Phytochrome A Overexpression in Transgenic Tobacco

Correlation of Dwarf Phenotype with High Concentrations of Phytochrome in Vascular Tissue and Attenuated Gibberellin Levels

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Phytochromes are a family of related chromoproteins that regulate photomorphogenesis in plants. Ectopic overexpression of the phytochrome A in several plant species has pleiotropic effects, including substantial dwarfing, increased pigmentation, and delayed leaf senescence. We show here that the dwarf response is related to a reduction in active gibberellins (GAs) in tobacco (Nicotiana tabacum) overexpressing oat phytochrome A under the control of the cauliflower mosaic virus (CaMV) 35S promoter and can be suppressed by foliar applications of gibberellic acid. In transgenic seedlings, high concentrations of oat phytochrome A were detected in stem and petiole vascular tissue (consistent with the activity of the CaMV 35S promoter), implicating vascular tissue as a potential site of phytochrome A action. To examine the efficacy of this cellular site, oat phytochrome A was also expressed using Arabidopsis chlorophyll a/b-binding protein (CAB) and the Arabidopsis ubiquitin (UBQ7) promoters. Neither promoter was as effective as CaMV 35S in expressing phytochrome in vascular tissue or in inducing the dwarf phenotype. Collectively, these data indicate that the spatial distribution of ectopic phytochrome is important in eliciting the dwarf response and suggest that the phenotype is invoked by elevated levels of the far-red-absorbing form of phytochrome within vascular tissue repressing GA biosynthesis.

Phytochromes are a family of regulatory photoreceptors in plants that measure both the quantity and quality of available light (for reviews, see Quail, 1991; Vierstra, 1993; Kendrick and Kronenberg, 1994). They are dimeric, cytoplasmic proteins, with each subunit consisting of a linear tetrapyrrole chromophore covalently linked to an approximately 120-kD polypeptide. The regulatory functions of phytochromes require that they switch between two photo-isomerized forms, a Pr form that primarily absorbs red light and a Pfr form that primarily absorbs far-red light. They are initially synthesized as Pr, which in most cases is biologically inactive (Liscum and Hangarter, 1993; Kendrick and Kronenberg, 1994). Upon photoconversion to Pfr, phytochromes become biologically active and govern a diverse array of physiological and developmental responses that span the life cycle of plants, including initiation of seed germination, chloroplast biogenesis, regulation of stem growth, floral induction, and timing of senescence. In many cases, these responses can be cancelled when Pfr is photoconverted back to Pr. As a result of this unique interconversion between inactive Pr and active Pfr, phytochromes operate as light-regulated, reversible switches in photomorphogenesis. The mechanism(s) by which phytochromes control morphogenesis is unclear, but recent models have implicated protein kinase signaling cascades, heterotrimeric G-protein activation, and calcium/calmodulin (Vierstra, 1993; Bowler et al., 1994).

Attempts to elucidate the molecular mechanism(s) of phytochrome action have focused, in part, on a biochemical characterization of the photoreceptors and on identifying structural domains that distinguish Pfr from Pr. However, this approach has been hampered by the lack of a relevant in vitro assay of Pfr activity (Quail, 1991) and by the complicated assembly of the chromoprotein that is difficult to reproduce with high yields in vitro or in microorganisms (Wahleithner et al., 1991; Edgerton et al., 1993; Furuya and Song, 1994). To overcome these limitations, one strategy now used is to ectopically express phytochrome genes in transgenic plants (for review, see Cherry and Vierstra, 1994). In a variety of plant species, expression of unmodified PHY genes results in the accumulation of chromoproteins, which are photochemically indistinguishable from the endogenous photoreceptors. When expressed to sufficient levels, the introduced phytochromes are also functional and impart a light-exaggerated phenotype, charac-

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Abbreviations: CAB, chlorophyll a/b binding protein; CaMV, cauliflower mosaic virus.
amplified by substantially elongated stems and hypocotyls. Elongated internodes are also observed in transgenic tomatoes expressing high levels of oat phytochrome A (Boylan and Quail, 1989) and elevated levels of GAs (Rood et al., 1990; Boylan et al., 1994). Almost all work to date has utilized the CaMV 35S promoter and not the native PHY promoters to drive expression of exogenous phytochromes. As a result, it has been argued that the light-exaggerated phenotype is induced by artificially high levels of phytochrome in tissues that normally lack the native chromoprotein and thus may not reflect phytochrome functioning in its normal capacity. However, a similar phenotype was recently observed when an Arabidopsis phytochrome B was expressed in Arabidopsis under its own promoter. This supports the possibility that the light-exaggerated phenotype represents the photoreceptor functioning in its correct context and thus is relevant to normal phytochrome action (Wester et al., 1994).

Important clues concerning phytochrome function may be uncovered by examining the physiological basis of the light-exaggerated phenotype. Its pleiotropic nature suggests that elevated levels of phytochrome alter hormone balances and/or sensitivities (Cherry and Vierstra, 1994). Several lines of evidence have specifically implicated GAs. For example, transgenic tomatoes expressing high levels of oat phytochrome A (Boylan and Quail, 1989) are remarkably similar in phenotype to tomato mutants defective in GA biosynthesis (Koornneef et al., 1990). Both types of plants display a shortened stature, curled leaves, and increased leaf and fruit pigmentation. In contrast, the Brassica napus mutans, which have both undetectable levels of phytochrome B (Childs et al., 1992; Devlin et al., 1992) and elevated levels of GAs (Rood et al., 1990; Beall et al., 1991), have an opposite phenotype characterized by substantially elongated stems and hypocotyls. Elongated internodes are also observed in Pisum mutants that have putative defects in the phytochrome signal transduction chain (bo [Nagatani et al., 1990] and lip1 [Frances et al., 1992]) or that contain aberrantly high levels of GAs (shn [Reid et al., 1992a]). Taken together, these data suggest that phytochrome responsiveness and GAs are inversely related: elevated Pfr levels repress GA biosynthesis and/or sensitivity, which in turn retards stem elongation, whereas reduced Pfr levels elevate GA biosynthesis and/or sensitivity, which then increases stem elongation. Although some data implicate phytochromes in altering the sensitivity of plants to GAs (e.g. Pisum), most data suggest that phytochromes act by altering GA biosynthesis (Weller et al., 1994).

Here we provide additional evidence supporting the regulation of GA biosynthesis by phytochrome. We show that tobacco (Nicotiana tabacum) plants, dwarfed by overexpression of oat phytochrome A under the control CaMV 35S promoter (Keller et al., 1989; Cherry et al., 1991), have substantially lower levels of active GAs. Foliar application of GA3 was able to reverse the dwarf phenotype. In these transgenic plants, high concentrations of oat phytochrome A were found in vascular tissue, implicating vascular tissue as a potential cellular site for altering GA levels. Comparison of the light-exaggerated phenotype induced by the CaMV 35S promoter to those induced by two other promoters show that this spatial distribution of ectopic phytochrome is important for eliciting the light-exaggerated phenotype.

**MATERIALS AND METHODS**

**Plant Material**

Tobacco lines (Nicotiana tabacum, cv Xanthi) expressing full-length oat phytochrome A under the control of the CaMV 35S promoter were those described by Keller et al. (1989) and Cherry et al. (1992). The Arabidopsis UBQ1 promoter was as described by Callis et al. (1993) and the CAB140 promoter was obtained courtesy of Dr. Anthony Cashmore (Leutwiler et al., 1986). A 1.4-kb EcoRI/PstI fragment containing the entire CAB promoter was cloned from pAB140160S into pBluescript KS+ (Stratagene, La Jolla, CA) containing a 40-bp EcoRI/BamHI linker. The linker (5'-GAATTCGGAGCTGCAGATGTCT 3') provided codons for the first five amino acids of oat phytochrome A ending in a Stul site and introduced a PstI site immediately 5' to the initiation codon. An EcoRI/Stul fragment containing the CAB promoter and linker was attached in a tripartite ligation to a 323-bp Stul/KpnI fragment encoding amino acids 6 to 113 of oat phytochrome A derived from pBinphyt (Cherry et al., 1992) and pBluescript KS+ digested with EcoRI/KpnI. The CAB promoter/phytochrome-encoding region was then cloned as a 1.7-kb EcoRI/KpnI fragment into the 14.3-kb EcoRI/KpnI fragment of pBinphyt containing the rest of the phytochrome-encoding region.

The UBQ1 promoter consisted of a 2.5-kb HindIII/MluI fragment (Callis et al., 1990). This region was appended to the phytochrome A-coding region using a 93-bp Sall/Xhol oligonucleotide linker. The linker was constructed by annealing and extending two overlapping oligonucleotides of 61 and 62 bp (5'-GGGTTCAGACCGG-3' and 5'-GGGTTCAGACCGG-3'). The resulting double-stranded linker was inserted into the Sall/Xhol sites of pBluescript KS+ and the UBQ1 promoter was added following digestion of the plasmid with HindIII and MluI. The coding region for oat phytochrome A was obtained from a 5' reconstruction of
HindIII/XhoI fragment containing the UBQZ promoter and pCV35phyt (Keller et al., 1989) in which a translationally silent Xhol site was introduced 8 bp downstream from the initiator ATG (Cherry et al., 1992; J.R. Cherry and R.D. Vierstra, unpublished data). In a tripartite ligation, a 2.6-kb HindIII/Xhol fragment containing the UBQ1 promoter and the N-terminal codons of oat phytochrome A, a 5.0-kb Xhol/Sall encoding the remainder of oat phytochrome A, and p8Bin19 digested with Sall/HindIII were ligated together to create an oat phytochrome A gene under the control of the UBQ1 promoter. For all genes used, introduced changes in nucleotide sequence were verified by DNA sequence analysis of the affected regions using the dideoxy chain termination method.

Genes were introduced into tobacco (cv Xanthi) by Agrobacterium-mediated transformation (Cherry et al., 1992). Kanamycin-resistant plants were screened for oat phytochrome A expression by immunoblot analysis with polyclonal antibodies against oat phytochrome A (Shanklin et al., 1987; Cherry et al., 1992). Plants expressing oat phytochrome A under the control of the CaMV 35S, CAB, and the UBQ1 promoters were designated 35S-PHYA, CAB-PHYA, and UBQ-PHYA, respectively. Three independent lines of each were used as the starting material in this study. Progenies of initial transformants homozygous for the inserted genes were identified by their ability to completely retain oat phytochrome A expression in subsequent generations. Heterozygous plants used in the dose-response analysis were progenies of the initial transformants.

All plants were grown in a greenhouse under natural diurnal light cycles. The effects of GA were tested by foliar application of various concentrations of GA3 (0-100 μM solutions containing 100 μL/L of Triton X-100). Spraying began 40 d after sowing when the wild-type plants were approximately 5 cm tall and the 35S-PHYA plants were approximately 1 cm tall and continued at 3- to 4-d intervals. Plant height was measured every 3 to 4 d until the onset of flowering, as determined by the appearance of flower primordia. For the dose-response analysis of phytochrome content, all tissue from the apex to the first 13-cm-long leaf (measured from the base of the leaf to the tip along the midvein) of mature 74-d-old plants was used. For analysis of the spatial distribution of phytochrome, tissue was harvested at seven different positions: position 1, the apex to leaves 5 to 6 cm in length; position 2, leaves 6 to 7 cm in length; position 3, leaves 10 to 11 cm in length (youngest expanded leaves); position 4, leaves 16 to 17 cm in length; position 5, leaves 19 to 20 cm in length; position 6, leaves 22 to 23 cm in length (old leaves); and position 7, stem section between positions 3 and 4.

Phytochrome Extracts

Phytochrome extracts and analyses were performed under a dim green safelight unless otherwise noted. Frozen tissue was pulverized at liquid nitrogen temperatures in a mortar and pestle. The powder was homogenized at a ratio of 2 mL/g fresh weight in 37.5% ethylene glycol, 75 mM Tris-HCl (pH 8.3, 4°C), 105 mM ammonium sulfate, and 7.5 mM Na2EDTA and made 20 mM sodium metabisulfite and 2 mM PMSF prior to use (Cherry et al., 1993). Samples for spectrophotometric assays were made 0.1% polyethyleneimine and clarified by centrifugation for 10 min at 48,000 g. Phytochrome was concentrated by ammonium sulfate (0.26 g/mL extract) precipitation and resuspended in one-tenth to one-twentieth of the original volume in 25% ethylene glycol, 50 mM Tris-HCl (pH 7.8, 4°C), 5 mM Na2EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF. Protein content in the clarified extracts was determined by the Bradford method (Bradford, 1976). Spectrophotometric analyses were conducted using a Shimadzu (Kyoto, Japan) UV3000 dual-wavelength spectrophotometer. Phytochrome content (ΔΔΔΔ) was measured as (ΔA730 - ΔA800) following saturating irradiation with red and far-red light and converted to (ΔA665 - ΔA730) using the equivalence factor 2.18 (Cherry et al., 1991).

Immunological Techniques

Oat phytochrome A content was determined in triplicate by sandwich ELISA (Cherry, et al., 1992). Affinity-purified rabbit polyclonal antibodies against oat phytochrome A (Shanklin et al., 1987) were used as the capture antibody and the murine monoclonal antibody Oat-22, specific for oat phytochrome A (Cordonnier et al., 1985), was used as the detection antibody. In this case, the initial crude extracts were clarified twice at 48,000 g for 10 min and used directly. Purified oat phytochrome A, quantitated by dual-wavelength difference spectroscopy (ΔΔΔΔ) was used as the standard.

Tissue printing involved gently pressing 1-mm tissue slices onto CaCl2-treated nitrocellulose as described by Ye et al. (1992). Prints were incubated with either polyclonal rabbit antibodies against oat phytochrome A at 0.5 μg/mL or preimmune serum diluted 1:1000. Primary antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (0.25 μg/mL) and the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Cherry et al., 1992).

Quantification of GAs

For quantitative analysis of GAs, apical portions from the shoot tip to the first mature leaf were collected from 50 wild-type plants and 50 homozygous 35S-PHYA plants expressing high levels of oat phytochrome A (Cherry et al., 1991). The plants were grown for 63 d (following sowing) in a greenhouse. GA levels were quantified as described by Talon et al. (1991). Briefly, the tissue was lyophilized, weighed, and extracted in methanol. The GAs, [17,17-3H]GA3 (90% enrichment), [20-2H]GA4 (95.7% enrichment), [17,17,2H,19]GA19 (94.2% enrichment), [17,17,2H,20]GA20 (99.3% enrichment), and [17,17,2H]GA1 (99.2% enrichment) were added as internal standards. GAs were purified by chromatography via charcoal, silicic acid, and QAE Sephadex, followed by reverse-phase HPLC. The samples were methylated and trimethylsilylated, and the derivatized material was quantitated by GC-selected ion
monitoring with magnetic field switching. Yields of internal standards were used to quantitate endogenous GAs. Each analysis was performed in duplicate. The results in Table I are averages of two replicates that showed similar results.

RESULTS

In this study, we explored the possibility that the light-exaggerated phenotype induced by ectopic expression of phytochrome in tobacco is caused, in part, by reduced levels of GAs. As a simple first test, wild-type plants and plants expressing high concentrations of oat phytochrome A driven by the CaMV 35S promoter (35S-PHYA) were treated with foliar applications of GA\textsubscript{3} at 3- to 4-d intervals, and their growth rates were measured. (Although it is probable that GA\textsubscript{3} is not an active GA in tobacco, it is likely converted to the active form(s) once in the plants.) Typically, high levels of oat phytochrome A reduces concomitantly the growth rate and the final height of mature tobacco (cv Xanthi) by nearly 4-fold (Fig. 1; Cherry et al., 1991, 1992). As can be seen in Figure 1, GA\textsubscript{3} reversed this dwarf phenotype. After a lag of approximately 10 d following the start of GA\textsubscript{3} application, the growth rate of 35S-PHYA plants increased 386% (from 1.1 to 4.25 cm/d) when treated with 60 $\mu$m GA\textsubscript{3} as compared to untreated 35S-PHYA plants. As a result, treated plants eventually grew to heights comparable to those of untreated wild-type plants (Fig. 1A). The 35S-PHYA plants still retained elevated levels of oat phytochrome A after GA\textsubscript{3} treatment, thus eliminating the possibility that GA\textsubscript{3} application blocked ectopic phytochrome accumulation. We could detect significant growth enhancement at GA\textsubscript{3} levels $\geq 3$ $\mu$m; at higher concentrations of GA\textsubscript{3}, the growth rate asymptotically approached that of GA\textsubscript{3}-treated wild-type plants (Fig. 1B). For comparison, GA\textsubscript{3} application had little effect on wild-type plants; plants treated with $\leq 10$ $\mu$m showed no significant affect on growth, and the highest level applied (100 $\mu$m) increased growth rate by only 23% (from 4.3 to 5.15 cm/d; Fig. 1B). Chl content was also affected by GA\textsubscript{3} application; levels within 35S-PHYA plants treated with 50 $\mu$m GA\textsubscript{3} declined to those of wild-type plants (data not shown). Not all effects associated with the light-exaggerated phenotype were repressed by GA\textsubscript{3} application. Most notably, the decrease in apical dominance (as detected by the development of axillary shoots), a delay in senescence, and the reduction in leaf width were still evident in the GA\textsubscript{3}-treated 35S-PHYA tobacco even at the highest concentrations used (100 $\mu$m; data not shown).

Suppression of the dwarf phenotype by GA\textsubscript{3} suggested that oat phytochrome A represses GA biosynthesis and not the sensitivity of the plants to the hormone. This possibility was supported by comparing the endogenous GA levels within wild-type and 35S-PHYA plants. Both wild-type and 35S-PHYA plants were grown simultaneously for 63 d and harvested prior to the development of detectable flower primordia. At that time, the heights of wild-type and 35S-PHYA plants were 49.5 $\pm$ 6.7 and 12.3 $\pm$ 3.5 cm, respectively. The profile of GAs from apical tissue was then resolved by GC/MS and quantitated using internal standards (Table I). The GA profile in wild-type plants was indicative of a GA biosynthetic pathway involving conversion of the inactive precursors, GA\textsubscript{20} and GA\textsubscript{1}, into the biologically active GAs, GA\textsubscript{1} and GA\textsubscript{4}, respectively (Table I). A similar GA profile was detected in 35S-PHYA plants but the total levels of GAs were significantly reduced (approximately 40% of wild-type levels). Importantly, the levels of the two biologically active GAs, GA\textsubscript{1} and GA\textsubscript{4}, were reduced by nearly 4-fold (Table I).

To help identify the cellular site(s) where oat phytochrome A affects tobacco growth and possibly GA levels,
the tissue distribution of the oat chromoprotein in light-grown plants was determined. Gross analysis involved quantitation of phytochrome levels in various locations by sandwich ELISA using the monoclonal antibody Oat-22, which is specific for oat phytochrome A (Cordonnier et al., 1985; Cherry et al., 1992). As can be seen in Figure 2, oat phytochrome A was detected immunologically in all tissues examined from 35S-PHYA plants. Immature leaves contained high levels that declined as the leaves aged (cf. positions 1 and 6, Fig. 2). Phytochrome A was especially abundant in stem tissue, having nearly twice the levels found in leaves when calculated relative to total protein content (position 7).

More precise localization of oat phytochrome A used tissue printing of light-grown plants (Reid et al., 1992b). Because we were unable to obtain suitable tissue prints from leaves and meristems, the study was confined to the more rigid stem and petiole sections (Fig. 3; data not shown). Immunodetection used polyclonal antibodies against oat phytochrome which weakly cross-reacted with tobacco phytochromes (Cherry et al., 1991). In the absence of this primary antibody or when preimmune serum was used as a substitute, only a faint ring of brown staining was observed in the tissue prints that was coincident with the ring of vascular tissue (Fig. 3, A and F). This color was distinct from the blue staining derived from the histochemical reagents used and likely resulted from brown phenolic compounds leaching from the sections. In wild-type plants, weak but immunospecific blue staining of vascular tissue and the epidermis was reproducibly seen (Fig. 3D). Even though this labeling was not observed when wild-type stem sections were probed with preimmune sera (Fig. 3A), it was uncertain whether this pattern actually reflected the distribution of endogenous tobacco phytochromes or was artifactual, given the weak affinity of the antibody to tobacco phytochromes. However, when 35S-PHYA plants were analyzed, intense blue staining of vascular tissue was evident just inside and outside the vascular ring (Fig. 3C). This ring was coincident with phloem and the surrounding companion cells (Fig. 3F). As with wild-type plants, slight immunostaining was observed in the epidermis, but little staining was present in the cortex, xylem, and pith. Analogous results were obtained with several different transgenic lines expressing 35S-PHYA (data not shown).

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Table 1. GA levels in tobacco expressing oat phytochrome

<table>
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<th>GA</th>
<th>Wild Type</th>
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<th>35S/Wild Type</th>
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<tr>
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<td>17.3</td>
<td>50</td>
</tr>
<tr>
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<td>0.3</td>
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<td>2.7</td>
<td>29</td>
</tr>
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</table>

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Figure 2. Spatial distribution of oat phytochrome A expressed in tobacco under the control of either Arabidopsis CAB, Arabidopsis ubiquitin (UBQ1), or CaMV 35S (35S) promoters. For each promoter, three independently transformed lines were selected that expressed a range of phytochrome levels, and two plants from each line were analyzed for phytochrome content (represented by the individual bars in the graphs) at various positions within the plant. Horizontal lines at each position represent the average expression levels of all six plants in each group. Plants were grown in a greenhouse and tissue was harvested 72 d after sowing. Levels of oat phytochrome A were determined by sandwich ELISA using monoclonal antibody Oat-22, which is specific for the oat phytochrome A polypeptide, and plotted relative to total sample protein. No phytochrome was detected in wild-type plants by this assay (data not shown). Position 1, Apex to leaves 5 to 6 cm in length; position 2, leaves 6 to 7 cm in length; position 3, leaves 10 to 11 cm in length (youngest expanded leaves); position 4, leaves 16 to 17 cm in length; position 5, leaves 19 to 20 cm in length; position 6, leaves 22 to 23 cm in length (older leaves); and position 7, stem sections between positions 3 and 4.
equivalent to those in 35S-PHYA plants, the average levels of phytochrome A in the CAB-PHYA plants were nearly successfully generated; whereas the average levels of oat pollen (Callis et al., 1990) types, including epidermis, mesophyll, cortex, pith, and xylematic tissue (a predicted site of phytochrome action). The promoter was chosen because it directs expression in meristematic tissue, mainly leaf parenchyma. As expected, the UBQ-PHYA plants expressed the chromoprotein most strongly in the apex (position 1: apex to leaves 5–6 cm in length; Fig. 2). Accumulation was apparent in stems, although little was detected in mature or senescing tissues. Tissue printing of CAB-PHYA and UBQ-PHYA plants expressing the highest levels of oat phytochrome A showed that relatively little oat phytochrome A accumulated in vascular tissue (Fig. 3, B and E). In contrast to 35S-PHYA plants, neither the CAB-PHYA nor UBQ-PHYA plants displayed enhanced immunostaining of vascular tissue (or any other stem tissue) beyond that observed with wild-type plants.

The tissues of oat phytochrome A in vascular tissue (possibly phloem) suggested to us that the dwarf response of 35S-PHYA plants may be induced by artificially high levels of photoreceptor in this tissue type. To test this possibility, oat phytochrome A was also expressed under the control of two other promoters, Arabidopsis CAB and UBQ1, that were expected to generate a different tissue distribution of the chromoprotein. The tissue distribution of the oat phytochrome A protein under the direction of each promoter was then determined and correlated with the ability of each to induce the light-exaggerated phenotype. Given the involvement of the CAB gene product in photosynthesis, its promoter was expected to function primarily in photosynthetic tissue, mainly leaf parenchyma (Leutwiler et al., 1986). The CAB promoter is also induced by Pfr, thus creating a potential feedback loop where the activity of the CAB-PHYA gene would be enhanced further by increasing levels of oat phytochrome A. The UBQ1 promoter was chosen because it directs expression in meristematic tissue (a predicted site of phytochrome action [Pratt, 1994]) and expresses to some extent in most cell types, including epidermis, mesophyll, cortex, pith, and pollen (Callis et al., 1990).

Transgenic CAB-PHYA and UBQ-PHYA tobacco were successfully generated; whereas the average levels of oat phytochrome A in the CAB-PHYA plants were nearly equivalent to those in 35S-PHYA plants, the chromoprotein was found in the apex and young leaves (Fig. 2), consistent with the increased activity of the CAB promoter during chloroplast biogenesis (Leutwiler et al., 1986). In contrast to 35S-PHYA plants, only moderate levels were present in stem tissue. Phytochrome concentrations declined in more mature and senescing leaves in accord with their reduced photosynthetic capacity. As expected, the UBQ-PHYA plants expressed the chromoprotein most strongly in the apex (position 1: apex to leaves 5–6 cm in length; Fig. 2). Accumulation was apparent in stems, although little was detected in mature or senescing tissues. Tissue printing of CAB-PHYA and UBQ-PHYA plants expressing the highest levels of oat phytochrome A showed that relatively little oat phytochrome A accumulated in vascular tissue (Fig. 3, B and E). In contrast to 35S-PHYA plants, neither the CAB-PHYA nor UBQ-PHYA plants displayed enhanced immunostaining of vascular tissue (or any other stem tissue) beyond that observed with wild-type plants.

To assess the importance of phytochrome location in eliciting the light-exaggerated phenotype, the biological activity of oat phytochrome A under the control of the CAB and UBQ1 promoters was determined and compared to that using the CaMV 35S promoter. To accomplish this, a phytochrome dose-phenotype response curve was generated for all three, in which plant height at maturity was correlated with the amount of phytochrome present in the apical portions of the mature plants (Cherry and Vierstra, 1994). To maximize the range of oat phytochrome A that accumulated from each construct, homozygous and heterozygous plants, derived from three independently transformed lines, were used. The levels of phytochrome were determined either by red-minus-far-red difference spectrosopy (Fig. 4, left), which detects both oat phytochrome A and the endogenous tobacco phytochromes that bear chromophores, or by sandwich ELISA (Fig. 4, right), using Oat-22, which is specific for the oat phytochrome A polypeptide (Cherry et al., 1992, 1993). In all cases, the increased levels of spectrophotometrically detectable phytochrome was matched by a similar accumulation of the oat phytochrome A polypeptide and not by elevated levels of the endogenous tobacco phytochromes. This result supports previous data showing that a majority of the oat phytochrome A polypeptides associates with chromophore and assembles into a spectrally active chromoprotein in tobacco (Cherry et al., 1991, 1992, 1993).

As can be seen in Figure 4, plants expressing a wide range of oat phytochrome A levels under the direction of the CAB promoter were generated. Some contained spectrophotometrically detectable levels of total phytochrome that were 16 times greater than in wild-type plants, levels that even exceeded that synthesized in 35S-PHYA plants. The high phytochrome levels were caused by an increase in the growth of CAB-PHYA plants.
Phytochrome A Overexpression in Tobacco

Figure 4. Phenotypic response of tobacco to increasing levels of oat phytochrome A expressed under the direction of either the CaMV 35S promoter (C), CAB promoter (O), or the UBQ1 promoter (A). Wild-type (C) and transgenic plants expressing oat phytochrome A under the direction of the three promoters were grown concurrently under natural diurnal light cycles. When flower primordia were detected (74 d after sowing), apical leaf tissue was harvested, and the phytochrome was extracted and concentrated by ammonium sulfate precipitation. Plant height was plotted relative to phytochrome concentration as determined either by red-minus-far-red difference spectroscopy ($\Delta A_{665} - \Delta A_{330}$) (left), which detects both endogenous tobacco phytochromes and oat phytochrome A that bear photoreversible chromophores, or by sandwich ELISA (right) using the monoclonal antibody Oat-22 that is specific for the oat phytochrome A polypeptide. Top, Expression directed by the CAB promoter compared to the 35S promoter. Bottom, Expression directed by the UBQ1 promoter compared to the 35S promoter.

was never inhibited to the same extent as that of 35S-PHYA plants. 35S-PHYA plants containing high levels of oat phytochrome A grew only to an average height of 24 cm (or 28% the height of wild-type plants) with substantial dwarfing evident even with modest increases in spectrally detectable chromoprotein. However, the failure of CAB-PHYA and UBQ-PHYA plants to respond as effectively as 35S-PHYA plants to exogenous GA$_3$ discounts the possibility that Pfr represses sensitivity to active GAs. The involvement of GAs is consistent with the phenotypic similarity of various plant species altered in phytochrome levels or responsiveness to those defective in GA metabolism (see above). The dwarf response observed in 35S-PHYA plants results from a decrease in cell expansion and not from a decline of cell division (Nagatani et al., 1991): an effect that agrees well with the specific role of GAs in promoting cell elongation (Jones, 1982).

The major GAs in wild-type tobacco belong to the early 13-hydroxylation pathway (Reid, 1993). The presence of GA$_3$ and GA$_4$ suggests that the non-3,13-hydroxylation pathway also operates in this species. The mechanism by which excess Pfr affects the production of active GAs, GA$_1$ and GA$_4$, is unclear. Since none of the GAs analyzed were individually increased in 35S-PHYA plants, it suggests that the block in GA biosynthesis occurred prior to the synthesis of GA$_3$. The inability of GA$_3$ application to suppress all effects induced by oat phytochrome A overexpression (e.g. partial release of apical dominance and a delay in senescence) indicates that other hormonal factors may participate. Chory et al. (1994) recently showed that cytokinin application can phenocopy light-regulated development in dark-grown Arabidopsis. We have preliminary data that wild-type and 35S-PHYA plants have indistinguishable cytokinin levels, which suggests that excess phytochrome A does not alter cytokinin biosynthesis (M. Brenner, J. Cherry, and R.D. Vierstra, unpublished data).

This study and others (Boylan and Quail, 1989, Kay et al., 1989; Cherry et al., 1992, 1993; Boylan et al., 1994) clearly establish that ectopic phytochrome A can accumulate to high levels in transgenic plants, even in light-grown plants in which high rates of Pfr degradation occur (Vierstra, 1994). Moreover, when appropriate promoters are used, it is possible to create plants that accumulate the photoreceptor with different tissue distributions. The fact that high levels can be achieved by the CAB and the CaMV 35S promoters suggests that many cell types have the capacity to convert the apoprotein into a photoreversible chromoprotein. However, the failure of CAB-PHYA and UBQ-PHYA plants to respond as effectively as 35S-PHYA plants to elevated levels of oat phytochrome A indicates that not all cells/tissues are equally responsive. This is especially evident for tobacco leaves that failed to elicit the maximal dwarf response even when they contained high concentrations of oat phytochrome A. Thus, the biological activity of amounts of oat phytochrome A were compared, 35S-PHYA plants were shorter in almost all cases.

DISCUSSION

Here we provide evidence that the light-exaggerated phenotype induced by ectopic expression of phytochromes is elicited, at least in part, by alterations in GA biosynthesis. The involvement of GAs is implicated by (a) the ability of foliar applications of GA$_3$ to suppress the dwarf phenotype in 35S-PHYA plants and (b) a nearly 4-fold reduction in the active GAs, GA$_1$ and GA$_4$, from apical tissue in 35S-PHYA plants versus wild-type plants. The response of 35S-PHYA plants to exogenous GA$_3$ discounts the possibility that Pfr represses sensitivity to active GAs. The involvement of GAs is consistent with the phenotypic similarity of various plant species altered in phytochrome levels or responsiveness to those defective in GA metabolism (see above). The dwarf response observed in 35S-PHYA plants results from a decrease in cell expansion and not from a decline of cell division (Nagatani et al., 1991): an effect that agrees well with the specific role of GAs in promoting cell elongation (Jones, 1982).

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ectopic phytochromes appears dependent not only on the concentration of the photoreceptor but also on its spatial distribution.

Obviously, to completely understand the function(s) of phytochromes, information regarding their tissue distribution will be required. Whereas the distribution of phytochromes in dark-grown plants is known (at least for phytochrome A), the distribution in green plants is unknown, mainly because of the low levels of phytochromes present (Pratt, 1994). Ectopic expression of oat phytochrome A under the direction of the CaMV 35S promoter results in high phytochrome A concentrations in stem and petiole vascular tissue. This distribution is indistinguishable from that observed when the CaMV 35S promoter is used to drive expression of the histochemical marker, GUS (Jefferson et al., 1987). Thus, the expression patterns in 35S-PHYA plants seem dependent solely on the promoter and not on the capacity of vascular tissue to assemble the holoprotein or on its failure to degrade Prf. With respect to stem elongation, the enhanced phenotypic sensitivity of 35S-PHYA plants, compared with CAB-PHYA and UBQ-PHYA plants, implicates the high concentrations of phytochrome A in vascular tissue. If we assume that phytochromes regulate GA levels, this vascular tissue Prf may in turn affect GA synthesis, but whether this happens by altering GA synthesis within vascular tissue directly or within other tissue indirectly is unknown. Clearly, identification of the cellular site(s) of GA biosynthesis is needed to resolve such possibilities.

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