The critical region identified here is upstream of the region of potential homology with the two-component receptor kinases. Interestingly, the identified region overlaps with an area (around Lys-788 of phyB) that was highly susceptible to proteolysis only in the Pfr form (26, 27). These data indicate surface exposure of this region in the active form, consistent with the proposed involvement of the subregion in activation of downstream signaling components.

We are grateful to Dr. Margaret Boylan and Dr. Yong Xu for sharing unpublished data, to John Wagner and Dr. David Dailey for critical reading of the manuscript, to Dr. Craig Fairchild for helpful discussions, and to David Hantz and his greenhouse staff for expert tending to our plants. This research was supported by grants from U.S. Department of Energy (FG03-92ER13742) and U.S. Department of Agriculture CRIS (5335-21000-006-00D).

1. Kendrick, R. E. & Kronenberg, G. H. M., eds. (1994) *Photomorphogenesis in Plants* (Kluwer, Dordrecht, The Netherlands), 2nd Ed.
2. Furuya, M. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 617–645.
3. Quail, P. H. (1994) *Curr. Opin. Genet. Dev.* **4,** 652–661.
4. Quail, P. H. (1991) *Annu. Rev. Genet.* **25,** 389–409.
5. Vierstra, R. D. (1993) *Plant Physiol.* **103,** 679–684.
6. Smith, H. (1994) in *Photomorphogenesis in Plants*, eds. Kendrick, R. E. & Kronenberg, G. H. M. (Kluwer, Dordrecht, The Netherlands), 2nd Ed., pp. 377–416.
7. Elich, T. D. & Chory, J. (1994) *Plant Mol. Biol.* **26,** 1315–1327.
8. Jones, A. M. & Edgerton, M. D. (1994) *Semin. Cell Biol.* **5,** 295–302.
9. Clack, T., Mathews, S. & Sharrock, R. A. (1994) *Plant Mol. Biol.* **25,** 413–427.
10. Somers, D. E., Sharrock, R. A., Tepperman, J. M. & Quail, P. H. (1991) *Plant Cell* **3,** 1263–1274.
11. Wagner, D., Tepperman, J. M. & Quail, P. H. (1991) *Plant Cell* **3,** 1275–1288.
12. McCormac, A. C., Wagner, D., Boylan, M. T., Quail, P. H. & Smith, H. (1993) *Plant J.* **4,** 19–27.
13. Reed, J. W., Nagpal, P., Poole, D. S., Furuya, M. & Chory, J. (1993) *Plant Cell* **5,** 147–157.
14. Dehesh, K., Franci, C., Parks, B. M., Seeley, K. A., Short, T. W., Tepperman, J. M. & Quail, P. H. (1993) *Plant Cell* **5,** 1081–1088.
15. Vierstra, R. D. & Quail, P. H. (1986) in *Photomorphogenesis in Plants*, eds. Kendrick, R. E. & Kronenberg, G. H. M. (Nijhoff, Dordrecht, The Netherlands), pp. 35–60.
16. Edgerton, M. D. & Jones, A. M. (1993) *Biochemistry* **32,** 8239–8245.
17. Boylan, M., Douglas, N. & Quail, P. H. (1994) *Plant Cell* **6,** 449–460.
18. Cherry, J. R., Hondred, D., Walker, J. M. & Vierstra, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5039–5043.
19. Cherry, J. R., Hondred, D., Walker, J. M., Keller, J. M., Hershey, H. P. & Vierstra, R. D. (1993) *Plant Cell* **5,** 565–575.
20. Stockhaus, J., Nagatani, A., Halfter, U., Kay, S., Furuya, M. & Chua, N. H. (1992) *Genes Dev.* **6,** 2364–2372.
21. Reed, J. W., Nagatani, A., Elich, T. D., Fagan, M. & Chory, J. (1994) *Plant Physiol.* **104,** 1139–1149.
22. Parks, B. M. & Quail, P. H. (1993) *Plant Cell* **5,** 39–48.
23. Dellaporta, S., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1,** 1442–1447.
24. Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2116–2220.
25. Schneider-Poetsch, H. A. W., Braun, B., Marx, S. & Schaumburg, A. (1991) *FEBS Lett.* **281,** 245–249.
26. Grimm, R., Eckerskorn, C., Littspeich, F., Zenger, C. & Rüdiger, W. (1988) *Planta* **174,** 396–401.
27. Lagarias, J. C. & Mercurio, F. M. (1985) *J. Biol. Chem.* **260,** 2415–2423.
28. Mathews, S., Lavin, M. & Sharrock, R. A. (1995) *Ann. Mo. Bot. Gard.* **82,** 296–321.
29. Xu, Y., Parks, B. M., Short, T. W. & Quail, P. H. (1995) *Plant Cell* **7,** in press.