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Expression of a functional monocotyledonous phytochrome in transgenic tobacco

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A chimeric oat phytochrome structural gene with an uninterrupted coding region was constructed for expression of the monocot protein in transgenic plants. The structural gene was placed under the transcriptional control of either a light-regulated oat phytochrome promoter or the constitutively active cauliflower mosaic virus 35S promoter. These genes were then introduced into *Nicotiana tabacum* and *N.plumbaginifolia*. None of the regenerated plants showed expression of oat phytochrome RNA when transcription was controlled by the oat promoter. In contrast, RNA was obtained in plants when the structural gene was functionally linked to the 35S promoter. Transformants expressing oat phytochrome RNA produced a full length 124-kd polypeptide that was recognized by oat-specific anti-phytochrome monoclonal antibodies. The oat protein was a substrate for chromophore addition in tobacco as judged by its red/far-red photoreversible sensitivity to trypsin degradation. Production of oat phytochrome in transgenic plants gave rise to increased phytochrome spectral activity in both light- and dark-grown plants. This increased phytochrome content resulted in phenotypic changes in transformed plants, including semi-dwarfism, darker green leaves, increased tillering and reduced apical dominance. The possible significance of expressing a biologically active phytochrome in transgenic plants is discussed.

Key words: chimeric constructions/phenotypic changes/phytochrome expression/transgenic plants

Introduction

Plants perceive light in the environment using a number of photoreceptor systems. Phytochrome is the best characterized of these systems, having been shown to play a critical role in the light-mediated regulation of a wide range of growth and developmental processes throughout the life cycle of plants. Among the biological events controlled at least in part by the phytochrome system are seed germination, de-etiolation of germinating seedlings, biosynthesis of many plastid proteins including those of the photosynthetic apparatus, control of shade tolerance and the timing of flowering and fruit production (Kendrick and Kronenberg, 1986).

Phytochrome is a soluble protein that is thought to exist *in vivo* as a dimer of identical subunits, each with a molecular mass of 120–127 kd depending on the plant species (Vierstra *et al.*, 1984). Each monomer contains a single linear tetrapyrrole chromophore covalently attached to a cysteine residue in the polypeptide via a thioether linkage. Phytochrome can exist in either of two spectrally distinct forms; the Pr form that absorbs maximally in the red (λ_{max} = 666 nm) region of the spectrum and the Pfr form that absorbs maximally in the far-red (λ_{max} = 730 nm) region of the spectrum. The two forms are reversibly interconvertible by light; Pr is converted to Pfr after absorbing red light and Pfr is converted to Pr after absorbing far-red light. Photoconversion of Pr to Pfr *in vivo* induces a characteristic array of morphogenic responses whereas rapid reconversion of Pfr to Pr often cancels the induction of these responses. This property of repeatable red/far-red photo-interconvertibility allows phytochrome to function as a reversible regulatory molecule, with Pr and Pfr considered to be biologically inactive and active forms of the molecule, respectively.

Phytochrome levels in plant tissues are controlled by a complex light-mediated mechanism that regulates the transcription rate of phytochrome genes, the turnover rate of phytochrome mRNA and destruction of the protein through the differential degradation of Pr and Pfr. In the dark, phytochrome is synthesized *de novo* as Pr with a protein half life of >100 h. Photoconversion of Pr to Pfr causes a rapid loss of spectrophotometrically and immunochemically detectable phytochrome due to the rapid proteolytic degradation of Pfr (Pratt, 1978). This rapid loss of Pfr appears to result from the selective conjugation of this form of the photoreceptor with ubiquitin, targeting it for degradation (Shanklin *et al.*, 1987; Jabben *et al.*, 1989). Steady-state phytochrome levels in plants are also self-regulated at the level of cellular mRNA concentration, particularly in monocot species (reviewed in Quail *et al.*, 1986). Red light irradiation of etiolated oat (*Avena sativa* L) results in a 20-fold reduction of cellular phytochrome mRNA within 5 h (Colbert *et al.*, 1983, 1985). In addition, this irradiation initiates a decrease in the transcription rate of the phytochrome genes that is detectable within 2 min after Pfr formation and leads to a 3-fold reduction in phytochrome transcription within 15 min after light treatment (Quail *et al.*, 1986).

It is now widely accepted that phytochrome mediates many of its effects on plant development by controlling the expression of specific genes. However, despite considerable research effort, the chain of events leading from Pfr formation to changes in the transcriptional pattern of these genes remains unknown. One method for studying this signal transduction pathway has been to generate plants with reduced phytochrome levels or with impaired signal transduction pathways by *in vivo* mutagenesis (Koornneef *et al.*, 1980, 1985). However, mutations generated by this method have not yet led to an improved understanding of

how phytochrome functions (Parks *et al.*, 1987). The availability of cloned phytochrome genes now makes it possible to study phytochrome-regulated gene expression by making specific modifications in the structural genes for the photoreceptor *in vitro* and then observing the effects that result from expressing these altered phytochromes in transgenic plants. This method will allow the identification of structural domains of the photoreceptor required for specific biological action and facilitate assessing the impact of changing phytochrome levels on specific phytochrome-controlled events in various plant tissues at different stages of development.

In a preliminary effort to express phytochrome in transgenic plants and to determine the effects that altering cellular levels of the photoreceptor might have in these plants, we have generated plant transformants containing a recombinant oat phytochrome gene. The structural region of this gene was created by combining cDNA and genomic sequences derived from phytochrome genes expressed in etiolated oat seedlings (Hershey *et al.*, 1985, 1987). Expression of the resulting structural gene was placed under the control of either a light-repressible oat phytochrome promoter (Colbert *et al.*, 1985; Hershey *et al.*, 1984, 1985) or the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The results obtained in this work demonstrate that transgenic tobacco plants did not produce a detectable monocot phytochrome when the chimeric oat gene was linked to its own promoter. However, a biologically active oat phytochrome was produced when expression of the structural gene was controlled by the 35S promoter. Production of the oat photoreceptor in transgenic plants gave rise to abnormally high phytochrome levels in the tissues of both light- and dark-grown plants. Phytochrome overexpression in these plants led to distinct heritable phenotypic alterations in a number of morphological traits known to be influenced by phytochrome.

Results

Introduction of chimeric oat phytochrome genes into Nicotiana tabacum and N.plumbaginifolia

Studies comparing the phytochrome gene sequences from oat and zucchini have shown that the monocot and dicot proteins share only 67% homology (Sharrock *et al.*, 1986). It was of interest to determine if an oat phytochrome gene could be expressed in dicot plants and if the resulting polypeptide would serve as a substrate for chromophore addition to generate a spectrally active photoreceptor. Chimeric phytochrome genes were created using oat cDNA and genomic clones pGP8.2-2, pGP2.4-1 and pAP3.1 (Hershey *et al.*, 1985, 1987) for introduction into transgenic tobacco. A structural gene encoding the N-terminal portion of type 3 phytochrome and the C-terminal portion of type 4 phytochrome was made. A polyadenylation signal from a type 4 gene was used in this construction (Figure 1A). This structural gene eliminates all of the introns normally found in the coding region of the known oat phytochrome genes, and therefore should obviate any potential problems associated with proper processing of a monocot primary transcript in dicots (Keith and Chua, 1986). This structural gene was linked to either a 960-bp CaMV 35S promoter fragment (Odell *et al.*, 1985) to generate the gene designated CV35phyt, or to a 1.1-kbp promoter fragment from the light-regulated type 3 oat phytochrome gene to generate the CV5B3 gene (Figure 1B).

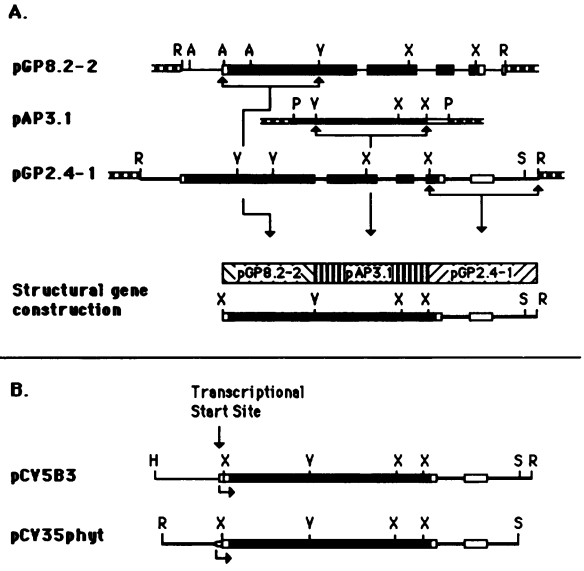

Fig. 1. Construction of chimeric oat phytochrome genes. Plasmids pCV5B3 and pCV35phyt were constructed by combining DNA fragments from each of three oat phytochrome clones, the cDNA clone, pAP3.1 and the genomic clones, pGP2.4-1 and pGP8.2-2 in such a way that all introns in the coding region were removed. The chimeric phytochrome coding sequence was created by using a 1.9-kbp *Eco*RV–*Xba*I fragment from pAP3.1 to provide the coding region for the central 630 amino acids of phytochrome. A 1.4-kbp *Acc*I–*Eco*RV fragment from pGP8.2-2 providing 5′-untranslated and N-terminal phytochrome coding sequences and a 1.7-kbp *Xba*I–*Eco*RI fragment from pGP2.4-1 providing the remaining C-terminal coding sequence and 3′-processing signals were ligated to this fragment to produce a promoterless phytochrome gene. This construction was linked either to a 1.1-kbp type 3 phytochrome promoter fragment to generate pCV5B3, or to a 960-bp 35S promoter fragment to generate pCV35phyt. The regions of the plasmids used to make the constructions are underlined with arrows indicating the fragment borders. Solid black regions in the maps denote coding sequences, white boxes denote transcribed but untranslated regions of the genes and lines represent control regions of the gene which do not appear in the final mRNA sequence. The partially filled, open ended boxes indicate plasmid sequences surrounding the DNA sequences of interest. A = *Acc*I, R = *Eco*RI, V = *Eco*RV, H = *Hin*dIII, P = *Pst*I, S = *Sal*I, X = *Xba*I.

The CV5B3 and CV35phyt constructions were mobilized from *Escherichia coli* HB101 into *Agrobacterium tumefaciens* strain GV3850 (Zambryski *et al.*, 1983) by bacterial conjugation, and the resulting *Agrobacteria* were used to infect *N.tabacum* (cv xanthi) and/or *N.plumbaginifolia* leaf disks (Horsch *et al.*, 1985). Only four of the 53 kanamycin-resistant shoots excised from the leaf disks infected with the CV35phyt gene were able to form roots when placed on rooting medium. In contrast, a much higher percentage of shoots transformed with the CV5B3 gene were able to form roots. Attempts to improve the rooting efficiency of the CV35phyt-transformed shoots by removal of Pfr through dark adaptation of shoots were unsuccessful. Six tobacco and 11 *N.plumbaginifolia* plants transformed with the CV5B3 gene and four plants transformed with the CV35phyt gene were transferred to soil and grown to maturity. Expression of the oat phytochrome gene in these plants was assessed at three levels; cellular RNA, immunologically recognizable protein and spectrally detectable phytochrome.

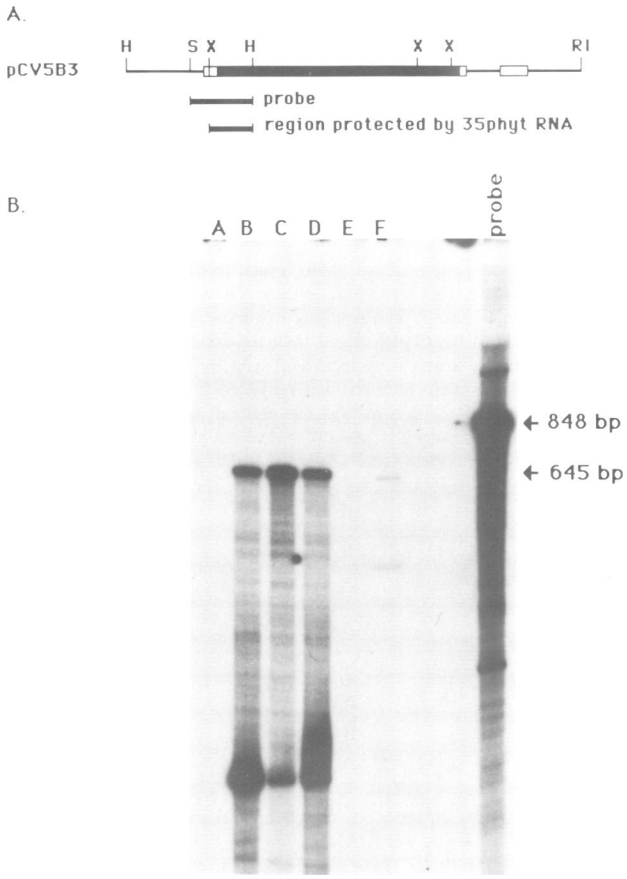

Fig. 2. RNase protection analysis of oat phytochrome gene expression in transgenic tobacco. An 800-bp *SalI*−*Hin*dIII fragment from the CV5B3 gene overlapping the transcriptional start site of the CV35phyt gene was cloned into pGEM 3 such that an antisense oat phytochrome RNA probe was generated by SP6 RNA polymerase. Total RNA was isolated from wild-type tobacco and transformed plants 7A, 9A, 9B and 54A grown in the light and from etiolated oat seedlings which had been exposed to red light 2 h prior to RNA isolation. Fifty μg of tobacco RNA from plants 7A, 9A, 9B, 54A and wild-type tobacco (**lanes A−E**) and 10 μg of oat RNA (**lane F**) were mixed with 1×10^6 c.p.m. of probe, hybridized overnight at 45.5°C and digested with RNases T1 and A at 30°C (see Materials and methods). The digestion products were electrophoresed in a 4.5% acrylamide gel containing 8 M urea, and subjected to autoradiography. Undigested probe was included for size comparison.

RNA analysis of oat phytochrome in transgenic tobacco

Cellular oat mRNA levels in transgenic plants were measured by RNase protection (Melton *et al.*, 1984; Zinn *et al.*, 1983). For this analysis, an 800-bp *Hin*dIII−*Sal*I fragment overlapping the transcription start site of the oat phytochrome type 3 gene was isolated from the CV5B3 gene and subcloned into pGEM3. SP6 polymerase was then used to generate an 800-base antisense RNA probe that was complementary to 645 bases at the 5′ end of the RNA transcribed from the CV35phyt gene. Figure 2 shows the results of a typical experiment in which expression of the CV35phyt gene in transformed tobacco grown to the 10 leaf stage was analyzed. No protection of the probe was observed following its hybridization to 50 μg of total RNA from either wild-type tobacco or transformant 7A (Figure 2, lanes E and A). However, RNA from plants 9A, 9B and 54A all protected the expected 645-base fragment from RNase digestion, indicating that these plants were expressing the oat phytochrome gene (Figure 2, lanes B, C and D). Total RNA isolated from etiolated oat seedlings 2 h after red light irradiation and known to contain low levels of oat phytochrome RNA served as a positive control in the protection procedure (Figure 2, lane F). The results show that tobacco transformants 9A, 9B and 54A produced high levels of oat phytochrome RNA, even in the light where the level of the native phytochrome RNA is typically reduced.

Tobacco and *N.plumbaginifolia* transformed with the CV5B3 gene were also analyzed for expression of oat phytochrome RNA. Plants were grown to the 10 leaf stage in constant light, and then placed in total darkness for 10 days prior to harvesting of tissue to allow maximal expression of oat phytochrome from its own light-repressible promoter. RNase protection analysis performed with these dark-adapted plants showed that none of the 17 individual transformants gave any protection of the 800-bp probe under conditions where RNA from red-light irradiated etiolated oat gave a strong hybridization signal (data not shown). These results lead us to speculate that the oat promoter may be inactive in tobacco and *N.plumbaginifolia* since stable phytochrome RNA was obtained when the oat phytochrome promoter in the CV35B gene was replaced with the CaMV 35S promoter. We are currently performing further experiments to assess the transcriptional activity of the oat phytochrome promoter in dicotyledonous plants.

Immunoblot analysis of oat phytochrome in transgenic tobacco

An immunoblot analysis procedure was used to show that transformed plants expressing the CV35phyt gene produced a full-length oat phytochrome apoprotein. Protein extracts from transformed plants were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), electroblotted to nitrocellulose and probed with two different monoclonal antibody preparations. One preparation was a mixture of five monoclonal antibodies generated against purified pea phytochrome that recognizes both dicot and monocot forms of the photoreceptor. The other preparation was a single monoclonal antibody generated against oat phytochrome that only recognizes the oat photoreceptor. The results of one such immunoblot analysis are shown in Figure 3. Two phytochrome species with molecular masses of 118 and 120 kd were detected in extracts of dark-grown wild-type tobacco. These two phytochrome species were not found in extracts of tobacco plants grown in the light (Figure 3A). However, plants expressing the CV35phyt gene contained a third, 124-kd species in both light- and dark-grown plants in addition to the 120 and 118 kd species found in dark-grown plants. This 124-kd species was shown to be oat phytochrome by its co-migration with purified 124-kd oat phytochrome in SDS−polyacrylamide gels (data not shown) and by recognition of the protein with a monoclonal antibody specific to the oat protein (Figure 3B). The level of oat phytochrome in tobacco was reduced in light-grown plants, suggesting that, as in oat, the protein may be more readily degraded in light.

Differential red/far-red trypsin sensitivity of oat phytochrome in tobacco

The synthesis of spectrally active phytochrome involves post-translational attachment of a linear tetrapyrrole chromophore

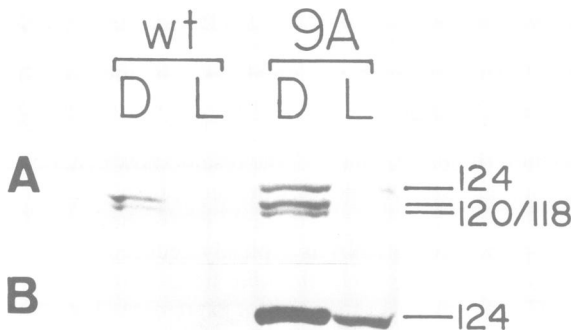

Fig. 3. Immunoblot detection of oat phytochrome in transgenic tobacco. Seeds from wild-type (wt) tobacco and self-fertilized tobacco transformant 9A which expresses oat phytochrome RNA were grown for 10 days in the dark (D) or 10 days in the light (L). Extracts from seedlings were concentrated by ammonium sulfate precipitation and subjected to SDS–PAGE. Immunoblot analysis was performed with a mixture of monoclonal antibodies against pea phytochrome that recognizes either both oat and tobacco phytochrome (**A**) or a monoclonal antibody specific to oat phytochrome (**B**). Sample volumes were adjusted to contain equal amounts of protein extracted from wt and transformed tissue. Approximately 10-fold more protein was used in the L lanes than in the D lanes to enable visualization of phytochrome from light-grown plants. The molecular mass of the various phytochrome polypeptides in kd is shown on the right.

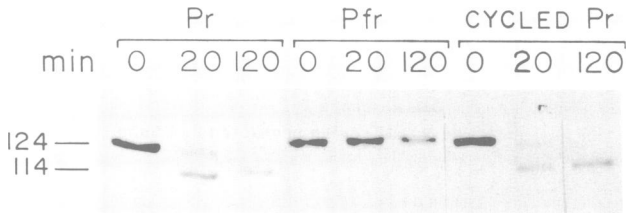

Fig. 4. Tryptic digestion of oat phytochrome from transgenic tobacco. Dark-grown tissue from transgenic tobacco 9A was grown and extracted as in Figure 3. The resulting extract was irradiated with far-red light (Pr), red light (Pfr) or red light followed by far-red light (cycled Pr). The extracts were incubated with trypsin. At the times indicated, aliquots were removed and subjected to SDS–PAGE and immunoblot analysis with a monoclonal antibody specific to oat phytochrome. The molecular mass of native oat phytochrome (124 kd) and its initial tryptic cleavage fragment (114 kd) are shown on the right.

Table I. Spectrophotometric analysis of phytochrome content in transgenic tobacco seedlings

		$\Delta(\Delta A)$/gFwt X 10^3	% of wt dark
Dark-grown	wt	2.3	100
	9A	6.0	261
Light-grown	wt	<0.01[a]	<0.4[a]
	9A	0.24	10

Wild-type (wt) tobacco seedlings or seedlings derived from self-fertilization of plant 9A were grown using the lighting conditions indicated for 8–10 days. Tissue was harvested, extracted and proteins were concentrated by ammonium sulfate precipitation as described in Materials and methods. Phytochrome content was assayed by dual wavelength difference spectroscopy ($A730/A660$) and expressed on a $\Delta(\Delta A)$/g fresh weight (Fwt) basis. Results represent the mean of two separate experiments.

[a]Phytochrome was undetectable in extracts from light-grown wild-type seedlings. The detection limit of the assay is 0.01.

to the apoprotein. The differential red/far-red sensitivity of the monocot protein to trypsin was examined to determine if the linear tetrapyrrole chromophore was added to oat apophytochrome in tobacco. This approach is based on the observation that functional Pr and Pfr display different sensitivities to various proteases (Vierstra and Quail, 1982; Lagarias and Mercurio, 1985). In the case of oat phytochrome, it has been shown that Pr is more sensitive to cleavage by trypsin than is Pfr, producing a 114-kd digestion product. Extracts of transgenic tobacco plants were given saturating irradiations with far-red light to generate Pr, red light to generate Pfr or red light followed by far-red light to generate cycled Pr. Extracts were then incubated with trypsin for various periods of time. Immunoblot analysis of the phytochrome digestion products in these extracts showed that trypsin rapidly cleaved both Pr and cycled Pr forms of oat phytochrome to a 114-kd species (Figure 4). In contrast, oat Pfr was cleaved less rapidly. This differential trypsin sensitivity is identical to that observed for purified oat phytochrome, indicating that a chromophore is added to the oat apoprotein synthesized in transgenic tobacco and that the protein is then able to undergo light-induced conformational changes.

Measurement of oat phytochrome spectral activity

Seedlings derived from the self-fertilization of both wild-type tobacco and transgenic tobacco expressing oat phytochrome were assayed for phytochrome spectral activity (Table I). Dark-grown plants expressing the CV35phyt gene were found to have 2.6 times more spectrally detectable phytochrome per gram fresh weight than did wild-type plants. Transfer of these plants to the light caused a substantial lowering of phytochrome levels in both wild-type and transgenic plants. However, light-grown transgenic plants contained 20 times more spectrally active phytochrome than did their wild-type counterparts. Since immunoblot analysis showed that oat photoreceptor was the sole detectable phytochrome species in light-grown transgenic tobacco (Figure 3), the increased spectral activity in these plants must be due to the presence of photoreversible oat phytochrome.

Phenotypic changes in plants overexpressing oat phytochrome

It became readily apparent as plants 9A, 9B and 54A matured that their increased phytochrome content caused striking changes in their morphology (Figure 5). Plant 7A, a kanamycin-resistant transformant that was not expressing oat phytochrome, was morphologically indistinguishable from wild-type tobacco. However, the three transformants that were overexpressing phytochrome showed increased green pigmentation, increased tillering (more stems formed from the base of the plant), reduced rooting capacity in the presence of kanamycin, reduced apical dominance and reduced internodal distance when compared with wild-type plants. The latter trait resulted in plants which had the same number of leaf nodes to inflorescence but which were approximately half the height of normal plants. Plants 9A, 9B and 54A had slightly smaller and thicker leaves than wild-type plants and plant 7A, but flowered at approximately the same time and had comparable seed yields per inflorescence. The phenotypic changes were found to be stably transmit-

Fig. 5. Phenotypic changes exhibited in transgenic tobacco expressing oat phytochrome. The non-expressing transformed plant 7A (left) is shown next to the phytochrome overexpressing transformant, 9A (right). Plant 9A shows the characteristic phenotype seen in all phytochrome overexpressors. These include darker green pigmentation, shorter height, smaller leaf size, and increased tillering. The plants have approximately the same number of internodes to inflorescence.

ted to later generations and segregated in the expected 3:1 ratio when the transformants were self-fertilized.

Discussion

In experiments described here, we show that a monocot phytochrome gene can be expressed in a dicot, that the monocot apoprotein can be a substrate for chromophore addition and that the resulting molecule can act as a functional photoreceptor. By all criteria examined, including SDS−PAGE mobility, spectral activity, differential red/far-red trypsin sensitivity and monoclonal antibody recognition, the oat chromoprotein in transgenic tobacco is indistinguishable from that found in etiolated oat seedlings. The functional nature of the monocot molecule in transgenic plants was demonstrated by its ability to effect changes in a number of morphological characteristics known to be under phytochrome control.

The results of the work presented here have led us to some interesting conclusions. They indicate that the biosynthetic pathway responsible for post-translational modification of the phytochrome apoprotein to yield a functional photoreceptor is common to both oat and tobacco. The commonality of this pathway is significant since the undecapeptide surrounding the chromophore attachment site is one of the few amino acid sequences found to be conserved when known monocot and dicot phytochrome sequences are compared (Sharrock *et al.*, 1986; Mercurio *et al.*, 1986). It remains to be determined if the undecapeptide sequence itself is sufficient for targeting chromophore attachment. The lower level of oat phytochrome in light-grown versus dark-grown transgenic tobacco suggests that the chromoprotein is degraded in a fashion similar to that in oat (Qauil *et al.*, 1973; Pratt *et al.*, 1974). Therefore, the pathway responsible for the selective degradation of Pfr may also be conserved between monocots and dicots.

The phenotypic changes resulting from constitutive expression of etiolated oat phytochrome in transgenic tobacco have several important physiological implications. First, these changes show that a monocot photoreceptor can function in a dicot, providing the first evidence that phytochromes from distantly related species function in a similar manner. The functional nature of oat phytochrome in tobacco also indicates that domains in the protein responsible for biological action have been conserved through evolution, lending support to an experimental approach using amino acid sequence comparison among phytochromes to identify these functional domains (Sharrock *et al.*, 1986). Second, our results demonstrate that an etiolated phytochrome functions as a biologically active photoreceptor in light-grown plants. This biological activity in the light is significant since the recent discovery of two types of phytochrome in plants, one expressed mainly in etiolated tissue and the other expressed mainly in light-grown tissue (Shimizaki and Pratt, 1985, 1986; Tokuhisa *et al.*, 1985; Tokuhisa and Quail, 1983, 1987) might indicate that etiolated phytochrome is inactive in plants following de-etiolation. Third, the results show that overexpression of phytochrome can be achieved in transgenic plants and leads to an exaggerated light-grown phenotype. Whether increased phytochrome content was accomplished solely by circumventing the light-induced transcriptional down-regulation of the phytochrome genes, or occurred in conjunction with a reduced ability of tobacco to degrade oat phytochrome must be determined. The phenotypic effects of this overexpression lend support to the hypothesis that the regulation of phytochrome-controlled processes in light-grown plants is due, at least in part, to maintenance of cellular phytochrome levels through a delicate balance between biosynthesis and degradation of the photoreceptor. The ability to induce a similar phenotype in transgenic plants by inhibiting Pfr-dependent degradation of the photoreceptor is currently under investigation.

No stable phytochrome RNA was detected in transformed tobacco or *N.plumbaginifolia* plants when expression of the chimeric phytochrome gene was regulated by its own promoter. There are a number of possible explanations for this result. The oat phytochrome RNA transcript might be less stable in tobacco due to sequence divergence between the oat and tobacco phytochrome RNAs (Sharrock *et al.*, 1986). However, our ability to obtain phytochrome RNA when expression of the oat phytochrome gene was under the control of the 35S promoter suggests that oat RNA is stable in tobacco. A more likely explanation for the lack of expression lies in the relative weakness of the oat phytochrome promoter (Colbert *et al.*, 1985). It is quite possible that this promoter may not be functional in the heterologous dicotyledonous plants. Further experiments to determine the

reason(s) for the lack of expression using phytochrome promoter/reporter gene fusions are in progress.

The overexpression of phytochrome in transformed tobacco plants yielded some interesting inverse correlations with the work of Koornneef and colleagues (1980, 1985) who studied tomato mutants with decreased levels of spectrally detectable phytochrome. Their mutants were yellow green, taller than normal and displayed increased apical dominance. In contrast, our transformed plants overexpressing phytochrome displayed characteristics at the other extreme, i.e. they were shorter than normal, more green and displayed reduced apical dominance.

The phenotype of transgenic plants overexpressing phytochrome is consistent with a model of phytochrome action in which the photoreceptor participates in regulating phytohormone balances within plant tissues. It is clear from numerous studies that the levels of a number of phytohormones in plants can be modulated by phytochrome (De Greef and Fredericq, 1983). For example, red light irradiation of etiolated seedlings transiently increases the levels of cytokinin and gibberellins and decreases the levels of extractable auxins and emitted ethylene. Exogenous application of gibberellic acid and ethylene can substitute for Pfr in inducing the germination of seeds from light-sensitive plant species such as lettuce (Frankford and Taylorson, 1983). In addition, treatment of dark-grown plants with cytokinins promotes chloroplast development, and in particular stimulates the expression of the phytochrome-regulated genes for ribulose bisphosphate carboxylase small subunit and chlorophyll *a/b*-binding protein (Flores and Tobin, 1986).

Further support for the hypothesis that phytochrome overexpression causes phenotypic changes by disrupting phytohormone balances in the transgenic plants is provided by the finding that phenotypic changes similar to those observed here occur when plants are treated with gibberellin antagonists (Steffens *et al.*, 1985; Wang *et al.*, 1985). Similar phenotypic alterations occur when cytokinin levels are increased in plants by transforming them with a functional cytokinin biosynthetic gene from the *A.tumefaciens* Ti plasmid (Peerbolte *et al.*, 1987). Analysis of the hormone levels in plants overexpressing phytochrome is in progress, and the results should help provide additional insights into the interaction of phytochrome with phytohormone-mediated plant development.

The discovery that functional oat phytochrome can be expressed in transgenic tobacco presents a unique opportunity to further investigate the structure and function of this photoreceptor system. Site-directed mutagenesis of phytochrome genes can be used to identify peptide sequences essential for their biological activity and for the protein's photoreversible spectral properties. In addition, this approach can be used to determine both the specific amino acid sequence(s) required for chromophore attachment, and those sequences required for the ubiquitin binding that initiates Pfr-dependent protein degradation. Deletion analysis and mutagenesis of the C-terminal end of the molecule may also help to identify the domains responsible for phytochrome dimerization (Jones and Quail, 1986).

The phenotypic changes generated through overexpression of phytochrome may be of agricultural importance. In plants such as rice, wheat, soybean and sunflower, semi-dwarfism has been found to enhance yields by reducing lodging (Fehr, 1987). Overproduction of phytochrome might also alleviate photoperiod sensitivity in crops such as wheat, thus allowing them to mature earlier in the season. Furthermore, if the heightened green pigmentation indicates a net increase in photosynthetic activity, transfer of this trait to forage plants such as alfalfa or clover might increase the deposition of biomass into their leaves and thereby make them more valuable foodstuffs.

Materials and methods

Plasmid constructions

A chimeric phytochrome gene with an uninterrupted coding region was created by linking coding sequences from types 3 and 4 phytochrome genes to a type 3 promoter/5′-untranslated leader, and a type 4 3′-flanking sequence (Figure 1). The 5′-transcribed portion of the phytochrome type 3 gene was subcloned into pUC18 from pGP8.2-2 (Hershey *et al.*, 1985) as a 2.56 kb *Eco*RI−*Pst*I fragment. The resulting plasmid, designated pGP8.2-21, was partially digested with the restriction endonuclease *Acc*I, the 5′ ends were rendered blunt with the Klenow fragment of DNA polymerase I and the resulting DNA was cleaved with *Pst*I. A 1.9 kbp *Acc*I−*Pst*I DNA fragment containing 38 bp of the 5′-untranslated region and 1.86 kbp of protein coding sequence was then subcloned into pUC18 that had been digested with *Pst*I and *Hin*dIII to create plasmid pGP8.2-22.

A fragment of the phytochrome type 3 gene containing 1 kbp of sequence 5′ to the transcription start site and 85 bp of the 5′-untranslated leader sequence was obtained by digesting pGP8.2-1 (Hershey *et al.*, 1985) with *Kpn*I, blunting the ends with Klenow fragment and then digesting with *Hin*dIII. The resulting DNA was ligated to pUC18 that had been digested with *Hin*cII and *Hin*dIII to create plasmid pJ-I. A 1.1-kbp *Hin*dIII−*Xba*I promoter fragment was subcloned from pJ-I into pMSP^{r}K that had been digested with the same enzymes to generate pCV5. pMSP^{r}K is a plasmid vector containing the pUC19 polylinker, a pBR322 origin of replication, a bacterial selection marker and a neomycin phosphotransferase II (NPT II) coding sequence linked to the promoter region and downstream polyadenylation signals from the CaMV 35S transcript.

The phytochrome fragment in pGP8.2-22 was excised by digestion with *Pst*I and *Xba*I, and ligated into the *Pst*I and *Xba*I sites of pCV5 to generate plasmid pCV5-8.2. A 3.0-kbp *Eco*RV fragment containing the majority of the phytochrome-coding region was obtained from pAP3.1 (Hershey *et al.*, 1985) and ligated into the *Eco*RV site in pCV5-8.2 to create pCV5B. Plasmid pCV5B was partially digested with *Xba*I and then digested to completion with *Eco*RI. A 1.7-kbp *Xba*I−*Eco*RI fragment containing the C-terminal coding region and 3′-processing signals from the phytochrome type 4 gene was excised from pGP2.4-1 and ligated into pCV5B to generate pCV5B3 (see Figure 1).

Plasmid pCV35phyt was created in pMSPKN (a plasmid similar to pMSP^{r}K with the NPT II gene under the control of the nopaline synthetase gene promoter and 3′ sequences). A region from nucleotide 6493 to 7454 of the CaMV 35S transcript containing the 35S promoter was obtained from plasmid pUC9 by cleaving it with *Hin*dIII, filling in the resulting 5′ ends with Klenow fragment and digesting with *Eco*RI. A 960-bp fragment was gel purified and ligated into the pMSPKN polylinker between a blunt-ended *Kpn*I site and the *Eco*RI site to generate pCV35P. pCV5B3 and pCV35P were both digested with *Xba*I and ligated together to generate pCV35phytA, which contains the 2.8-kbp promoter-proximal *Xba*I fragment from pCV5B3 operably linked to the 35S promoter. The C-terminal protein coding sequence and the 3′-untranslated region were excised from pCV5B3 as a *Bcl*I−*Sal*I fragment and ligated with pCV35phytA that had been cleaved with the same enzymes. Both plasmids were grown in the dam− *E.coli* strain NS2626 to obtain unmethylated *Bcl*I sites. The resulting plasmid, called pCV35phyt, contains the complete phytochrome coding sequence from pCV5B3 operably linked to the CaMV 35S promoter (see Figure 1).

Transformation of plants

pCV35phyt and pCV5B3 were mobilized from *E.coli* strain HB101 into *A.tumefaciens* strain GV3850 using a pRK2013 in HB101 for plasmid mobilization in a triparental conjugation (Ditta *et al.*, 1980). Conjugates were selected after 16 h of growth at 30°C on non-selective medium by plating on LB agar plates containing 100 μg/ml each of spectinomycin, streptomycin and rifampicin. Constructions were mobilized into the plant genome via *A.tumefaciens* infection of *N.tabacum* (cv. xanthi) or *N.plumbaginafolia* leaf disks and plants were regenerated as described by Horsch *et al.* (1985).

Plant RNA isolation

Potted transformed plants were grown to approximately the 10 leaf stage and 1 g of leaf tissue was harvested and frozen in liquid nitrogen. The frozen leaf tissue was ground with a mortar and pestle, suspended in 4 ml of proteinase K buffer (250 μg/ml proteinase K in 50 mM Tris−HCl pH 9.0, 10 mM EDTA and 2% SDS) for 10 min at 50°C, and then extracted twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1). Nucleic acids were collected by isopropanol precipitation and RNA was differentially precipitated from the DNA by resuspending the pellet in 1.5 ml H_2O and adding 0.5 ml of 8 M LiCl. After 2 h on ice, RNA was collected by centrifugation at 4°C for 20 min at 12 000 *g*. The RNA was resuspended in H_2O and the LiCl precipitation was repeated. The RNA was then resuspended in H_2O and precipitated with ethanol.

RNase protection analysis

An RNase protection procedure (Zinn *et al.*, 1983; Melton *et al.*, 1984) was used to detect oat phytochrome mRNA in transformed tobacco plants. An 800-bp *Hin*dIII−*Sal*I fragment overlapping the transcription start site of the oat phytochrome type 3 gene was isolated from pCV5B3 and ligated into *Hin*dIII−*Sal*I-digested pGEM 3 (Promega Biotec) to generate pSP3.3. Plasmid pSP3.3 was linearized with *Eco*RI and a probe was synthesized in the presence of [α-^{32}P]UTP (>3000 Ci/mM) using a Promega Biotec kit as specified by the manufacturer. Fifty micrograms of total RNA from either transformed or control plants were mixed with 1×10^6 c.p.m. of probe and hybridized for 16 h at 45.5°C. Hybrids were digested at 30°C with 40 μg/ml RNase A and 2 μg/ml RNase T1. The RNases were then inactivated by digestion with proteinase K in the presence of SDS (Zinn *et al.*, 1983). The RNA samples were analyzed on a 4.5% acrylamide gel containing 8 M urea.

Immunoblot analysis

The synthesis of the oat phytochrome protein in transformed plants was assessed by immunoblot analysis using anti-phytochrome monoclonal antibodies (Shanklin *et al.*, 1987). Proteins were extracted from frozen tobacco seedlings by homogenization for 30 s at 4°C in a solution of 50% ethylene glycol, 100 mM Tris−HCl (pH 8.3), 140 mM ammonium sulfate, 20 mM sodium metabisulfite, 10 mM EDTA, and freshly added 4 mM phenylmethylsulfonyl fluoride (Buffer A). The extract was made to 0.1% (w/v) in poly(ethylenimine) by the addition of a 10% (w/v) solution (pH 7.8), stirred for 5 min and clarified at 50 000 *g* for 20 min. The protein concentration of the supernatant was determined by the Bradford assay. A volume of each extract containing 35 μg of protein was boiled for 3 min with an equal volume of SDS−PAGE sample buffer (Vierstra *et al.*, 1985) and subjected to SDS−PAGE on a 7% SDS−polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose (HAHY 304 FO, Millipore). Immunoreactive phytochrome species were visualized colorimetrically using anti-phytochrome monoclonal antibodies in conjunction with alkaline phosphatase-conjugated goat IgGs directed against mouse immunoglobulins and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Both tobacco and oat phytochromes were detected with a mixture of five monoclonal antibodies against pea phytochrome (Nagatani *et al.*, 1987). Oat phytochrome was specifically detected by a monoclonal antibody that recognizes only the oat protein.

Spectral analysis

Spectral analysis of phytochrome activity was performed using protein extracts that were prepared as described above with the following modifications. After poly(ethylenimine) clarification of crude extracts, proteins were concentrated by ammonium sulfate precipitation (250 mg/ml) and resuspended in 0.5 × buffer A with 14 mM β-mercaptoethanol replacing sodium metabisulfite. Spectrophotometric measurements were performed using a Shimadzu UV3000 dual wavelength (A730/A666) spectrophotometer following saturating red or far-red irradiations of the extracts. The extinction coefficient of 1.2×10^5 l/mol/cm for Pr (Litts *et al.*, 1983) and a photoequilibrium of 86% Pfr in red light (Vierstra and Quail, 1983) were used for all calculations of phytochrome concentration.

Tryptic mapping

Tobacco extracts were irradiated either with red light (I = 35 W/m^2), far-red light (I = 14 W/m^2) or red light followed by far-red light. Trypsin (Sigma type XI) was added at a ratio of 1/500 (w/w) trypsin/total protein and the digestion mixtures incubated at 20°C in darkness. At various times, aliquots were boiled for 2 min in an equal volume of SDS−PAGE sample buffer, cooled on ice and mixed with a 5-fold excess (w/w) of soybean trypsin inhibitor. Samples were analyzed by immunoblot analysis as above.

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