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Carboxy-Terminal Deletion Analysis of Oat Phytochrome A Reveals the Presence of Separate Domains Required for Structure and Biological Activity

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A series of seven carboxy-terminal deletion mutants of oat phytochrome A were stably expressed in transgenic tobacco to localize phytochrome domains involved in chromophore attachment, spectral integrity, photoreversibility between the red light (Pr)- and far-red light (Pfr)-absorbing forms, dimerization, and biological activity. Amino acids necessary for chromophore attachment in vivo were localized to the amino-terminal 398 residues because mutant proteins this small had covalently bound chromophore. Deletion mutants from the carboxy terminus to residue 653 were spectrally indistinguishable from the full-length chromoprotein. In contrast, further truncation to residue 399 resulted in a chromoprotein with a bleached Pfr absorbance spectrum, Pr and Pfr absorbance maxima shifted toward shorter wavelengths, and reduced Pfr to Pr phototransformation efficiency. Thus, residues between 399 and 652 are required for spectral integrity but are not essential for chromophore attachment. The sequence(s) between residues 919 and 1093 appears to be necessary for dimerization. Carboxy-terminal mutants containing this region behaved as dimers under nondenaturing conditions in vitro, whereas truncations without this region behaved as monomers. None of the plants expressing high levels of deletion mutants lacking the 35 carboxy-terminal amino acids displayed the light-exaggerated phenotype characteristic of plants expressing biologically active phytochrome A, even when the truncated phytochromes were expressed at levels 6- to 15-fold greater than that effective for the full-length chromoprotein. Collectively, these data show that the phytochrome protein contains several separable carboxy-terminal domains required for structure/function and identify a domain within 35 residues of the carboxy terminus that is critical for the biological activity of the photoreceptor in vivo.

INTRODUCTION

Phytochrome is a cytoplasmic photoreceptor that initiates a wide variety of growth and developmental responses in plants. These responses span the plant's life cycle from the germination of seeds to deetiolation and chloroplast development, shade avoidance, flowering, and finally senescence (Kendrick and Kronenberg, 1986). The biologically active molecule consists of a soluble dimer of identical ~120-kD (1100 amino acids) subunits (Vierstra and Quail, 1986; Furuya, 1989; Quail, 1991). Each subunit contains a covalently bound linear tetrapyrrole chromophore that is autocatalytically attached via a thiol-ether linkage to cysteine-322 in the polypeptide (Lagarias and Lagarias, 1989). As a result of a *cis*-to-*trans* isomerization of the chromophore and unique interactions between chromophore and polypeptide, phytochrome can assume two spectrally distinct conformations that are repeatedly photointerconvertible: a red light–absorbing form, Pr (λ_{max} = 665 nm), and a far-red light–absorbing form, Pfr (λ_{max} = 730 nm) (Rudiger, 1986). The photoreceptor is synthesized as Pr, which is both biologically inactive and metabolically stable (half-life [t½] ≥100 hr). However, upon photoconversion of Pr to Pfr by red light, phytochrome becomes biologically active, initiating the diverse array of responses under phytochrome control, and coincidentally becomes metabolically unstable (t½ ~1 hr) (Jabben et al., 1989; Quail, 1991). This ability to interconvert between Pr and Pfr allows phytochrome to act as a light-regulated switch during plant development. Although the molecular mechanism of phytochrome action is still unclear, analysis of a number of phytochrome-mediated responses indicates that alterations of plant gene expression represent an early event in the signal transduction chain (Quail, 1991).

Because the biological activity of phytochrome must initially result from conformational change(s) in the chromoprotein upon photoconversion of Pr to Pfr, much effort has been directed toward characterizing the structure of purified phytochrome and locating domains that change upon photoconversion. Immunochemical and molecular studies have

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established that several types of phytochrome exist that are encoded by a small family of divergent *phy* genes (*phyA* to *phyE* [Sharrock and Quail, 1989; Quail, 1991]). The best characterized of these are the *phyA* gene products that accumulate in etiolated seedlings. Small angle x-ray scattering and various biochemical analyses of proteolytic fragments indicate that phytochrome A consists of two discrete globular domains: a 74-kD region proximal to the amino terminus that binds the chromophore and is spectrally identical to the intact 124-kD chromoprotein, and a 55-kD carboxy-terminal region that contains contact sites for dimerization (Jones et al., 1985; Jones and Quail, 1986; Vierstra and Quail, 1986; Tokutomi et al., 1989). The first ~70 residues of the amino terminus also contain amino acids required for maintaining spectral integrity and conformational stability of Pfr (Vierstra and Quail, 1983). This amino-terminal region as well as other regions throughout the molecule contain residues that change conformation between Pr and Pfr (Lagarias and Mercurio, 1985; Vierstra and Quail, 1986; Grimm et al., 1988).

Continued structural studies on purified phytochrome have been hampered by an inability to generate precisely defined polypeptide fragments or altered polypeptides with specific amino acid substitutions, coupled with the lack of an in vitro assay suitable for assessing the biological activity of the mutant chromoproteins. The availability of phytochrome genes from several plant species has facilitated an alternative approach to analyzing photoreceptor structure/function through their expression in transgenic plants (for review, see Cherry and Vierstra, 1993). The transgenically expressed proteins bind chromophore and are biologically active; when expressed to sufficient levels, they confer a "light-exaggerated" phenotype in several plant species characterized by reduced stem elongation, increased leaf chlorophyll content, decreased apical dominance, and delayed senescence (Boylan and Quail, 1989, 1991; Kay et al., 1989; Keller et al., 1989; Cherry et al., 1991; McCormac et al., 1991; Nagatani et al., 1991; Wagner et al., 1991). By exploiting this system as an in vivo assay of phytochrome function, it is possible to precisely generate phytochrome variants in vitro by using site-specific mutagenesis and to subsequently correlate perturbations in structure with biological activity. This approach permits us to assess the functional importance of specific regions of the molecule and their involvement in many of the physicochemical properties of phytochrome, including dimerization, chromophore–protein interactions, conformational changes between Pr and Pfr, and Pfr stability (Cherry and Vierstra, 1993). It also allows us to extend our studies to dynamics of the photoreceptor that require additional factors within the plant, such as chromophore attachment, Pfr sequestering, and Pfr-enhanced degradation. Recent successes with this approach include the demonstration that the first 69 residues of the amino terminus, previously identified as critical to the spectral integrity of the molecule (Vierstra and Quail, 1986), are also required for its biological activity (Cherry et al., 1992; Stockhaus et al., 1992).

In this study, we have examined the role of the carboxy terminus in phytochrome structure and function by expressing various carboxy-terminal deletions of oat phytochrome A in tobacco. Of interest is the location of the contact site(s) necessary for dimerization within the ~55-kD carboxy-terminal domain (Jones and Quail, 1986) and testing the hypothesis that the carboxy terminus contains sequences necessary for function (Schneider-Poetsch, 1992; Thümmler et al., 1992). From analysis of seven deletion mutants of the protein, several distinct domains were detected that are involved in chromophore attachment, maintenance of spectral integrity, dimerization, and biological activity. Of particular interest is the discovery that removing as few as 35 residues from the carboxy terminus renders oat phytochrome A biologically inactive even though its physicochemical properties appear unaltered in vitro.

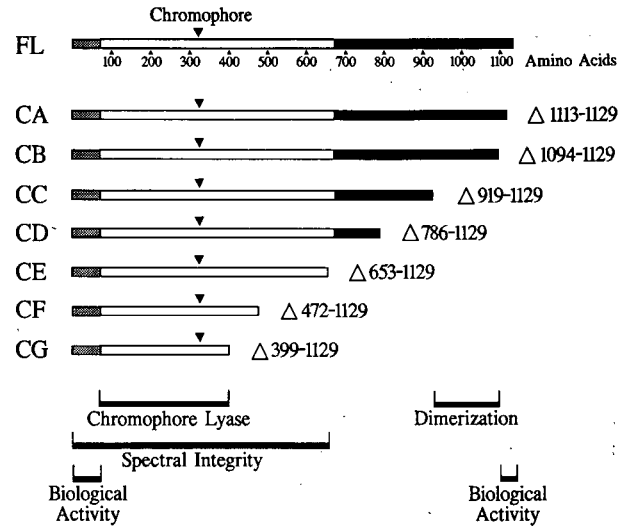

Figure 1. Diagrammatic Representation of the Various Carboxy-Terminal Deletions CA to CG of Full-Length Oat Phytochrome A Used in This Study.

Procedures used for synthesizing the various mutants are described in Methods. Numbering of the amino acids begins with the initiator methionine. The position of cysteine-322 involved in chromophore attachment is identified by an arrowhead. Open boxes denote the domain encompassing a small, 60-kD phytochrome previously characterized (Vierstra and Quail, 1986); shaded boxes denote the regions within 69 residues of the amino terminus previously shown to be required for phytochrome action (Cherry et al., 1992). Additional regions identified in this study as being required for specific phytochrome functions are indicated. FL, full-length oat phytochrome A.

RESULTS

Expression of Carboxy-Terminal Mutants

As a first step in structure/function analysis of the carboxy-terminal portion of phytochrome, a series of seven genes were

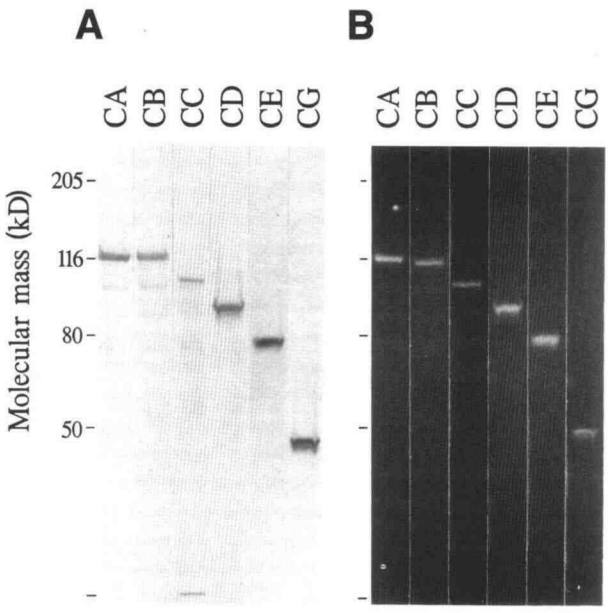

Figure 2. Synthesis of Carboxy-Terminal Deletions (CA to CE and CG) of Oat Phytochrome A in Transgenic Tobacco.

The mutant phytochromes (see Figure 1) were partially purified from etiolated seedlings as Pfr and subjected to SDS-PAGE.
(A) Immunoblot analysis with polyclonal oat phytochrome antibodies.
(B) UV-induced chromophore fluorescence in the presence of Zn^{2+}.

synthesized that encode sequential carboxy-terminal deletions of oat phytochrome A. The genes were generated by removing coding sequence between selected restriction sites within the 3′ end of the coding region and from a unique NdeI site immediately distal to the translational termination codon. The sequences were replaced with a double-stranded oligonucleotide containing translational termination signals in all three reading frames. These deletions were designated CA (Δ1113 to 1129), CB (Δ1094 to 1129), CC (Δ919 to 1129), CD (Δ786 to 1129), CE (Δ653 to 1129), CF (Δ472 to 1129), and CG (Δ399 to 1129), as shown in Figure 1. In most cases, the final construct encoded a protein with several additional amino acids appended to the indicated carboxy-terminal residue (see Methods).

All seven coding regions were placed under the transcriptional control of the cauliflower mosaic virus 35S promoter and stably expressed in tobacco. RNA gel blot analysis indicated that each gene was properly expressed and produced mRNA of the expected size (data not shown). Likewise, immunoblot analysis with the monoclonal antibody Oat-22, specific for oat phytochrome A, revealed that the transgenic lines accumulated truncated phytochrome proteins of the expected mass for each deletion. As shown in Figure 2 and Table 1, these sizes were CA, 122 kD; CB, 120 kD; CC, 102 kD; CD, 86 kD; CE, 72 kD; CF, 52 kD; and CG, 44 kD. Immunoblot analysis of the soluble and particulate fractions of crude extracts indicated that all mutant proteins were soluble as either Pr or Pfr. With the exception of CF, transgenic plants were obtained that expressed each deletion to sufficient levels for most analyses. That plants containing CF expressed relatively high levels of mRNA but low levels of the corresponding polypeptide suggested to us that the CF protein is highly unstable in vivo. The potential reason(s) for this instability is unclear, although it may be related to the absence of a highly conserved, strongly hydrophilic domain centered at residue 540 (Parker et al., 1991; Quail et al., 1991).

Photochemical Properties

Biochemical characterization of the six mutant proteins that were adequately expressed (i.e., CA, CB, CC, CD, CE, and CG) revealed that all were capable of covalently binding chromophore and producing a red light/far-red light–photoreversible product in vivo. Preliminary observations indicated that the various carboxy-terminal deletions, like full-length oat phytochrome A, are degraded more slowly in vivo following red light irradiation than the endogenous tobacco chromoproteins ($t_{1/2} \geq 4$ hr versus 1 hr [data not shown; Cherry et al., 1991]). Thus, to minimize contamination of endogenous tobacco phytochrome in the oat phytochrome preparations (notably CA, CB, and CC), etiolated seedlings were first irradiated for 2 hr with red light prior to extraction. Most of the endogenous tobacco phytochrome was degraded prior to purification as a result of this light treatment, while not significantly affecting the level of the oat proteins. Chromophore attachment was confirmed by detection of Zn^{2+}-induced fluorescence from the various species following SDS-PAGE (Figure 2). The stoichiometry of chromophore bound per polypeptide could not be accurately

Table 1. Native Size of Phytochrome Carboxy-Terminal Deletions

Protein	Size (kD) Nondenaturing[a]	Monomeric Mass[b] (kD)	Ratio
Tobacco	350	120/118	2.9
FL[c]	360	124	2.9
CA	500	122	4.0
CB	450	120	3.8
CC	120	102	1.2
CD	105	86	1.2
CE	85	72	1.2
CF	60	52	1.2
CG	50	44	1.1

[a] Determination was made by size-exclusion chromatography under nondenaturing conditions using either a BioGel A1.5M column or an HPLC Bio-Sil SEC400 column. Phytochrome-containing fractions were determined by immunoblot analysis with monoclonal antibody Oat-22.
[b] Monomeric molecular mass of oat phytochrome A and its various carboxy-terminal deletions were calculated from the derived amino acid sequence of genetic constructions, whereas that of tobacco phytochrome was determined by SDS-PAGE (Keller et al., 1989).
[c] Full-length oat phytochrome A.

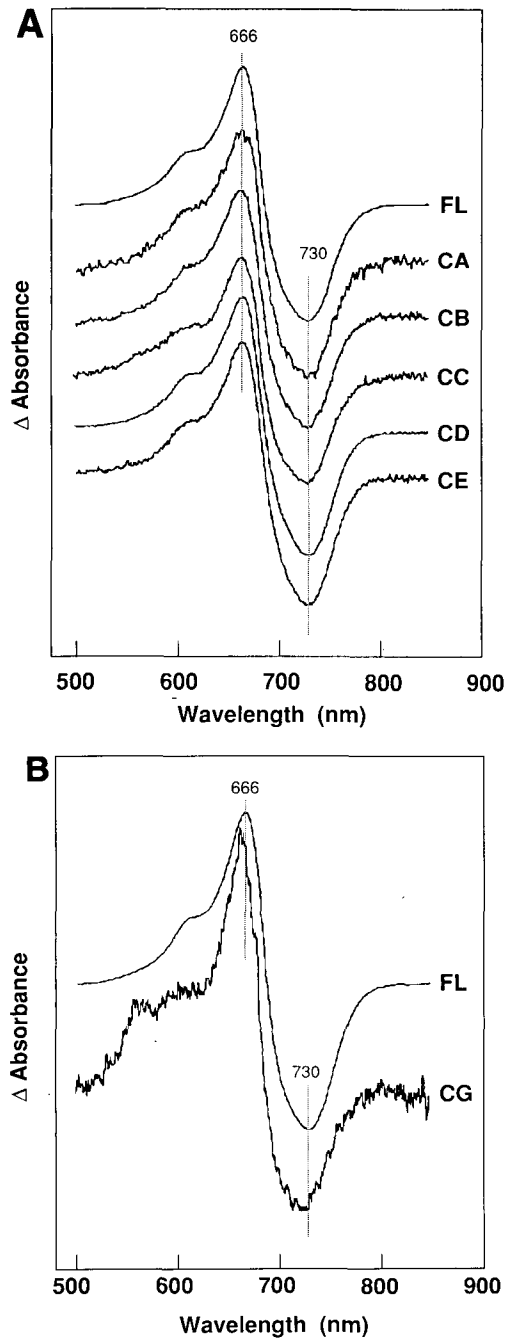

Figure 3. Far-Red Irradiated Minus Red Irradiated Difference Spectra of the Carboxy-Terminal Deletions (CA to CE and CG) of Oat Phytochrome A Expressed in Tobacco.

The mutant phytochromes were partially purified from etiolated seedlings. SDS-PAGE analysis of the samples is presented in Figure 2.
(A) Carboxy-terminal deletions CA to CE.
(B) Carboxy-terminal deletion CG.
The difference spectrum of full-length oat phytochrome (FL) expressed in tobacco and its maxima and minima are included in each panel for comparison.

quantitated, but comparison of signals generated from the immunoblots with those from Zn^{2+} fluorescence demonstrated that even deletion CG was reasonably effective in attaching chromophore in vivo.

As shown in Figure 3, all the truncated chromoproteins were capable of generating Pr and Pfr. Red-minus-far-red difference spectra of CA, CB, CC, CD, and CE were indistinguishable from that of full-length phytochrome having maxima and minima at 666 and 730 nm, respectively, and a spectral change ratio ($A_{666} - A_{730}$) of 1.02 to 1.17 (Figure 3A). In contrast, the difference spectrum of CG was substantially altered; the absorbance maximum and minimum were shifted to shorter wavelengths, and the spectral change ratio was increased to greater than 2 (Figure 3B). Absorbance calculated on an equimolar basis of immunodetectable protein was also reduced for CG, suggesting that the Pr and Pfr extinction coefficients for this 398–amino acid chromoprotein were substantially lower than for its full-length counterpart (see below). Mutant CG was also selectively affected in its ability to phototransform from Pfr to Pr. As seen in Figure 4, the efficiency of phototransformation from Pr to Pfr by red light was similar for all the mutants including CG. However, when comparing the phototransformation efficiency from Pfr to Pr by far-red light, mutant CG was noticeably reduced, requiring almost five times more light than the other mutants CA to CE to achieve 50% conversion.

Dimerization

A number of studies have drawn the conclusion that the carboxy-terminal half (~55 kD) of phytochrome contains a contact site(s) for dimerization between phytochrome molecules (Vierstra and Quail, 1985; Jones and Quail, 1986; Edgerton and Jones, 1992). Size-exclusion chromatography of the seven carboxy-terminal deletions CA to CG allowed further definition of sequences necessary for this quaternary structure (Table 1). Using two different column systems, full-length oat phytochrome and deletions CA and CB behaved as dimers under nondenaturing conditions with apparent molecular masses ranging from 360 to 500 kD. The three- to fourfold difference in the apparent mass of native full-length phytochrome from that calculated from the polypeptide length agreed well with previous determinations (Jones and Quail, 1986; Lagarias and Mercurio, 1985) and structural analyses showing that the intact phytochrome dimer is asymmetric (Jones and Erickson, 1989; Tokutomi et al., 1989). In contrast, deletions CC, CD, CE, CF, and CG exhibited little difference between their apparent molecular mass to the calculated mass, consistent with a monomeric quaternary structure (Table 1). These data define a region between residues 919 and 1094 as potentially important for dimerization.

Biological Activity

The constitutive expression of full-length phytochrome A induces a number of phenotypic changes in transgenic tobacco,

including delayed leaf senescence, increased chlorophyll content, reduced apical dominance, and reduced elongation of both hypocotyls and stems (Keller et al., 1989; Cherry et al., 1991; Nagatani et al., 1991). As shown in Figure 5, selected high-expressing plant lines for each of the deletions, CB, CD, CE, and CG, exhibited none of these characteristics. All of the plants were phenotypically identical to their nontransformed counterparts (wild type), regardless of whether the plants were heterozygous or homozygous for the introduced genes, suggesting that a region within the carboxy terminus is necessary for full biological activity. A similar phenotype was observed for plants expressing CA, CC, and CF, but the low level of chromoproteins that accumulated (less than two times endogenous phytochrome levels) precluded an accurate assessment of their biological activity.

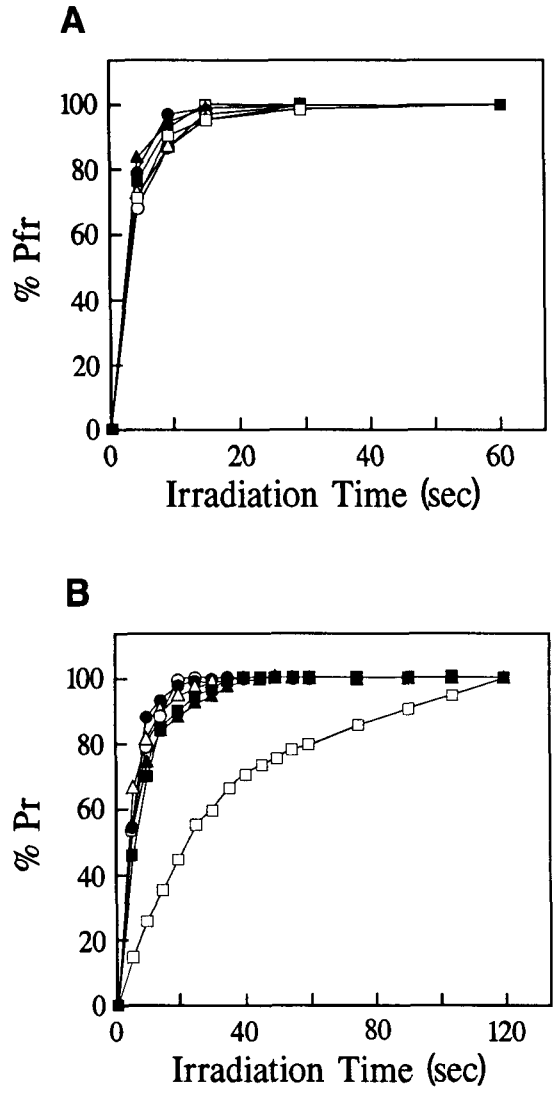

Figure 4. Phototransformation Efficiency of the Carboxy-Terminal Deletions of Oat Phytochrome A.

(A) Photoconversion of Pr to Pfr by red light.

(B) Photoconversion of Pfr to Pr by far-red light.

Each phytochrome was partially purified via size-exclusion chromatography and diluted to 0.05 $\Delta(\Delta A)$/mL. Preparations were exposed to saturating pulses of red or far-red light, followed by repeated 5-sec pulses of either far-red or red light, respectively. Photoconversion was assessed by measuring $A_{666} - A_{730}$ after each pulse and expressed as a percentage of the value obtained after complete photoconversion: CA (○); CB (●); CC (■); CD (△); CE (▲); and CG (□) (see Figure 1).

To more accurately characterize the biological activity of carboxy-terminal deletions of phytochrome A, a phytochrome dose–phenotypic response curve was generated for four of the deletion mutants that expressed sufficient levels of chromoprotein (CB, CD, CE, and CG), as shown in Figure 6. In each case, the phenotypic effects of the deletions were compared to those generated with the active full-length chromoprotein. Three to five independent transformants, maintained as either homozygous or heterozygous lines, were used for each deletion to maximize the range of chromoprotein accumulation. The effect on plant height at maturity was assayed, because it is an easily quantifiable phenotypic alteration (Cherry et al., 1992). (Similar relationships between phytochrome content and either chlorophyll content or growth of axillary meristems were also observed [data not shown].) The level of chromoprotein was measured from the mature plants either by red-minus-far-red difference spectroscopy (which includes both oat and tobacco phytochromes) or by sandwich ELISA using the monoclonal antibody Oat-22, which is specific for oat phytochrome A (Cherry et al., 1992). Spectroscopic and immunochemical quantitation of oat phytochrome in plants expressing deletions CB, CD, and CE were similar, whereas a wide discrepancy existed for the values obtained for CG; in this case, the levels measured immunologically were far greater than those measured spectroscopically (Figure 6). This difference reflects the previously described spectral aberrations of the CG chromoprotein, including its reduced extinction coefficient (especially for Pfr, see above). It also likely reflects the possibility that not all CG proteins contained chromophore.

As observed previously, there was a strong nonlinear relationship between plant height and amount of full-length oat phytochrome A (Figure 6; Cherry et al., 1992). Light-grown transgenic plants containing as little as two times more spectrally detectable full-length phytochrome than wild-type plants showed dramatic reductions in height, growing only one-quarter as tall (Figure 6). Additional increases of chromoprotein, up to 20 times that of wild-type plants, had no additional effect, indicating that the response of plants was insensitive to further increases in Pfr. Immunochemical quantitation showed that the increase in spectrally detectable phytochrome was completely accounted for by the introduction of full-length oat phytochrome A and not by elevated levels of tobacco photoreceptor. In contrast, when the carboxy-terminal mutants CB, CD, CE, and CG were analyzed, none was effective in reducing plant height (Figure 6). The plants grew as tall as wild-type tobacco despite containing levels of chromoprotein that were well in excess of those effective with full-length phytochrome.

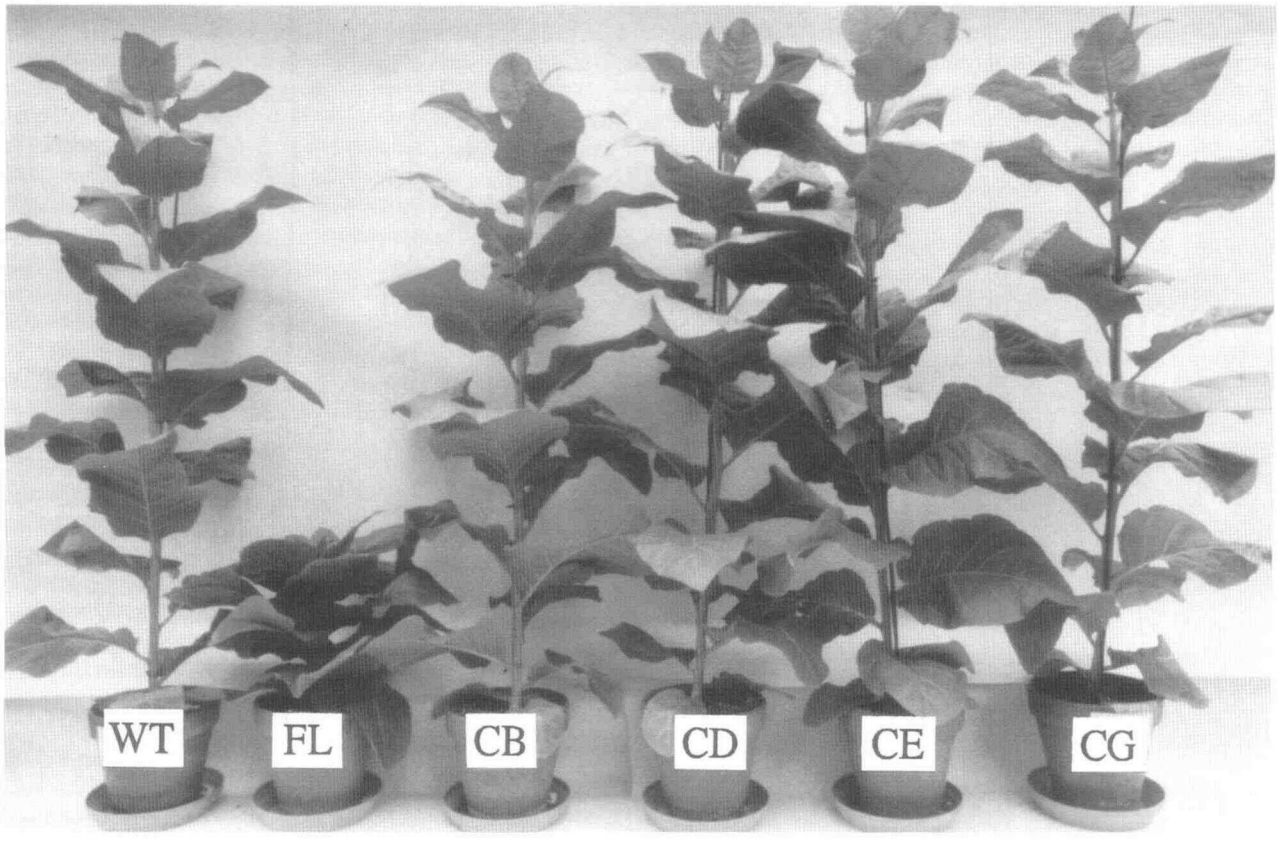

Figure 5. Phenotypes of Xanthi Tobacco Expressing High Levels of Various Carboxy-Terminal Deletions of Oat Phytochrome A.

Plants were grown to maturity under natural light/dark cycles in a greenhouse. WT, nontransformed wild-type plant; FL, plants expressing full-length oat phytochrome; CB, CD, CE, and CG, plants expressing carboxy-terminal deletions of oat phytochrome (see Figure 1).

For deletions CB, CD, CE, and CG, the highest levels of immunochemically detectable protein represented 15, 12, 6, and 10 times, respectively, the level needed by full-length phytochrome for saturating the phenotypic response (~4 μg/g protein). The lack of biological activity for CB was surprising, considering that this deletion is missing only 35 residues from the carboxy-terminal end of the protein.

DISCUSSION

In this report, we provide a detailed analysis of the contribution of the carboxy-terminal end of phytochrome A to the structure and function of the photoreceptor. Essential regions required for chromophore attachment, photoreversibility between Pr and Pfr, spectral integrity, dimerization, and biological activity were localized (see Figure 1). However, we note that the deletions chosen in this study were somewhat arbitrary and, thus, do not necessarily reflect the boundaries of each domain. Like other complex proteins, we found that at least some of the biochemical functions (i.e., dimerization and spectral integrity) can be removed through molecular genetic dissection without affecting other functions. In conjunction with similar recent analyses of the amino terminus (Boylan and Quail, 1991; Cherry et al., 1992; Stockhaus et al., 1992), these data confirm previous biochemical studies on proteolytic fragments of phytochrome (Vierstra and Quail, 1986); namely, this large chromoprotein can be subdivided into specific regions that each contribute distinct functions to the holoprotein. For example, at least 70 residues can be removed from the amino terminus and 476 residues from the carboxy terminus without substantially altering the photochemical properties of Pr and Pfr. The ability to dissect the polypeptide into discrete functional regions using in vitro mutagenesis should greatly facilitate further refined mapping of various structural domains.

The ability of carboxy-terminal phytochrome deletions as small as CG to bind chromophore in vivo indicates that the chromophore lyase activity responsible for attaching the linear tetrapyrrole to cysteine-322 is, for the most part, contained within the first 398 residues. These data refine previous studies on pea phytochrome A protein expressed in yeast in which

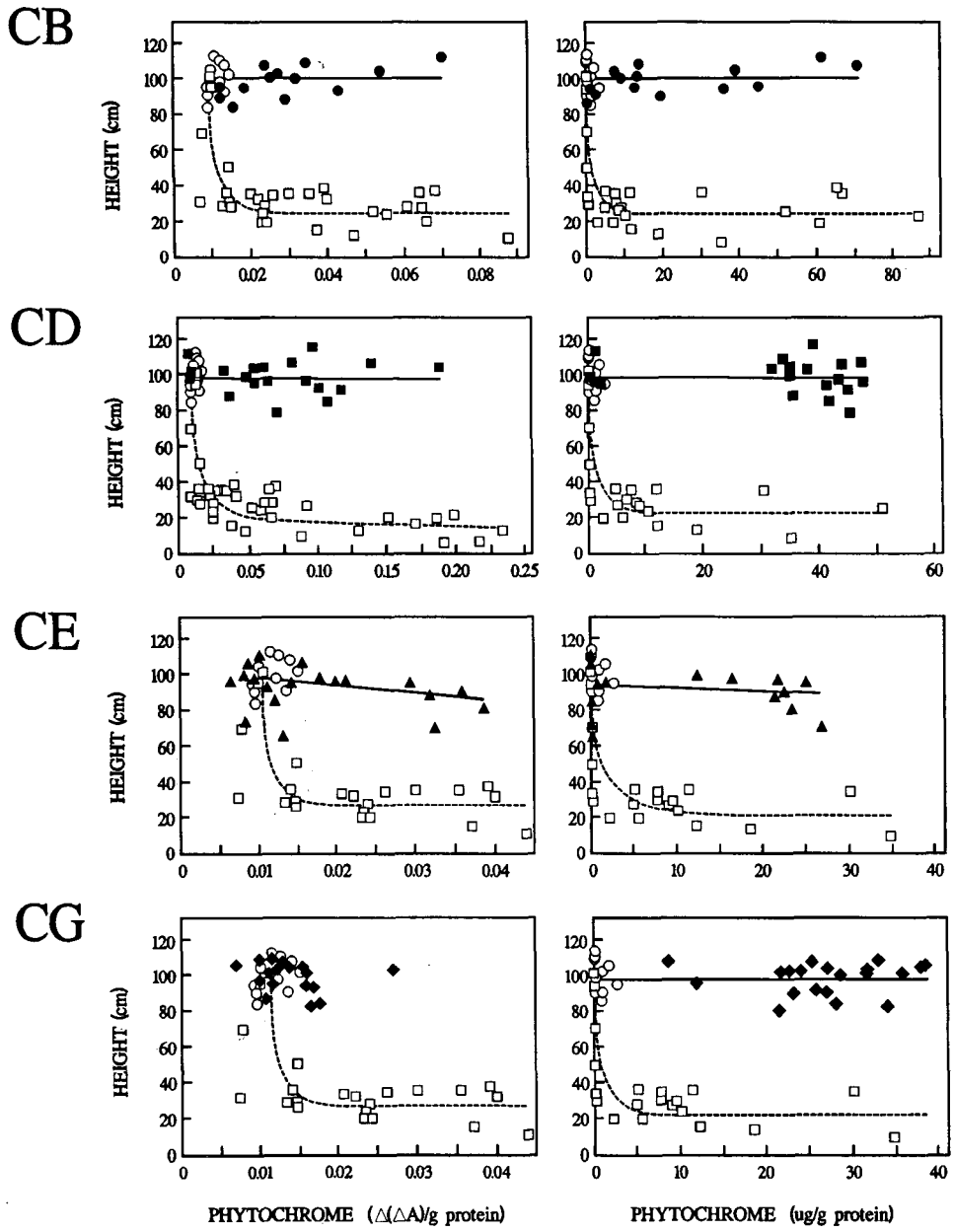

Figure 6. Phenotypic Response of Xanthi Tobacco to Increasing Levels of Full-Length Oat Phytochrome A or Various Carboxy-Terminal Deletions.

The carboxy-terminal deletions include CB (Δ1094 to 1129, ●), CD (Δ786 to 1129, ■), CE (Δ653 to 1129, ▲), and CG (Δ399 to 1129, ♦) (see Figure 1). Nontransformed wild-type plants, (○); full-length oat phytochrome A, (□). Transgenic plants expressing various levels of the full-length oat phytochrome or mutant chromoproteins were grown simultaneously to maturity under natural light/dark cycles. After detection of flower primordia (72 days), leaf tissue was collected from each plant and phytochrome was extracted and concentrated by ammonium sulfate precipitation. Plant height was plotted versus phytochrome content measured either by red-minus-far-red difference spectroscopy ($\Delta(\Delta A)$, which includes both the introduced oat chromoprotein and the endogenous tobacco phytochromes (panels at left) or by sandwich ELISA using monoclonal antibody Oat-22, which is specific for oat phytochrome (panels at right). Portions of the phytochrome dose–phenotypic response curve obtained with full-length oat phytochrome A are included in each panel for comparison.

carboxy-terminal deletions to residue 548 were still capable of binding chromophore in vitro (Deforce et al., 1991). These observations, along with the fact that amino-terminal deletions Δ7 to 69 of oat phytochrome A can bind chromophore in vivo, whereas pea phytochrome A deletion Δ4 to 222 cannot bind in vitro (Deforce et al., 1991; Cherry et al., 1992), lead us to conclude that the minimal sequences necessary for the chromophore lyase activity must reside between amino acids 70 and 398.

Most of the spectral properties of phytochrome, including its unique photoreversibility, are a result of interactions between the chromophore and the protein (Rudiger, 1986; Parker et al., 1991). The spectral properties of phytochrome A remain unaltered despite removal of 476 residues from the carboxy terminus, indicating that most, if not all, amino acids involved in this interaction must be contained within the amino-terminal 672 residues (deletion CE). Previously, one important region has been mapped to the extreme amino terminus. A deletion of only 62 amino-terminal residues (Δ7 to 69) was sufficient to alter the absorbance maximum of Pfr and increase the rate at which Pfr reverts nonphotochemically to Pr (Cherry et al., 1992). Our results, indicating that residues 1 to 672 define a minimal region required for maintenance of spectral integrity, are in agreement with previous studies by Jones et al. (1985) using the purified photoreceptor. They showed that proteolytic removal of ~55 kD from the carboxy terminus to generate a small, 74-kD phytochrome had little or no effect on the spectral properties of the truncated chromoprotein. Although we were able to generate a photoreversible chromoprotein with only 398 residues (CG), this deletion exhibited a blue shift in the absorbance spectra for both Pr and Pfr and an impaired Pfr–Pr phototransformation efficiency. This defines a region between residues 399 and 672 as essential for proper chromophore–protein interactions. The spectral properties of CG were similar to those of a 39-kD chromoprotein generated by proteolytic digestion of pea and oat phytochrome A (Yamamoto and Furuya, 1983; Reiff et al., 1985). Like CG, this species exhibits a substantially bleached spectrum for Pfr and a shift in the Pr maximum to shorter wavelengths. At present, its location in the full-length polypeptide is not known, but it is likely that the fragment substantially overlaps with that of the CG chromoprotein.

Size measurements of various proteolytic fragments of purified phytochrome A revealed that the site or sites required for phytochrome dimerization reside in the carboxy-terminal 55 kD of the protein. From modeling studies of phytochrome sequences, the contact sites have been proposed to be between residues 730 and 810 (Romanowski and Song, 1992). To empirically locate these contact sites, Edgerton and Jones (1992) scanned the carboxy-terminal sequence of oat phytochrome A for domains that would facilitate dimerization when fused to a monomeric λ repressor fragment. They identified two regions encompassing residues 623 to 673 and 1049 to 1129 as potentially important in phytochrome–phytochrome interactions. In contrast, our present study using size measurements of native chromophore-containing, carboxy-terminal deletions has identified a carboxy-terminal region between residues 919 and 1093 that is essential for phytochrome dimerization. The combined data of the two analyses suggest that a region between residues 1049 and 1094 is necessary but not sufficient for dimerization by itself and that a second site between residues 623 and 673 may also be required. Notably, the domain between 1049 to 1094 is predominantly hydrophilic and, thus, is likely to be present on the surface of the chromoprotein (Hershey et al., 1985). Neither of these domains coincides with the predicted location of such contact sites using helical hydrophobic moment analysis (Romanowski and Song, 1992).

With respect to the role of the carboxy terminus in the biological activity of phytochrome A, we found that removal of as little as 35 amino acids from the carboxy terminus renders the molecule nonfunctional. By all criteria measured, deletion CB is biochemically indistinguishable from the intact chromoprotein, behaving as a dimer, having unaltered Pr and Pfr absorbance spectra, and showing equivalent efficiency in the phototransformation between Pr and Pfr. Therefore, the CB deletion would seem to be inactive not because of general physicochemical perturbations of the molecule (including the addition of three amino acids to the carboxy terminus as a result of its construction), but because it is missing an essential component of the active site. Clearly, our results indicate that the carboxy terminus of phytochrome contains sites necessary for biological activity beyond those needed to maintain the quaternary structure of the chromoprotein. They strengthen the recent hypothesis that the carboxy terminus has a catalytic activity based on its amino acid sequence homology with a group of bacterial sensor proteins that act as protein kinases (Schneider-Poetsch, 1992; Thümmler et al. 1992). While the 35–amino acid stretch identified here is within the proposed catalytic domain, it is distal to the specific residues proposed to be directly involved in protein phosphorylation. In our results, together with those of Cherry et al. (1992), two domains involved in biological activity have been located, one within the first 69 amino acids of the amino terminus and another within the final 35 residues of the carboxy terminus (Figure 1). However, we think it is unlikely that these are the only biologically essential regions and expect that other essential regions will be identified as functional mapping of phytochrome progresses.

The biological function of the carboxy-terminal domain is unclear. Interestingly, despite its importance, its amino acid sequence is not well conserved when compared to various phytochromes (A, B, and C) from other species (Quail et al., 1991). Even though the domain is predicted to be hydrophobic and not to reside on the surface, it is readily accessible to proteolytic attack, especially in the Pr form (Grimm et al., 1988). This Pr-enhanced sensitivity is reminiscent of that for the extreme amino terminus which rapidly loses ~6 kD when digested as Pr with purified or endogenous proteases (Vierstra and Quail, 1983; Lagarias and Mercurio, 1985). This similar sensitivity could indicate that the two domains are in close proximity to each other in the holoprotein and undergo a conformational change in relation to each other during

photoconversion of Pr to Pfr, making both less accessible to proteolytic attack as Pfr. Clearly, more refined mapping of this region is essential to identify critical residues. Initial attempts in this direction have been hampered by our inability to generate transgenic plants expressing sufficient quantities of CA phytochrome for phenotypic analysis.

In our phenotypic characterization of the various amino- and carboxy-terminal mutants, it is interesting to note that none of the mutants exhibited any dominant negative effects on the function of the endogenous tobacco photoreceptor (Cherry et al., 1992; this report). One would expect such dominant negative effects to be manifested by an increase in tobacco stem length as the amount of mutant phytochrome is increased. Under the assumption that Pfr stably interacts directly with a signal transduction component, it is reasonable to expect that some inactive forms could compete with the functional photoreceptor for such interactions or that cross-dimerization with active forms could render the whole complex inactive (Cherry and Vierstra, 1993). This lack of interference is especially surprising for CB, which appears biochemically indistinguishable from the full-length active molecule. It is possible that the amounts of inactive forms in specific photoresponsive cells were insufficient to elicit such inhibitory effects. However, it may also imply that phytochrome works not by stable interactions with other factors, but through more transient interactions or by enzymatically generating its own signal molecule.

METHODS

Construction of Transgenic Plant Lines

The oat *phyA* gene used in this study was derived from plasmid pBinphyt (Cherry et al., 1992) that includes a chimeric, intronless gene containing the entire phytochrome coding region and ~1 kb of 3′ untranslated sequence under the control of the cauliflower mosaic virus 35S promoter. The various carboxy-terminal deletions were created by removing coding sequence from selected restriction sites within the 3′ end of the gene to a unique NdeI site present 18 bp downstream of the natural stop codon. When necessary, the gaped plasmid was rendered blunt ended, and a double-stranded, blunt-ended oligonucleotide (CTAGTCTAGACTAGCTAGTCTAGACTAGCTAGTCTAGAGGATCCCC), which was designed to terminate translation in all three reading frames, was ligated in place of the deleted coding sequence. Final deletions contained a truncated protein coding sequence terminated by an artificial stop codon followed by the oat *phyA* 3′ untranslated sequence. Codons missing from the deletion mutants (with numbering starting at the initiator ATG) as well as the restriction enzyme used to make the deletion are as follows: CA, Δ1113 to 1129 (HincII); CB, Δ1094 to 1129 (XbaI); CC, Δ919 to 1129 (XbaI); CD, Δ786 to 1129, (HincII); CE, Δ653 to 1129 (HincII); CF, Δ472 to 1129 (EcoRV); and CG, Δ399 to 1129 (HincII) (see Figure 1). After generation, the predicted coding regions were verified by DNA sequence analysis of the 3′ end of each deletion.

Because translation termination for each phytochrome deletion typically did not occur precisely at the deletion–termination oligonucleotide junction, most of the initial translation products contained a few additional amino acids appended to the carboxy terminus. The additional amino acids, expressed in the single letter code, are: CA, +GIL; CB, +GIL; CC, +GIL; CD, +GIL; CE, no additions; CF, +GDPLD; and CG, +GDPLD.

Transgenic tobacco (*Nicotiana tabacum* cv Xanthi) lines were created by Agrobacterium-mediated transformation with the Ti plasmid vector BIN19 containing either full-length phytochrome or carboxy-terminal deletion mutants (CA to CG) as previously described (Cherry et al., 1992). Kanamycin-resistant plants were screened for the presence of the 124-kD full-length or truncated phytochrome proteins by immunoblot analysis using polyclonal anti-oat phytochrome antibodies. Six independent transgenic plant lines expressing various levels of full-length phytochrome and at least two independent lines of plants expressing each deletion were used in this study. Progenies of initial transformants homozygous for the inserted gene were identified by their ability to completely retain oat phytochrome A expression in subsequent generations. Heterozygous plants used in the generation of the phytochrome dose–phenotypic response curves were the progenies of initial transformants. Plants were grown from seed in the greenhouse under natural diurnal light/dark cycles.

For physicochemical analyses, the various phytochromes were purified from etiolated tobacco seedlings. They were grown aseptically in total darkness for 5 to 6 days at 25°C on 1.5% agar in Murashige-Skoog media (Gibco–Bethesda Research Laboratories) covered with sterile cellophane. Plants expressing the deletion mutants were exposed to red light for 2 hr just prior to harvest.

Phytochrome Extraction and Analysis

Biochemical studies with etiolated tobacco utilized whole seedlings, whereas those with green plants used the youngest fully expanded leaves of mature plants. Tissue was rapidly frozen at liquid N_2 temperature and pulverized in a mortar with pestle while frozen. Phytochrome (as Pfr) was extracted at 4°C in an appropriate dilution of homogenization buffer (1 × HB is 50% [v/v] ethylene glycol, 100 mM Tris-HCl [pH 8.2, 4°C], 140 mM ammonium sulfate, 10 mM Na_4EDTA). Green tissue was homogenized in three-quarter strength HB at a ratio of 2 mL/g fresh weight of tissue, and etiolated tissue was homogenized in two-third strength HB at a ratio of 3 mL/g fresh weight of tissue. Just prior to use, phenylmethylsulfonyl fluoride (PMSF) and sodium metabisulfite were added to the diluted HB (final concentrations of 4 and 20 mM, respectively), and the pH was adjusted to 8.2. Extracts were clarified at 48,000*g*, stirred with polyethyleneimine (0.1% [v/v] final concentration), and reclarified at 48,000*g*. Protein was precipitated by addition of ammonium sulfate and collected by centrifugation at 48,000*g*. The amount of ammonium sulfate used varied with the size of the deletion: full-length phytochrome, CA, and CB, 0.25 g/mL; CC, CD, and CE, 0.32 g/mL; CF, 0.34 g/mL; and CG, 0.35 g/mL.

The phytochrome-containing precipitate was resuspended in 25% ethylene glycol, 50 mM Tris-HCl, 5 mM Na_4EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF (pH 7.8, 4°C) (Vierstra and Quail, 1983) and subjected to size-exclusion chromatography with either a BioGel A1.5M column (2.5 × 96 cm, Bio-Rad) or an HPLC Bio-Sil SEC400 column (7.8 × 300 mm, Bio-Rad). The BioGel A1.5M column was equilibrated in 50 mM Tris-HCl (pH 7.8, 4°C), 1 mM Na_4EDTA, 150 mM NaCl, and 7 mM 2-mercaptoethanol, whereas the HPLC column was equilibrated in 50 mM Tris-HCl (pH 7.0, 4°C), 5 mM EDTA, 150 mM NaCl, and 10% (v/v) glycerol. Peak fractions, as determined by immunoblot analysis with monoclonal antibody Oat-22 (Cherry et al., 1991), were pooled and precipitated by the addition of 0.35 g/mL ammonium sulfate. This partially purified phytochrome was resuspended in 5% glycerol, 50 mM potassium phosphate, 14 mM 2-mercaptoethanol, and 2 mM PMSF

(pH 7.8, 4°C) prior to spectrophotometric characterization. Size-exclusion columns were calibrated with Sigma high molecular mass protein standards consisting of thyroglobulin (669 kD), apoferritin (443 kD), α-amylase (200 kD), alcohol dehydrogenase (150 kD), albumin (66 kD), and carbonic anhydrase (29 kD).

Spectrophotometric analyses were performed with a dual-wavelength spectrophotometer (model UV3000; Shimadzu, Kyoto, Japan). Phytochrome content ($\Delta(\Delta A)$) was determined by dual-wavelength difference spectroscopy following saturating red (PR) and far-red (Pfr) light irradiations. Phytochrome isolated from etiolated tissue was assayed by measuring $\Delta(\Delta A_{666} - \Delta A_{730})$. Phytochrome from green tissue was assayed by measuring $\Delta(\Delta A_{730} - \Delta A_{800})$ and converted to $\Delta(\Delta A_{666} - \Delta A_{730})$ equivalence (Cherry et al., 1991). Protein was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard after trichloroacetic acid precipitation of total protein from clarified crude extracts.

The efficiency of phototransformation from Pr to Pfr and from Pfr to Pr was determined with phytochrome preparations partially purified through the gel filtration step. Pr and Pfr preparations, diluted to 0.05 $\Delta(\Delta A)$/mL, were exposed to saturating pulses of red or far-red light, followed by repeated 5-sec subsaturating pulses of either far-red or red light, respectively. Photoconversion was assessed by measuring $A_{666} - A_{730}$ after each pulse until complete photoconversion.

Visualization of Chromoproteins

Chromophore-containing phytochrome was detected by zinc acetate–induced UV fluorescence according to the method of Berkelman and Lagarias (1986), with some modifications. Proteins were separated by SDS-PAGE on 7 or 8% acrylamide gels (Laemmli, 1970) and transferred onto a polyvinylidene difluoride membrane. Membranes were soaked for 2 hr in 1 M zinc acetate in the dark, and biliprotein-containing proteins were visualized by fluorescence during excitation with 302 nm light and photographed using a red filter (Hoya 25A).

Immunological Techniques

Polyclonal phytochrome antibodies were raised in rabbits against etiolated oat phytochrome and purified by affinity chromatography (Shanklin et al., 1987). Monoclonal antibody Oat-22, which reacts exclusively with oat phytochrome and has been described by Cordonnier et al. (1985), was affinity purified from culture supernatants using protein A Sepharose. Immunoblot analysis was performed using polyclonal or Oat-22 antibodies in conjunction with alkaline phosphatase–labeled goat anti-rabbit or goat anti-mouse immunoglobulins, respectively (Kirkegaard and Perry Laboratories, Gaithersburg, MD) (Cherry et al., 1992). Immunoreactive species were detected using the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Oat phytochrome content in transgenic tobacco plants was quantitated in triplicate by sandwich ELISA (Cherry et al., 1992). Polyclonal oat phytochrome antibodies (100 μL per well of a 0.02 mg/mL solution) were incubated overnight at 22°C in microtiter plates (Immulon II; Dynatech Labs, Chantilly, VA). Plates were then rinsed twice with TBS (10 mM Tris-HCl, pH 7.8, 0.9% NaCl, 0.02% NaN_3) and incubated at 22°C for 2 hr in block solution (TBS plus 1% bovine serum albumin). Ammonium sulfate–purified phytochrome extracts (as Pfr) were diluted 10-fold in TBS and incubated in blocked wells for 1.5 hr at 22°C in the dark. After washing four times with ELISA wash buffer (10 mM $KHPO_4$, pH 7.5, 150 mM NaCl, 0.05% Triton X-100, 0.02% NaN_3), 50 μL of Oat-22 antibody solution (0.017 mg/mL) was incubated in each well for 1.5 hr. Wells were then washed four times with ELISA wash buffer and incubated for 15 min in block solution. Alkaline phosphatase–labeled goat anti-mouse immunoglobulins (50 μL of 0.01 mg/mL solution) were then added and incubated for 1.5 hr. After washing, 50 μL of a development solution containing 10 mg/mL *p*-nitrophenyl-phosphate in 10 mM ethanolamine, pH 9.5, 0.5 mM $MgCl_2$, 0.02% NaN_3 was added to each well. Color development was quenched by the addition of 50 μL of 0.1 M Na_4EDTA and absorbance at 405 nm was determined using a microplate reader. Purified 124-kD oat phytochrome (Vierstra and Quail, 1983) was used as a standard and was quantified by dual-wavelength difference spectroscopy ($\Delta(\Delta A_{666} - \Delta A_{730})$) using the extinction coefficient of $1.32 \times 10^5\ M^{-1}cm^{-1}$ for Pr (Lagarias et al., 1987).

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