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PHYTOCHROMES IN PHOTOSYNTHETICALLY COMPETENT PLANTS

FINAL REPORT

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DISCLAIMER

FINAL REPORT

INTRODUCTION

This progress report refers extensively to the appended publications, which provide additional detail. Frequent reference will be made to figures in these appended publications. The appended publications, all of which describe work funded by this DOE grant, are:

Cordonnier, M.-M. and L. H. Pratt. 1991. Phytochrome from green *Avena* characterized with monoclonal antibodies directed to it. In: *Photobiology: the Science and its Applications*, E. Riklis, ed., Plenum Press, New York (11 pages).

Pratt, L. H., Y. Shimazaki, S. J. Stewart and M.-M. Cordonnier. 1991a. Large-scale partial purification of phytochrome from green leaves of *Avena sativa* L. *Planta* 184:81-86.

Pratt, L. H., S. J. Stewart, Y. Shimazaki, Y.-C. Wang and M.-M. Cordonnier. 1991b. Monoclonal antibodies directed to phytochrome from green leaves of *Avena sativa* L. cross react weakly or not at all with the phytochrome that is most abundant in etiolated leaves of the same species. *Planta* 184:87-95.

Wang, Y.-C., S. J. Stewart, M.-M. Cordonnier and L. H. Pratt. 1991. *Avena sativa* L. contains three phytochromes, only one of which is abundant in etiolated tissue. *Planta* 184:96-104.

Pratt, L. H., M.-M. Cordonnier, Y.-C. Wang, S. J. Stewart and M. Moyer. 1991c. Evidence for three phytochromes in *Avena*. In: *Phytochrome Properties and Biological Action*, B. Thomas and C. B. Johnson, eds., Springer-Verlag, Berlin pp. 39-55.

Childs, K. L., L. H. Pratt and P. W. Morgan. 1991. Genetic regulation of development in *Sorghum bicolor*. VII. The ma3R allele results in abnormal phytochrome physiology. *Plant Physiology* 97:714-719.

Cordonnier-Pratt, M.-M., L. H. Pratt, S. J. Stewart and Y.-C. Wang. 1991. Multiple phytochromes in *Avena sativa* L.: discovery, characterization, and differential expression. In: *Plant Sciences Today*, Y. de Kouchkovsky, ed., INRA Editions, Paris, pp. 57-60.

Wang, Y.-C, M.-M. Cordonnier-Pratt and L. H. Pratt. 1992. Detection and quantitation of three phytochromes in unimbibed seeds of *Avena sativa* L. *Photochemistry and Photobiology*, in press pending minor revision.

Stewart, S. J., L. H. Pratt and M.-M. Cordonnier-Pratt. 1992. Phytochrome levels in light-grown *Avena* change in response to end-of-day irradiations. *Plant Physiology* (to be submitted).

Definitions. An initial objective of this DOE-funded research program, beginning about a decade ago, was to determine whether there might be more than one kind of phytochrome. Initial work established that the phytochrome isolated from a light-grown plant differs markedly from that isolated from etiolated tissue of the same organism. The term `etiolated-plant phytochrome' will be used to refer to the chromoprotein that predominates in etiolated tissue and that has been well characterized over the past three decades. The phytochrome that predominates in light-grown tissue will be referred to as `green-plant phytochrome.' Use of these terms is not meant to imply that etiolated-plant phytochrome is found only in etiolated tissue, or that green-plant phytochrome is present only in light-grown tissue.

An even more recent complication is the discovery that green-oat phytochrome is itself composed of two types (see below). One green-oat phytochrome has a monomer size of 125 kDa, the other 123 kDa. The more general term `green-oat phytochrome' will be used when it is uncertain whether the phytochrome in question is 125 kDa, 123 kDa, or a mixture of the two. Otherwise, reference will be to either `125-kDa' or `123-kDa' phytochrome as appropriate.

BACKGROUND

Both the physicochemical properties of phytochrome and its biological functions are best understood with reference to etiolated plant tissues. Phytochrome plays, however, an important role in green plants as well. For many years it was tacitly assumed that phytochrome in green plants was the same as that in etiolated seedlings, primarily because it is exceedingly difficult to isolate and characterize phytochrome from green plants. Nevertheless, it was occasionally hypothesized that phytochrome should be heterogeneous, with different types of phytochrome predominating in dark- and light-grown tissue. Largely as the result of DOE-funded research programs over the past decade, these early hypotheses have proven to be valid, at least in part. It is now evident that etiolated- and green-plant phytochromes differ markedly from one another.

Our DOE-funded research program prior to the most recent three-year period had led to the following major observations.

(1) Using newly produced monoclonal antibodies (MAbs) to etiolated-oat phytochrome, we devised a positive, heterologous sandwich, enzyme-linked immunosorbant assay (ELISA) for phytochrome that detects as little as 100 pg (< 1 fmol) of the chromoprotein (Shimazaki *et al.*, *Planta*, 159:534-544, 1983). This ELISA is as much as 1000-fold more sensitive than widely used photoreversibility assays. While we used it to quantitate phytochrome in crude extracts of light-grown plants, it quickly became apparent that it detected only a small portion of the phytochrome that was present.

(2) Both the polyclonal antibodies (PAbs) and the MAbs that were used for the immunoquantitation ELISA were directed to etiolated-oat phytochrome. We found that most of these antibodies would not bind to green-oat phytochrome (Shimazaki and Pratt, *Planta* 164:333-344, 1985). While Shimazaki and Pratt had developed an initial protocol for partial purification of green-oat phytochrome, it did not permit its extensive characterization.

(3) We subsequently took advantage of a MAb to phytochrome from etiolated pea shoots (Pea-25). This MAb cross reacts with phytochrome from green oat leaves, as well as phytochrome (or a protein the size of phytochrome) from a wide variety of other organisms, including other angiosperms, a moss, and three algae (Cordonnier *et al.*, *Plant Physiol.* 80:982-987, 1986). With this MAb, Cordonnier *et al.* (*Biochemistry* 25:7657-7666, 1986) initiated a physicochemical characterization of green-oat phytochrome. This work helped establish the magnitude of the differences between etiolated- and green-oat phytochrome, reinforcing the hypothesis that they derive from different genes. It was also with the aid of this MAb that we were able to devise a purification protocol useful for obtaining both MAbs and PAbs directed to the immunochemically unique phytochrome isolated from green oats, as described below. Unfortunately, Pea-25 itself does not function for immunoaffinity purification of phytochrome.

(4) With two other MAbs (Oat-9 and Oat-16), an epitope was identified on etiolated-oat phytochrome that is also found on at least some green-oat phytochrome. This epitope is of special interest because it undergoes a conformational change upon phototransformation (Shimazaki *et al.*, *Plant Physiol.* 82:109-113, 1986).

(5) Several observations indicated that green-oat phytochrome might itself be heterogeneous. Specifically, antibodies directed to etiolated-oat phytochrome failed at saturation to precipitate more than a fraction of the photoreversibility from green-oat phytochrome preparations (Shimazaki and Pratt, *Planta* 164:333-344, 1985, *Planta* 168:512-515, 1986). The green-oat phytochrome that was precipitated was not detected by a MAb (Pea-25) that, while not being able to precipitate phytochrome, nevertheless could detect green-oat phytochrome by immunoblot assay (Cordonnier *et al.*, *Biochemistry* 25:7657-7666, 1986).

Thus, four years ago the evidence was strong that etiolated- and green-plant phytochromes were markedly different, and that they probably derived from different genes. Moreover, there were indications that green-oat phytochrome itself might be heterogeneous. Progress made since then will be summarized below.

PROGRESS

Improve protocol for purification of green-oat phytochrome

The exceedingly low abundance of green-oat phytochrome, estimated to be about 0.002-0.003% of the protein in an aqueous crude extract (Pratt *et al.*, 1991a), makes its purification correspondingly difficult. In particular, methodology that could be employed on a large scale and that was significantly more rapid than prior protocols was essential. In addition, we learned that green-oat phytochrome samples prepared essentially as described by Shimazaki and Pratt (*Planta* 164:333-344, 1985) contained an immunodominant contaminant of about the same monomer size (115 kDa) as proteolytically degraded green-oat phytochrome (Fig. 1 in Cordonnier and Pratt, 1991). It was therefore imperative that we also eliminate this contaminant.

Improvements in our earlier protocol are described in Pratt *et al.* (1991a). Compared to earlier protocols, the improved protocol is significantly more rapid, provides improved yield and purity, permits beginning with larger quantities of tissue (Table 1 in Pratt *et al.*, 1991a, and compare lanes 2 and 4 in Fig. 2 in Pratt *et al.*, 1991a), and eliminates the immunodominant contaminant referred to above (compare lane 1 to lanes 2 and 3 in Fig. 1c in Cordonnier and Pratt, 1991; MAb G3-12H8 is one of a large number directed to the contaminant). While still only about 0.6% pure the green-oat phytochrome thus obtained proved suitable for generating both monoclonal and polyclonal antibodies (see below).

Other approaches. As suggested in the previous proposal, we prepared both MAbs and PAbs to the major contaminating proteins in hydroxyapatite-purified green-oat phytochrome samples. Affi-Gel 10 affinity columns were prepared with immobilized antibodies to these contaminants. Somewhat unexpectedly, while these columns did immunoadsorb contaminating proteins, enough green-oat phytochrome was lost during the adsorption process that there was no effective purification. Thus, while this approach should work in theory, it did not work in practice.

We also attempted the approach used by Kidd and Lagarias (*J. Biol. Chem.* 265:7029-7035, 1990) to purify phytochrome from *Mesotaenium.* Whereas *Mesotaenium* phytochrome bound differentially to an anion exchange column, the same was not true for green-oat phytochrome. Thus, this differential approach was no better for our application than more conventional elution protocols.

Similarly, initial attempts to purify hydroxyapatite-purified green-oat phytochrome further by conventional means (*e.g.*, ion exchange chromatography, size exclusion chromatography, HPLC, FPLC) was not helpful. While modest 2-5-fold increases in purity were achieved, yields were typically 50% or less. Given the exceedingly low concentration of green-oat phytochrome (estimated to be 1 part in 50,000 parts of protein in a crude, clarified homogenate), these losses were unacceptable. Note that in the purest hydroxyapatite fractions

(Table 1 in Pratt *et al.*, 1991a) there is only *ca.* 100 μg of green-oat phytochrome per kilogram fresh weight. While we could eventually obtain green-oat phytochrome that is estimated to be 5-8% pure, the yield was only 15-20 μg/kg fresh weight.

Prepare and characterize antibodies to green-oat phytochrome

Immunogen preparation. Because the HA-purified green-oat phytochrome was still too impure to function well as an immunogen, it was purified further by SDS PAGE. Aliquots containing about 1 μg phytochrome were subjected to SDS PAGE. Phytochrome was identified by Zn^{2+}-induced fluorescence and staining with Coomassie blue. Phytochrome bands were excised and applied seven at a time to lanes of a second SDS polyacrylamide gel. After this second electrophoretic separation, which includes concentration of the phytochrome, proteins were electrotransferred to nitrocellulose. Phytochrome was identified on the nitrocellulose both by Zn^{2+}-induced fluorescence and by staining with Ponceau S. Phytochrome-containing bands were cut from the nitrocellulose, washed exhaustively with H_2O, dissolved in DMSO, emulsified with adjuvant, and injected into both mice and rabbits.

MAbs. Hybridomas secreting MAbs directed to green-oat phytochrome were prepared from immunized mice as described in Pratt *et al.* (1991b). Cell lines of interest were identified by screening by both ELISA and immunoblotting. For ELISA screening it was necessary to adapt an amplification strategy to our application, because the purity of the green-oat phytochrome with which microtiter wells were coated was low. This amplification utilizes NADP as the initial substrate. NAD formed by the alkaline phosphatase used as a label then functions as a cofactor in the cyclic transfer of electrons from ethanol (via alcohol dehydrogenase) to a tetrazolium dye (via a non-specific diaphorase). The details are presented in Pratt *et al.* (1991b). Five new MAbs directed to green-oat phytochrome were generated, to be added to two obtained previously (Pratt and Cordonnier, in *Phytochrome and Photoregulation in Plants*, M. Furuya, ed., Academic Press, pp. 83-94, 1987). Three of the MAbs (GO-4, GO-5, GO-6) were shown unambiguously to be directed to phytochrome by their ability to immunoprecipitate photoreversibility (Fig. 1 in Pratt *et al.*, 1991b). A fourth (GO-1) is clearly directed to phytochrome by its ability to immunostain etiolated-oat phytochrome apoprotein expressed as a fusion protein in *E. coli* (Fig. 5 in Pratt *et al.*, 1991b). As discussed in Pratt *et al.* (1991b) this MAb is, however, clearly directed to green- rather than etiolated-oat phytochrome. Because GO-1 cross reacts with etiolated-oat phytochrome, its epitope could be mapped. It lies in the region between amino acids 618 and 686 in the etiolated-oat phytochrome sequence (Fig. 6 in Pratt *et al.*, 1990b). While the other three MAbs (GO-2, GO-7, GO-8) are also directed to green-oat phytochrome, establishing this point unequivocally is inextricably linked to the observation that there are two green-oat phytochromes. Thus, this issue will be dealt with below.

Of the seven MAbs to green-oat phytochrome, only one (GO-1) cross reacts with etiolated-oat phytochrome. The other six fail to detect by immunoblot assay etiolated-oat phytochrome apoprotein expressed in *E. coli* even when the SDS polyacrylamide gel is overloaded (Fig. 5 in Pratt *et al.*, 1991b). None of these MAbs appears to be directed to a carbohydrate-containing epitope (Table 1 in Pratt *et al.*, 1991b). Data obtained with these antibodies reinforce earlier observations made with antibodies to etiolated-oat phytochrome, indicating that phytochrome from etiolated and green oats are markedly distinct from one another immunochemically.

Each of the new MAbs has a kappa light chain and all but GO-1 and GO-6 is an IgG_1. GO-6 is an IgM while the heavy chain of GO-1 does not react with any of the isotyping antibodies that we used. These seven new MAbs detect four different epitopes as elucidated by immunoblot analysis of proteolytic digests (Fig. 1 in Wang *et al.*, 1991). None of these epitopes is equivalent to that detected by Pea-25, which cross reacts with green-oat phytochrome.

PAbs. Rabbits immunized with SDS PAGE-purified green-oat phytochrome yielded antisera that appear specific to green-oat phytochrome (Fig. 10 in Wang *et al.*, 1991). These antisera cross react poorly or not at all with etiolated-oat phytochrome. Unfortunately, as is the case with many of the MAbs, these PAbs do not bind well to phytochrome in solution. Thus, as is the case for all MAbs except GO-4, GO-5, and GO-6 (which might detect the same epitope-*cf.* Fig. 1 in Wang *et al.*, 1991), these PAbs are not useful either for immunoprecipitation of green-oat phytochrome, for its immunopurification, or for ELISAs. Given that SDS-denatured phytochrome was used as immunogen, this outcome is perhaps not too surprising.

Antibodies to a synthetic peptide. As discussed further below, we have obtained an amino acid sequence for green-oat phytochrome. Rabbit antibodies have been prepared to a synthetic peptide consisting of this sequence. Unfortunately, the rabbit antibodies fail to detect intact green-oat phytochrome, either by immunoblot assay or by immunoprecipitation of photoreversibility. Since only about one-half of randomly selected peptides yield antibodies capable of detecting the protein from which the peptides were derived, however, this result is not surprising.

Immunoaffinity purification of green-oat phytochrome. Affinity columns of immobilized MAb to green-oat phytochrome have been prepared. Those made with GO-4 and GO-5 have been effective (Fig. 5 in Wang *et al.*, 1991; Fig. 1 in Pratt *et al.*, 1991c). When hydroxyapatite-purified green-oat phytochrome is adsorbed to one of these columns, however, it must be eluted by low pH. Moreover, these MAbs only purify one of the two green-oat phytochromes now known to exist (see below). The transient exposure to low pH irreversibly denatures what is eluted, which limits its utility. It was this immunoaffinity-purified green-oat phytochrome that was used for microsequencing, as summarized below.

Characterize green-oat phytochrome

Absorbance spectra. The increased purity of green-oat phytochrome obtained by the improved protocol, together with a large decrease in contaminating pigments, permitted measurement of absorbance spectra in the blue region for the first time. While the previously observed blue shift of Pr absorbance was seen in the red spectral region, no significant difference was detected in the blue (Fig. 4 in Pratt *et al.*, 1991a). In addition, the blue shift in the red region, which previously had been reported for difference spectra, was also observed for absolute spectra (Fig. 3 in Pratt *et al.*, 1991a).

Two green-oat phytochromes. Whereas previously we did not feel the evidence indicating that there are two green-oat phytochromes was wholly convincing, we now feel that this is the case (Wang *et al.*, 1991; Pratt *et al.*, 1991c; Wang *et al.*, 1992). Demonstrating that this is the case is inextricably linked to the evidence establishing unambiguously that MAbs GO-2, GO-7 and GO-8 are also directed to green-oat phytochrome. This evidence is summarized in the first paragraph of the discussion in Wang *et al.* (1991), and will not be repeated here.

Having established rigorously that GO-2, GO-7 and GO-8 are also directed to green-oat phytochrome (Wang *et al.*, 1991), it becomes immediately evident that there are two green-oat phytochromes. There are three reasons for reaching this conclusion. First, green-oat phytochromes have different apparent monomer sizes. GO-4, GO-5 and GO-6 immunostain a polypeptide marginally but significantly larger than that of etiolated-oat phytochrome (125 *vs.* 124 kDa), while GO-1, GO-2, GO-7 and GO-8 immunostain a slightly smaller polypeptide of 123 kDa (Figs. 2, 3 in Wang *et al.*, 1991). The sizes of these green-oat phytochrome monomers detected in HA-purified samples are the same as those observed for phytochrome in SDS sample buffer extracts of rapidly frozen, lyophilized oat leaves, indicating that these sizes do not reflect any artifactual modification *in vitro* (Fig. 4 in Wang *et al.*, 1991). These data also indicate that the 120-kDa polypeptide immunostained by GO-4, GO-5, and GO-6 is a degradation product of the 125-kDa polypeptide. Apparently, the iodoacetamide that prevents proteolytic cleavage of 123-kDa green-oat phytochrome (Wang *et al.*, 1991) does not protect

against cleavage of the 125-kDa species. Second, the two green-oat phytochromes are immunochemically distinct from one another. GO-4, GO-5, and GO-6 do not recognize the green-oat phytochrome detected by the other four MAbs, while GO-2 and GO-7 do not recognize the green-oat phytochrome detected by the first three MAbs (Figs. 5, 9 in Wang *et al.*, 1991; Fig. 5 in Pratt *et al.*, 1991c). Third, 123-kDa, but not 125-kDa, green-oat phytochrome undergoes a Zn^{2+}-induced mobility shift during SDS PAGE (Figs. 2, 6-8 in Wang *et al.*, 1991; Fig. 3 in Pratt *et al.*, 1991c). The significance of this mobility shift, if any, is unknown. Analyses of predicted amino acid sequences for the two green-*Arabidopsis* phytochromes, however, indicates that neither exhibits a recognized Zn^{2+}-finger protein motif, although each has a sequence indicative of a single, possible Zn^{2+}-binding domain.

Microsequencing. A proteolytically derived peptide from green-oat phytochrome that had been immunopurified with a column of immobilized GO-5 yielded a sequence of 19 residues with 53% identity to etiolated-oat phytochrome (Fig. 2 in Pratt *et al.*, 1991c). Of the nine non-identical residues, eight are evolutionarily conserved substitutions. This green-oat phytochrome sequence exhibits similar homology to sequences for green-*Arabidopsis* phytochromes, but in neither case is the homology any better than it is for etiolated-oat phytochrome. An homology search of a large protein data base revealed no other matches, verifying that the homology observed with etiolated-oat phytochrome is significant. This unique sequence for 125-kDa green-oat phytochrome confirms that it is from a gene other than one that encodes etiolated-oat phytochrome.

How much etiolated-oat phytochrome is in an extract of green oat leaves? When green oat leaves are harvested at the end of a natural photoperiod, regardless of the time of year, extracts derived from them contain exceedingly little if any etiolated-oat phytochrome. The initial ELISA data of Shimazaki *et al.* (*Planta* 159:534-544, 1983) indicate that such extracts have at least 1000-fold less etiolated-oat phytochrome than does etiolated tissue. Because the ELISA performed by Shimazaki *et al.* included antibodies cross reacting with some green-oat phytochrome, this value represented an upper limit. More recent data obtained with single MAbs known not to cross react with etiolated-oat phytochrome indicate that leaves harvested at the end of day contain no more than about 0.02% as much etiolated-oat phytochrome as do etiolated shoots (*i.e.*, at least a 5000-fold reduction; Fig. 4 in Pratt *et al.*, 1991c; Fig. 2 in Pratt *et al.*, 1991b). This upper limit is set by the detection limit of the ELISA used for this purpose, meaning that the reduction could be much greater.

Do heterodimers of one green-oat and one etiolated-oat phytochrome monomer exist? One year ago, data indicated that green- and etiolated-oat phytochrome heterodimers might exist. Data obtained during the past year confirm that such heterodimers do exist (Pratt *et al.*, 1991b). MAbs directed to green-oat phytochrome detect antigen in highly purified etiolated-oat phytochrome samples, prepared under conditions that ensure the absence of any green-oat phytochrome dimers unless they were present by virtue of dimerization with an etiolated-oat phytochrome monomer (Fig. 4 in Pratt *et al.*, 1991b). All but one of these MAbs, however, fails to detect full length etiolated-oat phytochrome apoprotein expressed in *E. coli*, even when gels are heavily loaded (Fig. 5 in Pratt *et al.*, 1991b). These MAbs therefore are not simply detecting etiolated-oat phytochrome by cross reacting with it with low affinity. They must instead be detecting green-oat phytochrome monomers that are present in the form of heterodimers with etiolated-oat phytochrome (see Pratt *et al.*, 1991b, for further discussion and details).

Green-oat phytochrome accumulation is a function of end-of-day irradiation. The amount of phytochrome obtained after PEI and $(NH_4)_2SO_4$ fractionation of crude homogenates of green oat leaves varies by as much as a factor of four depending upon where plants are grown (*e.g.*, in greenhouses either at the University of Georgia or at CIBA-GEIGY Biotechnology in North Carolina). After eliminating many of the more likely explanations for these differences, such as use of different lots of seed, we initiated a series of experiments that utilized growth chambers and irradiation boxes available through a collaboration with Mr. Sandy Stewart at CIBA-GEIGY (Stewart *et al.*, 1992). In all cases,

plants were grown on a diurnal light:dark cycle over a period of 9 or 10 days prior to harvest. The only significant factor that could be identified was an end-of-day irradiation effect. Plants given far-red light at the end of day and then placed overnight into darkened growth chambers yielded almost twice as much phytochrome, all of which was green-plant phytochrome, than did plants given a red light end-of-day treatment, or a far-red followed by red light end-of-day treatment prior to being placed into a dark chamber for the night. Thus, there is feedback regulation of green-oat phytochrome accumulation, although it is considerably less than that observed for etiolated-oat phytochrome.

Unimbibed seeds of Avena contain all three phytochromes. Phytochromes in unimbibed oat seed have been quantitated independently by immunoblot assay (Wang *et al.*, 1992). All three phytochromes are readily detected in embryo-containing portions of the seed, but are virtually absent from embryo-free portions. Photoreversibility assays indicate that at least one of these phytochromes is present as chromophore-containing holoprotein. We found per seed 1.4, 1.6 and 6.3 ng of 125-kDa green-oat, 124-kDa etiolated-oat and 123-kDa green-oat phytochrome, respectively. It is evident that each of the three is present in sufficient quantity to play an important role in early events associated with seed germination and seedling development. These data also help resolve an earlier discrepancy concerning whether the 124-kDa etiolated-oat phytochrome is present in unimbibed oat seed.

A photoperiodically insensitive genotype of Sorghum lacks a homologue of 123-kDa green-oat phytochrome. In collaboration with Dr. Page Morgan and his graduate student, Mr. Kevin Childs, we investigated the phytochrome status of a *Sorghum* genotype that is photoperiodically insensitive, whereas *Sorghum* is normally considered a short-day plant. While this genotype has a normal amount of photoreversible phytochrome in etiolated seedlings (Table 4 in Childs *et al.*, 1991), initial data indicated that one of the minor phytochromes that predominates in green *Sorghum* might be lacking (Fig. 3 in Childs *et al.*, 1991). In subsequent work not supported by DOE (Childs *et al.*, 1992), this preliminary conclusion has been confirmed. This non-photoperiodic *Sorghum* genotype is missing a phytochrome polypeptide equivalent to 123-kDa green-oat phytochrome. This correlation between the absence of a specific phytochrome and the loss of photoperiodic sensitivity is especially intriguing given that the discovery of phytochrome is so closely linked to the investigation of photoperiodism.

Molecular genetic approach to the characterization of green-oat phytochrome

Four years ago, this goal was given a very low priority for two reasons. First, the tools (*i.e.*, antibodies) needed to verify that a gene or cDNA encoded green-oat phytochrome were lacking. Second, it was then not clear that green- and etiolated-oat phytochromes were products of different genes. One could have been a co- or post-translational modification of the other. During the past few years, however, convincing data indicate that at least one, and possibly two, green-plant phytochrome genes exist.

Screening of lambda gt11 library. A lambda gt11 expression library containing about 10^5 independent inserts was prepared from size-enriched, poly(A)$^+$-RNA isolated from young, rapidly growing, light-grown oat leaves. This library has been screened with MAb Pea-25, which detects an epitope near the C terminus of phytochrome and works well for this application. Pea-25 binds effectively to 123-kDa green-oat phytochrome by immunoblot assay. The library has also been screened by all seven GO MAbs and by our PAbs to green-oat phytochrome. While a number of potentially positive clones have been identified, all have been found to be artifacts. This library has also been screened with degenerate oligonucleotides corresponding to the epitope of Pea-25, to the highly conserved domain at the site of chromophore attachment, and to the green-oat phytochrome amino acid sequence. In addition, the library has been screened by full length etiolated-oat phytochrome cDNA and cRNA. As with the antibodies, potential phytochrome-containing clones were identified, but all proved to be artifacts.

Screening of lambda DASH library. A genomic library was prepared in lambda DASH with oat DNA. We screened this library with all of the hybridization probes described above. In one instance (screening with cDNA encoding etiolated-oat phytochrome in collaboration with Dr. Cordonnier at CIBA-GEIGY) 22 clones were identified, some of which hybridized only weakly with the probe. All 22 were plaque-purified and characterized by restriction mapping, which indicated that nine independent cloning events were represented among them. Partial sequence data were obtained for all nine. None appear to be candidates for a green-oat phytochrome gene, whether obtained by this or any other approach so far tried. Given the large size of the oat genome as compared, for example, to *Arabidopsis* (13.2 *vs.* 0.2 pg DNA per haploid genome), this failure is not too surprising, assuming that our probes are only about 50% homologous, as recent data indicate is likely to be the case.

Attempts to identify green-oat phytochrome mRNA. If we could identify green-oat phytochrome mRNA unequivocally, we could then make a cDNA library that should contain green-oat phytochrome clones. We have used two approaches. First, we have isolated poly(A)$^+$-RNA from green oat leaves and translated it *in vitro*. Efforts to detect green-oat phytochrome by immunoprecipitation, however, were unsuccessful, presumably because of the exceedingly low abundance of the mRNA for which we are searching, and the inability of any of our antibodies to precipitate 123-kDa green-oat phytochrome. Second, we have attempted to identify green-oat phytochrome mRNA as follows. Poly(A)$^+$-RNA was prepared from oat seedlings grown (1) for 10 days on a normal day:night cycle in the greenhouse, (2) for 4 days in darkness and then for 12 h in continuous white light, and (3) for 3 days in darkness followed by (a) 3, 6, 12 or 24 h continuous white light, (b) two red-light pulses separated by 3 h darkness, (c) or four red-light pulses separated by 6 h darkness. Northern blots were prepared with these different RNA samples and probed with a cRNA that was transcribed from full-length cDNA encoding etiolated-oat phytochrome. Small amounts of phytochrome mRNA could be detected in every sample. By hybridizing replica blots over a wide range of stringencies, however, we found that the probe bound as tightly to the mRNA that was being detected as it does to authentic etiolated-oat phytochrome mRNA transcribed *in vitro*. It therefore appears that in each case the cRNA probe was detecting residual etiolated-oat phytochrome mRNA rather than green-oat phytochrome mRNA.

Implications of present work with respect to previous publications

The realization that there are two green-oat phytochromes, each of which differs from etiolated-oat phytochrome, resolves several apparent discrepancies in the literature (see Wang *et al.*, 1991, and Pratt *et al.*, 1991c, for discussions). Major points will only be summarized here. (1) It is evident that most previous characterizations of green-oat phytochrome were done essentially exclusively with the 123-kDa species. (2) The `etiolated-oat-like' phytochrome observed by Tokuhisa *et al.* (*Planta* 164:321-332, 1985) was probably the 125-kDa green-oat phytochrome referred to here, and not etiolated-oat phytochrome at all. (3) The failure of Pea-25 to immunostain the green-oat phytochrome precipitated by Oat-9 (Shimazaki and Pratt, *Planta* 164:333-344, 1985) is consistent with the observations that Oat-9 immunoprecipitates 125-kDa phytochrome, while Pea-25 selectively recognizes 123-kDa green-oat phytochrome (Fig. 6 in Pratt *et al.*, 1991c). (4) The variable electrophoretic mobility detected for green-oat phytochrome likely reflects the susceptibility of 123-kDa green-oat phytochrome to mobility shifts such as that observed in the presence of added Zn^{2+} (Wang *et al.*, 1991). Thus, while the recognition that there are two (or more) green-oat phytochromes complicates efforts to elucidate its immediate mode of action, the same information resolves a number of apparently conflicting observations that have appeared over the past five years.

SUMMARY

A separate list of major accomplishments during the previous funding period follows.

1) Major improvements have been made in the purification of green-oat phytochrome. An effective protease inhibitor has been incorporated, the scale of preparations has been increased greatly, an immunodominant contaminant has been eliminated, and the extent of purification has been increased by at least a factor of ten.

2) Five new MAbs and rabbit PAbs to green-oat phytochrome, as well as rabbit PAbs to a synthetic, putative green-oat phytochrome peptide, have been produced and characterized, together with two MAbs to green-oat phytochrome that had been identified previously.

3) Light-grown oat leaves contain several thousand-fold less etiolated-oat phytochrome than a comparable fresh weight of dark-grown shoots.

4) The level of green-oat phytochrome in light-grown plants can vary by as much as four fold, at least in part as a result of differing end-of-day light treatments.

5) Green-oat phytochrome has been characterized further, both spectrally and immunochemically.

6) An hypothesis that green-oat phytochrome itself consists of two types was found to be true. One type of green-oat phytochrome has an apparent monomer size of 125 kDa while the other is 123 kDa. The latter, but not the former, undergoes a Zn^{2+}-induced mobility shift during SDS PAGE and the two phytochromes are immunochemically distinct from one another.

7) Neither 123- nor 125-kDa green-oat phytochrome is equivalent to etiolated-oat phytochrome, leading to the conclusion that in oat at least there are three phytochromes.

8) Affinity columns prepared with MAbs to green-oat phytochrome have been used to purify 125-kDa green-oat phytochrome to near homogeneity. A proteolytically derived peptide has been isolated from immunopurified green-oat phytochrome and 19 residues have been determined by microsequencing. The results verify that in monocotyledons as well as dicotyledons green- and etiolated-oat phytochromes derive from different genes.

9) The discovery that there are two green-oat phytochromes resolves several discrepancies that have accumulated in the literature since green-oat phytochrome was discovered.

10) A *Sorghum* genotype that is non-photoperiodic is lacking a green-plant phytochrome, homologous to the 123-kDa green-oat phytochrome.

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