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Phytochrome in green tissue: Spectral and immunochemical evidence for two distinct molecular species of phytochrome in light-grown *Avena sativa* L.

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Abstract. A method is described for the extraction of phytochrome from chlorophyllous shoots of *Avena sativa* L. Poly(ethyleneimine) and salt fractionation are used to reduce chlorophyll and to increase the phytochrome concentration sufficiently to permit spectral and immunochemical analyses. The phototransformation difference spectrum of this phytochrome is distinct from that of phytochrome from etiolated shoots in that the maximum in the red region of the difference spectrum is shifted about 15 nm to a shorter wavelength. Immunochemical probing of electroblotted proteins (Western blotting), using a method sensitive to 50 pg, demonstrates the presence of two polypeptides in green tissue that bind antiphytochrome antibodies: a predominant species with a relative molecular mass (M_r) of 118000 and a lesser-abundant 124000-M_r polypeptide. Under nondenaturing conditions all of the 124000-M_r species is immunoprecipitable, but the 118000-M_r species remains in the supernatant. Peptide mapping and immunochemical analysis with monoclonal antibodies show that the 118000-M_r species has structural features that differ from etiolated-oat phytochrome. Mixing experiments show that these structural differences are intrinsic to the molecular species from these two tissues rather than being the result of post-homogenization modifications or interfering substances in the green-tissue extracts. Together the data indicate that the phytochrome that predominates in green-tissue has a polypeptide distinct from the well-characterized molecule from etiolated tissue.

Abbreviations and symbols: Ig = immunoglobulin; M_r = relative molecular mass; Pfr, Pr = far-red-absorbing and red-absorbing forms of phytochrome respectively; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; λ^{R}_{max}, λ^{FR}_{max} = maxima of the phototransformation difference spectrum in the red and far-red region

Key words: *Avena* (phytochrome) – Immunoprecipitation – Monoclonal antibody – Phytochrome from green tissue – Western blotting (immunoblot analysis).

Introduction

Phytochrome regulates growth and development throughout the life cycle of green plants, including the vegetative and floral stages (for a recent review, see Shropshire and Mohr 1983). However, understanding of the molecular properties of the photoreceptor has been derived exclusively from studies on the apparently homogeneous pool of phytochrome extracted from dark-grown tissue (Smith 1983; Vierstra and Quail 1983). While the possibility exists that there is only one species of phytochrome whose properties remain constant throughout the life cycle, this has never been established directly in biochemical studies. Indeed, the existence of more than one pool of phytochrome has been suggested in the literature over the years to explain a variety of physiological and in-vivo spectroscopic data (Hillman 1967; Jabben and Holmes 1983). Thus, the question has remained open as to whether the phytochrome molecule that functions in light-grown tissue is identical to that in etiolated tissue.

The principal reason for lack of information on phytochrome from chlorophyllous tissue is the technical difficulty of isolating and assaying the molecule from such tissue (Pratt 1982). Light-grown tissues (i) contain chlorophyll which interferes with the spectral analysis of phytochrome through screening, fluorescence and irreversible photobleaching (Siegelman and Butler 1965; Jose et al. 1977; Bolton and Quail 1981), and (ii) con-

tain approx. 50-fold lower levels of the photoreceptor than etiolated tissue (Pratt 1982). Previous approaches to this problem have included the use of achlorophyllous tissue (Butler et al. 1963; Jabben and Deitzer 1978) or extracts of chlorophyllous tissue treated either with organic solvents (Taylor and Bonner 1967), with various precipitating agents (Hunt and Pratt 1979; Bolton and Quail 1981), or simply with repetitive fractionation using $(NH_4)_2SO_4$ and chromatography (Lane et al. 1963). These extracts required extensive preparation time or the use of a scattering agent such as $CaCO_3$ (Butler and Norris 1960) to amplify the absorbance changes in order that phytochrome could be detected spectrally. Thus, the limited sensitivity of the assay and the interference by chlorophyll have prevented the use of the spectrophotometric assay for detailed analysis of the molecule.

The advent of the immunochemical assay for phytochrome, developed by Pratt and coworkers (Pratt 1982), circumvented the two major limitations to the spectrophotometric assay. This technology has recently permitted preliminary characterization of phytochrome from light-grown plants using enzyme-linked immunosorbent assays (ELISA) in microtiter plates (Shimazaki et al. 1983; Thomas et al. 1984). However, because these current immunochemical assays neither measure structural integrity nor permit the detection of the chromophore, the spectrophotometric assay is still needed.

We describe here a rapid, simple procedure for the extraction of phytochrome from chlorophyllous tissue and a highly sensitive immunoblot procedure for the detection of phytochrome polypeptides following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These procedures reduce chlorophyll to a level that does not interfere significantly with spectrophotometric analysis and permit detection of as little as 50 pg of phytochrome in a gel band. With these methods, the spectral, immunochemical and structural properties of phytochrome from green and from etiolated tissue of oat have been compared and found to be different. Preliminary reports of this research have been presented elsewhere (Bolton and Quail 1981; Tokuhisa and Quail 1983; Tokuhisa et al. 1984).

Material and methods

Chemicals and supplies. Ethylene glycol and poly(ethyleneimine) (PEI) were obtained from Eastman Kodak (Rochester, NY, USA). Acrylamide, bisacrylamide and sodium dodecyl sulfate (SDS) were purchased from BioRad Laboratories (Richmond, Cal., USA). Bovine serum albumin, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), gelatin, nitro blue tetrazolium, phenylmethylsulfonyl fluoride (PMSF), *Staphylococcus aureus* V8 protease and Tween 20 (Polyoxyethylenesorbitan monolaurate) were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Goat-antirabbit and rabbit-antimouse antibodies, and rabbit-anti-goat and goat-antirabbit immunoglobulins (Ig) conjugated with alkaline phosphatase were purchased from Kirkegaard & Perry (Gaithersburg, Md., USA).

Plant material. Oat seeds (*Avena sativa* L. cv. Garry; Olds Seed Co., Madison, Wis., USA) were planted on a 6-cm bed of coarse vermiculite in plastic boxes ($19 \cdot 28$ cm^2), using 150 ml of seeds and 1200 ml of tapwater. Etiolated seedlings were grown in the dark for 4 d at 25° C. Aerial portions were harvested under dim green light (Bolton and Quail 1982). Chlorophyllous seedlings were grown under continuous light from white fluorescent lamps (F96T12/CW/SHO, Westinghouse Electric, Pittsburgh, Pa., USA) supplemented with light from incandescent lamps (11 Wm^{-2} PAR) for 7 d at 25° C. Aerial portions were harvested under laboratory lighting. Both tissue types then were processed identically. The tissue was chilled to 4° C and irradiated with red light for 20 min ($\lambda_{max} = 660$ nm; Vierstra and Quail 1982b). It was then frozen at −80° C, lyophilized, ground to a powder (Wiley-Mill No. 1; A.H. Thomas Co., Philadelphia, Pa., USA) and stored in air-tight jars at −20° C.

Extraction procedure. All manipulations were performed under dim green light with samples maintained at or below ice temperatures unless stated otherwise. Powder of lyophilized, green-oat tissue (9.0–9.5 g dry weight = 100 g fresh weight) was mixed with extraction buffer at a ratio of 1:1.5 (g fresh weight equivalents to ml of buffer). Extraction buffer contained 25% ethylene glycol, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-HCl), 75 mM $(NH_4)_2SO_4$, 5 mM tetrasodium ethylenediaminetetraacetic acid (Na_4EDTA) and was adjusted to 25 mM $NaHSO_3$, pH 8.5 (4° C), and 2 mM PMSF just prior to use.

The brei was mixed with 5 ml of 10% (v/v) PEI (pH 7.8; Jendrisak and Burgess 1975) per 100 ml extract, incubated for 10 min, and centrifuged at 27000 *g* for 30 min. The supernatant was filtered through Miracloth (Calbiochem, San Diego, Cal., USA), adjusted to 4 mM PMSF, and irradiated with red light for 10 min (16 J m^{-2} s^{-1}; 660 nm interference filter, PTR Optics, Waltham, Mass., USA). Then 25 g of $(NH_4)_2SO_4$ were added per 100 ml of PEI supernatant. The supernatant was stirred for 10 min and centrifuged at 27000 *g* for 30 min. The pellet was resuspended in 5 ml of extraction buffer minus $(NH_4)_2SO_4$ per 9 g dry weight of tissue and clarified at 48000 *g* for 20 min. Etiolated-oat extract was prepared in the identical fashion.

Protein and chlorophyll assay. The method of Lowry et al. (1951) was used to quantitate protein precipitated in 5% trichloroacetic acid. Bovine serum albumin was used as the standard. Chlorophyll concentration was estimated as described by Vernon (1960).

Spectral assay of phytochrome. Continuous spectra (525–815 nm) were recorded with a Perkin-Elmer 557 Spectrophotometer (Perkin-Elmer Co., Norwalk, Conn., USA) as described by Vierstra and Quail (1982a). Wavelengths were calibrated with a holmium oxide filter (Beckman Instruments, Fullerton, Cal., USA). Alternatively, phytochrome was measured in a custom-built, dual-wavelength spectrophotometer (L.H. Pratt, University of Georgia, Athens, USA) using $CaCO_3$ as a scattering agent (Butler and Norris 1960). The monitoring wavelengths were obtained with a 730-nm interference filter (Corion Corp., Hilliston, Mass., USA) and an 800-nm interference filter (Baird-Atomic, Bedford, Mass., USA).

Dark reversion. All phytochrome samples were in extraction buffer (pH 7.8) containing 5 mM sodium dithionite. At various times after the samples were irradiated with red light to maximize conversion from the red-absorbing form of phytochrome (Pr) to the far-red-absorbing form (Pfr), aliquots were removed from the sample that was kept in the dark at 4° C and assayed for unreverted Pfr (Pike and Briggs 1972a).

Etiolated-oat phytochrome: Purification and antibody purification. Phytochrome with a relative molecular mass (M_r) of 124000 from etiolated oats was purified by the Affigel-Blue procedure (Vierstra and Quail 1983) and used to generate polyclonal rabbit antisera (Vierstra et al. 1984) and mouse monoclonal antibodies (Daniels and Quail 1984). The 114000/118000-M_r phytochrome was purified by the immunoaffinity procedure (Hunt and Pratt 1979) and used to generate polyclonal antibodies in rabbit (Vierstra and Quail 1982b) and in eight-week-old, female ICR mice (Harlan Industries, Madison, Wis., USA). Polyclonal-antibody production in mice was amplified by the generation of ascites fluid (Sartorelli et al. 1966).

Immunoprecipitation. Immunoprecipitations were performed as described in Vierstra et al. (1983). The samples were incubated with antiphytochrome or preimmune Igs for 30 min at 4° C. Aliquots of 10% (v/v) *Staphylococcus aureus* Cowan I suspension, prepared according to Kessler (1976), were added as an immunoadsorbent at 3 µl:1 µg Ig, incubated for 15 min, and then centrifuged for 5 min (Beckman Microfuge B). The supernatants and washed pellets were assayed for phytochrome spectral activity.

Immunochemical detection of electroblotted proteins. This procedure was performed as described by Vierstra et al. (1984) with modifications that enhance sensitivity 20-fold. Samples were subjected to SDS-PAGE according to the method of Laemmli (1970). The acrylamide gel consisted of a 5.3% resolving gel with a 4.5%, 1-cm-deep stacking gel (acrylamide:bisacrylamide ratio of 30:0.8). Specific lanes were marked by adding 0.1% methyl green in 50% glycerol to the wells; methyl green binds to nitrocellulose. The proteins were blotted onto nitrocellulose (HAHY 304 FO; Millipore Corp.) with a Trans-blot cell (BioRad Laboratories) at 150 mA constant current for at least 8 h (Burnette 1981). Remaining protein-binding sites on the nitrocellulose were quenched with saturation buffer (3% gelatin, 1% bovine serum albumin, 0.02% NaN_3 in Tris-buffered saline pH 9.0); for 1 h at 25° C or overnight at 4° C.

Molecular-mass markers (Vierstra and Quail 1982a) and other protein profiles were visualized by using Tris-buffered saline (TBS; 20 mM Tris (pH 7.5, 25° C), 150 mM NaCl) containing 0.1% Tween 20, incubating for 30 min followed by incubating with Coomassie stain (0.1% Coomassie Brilliant Blue R, 25% ethanol, 10% acetic acid) for less than 1 min, and lastly rinsing with H_2O. Alternatively, prestained molecular-mass markers (Bethesda Research Laboratories, Gaithersburg, Md., USA) were used.

The primary antibody solution was prepared by diluting the serum 1:200 with antibody dilution buffer (1% gelatin, 1% bovine serum albumin, 0.02% NaN_3 in TBS, pH 7.5, 25° C). The blot was incubated with the solution at room temperature for 2 h (monoclonal Igs for 3 h), removed, and incubated in three changes of TBS-0.1% Tween 20 for a total of 1 h followed by a rinse in TBS.

All subsequent antibody incubations were for 2 h at 25° C with antibody solutions diluted 1:200 as above and followed by incubation with three changes of TBS-0.1% Tween 20. Second antibody incubations were done with antibodies directed against the primary antibody (goat-antirabbit or rabbit-antimouse). Strong background stain was eliminated by using more stringent washes (TBS-0.1% Tween 20, 0.05% SDS). Third antibody incubations were done with rabbit-antigoat or goat-antirabbit Ig conjugated with alkaline phosphatase.

The enzyme substrate was prepared according to R. Dimond (University of Wisconsin, Madison, personal communication) and Leary et al. (1983). An aliquot of 15 mg of BCIP was dissolved in approx. 100 µl dimethyl sulfoxide, diluted with two volumes of 2.0 M Tris (pH 9.0) and adjusted to approx. 5 ml with color buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.0, 25° C). An aliquot of 30 mg of nitro blue tetrazolium was dissolved by suspending the powder in 1 ml of color buffer and adding 4 ml of boiling color buffer with very rapid mixing. The remaining particles of nitro blue tetrazolium were dissolved by additional heating. This solution was diluted rapidly to 75 ml with room-temperature color buffer and rapidly stirred during the addition of the BCIP solution. The total volume was adjusted to 100 ml. This amount was sufficient for two 13·15-cm^2 blots. The blots were rinsed in TBS and incubated in the color reagent for 10–60 min in the dark as the color reagent is light sensitive. The reaction was terminated by rinsing the blot in 0.02% (w/v) sodium azide. The developed blots were stored in 0.02% (w/v) NaN_3 or wrapped in plastic film. Azide minimizes nonspecific color development during storage.

Peptide mapping. Peptides were prepared and analysed according to Cleveland (1983). Purified oat phytochrome from etiolated tissue with M_rs of 124000 or 114000/118000 and green-oat extract containing spectrally measurable phytochrome were subjected to SDS-PAGE on a 5.3% slab gel (Laemmli 1970). The gel was stained (0.1% Coomassie Blue, 50% methanol, 10% acetic acid) for 30 min and destained (5% methanol, 10% acetic acid) for 60 min. The purified samples of etiolated-oat phytochrome were used as stainable markers to locate the region corresponding to 118000 M_r in the lanes containing green-oat extract. Gel pieces from relevant regions were excised, equilibrated for 1 h in three changes of equilibration buffer (0.125 M Tris, pH 6.8, 10% glycerol, 0.3% 2-mercaptoethanol, 0.1% sodium dodecyl sulfate, 1 mM Na_4EDTA), and loaded on an acrylamide gel consisting of a 15% resolving gel and a 1-cm-long, 4.5% stacking gel. The gel pieces in each lane were overlayed with 10 µl of a buffer (0.125 M Tris, pH 6.8, 20% glycerol, 0.3% 2-mercaptoethanol, 0.1% sodium dodecyl sulfate, 1 mM Na_4EDTA) and with 5–10 µl of *S. aureus* V8 protease diluted with equilibration buffer. The samples were subjected to electrophoresis until stacked at 20–35 mA/0.15-cm-thick gel, incubated without applied voltage for 30 min, and subjected to electrophoresis at 35–40 mA/0.15-cm-thick gel until resolved. The gel was processed for immunoblot analysis as above.

Terminology. The following terms are used to describe the two different types of mixtures of etiolated- and green-oat phytochrome. These mixtures contain etiolated-oat phytochrome that has been introduced into an environment that is predominantly green-oat extract.

Mixed extracts: An extract from lyophilized tissue of green oats and an extract from lyophilized tissue of etiolated oats were prepared separately according to the described procedure. Just before analysis, the two extracts were mixed to obtain a defined amount of spectrally measurable phytochrome contributed by each of the extracts. For a 1:1 ratio of spectral activity, the extract was 90% green-oat extract.

Mixed-tissues extract: Lyophilized tissue from etiolated oats and lyophilized tissue from green oats were mixed and processed together as a single extract from homogenization on-

ward according to the described procedure. Thus, etiolated-oat phytochrome experienced the same environment as green-oat phytochrome during the extraction procedure. The ratio of the tissues was varied to achieve the desired ratio of spectrally detectable phytochrome contributed by each of the tissues. For a 1:1 ratio of phytochrome spectral activity from green and etiolated tissue, the extract was 98% green-oat tissue.

The introduction of the etiolated-oat phytochrome at these two stages in the preparative procedure was designed to differentiate between any potential modifications induced by green-tissue components in the final $(NH_4)_2SO_4$ pellet ("mixed extracts"), and by the preparative manipulations in the presence of green-tissue components from the point of homogenization onward ("mixed-tissues extract") respectively.

Results and discussion

Extraction procedure. Four extraction procedures (Taylor and Bonner 1967; Hunt and Pratt 1979; Bolton and Quail 1981; Vierstra and Quail 1983) were assessed for their capacity to (i) provide undegraded 124000-M_r phytochrome from etiolated-oat tissue, (ii) yield consistent recovery of phytochrome from green-oat shoots, (iii) effectively remove chlorophyll from homogenates of green-oat shoots, and (iv) effect rapid extraction. Each of the procedures was deficient in at least two of the four criteria. The final method described under "Materials and methods" optimizes these four parameters. We have characterized phytochrome from green oats at the earliest stages of purification to minimize the possibility of time-dependent, in-vitro artifacts.

Both the extraction of phytochrome as Pfr and the selection of buffer conditions were based on experience with etiolated tissue. Vierstra and Quail (1983) have shown that extraction as Pfr increases the recovery of 124000-M_r phytochrome because Pfr is relatively resistant to the proteolytic degradation to 114000/118000 M_r that occurs as Pr. Inclusion in the buffer of PMSF, 75 mM $(NH_4)_2SO_4$ and 25% ethylene glycol and the exclusion of 2-mercaptoethanol also have been shown to maximize recovery of the 124000-M_r species (Litts et al. 1983; Vierstra and Quail 1983).

The use of green plant material that is lyophilized and then powdered results in the extraction of two- to threefold more phytochrome than that extracted from fresh plant tissue (data not shown). Consistent recoveries of phytochrome could not be obtained with homogenates of fresh tissue due to either incomplete cell breakage or extensive foaming of the homogenate. The advantages of using lyophilized tissue have been described by Smith (1983).

Treatment of the brei from green-oat shoots with poly(ethyleneimine) enhances the precipitation of chlorophyll and protein (Fig. 1, and Bolton and Quail 1981). The standard, final concentration of 0.5% poly(ethyleneimine) was found to reduce residual chlorophyll in the brei to 1.2% of the minus-poly(ethyleneimine) control. Although 0.1% poly(ethyleneimine) was almost as effective in eliminating chlorophyll, 0.5% removed the additional amount necessary for spectral characterization of phytochrome free of chlorophyll interference. The amount of chlorophyll remaining was 0.2 µg per ml of poly(ethyleneimine) supernatant.

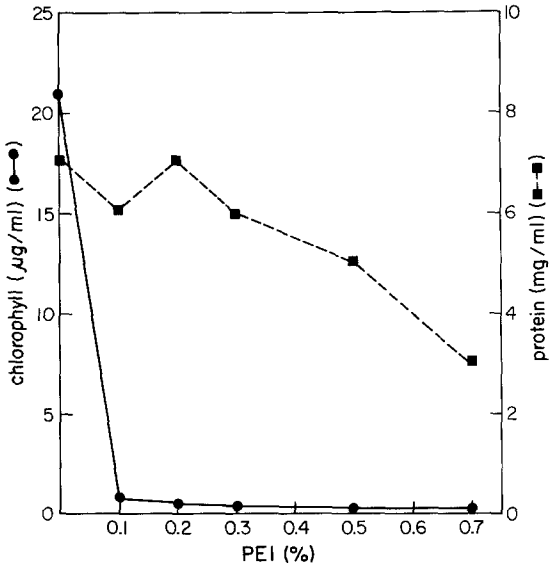

Fig. 1. Poly(ethyleneimine) (*PEI*) fractionation of chlorophyll and protein in crude extracts of green oat tissue. Powder from lyophilized, green-oat shoots was mixed with buffer (50 mM Tris pH 8.5, 25% ethylene glycol, 75 mM $(NH_4)_2SO_4$, 25 mM $NaHSO_3$, 5 mM Na_4EDTA, 2 mM phenylmethylsulfonyl fluoride. The extract was adjusted to various concentrations of poly(ethyleneimine) and centrifuged at 27000 *g* for 30 min. The supernatant was measured for chlorophyll (●) and protein (■)

Although poly(ethyleneimine) treatment enhances precipitation of some proteins, the data strongly indicate the quantitative recovery of phytochrome in the supernatant. Litts et al. (1983), and Vierstra and Quail (1983) have demonstrated 85–95% recoveries of phytochrome from extracts of etiolated-oat tissue that had been treated with poly(ethyleneimine). With the brei from green-oat shoots, concentrations of 0.35 or 0.70% poly(ethyleneimine) resulted in identical recoveries of phytochrome at the subsequent $(NH_4)_2SO_4$ stage while total protein concentration was halved in the post-poly(ethyleneimine) supernatant of the 0.7% treatment (data not shown).

Phytochrome is concentrated from the post-poly(ethyleneimine) supernatant by $(NH_4)_2SO_4$ fractionation. The addition of 25 g/100 ml of poly(eth-

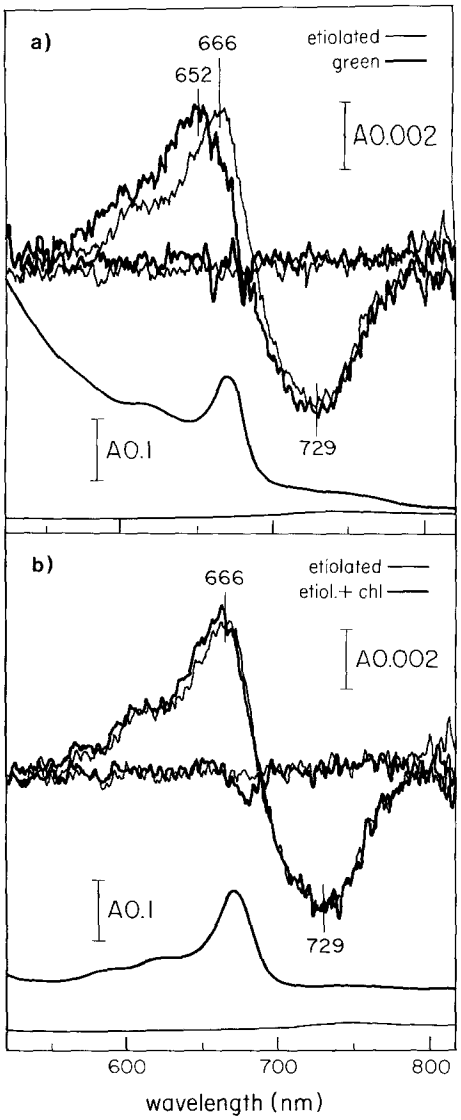

Fig. 2a, b. Absorbance and difference spectra of green-oat extract and of etiolated-oat extract with or without added chlorophyll. Crude extracts of green- and etiolated-oat tissue were concentrated with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitates were resuspended in a buffer (50 mM Tris, pH 8.1, 25% ethylene glycol, 25 mM $NaHSO_3$, 5 mM Na_4EDTA, 2 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation. Absorbance spectra (*lower curves* in each panel) and Pr-Pfr difference spectra (*upper curves* in each panel) were recorded. **a** The spectra of green-oat extract (*thick lines*) compared with those of etiolated-oat extract (*thin lines*). The spectra of etiolated-oat extract were obtained with a preparation diluted to equivalent phytochrome spectral activity with the above buffer (pH 7.8) containing 75 mM $(NH_4)_2SO_4$ (pH 7.8). **b** The spectra of etiolated-oat extract from panel **a** (*thin lines*) compared to the spectra of etiolated-oat extract to which has been added chlorophyll at the concentration found in the green-oat extract (*thick lines*). Chlorophyll was extracted from green oat shoots with 50% ethanol

yleneimine) supernatant permits recovery of $6 \cdot 10^{-3}$–$8 \cdot 10^{-3}$ $\Delta(\Delta A)$/ml of green-oat extract. This is 80–90% of the spectrally measurable phytochrome in the poly(ethyleneimine) supernatant (data not shown). Using the extinction coefficient for etiolated-oat phytochrome (Litts et al. 1983), the photoreceptor is 0.03% of the total protein found in this green-oat extract. These data indicate that soluble phytochrome in green-oat shoots is present at least at a concentration of $3 \cdot 10^{-4}$–$4 \cdot 10^{-4}$ $\Delta(\Delta A)$/g fresh weight or 270–360 ng/g fresh weight. This is approx. 2% of the phytochrome concentration found in etiolated-oat shoots (Vierstra and Quail 1983). The phytochrome preparation permits characterization by spectral assay in a clear solution without the use of a scattering agent.

Spectral characterization. Comparison of the phototransformation difference spectrum (Pr-Pfr) of phytochrome in green-oat extracts with phytochrome in etiolated-oat extracts shows differences between the two preparations (Fig. 2a). The difference-spectrum maximum in the red region (λ^R_{max}) of phytochrome from green-oat extracts is at 652 nm and the isosbestic point at 681 nm. These values deviate 14 and 8 nm, respectively, from those of the difference spectrum of phytochrome from etiolated oats (Fig. 2a; Vierstra and Quail 1982b). This shift in λ^R_{max} has also been observed in extracts from herbicide-treated, light-grown oats (Jabben and Deitzer 1978) as well as in vivo with herbicide-treated maize (Jabben 1980).

Other parameters such as the spectral change ratio ($\Delta A_R/\Delta A_{FR}$) of 1.09, and the λ^{FR}_{max} of 729 nm (Fig. 2a) are similar to those described for 124000-M_r phytochrome from etiolated oats. They are different, however, from those of degraded, 118000-M_r phytochrome from etiolated oats, indicating the absence of in vitro proteolysis and spectral denaturation of an etiolated-tissue-type molecule (Vierstra and Quail 1982b; Litts et al. 1983). In the absence of precautions against proteolysis, we have obtained preparations of green-oat phytochrome which exhibit the $\Delta A_R/\Delta A_{FR}$ and λ^{FR}_{max} more characteristic of the 114000/118000-M_r degraded phytochrome from etiolated oats, albeit with a λ^R_{max} of 652 nm.

The possibilities were tested that the shift observed in the difference spectrum was generated either by residual chlorophyll or by in vitro modifications of the phytochrome in the green-oat extract. First, the difference spectrum of an extract of etiolated oats was compared with that of an identical extract containing chlorophyll added such

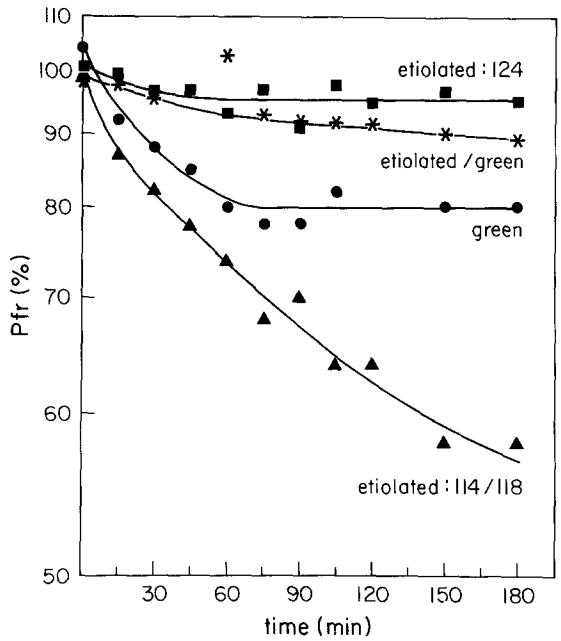

Fig. 3. Comparison of dark reversion rates for green- and etiolated-oat phytochrome. Phytochrome preparations were in extraction buffer adjusted to 5 mM sodium dithionite. Samples were irradiated to maximize Pfr and maintained in the dark (4° C). Aliquots were removed at various intervals and were measured for remaining Pfr. "Green" (●), "etiolated/green" (*) and "etiolated:124" (■) extracts were prepared according to the standard procedure. "Etiolated/green" extract was a mixed-tissues extract containing a 1:9 ratio of green- and etiolated-oat phytochrome as determined spectrally. "Etiolated:114/118" (▲) was a purified preparation of 114000/118000-M_r phytochrome from etiolated oats (Hunt and Pratt 1979)

that the absolute absorbance contributed by the chlorophyll was the same as in the extract from green oats (Fig. 2b). Secondly, a difference spectrum was obtained from a mixed-tissue extract which was prepared by mixing the powdered tissue of green and etiolated oats prior to extraction to generate a 1:9 ratio (green:etiolated) of spectrally detectable phytochrome (data not shown). In both cases, the difference spectra were similar to that of etiolated-oat phytochrome. These data indicate that the different λ^{R}_{max} of green-oat phytochrome results neither from (i) chlorophyll interference nor (ii) modification of the spectral properties of an etiolated-oat-type phytochrome by green-oat-extract components or the extraction procedure.

The extent of dark reversion, the nonphotochemical conversion of phytochrome from Pfr to Pr, can indicate the degree of NH_2-terminal proteolysis of etiolated-tissue phytochrome (Vierstra and Quail 1982b). Figure 3 demonstrates that when phytochrome-containing samples in ethylene glycol-containing buffers are maintained at 4° C in the presence of sodium dithionite, an accelerant of reversion (Pike and Briggs 1972a), 124000-M_r phytochrome from etiolated oats exhibits little reversion over 3 h whereas 114000/118000-M_r phytochrome from etiolated oats exhibits appreciable dark reversion. In green-oat extracts, limited dark reversion is observed (Fig. 3). Of the total photoreversible activity, 20% reverted in the first hour with kinetics similar to those of 114000/118000-M_r phytochrome. The remaining 80% of the spectral activity showed negligible rates of reversion up to 180 min. Adjusting the extract to 10 mM sodium dithionite at 120 min did not stimulate further reversion.

The occurence of limited dark reversion indicated the possibility that there was at least partial NH_2-terminal proteolysis of the type that occurs in vitro with etiolated-oat phytochrome. Pike and Briggs (1972b) demonstrated protease activity in extracts of green oats similar to the activity associated with the induction of dark reversion in phytochrome from etiolated oats. This possibility was tested by preparing a mixed-tissues extract and monitoring dark reversion (Fig. 3). Powder of green and etiolated shoots was mixed such that the final extract contained a 1:9 ratio of spectral activity from green- and etiolated-oat tissue (95% green-tissue environment). The rate and extent of dark reversion in this preparation (10% reversion) is more like that predicted by a 1:9 mixture of phytochrome from green oats and 124000-M_r phytochrome from etiolated oats (6%) than that predicted by phytochrome from green oats alone (20%) or a 1:9 mixture of phytochrome from green oats and 114000/118000-M_r phytochrome from etiolated oats (40%). This result demonstrates that minimal proteolysis of phytochrome from etiolated oats occurs in the environment of green-oat extract and indicates that the majority of the 20% dark-reverting phytochrome from green tissue is not generated by in vitro, proteolytic action on a phytochrome molecule of the type found in etiolated tissue.

Immunochemical characterization by immunoblot analysis. To detect the very low levels of phytochrome in green-oat extracts, we had to use a highly sensitive immunoblot procedure. To this end, we have modified the procedures of Dimond and colleagues (Knecht and Dimond 1984; Vierstra et al. 1984) such that the procedure described here can detect, under optimal conditions, as little as 50 pg of phytochrome from etiolated oats using polyclonal anti-phytochrome Igs, and 1 ng of phytochrome using monoclonal Igs. When immuno-

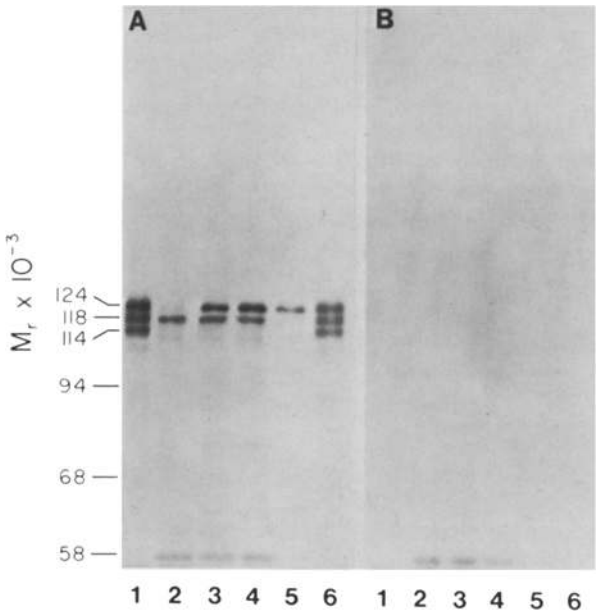

Fig. 4A, B. Immunoblot analysis of green-oat extract using polyclonal antiphytochrome Igs. Various phytochrome-containing samples were prepared and subjected to SDS-PAGE in a 5.3% acrylamide gel, and electroblotted onto nitrocellulose. The nitrocellulose blot was quenched with 3% gelatin and incubated with **A** polyclonal rabbit Igs directed against 124000-M_r phytochrome from etiolated oats or **B**, nonimmune serum from rabbit. Both blots were incubated sequentially with goat-anti-rabbit Ig and rabbit-antigoat Ig linked with alkaline phosphatase. *Lanes 1, 6* contain approx. 15 ng of purified 114000/118000-M_r and 124000-M_r, etiolated-oat phytochrome (Hunt and Pratt 1979; Vierstra and Quail 1983); *lane 2*, green-oat extract containing 7 ng of spectrally detectable phytochrome; *lane 3*, mixed extracts containing 12 ng of an approx. 1.4:1 ratio of spectrally detectable green- and etiolated-oat phytochrome; *lane 4*, 12 ng of phytochrome from a mixed-tissues extract containing a 1.4:1 ratio of spectrally detectable phytochrome prepared by mixing etiolated- and green-oat shoots and extracting according to the standard procedure; and *lane 5*, 5 ng of phytochrome from etiolated-oat extract

blots of green-oat extracts were probed with polyclonal Igs directed against 124000-M_r, etiolated-oat phytochrome, two principal bands were observed (Fig. 4a, lane 2): a major one at 118000 M_r and a minor one at 124000 M_r (barely visible). Other antiphytochrome preparations, namely rabbit and mouse Igs directed against 114000/118000-M_r phytochrome from etiolated oat, generate a similar pattern (data not shown). These data establish that green- and etiolated-oat phytochrome carry common epitopes.

The possibility was investigated that the 118000-M_r species from green oats was generated by the same in vitro proteolysis that produces the 118000-M_r degradation product from etiolated-oat phytochrome (Vierstra and Quail 1982a). A mixed-extracts preparation was made by mixing, just before electrophoresis, separate extracts to provide a 1:1.4 ratio of spectrally measurable phytochrome from green- and etiolated-oats (Fig. 4, lane 3). A mixed-tissues extract was prepared containing a 1:1.4 ratio of spectrally measurable phytochrome from green and etiolated oats (Fig. 4, lane 4). The mixed-tissues extract contains two bands corresponding to 124000- and 118000-M_r, with the band at 124000 M_r enhanced in intensity relative to green-oat extract alone (Fig. 4, lane 4). This pattern and the stain intensity are identical to those of the mixed extracts (Fig. 4, lane 3). The stain intensity of the band at 118000 M_r in the three preparations containing green-oat extract (Fig. 4, lanes 2–4) is constant and correlates with the constant amount of green-oat extract applied to each lane. These data demonstrate stability of the 124000-M_r, etiolated-oat phytochrome in the extract and indicate that the presence and amount of the 118000-M_r band in green-oat extracts is not due to time-dependent, in vitro proteolysis of the type that can occur with phytochrome from etiolated tissue. Immunoblot analysis of the post-poly(ethyleneimine) supernatant demonstrated an intensity and molecular-mass pattern identical with that shown here, again indicating that time-dependent modifications are not occurring between the two steps in the procedure (data not shown). This result further supports the notion that the two molecular species of phytochrome observed are intrinsic to shoots of green oats.

To define the immunochemical relationship between phytochrome from green and etiolated oats more precisely, the immunoblots of green-oat extracts were probed with monoclonal antibodies directed against three spatially distinct regions of 124000-M_r, etiolated-oat phytochrome (Daniels and Quail 1984). These three classes of monoclonal antibody, designated Types 1, 2 and 3, recognize, in this order, epitopes in the NH_2-terminal region, the central chromophore-bearing region and the COOH-terminal region of the polypeptide. Because of the lowered sensitivity inherent in probing immunoblots with monoclonal as opposed to polyclonal antibodies, higher nonimmunospecific backgrounds were experienced when we tested the green-oat extracts with the three monoclonal types (Fig. 5). Nevertheless, immunospecifically positive bands are readily identifiable by direct comparison of each lane with its corresponding lane in the nonimmune-serum control.

In this and numerous identical experiments, only monoclonal Type 3 was found to exhibit clear

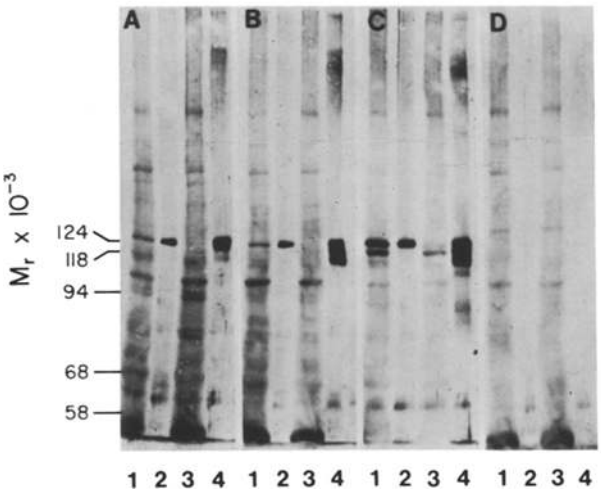

Fig. 5A–D. Immunoblot analysis of green-oat extract using monoclonal Igs directed against phytochrome (124000 M_r) from etiolated oats. Phytochrome-containing samples were subjected to SDS-PAGE in a 5.3% acrylamide gel and electroblotted onto nitrocellulose. Four replica blots (**A, B, C, D**) were prepared and probed with either one of three groups of monoclonal Igs specific for three spatially discrete epitopes on 124000-M_r phytochrome from etiolated oats (Daniels and Quail 1984): **A** Type 1 (NH_2-terminal region), **B** Type 2 (chromphore-bearing region), **C** Type 3 (COOH-terminal region), or **D** mouse nonimmune antiserum. The blot was then incubated with rabbit-antimouse Ig followed by goat-antirabbit Ig linked with alkaline phosphatase. *Lane 1*, 110 ng of phytochrome from mixed tissues extract (1:1 ratio of green- and etiolated-oat phytochrome); *lane 2*, 46 ng of phytochrome from etiolated-oat extract; *lane 3*, 70 ng of phytochrome from green-oat extract, and *lane 4*, 50 ng of purified 114000/118000-M_r and 124000-M_r, etiolated-oat phytochrome

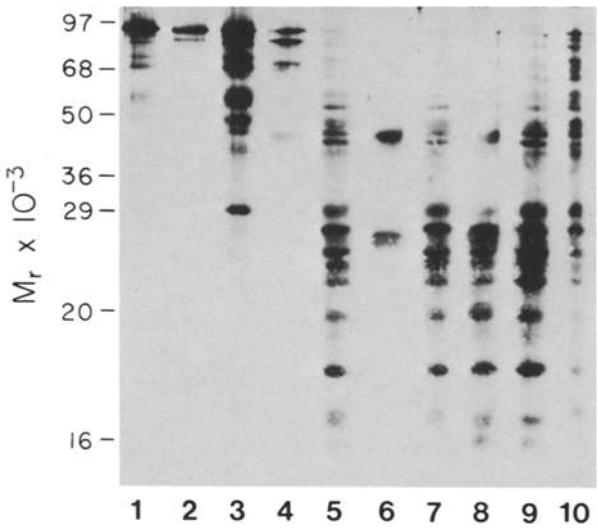

Fig. 6. Immunoblot analysis of peptides generated by *S. aureus* V 8 protease digestion of etiolated- and 118000-M_r green-oat phytochrome. Purified 124000-M_r and 118000-M_r phytochrome from etiolated oats and green-oat extract containing phytochrome were subjected to SDS-PAGE in a 5.3% SDS-acrylamide gel. The gel was stained with Coomassie Blue R and destained according to Cleveland (1983). Both the phytochrome bands from etiolated oats and the region corresponding to 118000 M_r in the lanes containing green-oat extract were cut from the gel. These gel pieces were loaded on a second 15% gel with various concentrations of *S. aureus* V 8 protease, subjected to electrophoresis until stacked, incubated without applied voltage for 30 min and subjected to electrophoresis to resolution. The gel was processed for immunoblot analysis using polyclonal rabbit antisera directed against 124000-M_r phytochrome from etiolated oats as the primary antibody. *Lanes 1, 3, 5, 7, 8* contained 200 ng of 124000-M_r, etiolated-oat phytochrome; *lanes 2, 4, 6, 8*, 200 ng of 118000-M_r, green-oat phytochrome; *lane 9*, 200 ng 118000-M_r, etiolated-oat phytochrome; *lane 10*, 100 ng 124000-M_r, etiolated-oat phytochrome; *lanes 1, 2*, 2 ng protease; *lanes 3, 4*, 20 ng, *lane 10*, 100 ng; and *lanes 5–9*, 200 ng

recognition of green-oat phytochrome (Fig. 5c, lane 3). Thus within the limits of resolution of the procedure as employed here, these data indicate that Type 1 and Type 2 antibodies have little or no affinity for green-oat phytochrome and that there is an epitope defined by Type 3 in common between green- and etiolated-oat phytochrome. Figure 5 demonstrates further that all monoclonal antibody types recognize etiolated-oat phytochrome that has experienced the extraction conditions of green-oat extract (lane 1). These data support the notion that the apparent lack of recognition of the 118000-M_r species by the Type 1 and 2 antibodies reflects inherent structural differences compared with etiolated-oat phytochrome rather than post-homogenization proteolysis of an etiolated-oat-type phytochrome.

Peptide mapping. Peptide mapping with *S. aureus* V 8 protease has been used to detect differences between proteins that differ only 2% in primary sequence (Cleveland 1983). This technique permitted further testing of the possibility that the 118000-M_r species from green oats was different from the 124000-M_r phytochrome from etiolated oats. The very low levels of 124000-M_r, green-oat phytochrome have thus far precluded direct comparison of the two green-oat polypeptides. Peptide fragments of the 118000-M_r species from green oats, generated by *S. aureus* V 8 protease (Cleveland 1983) and visualized by immunoblot analysis, exhibit a different pattern from that of 124000-M_r phytochrome from etiolated oats at all protease concentrations investigated (Fig. 6, lanes 1–7). In contrast, 118000-M_r and 124000-M_r phytochrome

from etiolated oats exhibit an identical pattern (lanes 7 and 9) which is also similar to that obtained by Gottman and Schäfer (1983). The fewer bands associated with the green-oat species may represent fewer antigenic determinants separately located on that peptide than on etiolated-oat phytochrome, or fewer peptide fragments generated by the partial proteolysis. In either case, this result indicates differences in the structures of green- and etiolated-oat phytochrome.

Figure 6, lane 8, shows that the pattern of protease digestion of a mixture of green- and etiolated-oat peptides is the composite of the individual patterns shown in Fig. 6, lanes 5 and 6. The pattern generated by 100 ng of etiolated-oat phytochrome (lane 10) is identical to the one generated by a 200-ng load (all other lanes). These data demonstrate that the different patterns are not caused by differences in the concentration of phytochromes nor different ratios of protein and protease. We conclude that the pattern differences between the green-oat and etiolated-oat phytochrome species reflect differences in the primary structure of the two polypeptides.

Immunoprecipitation of green-oat phytochrome. Immunoprecipitation experiments were performed to determine the degree of recognition of the photoreceptor in the undenatured state and to correlate directly immunochemical recognition of the chromopeptide with spectral activity. As we reported earlier (Tokuhisa and Quail 1983), immunoprecipitation of spectral activity from green-oat extract is incomplete using polyclonal Igs directed against 114000/118000-M_r phytochrome from etiolated oats (Fig. 7). Only 30% of the total spectral activity of green-oat phytochrome is immunoprecipitated under conditions which immunoprecipitate all of an identical amount of etiolated-oat phytochrome. The shape and extent of the antibody titration curve indicate reduced affinity and limited recognition of green-oat phytochrome. Other authors have now obtained similar indications of immunochemical differences from microtiter plate, enzyme-linked immunosorbent assays (Shimazaki et al. 1983; Thomas et al. 1984).

The possibility was investigated that the limited immunochemical recognition was because the extract from green oats either inhibited the immunochemical reaction or masked antigenic determinants. This possibility was addressed either by immunoprecipitating phytochrome from a mixed-extracts preparation containing a 1:1 ratio of spectral activity from green- and etiolated-oat extracts or by immunoprecipitating phytochrome from a

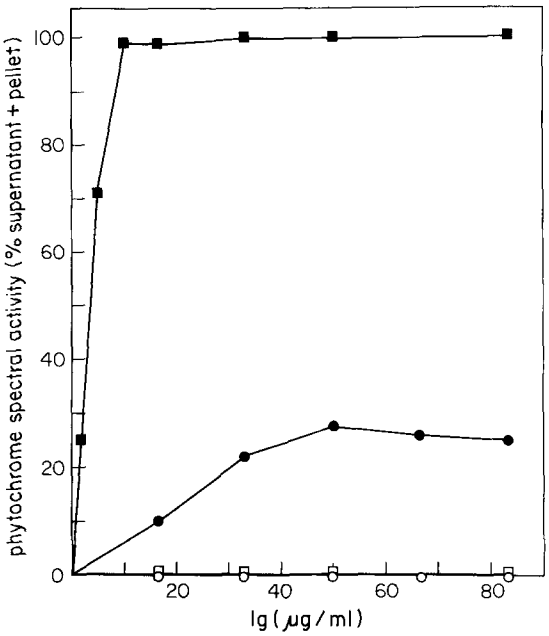

Fig. 7. Immunoprecipitation of spectrally measurable phytochrome from green-oat extract with polyclonal Igs. Separate green- (●, ○) and etiolated- (■, □) oat extracts were prepared and adjusted to equivalent phytochrome spectral activity with buffer (50 mM Tris pH 7.8, 25% ethylene glycol, 75 mM $(NH_4)_2SO_4$, 25 mM $NaHSO_3$, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride). Aliquots were incubated for 30 min (4° C) with various concentrations of either polyclonal rabbit Igs directed against 114000/118000-M_r phytochrome from etiolated oats (●, ■) or rabbit nonimmune serum (○, □). *Staphylococcus aureus* cells were added as an immunoadsorbent and after 15 min collected by centrifugation. Supernatants and washed pellets were assayed for phytochrome using $CaCO_3$ as a scattering agent. The data are presented as spectral activity precipitated as a percent of the total spectral activity in both fractions

mixed-tissues extract. This latter extract also contained a 1:1 ratio of spectral activity contributed by green- and etiolated-oat shoots. Figure 8 (a, b) shows that at the antibody concentrations used (17 μg of Ig/ml of extract), 11% of the spectrally detectable phytochrome from green-oat extract was precipitated (compare Fig. 7). In contrast, the spectral activity precipitated from either of the two mixed preparations (Fig. 8c, d, e, f) was well in excess of 11%. In both cases, the spectral activity precipitated was the sum of all the phytochrome contributed by the etiolated-oat shoots and the 11%-immunoprecipitable fraction of green-oat phytochrome. These data indicate that the lack of immunorecognition of spectral activity from green oats is a consequence neither of factors in the extract inhibiting immunoprecipitation nor of post-homogenization modifications of a phytochrome of the type extracted from etiolated-oat tissue. Thus there are two populations of spectrally de-

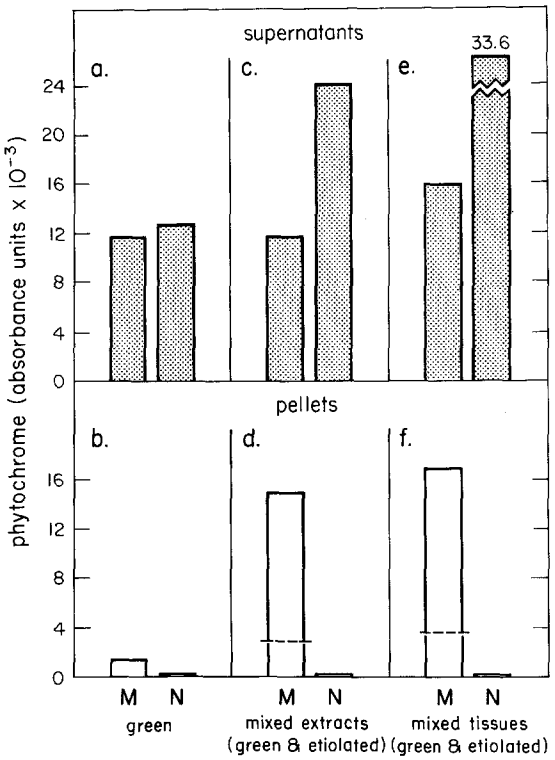

Fig. 8a–f. Immunoprecipitation of spectrally measurable phytochrome from mixed extracts and mixed-tissues extract. Three preparations: (1) green-oat extract, "Green" (**a, b**); (2) green-oat extract and etiolated-oat extract prepared in parallel and mixed in a 1:1 ratio of spectral activity, "Mixed extracts" (**c, d**); and (3) extract prepared from a mixture of etiolated and green oat shoots with a 1:1 ratio of spectral activity "Mixed-tissues extract" (**e, f**), were subjected to immunoprecipitation with either polyclonal rabbit Igs directed against 114000/118000-M_r phytochrome from etiolated oats (17 μg/l of extract) (*M*) or 17 μg/ml of nonimmune serum (*N*). Phytochrome spectral activity in the supernatants (**a, c, e**) and washed pellets (**b, d, f**) was measured using $CaCO_3$ as a scattering agent. The *dotted line* (**d, f**) indicates the amount of spectral activity that would have been immunoprecipitated (11%) if strictly proportional to that observed in the green-oat extract in **a, b**

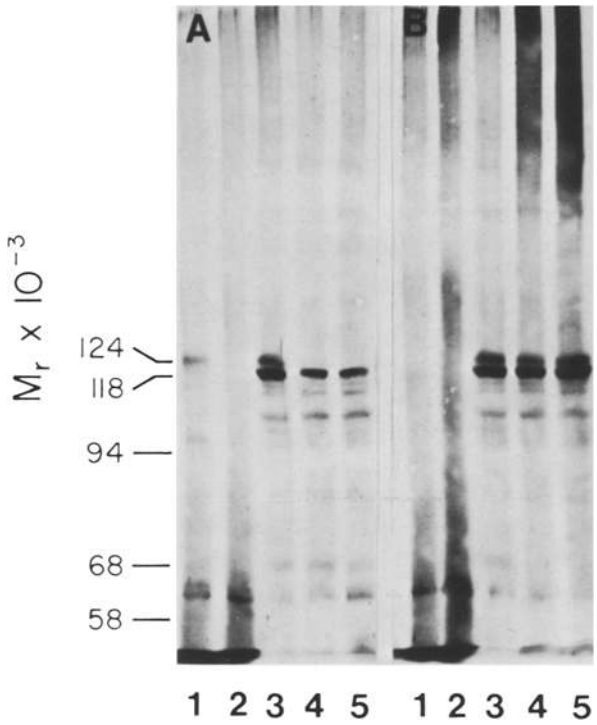

Fig. 9A, B. Immunoblot analysis of fractions obtained by immunoprecipitation from green-oat extract. Green-oat extract was subjected to immunoprecipitation with either **A** polyclonal rabbit Igs directed against 114000/118000-M_r phytochrome from etiolated oats (50 μg/ml of extract) or **B** a comparable amount of nonimmune Ig. The first supernatants were subjected to a second immunoprecipitation under identical conditions. The resultant fractions were each applied to the lanes of a 5.3% acrylamide gel and subjected to SDS-PAGE. The gel was processed for immunoblot analysis using monoclonal mouse Ig (Type 3) directed against 124000-M_r phytochrome from etiolated oats as the primary antibody. *Lane 1*, first immunoprecipitate; *lane 2*, second immunoprecipitate; *lane 3*, green-oat extract before immunoprecipitation; *lane 4*, first supernatant; and *lane 5*, second supernatant

tectable phytochrome in green oats that are distinguishable by the presence or absence of one or more epitopes recognizable in the undenatured form of the molecule.

Immunoblot analysis of immunoprecipitated fractions. To determine the relative molecular mass of the immunoprecipitable phytochrome from green oats, we subjected the various fractions obtained from immunoprecipitation to gel electrophoresis and immunoblot analysis. The blots show preferential immunoprecipitation of the 124000-M_r, green-oat species (Fig. 9A, lane 1). The supernatant from this immunoprecipitation contains exclusively the 118000-M_r species (Fig. 9A, lane 4). A second immunoprecipitate from the depleted supernatant did not recover any of the remaining spectral activity and contained no polypeptide immunospecifically recognizable on the blot (Fig. 9A, lane 2).

Thus the limited proportion of spectral activity that is immunoprecipitable from green-oat extracts under nondenaturing conditions is associated with the 124000-M_r band. These data indicate that this small population of the phytochrome of green oats is of the etiolated-tissue type. However, the apparently reduced affinity demonstrated in the antibody titration curve for the green-oat extract (Fig. 7) might mean that the 124000-M_r band from green-oat tissue is not identical to the 124000-M_r species from etiolated oats.

The remaining spectral activity from green-oat

Table 1. Comparison of phytochrome from green and etiolated oats

	Phytochrome source	
	Green	Etiolated
Concentration in plant tissue (μg/g FW)	0.3	19.0[a]
Difference spectrum maxima for Pr, Pfr (nm)	652, 729	665, 730[a]
Dark reversion (%)	20	6
Immunoprecipitation (%)	30	100
M_r	124000; 118000	124000
Reactivity with monoclonal antibodies		
Type 1	−	+
Type 2	−	+
Type 3	+	+
Peptide pattern	non-identical	

[a] From Vierstra and Quail 1983

extracts that is not immunoprecipitated appears to be associated with the 118000-M_r species of phytochrome. The immunochemical recognition of the 118000-M_r species by immunoblot analysis and the lack of recognition by immunoprecipitation are probably related to the conformation of the chromoprotein. The immunoblot procedure which involves denaturation of the protein, may expose an epitope on the 118000-M_r polypeptide that is inaccessible in the presumptively native conformation of the phytochrome during immunoprecipitation.

Conclusions

The extraction procedure described here provides preparations from chlorophyllous tissue that are suitable for investigations on the spectrophotometric and immunochemical characteristics of phytochrome from light-grown plant material. A laboratory with a standard refrigerated centrifuge and a spectrophotometer can use this procedure and obtain extracts within 4 h. The enhanced sensitivity of the immunoblot procedure described should be of general value in the detection of other low-abundance proteins in relatively crude plant extracts.

The data presented here indicate the presence of two distinct molecular species of phytochrome in green oats. One species is like etiolated-oat phytochrome in the similarity of its molecular mass and in the immunochemical recognition of this species in the undenatured state as demonstrated by immunoprecipitation. The second species, while exhibiting the diagnostic red/far-red photoreversibility that defines the photoreceptor, differs from etiolated-oat phytochrome to the extent that the relative molecular mass is lower, the peptide map is different, the nature and extent of immunochemical reactivity with anti-etiolated oat Ig is limited, and the absorbance maximum of Pr is shifted to shorter wavelength (Table 1). Various mixing experiments indicate that these differences are intrinsic to the phytochrome molecule present in green-oat cells. The presence of two species of phytochrome in green tissue and the preponderance of a species which is different in a number of respects from phytochrome from etiolated oats raises fundamental questions as to the function or functions of these two molecules in photomorphogenesis and as to the source of the heterogeneity. The heterogeneity could be a manifestation of two gene products or a post-translational modification of the 124000-M_r chromoprotein from etiolated oats (Colbert et al. 1983).

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Note added in proof: Recent data (Cordonnier et al. (1985) European Symposium Photomorphogenesis in Plants, Wageningen, The Netherlands, April 15–19, Book of Abstracts, p. 18) confirm that green-oat tissue contains a phytochrome species distinct from that in etiolated tissue, but indicate that this green-tissue-type molecule is preferentially susceptible to rapid, partial proteolysis in green oat extracts yielding the 118000-M_r species reported here. Etiolated-oat-type phytochrome appears to be comparatively resistant to this proteolysis in green-tissue extracts under the same conditions.