Phytochrome A overexpression inhibits hypocotyl elongation in transgenic Arabidopsis

(regulatory photoreceptor/plant transformation/photomorphogenesis/site-specific mutagenesis)

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ABSTRACT To develop a model plant system for efficient functional analysis of mutagenized phytochrome polypeptides, we have overexpressed oat phytochrome A in Arabidopsis thaliana. R1 seedlings from selfed primary transformants segregated for hypocotyl length, when grown in the light, with a ratio of 3 short to 1 of normal length. When homozygous lines were established from these two size classes, accumulation of immunologically detectable oat phytochrome cosegregated with the short-hypocotyl trait. The short-hypocotyl seedlings contained substantially more spectrally active phytochrome than their normal-sized siblings, indicating that the introduced oat protein was photoreversible. The short-hypocotyl phenotype was strictly light-dependent, since no morphological effects of phytochrome overexpression could be seen in etiolated seedlings. Overexpression of only the chromophore-bearing, N-terminal domain of phytochrome A did not induce short hypocotyls in light-grown seedlings, indicating that additional sequence is essential for photoreceptor function. Similarly, overexpression of a full-length sequence mutated at the chromophore attachment site had no effect on phenotype, indicating the absence of any detectable dominant negative effect of the chromophoreless polypeptide on the activity of endogenous Arabidopsis phytochrome. Thus, the readily scorable short-hypocotyl phenotype of Arabidopsis seedlings overexpressing phytochrome A provides a simple visual assay for rapidly monitoring the biological activity of mutagenized phytochrome A polypeptides.

Phytochrome is a regulatory protein that controls plant gene expression in response to light (1). At the molecular level, the photoreceptor functions as a switch that is reversibly interconvertible between its inactive, red-absorbing form (Pr, \[\lambda_{\text{max}} = 666 \text{ nm}\]) and active, far-red-absorbing form (Pfr, \[\lambda_{\text{max}} = 730 \text{ nm}\]) by sequential absorption of red and far-red light. Formation of Pfr initiates a transduction process that dramatically alters the pattern of gene expression and ultimately the growth habit of the plant. We are interested in understanding the molecular mechanism by which phytochrome functions since this protein constitutes an indispensable link between the plant and its environment.

The photoreceptor consists of two moieties: a polypeptide chain of 116–127 KDa and a covalently attached, linear tetrapyrrrole chromophore (1). Immunochemical and molecular studies have established that plants contain more than one type of phytochrome, whose polypeptides are encoded by a small family of divergent phy genes (2–4). The most abundant species of phytochrome in the plant is the form found in etiolated tissues, often referred to as type 1 phytochrome (5). Since type 1 phytochrome is encoded by phyA genes (6–9), we refer to it here as phytochrome A. Phytochrome A is synthesized in the Pr form and accumulates as a soluble cytoplasmic protein in dark-grown plants (10–12). Light-induced conversion to Pfr causes a rapid decrease in phytochrome A levels because Pfr is degraded much more rapidly than Pr (12).

The availability of cloned phytochrome genes has provided the opportunity to probe the functional importance of various regions of the polypeptide by in vitro mutagenesis combined with subsequent expression of the mutated proteins in transgenic plants. Although phytochrome-deficient mutant lines would be the ideal recipients for these experiments, the photomorphogenic mutants that have been studied thus far do not appear to carry lesions in the phyA structural gene itself (13–17). This lack of appropriate apoprotein mutants has stimulated efforts to overexpress the phyA polypeptide in heterologous transgenic plants as an alternative approach to defining functionally significant domains. Overexpression of oat or rice wild-type phyA sequences in tomato (18) and tobacco (19–22) leads to the accumulation of spectrally and biologically active monomeric phytochrome in the dicot tissue, with the induction of an easily recognized dwarf phenotype. The phenotypic effects of phytochrome overexpression provide a convenient visual assay for monitoring the biological activity of the introduced polypeptide in the transgenic plants. However, the inordinate amount of time and space needed to conduct these studies in tomato and tobacco renders these species less than ideal for our purposes.

We decided to exploit the experimental advantages offered by the small crucifer Arabidopsis thaliana (23). Rapid and efficient transformation procedures have been developed for this species (24). Arabidopsis completes its life cycle in 4–6 weeks, and individual plants can be grown to maturity in as little as 1 cm². We report here that (i) oat phytochrome A is faithfully synthesized in transgenic Arabidopsis, (ii) overexpression of this molecule dramatically inhibits hypocotyl elongation in light-grown seedlings, and (iii) induction of this phenotype can be abrogated by prior mutagenesis of the introduced oat sequence.

MATERIALS AND METHODS

Plant Transformation. The plasmid pFY123 (18), which contains an oat phyA CDNA fused to the cauliflower mosaic virus (CaMV) 35S promoter, was transferred into Agrobacterium strain 3111SE by triparental mating (25). Root explants of aseptically grown 18-day-old A. thaliana plants, Nossen ecotype, were transformed as described (24). Primary transgenic plants are designated the R0 generation, the first-generation progeny obtained by selfing the R0 plants are designated R1, and their progeny are designated R2.

Seedling Growth. Transgenic Arabidopsis seeds were sown aseptically on the appropriate media, and plates were chilled at 4°C for 24 hr and then exposed to white light for 30

Abbreviations: Pr and Pfr, red and far-red light-absorbing forms of phytochrome; CaMV, cauliflower mosaic virus.

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min to stimulate germination. Subsequent growth was at 21°C in either darkness or white light (0.6 W/m², 16/8-hr photoperiod). For light-pulse experiments, seedlings were grown in the dark for 2 days and then treated every 4 hr for the next 3 days with either 5 min of red light alone (0.1 W/m²), 5 min of red light followed by 10 min of far-red light (0.2 W/m²), or 10 min of far-red light alone. For measurement of hypocotyl length or phytochrome content, seedlings were grown on germination medium (24). Kanamycin-resistant seedlings were selected on germination medium supplemented with kanamycin (100 µg/ml).

**Immunological Detection of Phytochrome.** R2 seedlings from homozygous R1 plants were grown in complete darkness for 5 days. Harvesting of the tissue, preparation of crude extracts, SDS/PAGE, and immunoblotting were performed as described (26). Protein was visualized by using two monoclonal antibodies developed against oat phytochrome. One antibody, 1B5A, was monocot-specific (27). The other antibody, 073D, reacted with both monocot and dicot phytochromes (28).

**Spectrophotometric Measurements.** Seedlings were grown for 5 days either in complete darkness or in white light. Etiolated tissue was harvested and extracted as described above. Light-grown tissue was extracted as described (29). Difference spectroscopy was performed (Shimadzu UV3000 spectrophotometer) following saturating red (666 nm) or far-red (727 nm) irradiation. The phytochrome content of the extracts was also measured in a custom-built dual-wavelength spectrophotometer (30) (measuring beams, 660/730 nm) using CaCO₂ as a scattering agent.

**Mutant Constructions.** For the Cys⁴²² → Ser mutation, oligonucleotide mutagenesis (31) was used to substitute a serine codon (TCT) for a cysteine codon (TGT) at amino acid position 322 in the oat phyA coding sequence, to generate pFY332. The oligonucleotide used for this mutagenesis was 5'-CCACACAGTTCTCACCTTCAG-3'. For the ΔG17–1129 deletion, the 3.5-kilobase BamHI–EcoRI oat phyA cDNA insert in pFY122 (18) was cloned into pBluescript KS(+) (Stratagene) to create pFY371. A Pst I site was inserted upstream of the phyA termination codon in pFY371 by using a 5’ oligonucleotide (5’-CTGCAATGATGAGCCACAGTGA-3’) for PCR in conjunction with a 3’ oligonucleotide (5’-AACAGCTATGACCATG-3’). The PCR product was digested with Pst I and EcoRI and cloned into Pst I/EcoRI-digested pFY371 to generate pFY431, which lacks the sequence encoding C-terminal amino acids 617–1129. pFY332 and pFY431 were digested with BamHI–EcoRI, the phyA cDNA inserts were ligated to Bgl II/EcoRI-digested pMON316 to generate pFY333 and pFY432, respectively, and these were transferred into Agrobacterium as above.

**RESULTS**

**R1 Seedlings Segregate for Hypocotyl Length.** An oat phyA cDNA under the control of the CaMV 35S promoter, was introduced into *Arabidopsis* by Agrobacterium-mediated root transformation. R1 seedlings from 10 of the primary transformants segregated for kanamycin resistance, the selective marker linked to the oat gene on the T-DNA. When etiolated seedlings were examined by immunoblotting, of the 4 lines contained appreciable quantities of the oat phyA polypeptide, while substantially lower amounts of the monocot protein were found in the other 6 lines (data not shown).

When seedlings from the six lines that had relatively little detectable oat phytochrome A were grown in white light, they were phenotypically indistinguishable from wild-type *Arabidopsis* (data not shown). By contrast, light-grown seedlings from the four lines that expressed high levels of the oat protein segregated into two distinct groups on the basis of hypocotyl length. These R1 populations consisted of both tall seedlings, whose height was comparable to that of wild-type plants, and many seedlings with much shorter hypocotyls (Fig. 1A). In contrast to the changes in hypocotyl length, root growth was relatively unaffected in these seedlings, indicating that the decrease in stature was not due to some general reduction in plant vigor (Fig. 1B).

The average height of wild-type seedlings was 6.8 ± 1.4 mm (Fig. 2A). R1 populations, however, contained a substantial proportion of much shorter seedlings, 1–3 mm tall (Fig. 2B). The ratio of short to normal-sized R1 seedlings was 3.2:1 for this particular line. The ratio of kanamycin-resistant to sensitive seedlings was 3.5:1. Both ratios are consistent with there being a single-locus T-DNA insert (χ² = 0.11 and 0.29, respectively; P = 0.05) whose activity is associated with the short-hypocotyl phenotype.

**Short-Hypocotyl Phenotype Cosegregates with Phytochrome Overexpression.** Both short and normal-sized R1 seedlings were transferred to soil and allowed to self-fertilize. R2 progeny derived from the normal-sized seedlings were uniformly tall and 100% kanamycin-sensitive. R2 progeny from the small seedlings were either homozygous for the short-hypocotyl phenotype and 100% kanamycin-resistant or continued to segregate for both hypocotyl length and drug resistance. When these seedlings were analyzed by immunoblotting with a monocot-specific anti-phytochrome monoclonal antibody, expression of oat phytochrome A was strongly correlated with the short-hypocotyl phenotype (Fig. 3A). Oat phytochrome A could not be detected in either etiolated wild-type *Arabidopsis* (Fig. 3A, lane 2) or the normal-sized R2 seedlings (lanes 10–16). Authentic oat phytochrome A monoclonal antibody did not cross-react with the oat phytochrome A in the wild-type *Arabidopsis* seedlings (Fig. 3A, lane 2).
This type of oat phytochrome spectral content cannot be distinguished from its 124-kDa normal-sized polypeptide (25 kDa Arabidopsis phytochrome) and was used to probe monoclonal antibody 1.9B5A, which recognizes oat (monocot) phytochrome alone (A), or 073D, which recognizes both oat (monocot) and Arabidopsis (dicot) phytochromes (B). Extracts were prepared from wild-type (WT) Arabidopsis (lane 2) or from the short R2 seedlings (lanes 3–9) and their normal-sized siblings (lanes 10–16). Lane 1 contained 25 ng of purified oat phytochrome. The phytochrome content of the extracts (mean ΔΔA per g of fresh weight) was measured using a dual-wavelength spectrophotometer (C).

**Developmental Aspects of Phytochrome Overexpression.** Although the phytochrome A overexpressors could readily be distinguished from their normal-sized siblings as seedlings, the phenotypic differences between these two groups were no longer apparent once the true leaves expanded and the basal rosette growth form was established (data not shown). At maturity, the overexpressors were similar in height to wild-type plants. In addition, floral morphology and
fruit development were unaffected, indicating that phytochrome A overexpression did not interfere with sexual reproduction in Arabidopsis.

The short-hypocotyl growth habit of the phytochrome overexpressors was a conditional phenotype that showed a strict dependence on light (Fig. 5). When grown in the dark, the transgenic plants were indistinguishable in appearance from wild-type Arabidopsis. Both kinds of seedlings had the same etiolated morphology with elongated hypocotyl, unexpanded cotyledons, and lack of chlorophyll.

To test directly for the role of phytochrome in eliciting the short-hypocotyl phenotype, we examined the response of the homozygous phytochrome A overexpressors to pulses of red and far-red light (Fig. 5). The average hypocotyl length of dark-grown seedlings was 16.6 ± 2.0 mm. Seedlings subjected to pulses of far-red light were of a similar height, with an average hypocotyl length of 17.3 ± 3.2 mm. In contrast, the hypocotyls of phytochrome overexpressors pulsed with red light were substantially shorter, 8.3 ± 2.2 mm. The phenotypic response to red light was reversible by subsequent exposure to far-red light, since the hypocotyls of these seedlings were 17.1 ± 3.5 mm long. These results demonstrate directly that expression of the short-hypocotyl trait was dependent upon the photoconversion of oat phytochrome into its biologically active form, Pfr. The reason for the lack of response of the wild-type seedlings to red light pulses is unknown. On the other hand, the far-red and the red/far-red light treatments were each sufficient to cause the cotyledons of the phytochrome overexpressors to unfold. This apparent high sensitivity of cotyledon unfolding to overexpressed phytochrome has implications for the role of the photoreceptor in this crucial aspect of de-etiolation.

Overexpression of Mutant phyA Sequences. The short-hypocotyl phenotype caused by phytochrome A overexpression in Arabidopsis can be exploited for structure-function studies of the photoreceptor because this trait provides a biological assay for monitoring the activity of mutagenized phyA sequences. For this purpose, we have constructed homozygous transgenic lines that overexpress mutant forms of the oat protein. A point mutation (Cys322 → Ser) at the chromophore attachment site resulted in a 124-kDa polypeptide (Fig. 6, lane 2) that lacked photoreversibility, since spectral activity in these seedlings was similar to that of wild-type Arabidopsis (Fig. 4C). On the other hand, spectral activity in seedlings that overexpressed a 68-kDa N-terminal fragment (Fig. 6, lane 3) was similar to that found in seedlings that overexpressed authentic, full-length oat phyA protein (lane 1), demonstrating that the 68-kDa polypeptide was photochemically active. Overexpression of either mutant sequence failed to elicit the short-hypocotyl phenotype, indicating that these two mutations interfered with the biological activity of oat phytochrome A.

DISCUSSION

Conservation of photoreceptor function across divergent evolutionary boundaries has recently emerged as a central theme underlying the molecular basis of phytochrome action. Work with transgenic plants has shown that monocot phytochromes can function normally in tomato and tobacco, two dicot species that belong to the Solanaceae (18–22). Our current results extend these observations to Arabidopsis, a member of another dicot family, the Brassicaceae. Overexpression of wild-type oat phyA sequences led to the accumulation of spectrally active monocot phytochrome A in transgenic Arabidopsis plants, relative high levels of overexpression strongly inhibited hypocotyl elongation in light-treated seedlings, and overexpression of either the N-termi-

![Fig. 5. Comparison of the morphology of 5-day-old seedlings of wild-type (WT) Arabidopsis and the overexpressor line 13K7, grown either in the dark or under the indicated pulsed-light regimes (red light pulses alone, red followed by far-red light pulses, or far-red light pulses alone). (Bar = 3 mm.)](attachment:fig5.png)

![Fig. 6. Overexpression of mutant oat phyA polypeptides in homozygous transgenic Arabidopsis. Extracts of 5-day-old etiolated seedlings were analyzed on immunoblots probed with the monocot phytochrome-specific monoclonal antibody 1.9B5A (A). These plants overexpressed either the authentic, full-length oat phyA protein (lane 1), a polypeptide with a Cys322 → Ser point mutation (lane 2), or a 68-kDa N-terminal fragment that lacks amino acids 617–1129 (lane 3). Phytochrome in the extracts was measured using a dual-wavelength spectrophotometer (B). Hypocotyl length was measured for seedlings grown in white light for 7 days (C).](attachment:fig6.png)
nal domain alone or a chromophoreless full-length polypeptide failed to elicit this response.

The immunological and spectral properties of the oat protein synthesized in Arabidopsis were similar to those of authentic oat phytochrome A, emphasizing the fidelity with which dicot tissues can produce heterologous phytochromes. As with tomato and tobacco (18–20), the phytochrome chromophore does not appear to be limiting in Arabidopsis cells, since accumulation of the additional monocot photoreceptor did not change endogenous phytochrome levels.

Despite its photoreversibility, oat phytochrome A did not decrease in abundance when seedlings were exposed to light. In general, it has been found that the light-dependent degradation of the Pfr form of monocot phytochromes is much slower in transgenic dicot plants than expected from the half-lives observed in the parent monocot tissues (18, 22). It has been speculated that this anomalous behavior could reflect (i) a higher rate of oat phytochrome transcription driven by the CaMV 35S promoter than for the endogenous Arabidopsis polypeptide or (ii) alternatively, a slower rate of turnover of oat Pfr, either because of intrinsic structural differences between monocot and dicot phytochromes or because of ectopic expression of the oat sequence in cells not normally equipped to degrade the photoreceptor. Regardless of the actual mechanism, the persistence of high levels of spectrally active phytochrome in light-grown Arabidopsis may provide insight into the molecular basis of the selective turnover of Pfr in plant cells.

The observation that the inhibition of hypocotyl elongation in Arabidopsis seedlings was strictly light-dependent in a red/far-red reversible manner indicates that the normal regulatory properties of the monocot photoreceptor were retained. Further, the data show that the monocot protein recognizes components of the Arabidopsis signal-transduction system responsible for controlling extension growth. The effects of phytochrome overexpression on seedling size complement the phenotypic changes associated with phytochrome deficiency seen in the photomorphogenic mutants hy-1, hy-2, and hy-6 (17, 26). These mutants have abnormally long hypocotyls when grown in white light and contain little or no photoreversible phytochrome.

Although the overexpressor lines were readily distinguished from their nonexpressing siblings as seedlings, no obvious phenotypic differences were found in adult plants. This limited phenotypic effect contrasts with the pleiotropic consequences associated with phytochrome overexpression in other species (18–22). In tomato and tobacco, overexpression of monocot phyA sequences resulted in seedlings with short hypocotyls, while the adult plants were dwarfs with dark green leaves. In both these species, a number of transgenic lines were also obtained where even though hypocotyl elongation was inhibited, the plants grew to a normal height at maturity. Thus, hypocotyl length appears to be a more sensitive and consistent indicator of phytochrome A overexpression than adult plant morphology.

The distinctive phenotypic changes associated with phytochrome overexpression in dicot plants can be exploited to dissect the functional domains of the photoreceptor by introducing sequences that have been mutated in vitro. In an initial test of the feasibility of this approach, we have demonstrated that overexpression of the chromophore-containing N-terminal domain alone fails to elicit the short-hypocotyl phenotype, indicating that additional sequences in the dimerization-site-bearing, C-terminal domain are necessary for proper photoreceptor function. In addition, the failure of the chromophoreless, full-length oat polypeptide to perturb the wild-type phenotype indicates not only the expected absence of activity of the photoreceptor apoprotein but also the absence of any dominant negative effects on Arabidopsis phytochrome function. Such an effect is theoretically possible, for example, through cross-dimerization of the dicot and mutant monocot polypeptides. Arabidopsis provides an ideal model system for mutational analysis of the phytochrome A polypeptide because sufficient numbers of transformants can be produced in much less time and space than with either tomato or tobacco, the fast life cycle facilitates the process of proceeding from a segregating R1 population to a homozygous line for any particular mutant phytochrome sequence, and the short-hypocotyl phenotype that characterizes phytochrome overexpression in this plant provides a quick, reliable, and quantitative assay for photoreceptor function.

We dedicate this publication to the memory of Daria Aponte, who enriched our lives as a coworker and friend. We thank Drs. David Earp, Mary Honna, and Candace Waddell for their advice on Arabidopsis transformation; Dr. Brian Parks for his help with spectrophotometry; Drs. John Shanklin and Richard Vierstra for monoclonal antibody 073D; and Dr. Stephen Rogers for Agrobacterium vectors and strains. This work was supported by National Science Foundation Grant DCB-8796344 to P.H.Q.