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Phytochrome quantitation in crude extracts of *Avena* by enzyme-linked immunosorbent assay with monoclonal antibodies

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Abstract. An enzyme-linked immunosorbent assay (ELISA), which uses both rabbit polyclonal and mouse monoclonal antibodies to phytochrome, has been adapted for quantitation of phytochrome in crude plant extracts. The assay has a detection limit of about 100 pg phytochrome (<1 fmol of monomer) and can be completed within 10 h. Nonspecific interference by crude plant extracts was detected and corrected for. Quantitation of phytochrome in crude extracts of etiolated oat (*Avena sativa* L.) seedlings by ELISA gave values that agreed well with those obtained by spectrophotometric assay. When etiolated oat seedlings were irradiated continuously for 24 h, the amount of phytochrome detected by ELISA and by spectrophotometric assay in crude extracts of these seedlings decreased by more than 1000-fold and about 100-fold, respectively. This discrepancy indicates that phytochrome in light-treated plants may be antigenically distinct from that found in fully etiolated plants. Both a decrease in the light and an increase in the dark of phytochrome content was observed in crude extracts of light-grown oat shoots, both green and Norflurazon-bleached, in response to a 12:12-h light-dark cycle. When these light-grown oat seedlings were kept in darkness for 48 h, phytochrome content detected by ELISA increased by 50-fold in crude extracts of green oat shoots, but only about 12-fold in extracts of herbicide-treated oat shoots. Phytochrome reaccumulation in green oat shoots was initially more rapid in the more mature cells of the primary leaf tip than near the basal part of the shoot. The inhibitory effect of Norflurazon on phytochrome accumulation was much more evident near the leaf tip than the shoot base. A 5-min red irradiation of oat seedlings at the end of a 48-h dark period resulted in a subsequent, massive decrease in phytochrome content in crude extracts from both green and Norflurazon-bleached oat shoots. These observations eliminate the possibility that substantial accumulation of chromophore-free phytochrome was being detected and indicate that Norflurazon has a substantial effect on phytochrome accumulation during a prolonged dark period.

Key words: *Avena* (phytochrome) – Enzyme-linked immunosorbent assay (phytochrome) – Monoclonal antibody – Phytochrome (immunoquantitation).

Introduction

Since the discovery of phytochrome, quantitation methods for this chromoprotein have been based primarily on its unique spectral characteristics (Butler et al. 1959). More recently, phytochrome quantitation by an immunochemical technique, which depends upon rabbit antisera specific to phytochrome, has been developed (Hunt and Pratt 1979b). This radioimmunoassay (RIA) eliminates serious limitations inherent to spectrophotometric assay of phytochrome (see Pratt 1983 for review) and makes it possible to detect as little as 1 ng of phytochrome without interference by other pigments, most notably chlorophyll (Hunt and Pratt 1980).

Nevertheless, the RIA is cumbersome, requiring 3 d to obtain the final result. We report here the adaptation of a more sensitive and more convenient enzyme-linked immunosorbent assay (ELISA) to phytochrome quantitation. The assay can detect as little as 100 pg and can be completed within 10 h. We describe the initial use of this assay

Abbreviations: ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; RIA = radioimmunoassay

to quantitate phytochrome in both green and Norflurazon-bleached oat shoots. These initial observations provide new information about phytochrome in green plants and resolve an apparent discrepancy between earlier results obtained by RIA of phytochrome in shoots of light-grown, green oat seedlings (Hunt and Pratt 1980) and by spectrophotometric assay of phytochrome in comparable shoots of light-grown, but Norflurazon-bleached, oat seedlings (Jabben and Deitzer 1978b). The RIA had indicated a 50-fold increase in phytochrome content during a 48-h dark incubation of oat seedlings, while the spectrophotometric assay had indicated only a two-fold increase.

Materials and methods

Plants. Etiolated oats (*Avena sativa* L., cv. Garry; seeds from Whitney-Dickinson Seeds, Buffalo, N.Y., USA) and etiolated peas (*Pisum sativum* L., cv. Alaska; Leatherman's Seed Co., Canton, O., USA) were grown in total darkness at 25° C and harvested as described by Pratt (1973). Light-grown, green oats were obtained by germinating seeds for 3 d at 25° C in darkness and then transferring them to a 12:12-h light-dark cycle for 5 d (Hunt and Pratt 1980). Light was provided by closely spaced Cool-white, Gro-lux and Daylight fluorescent lamps (F48T12-CW-VHO, F48T12-GRO-VHO and F48T12-D-VHO; Sylvania, Winchester, Ky., USA) at a distance of about 80 cm above the seedlings. The seeds were sown on cellulose packing material and covered with water-saturated vermiculite to keep them from drying. To obtain light-grown, achlorophyllous oats, seeds before planting were imbibed for 1 h in 0.2 mM Norflurazon (Sandoz 9789, 4-chloro-5-(methylamino)-2-(α,α,α,-trifluoro-*m*-tolyl)-3(2H)-pyridazinone, provided by Dr. Gerald Deitzer, Smithsonian Radiation Biology Laboratory, Rockville, Md., USA) (Jabben and Deitzer 1978a). Otherwise, these herbicide-treated oats were handled as the green oats. Etiolated oat shoots were harvested close to the grain while light-grown oat shoots were harvested just above the coleoptilar node unless otherwise noted. Harvested tissue was stored in darkness at −20° C prior to extraction.

Crude extracts. Crude extracts of dark- or light-grown oat shoots were prepared by grinding 1.5 g of tissue in liquid N_2 with a mortar and pestle. The resultant powder was dispersed by homogenization with an Ultra Turrax (SDT Tissumizer with SDT100N shaft; Tekmar, Cincinnati, O., USA) in 3 ml of ice-cold 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-Cl, pH adjusted to 8.5 at room temperature, 0.2 M 2-mercaptoethanol. The extract was clarified by centrifugation at 40,000 *g* for 15 min. The resultant supernatant was used on the same day without further preparation, except for dilution when appropriate. Preparation of these clarified extracts was done under green light (Pratt 1973). Extracts were kept at 0–4° C.

Purified phytochrome. Oat phytochrome was purified from etiolated tissue by immunoaffinity chromatography, with elution from the antibody column induced by 3 M $MgCl_2$ at pH 7.8, as described previously (Hunt and Pratt 1979a; Cordonnier and Pratt 1982). The immunopurified phytochrome had a specific absorbance ratio (A_{667}/A_{280} with phytochrome in the red-absorbing form) of 0.77 and exhibited by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Weber and Osborn 1969) only a single, major band at about 118,000 dalton.

Pea phytochrome was purified from etiolated tissue by brushite, diethylaminoethyl-agarose (DEAE-Bio-Gel A; Bio-Rad, Richmond, Cal., USA), second brushite and Sephacryl S-300 (Pharmacia, Uppsala, Sweden) chromatography as described in Hunt and Pratt (1979b) and Pratt (in press). The pea phytochrome was about 40% pure (specific absorbance ratio = 0.23; Cordonnier and Pratt 1982) and had a monomer size near 115,000 dalton as judged by SDS-PAGE (Weber and Osborn 1969).

Phytochrome quantities were calculated from the extinction coefficient reported by Roux et al. (1982), who used quantitative amino-acid analyses for its determination. By this extinction coefficient, one unit of phytochrome, which is the quantity that in 1 ml has an $A_{667}^{1cm} = 1$, is equivalent to 1.2 mg. Phytochrome was diluted with Diluent (see below) to give a concentration of 1.32 µg/ml and stored at −80° C in 500-µl aliquots.

Phytochrome photoreversibility. Phytochrome photoreversibility (Butler et al. 1963) was measured with a custom-built dual-wavelength spectrophotometer using the same clarified extracts that were used for ELISA. Measurements were made at 667 nm versus 724 nm, or 724 nm versus 800 nm. Photoreversibility values ($\Delta\Delta A$) were converted to phytochrome units as described in Pratt (1983).

Rabbit antibodies to phytochrome. Rabbit antibodies to either oat or pea phytochrome were immunopurified from antisera to oat or pea phytochrome by adsorption to and elution from columns of agarose-immobilized oat or pea phytochrome, respectively (Hunt and Pratt 1979a; Cordonnier and Pratt 1982). Purified immunoglobulins were stored at −20° C in 30-µg aliquots so that each sample would be frozen and thawed only one time.

Monoclonal antibodies. Monoclonal antibodies produced by six different hybridomas, four of which secreted antibodies to oat phytochrome and two to pea phytochrome, were immunopurified from medium in which the hybridomas had been grown individually (see Cordonnier et al. 1983 for details). The purified antibodies were stored at 4° C in 50 mM Tris-Cl, pH 8.0 at room temperature, 40% glycerol, 0.1% NaN_3, and 1% bovine serum albumin.

Alkaline phosphatase-conjugated second antibody. Rabbit antibodies to mouse immunoglobulin G (IgG) were produced and immunopurified as described by Cordonnier et al. (1983). Five mg of alkaline phosphatase (type VII-S; Sigma Chemical Co., St. Louis, Mo., USA; specific activity = 1,100 unit mg^{-1}) was coupled by glutaraldehyde to 2 mg of the immunopurified antibodies as described in Voller et al. (1980). The alkaline phosphatase-conjugated rabbit antibodies to mouse IgG were stored at 4° C in 50 mM Tris-Cl, pH 8.0 at room temperature, 40% glycerol, 0.1% NaN_3, and 1% bovine serum albumin.

Standard ELISA protocol. After determining empirically optimal parameters for the ELISA (see Results), the following protocol was adopted as standard (Fig. 1). The wells of ethanol-washed vinyl assay plates (2596; Costar, Cambridge, Mass., USA) were coated with immunopurified rabbit antibodies to phytochrome (RAP in Fig. 1) by adding to each well 50 µl of a 5 µg ml^{-1} preparation, which was made by diluting a 30-µg stock aliquot into 6 ml of 0.2 M sodium borate, 75 mM NaCl, pH 8.5. After incubation overnight at 4° C, the wells were emptied and the plate was washed three times with 10 mM Tris-Cl,

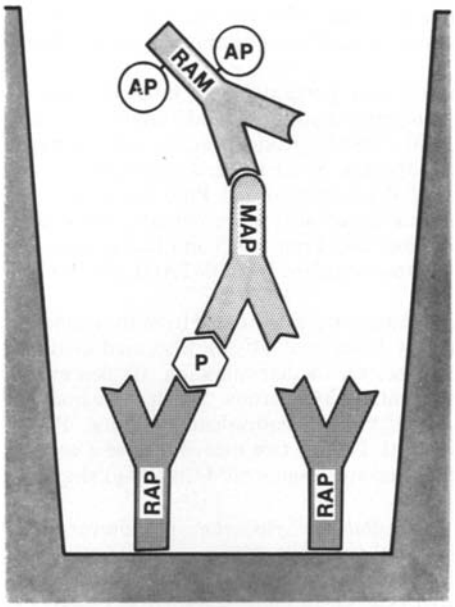

Fig. 1. Schematic illustration of the ELISA protocol used in this work. After coating the vinyl assay plate well with excess rabbit antibodies to oat phytochrome (RAP), phytochrome (P), monoclonal antibodies to oat phytochrome (MAP) and alkaline phosphatase-conjugated rabbit antibodies to mouse IgG (AP-RAM) were added in sequence, with intervening washes

Table 1. Immunoprecipitation of oat-phytochrome photoreversibility by the monoclonal antibodies used for the ELISA

Monoclonal antibody	Yield (mg)	Amount of monoclonal antibody used (μg)	
		50	200
Oat-3	13.5[a]	85.5[b]	85.1[b]
Oat-9	15.0	57.0	97.9
Oat-16	13.8	13.0	71.3
Oat-22	21.4	80.7	83.5
Non-immune mouse IgG	–	0.3	−4.0

[a] Quantity of antibody purified from 1 l of hybridoma medium using an immunoaffinity column of rabbit antibodies to mouse IgG

[b] Percentage of photoreversible phytochrome precipitated by monoclonal antibody. About 12 μg oat phytochrome (brushite preparation) was mixed with 50 or 200 μg monoclonal antibody and incubated for 4 h at 4° C. Subsequently, 150 or 600 μl, respectively, of a 10% suspension of *Staphylococcus aureus* cells, which had been washed with 0.2 M sodium borate, 75 mM NaCl, pH 8.5, and suspended in the same buffer, was added. After a 15-min incubation at 4° C, the mixture was centrifuged and photoreversible phytochrome remaining in the supernatant was assayed at 667 nm versus 724 nm with a dual wavelength spectrophotometer

pH 8.0 at room temperature, 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma), and 0.02% NaN_3 (wash solution). Remaining non-specific protein binding sites were blocked by filling the wells with 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, containing 1% bovine serum albumin and 0.02% NaN_3, and incubating for 30 min at room temperature. After washing the plate once with wash solution, 50 μl of test sample was added to each well under green light. When required, test samples were diluted with 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, containing 0.05% Tween 20, 0.02% NaN_3, 1% bovine serum albumin (Diluent). The plate was incubated for 3 h at 4° C in darkness. It was then washed three times with wash solution and 50 μl of a mixture of four monoclonal antibodies to oat phytochrome (oat-3, oat-9, oat-16, oat-22; see Cordonnier et al. 1983) was added to each well (MAP in Fig. 1). The monoclonal antibodies were diluted in Diluent and each was present at 10 μg ml^{-1}. Since phytochrome is a large protein, it is reasonable to expect that even after one epitope is bound by a rabbit antibody, with which the plate is coated, several should remain available for binding by the monoclonal antibodies. After an additional 2-h incubation at 37° C in a humidified chamber, the plate was again washed three times with wash solution. Fifty μl of alkaline phosphatase-conjugated rabbit antibody to mouse IgG (AP-RAM in Fig. 1) was added next to each well. This second antibody preparation was diluted with Diluent by 500-fold from the concentration obtained following enzyme conjugation as described by Voller et al. (1980). After incubation at 37° C for 2 h in a humidified chamber, the plate was washed three times with wash solution and 50 μl of substrate solution was added to each well. Substrate solution was prepared by dissolving *p*-nitrophenyl phosphate at a concentration of 0.6 mg ml^{-1} in 9.6% (v/v) diethanolamine, 0.5 mM $MgCl_2$, pH 9.6 with HCl. The reaction was allowed to proceed at room temperature for 30 min and was stopped by addition of 50 μl of 3 N NaOH to each well. Extent of enzyme activity was monitored by measuring absorbance at 410 nm with a Minireader MR 590 (Dynatech Instruments, Alexandria, Va., USA).

Non-specific interference, if any, of crude oat extracts in the ELISA was detected and corrected for as follows. In the same vinyl assay plate as used for oat phytochrome quantitation, some of the wells were coated with immunopurified rabbit antibodies to pea phytochrome exactly as described for antibodies to oat phytochrome. Two standard curves for pea phytochrome quantitation were then prepared in exactly the same way as for oat phytochrome except that two monoclonal antibodies to pea phytochrome (I-15a3 and I-18a1, see Cordonnier et al. 1983) were used in place of the monoclonal antibodies to oat phytochrome. One standard curve was prepared by diluting purified pea phytochrome with Diluent; the other by diluting the same pea phytochrome with a crude oat extract at the same dilution used in the oat-phytochrome ELISA. The extent of interference by the crude oat extract was assumed to be the same for both the oat and pea phytochrome ELISAs. The standard curve for the oat phytochrome ELISA could thus be corrected by the amount indicated in the pea phytochrome ELISA (see Fig. 6 below for example).

Results and discussion

Specificity of monoclonal antibodies. Immunoaffinity purification of monoclonal antibody from medium in which hybridomas had been grown yielded 13–21 mg of immunoglobulin per liter of medium (Table 1). The high purity of the oat phytochrome that was used for immunizing mice and for screen-

Table 2. Ability of monoclonal antibodies to bind oat phytochrome as determined with rabbit antisera to oat phytochrome. Values represent absorbance at 410 nm × 100. Each assay well was coated individually with 50 µl of 0.3 µg ml^{-1} monoclonal antibody. After addition of phytochrome and incubation for 4 h, one of three rabbit antisera to oat phytochrome was added at 1000-fold dilution. Alkaline phosphatase-conjugated goat antibody to rabbit IgG was used as second antibody at 500-fold dilution. Incubation times and temperatures were the same as the standard ELISA procedure except for the 4-h incubation after adding phytochrome

Monoclonal antibody	Rabbit antiserum to oat phytochrome	Phytochrome added (ng)	
		0	60
Non-immune mouse IgG	Ab-W	5	6
	Ab-G	5	8
	Ab-271	5	8
Oat-3	Ab-W	6	46
	Ab-G	6	49
	Ab-271	7	45
Oat-9	Ab-W	2	101
	Ab-G	1	100
	Ab-271	1	118
Oat-16	Ab-W	3	49
	Ab-G	3	59
	Ab-271	3	58
Oat-22	Ab-W	7	181
	Ab-G	7	186
	Ab-271	7	176

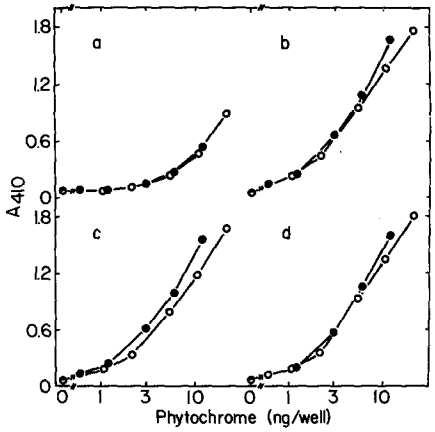

Fig. 2a–d. Comparison of ELISA standard curves with immunopurified oat phytochrome (●) and an appropriately diluted crude extract of etiolated oats (○) using individual monoclonal antibodies to oat phytochrome: oat-3 (**a**), oat-9 (**b**), oat-16 (**c**) and oat-22 (**d**). Phytochrome quantity in the crude extract was determined by photoreversibility measurements at 667 nm versus 724 nm

ing resultant hybridomas minimized the possibility that one of the monoclonal antibodies used here might bind to a contaminating antigen (Cordonnier et al. 1983). Nevertheless, it is still essential for this application to verify that the monoclonal antibodies detect only phytochrome.

As an initial test of specificity, the four monoclonal antibodies used for quantitation of oat phytochrome by ELISA (oat-3, oat-9, oat-16, oat-22) were tested for their ability to adsorb oat phytochrome from a partially purified extract as described by Cordonnier et al. (1983). More than 70% of the added phytochrome photoreversibility could be precipitated (Table 1), indicating that all of the monoclonal antibodies tested were to oat phytochrome. When non-immune mouse IgG (I-5381; Sigma) was added as a control, no significant precipitation of phytochrome photoreversibility was observed (Table 1).

As a second test, vinyl assay plate wells were coated with the four monoclonal antibodies individually, rather than with rabbit antibodies to phytochrome as in the usual assay (Fig. 1). The assay was then continued by the standard protocol except that (1) rabbit antibodies to oat phytochrome were added in place of the usual addition of monoclonal antibodies and (2) the alkaline-phosphatase label was conjugated to goat antibodies to rabbit IgG (TAGO, Burlingame, Calif., USA) rather than to rabbit antibodies to mouse IgG. Since the monospecificity of these rabbit antibodies to oat phytochrome had been established earlier (Pratt 1973; Hunt and Pratt 1979b), and since the results indicated in each case that the monoclonal antibodies had bound phytochrome to the assay wells (Table 2), it is again evident that each of the monoclonal antibodies recognizes phytochrome as its antigen. As controls, non-immune mouse IgG was used to coat assay wells in place of the monoclonal antibodies (Table 2) or non-immune rabbit serum was used in place of the rabbit antisera, in which case the maximum value observed was 0.02*A*. Neither control resulted in significant activity.

In a third test, the standard ELISA protocol was used, but each monoclonal antibody was tested separately against both immunopurified oat phytochrome and phytochrome in crude extracts from etiolated oats (Fig. 2). Although the activity exhibited by each monoclonal antibody was quantitatively different, the two standard curves obtained for each were virtually identical. Because it is highly unlikely that a contaminating antigen would be present at the same ratio to phytochrome in both the immunopurified sample and the crude extract, or that a potential contaminant would be recognized equally well by all four monoclonal an-

tibodies, the data indicate not only that the monoclonal antibodies bind phytochrome but also that they do so monospecifically.

As a final test, the detection limit of the standard ELISA was determined with both immunopurified oat phytochrome and a crude extract of etiolated oats (Fig. 3). When the phytochrome added to a well was less than 1 ng, the resultant activity was related linearly to the amount of phytochrome added. A highly significant increase in absorbance at 410 nm was detected even when less than 100 pg of immunopurified phytochrome was added. Since in one case this phytochrome is highly purified (see Fig. 1 in Cordonnier et al. 1983 for sodium dodecyl sulfate polyacrylamide gel profile of this sample), it would be highly unlikely that there would be a potential contaminating antigen present in sufficient quantity to be detected by the assay in the range used here.

As discussed elsewhere (Cordonnier et al. 1983), the probability that these mouse antibodies are derived from hybridomas that secrete immunoglobulins that bind to only a single epitope is well in excess of 99%. Taken together with the evidence presented here, it is clear that the monoclonal antibodies not only are to phytochrome (Tables 1, 2; Figs. 2, 3) but are also highly specific for phytochrome (Figs. 2, 3). This conclusion is reinforced by the observations presented below that the monoclonal antibodies at no time detect quantities of antigen greater than what might be anticipated given known properties of phytochrome. We have chosen to use all four of these monoclonal antibodies primarily because of the increased sensitivity that they provide as compared to the use of any one alone (compare Fig. 3 to Fig. 2, noting that abscissa scales are different). Additionally, the possibility is reduced, even though not eliminated, that an outcome of ELISA might reflect a property unique to one epitope of phytochrome and therefore be correspondingly biased.

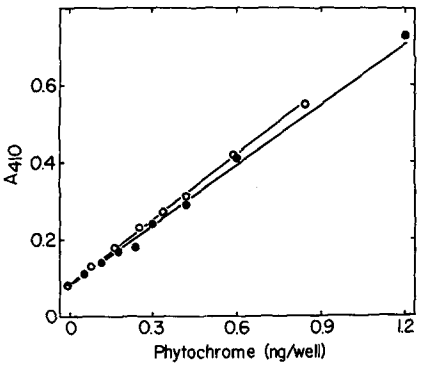

Fig. 3. ELISA standard curves with immunopurified oat phytochrome (●) and an appropriately diluted crude extract of etiolated oats (○) using the standard mixture of four monoclonal antibodies to oat phytochrome. Photoreversibility measurements were made at 667 nm versus 724 nm

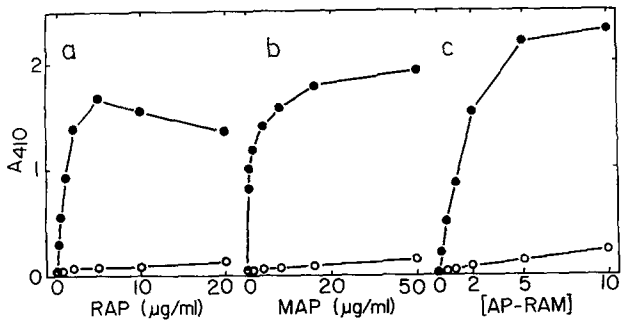

Fig. 4a–c. ELISA activity as a function of the concentrations of (**a**) rabbit antibodies to oat phytochrome (RAP), (**b**) monoclonal antibodies to oat phytochrome (MAP), and (**c**) alkaline phosphatase-conjugated rabbit antibodies to mouse IgG (AP-RAM). For AP-RAM, relative concentrations × 1000 are given. Three ng of immunopurified oat phytochrome (●) or Diluent as a control (○) was assayed by varying the concentration of the antibody in question. Otherwise, 50 μl of 5 μg ml^{-1} of rabbit antibodies to phytochrome, a mixture of 10 μg ml^{-1} of each of the four monoclonal antibodies and a 500-fold dilution of enzyme-linked second antibody were added. Incubation times and temperatures were those of the standard ELISA procedure

Standard ELISA protocol. Optimal concentrations of rabbit antibodies to phytochrome, with which the assay wells were coated (RAP in Fig. 1), monoclonal antibodies to phytochrome (MAP in Fig. 1), and alkaline phosphatase-conjugated second antibody (AP-RAM in Fig. 1) were determined by varying independently the concentration of each over a wide range (Fig. 4). The concentrations chosen as standard (see Materials and methods) represented compromises among (1) conserving relatively scarce reagents, (2) yielding minimum background activity for controls so that maximal sensitivity could be achieved, and (3) providing high activity. In addition, the relatively high concentration of rabbit antibodies to phytochrome was chosen to minimize the time needed to bind phytochrome from a highly diluted sample and to maximize the amount of phytochrome bound from such a sample.

Incubation times were determined from time course experiments for each phase of the protocol, with the exception of the coating step with rabbit antibodies to phytochrome, which was presumed to go to completion during the standard overnight

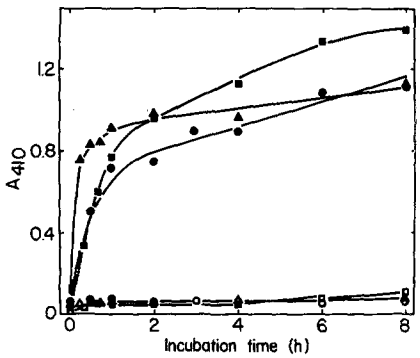

Fig. 5. Effect of increasing incubation times on ELISA activity. Time courses for each incubation were determined both with 3 ng immunopurified oat phytochrome (●, ▲, ■) and with Diluent as a control (○, △, □). Incubation times were varied following addition of immunopurified phytochrome or Diluent (●, ○), a mixture of 10 μg ml^{-1} of each of the monoclonal antibodies to oat phytochrome (▲, △), and 500-fold diluted alkaline phosphatase-conjugated rabbit antibody to mouse IgG (■, □). Except for the incubation time being varied, others were done under constant conditions. Incubation with phytochrome, monoclonal antibodies and enzyme-linked second antibody were for 4 h at 4° C, 1 h at 37° C, and 2 h at 37° C, respectively

incubation (Fig. 5). Each step appears to proceed in two phases, an initial rapid binding followed by a slower interaction that does not seem to be complete even after 8 h. Background activity appeared relatively constant over the entire period. To minimize (a) experimental error resulting from potential deviations in incubation times, (b) the possibility of proteolysis, and (c) total assay time, the following incubation times were chosen as standard: 3 h at 4° C for phytochrome and 2 h at 37° C for both monoclonal antibody and enzyme-linked second antibody (Fig. 5). Thus, even with up to 192 samples in two assay plates, the entire protocol (excluding the overnight coating step) can be completed within 10 h.

By using the standard protocol and immunopurified oat phytochrome, it is possible to prepare a standard curve for quantitation of phytochrome in crude extracts over a range from about 100 pg (Fig. 3) to 6 ng (Fig. 6, curve a). When quantifying phytochrome in crude extracts of etiolated oat shoots, which had about 600 ng phytochrome in a 50-μl aliquot, the extracts had to be diluted 100- to 1000-fold prior to assay. At these extreme dilutions non-specific interference was not detected as indicated by the close correspondence between spectrophotometrically detectable phytochrome and that detected by ELISA (Fig. 3). In addition, after such dilution, these crude oat extracts also had no effect on quantitation of phytochrome in an ELISA intended for pea phytochrome.

When extracts were of green oat shoots, however, they were diluted at most tenfold for assay, in which case general interference with the ELISA was observed. The amount of interference, which was presumably an inhibition of phytochrome binding to the rabbit antibodies with which the wells were coated, varied as a function both of the extent of dilution and of the tissue being extracted. Neither ammonium sulfate fractionation of the extract nor filtration through Sephadex G-25 eliminated the interference. Furthermore, addition of the protease inhibitor phenylmethylsulfonyl fluoride had no effect.

Since simple procedures did not eliminate the non-specific interference and since important reasons exist for not partially purifying phytochrome from crude extracts prior to assay, we decided to use only the clarification step as described in Materials and methods. The reasons for avoiding preliminary purification of extracts are twofold. Firstly, we wanted to keep the procedure as simple as possible so that a maximum number of extracts could be assayed in a single experiment. Secondly, we wanted to minimize the possibility that phytochrome might be lost during sample preparation, which is an especially important point given that little information is available about methods for phytochrome purification from green oats. Thus, rather than eliminating the non-specific interference we decided to devise a method whereby we could correct for it.

The correction method that we developed is based on observations that neither phytochrome in appropriately diluted (100–1000 fold) crude extracts of etiolated oat shoots nor immunopurified oat phytochrome (even as much as 300–600 ng) had any effect on quantitation of pea phytochrome in an ELISA intended for pea phytochrome. Thus, even if oat phytochrome did bind to the rabbit antibodies to pea phytochrome, with which the wells were coated, it did not inhibit binding of pea phytochrome. Two standard curves for quantitation of pea phytochrome were prepared, one by diluting purified pea phytochrome with Diluent as in the standard protocol (Fig. 6, curve b), the other by diluting pea phytochrome with the crude oat extract at the same concentration as that used for assay in the oat-phytochrome ELISA (Fig. 6, curve c). It is apparent that in this case the crude oat extract exhibits significant non-specific inhibitory activity in the pea-phytochrome ELISA. By assuming that the extent of this inhibition was the same in the oat phytochrome ELISA, correction could then be done as follows. At each phytochrome concentration, the extent of inhibition is determined

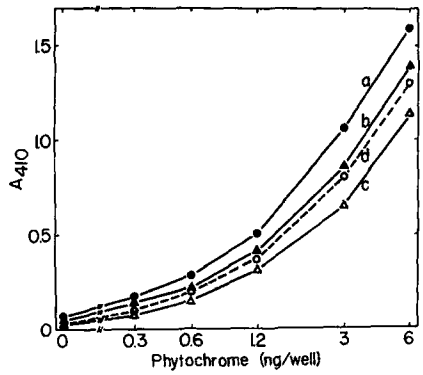

Fig. 6. An example of standard curves used both to correct for non-specific interference in the ELISA and to quantitate phytochrome in crude oat extracts. *Curve a:* immunopurified oat phytochrome diluted with Diluent was measured by the oat-phytochrome ELISA. *Curve b:* purified pea phytochrome diluted with Diluent was measured by the pea-phytochrome ELISA. *Curve c:* purified pea phytochrome was diluted with a crude oat extract at the same concentration as that used for oat-phytochrome assay, and measured by the pea-phytochrome ELISA. *Curve d:* corrected standard curve obtained by multiplying each absorbance value on curve a by the ratio of the absorbance value at the same phytochrome concentration on curve c to that on curve b

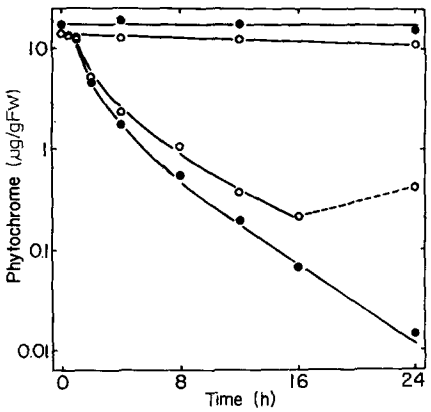

Fig. 7. Phytochrome content in crude extracts of etiolated oats as determined by ELISA (●) and by spectrophotometric assay (○) as a function of time after transferring 5-d-old etiolated plants to continuous illumination. Entire shoots were harvested after the illumination times indicated and frozen at −20° C prior to extraction. The upper two curves were obtained as controls with seedlings kept in darkness. Spectral assays were done with the same extracts as assayed by ELISA by measuring difference in absorbance between 724 nm and 800 nm after saturating irradiation with red and far-red light

by dividing the absorbance value on curve c by that on curve b. The result is then multiplied by the absorbance value on curve a and plotted as the corrected value for the oat phytochrome standard curve (Fig. 6, curve d), which is in turn used to estimate phytochrome amount in the crude oat extract. While the correction can sometimes be substantial as here, it is often negligible and in any case does not alter the relative outcome indicated by the data presented below.

To test the validity of the assay and the correction procedure, one or more known quantities of immunopurified oat phytochrome were added to aliquots of every crude oat extract that was assayed. Assay of these supplemented extracts led always to the expected increase in ELISA activity, verifying that the assumption made in the correction procedure is valid and that the ELISA quantitates accurately phytochrome in the crude extracts.

Phytochrome destruction under continuous illumination. Phytochrome content during continuous irradiation was followed by both ELISA and spectral assay of crude extracts of shoots harvested from etiolated oat seedlings as a function of time after transfer of the latter to light. As observed previously by radial immunodiffusion assay (Pratt et al. 1974) and by RIA (Hunt and Pratt 1979b), phytochrome levels measured by both immunochemical and spectral methods agreed reasonably well during the first 2 h (Fig. 7). After the seedlings had been 4 h in the light, however, phytochrome detected by ELISA in extracts of these seedlings had disappeared more rapidly than that detected by spectral assay, with half-lives of 2.9 and 3.5 h, respectively (Fig. 7). Consequently, with increasing time of the tissue in the light, the ELISA detected an increasingly smaller proportion of the spectrophotometrically assayable phytochrome. After seedlings were in the light for 16 h, spectrally detectable phytochrome reached a plateau at about 2–3% of the level observed with extracts of etiolated seedlings prior to any light exposure. In contrast, even after seedlings were in the light for 24 h, at which point phytochrome content detected by ELISA in crude extracts from those seedlings had decreased to less than 0.1% of the level observed with extracts of non-irradiated seedlings, there was no indication that a plateau had been reached.

Each monoclonal antibody, as well as the mixture of four used in the standard assay, detected equally about 20–30% of the spectrally measurable phytochrome found in crude extracts of oat shoots kept in the light for 8 h (Table 3). The ELISAs using individual monoclonal antibodies are as meaningful as the standard ELISA, which uses all four, because (a) standard curves like those in Fig. 2 were prepared separately for each monoclonal antibody, (b) only the initial linear part of these standard curves, comparable to the data in

Table 3. Phytochrome contents in crude extracts of 5-d-old dark-grown oat shoots as determined by spectrophotometric assay and by ELISA. For the ELISA each monoclonal antibody was tested independently as well as combined with the other three. Values are μg phytochrome per g fresh weight tissue. Results from two separate experiments are shown. Entire shoots were harvested from 5-d-old oat seedlings immediately before and 8 h after transferring the plants to continuous illumination. Harvested shoots were frozen at −20° C prior to extraction. Spectral assays were done with the same extracts as assayed by ELISA by measuring difference in absorbance between 724 nm and 800 nm after saturating irradiation with red and far-red light. Average photoreversibility values for a 1-cm light path were $0.0025A$ and $0.00033A$ for the extracts from the non-irradiated and the 8-h irradiated shoots, respectively

Assay method	Irradiation time (h)			
	0		8	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Spectrophotometric assay	17.0	15.1	1.96	2.49
ELISA with Oat-3	24.0	18.6	0.51	0.51
ELISA with Oat-9	18.2	16.6	0.73	0.68
ELISA with Oat-16	14.8	15.5	0.62	0.59
ELISA with Oat-22	17.1	18.2	0.58	0.49
ELISA with all four monoclonal antibodies	21.1	18.6	0.78	0.79

Fig. 3, was used, (c) all diluted phytochrome samples showed less than 10% non-specific inhibition in the pea-phytochrome ELISA, and (d) addition of known quantities of immunopurified oat phytochrome to every sample increased ELISA activity as expected.

There are several possible explanations for why the ELISA indicates the presence of less phytochrome in extracts of greening tissue than does the spectral assay. Since spectral assays and ELISAs were performed on the same crude extracts, however, neither sample variability nor extraction artifacts can serve as an explanation. Remaining possibilities include the following. (1) Spectrophotometric assay could yield artifactual values (a) because of its relative insensitivity, which is important here since phytochrome levels after keeping seedlings in the light for more than 8 h are near the limit of detectability of the spectral assay (less than 10^{-3} $\Delta\Delta A$), (b) because of the presence of increasing amounts of chlorophyll, or (c) because of an increase in phytochrome extinction during greening. (2) Alternatively, values obtained by ELISA could be distorted by an increase in either non-specific or specific interference with the assay. (3) Or, green oat shoots may contain phytochrome that is antigenically distinct from that found in dark-grown oats, either (a) because the pigment is modified in some way during, or as a consequence of, the greening process or (b) because it is the product of a different gene.

While the first possibility given above cannot be eliminated entirely, it appears unlikely. Chlorophyll-related artifacts typically lead to underestimates, rather than overestimates, of phytochrome content by spectral assay (see Pratt 1983 for review and discussion). Furthermore, even though phytochrome photoreversibility values are small, they are highly reproducible and thus seem highly significant. Finally, while it is theoretically possible that phytochrome extinction could increase, it seems highly improbable that it would increase by more than tenfold. The second possibility can be discarded for even sounder reasons. Non-specific inhibition in the experiments reported here never exceeded 11%. Inhibition of 90% or more would be needed to explain the greater than tenfold difference that is observed between the two assays. In addition, specific inhibition was never detected. Addition of 600 pg of immunopurified phytochrome to aliquots of every sample invariably led to the expected increase in ELISA activity.

The third possible explanation for the increasing disagreement between spectral and immunochemical assays seems most reasonable for two reasons. Firstly, the ELISA utilized polyclonal rabbit antibodies and monoclonal mouse antibodies that are directed to phytochrome purified from etiolated oat shoots. If phytochrome from green oats were antigenically different, then one would not expect the ELISA to quantitate it accurately. Secondly, Tokuhisa and Quail (1983) have recently observed that rabbit antibodies to phytochrome from etiolated oats will bind to only a fraction of the phytochrome obtained from green oats. Their observations have led them to suggest as well that green oats contain a pool of antigenically distinct phytochrome.

Assuming that the ELISA fails to detect all phytochrome isolated from irradiated oat shoots because of a difference in phytochrome antigenicity, two questions arise. (1) Does the limitation in the assay arise from inability of the polyclonal rabbit antibodies or the monoclonal mouse antibodies to bind phytochrome? Since the monoclonal antibodies presumably represent a selected fraction of the total antibody pool in the polyclonal preparation, it would seem most likely that either the monoclonal antibodies alone or both antibody preparations together are limiting. (2) Does activity in the ELISA decrease because of a selective change in only one or a few epitopes or because of a more general change in all antigenic properties

of phytochrome? Since the combination of any two monoclonal antibodies in the ELISA always gives greater sensitivity than does either one alone, it appears that each of the four recognizes a different epitope on phytochrome, which is reasonable given that phytochrome is a large chromoprotein. Furthermore, since each of the four monoclonal antibodies detects the same proportion of the photoreversible phytochrome isolated from greening shoots (Table 3), it is evident that the epitopes that they recognize are all decreasing in quantity or changing in properties at equivalent rates. It thus seems likely that the decrease in ability to detect phytochrome by ELISA represents a general difference in antigenicity of the phytochrome pool rather than a difference in only one or a few epitopes. This suggestion is consistent with the recent data of Tokuhisa and Quail (1983), who found that polyclonal rabbit antibodies to phytochrome from etiolated oat shoots bound no more than 30% of the photoreversible phytochrome obtained from green oat shoots, even when excess antibody was added.

It therefore appears likely that crude extracts of green or greening oat seedlings contain a unique pool of photoreversible phytochrome that is antigenically distinct from that found in crude extracts of etiolated oat shoots. As a consequence, immunochemical assays that depend upon antibodies to phytochrome purified from etiolated oats, which are the only antibodies presently available, give results that must be expressed in terms of equivalent amounts of etiolated-oat phytochrome. Immunochemical data, including those presented here, must be interpreted with this inherent limitation in mind.

Phytochrome levels in crude extracts of green and herbicide-bleached oat shoots. Phytochrome content as determined by ELISA of crude extracts of whole shoots harvested from both green and light-grown, Norflurazon-bleached oat seedlings increased two- to threefold during a 12-h dark period and decreased by the same amount during a subsequent 12-h light period (Fig. 8). When these seedlings were placed in darkness for 48 h, phytochrome content in crude extracts increased by more than 50-fold and by 12-fold for the green and Norflurazon-bleached seedlings, respectively. After the 48-h dark period, a 5-min irradiation of the seedlings followed by another dark incubation resulted in massive decreases in phytochrome content in both cases (Fig. 8).

During the first 48 h of a prolonged dark incubation of light-grown seedlings, assay of crude extracts indicated that phytochrome accumulated more rapidly in the tips of primary leaves than in the more basal portion of the shoot, which included part of the mesocotyl, the coleoptilar node, all of the coleoptile and the basal region of the leaves (Fig. 9). Norflurazon inhibited phytochrome accumulation most strongly near the primary leaf tip, resulting in only a fourfold increase in this region during the 48-h dark incubation.

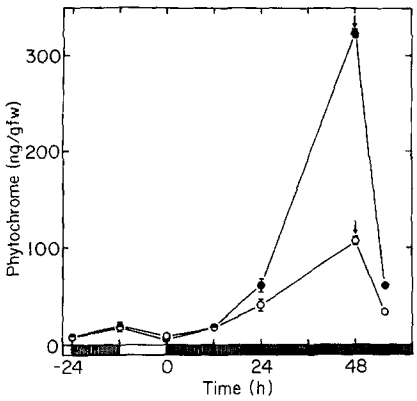

Fig. 8. Phytochrome content in crude extracts of whole shoots harvested from green (●) and Norflurazon-treated (○) oat seedlings as a function of time during both a 12:12-h light-dark cycle and a subsequent prolonged dark incubation of the seedlings. Three-d-old etiolated oats were subsequently grown for 5 d under 12:12-h light-dark cycles before being placed in continuous darkness at zero time. Light and dark periods are indicated by horizontal bars: *open*=light; *closed*=dark. *Arrows* indicate a 5-min irradiation of the plants with light provided by Gro-lux (Sylvania, Winchester, Ky., USA) lamps. Bars=SE of four replicates from two independent experiments

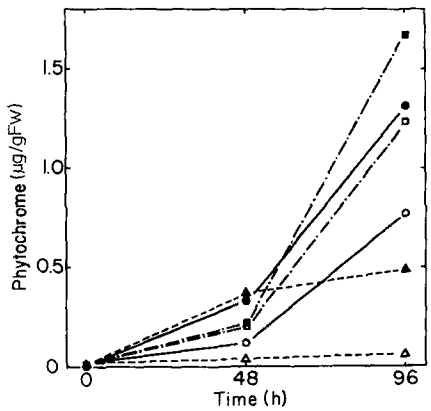

Fig. 9. Phytochrome content in crude extracts of green (●, ▲ and ■) and Norflurazon-treated (○, △ and □) oats as a function of time during prolonged dark incubation. Three-d-old etiolated oats were subsequently grown for 5 d under 12:12-h light-dark cycles before being placed in the dark at zero time. Whole shoots (●——● and ○——○), 20-mm-long tips of first leaves (▲——▲ and △——△) or 20-mm-long basal parts of shoots (■-·-·-■ and □-·-·-□) were harvested after the incubation times indicated, and frozen at −20° C prior to extraction

After the seedlings had been 48 h in darkness, phytochrome accumulation almost ceased in the primary leaf tip while it continued at an accelerated rate in the basal region of both green and herbicide-bleached shoots. Nevertheless, phytochrome levels detected by ELISA were at most only about 10% of the levels found in extracts of plants that were never exposed to light, even after light-grown seedlings were kept for 96 h in darkness (compare Fig. 9 with Fig. 7).

The alternation in phytochrome content during a 12-h light, 12-h dark cycle (Fig. 8) is qualitatively consistent with the earlier RIA data of Hunt and Pratt (1980). Hunt and Pratt, however, reported absolute phytochrome levels about 20-fold higher (approx. 200 versus 6–9 ng/g fresh weight) than those observed here for extracts of comparable, light-grown oat shoots. Although there is a possibility that the polyclonal rabbit antibodies used by Hunt and Pratt detected all phytochrome from green oats, it seems unlikely because of the recent data of Tokuhisa and Quail (1983), which have already been discussed above. The reason for the disagreement between the previous RIA and the present ELISA data must therefore remain unresolved.

While Hunt and Pratt (1980) observed a 50-fold increase in phytochrome content in extracts of green oat shoots that were incubated in darkness for 48 h, Jabben and Deitzer (1978b) observed by spectrophotometric assay only a twofold increase in phytochrome content of comparable light-grown, but Norflurazon-bleached oat shoots similarly incubated in darkness for 48 h. The RIA used extracts prepared from whole shoots of green seedlings while the spectral assays used primary leaf tips. Since ELISA quantitation of phytochrome extracted from both green and herbicide-bleached oat shoots indicates unambiguously that Norflurazon inhibits phytochrome accumulation during a prolonged dark incubation of the light-grown seedlings (Fig. 8), and since the inhibition is greatest in primary leaf tips (Fig. 9), it is evident that the earlier discrepancy was a consequence of the use of Norflurazon to make spectral assay possible (Jabben and Deitzer 1978a). Although Gottmann and Schäfer (1983) have argued in contrast that phytochrome accumulation in darkness is identical for green and herbicide-bleached oats, their data are not convincing. They used RIA for quantitation of phytochrome in green oat shoots and spectral assay with bleached oat shoots. Furthermore, their data as presented do not permit one to calculate absolute phytochrome quantities in either case. Consequently, it is not possible to compare adequately the RIA and spectral data, which was the problem one had originally when trying to compare the data of Hunt and Pratt (1980) and those of Jabben and Deitzer (1978b). The data of Gottmann and Schäfer therefore provide no reason to discard the conclusion reached here.

Norflurazon has been used to permit spectral quantitation of phytochrome in light-grown seedlings of several other species as well. Prolonged dark incubation of Norflurazon-treated maize (Jabben 1980), *Amaranthus* (Heim et al. 1981), mung bean (Kilsby and Johnson 1981) and mustard (Kilsby and Johnson 1982), as well as of *Avena* (Jabben and Deitzer 1978b; Gottman and Schäfer 1983), has led to increases in phytochrome content of only 1.2–5.7-fold. Since the present data (Figs. 8, 9) indicate that Norflurazon can have a marked effect on the rate of phytochrome accumulation in darkness, these spectral measurements of phytochrome levels in Norflurazon-bleached tissues may not be applicable to normal, green tissues. Thus, even though it has been shown that, under at least some conditions, phytochrome-mediated responses are not influenced by Norflurazon treatment (Jabben and Deitzer 1979), one must still be cautious in interpreting data derived by use of this herbicide.

The massive decline in phytochrome content in light-grown oat seedlings following a brief irradiation (Fig. 8) is similar to the decline observed by Hunt and Pratt (1980), who used the RIA. They used, however, continuous illumination which prevented any conclusion about whether phytochrome was photoreversible at the onset of light. The present data indicate that the phytochrome accumulated in both green and herbicide-bleached oats either possesses its chromophore or obtains it within only 5 min of the onset of irradiation.

Conclusions. The ELISA described here is shown to be highly specific for phytochrome (Figs. 2, 3; Tables 1, 2) and to quantitate the pigment accurately in crude extracts of etiolated oat shoots (Figs. 2, 3). Optimization of the assay protocol (Figs. 4, 5) provides sensitivity at the subfemtomol level (Fig. 3), which is about 1000-fold more sensitive than widely used spectral assays. As for the RIA, however, the ELISA has two limitations in its present form. (a) It is limited to quantitation of soluble phytochrome. Since the extraction conditions used here are expected to solubilize phytochrome from both etiolated (Hunt and Pratt 1980) and green plants (Bolton and Quail 1981; Shimazaki et al. 1981) this limitation does not seem serious. (b) The assay in an absolute sense quantitates

phytochrome accurately only if it is antigenically identical to that isolated from etiolated oats. Even within these limitations, however, a number of conclusions may be derived from the data presented here. (1) As indicated also by the data of Tokuhisa and Quail (1983), green oats contain a pool of phytochrome that appears to be antigenically distinct from that found in etiolated oats (Table 3, Fig. 7). (2) Phytochrome that is accumulated during a prolonged dark incubation of light-grown oat seedlings is found not only in meristematic or recently meristematic tissue, but also in the mature cells of primary leaf tips (Fig. 9). In fact, phytochrome accumulation during at least the first 48 h of darkness is most rapid in the more mature tissue. (3) Phytochrome that reaccumulates in darkness is immediately photoresponsive, irrespective of Norflurazon treatment (Fig. 8). (4) Norflurazon has a strong inhibitory effect on phytochrome accumulation during a prolonged dark period (Figs. 8, 9), which can explain the previously reported discrepancy between phytochrome accumulation in green and Norflurazon-bleached oats. Since the inhibition is greatest in tissue furthest removed from the seedling's food reserve, it is likely that the inhibition is a non-specific outcome of a nutritional deficiency that results from an inability to perform photosynthesis. These same data also serve as a warning that Norflurazon can under some circumstances have a strong influence on phytochrome levels and must therefore be used with caution as a tool to permit phytochrome assay in light-grown seedlings.

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References

Bolton, G.W., Quail, P.H. (1981) A method for preparing green plant tissue extracts for spectrophotometric measurement of phytochrome. (Abstr.) Plant Physiol. **67**, Suppl., 104

Butler, W.L., Lane, H.C., Siegelman, H.W. (1963) Nonphotochemical transformations of phytochrome in vivo. Plant Physiol. **38**, 514–519

Butler, W.L., Norris, K.H., Siegelman, H.W., Hendricks, S.B. (1959) Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants. Proc. Natl. Acad. Sci. USA **58**, 1703–1708

Cordonnier, M.-M., Pratt, L.H. (1982) Immunopurification and initial characterization of dicotyledonous phytochrome. Plant Physiol. **69**, 360–365

Cordonnier, M.-M., Smith, C., Greppin, H., Pratt, L.H. (1983) Production and purification of monoclonal antibodies to phytochrome. Planta **158**, 369–376

Gottman, K., Schäfer, E. (1983) Analysis of phytochrome kinetics in light-grown *Avena sativa* L. seedlings. Planta **157**, 392–400

Heim, B., Jabben, M., Schäfer, E. (1981) Phytochrome destruction in dark- and light-grown *Amaranthus caudatus* seedlings. Photochem. Photobiol. **34**, 89–93

Hunt, R.E., Pratt, L.H. (1979a) Phytochrome immunoaffinity purification. Plant Physiol. **64**, 332–336

Hunt, R.E., Pratt, L.H. (1979b) Phytochrome radioimmunoassay. Plant Physiol. **64**, 327–331

Hunt, R.E., Pratt, L.H. (1980) Radioimmunoassay of phytochrome content in green, light-grown oats. Plant Cell Environ. **3**, 91–95

Jabben, M. (1980) The phytochrome system in light-grown *Zea mays* L. Planta **149**, 91–96

Jabben, M., Deitzer, G.F. (1978a) A method for measuring phytochrome in plants grown in white light. Photochem. Photobiol. **27**, 799–802

Jabben, M., Deitzer, G.F. (1978b) Spectrophotometric phytochrome measurements in light-grown *Avena sativa* L. Planta **143**, 309–313

Jabben, M., Deitzer, G.F. (1979) Effect of the herbicide SAN 9789 on photomorphogenic responses. Plant Physiol. **63**, 481–485

Kilsby, C.A.H., Johnson, C.B. (1981) The influence of light quality on the phytochrome content of light grown *Sinapis alba* L. and *Phaseolus aureus* Roxb. Planta **153**, 109–114

Kilsby, C.A.H., Johnson, C.B. (1982) The in vivo spectrophotometric assay of phytochrome in two mature dicotyledonous plants. Photochem. Photobiol. **35**, 255–260

Pratt, L.H. (1973) Comparative immunochemistry of phytochrome. Plant Physiol. **51**, 203–209

Pratt, L.H. (1983) Assay of photomorphogenic photoreceptors. In: Encyclopedia of plant physiology, N.S., vol. 16A: Photomorphogenesis, pp. 152–177, Shropshire, W., Jr., Mohr, H., eds. Springer, Berlin Heidelberg New York

Pratt, L.H. (in press) Phytochrome purification. In: Techniques in photomorphogenesis, Smith, H., ed. Academic Press, London New York

Pratt, L.H., Kidd, G.H., Coleman, R.A. (1974) An immunochemical characterization of the phytochrome destruction reaction. Biochim. Biophys. Acta **365**, 93–107

Roux, S.J., McEntire, K., Brown, W.E. (1982) Determination of extinction coefficients of oat phytochrome by quantitative amino acid analyses. Photochem. Photobiol. **35**, 537–543

Shimazaki, Y., Moriyasu, Y., Pratt, L.H., Furuya, M. (1981) Isolation of the red-light-absorbing form of phytochrome from light-grown pea shoots. Plant Cell Physiol. **22**, 1165–1173

Tokuhisa, J.G., Quail, P.H. (1983) Spectral and immunochemical characterization of phytochrome isolated from light grown *Avena sativa*. (Abstr.) Plant Physiol. **72**, Suppl., 85

Voller, A., Bidwell, D., Bartlett, A. (1980) Enzyme-linked immunosorbent assay. In: Manual of clinical immunology, 2nd edn., pp. 359–371, Rose, N.R., Friedman, H., eds. Am. Soc. Microbiol., Washington, D.C.

Weber, K., Osborn, M. (1969) The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**, 4406–4412

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