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PHYTOCHROME AND PHOTOMORPHOGENESIS IN EMERGING SEEDLINGS OF *ZEA MAYS* L.

by

Brian Manfred Parks

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ABSTRACT

PHYTOCHROME AND PHOTOMORPHOGENESIS IN EMERGING SEEDLINGS OF ZEA MAYS L.

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A sensitive non-destructive spectrophotometric assay was developed for the localized measurement and characterization of phytochrome at different points along an etiolated corn seedling. Phytochrome, as measured by difference spectra analysis, showed an absorption minimum and maximum at 667 nm and 732 nm, respectively, after a saturating red stimulus. The largest concentrations were measured in a 3 mm region just below the coleoptilar node of 3-6 day old etiolated seedlings.

Reciprocity was valid for phytochrome conversion just below the coleoptilar node, permitting the utilization of phytochrome conversion as an in situ quantum counter. Phytochrome could be converted by a red-light stimulus (632 nm), incident on the shoot away from the point of measurement. Using this feature, and the dose-response for phytochrome conversion, various axial (i.e. longitudinal) light gradients along the seedling were measured. A 50% reduction in transmitted fluence was seen for every 1.80,

1.60, and 1.15 mm of mesocotyl, whole shoot (coleoptile plus inner leaf), and inner leaf alone, respectively.

The axial red-light gradient for the whole shoot was increased by staining the periphery of the coleoptile with a red-light absorbing dye (methylene blue). Based on these effects of localized staining, it was concluded that axial light transmission occurs primarily along the coleoptile. This conclusion was supported by the demonstration that chlorophyll synthesis in the inner leaf did not decrease the axial red-light gradient of the whole shoot (coleoptile plus inner leaf).

Increasing the axial red-light gradient via methylene blue staining shifted the dose-response curve for light-inhibited mesocotyl elongation to higher fluence for seedlings emerging from soil under natural lighting conditions. This shift in photomorphogenic sensitivity resulted solely from the alteration of the axial red-light gradient. These effects on the photomorphogenesis of emerging corn seedlings suggest strongly that a single photoperceptive site for mesocotyl inhibition exists in a buried plant structure, and that the perceived quanta for this photomorphogenesis travel primarily along the coleoptile.

To Marcel Duchamp and the many artists who
prove consistently that our surroundings
always comprise more than what we "know"

Marcel Duchamp: Fountain, 1917

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The experiences with my KP contemporaries; Rick, Donna, Choo, Carol, Zhangling, and also Mary will provide me with many moments of interesting and joyous reflections. In particular, two crazy and caring individuals contributed greatly to the various avenues of my development; Thérèse Best and Doug "der Slug" DeGaetano. A special niche is etched for Bob Creelman, Ted John, and Jim Smith who were three groovy companions during our mutually shared times of depression, delerium, and delight. In effect, our distances of separation will be equivalent to light-years*.

Without question, my Mom and Dad share in this present accomplishment. Their continual guidance and love permitted the completion of this work so that the cover of this thesis essentially bears their names as well.

* 1 light-year = 6 trillion miles

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INTRODUCTION

The study of sensory transduction in plants centers on the desire to understand how individuals in this kingdom respond and adapt to environmental stimuli. The inability of plants to alter their local environment has led to the evolution of acutely sensitive mechanisms to sense, respond, and frequently adapt to numerous environmental parameters, including light. Since it is known that the morphology of the plant can itself affect light quality and quantity in various regions of the plant tissue, it follows that many light-regulated plant growth and developmental responses can be inherently dependent on the optical dynamics of the plant tissues.

Young seedlings of Zea mays are ideal for studying the affects of tissue optics on the light-regulated responses of plants. An abundance of information is available for the photoreceptor (phytochrome) and related photoresponses of the grasses. Additionally, the assessments of corn seedling optical qualities are facilitated by a relatively generous seedling size and simple morphology. This dissertation comprises an investigation of phytochrome, plant tissue optics, and their relation to the photomorphogenic events which are initiated at the time of emergence.

This dissertation is divided into separate sections which have been composed so that they are essentially autonomous. It begins with a review of related literature, which describes a brief history of the study of photomorphogenesis, phytochrome, and the mechanisms of phytochrome action. Chapters 1 and 2 describe the development of a sensitive spectrophotometric assay for phytochrome and the use of this assay for the analysis of axial (i.e. longitudinal) light gradients in corn. The final chapter combines light gradient analysis with photomorphogenic studies to demonstrate the importance of axial light transmission in the light-regulated inhibition of mesocotyl elongation. A conclusion following this chapter discusses the environmental importance of an axial light gradient, and also addresses a description of the latter steps of the photoresponse sequence in the mesocotyl. Two appendices examine the tissue specific nature of axial light transmission within the young corn shoot.

REVIEW OF LITERATURE

Plants depend on light as a primary source of energy for normal growth and development. Light energy for these two processess is utilized in two very distinct ways. The vast majority of absorbed light quanta drive photosynthetic electron transport, permitting the plant to incorporate radiant energy into chemical energy for later use during growth. The remaining portion of actinic quanta provide a vital informational signal, which helps affect the normal growth and development of the organism.

Two plant pigments are responsible for processing the informational aspect of light. One, the blue-light receptor, is integrally linked to plant responses such as phototropism (Dennison, 1979) and stomatal regulation (Sharkey and Raschke, 1981a,b). Presently, we know little about this photoreceptor system, largely due to the lack of a definite pigment identification. The other photoreceptor molecule is phytochrome. Although considerably more is known about this developmental pigment (e.g. pigment identity and photochemistry), the quest for the determination of its mode of action in plant development has been difficult. Phytochrome has been recognized and described for over thirty years. However, the precise nature

of its response mechanism can still not be defined with any certainty.

Classically, phytochrome-mediated responses in higher plants are separated into three photomorphogenic classes; flowering, prevention of etiolation, and seed germination. Scientific records of these photomorphogenic responses have been developed for over a century. Modern studies on light and plant development were initiated by Sachs (1865). The effects of light duration on maturation were explored first by Kjellman (1885) when he conducted summer experiments above the Arctic Circle. Klebs (1913) was the first to propose that light acts as a signal in the promotion of flowering. Early observations on etiolation were noted by Rothert (1894) and MacDougal (1903), while light sensitive seed germination was observed in 1860 by Caspary. The effects of colored light on germination were noted as early as 1883 by Ciesler.

Discovery

The events which led to the identification and eventual isolation of phytochrome comprise one of the most exciting eras of plant research. The most significant steps toward increasing an understanding of photomorphogenesis were taken with the development of action spectra analysis. Through this technique, it was possible to define the effectiveness of specific spectral regions for many known photoresponses. This allowed investigators to categorize different responses under the control of particular photoreceptor pigments.

Early examples of this type of research include studies of internode elongation (Johnston, 1937) and lettuce seed germination (Flint and McAlister, 1937). Action Spectra for these responses revealed that they were sensitive to the same red spectral region. Interestingly, Flint and McAlister (1935) also had demonstrated that far-red light could lower the germination rate of red-light-promoted seeds. However, they did not connect these two observations. In fact, they believed that the red absorbing pigment might be chlorophyll.

A few years later, flowering studies were initiated at the Plant Physiology Laboratory in Beltsville, MD to explore the nature of the photoperiodic response, which had been previously defined by Garner and Allard (1920). It was already known that short day plants, placed in an inductive photoperiod, would not flower if the required dark period was interrupted by a very brief light stimulus (Hamner and Bonner, 1938).

Using high intensity/broad area spectral emission, the Beltsville group was able to describe the action spectrum for the dark interruption phenomenon. They found that red light was responsible for this effect (Parker et al., 1945). This red light response was also noted for floral promotion in long day plants (Borthwick et al., 1948), establishing the similarity between the two floral systems. A definition of the action spectrum for stem elongation in albino seedlings (Borthwick, et al., 1951) not only helped

demonstrate the ubiquity of the red-light effect, but also suggested that the pigment was not chlorophyll and was present in very low concentrations.

The Beltsville group then focused on the simpler seed germination system. The light treatments used by Flint and McAlister on studies of lettuce seed germination had been of long-saturating fluence. A re-examination of this system showed that wavelength specific action would still occur for lower fluences (Borthwick et al., 1952b). A brief exposure to red light promoted germination while far-red light inhibited it. Having two defined wavelengths for two opposite responses, they next performed a crucial experiment. They irradiated the same seeds with a promotive (red) and inhibitory (far-red) flash sequence and found that the latter flash completely reversed the expected promotive effect of the former. They also noted that regardless of the number of red/far-red cycles, the outcome of the response always depended on the identity of the last flash. This photoreversible effect permitted them to propose a regulatory pigment, later named phytochrome, which existed in two photointerconvertible forms.

Using a similar experiment to that of lettuce, it was soon found that red-interrupted short day plants would flower if the red treatment was followed immediately by a far-red flash (Borthwick et al.,1952a). This discovery, and similar confirmations for other red-affected responses,

demonstrated that phytochrome was responsible for many red-light developmental responses.

With the knowledge that phytochrome existed in two interconvertible chromic forms, investigators reasoned that this unique property could be used to assay directly for the molecule. Butler et al. (1959) became the first group to detect phytochrome by spectrophotometrically checking for reversible light-induced absorbance changes at 660 nm and 730 nm. The development of this assay made it possible to design procedures for phytochrome isolation and purification (Siegelman and Firer, 1964). In the following years, the information on the molecule, its photochemistry, and the responses it controls, has increased at a startling pace.

The Phytochrome Molecule

Molecular Purification

Initial work by Butler et al.(1959) indicated that phytochrome existed as a chromoprotein. Early purification attempts yielded a product with a molecular weight of about 60 kilodaltons (Mumford and Jenner, 1966). However, later investigations demonstrated that this isolated form was likely a degradation product of endopeptidase activity encountered during isolation (Gardner et al., 1971; Pike et al., 1972). These studies established a refined molecular value of ca. 120 kDa. This reported value was believed valid until very recently. It has now been demonstrated that specific degradation of a 6-10 kDa region from the amino terminus of the protein occurred during isolation of the ca. 120 kDa molecule in Pr form (Vierstra and Quail, 1982a). The currently established value for the native form of oat phytochrome is ca. 124 kDa. The similarity between this value and that expected from in vitro translation products of oat phytochrome mRNA (Bolton and Quail, 1982) argued that this purified molecule was the true native form.

Various methods have been used in the purification protocols for phytochrome. In general, these approaches are based on the original methods described by Siegelman and Firer (1964), and included various lengthy degrees of fractionation and chromatography (see Pratt, 1982). The substantial time (days) required for phytochrome isolation was responsible in part for the large protein degradation

which had been encountered earlier. Approximately ten years passed between the first attempt at purification and when phytochrome was first attained in uncontaminated large molecular weight form (ca. 120 kDa) (Rice et al., 1973). The spectral similarity between large and small molecular weight phytochrome was a major reason for this delay (Pratt and Cundiff, 1975; Rice and Briggs, 1973).

Affinity purification became an alternative method for isolation, yielding a homogenous preparation in relatively rapid time. Hunt and Pratt (1979) were the first to utilize this procedure successfully by using previously developed antiphytochrome immunoglobulins as the affinity source. Although initial yields were low (ca. 15%), they have now been improved greatly. Other affinity methods which did not utilize immunological techniques have also been employed with some degree of success (Smith and Daniels, 1981). They offer the advantage of not requiring the costly and cumbersome production of antibodies.

The Protein

Although the size of oat phytochrome has been established at 124 kDa, substantial variability exists between different plant species (Vierstra et al., 1984). These reported values range from as low as 120 kDa for Cucurbita pepo to 127 kDa for Zea mays. Amino acid analysis of phytochrome protein from various species indicated that about 46% of the residues are polar with no great abundance of any one amino acid (Hunt and Pratt, 1980; Cordonnier and

Pratt, 1982). Circular dichroic studies have shown that the secondary structure probably is composed of equal amounts of alpha helix and beta pleated sheet with the remainder being aperiodic (Tobin and Briggs, 1973; Hunt and Pratt, 1981). The tertiary nature of the molecule appears to be globular (see Pratt, 1982).

Peptide mapping has provided information on the various functional regions of the protein moiety. Previous results suggested that the amino terminus of the protein affected the spectral properties of phytochrome (Vierstra and Quail, 1982b; Vierstra and Quail, 1983). It has now been confirmed that the chromophore is located in the 74 kDa portion of the protein (Jones and Quail, personal communication). Additionally, the remaining 55 kDa portion appears to be involved in the proposed dimerization of the intact molecule (Jones and Quail, personal communication).

The Chromophore

The action spectrum for night interruption of short day plants indicated a similarity between phytochrome and allophycocyanin (Parker et al.,1950). This suggested that phytochrome was related structurally to the biliproteins. Later on, careful degradation analysis of the phytochrome chromophore, bile pigments, and other structurally different tetrapyrroles unequivocally revealed that the Pr (red-absorbing phytochrome) chromophore was related structurally to the bile pigments (Rüdiger and Correll, 1969). Eventual total synthesis of racemic phytochromobilin

confirmed the chromophore structure (Weller and Gossauer, 1980).

Information on the structural relationship between the chromophore and the protein moiety was aided by the production of phytochromobilinpeptides (Fry and Mumford, 1971). Extensive studies by Grombei et al. (1975) showed that the photoisomerism between Pr and Pfr (far-red absorbing phytochrome) caused ligand changes in ring A of the tetrapyrrole. This same work showed that covalent attachment occurred between the chromophore and the protein through a thioether linkage between cysteine and the C_2 position of the side chain on ring A. It was later confirmed by Lagarias et al. (1980) that this was the sole linkage in the molecule between the protein and chromophore. Although the structure of the native Pr chromophore has been proposed, a complete definition for Pfr is still lacking (see Rüdiger and Scheer, 1983).

Molecular Function

Theoretically calculated and measured positions of absorption maxima and polarizabilities for various configurations of Pr permitted Song et al. (1979) to propose a configuration (mainly planar) for the chromophore within its protein surrounding. Linear dichroism studies in the same analysis suggested a modest conformational difference between native forms of Pr and Pfr. However, this direct comparison is difficult since a defined structure for native Pfr is not yet available.

There have been reports that Pfr is more chemically reactive *in vitro* (see Pratt, 1982). Binding differences between the two chromic forms have also been reported (see Pratt, 1982). A proposed model to explain this reactivity has suggested in part that conversion from one form to the other causes a reorientation of the chromophore with respect to the protein and subsequent exposure of a hydrophobic binding domain (Song *et al.*, 1979). However, this is still conjecture, especially since the present *in vitro* observations have not been linked unequivocally to any possible photoreceptor mechanism.

Other indications of reactive differences between Pr and Pfr have included the demonstration of *in vivo* photoreversible pelletability and sequestering of phytochrome. The rapidity of these reactions has made them very inviting as model systems of the phytochrome mechanism. Light enhanced pelletability of phytochrome is indicated by the increase in yield (5% to 65%) of phytochrome in the pellet of crude plant extracts if the plant tissue is red-irradiated immediately prior to extraction (Quail *et al.*, 1973; Pratt and Marmé, 1976). Unfortunately, the exact nature of this pelletable effect is still not certain. Phytochrome sequestering was discovered by using immunocytological techniques (Mackenzie *et al.*, 1978). It was shown that phytochrome appeared to be distributed diffusely within the cytosol while in the Pr form. The conversion of phytochrome to the Pfr form by red

irradiation caused the localization (i.e. sequestering) of phytochrome in discrete areas within the cell. Although both pelletability and sequestering are interesting observations, it must be noted that neither has been linked to any known photomorphogenic responses.

It is very probable that the protein portion of phytochrome is integrally involved in the photoreceptor mechanism. It has been demonstrated that minute phytochrome protein degradation yields a molecule with altered spectral characteristics from those normally found in vivo (Vierstra and Quail, 1982b; Vierstra and Quail, 1983). The necessity of the protein for proper photoreceptor function was further indicated by the observation that this degradation altered the in vitro reactivity of the phytochrome molecule (Hahn et al., 1984).

Very recent findings have suggested that there are two different types of phytochrome in plants, "green" and "etiolated" (Shimazaki et al., 1983; Tokuhisa et al., 1985). Immunological evidence from both groups indicates that phytochrome from light-grown plants may be structurally distinct from phytochrome isolated from etiolated tissues. These findings expose the uncomfortable yet interesting possibility that the phytochrome molecule may also possess different action mechanisms as a result of differences in its molecular form.

Response Studies

Background

Phytochrome responses are divided into two primary categories (see Mohr, 1972). The first type is termed an induction-reversion reaction, and is characterized typically by photoreversibility at low irradiance and agreement with the Bunsen-Roscoe law of reciprocity. Examples of this type of reaction are seen in seed germination and some light-induced changes in elongation. The second class of response is termed the high irradiance reaction (HIR) and is characterized by a lack of complete photoreversibility, a high light irradiance requirement, and lack of reciprocity. Two common examples of this type include hypocotyl elongation and anthocyanin formation. It must be noted that the same response can often demonstrate induction-reversion or HIR characteristics depending on the experimental protocol (e.g. Mohr, 1957). This suggests a degree of similarity exists between the two types. However, they appear to be distinct substantially and thus are discussed separately.

Induction-Reversion Responses

During early analyses of phytochrome responses, Borthwick et al. (1954) demonstrated that the light reactions in both photochemical directions were temperature independent. This implied that a single photoreceptive molecule was controlling the observed responses. The demonstration that both photoreactions followed first

order kinetics argued conclusively that only one photoreceptor contributed to these reversible responses (Hendricks et al., 1956).

This same group also demonstrated that far-red reversion of a response could be lost if an interposed dark period between the red/far-red cycle were too long (Borthwick et al., 1954). This phenomenon, termed "escape time", has been found for virtually all photoreversible responses and varies considerably from seconds (Leung and Bewley, 1981) to hours (King et al., 1982). The phenomenon demonstrated the vast time range over which phytochrome can react. Traditionally, responses with smaller escape times have been more inviting for research, since they likely possess a minimum number of steps between excitation and coupling with the transduction chain.

The nature of the phytochrome molecular mechanism was first suggested by Haupt in connection with his studies on the filamentous alga, Mougeoutia. It had been shown previously that the single planar chloroplast in each cell of this organism oriented itself with respect to dim light (Oltmanns, 1922). Haupt proved that this was a reversible phytochrome-mediated reaction. Through a series of elegant experiments with plane polarized microbeams, Haupt (1960) concluded that the photoreceptor was associated integrally with and precisely oriented on the cell surface. The Pr and Pfr forms had opposite absorption vectors, indicating a spatial shift in the chromophore upon quantum

absorption by either form. He also demonstrated that the response was strictly localized at the point of irradiation. His studies proposed that phytochrome actions were related integrally to cellular membranes, although how still is not known.

The relationship between phytochrome and membrane phenomena was enhanced by studies of light-regulated leaf movements. This type of phytochrome response was first noted in Mimosa pudica (Fondeville, 1966), which was the most rapid phytochrome reaction known at that time (ca. 5 min). Based on the rapidity and nature of this response, the authors suggested that phytochrome was directly involved with membrane phenomena. Similar phytochrome responses have also been noted and described biophysically for the pulvini of Albizzia julibrissin and Samanea samen (see Satter and Galston, 1981). Red-light-mediated leaf movements in these species were shown to result from turgor changes in the pulvini which resulted from K^+ movement. These electrical changes occurred in seconds after irradiation, which suggested that phytochrome affected the protonmotive process directly. Observations by Tanada (1968) on the light-regulated affinity of barley or mung bean root tips to charged glass surfaces has also argued that phytochrome affects membrane charge. But despite these examples, it has still been argued logically that unequivocal proof of a membrane interaction mechanism is lacking (see Quail, 1983).

It has also been suggested that phytochrome might affect responses by acting directly on the genomic structure (Mohr, 1966). There have been many reports of phytochrome-induced changes in the levels of translatable mRNAs (eg. Silverthorne and Tobin, 1984). It has also been shown that activated phytochrome can affect its own translatable mRNA by affecting message abundance (Colbert et al., 1983; Quail, personal communication). However, effects on expression are not conclusive proof that phytochrome can interact directly with the genetic material.

High Irradiance Responses

The high irradiance reactions are very complex and not well understood. The importance of the HIR is evident, however, since it is believed generally that these responses represent the action of phytochrome under daily growing conditions. Thus, it is likely that plant development can not be explained without an understanding of the HIR transduction sequences.

The HIR was first suggested by Mohr (1957) to distinguish it from inductive phenomena. He noted that red/far-red control of cotyledon expansion in Sinapis alba lost photoreversibility given a long far-red stimulus. Moreover, this far-red treatment would promote the response over that found with brief red light alone. An action spectrum for this response showed that response maxima occurred in both the far-red and blue regions of the spectrum (Mohr, 1964). The occurrence of these maxima were

troublesome since neither occurred in regions of maximum Pr absorption. Thus it was possible that this type of response did not operate through the action of phytochrome.

However, direct evidence was obtained eventually for the involvement of phytochrome in the HIR by Hartmann (1966) during his studies on lettuce hypocotyl elongation. It had been suggested that the far-red response was the result of an optimal photostationary state between Pr and Pfr (see Schäfer, 1976). Absorption spectra for the chromic forms indicated an equilibrium of 0.03% Pfr/Ptot at the far-red response optimum. Using simultaneous irradiation at two separate non-active wavelengths, Hartmann was able to mimic the predicted photostationary state and the response which would normally only occur with narrow band far-red light. This agreement between predicted and experimental results argued for direct involvement of phytochrome in the HIR. Although other observations were not in complete agreement with Hartmann's findings (Fondeville et al., 1967; Borthwick et al., 1969), the involvement of phytochrome in the HIR is still well accepted.

The development of more precise action spectra (e.g. Hartmann, 1967) for high irradiance responses has demonstrated well defined action maxima in the blue spectral region. This actinic region does not correspond to phytochrome absorption. Therefore, it has been proposed generally that another blue absorbing photoreceptor might be operating with phytochrome in the HIR (eg. Drumm and

Mohr, 1978; Thomas and Dickinson, 1979; Holmes and Schäfer, 1981; and Gabrys et al., 1984). However, There is still no conclusive proof for the second receptor model of the HIR.

CHAPTER 1

The *In Situ* Measurement of Phytochrome within a Single Undamaged Corn Seedling

Introduction

Phytochrome is one of the major sensory pigments in plants regulating growth and development. It is a 124 kilodalton chromoprotein which is uniquely photoreversible between two chromic forms, Pr (red absorbing phytochrome) and Pfr (far-red absorbing phytochrome) (Vierstra and Quail, 1982a,b). This photoreversible nature has been linked to a number of physiological responses (see Shropshire and Mohr, 1983) and was the primary characteristic which permitted its initial *in vivo* spectral detection (Butler *et al.*, 1959) and eventual purification (Siegelman and Firer, 1964).

The majority of the spectrophotometric characterization of phytochrome has been *in vitro* and *in vivo* using excised, pooled tissue samples. These analyses have provided valuable information on phytochrome localization (eg. Briggs and Siegelman, 1965) and spectral characteristics (eg. Vierstra and Quail, 1982b; Vierstra and Quail, 1983). However, little has been offered on the spectral quantity or quality of phytochrome in the diverse optical micro-environments of an individual seedling. Additionally, pooled assays can not assess pigment variablility among

individuals in a given species population, which compounds any simple analysis of phytochrome genetics.

There have been a few notable exceptions to the more common *in vitro* and *in vivo* phytochrome assays. Kondo *et al.* (1973) measured *in situ* photoreversible absorbance changes at the Pr and Pfr absorption maxima along a single intact oat shoot. Although these results provided one of the first *in situ* measurements of phytochrome, the inability to report difference spectra prevented any analysis of the *in situ* phytochrome spectral profile. Conversely, an *in situ* phytochrome difference spectrum for whole tomato fruit has been reported (Jen *et al.* 1977). But the lack of appreciable spectral resolution prevented an adequate appraisal of *in situ* spectral definition of phytochrome.

This present work describes an accurate technique for the non-destructive assessment of phytochrome within a single seedling. Its development permitted a highly resolved *in situ* global assessment of phytochrome in the corn plant. This analysis reports on the spectral characterization, localization, photochemical dose requirement, and age dependency of phytochrome content within individual undamaged seedlings.

Materials and Methods

Plant material. Hybrid corn (WF9 x Bear 38, custom Farm Seed Res., Decatur, IL) was used in all experiments. Seeds were soaked overnight in distilled water, germinated and grown in darkness at 23.5 $\pm$ 0.5°C in plastic trays

(ca. 40 seeds/tray) on Kimpak (Kimberly-Clarke, Neenah, WI) soaked with distilled water. Except where specifically noted, only three to four day old etiolated seedlings (6 cm ± 1 cm seed to shoot tip; primary leaf fully enclosed within the coleoptile) were selected and prepared for spectrophotometric analysis.

Spectrophotometry. To obtain in situ spectra, a plant was anchored so that a given position along the shoot was situated beneath a 1-2 mm diameter hole in a 2 mm thick aluminum plate (Fig. 1A). Much of the shoot was inserted through a metal sleeve which helped to anchor the seedling. The plate and plant were placed into a sample compartment so that the measuring or actinic beams (see Light sources) passed through the hole, through the desired shoot segment and onto the cathode (5 cm dia.) of the photomultiplier tube, which was located 2 cm below the sample. The measuring and actinic beams were focused by means of a lens (4 cm focal length) mounted 4 cm above the sample (Fig. 1A). All absorption spectra were measured with a computerized single beam spectrophotometer consisting of a light source, the monochromator from a Cary 14 spectrophotometer, an EMI 9659 QA photomultiplier tube, and a log amplifier (Davis et al., 1973). Absorbance readings were recorded as the average of 1024 separate readings over each successive 0.2 nm wavelength interval. These readings were stored in a Hewlett Packard 2108mx minicomputer programmed so that spectra could be manipulated to obtain

Figure 1. Schematic diagram of sample holder compartment. (A) Side view. The actinic lamp was situated directly above the sample so that the light passed through the desired interference filter, a half-silvered mirror, a focusing lens, a small hole, and then was incident on the spectrophotometrically measured area. The measuring beam from the monochromator reflected off the half-silvered mirror and then followed an identical path to the PM tube. (B) Side view of sample holder adapted for spectral measurements at cold temperature. All dimensions and light paths are identical to those in (A). A copper tube containing circulated cold methanol was brazed to top of the brass plate. The foam cover served as a thermal insulator. The sleeves surrounding the shoot served partially to anchor the seedling. Plant roots were wrapped in moist tissue paper (not shown) to maintain plant turgidity during the experiments. The plant was held in place with black vinyl tape (not shown).

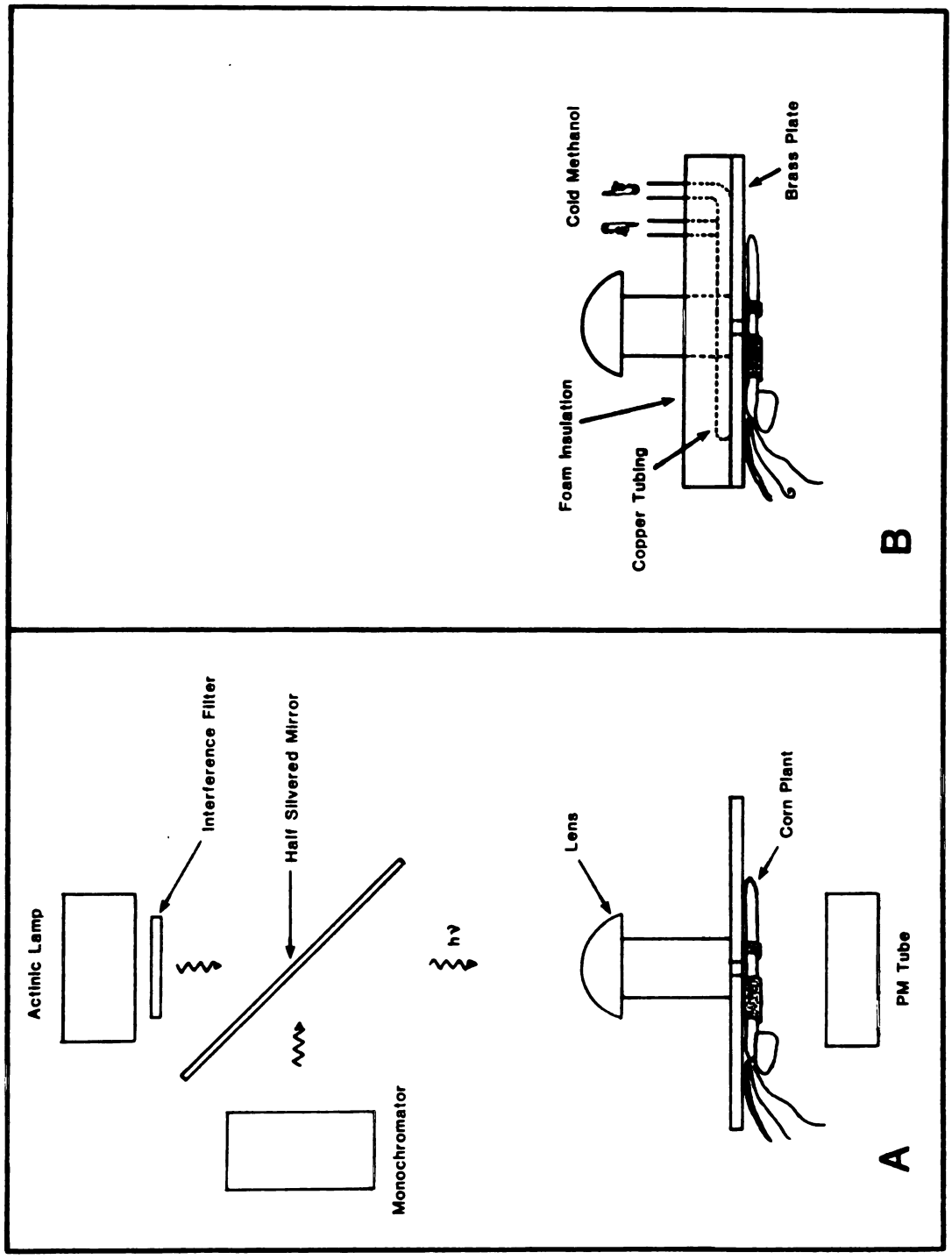
Actinic Lamp
Interference Filter
Half Silvered Mirror
Monochromator
hν
Lens
Corn Plant
PM Tube
A
Cold Methanol
Brass Plate
Foam Insulation
Copper Tubing
B

Figure 1

difference spectra and directly recorded on paper by the computer with a Hewlett Packard x-y recorder. Wavelength was calibrated with a Hg lamp and is accurate to less than 0.1 nm. The measurement of an individual absorbance spectrum required 80 s. The entire analysis (consisting of many absorbance spectra) of one area of corn shoot required a minimum of 12-15 min. Although the seedlings were very similar with respect to morphology and age, the total amount of photoconvertible pigment measured in the same region could vary in different plants by as much as two-fold. To compensate for this natural variation, the data are sometimes expressed as a percentage of the maximal photoconversion for that particular tissue sample or arithmetically

$$(NS/S) \times 100 = \% \text{ Conversion}$$

where NS equals the measured absorbance change after a non-saturating fluence and S equals the total absorbance change after a subsequent saturating fluence. In particular, data for percent conversion of phytochrome bear no reference to its photostationary state, which should be constant at *ca*. 80% Pfr after a saturating fluence (Pratt and Briggs, 1966).

Cold temperature spectra. To obtain *in situ* absorbance measurements below room temperature, a temperature controlled brass sample holder was substituted for the

normally used aluminum holder (Fig. 1B). Methyl alcohol was cooled within a Neslab refrigerated bath (model RTE-8; Portsmouth, NH) and circulated through copper tubing, which had been brazed to the upper surface of the brass plate. Temperatures reported for the spectral measurements correspond to the lower surface temperature of the brass sample holder and were measured with a YSI remote thermal probe (model 42SC; Yellow Springs, OH). Adequate thermal contact between the probe and the sample holder surface was obtained by coating the probe with silicone grease. A seedling was held in direct contact with the sample holder at all times during the spectrophotometric analysis. Seedling viability was verified after the spectral measurements at low temperature by transferring the young plant to an area supplied with white fluorescent lighting and maintained at room temperature (ca. 24°C). Seedlings were maintained under these growing conditions for a minimum of two days. All seedlings always remained viable at all temperatures.

Light sources. The actinic light source used to irradiate the spectrophotometrically measured area was provided by a Unitron incandescent lamp fitted with either of three interference filters [red: PTR Optics 628 nm, 10.3 nm 1/2 band, 25.5 W/m^2, and 669 nm, 9.8 nm 1/2 band, 21.7 W/m^2, or far red: PTR Optics 731 nm, 10.4 nm 1/2 band 41.0 W/m^2; beam diameter = 5 mm; beam incident upon a 1.2 mm diameter hole (Fig. 1A)].

All light intensities were measured with a Kettering radiometer (model 68; Laboratory Data Control, Riviera Beach, FL). Reported values have been corrected for radiant flux density based on the area of the plant tissue irradiated (0.011 cm^2 for actinic irradiation). All spectrophotometric measurements were performed in darkness with the aid of a green safelight.

Results and Discussion

Pigment distribution. Difference spectra were derived from absorbance measurements recorded before and after exposure to actinic red and far-red light given at specified points along an intact seedling shoot. The analysis of difference spectra showed that, below the coleoptilar node, red light induces an increase in absorption at 732 nm and a concomittant decrease in absorption at 667 nm (Fig. 2 - left column). Far-red light generally reversed these red-induced absorbance changes (Fig. 2 - right column). These reversible light-induced absorbance changes are similar to those expected for phytochrome (Butler et al., 1959). This in situ analysis demonstrated that phytochrome was present in its largest amounts just below the node in the region of elongation and high meristematic activity (Sharman, 1942; Duke and Wickliff, 1969), and is in agreement with data on sectioned and pooled samples (Briggs and Siegelman, 1965).

An identical analysis above the coleoptilar node revealed light-induced absorbance changes which were

Figure 2. Difference spectra as a function of position along a corn shoot. The shoot was spectrophotometrically analyzed along its axis at positions indicated by letters a-i. At each position, an absorption spectrum was measured before actinic irradiation (dark), after a 2 min red irradiation, and following a 2 min far-red irradiation. For each position, red-minus-dark difference spectra are presented in the left column, and far-red-minus-red difference spectra are presented in the right column. The spectra for (h) were measured across the whole shoot. The spectra for (i) were measured across the coleoptile alone. Positions are approximately: (a) 27 mm below the node; (b) 14 mm below; (c) 8 mm below; (d) 3 mm below; (e) at the node; (f) 2 mm above the node; (g) 11 mm above; (h,i) 17 mm above. $\Delta A = 0.01$ for a,b,c,d,e,and i. $\Delta A = 0.04$ for f,g,and h.

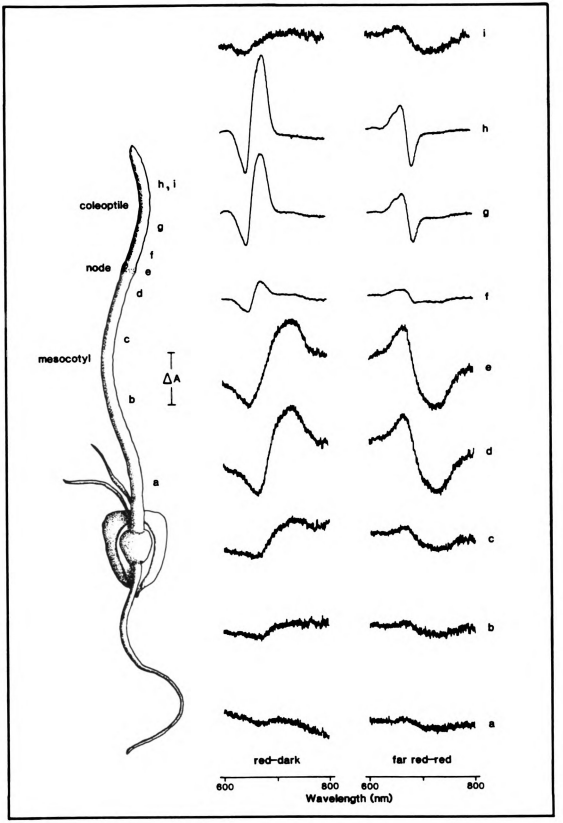
coleoptile
node
mesocotyl
h, i
g
f
e
d
c
b
a
ΔA
i
h
g
f
e
d
c
b
a
red-dark
far red-red
600
800
600
800
Wavelength (nm)

Figure 2

considerably more complex. In general, red light induced an increase in absorption at 680 nm and a decrease at 650 nm, while far-red light promoted an increse mainly at 666 nm and a decrease at 686 nm (Fig. 2). The profile of absorbance changes above the coleoptilar node were similar to those expected for the protochlorophyllide to chlorophyll phototransformation (650 nm to 680 nm) and a subsequent Shibata shift (686 nm to 666 nm) (Shibata, 1957). These absorption changes were greater for points further away from the coleoptilar node, and showed that the largest concentrations of photosynthetically related pigments were located in the more distal and mature sections of leaf tissue. The magnitude of these absorbance changes prevented any measurements of phytochrome in leaf tissue at room temperature (ca. 24°C). However, small amounts of phytochrome were measured in the coleoptile where photosynthetic pigments are reduced greatly (Fig. 2i).

Spectral characterization. A detailed examination of the absorbance changes measured ca. 3 mm below the node unequivocally defined the identity of the photoconvertible pigment and the degree of spectral purity (Fig. 3). No absorbance changes were induced by the measuring beam of the spectrophotometer (Fig. 3a). A far-red-minus-red difference spectrum (Fig. 3e) was essentially the reverse of the red-minus-dark difference spectrum (Fig. 3b) and a red #3-minus-far-red difference spectrum (Fig. 3f) which indicated that phytochrome is the only light-inducible

Figure 3. Light-induced absorbance changes measured across the mesocotyl 3 mm below the coleoptilar node in a single seedling. Absorption spectra were measured sequentially as follows: two spectra were measured before direct actinic irradiations (dark 1 and dark 2), and further spectra were measured after a 2 min actinic red irradiation (red 1), after 5 min in darkness (dark 3), after 2 min additional red actinic irradiation (red 2), after 2 min actinic far-red irradiation (far-red 1), and after 2 min additional red irradiation (red 3). Difference spectra presented are: (a) dark 2-minus-dark 1; (b) red 1-minus-dark 1; (c) dark 3-minus-red 1; (d) red 2-minus-dark 3; (e) far-red 1-minusred 1; (f) red 3-minus-far-red 1; (g) far-red 1-minus-dark 1. Fluence = 18 nE for 2 min of red at 628 nm; fluence = 33 nE for 2 min of actinic far-red at 731 nm.

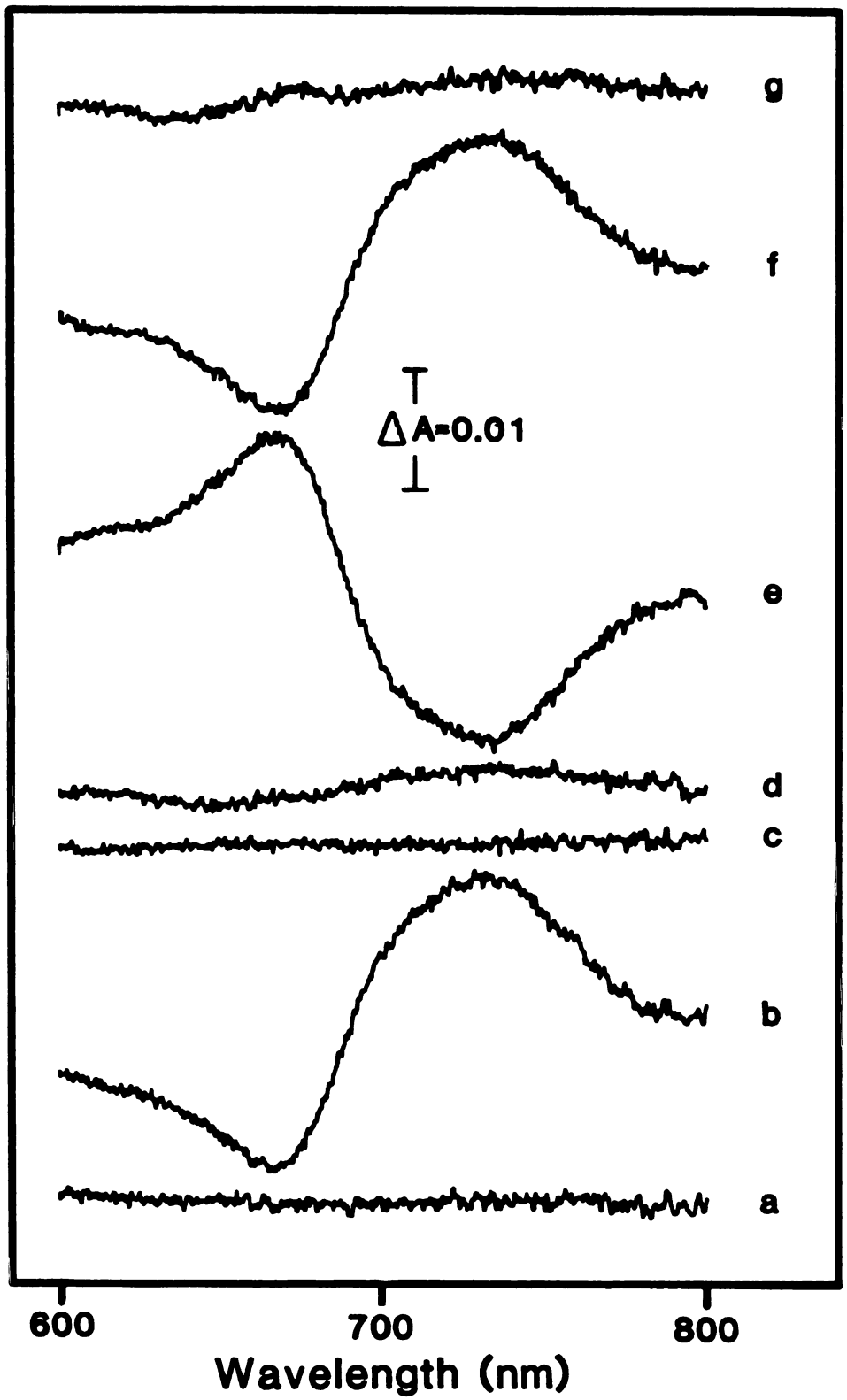

Figure 3

pigment in this general region and that it is completely photoreversible. The initial two minute red actinic irradiation resulted in a stable conversion of Pr to Pfr (Fig. 3b,c) with little further conversion by an additional two minute irradiation (Fig. 3d). Finally, the photochemical purity of this region with respect to red and far-red irradiations was established by the fact that no apparent nonreversible spectral changes were induced as a result of these acitinic treatments (Fig. 3g).

The measured wavelength maximum and minimum for phytochrome (Pr-Pfr) in situ are approximately 667 nm and 732 nm, respectively (Fig. 4). These values are similar to other reports using a purified sample (Vierstra and Quail, 1983). It is known that changes which occur in a pigment's microenvironment can affect alterations in the pigment's absorption profile (Song and Moore, 1974). However, these results suggest that the complex optical nature of the seedling does not alter severely the spectral qualities of phytochrome.

The percentage of phytochrome conversion was measured as a function of actinic exposure time. This type of analysis verified that a two-minute actinic exposure at the given fluence rate was saturating for pigment conversion (Fig. 5A). Although these data also demonstrated an exponential relationship (Fig. 5B), it must be noted that the percentage conversion measured after any given irradiance is the result of opposing Pr and Pfr

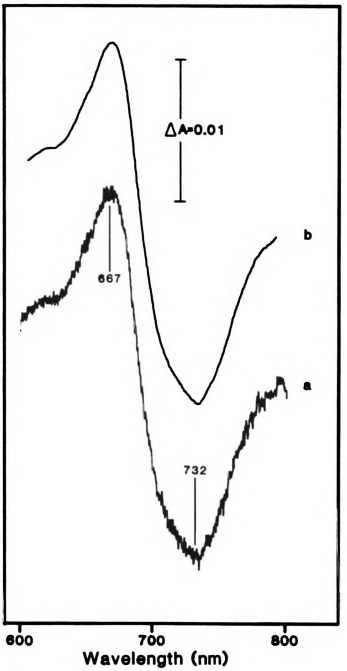

Figure 4. Phytochrome difference spectrum before and after incorporation of a mathematical smoothing function. (a) Pr - Pfr difference spectrum from a point 3 mm below the coleoptilar node (taken from figure 3). Wavelength maxima and minima were determined by inspection and are presented to the nearest 1 nm. (b) The same difference spectrum following employment of an 8 nm equal weight smoothing function such that each resulting absorbance value becomes the average of the unsmoothed values 4 nm to each side of that wavelength.

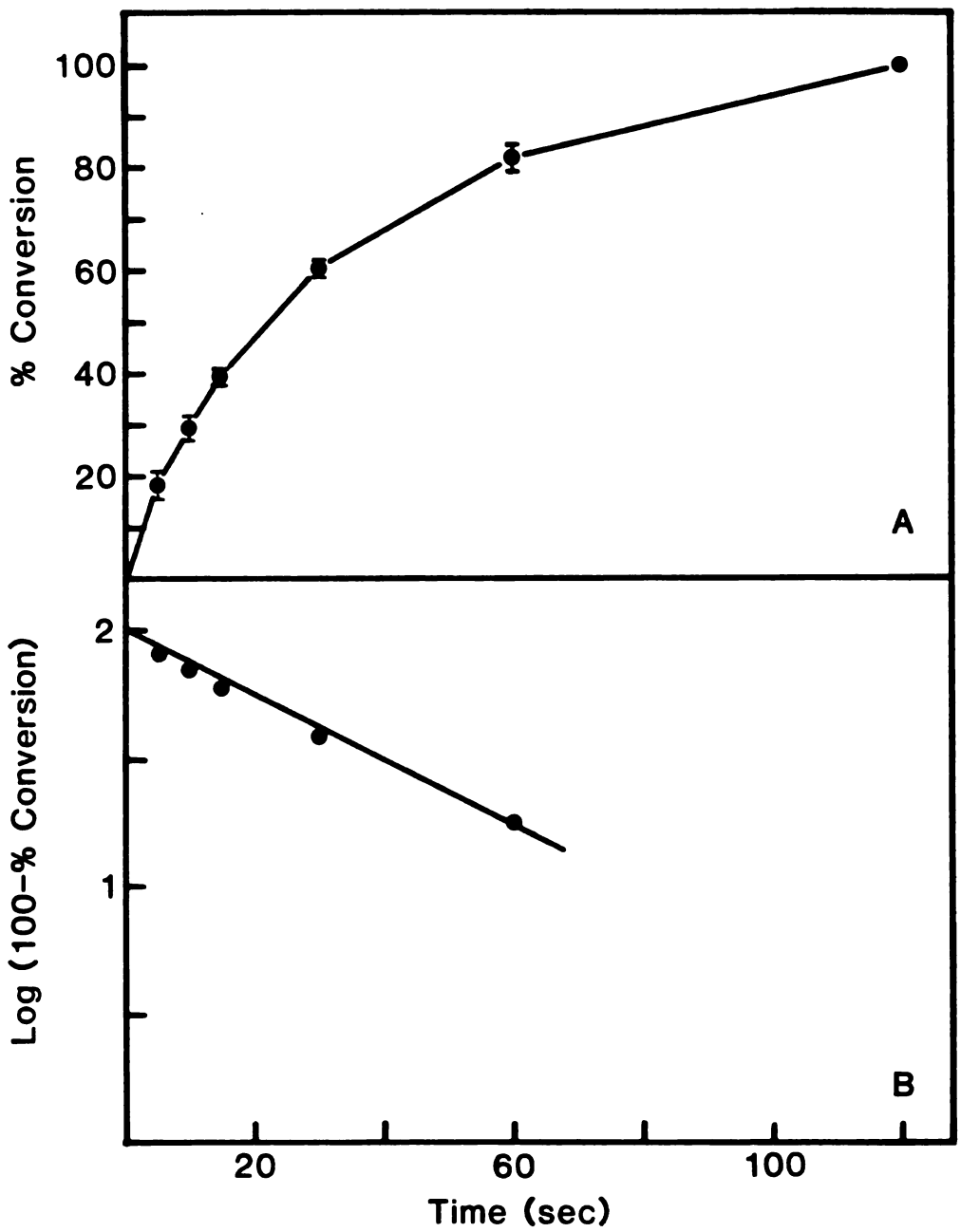

Figure 5. Light saturation for phytochrome conversion. (A) All of the points are averages of phytochrome conversion in three seedlings. For each seedling, the amount of phytochrome converted 3 mm below the coleoptilar node was calculated following a red irradiation at 25.5 W/m^2. Phytochrome was measured following successive irradiations at this fluence rate for various times for a total of 2 min of irradiation. The amount of phytochrome is expressed at each point as a percentage of the maximum difference ($\Delta A_{732nm} - \Delta A_{667nm}$) obtained during the entire 2 min of irradiation. The veritcal bars represent $\pm$ one standard error. (B) Logarithmic representation of the data from (A).

photoconversions. Therefore, the saturation of percentage conversion at long irradiance time is actually the point at which photoequilibrium is established at ca. 80% Pfr (Pratt and Briggs, 1966).

The total convertible phytochrome located below the coleoptilar node changed as a function of seedling age (Fig. 6). For the growth conditions used, 3-6 day old corn seedlings possessed the maximum amount of phytochrome. After the sixth day, the total amount of convertible phytochrome present dropped rapidly. Plant flats were rewatered lightly after the sixth day to examine whether the decrease in phytochrome content was a result of water loss. However, the values did not increase, and thus suggested that the decrease in phytochrome concentration was a function of aging alone (Fig. 6 - dotted line).

Phytochrome in leaf tissue. Since the spectral interference of photosynthetic pigments prevented an accurate appraisal of the amount of phytochrome within leaf tissue, it was necessary to measure phytochrome in this tissue at low temperature. The photochemical nature of protochlorophyllide conversion induced by red light is temperature independent (Fig. 2f,g,h - left column). However, the shift in light absorption after chlorophyll formation (Shibata shift) corresponds to the incorporation of chlorophyll into the lamella structures (see Ogawa et al., 1973). This change in the chlorophyll microenvironment affects a change in its absorption profile. Since the

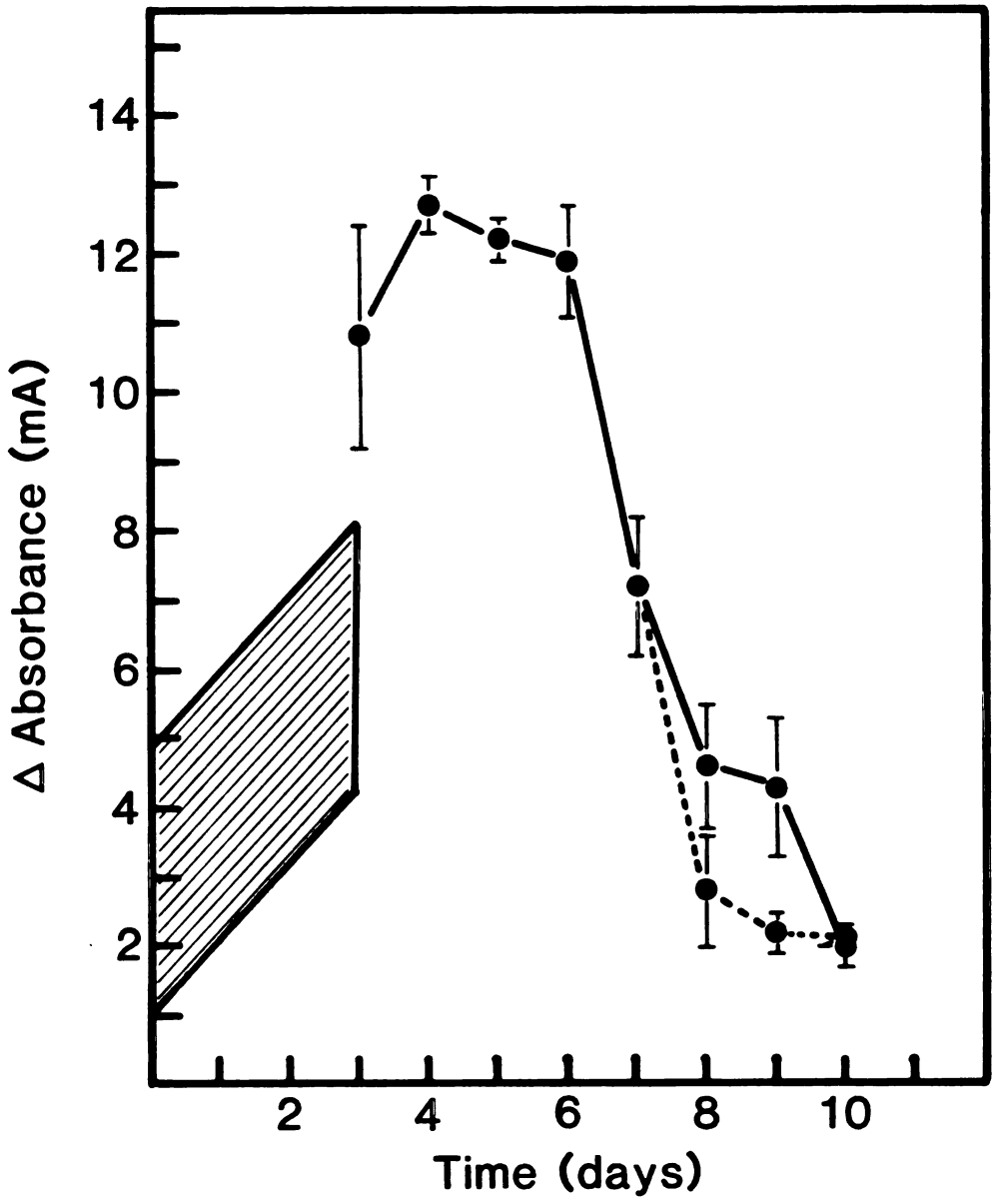

Figure 6. Phytochrome content as a function of seedling age. Phytochrome was measured 3 mm below the coleoptilar node after a 2 min red irradiation at 669 nm (16 nE). Data are expressed as the change in absorbance between 667 nm and 732 nm resulting from actinic red irradiation. Each plotted point represents three individual measurements. Age is measured from the time of initial planting. The dotted line represents seedlings which had been rewatered after the sixth day of growth and then subsequently measured on the 8, 9, and 10th days. The shaded area denotes the time when seedlings were too small to be measured. Vertical bars represent $\pm$ 1 standard error.

nature of this shift in absorbance is not photochemical, it can be retarded by lowering the sample temperature.

The measurement of far-red-induced phytochrome conversion (Pr-Pfr) could not be resolved at low temperature until the majority of protochlorophyllide had been converted to chlorophyll with red light (data not shown). Even relatively small amounts of protochlorophyllide hampered the measurement of the far less abundant phytochrome. In order to avoid this problem, the saturating red light dose for the photoconversion of protochlorophyllide at low temperature was determined. A measurement of the percent change in absorption (ΔA_{680nm} - ΔA_{650nm}) vs. actinic dose demonstrated that eight minutes of red light at the specified fluence rate depleted most of the protochlorophyllide (Fig. 7).

Having reduced the protochlorophyll pool with a long exposure to red light, phytochrome now could be assayed at low temperature. At successively lower seedling temperatures, thermally dependent absorption changes became progressively less pronounced (Fig. 8). When the seedling temperature was adjusted to -2°C, the far-red induced absorbance change exactly represented a Pfr to Pr phototransformation (Butler et al., 1959).

Using cold temperature analysis, phytochrome was assayed spectrophotometrically at two points along the leaf tissue of four day old seedlings. The analysis indicated that phytochrome is more abundant in the region most proximal to the coleoptilar node (3.5 $\pm$ 0.3 mA @ 2 mm above

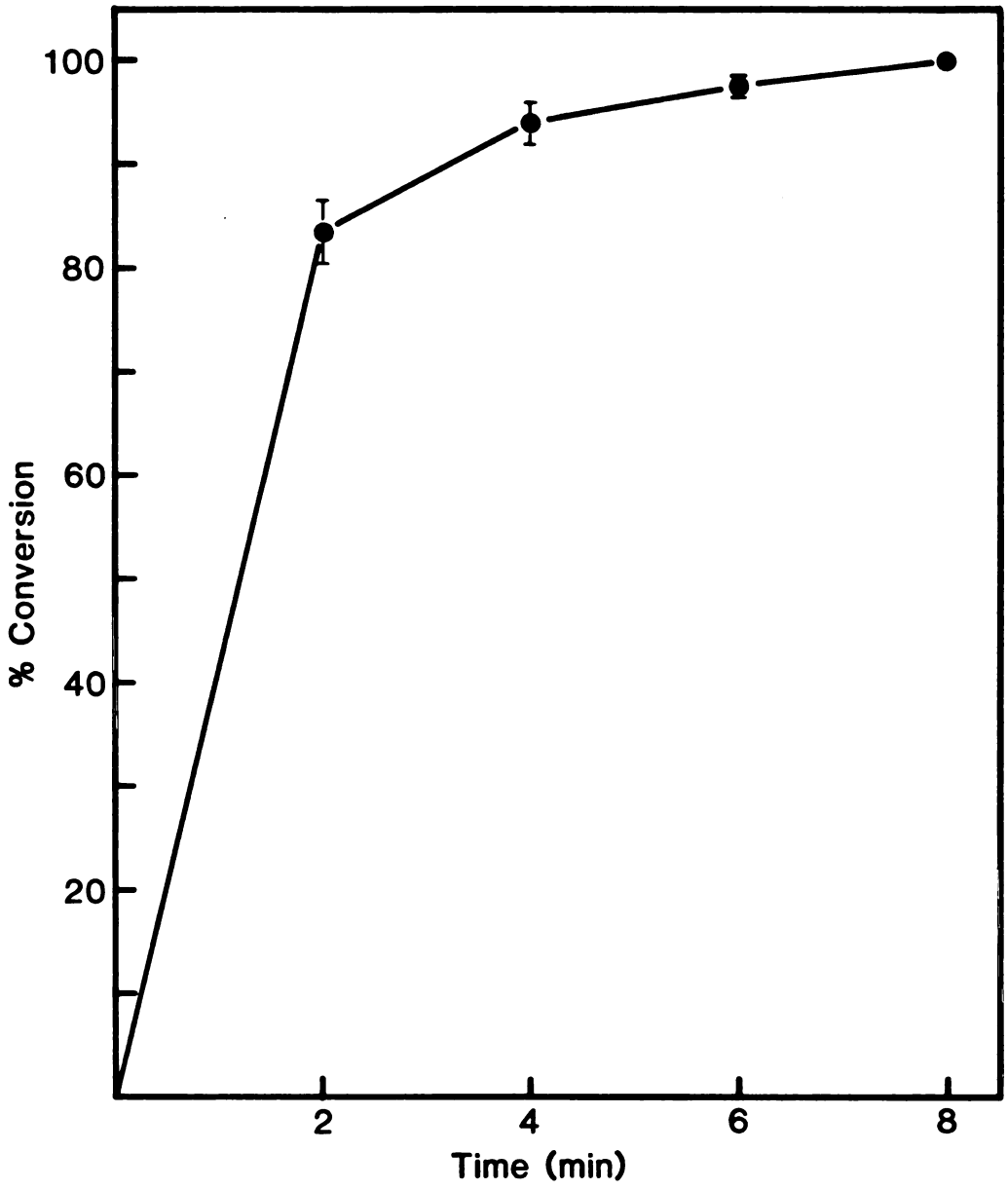

Figure 7. Red-light-induced chlorophyll formation at -2°C. The coleoptile was removed prior to all measurements. Samples were measured spectrophotometrically 6 mm above the coleoptilar node in a sequential fashion. An initial dark absorption spectrum was measured followed by an additional absorption spectrum measured after a 2 min red light irradiation. This sequence was repeated until 8 min of accumulated red exposure was attained. The change in absorbance ($\Delta A_{680nm} - \Delta A_{650nm}$) from the initial dark spectrum was calcualted after each successive red exposure. The data are plotted as the percentage of the total change in absorption after 8 min of total irradiation. Three separate seedlings were analyzed. Dose = 18 nE for 2 min red at 628 nm. Vertical bars represent $\pm$ 1 standard deviation.

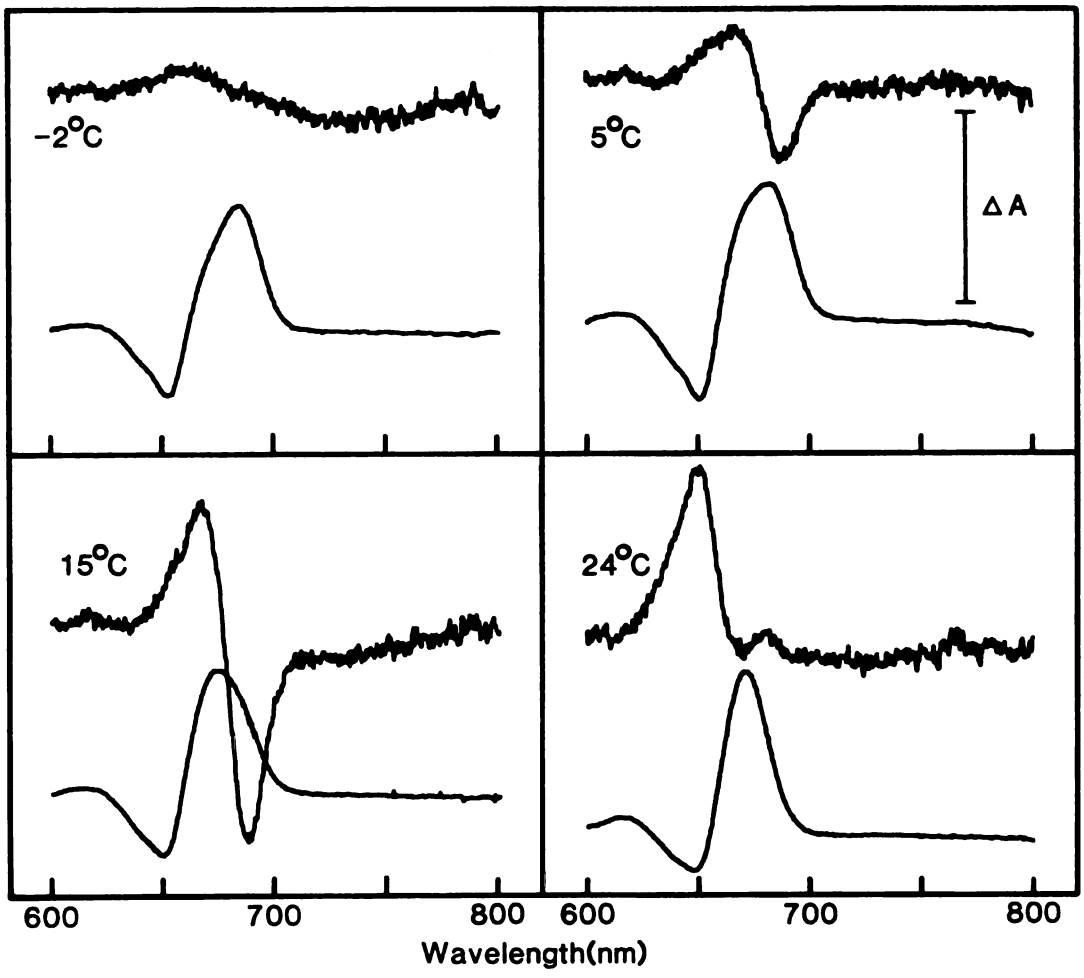

Figure 8. Light-induced absorbance changes measured 6 mm above the coleoptilar node at different seedling temperatures. The coleoptile was removed prior to all measurements. Absorption spectra were measured sequentially as follows: an initial dark absorption spectrum (a) was measured, followed by an additional absorption spectrum (b) measured after an 8 min red irradiation (72 nE at 628 nm), and a final absorption spectrum (c) measured after a 2 min far-red irradiation (33 nE at 731 nm). Difference spectra presented in each section of the figure are: b-minus-a for the lower spectrum; c-minus-b for the upper spectrum. $\Delta A = 0.1$ for b-a. $\Delta A = 12.5 \times 10^{-3}$ for c-b.

the node *vs*. 2.5 ± 0.1 mA @ 6 mm above the node). However, this difference was minor in comparison to the change seen along a similar distance of the mesocotyl (Fig. 2c,d), and is in agreement with previous reports using immunological techniques (Pratt and Coleman, 1974). Since the cross sectional width for both points above the node were similar (*ca*. 1 mm), the quantitative values at these two points are directly comparable. Conversely, mesocotyl tissue (*ca*. 1.6 mm dia.) was approximately 40% wider than leaf tissue. Accounting for this difference, leaf tissue contains about one third more measurable phytochrome than in the most abundant region of the mesocotyl.

In conclusion, the measurements provided here substantiate the assessments of phytochrome made previously with destructive methods in monocotyledonous seedlings (Briggs and Siegelman, 1965; Kondo *et al*., 1973). The present study has established the identity, location, and dose requirement for maximal absorbance changes attributable to phytochrome.

In addition, it appears that the percentage conversion of phytochrome is strictly dependent on the applied actinic dose, so that phytochrome acts essentially as an *in situ* quantum counter. This feature of phytochrome will be utilized in the subsequent chapters to investigate the internal light gradients of individual seedling structures, and the relationship of these gradients to the development of an emerging corn seedling.

CHAPTER 2

Phytochrome Conversion as an *In Situ* Assay for Effective Light Gradients in Etiolated Seedlings of *Zea mays* L.

Introduction

Growth and development in plants is often directed by the manner and amount of light passage through the tissue structures. Thus, many light-mediated responses are largely dependent on the optical qualities of the plant tissues in question. A common example of this is phototropism. It can be described as a spatially differential growth response resulting from a varied light stimulus developed across the shoot tissue (Dennison, 1979; Poff, 1983). With respect to photomorphogenesis, an axial conduction of light along shoot tissue has been proposed recently to explain how light-mediated growth inhibition might be effected beneath the soil surface (Mandoli and Briggs, 1982b). From these two reponses alone, it is apparent that an accurate appraisal of internal light gradients with respect to quantity and quality is important for understanding how a plant reponds to environmental light stimuli.

There have been many attempts to quantitate internal light gradients in plant tissues (Mandoli and Briggs, 1982a; Seyfried and Fukshansky, 1983; Seyfried and Schäfer, 1983; Vogelmann and Björn, 1984; Parsons *et al.*, 1984). Those

measurements were accomplished by directly probing within the tissue using radiometric techniques. However, because of the intrusive nature of those methods, the accuracy of the resulting measurements was unknown since the degree of optical variation caused by tissue disruption could not be determined unequivocally. Additionally, those methods could not assess the photochemical ramifications of tissue-conducted light. Thus, the amount of conducted light, whether part or all, that could effect photochemistry was not known. It is important to understand the relationship between conducted light and photochemistry in order to address any questions with respect to growth and development. This present work describes the first non-intrusive assessment of effective light gradients within plant tissue using the *in situ* photoconversion of phytochrome as an internal radiometric probe.

Materials and Methods

Plant material. Hybrid corn (WF9 x Bear 38, custom Farm Seed Res., Decatur, IL) was used in all experiments. Seeds were soaked overnight in distilled water, germinated and grown in darkness at 23.5 $\pm$ 0.5°C in plastic trays (*ca*. 40 seeds/tray) on Kimpak (Kimberly-Clarke, Neenah, WI) soaked with distilled water. Between the third and fourth days after planting, etiolated seedlings of the same developmental stage and size (6 cm $\pm$ 1 cm seed to shoot tip; primary leaf fully enclosed within the coleoptile) were selected and prepared for spectrophotometric analysis.

Spectrophotometry. To obtain in situ spectra, a plant was always situated so that the region 3 mm below the coleoptilar node was placed beneath a 1.2 mm diameter hole in a 2 mm thick aluminum plate (Fig. 9A). Much of the shoot was inserted through a metal sleeve to protect the spectrally measured region from reflected laser light during tests requiring this type of irradiation (see Light sources and Fig. 9B). The plate and plant were placed into a sample compartment so that the measuring or direct actinic beams (see Light sources) passed through the hole, through the desired shoot segment and onto the cathode (5 cm dia.) of the photomultiplier tube, which was located 2 cm below the sample. The measuring and direct actinic beams were focused by means of a lens (4 cm focal length) mounted 4 cm above the sample (Fig. 9A). All absorption spectra were measured at 24°C with a computerized single beam spectrophotometer consisting of a light source, the monochromator from a Cary 14 spectrophotometer, an EMI 9659 QA photomultiplier tube, and a log amplifier (Davis et al., 1973). Absorbance readings were recorded as the average of 1024 separate readings over each successive 0.2 nm wavelength interval. These readings were stored in a Hewlett Packard 2108mx minicomputer programmed so that spectra could be manipulated to obtain difference spectra and directly recorded on paper by the computer with a Hewlett Packard x-y recorder. Wavelength was calibrated with a Hg lamp and is accurate to less than 0.1 nm.

Figure 9. Schematic diagram of sample holder compartment. (A) Side view. The actinic lamp was situated directly above the sample so that the light passed through the desired interference filter, a half-silvered mirror, a focusing lens, a small hole, and then was incident on the spectrophotometrically measured area. The measuring beam from the monochromator reflected off the half-silvered mirror and then followed an identical path to the PM tube. (B) Bottom view. The actinic source (laser) was oriented such that the beam was incident upon the shoot at a given distance away (x) from the point of measurement and at an angle of 60^{o} normal to the long axis of the shoot. The sleeves surrounding the shoot served partially to anchor the seedling and protect the spectrophotometrically measured area from reflected laser light. Plant roots were wrapped in moist tissue paper (not shown) to maintain plant turgidity during the experiments. The plant was held in place with black vinyl tape (not shown).

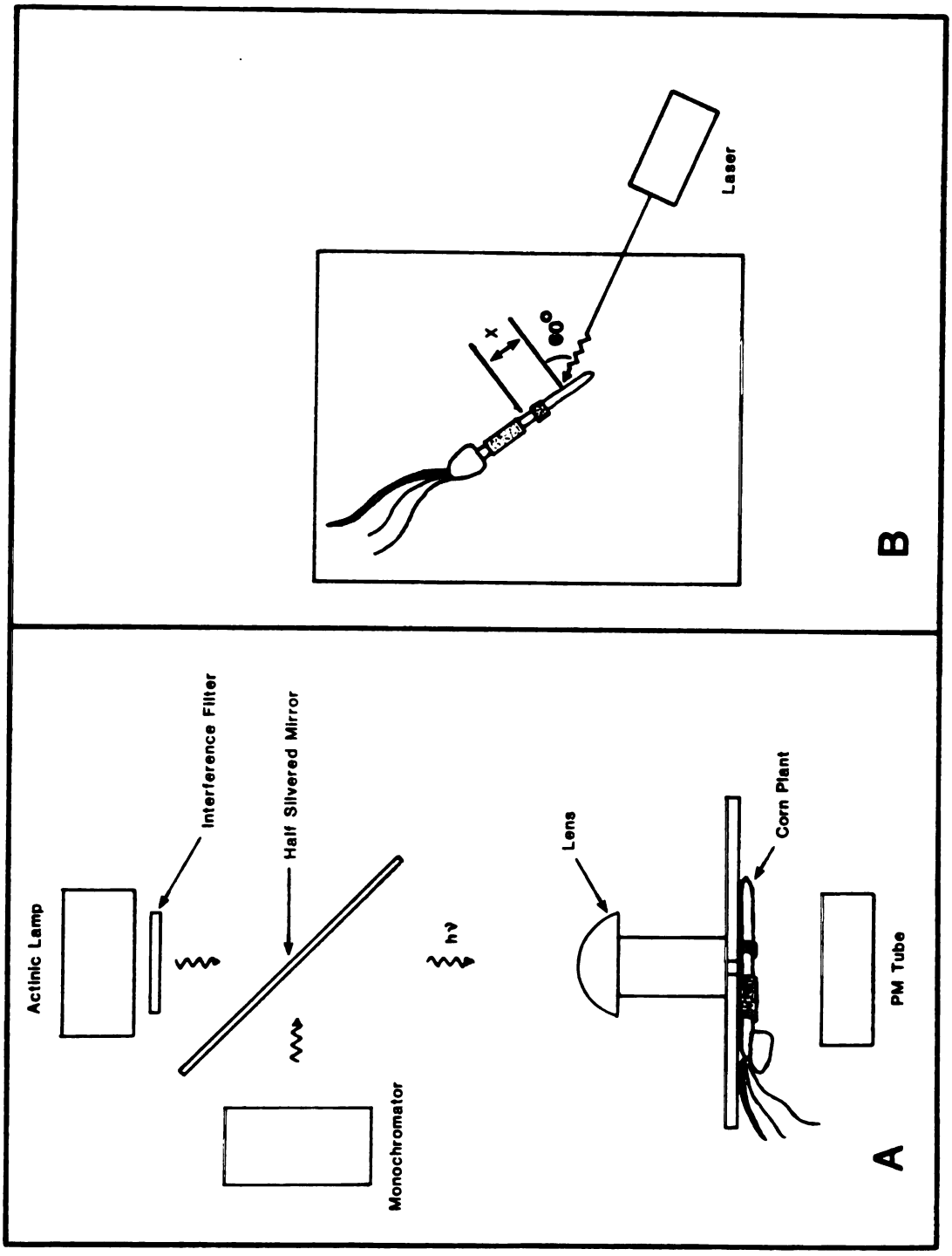
Actinic Lamp
Interference Filter
Half Silvered Mirror
hν
Monochromator
Lens
Corn Plant
PM Tube
A
Laser
x
60°
B

Figure 9

The measurement of an individual absorbance spectrum required 80 s. The entire analysis (consisting of many absorbance spectra) of one area of corn shoot required 12-15 min. Although the seedlings were very similar with respect to morphology and age, the total amount of photoconvertible phytochrome measured in the same region could vary in different plants by as much as two-fold. To compensate for this natural variation, the data are expressed as a percentage of the maximal phytochrome conversion (Pr to Pfr) for that particular tissue sample. Specifically, data are expressed as a percentage of the maximum difference ($\Delta A_{732nm} - \Delta A_{667nm}$) in the area measured as derived directly from the Pfr-minus-Pr difference spectra, or arithmetically

$$(NS/S) \times 100 = \% \text{ Conversion}$$

where NS equals the measured absorbance change after a non-saturating fluence and S equals the total absorbance change after a subsequent saturating fluence. Data for percent conversion bear no reference to the photostationary state, which should be constant at ca. 80% Pfr after a saturating fluence (Pratt and Briggs, 1966).

Light sources. The actinic light source for direct irradiation of the spectrophotometrically measured area was provided by a Unitron incandescent lamp fitted with either of two interference filters [red: PTR Optics 628 nm, 10.3 nm 1/2 band, 25.5 W/m^2, or far red: PTR Optics 731 nm, 10.4 nm

1/2 band 41.0 W/m^2; beam diameter = 5 mm; beam incident upon a 1.2 mm diameter hole (Fig. 9A)]. Indirect actinic irradiation was accomplished with a beam from an Oriel He-Ne laser (Model 6611) with a 632 nm line emission. The laser beam (2.6×10^4 W/m^2; 0.8 mm beam diameter) was directed onto the shoot a given distance from the area of spectrophotometric measuremnt and at a fixed angel of 60° from normal to the shoot axis of a corn shoot (Mandoli and Briggs, 1982a). Indirect irradiations of the leaf were accomplished by gently removing the coleoptile just above the node, while irradiations of the mesocotyl were accomplished by reversing the orientation of the plant in the sample holder (Fig. 9B).

All light intensities were measured with a Kettering radiometer (model 68; Laboratory Data Control, Riviera Beach, FL). Reported values have been corrected for radiant flux density based on the area of the plant tissue irradiated (0.011 cm^2 for direct irradiation; 0.005 cm^2 for laser irradiation). All spectrophotometric measurements were performed in darkness with the aid of a dim green safelight.

Results

Three requirements must be satisfied in order to use any photochemical conversion as an *in situ* assay for axial light conduction. They are: (1) the identity of the convertible pigment must be known; (2) the degree of photochemical purity (i.e. the relative amount of the

measureable photoconversion contributed by the pigment in question) must be established; and (3) reciprocity must hold for the photochemical conversion under the experimental conditions. The most sensitive region (high signal-to-noise ratio) for phytochrome measurement along an etiolated monocot is the region just below the coleoptilar node (Briggs and Siegelman, 1965; Kondo et al., 1973; Pratt and Coleman, 1974; Chapter 1). The unequivocal identification and degree of photochemical purity for phytochrome in this shoot region has been established previously (Chapter 1). Reciprocity was valid for phytochrome conversion just below the coleoptilar node (Fig. 10). Over the fluence range used, the percentage conversion was dependent only on the number of incident quanta (Fig. 10). It was possible to use the percentage of phytochrome conversion as a non-intrusive assay for the amount of light conducted along the tissue from a remote entry point to the point of phytochrome measurement just below the node. For a constant incident fluence, the percentage of conversion decreased as the distances increased (Fig. 11). No detectable absorbance change was measured when the shoot (not including the spectrally measured area) was wrapped with black vinyl electrical tape and irradiated with the actinic laser at a distance that normally would induce phytochrome conversion (data not shown). This observation indicated that conversion of phytochrome by indirect light was solely a result of light conducted along the plant axis.

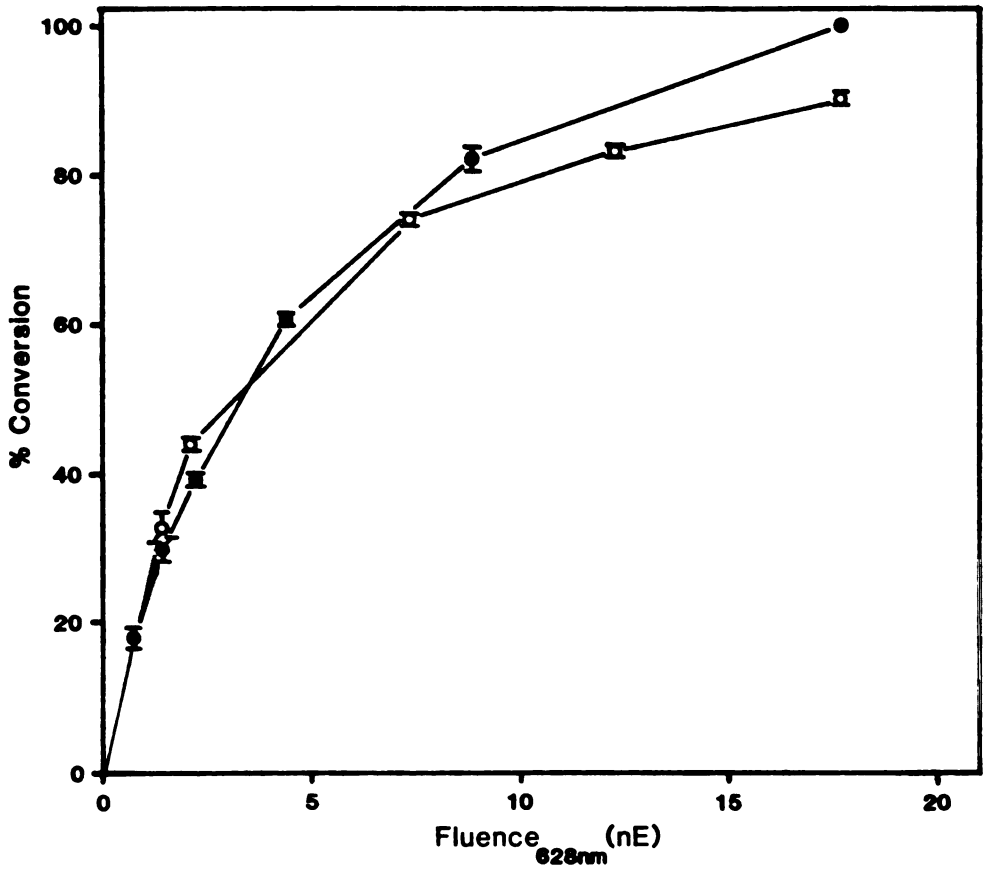

Figure 10. Reciprocity for the in situ photoconversion of Pr to Pfr. Each point represented by (○) was obtained by irradiating three separate plants 3 mm below the coleoptilar node with 2 min direct red irrdiation at the flux rate necessary to obtain the indicated fluence. The amount of phytochrome converted is expressed as a percentage of the average maximum amount obtained with a saturating fluence (18 nE at 628 nm). All of the points represented by (●) are averages of phytochrome conversion in three seedlings. For each seedling, the amount of phytochrome converted 3 mm below the coleoptilar node was calculated following a direct red irradiation at 25.5 W/m^2. Phytochrome was measured following successive irradiations at this fluence rate for various times for a total of 2 min of irradiation. The amount of phytochrome is expressed at each point as a percentage of the total converted during the entire 2 min of irradiation. The vertical bars represent ± one standard error.

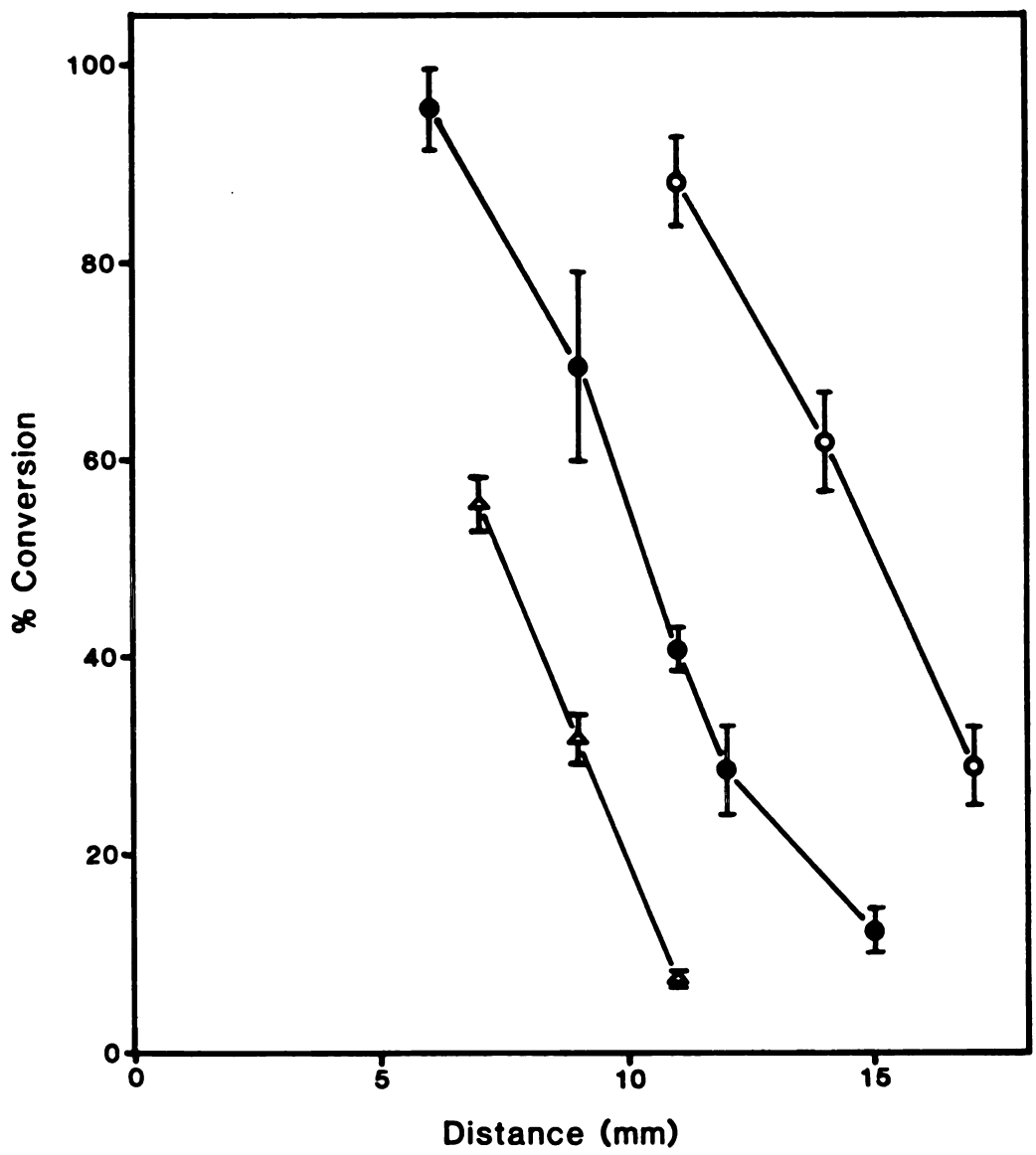

Figure 11. Phytochrome conversion by indirect actinic irradiation. Conversion of Pr to Pfr was measured after a 30 s indirect irradiation of 632 nm (2.05 nE). The calculated percentage conversion was compared to conversion induced by a subsequent saturating direct irradiation (18 nE at 628 nm). The indirect irradiation was obtained using the beam of a laser and was incident on the desired shoot tissue as described in the text and in Fig. 9B at an angle of 60° from normal and at a variable distance from the point of measurement. Mesocotyl (○). Coleoptile plus primary leaf (●). Inner leaf alone (△). Each point is the mean of 3-5 separate measurements on separate plants. The vertical bars represent ± 1 standard error.

A quantitative decription of axial light attenuation was determined by combining the experimental data. The direct fluence required for a particular conversion at the measurement point was known (Fig. 10). The distance from the site of indirect irradiation to that measurement point was determined for an equivalent percentage conversion (Fig. 11, Table 1). Therefore, the conducted fluence through each tissue type was derived as a function of increasing distance between the irradiance and measurement points. A log plot of this conducted fluence vs. distance revealed that 632 nm light was attenuated by 50% over the passage through each 1.80, 1.60, and 1.15 mm of mesocotyl, coleoptile plus primary leaf, and inner leaf alone, respectively (Fig. 12).

For this analysis, we assumed that a 628 nm and a 632 nm actinic irradiation were equivalent with respect to quantum efficiency. While not exactly identical, we believe the difference to be trivial in this case.

Discussion

The log-linear nature of the relationships in Fig. 5 is expected since all of the obvious mechanisms for light attenuation through a tissue are exponential. These main mechanisms for light loss are absorbance, scatter, and the inherent losses in "optical piping" resulting from incomplete reflectance at the piping interface (e.g. the tissue-air interface). Since the effect of these factors may be described by similar exponential functions, any

Table 1. Actinic irradiation required for phytochrome conversion. The beam 1 doses required for given percentages of conversion were determined from Fig. 10. The distances of beam 2 propagation required for given percentages of conversion were determined from Fig. 11.

Phytochrome conversion (% of maximum)	Beam 1* dose (nE)	Beam 2+ distance propagated (mm)		
		Leaf	Coleoptile/leaf	Mesocotyl
10	0.45	10.8	-	-
20	0.9	9.9	13.5	-
30	1.4	9.1	11.9	16.9
40	2.1	8.3	11.1	16.0
50	3.3	7.5	10.4	15.1
60	4.7	-	9.7	14.2
70	6.6	-	8.9	13.1
80	9.5	-	7.8	12.0
90	15.2	-	6.6	-

*628 nm beam incident at the point of measurement.
+632 nm beam incident at a variable distance from the point of measurement.

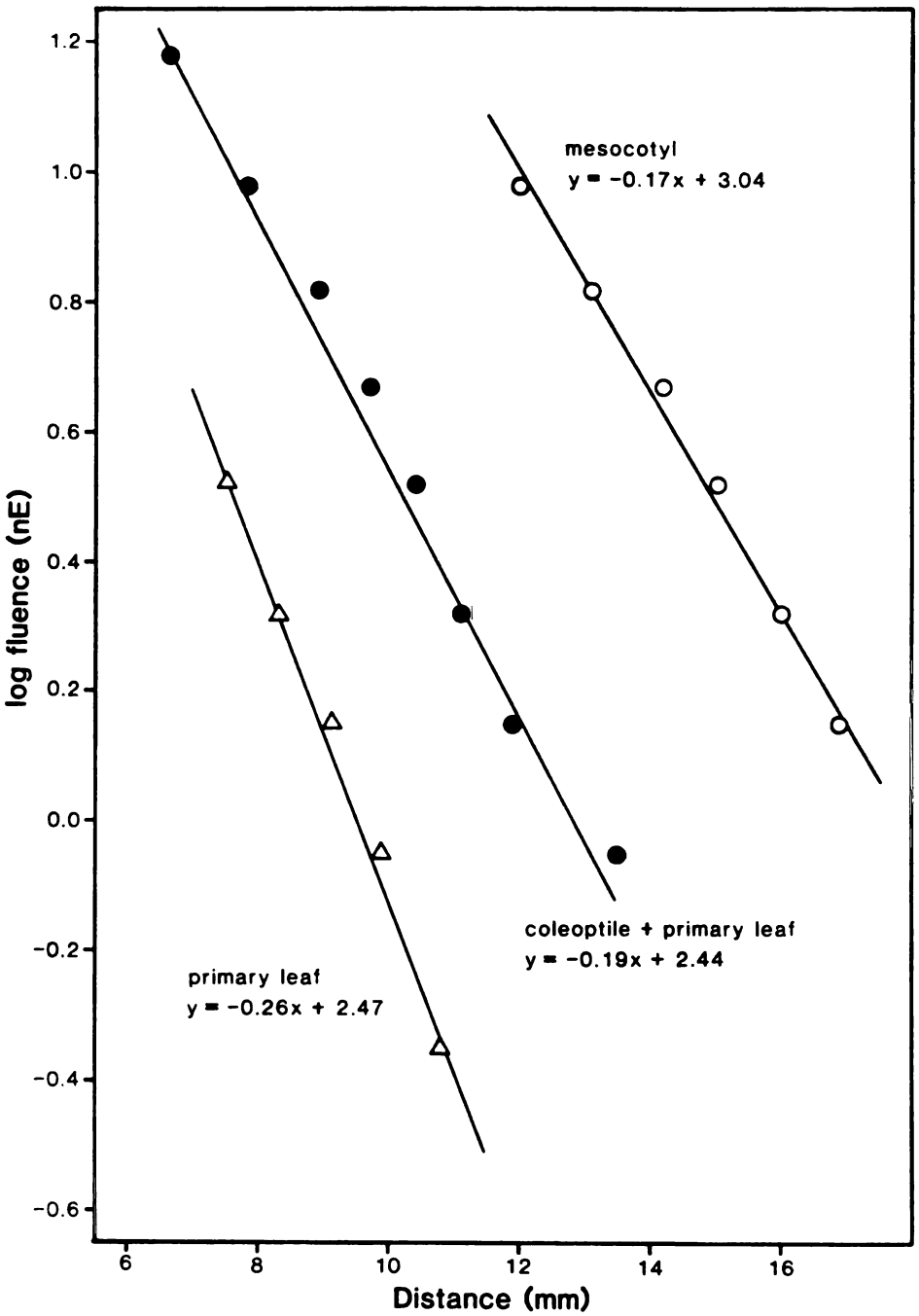

Figure 12. Attenuation of conducted light for various shoot tissue types as a function of distance conducted along the corn shoot. Fluence is derived from Table 1. Symbols correspond to the same tissues as for Fig. 11. These lines represent the best fit calculated by linear regression method. All have a correlation coefficient of r = -0.99.

combination of them would have the same effect on the shapes of the relationships shown in Fig. 12. Thus, one can not distinguish among these mechanisms of attenuation based on these data.

The precision of the log-linear relationships in Fig. 12 permits the derivation of linear equations decribing light attenuation through the experimental tissues. The equation described for the mesocotyl is $y = -0.17x + 3.04$, while for the coleoptile plus primary leaf, it is $y = -0.19x + 2.44$. The slopes of these two lines correspond to a fluence loss of 50% over 1.80 mm and 1.60 mm of tissue, respectively. These determinations are similar to recent values reported for oats, which partly validates the effectiveness of those previous measurements (Mandoli and Briggs, 1982b). The equation for leaf tissue alone is $y = -0.26x + 2.47$, and indicates a 50% reduction influence for every 1.15 mm length of tissue. The difference in the slopes for the coleoptile plus primary leaf and leaf alone (-0.19 vs. -0.26) is in agreement with a proposed primary light path along the coleoptile for increasing distance (Mandoli and Briggs, 1984). The greater attenuation seen for the leaf is due at least in part to the higher concentrations of photosynthetically related pigments (e.g. protochlorophyllide). An increased amount of scatter may also be responsible for this larger attenuation. This greater rate of attenuation seen for the leaf is not due to structural damage incurred during coleoptile removal, since

the similarity of the y-intercepts for the leaf and coleoptile plus primary leaf implies no extraneous fluence loss.

Since the rate of attenuation for the mesocotyl and the coleoptile plus primary leaf are essentially equal, they should extrapolate to the same fluence at zero distance (Fig. 12). This point describes the hypothetical situation in which the laser is directly incident upon the point of spectral measurement. The fluence value of the y-intercept for mesocotyl tissue is 1.10 mE, which is similar to the laser fluence value (2.05 mE). This smaller extrapolated value could be due in part to reflective light loss at the shoot surface. The lower value seen for the coleoptile plus primary leaf (0.27 mE) can be explained by a higher reflective light loss and/or a greater attenuation rate as the actinic light passed through the coleoptilar node, a situation not encountered in mesocotyl measurements (see Light sources in Materials and Methods).

Assuming that the reflective properties of the mesocotyl and coleoptile are similar, the percentage of light loss through the node can be derived by comparing the difference in conducted fluence values (y) at x = 3 mm for the mesocotyl and coleoptile plus primary leaf. Since all spectral measurements occurred 3 mm below the node, then theoretically, the actinic beam would be incident on and travel solely through mesocotyl tissue for all attenuation measurements when x = 3 mm. Therefore, the difference

between the derived fluences (mesocotyl minus coleoptile plus primary leaf) for x = 3 mm corresponds to the light loss contributed by the node. The derived value for the coleoptile plus primary leaf is 22% of the mesocotyl, or a 78% fluence loss over a ca. 1 mm long node, which is greater than previously reported for oats (Mandoli and Briggs, 1982b). One source of this difference could be morphological. Generally, corn is larger than oats, which could yield a greater percentage light loss if all structures were proportionally larger. Another explanation may involve back scatter at the mesocotyl-node interface. For these mesocotyl attenuation measurements, any non-absorbed indirect light at the measurement point might be absorbed there after back scatter from the mesocotyl-node interface. This would cause a higher percentage of photoconversion, correctly interpreted as a higher fluence at the measurement point. However, this fluence increase would be disproportionally larger than coleoptile plus primary leaf conducted light, where a back scatter fluence enhancement would not occur. Similar fluence increases have been encountered and discussed in other systems (Seyfried and Fukshansky, 1983; Vogelmann and Björn, 1984).

The numerical constants for these unidirectional attenuation equations could be affected by two factors: (1) the aforementioned back scatter; and (2) light channeling along the longitudinal plant axis (Fukshansky, 1981). As previously mentioned, back scatter would increase the

fluence at the measurement point, yielding an increased photoconversion. Therefore, the fluence value for unidirectional light attenuation (e.g. base of mesocotyl to node only) should be lower than the value that we could obtain through our analysis. Light channeling or coherent light conductance (Mandoli and Briggs, 1982a) would affect the relative absorption by Pr and Pfr if these two forms of phytochrome were differentially localized throughout the tissue structure as has been proposed (Mackenzie *et al.*, 1978). This phenomenon could alter the absorption rates by Pr and Pfr such that the amount of measured conversion ($\Delta A_{732nm} - \Delta A_{667nm}$) might be affected. If these arguments are valid, then the numerical assessment of a unidirectional light gradient *via in situ* photoconversion may not be accurate. In contrast, although radiometric measurements with physical probes may quantitate a unidirectional gradient with greater precision, the measurement of an "effective" photochemical gradient would now be difficult. However, it is apparent that the relationship between the location of the photoreceptor and the path of the conducted light should be considered in any evaluation of the importance of gradients in photomorphogenesis.

The present work has described how an *in situ* photoconversion can be used to measure an internal light gradient non-intrusively. In general, these results have validated some of the measurements obtained by disruptive methods. Additionally, it is now apparent that conducted

light can be photochemically significant, although earlier arguments presented here tend to indicate that the complete relationship between a light gradient and photochemistry may be quite complex. At this point, however, these results support the general hypothesis that light conduction can act as a partial means for affecting photomorphogenesis.

CHAPTER 3

Altering the Axial Light Gradient Affects Photomorphogenesis in Emerging Seedlings of *Zea mays* L.

Introduction

Light-inhibited elongation of cereal seedling mesocotyls has been well studied as a model system for plant photomorphogenesis. It is known that red light initiates the inhibition of mesocotyl elongation (eg. Johnston, 1937; Goodwin, 1941), and that the pigment responsible for this action is phytochrome (Edwards and Klein, 1964; Loercher, 1966; Duke *et al.*, 1977). However, the mechanism of pigment action and the later steps in the transduction sequence are still not defined completely. This has been due in part to confusion concerning the site(s) of photoperception for the measured response.

Initial studies demonstrated that mesocotyl inhibition could be affected by irradiation of either the region just below the coleoptilar node (Araki and Hamada, 1937; Goodwin, 1941), or the coleoptile tip (Goodwin, 1941; Duke and Wickliff, 1969). Early and recent evidence suggested that light inhibits mesocotyl growth by limiting the supply of auxin from the coleoptile (Went, 1928; van Overbeek, 1936; Iino, 1982). Thus, it seemed reasonable that a photoperceptive site for mesocotyl inhibition could be

located distally in the coleoptile. It was still suggested, however, that light received by the coleoptile actually was transmitted downward within the shoot to a distal perceptive site (Goodwin, 1941; Mer, 1969).

The role of tissue optical qualities in growth and developmental responses has long been recognized. Phototropism, for example, results from a spatially differential growth response based on a light gradient developed across the shoot tissue (Dennison, 1979; Poff, 1983; Parsons et al., 1984). Optically dependent models for photomorphogenesis also have been proposed (e.g. Jose and Schäfer, 1978). For example, the extreme light sensitivity of mesocotyl tissue (Mandoli and Briggs, 1981), and the axial light transmitting qualities of cereal seedling tissue (Mandoli and Briggs, 1982a,b; Chapter 2), together support the contention that light perception occurs solely within the mesocotyl. A correlative relationship between red light-initiated photomorphogenesis and the light transmitting qualities of the shoot tissue provided further evidence in support of this contention (Mandoli and Briggs, 1982b). Studies by our group have also demonstrated that light incident on the coleoptile could effect photoconversion of phytochrome within the mesocotyl tissue (Chapter 2). Combined, these data strongly favor the existence of a single light perceptive site within the mesocotyl tissue. However, no experiments have shown this directly under natural growing conditions.

To demonstrate that axially transmitted light significantly affects morphogenesis in the non-emergent mesocotyl, tests were conducted under growing conditions in which shoots emerged from soil under a natural light regime. Under the fluence and spectral ranges used in previous investigations (Mandoli and Briggs, 1982b), it remains possible that light-induced responses within shoot tissues, normally more proximal to direct light, had not been activated. Experiments under natural growing conditions permitted a complete assessment of the possible factors which could regulate mesocotyl photomorphogenesis.

This present work describes a method for selectively altering the axial light gradient of etiolated corn seedling shoots. Seedlings with altered optical qualities have been used to examine the role of axial light transmittance in the morphogenesis of the buried mesocotyl.

Materials and Methods

Plant material. Hybrid corn (WF9 x Bear 38, Custom Farm Seed Res., Decatur, IL) was used for all experiments. After overnight imbibition in distilled water, seeds were planted according to the type of experimental assay employed: (a) for spectrophotometric analyses, seeds were allowed to germinate and grow in darkness for 4 days at 24.0 $\pm$ 0.5°C in covered plastic trays (ca. 40 seeds/tray) on Kimpak (Kimberly-Clarke, Neenah, WI) soaked with distilled water; (b) for growth rate measurements, imbibed seeds were sown individually ca. 2 cm deep in glass shell vials (9 x 2

cm I.D.) filled with vermiculite and moistened with distilled water. Individual vials were then placed in a dark humid chamber (24.0 ± 0.5°C) for 3 days; (c) for photomorphogenesis experiments, presoaked seeds were sown evenly in plastic trays half-filled with vermiculite and soaked with distilled water. Trays containing planted seeds were covered with plastic wrap and placed in a dark room for 3 days at 24.0 ± 0.5°C. On the third day after planting, selected seedlings were treated and transplanted as described below (see Photomorphogenesis in Materials and Methods).

Spectrophotometry. All assessments of the light attenuating qualities of corn seedlings were performed as described previously (Chapter 2). In brief, axial light attenuation was measured at 632 nm along single etiolated corn seedlings using localized phytochrome conversion as an *in situ* quantum counter. Photochemical conversion was assayed across a seedling at a point 1 mm below the coleoptilar node. By changing the distance between the point of irradiation and the point of phytochrome measurement, the amount of photoconversion was altered. These measurements, and a dose-response curve for phytochrome conversion in this system, were used to calculate the number of quanta transmitted along a given length of shoot tissue (coleoptile plus inner leaf) (Chapter 2).

In order to account for stray actinic quanta, control measurements were conducted when the distance between the irradiance and measurement point was 5 mm or less. These measurements were accomplished by wrapping the coleoptilar region with black tape and then proceeding with attenuation measurements for 4 and 5 mm distances. Measurements at these same distances could then be corrected for stray light effects on phytochrome conversion. At points equal to or greater than 6 mm, stray light photoconversion was negligible.

Absolute absorption spectra of specific tissues above the coleoptilar node were measured spectrophotometrically across a given point along a single seedling as described above and in detail previously (Chapter 2). For these measurements, a rolled white tissue served as a reference.

Seedlings used for axial attenuation and absorption measurements often required tissue preparation and selective staining of the coleoptile. Under dim green light, the entire coleoptile, from the node to the shoot apex, of 4 day old dark-grown seedlings was prepared for staining by lightly abrading this area with a cotton applicator impregnated with wetted carborandum powder. After abrasion, seedlings were rinsed with distilled water and immersed in one of two staining solutions (0.05% methylene blue: abs.max. = 663 nm; 0.1% metanil yellow: abs.max. = 434 nm) for 1 or 4 minutes. Stained plant tissues were blotted with paper tissue and coated lightly with a thin film of

silicone grease to prevent evaporative water loss from the abraded and stained area during the spectral measurements necessary for the assay of light attenuation. Abraded controls were prepared as described above with the omission of the staining step. To obtain a seedling with a partially stained coleoptile, only the lower portion (ca. 4 mm) of the coleoptile was abraded and then stained for 4 min. The remaining cutinous layer enveloping the coleoptilar tip was impermeable to the tissue stains.

Growth rate measurements. Three day old dark-grown seedlings in shell vials were selected for proper size (3-4 cm shoot length) and, when necessary, were stained as before for 4 min (see Spectrophotometry). Individual vials containing treated seedlings were placed in a rack situated 40 cm away from a Minolta automatic camera (model X-700; Minolta Corp., Ramsey, NJ) equipped with an automatic flash wrapped with a Kodak infra red filter (wratten 87C; Eastman Kodak Co., Rochester, NY). The camera was programmed to take infra red photographs every 2 hours. With the exception of the infra red flash, time lapse photography was conducted in complete darkness in a temperature controlled room (24.0 $\pm$ 0.5°C). Unilateral actinic light exposures, when used, were provided by a red fluorescent lamp (F15T12-R; Sylvania Inc., Danvers, MA) wrapped with red cellophane ("K" 210FC; DuPont Co., Wilmington, DE) (Fig. 13). The actinic lamp was located 20 cm away from the seedlings. During the growth experiments, the seedlings grew within a 1.5 cm wide clear

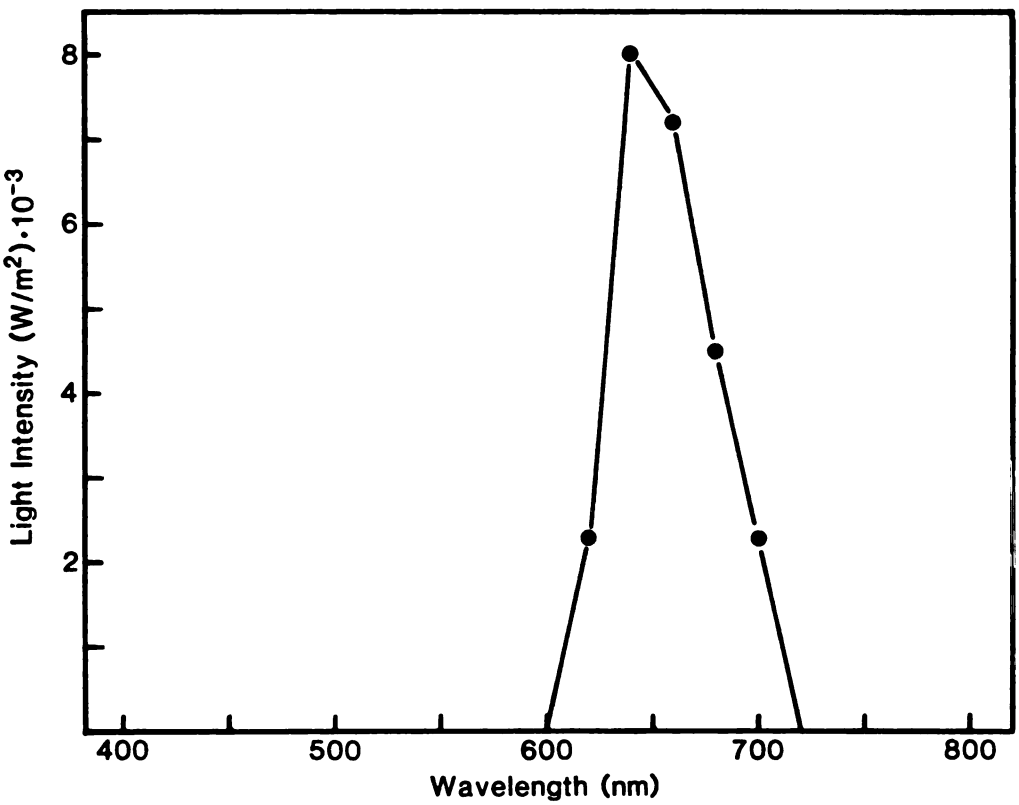

Figure 13. Spectral distribution of the red fluorescent light source used during growth rate experiments.

plastic corridor, which maintained the seedlings in the focal plane of the camera. Data were recorded by mounting the developed negatives and then projecting the images on a screen from which structure lengths were measured and corrected for the actual size.

Photomorphogenesis. Seedlings which had grown for 3 days in total darkness in covered trays were selected for size (ca. 3.5 cm shoot length) and stained, when necessary, for 4 min as before (see Spectrophotometry). Under dim green light, experimental and control seedlings were transplanted ca. 4 cm deep into moist vermiculite in black plastic pots (9 x 14 cm). A 1 cm layer of soil consisting of finely sifted peat and loam was used to fill the remaining volume of the pots and completely cover the transplanted seedlings. A clear plastic dome was placed over each individual pot to maintain a high humidity.

Pots containing treated and control seedlings were placed in a greenhouse (ca. 27°C) for 3 days. Pots were distributed evenly within an tiered rack consisting of 3 levels separated by metal screens to provide successively lower light intensities. Light was provided by a 1000 watt multi-vapor metal halide lamp (MV 1000/U; General Electric, Hendersonville, NC) suspended 60 cm above the top growing level of the rack (16 hour day cycle). A distilled water tank (7 cm deep) was placed between the samples and the light source to absorb excess infra red irradiation from the lamp. The entire lamp and rack was shrouded in black cloth

to ensure that the samples were irradiated only by diffuse sunlight and direct light from the lamp. This ensured an even balance of irradiation on the experimental pots. Blue and red light filters, when used, consisted of colored Plexiglas (red-#2423; blue-#2424; Rohm and Haas, Philadelphia, PA) placed underneath the water tank. Spectral outputs were recorded for the various light regimes used (Fig. 14).

Plants were watered lightly with distilled water every day. On the fifth day, individual plastic covers were removed from each pot to permit unhindered leaf elongation. After 6 days, data were recorded by clipping the seedlings at soil level and measuring the distance to the coleoptilar node above or below the point of cutting. All elongation of mesocotyl tissue had occurred before the sixth growing day so that the seedling node was stable at its position at the time of measurement.

All integrated light intensities were measured with a Kettering radiometer (model 68; Laboratory Data Control, Riviera Beach, FL), while spectral outputs were measured with an IL spectroradiometer (model 700A; International Light Inc., Newburyport, MA).

Results and Discussion

Axial light attenuation. To examine directly the role of a light gradient in light-induced mesocotyl inhibition, one must change the axial light gradient without significantly perturbing other functions of the seedling.

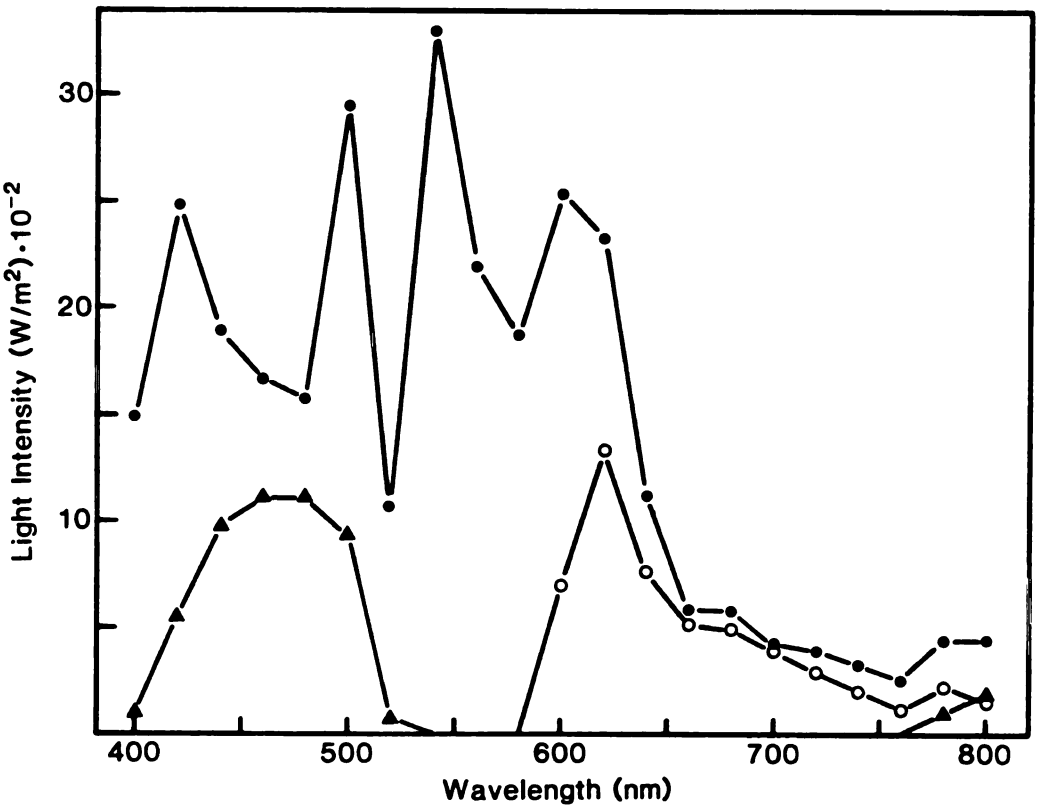

Figure 14. Spectral distribution of the light sources used during photomorphogenesis experiments. Red light source (○). Blue light source (▲). White light source (●).

Previous results indicated that the coleoptile was the structure along which light was transmitted particularly for relatively long distances (Chapter 2). Thus, changing the axial gradient likely would necessitate the alteration of coleoptilar pigmentation.

Alteration of the effective light gradient was accomplished by dyeing the entire coleoptile of young etiolated shoots with tissue stains. Only the red (632 nm) light attenuating qualities of the treated shoots were measured, since this wavelength is within the primary spectral region of Pr photosensitivity. The photoequilibrium of phytochrome should be constant at ca. 80% Pfr at this wavelength (Pratt and Briggs, 1966). Only dyes that absorb 632 nm light should affect the magnitude of axially transmitted red light. Neither coleoptilar abrasion nor staining with metanil yellow changed the in vivo light absorption of the coleoptile at 632 nm (Fig. 15A,B,D). Thus, as expected, the optical qualities of these treated shoot tissues were not different substantially from previously reported values (Fig. 16) (Mandoli and Briggs, 1982b; Chapter 2). Conversely, coleoptilar staining with methylene blue yielded a significant increase in the red region of both the magnitude of the in vivo absorption spectrum and the magnitude of axial light attenuation (Figs. 15C,16).

It was possible to affect the axial light gradient of corn shoots by introducing stains into a short length (ca. 4

Figure 15. Cross sectional absorbance of coleoptile and leaf tissue. Absorption measurements were recorded across individual tissues ca. half-way along the excised coleoptile or primary leaf of 4 day old seedlings. Where applicable, seedlings were stained for 4 min. (see Spectrophotometry in Material and Methods). The measured coleoptilar spectra represent the absorption of light across half of a longitudinally cut coleoptile. Specific mean absorbances listed in the upper right corner of each block are the average values from four separate seedling measurements ± 1 standard error. The separate sections of the figure are: (A) unabraded coleoptile; (B) abraded coleoptile; (C) methylene blue-stained coleoptile; (D) metanil yellow-stained coleoptile. Individual spectra are identified by: (L) leaf; (C) coleoptile.

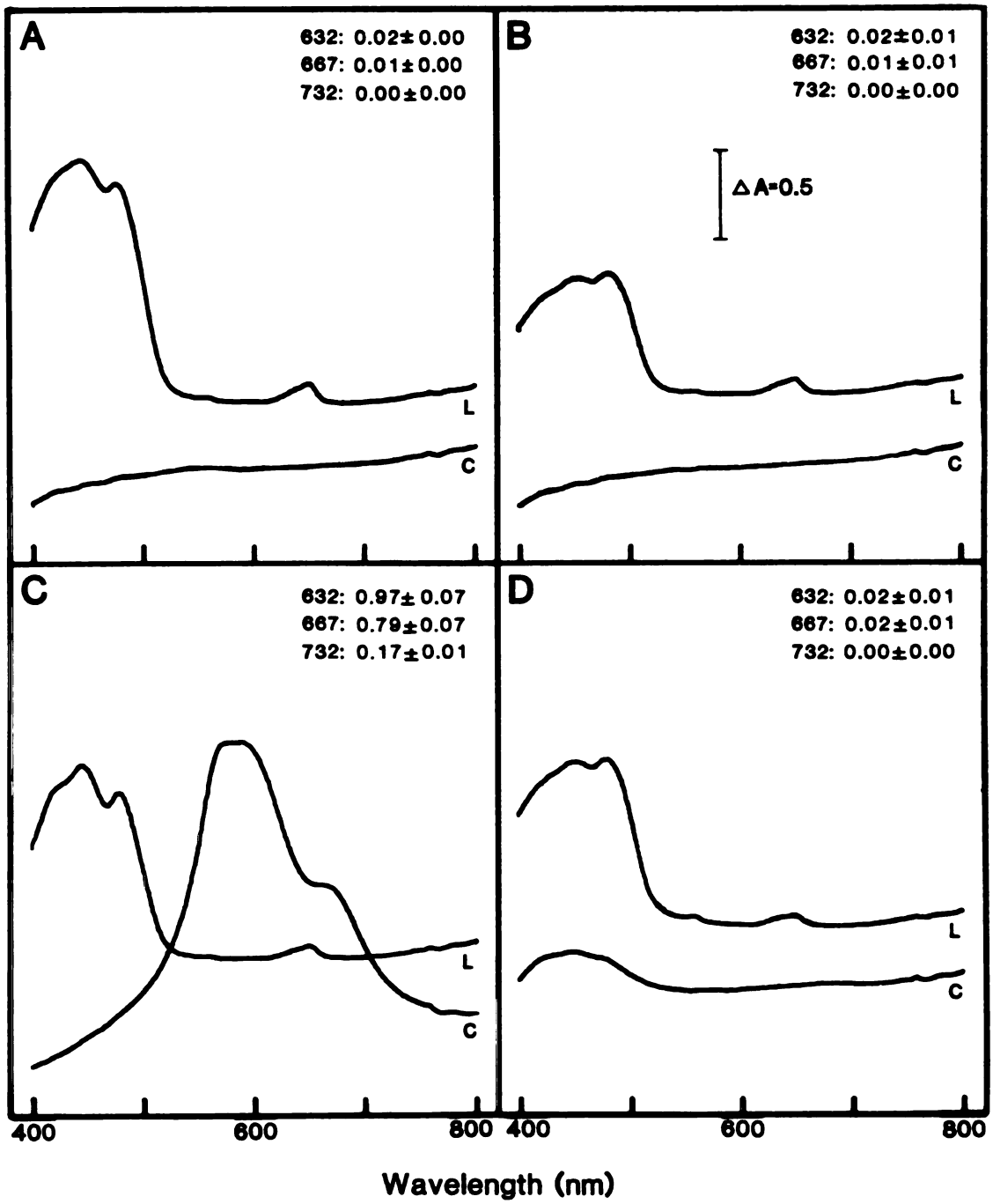
A
632: 0.02±0.00
667: 0.01±0.00
732: 0.00±0.00
L
C
B
632: 0.02±0.01
667: 0.01±0.01
732: 0.00±0.00
ΔA=0.5
L
C
C
632: 0.97±0.07
667: 0.79±0.07
732: 0.17±0.01
L
C
D
632: 0.02±0.01
667: 0.02±0.01
732: 0.00±0.00
L
C
400
600
800
400
600
800
Wavelength (nm)

Figure 15

Figure 16. Effects of coleoptilar staining on *in situ* axial light transmission at 632 nm. Data are plotted as the amount of actinic quanta transmitted over a given distance between the point of irradiance and the point of phytochrome measurement. Phytochrome conversion served as the assay for light quantitation (see Chapter 2). Each plotted value represents the mean transmitted dose from a minimum of 3 separate seedling measurements. One standard error for any given value never exceeded ± 0.3 nE. Abraded coleoptile (●). Metanil yellow-stained coleoptile (○). Coleoptile stained with methylene blue for 1 min (△). Coleoptile stained with methylene blue for 4 min (▲).

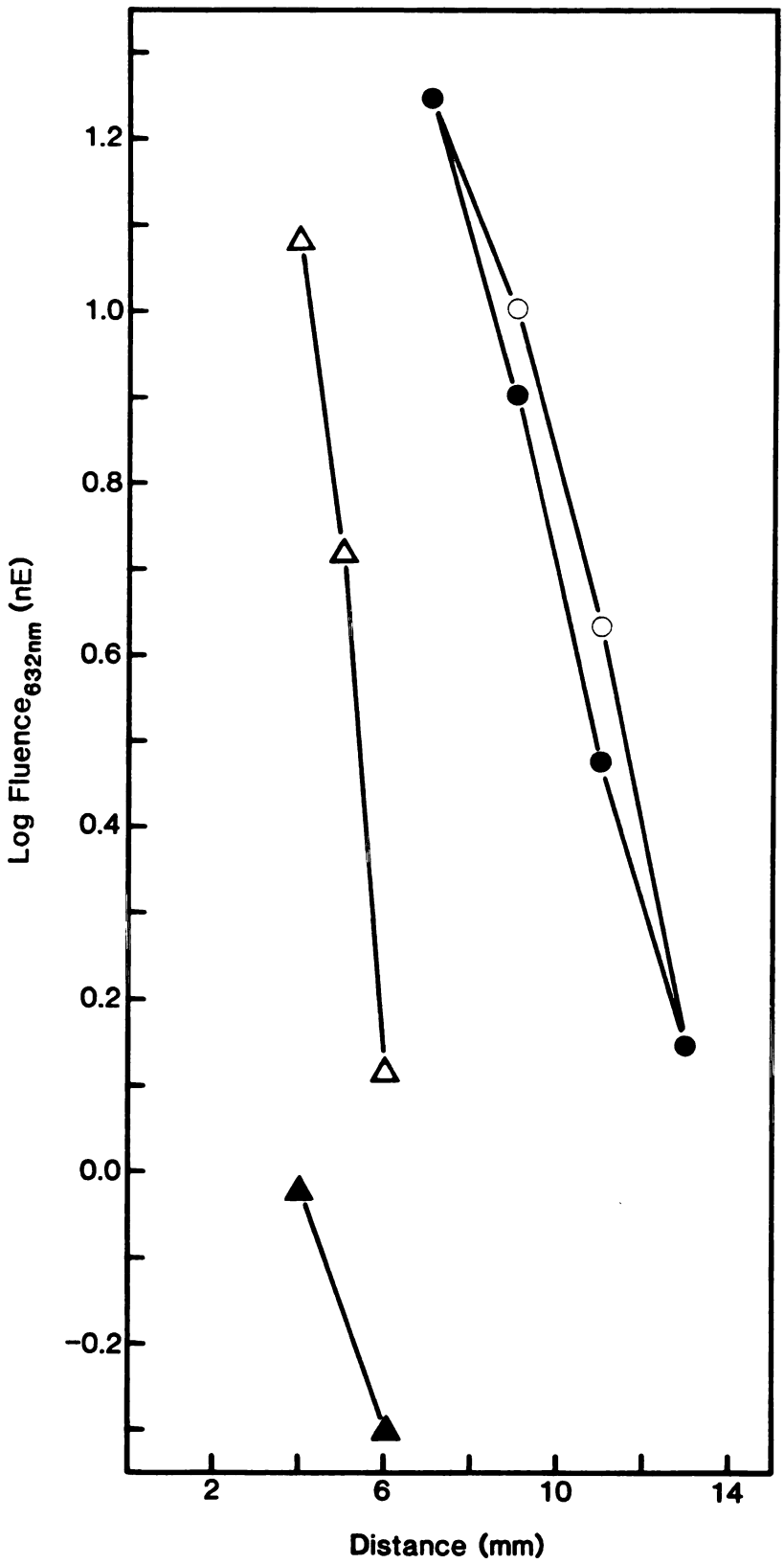

Figure 16

mm) of the coleoptile beginning 1 mm above the coleoptilar node. Although the tissue-transmitted light was incident initially on the unstained apical region, light transmittance still was affected noticeably for blue-stained seedlings (Table 2). This result indicated that the increase in attenuation observed for seedlings with coleoptiles which were dyed entirely resulted primarily from a decrease in axial light transmission and not from absorption of light at the point of incidence.

Microscopic analysis of tissue cross sections immediately after staining revealed that the stains were localized mainly within the peripheral cells (Fig. 17). There was little or no stain present inside the internal cells of the coleoptile. Moreover, the dye remained concentrated mostly within the originally stained cells for at least 14 hours after staining (Fig. 17), indicating that internal diffusion of the stains was minimal.

These results permit an analysis of the mode of axial light transmission. The effect of specifically localized staining on axial light gradients supported the idea that the internal regions of the shoot contribute minimally to the path of light propagation (Mandoli and Briggs, 1982a; Mandoli and Briggs, 1984). It would appear that unstained inner leaf tissues do not provide a primary route for light. The normally high attenuation values for leaf tissue tend to support this conclusion (Chapter 2). Previous photomorphogenic studies have indicated that light

Table 2. Light transmission along a partially stained corn seedling. The coleoptile section of a whole seedling was abraded and stained along a 4 mm section beginning 1 mm above the coleoptilar node. Axial light transmission was assessed along a 10 mm shoot portion as described previously (see Spectrophotometry in Materials and Methods). The actinic source was never incident on a stained portion of the shoot. Each value presented is the mean of at least 3 separate measurements. Error listed is ± one standard error.

Dose Transmitted (nE)		
Metanil yellow	Methylene blue	Abraded control
2.8 ± 0.2	0.2 ± 0.2	2.9 ± 0.2

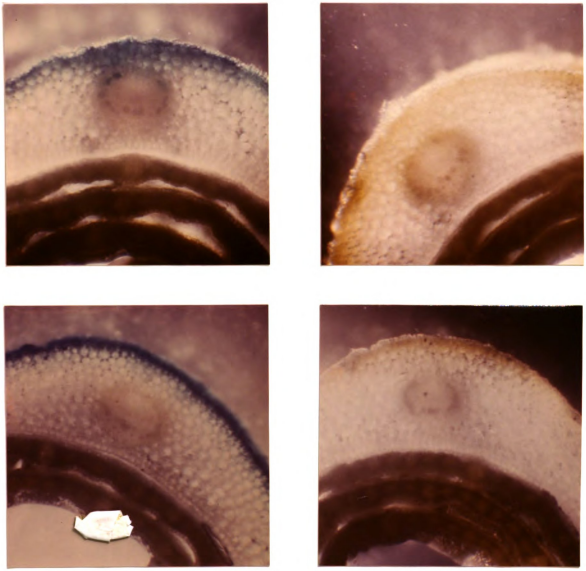

Figure 17. Photographs of tissue cross sections after coleoptilar staining. Coleoptiles were stained for 4 min prior to sectioning according to the previously mentioned procedure (see Spectrophotometry in Materials and Methods). Photographed sections are from a point half-way along the shoot region above the coleoptilar node. The left column shows seedlings stained with methylene blue, while the right column shows metanil yellow-stained seedlings. The lower row denotes seedlings which were sectioned immediately after staining, while the upper row denotes seedlings which were sectioned 14 h after staining. Scale is ca. 70:1.

absorption by a leaf can affect a response (Jose and Schäfer, 1978). However, these experiments utilized dicotyledonous seedlings, which are optically dissimilar from corn.

Two possibilities emerge from the observation that seedlings were optically altered as a result of staining the peripheral cells of the coleoptile. Either axial light transmission is limited exclusively to the outer periphery of coloeoptilar cells, or quanta entering internal coleoptilar regions are scattered to and absorbed within the stained periphery. Although our data can not distiguish between these two possibilities, recent analyses of light gradients across maize shoots have suggested that the coleoptile is a highly light scattering structure (Vogelmann and Haupt, 1985).

Growth rate measurements. To test for any extraneous effects of the various tissue treatments on photomorphogenic sensitivity, time course responses were measured for mesocotyl inhibition after direct unilateral red-light irradiation. This irradiation was not active phototropically (Iino et al., 1984). Over the fluence range tested, all mesocotyls showed sensitivity to light (Fig. 18). The lower fluence used was just within the saturation range for the low irradiance response for oats (Mandoli and Briggs, 1981). If corn responds similarly, then a difference in light sensitivity between tissue treatments would have been reflected in the growth

Figure 18. Affects of red light and coleoptilar staining on coleoptile and mesocotyl elongation. Each section of the figure represents the elongation of the coleoptile and mesocotyl in one particular representative plant. Bottom row - no red irradiation. Middle row - 25 min red irradiation at 0.68 W/m^2. Top row - 60 min red irradiation at 0.68 W/m^2. Mesocotyl (●). Coleoptile (○). Upward arrow indicates the time at which the red light treatment was initiated. Downward arrow denotes the time at which the primary leaf emerged from the coleoptile. Time is the number of hours elapsed after the time of planting.

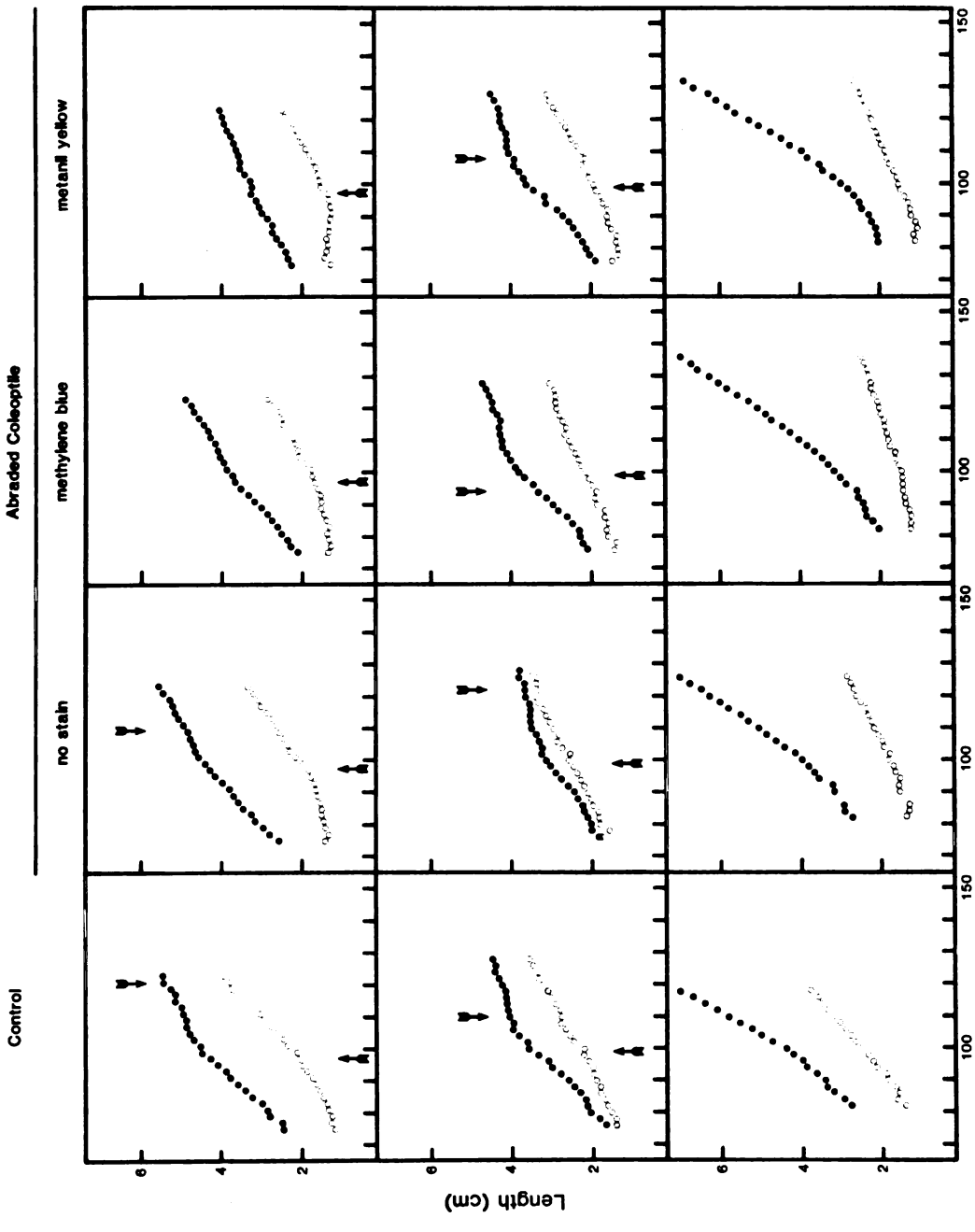
Control
Abraded Coleoptile
no stain
methylene blue
metanil yellow
Length (cm)
Time (h)

Figure 18

curves. However, this was not seen and thus, the tissue preparations did not appear to alter the light sensitivity of mesocotyl tissue. Under these conditions, the light-promotive response of coleoptiles was different from mesocotyls, responding only at the highest fluence used (Fig. 18). This result provided further evidence that the general light sensitivity of the plant tissues was not affected by tissue manipulations. Red-light stimulation of coleoptile elongation, which was also seen for blue-stained coleoptiles, is expected given previous evidence suggesting a perceptive site within the mesocotyl or the coleoptile base (Mandoli and Briggs, 1982b). Although only growth rates of individual seedlings are presented, repetitions revealed these responses to be typical. The slight differences between individual plants (e.g. inner leaf emergence) within a particular tested fluence range probably reflects a normal variance in the population rather than a specific effect induced by a particular treatment.

Photomorphogenesis. All types of seedlings grown under natural lighting conditions in a greenhouse showed an approximately direct linear relationship between light intensity and the degree of mesocotyl inhibition (Fig. 19) (Inge and Loomis, 1937). Lower light intensities yielded seedlings with longer mesocotyls. If the photoperceptive site resides within the buried mesocotyl, then a seedling with an altered red-light gradient should respond like a normal plant which had grown under lower light intensity

Figure 19. Coleoptilar node position as a function of tissue treatment, spectral regime, and light intensity. Node position denotes the point where this structure was located with respect to the soil surface (dotted line). Light intensity was measured at the soil surface of pots containing the transplanted seedlings. Each section of the figure represents one separate experiment with 4 separate seedling treatments and 3 different light intensities. Each experiment was repeated at least twice. The sections of the figure are: (A) white irradiation; (B) red irradiation; (C) blue irradiation; (D) white irradiation of partially stained seedlings (see Spectrophotometry in Materials and Methods). Symbols represent: (●) unabraded coleoptile; (○) abraded coleoptile; (△) metanil yellow-stained coleoptiles; (▲) methylene blue-stained coleoptiles. Each plotted value is the mean of 4 individual seedlings. Vertical bars represent $\pm$ 1 standard error.

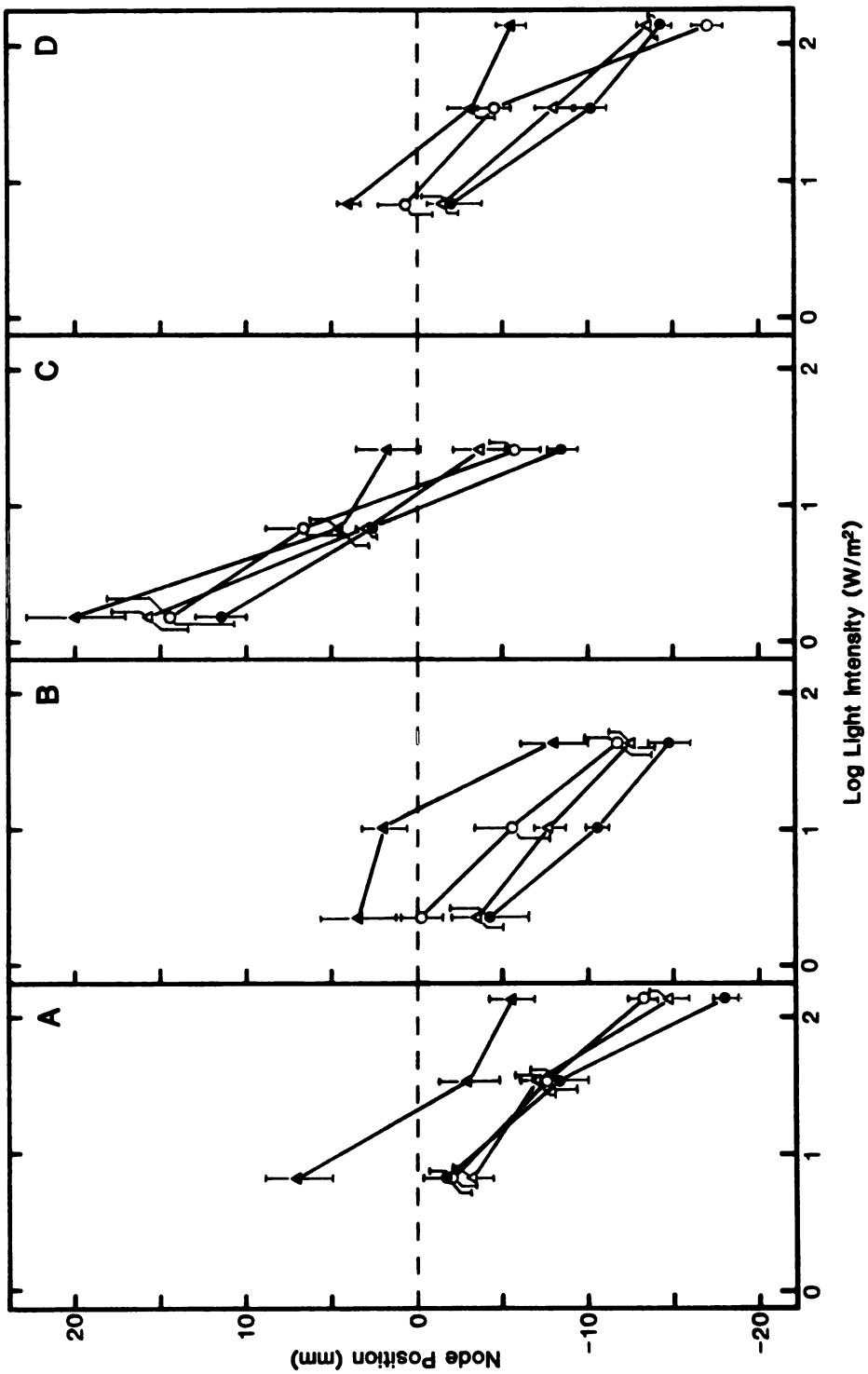
A
B
C
D
Node Position (mm)
20
10
0
-10
-20
0
1
2
Log Light Intensity (W/m²)

Figure 19

(i.e. the curve would be shifted toward higher fluence rate). To test this, photomorphogenesis was analyzed in stained seedlings. Under white or red light conditions, all plants responded similarly to untreated controls except for methylene blue-stained individuals (Fig. 19A,B). Under these white and red-light regimes, the response of blue-stained seedlings shifted toward lower light sensitivity. Since the axial red-light gradient was affected under these conditions, it follows that this alterated response likely resulted from a lower transmitted light fluence at the photosensitive region below the soil surface.

No differences in the response were seen between the treatments under blue-light growing conditions (Fig. 19C). This was expected, given a low blue-absorption for phytochrome, and the lower fluence rates for our blue irradiations. It would appear that the mesocotyls responded to this lower absorbed fluence by elongating above the soil surface. At that point, any effects of an altered axial light gradient could not occur since the mesocotyl tissue was now exposed directly to light. If we had grown seedlings under a higher blue-light intensity, then the response curve for metanil yellow-stained seedlings may have been shifted with respect to the other seedling treatments. This prediction assumes that the blue-light axial gradient is affected in yellow-stained seedlings.

The shift in the dose-response (Fig. 19A,B) seen for blue-stained shoots probably was not a result of absorption of red actinic quanta at the incident surface. An altered response was still seen when the tissue was stained blue in a small section (ca. 4 mm) just above the node leaving the apical region untreated (Fig. 19D). This indicated that apical light reactions, if any, did not effect morphogenic changes within the buried mesocotyl. Additionally, elongation of transplanted seedlings caused a thinning of the stain along the coleoptilar surface (data not shown), so that the absorptive barrier (A_{667nm}) across the coleoptile was lower upon emergence than it was initially (Fig. 15C).

The likelihood that light transmittance through the soil affected mesocotyl elongation is minimal since the light attenuating qualities of the soil are much greater than those of the shoot tissue (Wooley and Stoller, 1978; Mandoli *et al.*, 1981). Measurements for our system indicated that light atteuation by soil is *ca.* 10 times greater than that of the shoot tissue (Fig. 20). Given the magnitude of light attenuation seen for soil, photoinhibition of mesocotyl elongation *via* axially transmitted light would also be significant for more shallow planting depths than those used in these experiments.

Went (1928) and van Overbeek (1936) initially proposed that light affects mesocotyl inhibition by reducing the amount of auxin from the coleoptile. Although at least one study has indicated that a coleoptilar supply of auxin may

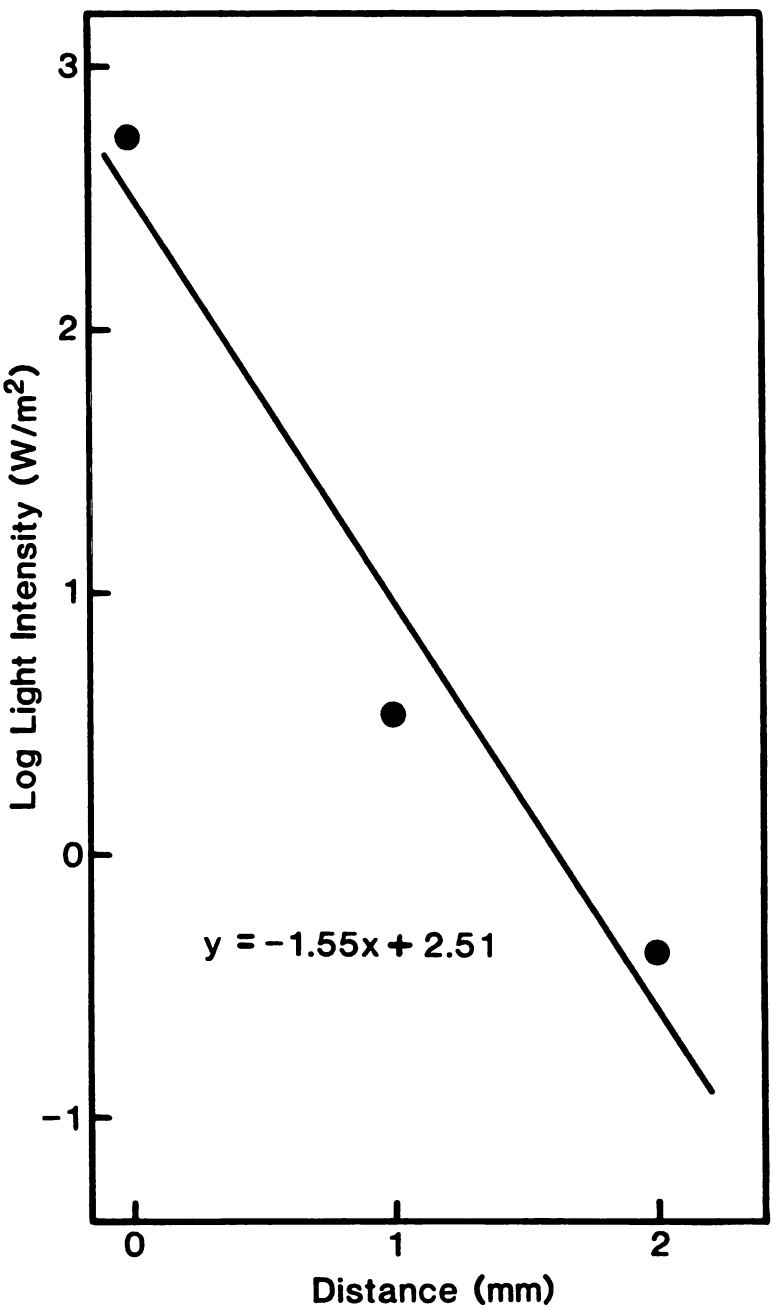

Figure 20. Light intensity as a function of depth beneath the soil surface. White light transmission through the soil was measured by covering the probe of a Kettering radiometer with a thin layer of moist finely sifted peat and loam (particle size < 0.5 mm). The soil surface was located 50 cm below the same lamp used during photomorphogenesis experiments. Each point represents the mean of 3 separate measurements. One standard error never exceeded 30 W/m^2 for any plotted value. $r = -0.97$.

not be required for the response (Schneider, 1941), current literature supports the former proposal (Vanderhoef and Briggs, 1978; Iino, 1982). It is unlikely that the experimental treatments described here affected the supply of auxin from the coleoptile. If tissue abrasion were to have affected auxin transport, then it most likely would have lowered it. An injury-induced reduction in auxin supply would yield shorter mesocotyls (Vanderhoef and Briggs, 1978), which was not seen. This leaves us with the conclusion that either there is an injury-induced effect on auxin supply which is promoted specifically by methylene blue, which is unlikely, or that light is perceived within the mesocotyl and affects coleoptilar auxin supply through some unknown mechanism.

The present results indicate that the site of photoperception for mesocotyl inhibition resides in tissue which is located beneath the soil surface. This site appears to be photostimulated by light which has reached it mainly <u>via</u> transmission through coleoptilar tissue rather than through the far more opaque soil. Thus, the coleoptile not only provides a protective sheath for the frail primary leaves during emergence, but likely serves as an optical structure through which light can pass and effect photomorphogenesis in plant regions which normally are not exposed to direct solar irradiation.

GENERAL DISCUSSION

Experiments directed toward defining photoreceptive sites for mesocotyl inhibition demonstrated that shoot tip irradiation initiated the inhibitory response of the mesocotyl (Goodwin, 1941; Duke and Wickliff, 1969), possibly through a reduction in auxin supply from the coleoptile (Went, 1928; van Overbeek, 1936; Iino, 1982). A logical conclusion from these observations held that light perception occurred in the shoot tip (Goodwin, 1941). Later experiments under controlled light conditions indicated that shoot tip "perception" of light actually might result from tip reception and subsequent light transmission to the true perceptive site in the buried mesocotyl. By examining the optics and light sensitivity of Zea mays, this dissertation has characterized the axial light gradients of corn and their relationship with a single photoperceptive site for mesocotyl inhibition.

It is apparent that photomorphogenic events can be initiated in regions beneath the soil. During the early developmental stages of grasses, a large degree of meristematic activity is localized in the region about the coleoptilar node. This region includes the dividing and elongating cells of the mesocotyl and the leaf primordia. Thus, by effecting the inhibition of the mesocotyl upon

shoot tip emergence, these delicate meristematic regions of the developing shoot will remain below ground. It is possible that this characteristic of young grasses is selectively beneficial. The soil could act as a protective buffer of the more aerial environment. Below the soil surface, temperatures would tend to be more stable, thus protecting the developing tissues from severe thermal fluctuations. Additionally, the structural integrity of the soil might offer a protective barrier against other forms of physical abuse, such as the damages incurred by defoliation.

The buried location of the photoperceptive site is puzzling regardless of whether or not underground photomorphogenesis is selectively advantageous. Since the plant tip is the first area exposed to direct sunlight, it would seem reasonable that photoperception should occur in that tip area. However, it is possible that the perceptive and responsive zones must be identical for certain photoresponses. Many photoresponses are known which support this concept (Haupt, 1960; Satter et al., 1970; Macleod et al., 1984). If this stipulation is also true for mesocotyl elongation, it could explain partially why phytochrome is so abundant just below the coleoptilar node. If mesocotyl inhibition is required below the soil surface, and if photoperception must occur at this buried response site, then large concentrations of phytochrome in this buried region might be necessary provided that a substantial amount of Pfr is required to initiate the photoresponse.

A high concentration of phytochrome within the nodal region would increase the capture cross section so that effective absorption would be maximized. This feature would permit the plant tissue to respond to the more lower fluences normally encountered in buried regions.

The transduction sequence for the inhibitory response appears complex. Our present understanding is that light passes into the coleoptile tip at the moment of emergence, and then is transmitted down the length of the shoot to the perceptive site beneath the soil. The few quanta perceived in this region must then initiate a sequence of events in the coleoptile which limit the auxin supply and thereby limit the elongation of the mesocotyl. This proposed transduction sequence requires the longitudinal transport of multiple signals before inhibition of growth can be effected. Light perception below the node could promote the synthesis and/or release of an inhibitor which would restrict auxin supply from the apical region. However, no light-induced inhibitor is known to occur in the nodal region at present.

A alternative hypothesis is that phytochrome may be involved directly in the hormonal mechanism. Activated phytochrome (Pfr) could act directly on inhibition by affecting the avaliability of hormone receptor sites. It has been proposed and shown that red light can inhibit elongation by decreasing the number of auxin binding sites below the coleoptilar node (Walton and Ray, 1981).

This decrease could be effected by a direct blocking of the receptor sites by Pfr. In vitro studies on sequestering and pelletability indicate that Pfr may associate with membrane components. Membrane binding of Pfr may obscure hormone receptor sites by direct blocking or by pigment-induced changes in membrane conformation. A light-induced limitation of receptor sites would yield a surplus in the pool of available auxin. This surplus might in turn inhibit the rate of further hormone synthesis. An implication of this hypothesis is that the inhibitory response would occur before the decrease in hormone supply (see Firn and Digby, 1980 for discussion), therefore that inhibition is actually caused by the photoreceptor pigment itself and not by a change in growth regulator supply.

These last arguments suggest that the transduction sequence for the inhibition of the mesocotyl may be shorter than previously thought, and thus emphasize the importance of defining the initial events concerning light reception and detection.

Summary

This dissertation has encompassed a study of phytochrome and related photomorphogenesis in a developing grass seedling. The work presented here has demonstrated:

1. The development of an in situ spectrophotometric assay for the characterization of small amounts of phytochrome present within a single undamaged seedling.

2. That a photochemical reaction can be utilized as an *in situ* quantum counter, thereby permitting the non-destructive assessment of light fluxes inside plant tissue.

3. A quantitative analysis of the axial red-light gradients along particular plant structures.

4. That the primary route for axial red-light transmission is along the coleoptile of etiolated and greened tissue (see Appendix A).

5. That light transmitted axially along the coleoptile can affect photomorphogenesis in regions of the plant that are not exposed to direct solar irradiation.

APPENDICES

APPENDIX A

The Internal Primary Leaf of an Emerging Corn Shoot: Its Contribution to Axial Light Transmission and Resultant Mesocotyl Photomorphogenesis

Introduction

Direct evidence now exists to suggest that light-induced mesocotyl inhibition depends on the optical qualities of the seedling shoot (Mandoli and Briggs, 1982b; Chapter 3). The emerging shoot of an etiolated grass seedling acts as an optical structure along which light can travel to a photoperceptive site within the buried mesocotyl. The activation of phytochrome at this site causes the eventual inhibition of mesocotyl growth as well as the likely promotion of coleoptile elongation (Mandoli and Briggs, 1981; Mandoli and Briggs, 1982b; Chapter 3).

At present, little is known about the optical properties of intact plant tissues. This is due in part to the complex nature of light absorption by pigments which are heterogeneously distributed throughout scattering plant tissue (Fukshansky, 1981; Seyfried and Fukshansky, 1983). A comprehensive understanding of the events which control photomorphogenesis is logically dependent on a thorough understanding of the optical qualities of the tissues in question. Thus, many studies have been initiated to obtain a physical descriptions of the changing qualities and

quantities of light once it enters plant tissues (Seyfried and Schäfer, 1983; Mandoli and Briggs, 1984; Vogelman and Björn, 1984). Unfortunately, the optical properties of various plant tissues depend heavily on individual plant morphology, so that a general description for all species is impossible.

Despite these complications, accurate measurements are now available which physically describe the axial light transmitting qualities of young grass seedlings (Mandoli and Briggs, 1982a,b; Mandoli and Briggs, 1984; Chapter 2). Information From these studies and the morphological evidence presented earlier (Chapter 3) indicated that the light transmitting qualities of the inner primary leaf tissue are unimportant with respect to the photomorphological response of the buried mesocotyl. However, experimental evidence is still lacking to demonstrate that light transmission through the immature leaf does not contribute significantly to a photomorphogenic response. The present study was initiated to assess physically the inner leaf's contribution to axial light transmission and the consequential growth inhibition of the buried mesocotyl.

Materials and Methods

Plant material. Two varieties of corn were utilized for these experiments. Unless specified otherwise, the type used most frequently was a hybrid (WF9 x Bear 38, Custom Farm Seed Res., Decatur, IL). On occasion, carotenoidless

mutant and normal phenotype seedlings from the same parent line (lw2 - Gift of D.S. Robertson, Iowa State Univ., Ames, IA) were employed. Seeds were imbibed overnight in distilled water or 2 x 10^{-4}M Norflurazon (SAN 9789; Sandoz, Inc., San Diego, CA). Norflurazon was used exclusively with the hybrid, and always at this concentration, which decreases the carotenoid content in this variety by 80% (Piening, 1984). After imbibition, seeds were planted according to the type of experimental assay utilized: (a) for spectrophotometric analyses, hybrid seeds were allowed to germinate and grow in darkness for 4 days at 24.0 $\pm$ 0.5°C in covered plastic trays (ca. 40 seeds/tray) on Kimpak (Kimberly-Clarke, Neenah, WI) soaked with either distilled water or the carotenoid inhibitor; (b) for growth rate measurements, imbibed seeds were sown individually ca. 2 cm deep in glass shell vials (9 x 2 cm I.D.) filled with distilled water- or inhibitor-moistened vermiculite. The individual vials were then placed within a dark humid chamber (24.0 $\pm$ 0.5°C) for 3 days; (c) for photomorphogenesis experiments, imbibed hybrid, or albino and normal seeds were evenly sown in distilled water- or inhibitor-moistened vermiculite in dark plastic pots (9 x 14 cm; 16 seeds/pot). A 1 cm layer of soil consisting of finely sifted peat and loam was used to fill the remaining volume of the pots, so that the total seed depth was 4 cm. The pots were then placed in a greenhouse (ca. 27°C) for 6 days (see Photomorphogenesis).

Spectrophotometry. All assessments of the light attenuating qualities of corn seedlings were performed as described previously (see Chapter 2). In brief, light attenuation was measured at 632 nm in a single etiolated corn seedling using localized phytochrome conversion as an *in situ* quantum counter. Photochemical conversion was assayed across a seedling at a point 1 mm below the coleoptilar region. By adjusting the distance between the point of actinic irradiation and the point of phytochrome measurement, a given percentage of phytochrome conversion could be attained for a given applied dose from the actinic light source. Use of the previously established dose-response curve for phytochrome conversion in this system permitted a determination of the total quanta transmitted over this given length of tissue.

Absolute absorption spectra of specific tissues above the coleoptilar node were measured spectrophotometrically across a point along a single seedling as described above and in detail previously (see Chapter 2). For these measurements, a rolled white tissue served as a reference.

Light attenuation was measured in both 4 day old dark grown control and inhibitor treated seedlings, either etiolated or pretreated with an actinic blue-light stimulus given just prior to the assay. [Light attenuation was measured over 8 mm along leaf tissue alone, or over 10 mm along the whole shoot (coleoptile plus primary leaf). These distances were optimal for attenuation measurement in

these two respective tissue types (see Chapter 2).] The blue-light pretreatment promoted the formation of chlorophyll within the enclosed primary leaf, yet minimized most phytochrome conversion and its subsequent breakdown. The preparatory blue-light treatment involved wrapping the plant roots in moist tissue and achchoring the shoot onto the surface of a metal plate. The shoots were positioned so that only the region directly above the coleoptilar node was exposed directly to unilateral blue light irradiation. The blue light (1.15 W/m^2) was supplied by an Oriel high intensity xenon lamp (model 6142; Stamford, CT) fitted with a light filter consisting of a sheet of blue plexiglas (#2424; Rohm and Haas, Philadelphia, PA), a DT Blau light filter (Balzers Corp., Hudson, NH), and an 8 cm pathlength of 0.6% aqueous $CuSO_4$ (Fig. 21).

Measurements of attenuation along leaf tissue alone were performed in the same manner as for the whole shoot with the exception that the coleoptile was removed gently just prior to spectrophotometric analysis. All spectrophotometric measurements were perofrmed in darkness with the aid of a green safelight.

Growth rate measurements. Three day old dark grown hybrid seedlings were selected for proper size (3-4 cm shoot length). In darkness, individual vials containing inhibitor-treated or control seedlings were then placed in a rack situated 40 cm away from a Minolta automatic camera (model X-700; Minolta Corp., Ramsey, NJ) equipped with an

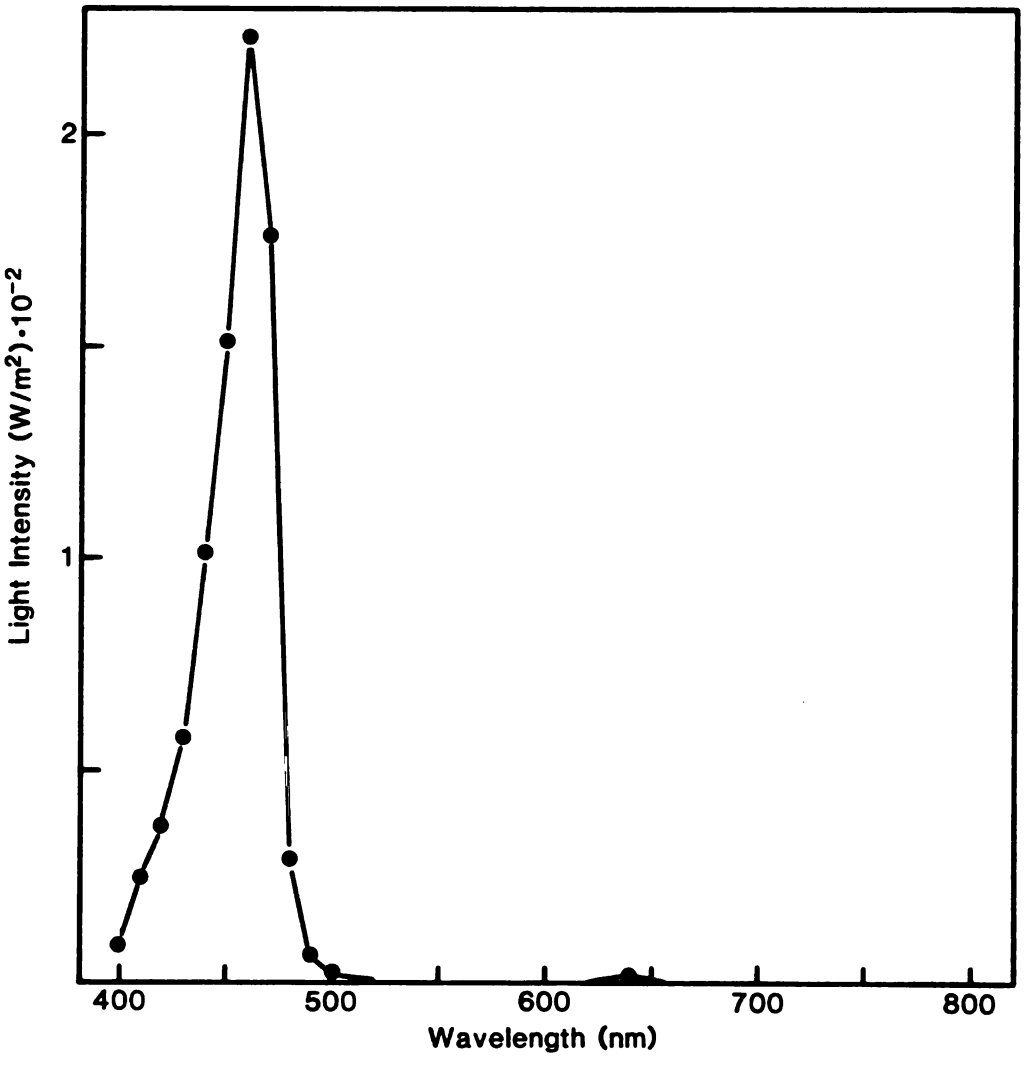

Figure 21. Spectral distribution of high intensity light source used to promote chlorophyll formation in corn seedlings. Integrated light intensity = 1.15 W/m^2.

automatic flash wrapped with a Kodak infra red filter (wratten 87C; Eastman Kodak Co., Rochester, NY). The camera was programmed to take infra red photographs every 2 hours. Time lapse photography was conducted in complete darkness in a temperature controlled room (24.0 $\pm$ 0.5°C). Unilateral actinic light exposures, when used, were provided by a red fluorescent lamp (F15T12-R; Sylvania Inc., Danvers, MA) wrapped with red cellophane ("K" 210FC; DuPont Co., Wilmington, DE) (0.68 W/m^2; see Fig. 13, Chapter 3). This red light treatment did not promote a phototropic response (Iino et al., 1984). The actinic lamp was located 20 cm away from the seedlings. Seedlings grew within a 1.5 cm wide clear plastic corridor during growth experiments. This structure maintained the seedlings within the focal plane of the camera for the experimental duration. Data were recorded by mounting the developed negatives and then projecting the images on a screen from which structure lengths were measured and corrected for the actual size.

Photomorphogenesis. In a greenhouse, pots containing control and inhibitor-treated seeds were distributed evenly in an tiered rack consisting of different levels separated by metal screens, which generated successively lower light intensities. White light (see Fig. 14, Chapter 3) was provided by a 1000 watt multi-vapor metal halide lamp (MV 1000/U; General Electric, Hendersonville, NC) suspended 60 cm above the top growing level of the rack (16 hour day cycle). A distilled water tank (7 cm deep) was placed

between the samples and the light source to absorb excess infra red irradiation from the lamp.

Plants were watered lightly with distilled water or inhibitor every day. After 6 days, data were recorded by clipping the seedlings at soil level and measuring the distance of the coleoptilar node above or below the point of cutting. All elongation of mesocotyl tissue had occured before the sixth growing day so that the seedling node was stable at its position at the time of measurement (data not shown).

Whenever necessary, all integrated light intensities were measured with a Kettering radiometer (model 68; Laboratory Data Control, Riviera Beach, FL). The spectral distributions of all light sources were measured with a spectroradiometer (model 700A; International Light Inc., Newburyport, MA). All dark manipulations were performed with the aid of dim green safelight.

Results and Discussion

It was demonstrated previously (see Chapter 3) that light transmitted axially along the shoot (coleoptile plus inner leaf) is perceived within the buried plant structures. It also was concluded that the path of light along the shoot likely was through the coleoptile alone. To further test this proposal, the effects of changes in leaf pigmentation on the axial light gradient and related photomorphogenesis were assessed. Since chlorophyll significantly absorbs red light (Shibata, 1957), we decided

to compare the axial light gradients in etiolated and partially greened shoots. If the synthesis of chlorophyll alters the light gradient, then the inhibition of chlorophyll accumulation should alter the photomorphogenic response of the buried mesocotyl.

Light transmission in green tissue. When etiolated seedlings were subjected to a long duration light treatment, the red light absorption of the inner leaf increased (Table 3). This absorption of red light by newly formed chlorophyll also may have caused the decrease seen for axial light transmission of the leaf tissue alone (coleoptile removed) at 632 nm (Fig. 22A).

Norflurazon-treated seedlings were used to check that the above changes in atteuation values were only a result of pigment synthesis. Norflurazon-treated seedlings were unable to accumulate substantial amounts of chlorophyll (Table 3). If attenuation measurements were only affected by pigment synthesis, then a "greening" stimulus should not affect the axial light attenuation of the leaf alone in Norflurazon-treated seedlings. However, examinations of inhibitor-treated leaf tissue indicated that a light pretreatment could effect a partial decrease in axial light transmission along the leaf alone (Fig. 22A). Therefore, it must be concluded that a portion of the pretreatment effect on light transmission measurements for leaf tissue was due to changes which were not associated with chlorophyll synthesis.

Table 3. Absorbances of inner leaf and coleoptile as a function of blue-light preirradiation and Norflurazon treatment. Absorbances were measured separately across the inner leaves and the removed coleoptiles of seedlings (WF9 x Bear 38) which had received a given chlorophyll-inducing stimulus. Exposure time is the amount of time seedlings were irradiated unilaterally with blue light (1.15 W/m^2). Norflurazon concentration was $2 \times 10^{-4}M$. Values listed are the means of at least three separate measurements. Bracketed values represent $\pm$ 1 standard error of the mean.

	Absorbance (mA)							
	Control				Norflurazon			
	Coleoptile		Leaf		Coleoptile		Leaf	
Pretreatment time (h)	632nm	667nm	632nm	667nm	632nm	667nm	632nm	667nm
0	0 (0)	0 (0)	16 (1)	64 (5)	0 (0)	0 (0)	14 (3)	29 (6)
2	0 (0)	0 (0)	200 (40)	559 (71)	-	-	-	-
4	0 (0)	0 (0)	560 (100)	1213 (175)	-	-	-	-
6	0 (0)	0 (0)	470 (30)	1043 (54)	0 (0)	0 (0)	33 (6)	109 (36)

Figure 22. Fluences of 632 nm light transmitted axially along blue-light pretreated tissues. (A) Transmission along leaf tissue only (the coleoptile was removed just prior to transmission measurement). Bars represent the dose transmitted over an 8 mm distance from the point of irradiance on the leaf (diagonal arrow) to the point of measurement (x) 1 mm below the coleoptilar node. (B) Light transmission along shoot tissue (coleoptile plus inner leaf). Bars represent the dose transmitted over a 10 mm distance from the point of irradiance in the shoot to a point 1 mm below the coleoptilar node. Time along the x-axis denotes the amount of time the seedlings were preirradiated unilaterally with blue light. For both sections of the figure, closed bars denote control seedlings, while open bars denote seedlings treated with 2 x 10^{-4}M Norflurazon. Each bar represents the mean of at least three separate measurements. Error bars represent $\pm$ one standard error of the mean.

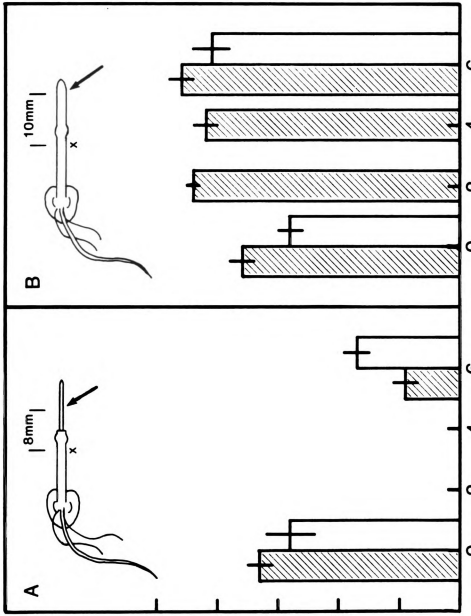

Figure 22

The axial light transmission of whole shoot tissue (coleoptile plus inner leaf) was not decreased after long duration light pretreatment irrespective of inhibitor treatment (Fig. 22B). Red-light absorption by coleoptiles was not affected by the greening treatment (Table 3). However, red-light absorption by leaves in control seedlings increased greatly (Table 3), supporting the idea that pigment synthesis in the leaf tissue does not affect axial red-light transmission along the whole shoot (coleoptile plus inner leaf) (Mandoli and Briggs, 1984). Since the initial concentration of Pr was not known for attenuation measurements in light-pretreated tissue, we can only conclude that the light-pretreatment increased the proportion of light transmitted along the coleoptile vs. the greened inner leaf.

Measurements of light attenuation were limited to assessments at 632 nm. It is likely that any increase in light attenuation through the inner leaf of plants not treated with inhibitor is greater at the Pr absorption maximum (667 nm), since chlorophyll absorption at this wavelength is greater than at 632 nm (Table 3). Therefore, at this more efficiently absorbed wavelength, the relative difference in light attenuation values between the whole shoot and the leaf tissue alone should be greater.

Photomorphogenesis. The axial light gradient of a whole shoot (coleoptile plus inner leaf) was not affected by the synthesis of chlorophyll in the inner leaf (ie. light is

transmitted mainly through the coleoptile). Since, the importance of this light gradient in photomorphogenesis has been established (Chapter 3), treatments which eliminate the chlorophyll content of the inner leaf should not influence the light-inhibited growth response of the mesocotyl. To test this proposal, Norflurazon-treated and control seeds were germinated and grown in a greenhouse under light intensities known to affect differentially the inhibition of the mesocotyl (see Chapter 3). The coleoptilar nodes of seedlings grown under successively higher light intensities position themselves at successively deeper points beneath the soil surface (Inge and Loomis, 1937; Chapter 3). An analysis of this fluence-response relationship for node position under greenhouse conditions revealed that inhibitor-treated plants responded differently from control seedlings (Fig. 23). The upward shift in the fluence-response line for Norflurazon-treated seedlings indicated that their axial light transmitting qualities had decreased. This observation was not expected, given the quantitative results concerning axial light gradients along whole shoot tissue after long duration light pretreatment. However, the photomorphogenic difference between the experimental and control seedlings may have resulted from a non-specific difference between experimental and control seedlings due to the inhibitor alone.

Control and inhibitor-treated seedlings were compared for differences in morphology, growth rate, and red-light

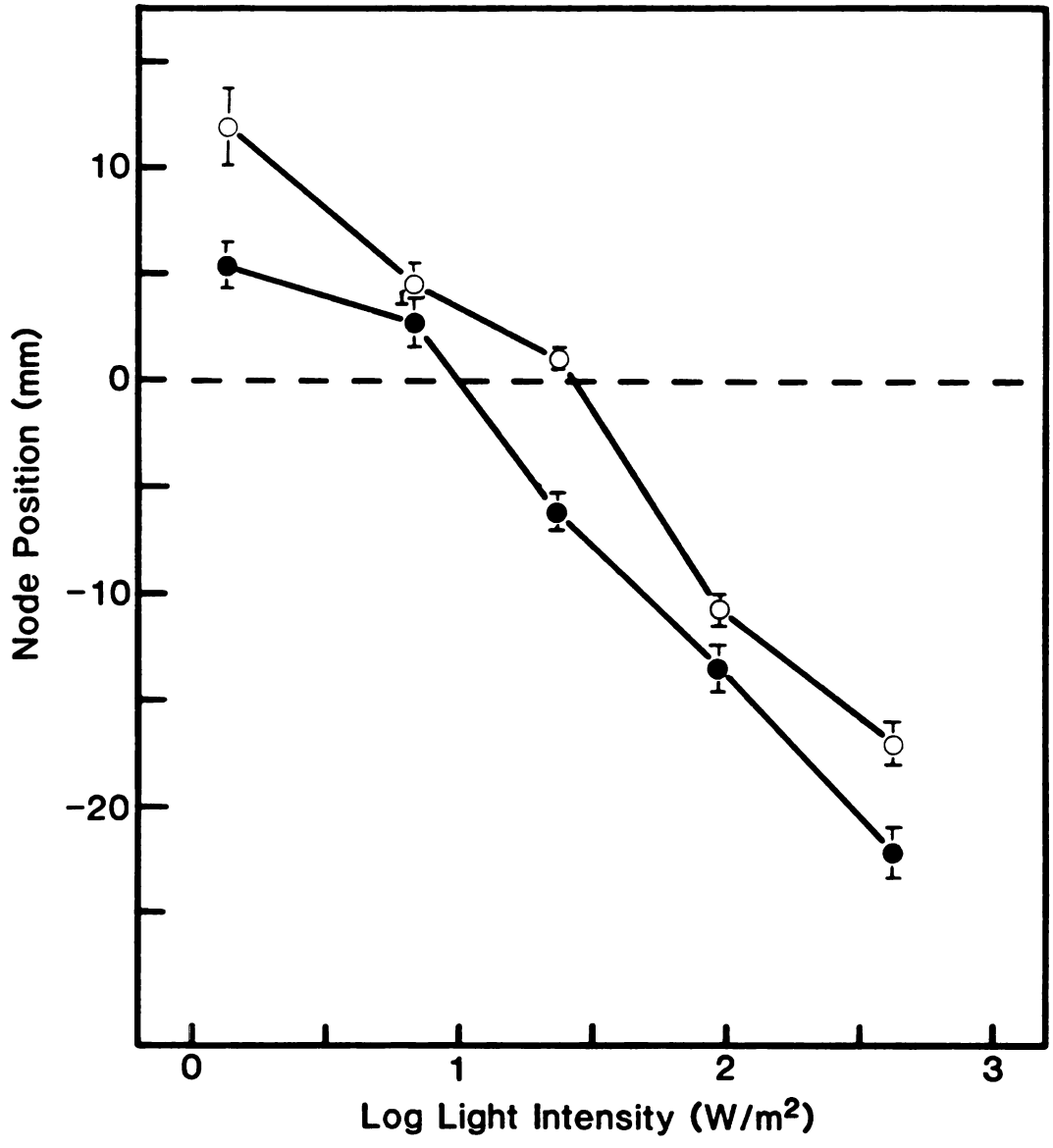

Figure 23. Node position with repsect to soil surface as a function of white light intensity and Norflurazon treatment. Ordinate and abscissa are the same as described for Fig. 19. Symbols represent: (●) control; (○) seedlings treated with 2 x 10^{-4}M Norflurazon. Each plotted value is the mean of at least 5 seedling measurements within a single experiment which was repeated twice. Vertical bars represent $\pm$ 1 standard error of the mean.

sensitivity. The mesocotyl of Norflurazon-treated seedlings showed similar sensitivity to a red light stimulus compared with controls (Fig. 24). It was also evident from the same data that the growth rate of an inhibitor-treated seedling was similar or slightly greater than the control, even after an inhibitory light stimulus. However, the ratios of coleoptile-to-mesocotyl lengths were different for control and inhibitor-treated seedlings (Table 4). These different ratios suggest that upon emergence from soil, a control seedling, initially planted 4 cm deep, should possess a 1.14 cm long coleoptile (ie. the node should be 1.14 cm below the soil surface). Conversely, the node of an inhibitor-treated seedling should located 0.88 cm below the surface at the time of emergence. It is assumed that light reactions are not initiated until emergence. Therefore, this difference in coleoptile lengths between emerging control and inhibitor-treated plants could account for the magnitude of the vertical dose-response shift seen in the photomorphogenic analysis (Fig. 23).

The alternative use of carotenoidless mutant and normal phenotype seedlings overcame this inhibitor-induced morphological difference. These two types of seedlings showed minimal disparity between the coleoptile/mesocotyl ratios (Table 4). The seedlings with a mutant phenotype are unable to accumulate substantial amounts of chloropyhll. When mutant and normal phenotype seedlings were examined under greenhouse conditions, the fluence-response

Figure 24. Effects of red light and norflurazon treatment on coleoptile and mesocotyl elongation. Descriptions of ordinate and abscissa are the same as for Fig. 18. Each section of the figure represents one sample seedling. Norflurazon concentration was 2×10^{-4}M. Bottom row - no red irradiation. Middle row - 25 min red at 0.68 W/m^2. Top row - 60 min red at 0.68 W/m^2. Mesocotyl (●). Coleoptile (○). Upward arrow represents the time at which red light stimulus was given. Downward arrow denotes the time at which the primary leaf emerged from the coleoptile. The experiment was repeated at least twice.

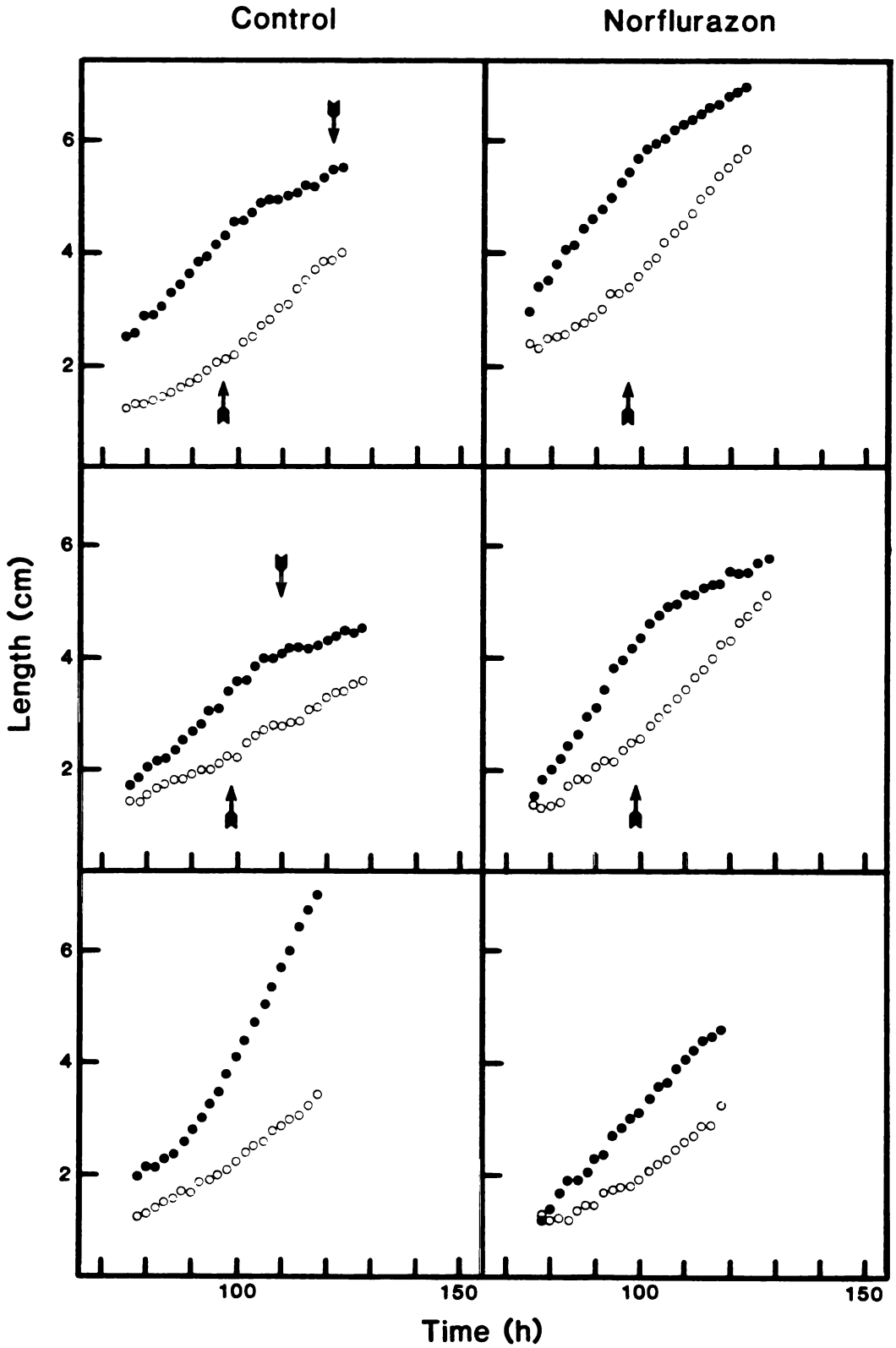

Figure 24

Table 4. Ratios of coleoptile-to-mesocotyl lengths as a function of Norflurazon treatment or altered phenotype. Measurements were taken from 4 day old dark-grown corn seedlings (see (a) - Plant material in Materials and Methods). Norflurazon concentration was 2×10^{-4}M. Values reported are the means of at least 26 individuals. Error reported is $\pm$ 1 standard error of the mean.

Ratio of coleoptile-to-mesocotyl lengths

WF9 x Bear 38		lw2	
Control	Norflurazon	Normal	Mutant
0.40 $\pm$ 0.02	0.28 $\pm$ 0.01	0.26 $\pm$ 0.01	0.26 $\pm$ 0.02

relationship of the mutant showed a small downward shift compared with the normal phenotype (Fig. 25). These results suggested that mutant seedlings transmitted light better than did the normal phenotype. This slight difference in response may have resulted from some unmeasurable difference between the optical qualities of the coleoptiles in the two types of seedlings. It has been shown that the red/far-red ratio measured along coleoptile tissue alone is about 50% smaller for plants grown under white light vs. dark conditions (Mandoli and Briggs, 1984). This difference likely is due to very small amounts of chlorophyll formation in the coleoptiles of light-treated seedlings. Thus, the coleoptiles of mutant seedlings may transmit more red light than the normal phenotype plants under these light conditions.

The photomorphogenic studies using mutant seedlings support the earlier prediction that the axial light transmitting qualities of the inner leaf do not contribute significantly to the morphogenic events within the mesocotyl. The mutant and normal phenotype seedlings may possess inherently different sensitivities to light and/or naturally dissimilar optical gradients in the etiolated state. Additionally, the photochemical events which effect mesocotyl inhibition may occur very shortly after emergence. Studies have shown that the response of the mesocotyl to light is very rapid (Vanderhoef et al., 1979). Therefore, the response of the mesocotyl might be

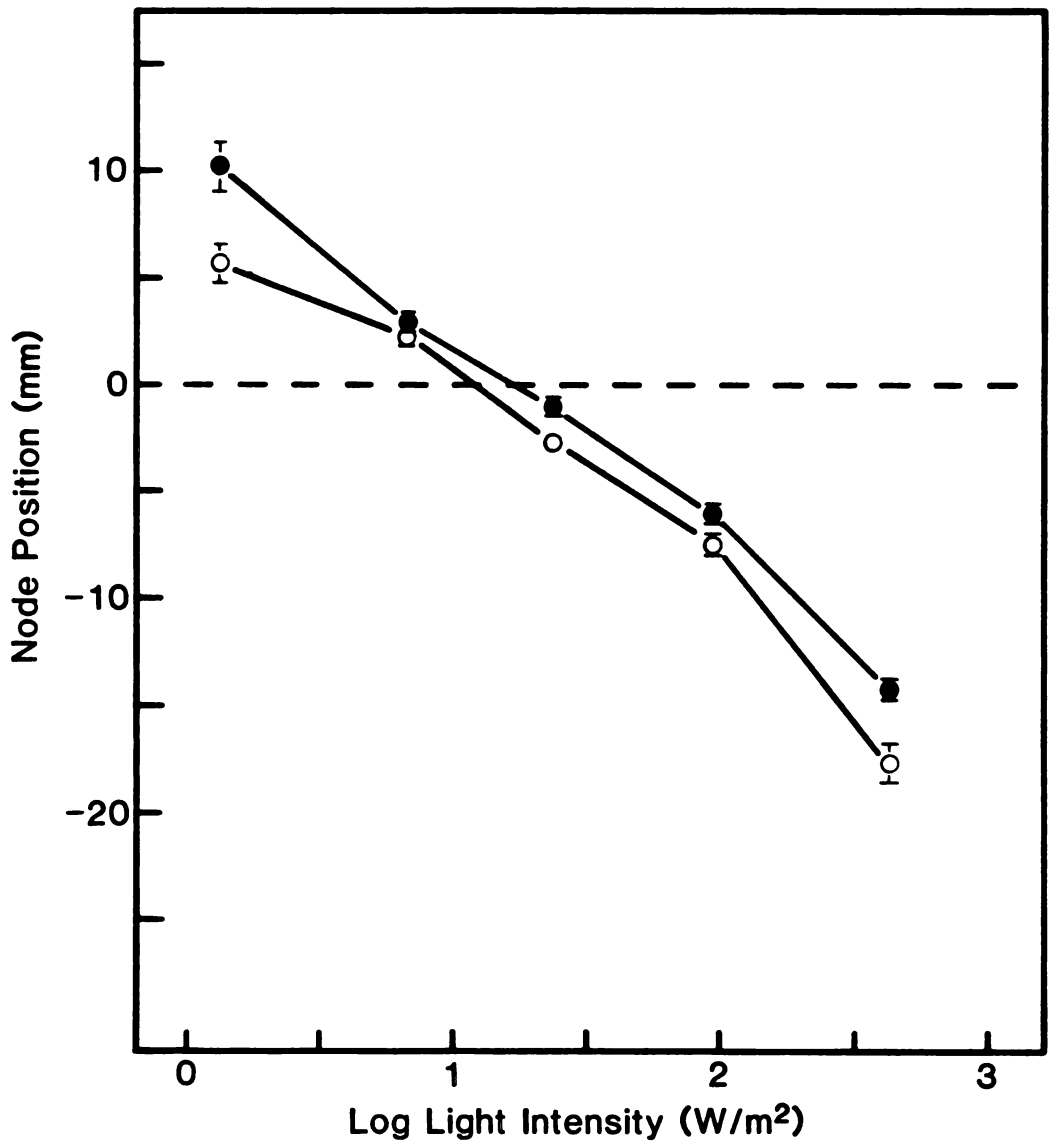

Figure 25. Node position with repsect to soil surface as a function of white light intensity and altered phenotype. Ordinate and abscissa are the same as described for Fig. 19. Symbols represent: (●) lw2 normal; (○) lw2 carotenoidless. Each plotted value is the mean of at least 10 seedling measurements within a single experiment which was repeated twice. Vertical bars represent ± 1 standard error of the mean.

determined before the chlorophyll content has increased enough to affect the axial light gradient. A significant contribution of these unknown factors is unlikely. But, their possibility only permits the very likely conclusion that leaf-transmitted light is insignificant for photomorphogenesis.

Few analyses exist that attempt theoretically to analyze light absorption in heterogeneous scattering material (eg. Fukshansky, 1981). It is known, however, that non-homogeneous pigment distribution within a sample decreases the effective absorption by that pigment (the "sieve effect") (Fukshansky, 1978). In this sense, the corn shoot (coleoptile plus inner leaf) presents itself as a severe example of inhomogeneity with respect to chlorophyll localization. These measurements that light is attenuated less through the coleoptile are consistent with expectations based on the "sieve effect". Recent results indicate that light scatters around the coleoptile without passing through the inner leaf (Vogelman and Haupt, 1985; Appendix B). Therefore, it follows that axial light transmission would be affected minimally by leaf pigmentation. This theoretical analysis combined with the experimental evidence presented here, support the proposal that the coleoptile is the primary path along which photomorphogenically significant light is transmitted axially.

APPENDIX B

A Model for the Mode of Axial Light Transmission

The effect of coleoptilar staining on axial light transmission and photomorphogenesis (Chapter 3) indicated that the coleoptile served as the main route for light transmission along the emerging shoot. Evidence has supported this conclusion by showing that chlorophyll formation within the inner leaf does not affect the axial red-light gradient of the whole shoot (Mandoli and Briggs, 1984; Appendix A). In general, the coleoptile contains less pigment and is more transparent than the inner leaf. Therefore, less attenuation of light should be encountered along the coleoptile.

At least two proposals can descibe theoretically how axial light is transmitted mainly through a particular plant organ. In one proposal, the coleoptile is viewed as a bundle of optical fibers (Mandoli and Briggs, 1982a). The parallel arrangement of coleoptilar cells along the longitudinal axis provides a homogeneous network along which light is reflected internally. Some evidence has suggested that axial light transmission procedes in a coherent fashion along the coleoptile (Mandoli and Briggs, 1982a), thus implying that light can occupy a preferential path in the

seedling shoot (i.e. that a portion of the incident quanta are excluded from the inner leaf). However, this model necessitates the difficult assumption that the coleoptile is structured homogeneously and scatters light minimally.

The main path of light through the coleoptile can also be explained by an analysis of the differences in light attenuation between separate seedling structures (coleoptile and inner leaf). The coleoptile and the inner leaf both possess unique absorption qualities. If the coleoptile attenuates light less over a given distance than does the inner leaf, then the fraction of the total quanta emitted would become progressively greater for the coleoptile as the shoot length increased. Therefore, for long lengths of shoot tissue practically all of the transmitted light could be conceivably within the coleoptile.

This previous analysis assumes that scatter attenuation and the cross-sectional areas of the inner leaf and coleoptile are equal. However, the incorporation of these two additional factors into the present model would only tend to favor primary light transmission along the coleoptile since this structure likely scaters less than the leaf and also possesses a greater cross-sectional area. Estimates of scatter attenuation are difficult. But, since a consideration of scatter coefficients would likely benefit this analysis, their contribution will be ignored by equating their values at zero.

It is possible to estimate the cross-sectional areas for the coleoptile and the inner leaf. If one pictures the whole shoot (coleoptile plus inner leaf; ca. 2 mm dia.) as a rod (the leaf; ca. 1 mm dia.) enclosed within a cylinder (the coleoptile), then by calculation, the cross-sectional area of the coleoptile will be ca. 3 times that of the inner leaf. This difference in area would mean that if light impinged uniformly on this cross sectional surface (for simplicity, the geometry of the shoot tip region is ignored), then ca. 75% of the total incident quanta would enter the coleoptile. Therefore, before any transmission has occurred along the shoot, three quarters of the total quanta are already present within the coleoptile.

Based on the previous geometric analysis and the use of the Beer-Lambert law, it was possible to gain a quantitative estimate for the amount of light transmitted along the coleoptile or the inner leaf as a function of shoot length. However, to make these estimates, one requires a measurement of the absorbance of each tissue per unit length. We measured separately the cross-sectional absorbance (we assume that cross-sectional and longitudinal absorbance are equal) of the coleoptile and inner leaf of an etiolated and a partially "greened" corn seedling in the manner described previously (see Materials and Methods in chapter 1). The pathlengths for both tissue types were approximately equal (1 mm). For one sample etiolated seedling, the absorption at 667 nm (Pr abs. max.) was ca. 7 mA for the leaf and ca. 5

mA for the coleoptile. In a partially greened seedling, the absorbance values were ca. 430 mA and 10 mA for the inner leaf and coleoptile, respectively.

Given these values, and correcting for the difference in cross-sectional area, transmission curves as a function of increasing shoot length were generated (Fig. 26). These curves demonstrate two important features. First, although the separate structures of an etiolated seedling do not absorb much light, the percentage of total quanta transmitted by the coleoptile is never less than 75% for any amount of shoot length (Fig. 26A - dotted line). Second, as the inner leaf accumulates chlorophyll, the transmitting qualities of this structure decrease drastically, so that the percentage of total quanta transmitted by the coleoptile increases beyond 99% after only 4 mm (Fig. 26B - dotted line).

The graphic display of figure 26 demonstrates that the differences in cross-sectional area and light attenuation between the coleoptile and the inner leaf can explain the development of a primary light path along the coleoptile of an emerging shoot. This analysis assumed that the incident radiant flux density was equal for both the leaf and the coleoptile. However, the coleoptile encases the inner leaf completely. Thus, the inclusion of this factor would increase the percentage of light transmitted along the coleoptile since this structure would shade the inner leaf.

Figure 26. Calculated red-light transmission as a function of distance transmitted along the inner leaf or coleoptile of sample etiolated or partially greened seedlings. An individual corn seedling was grown in darkness and measured spectrophotometrically measured as described previously (chapter 1). A partially greened seedling was generated by exposing an etiolated plant to 3 h of white fluorescent light (13.5 W/m^2) just prior to spectrophotometric measurement. Absorbance values (mA/mm) were used to generate the light transmitted for given lengths of tissue. The total number of quanta incident on the plant surface at zero distance (inset drawing within each section of the figure) was set at 100. (A) Etiolated. (B) Partially greened. Solid line - coleoptile. Dashed line - inner leaf. Dotted line represents the percentage of the total quanta which transmit along the coleoptile at a particular point along a hypothetical shoot (drawing at figure bottom).

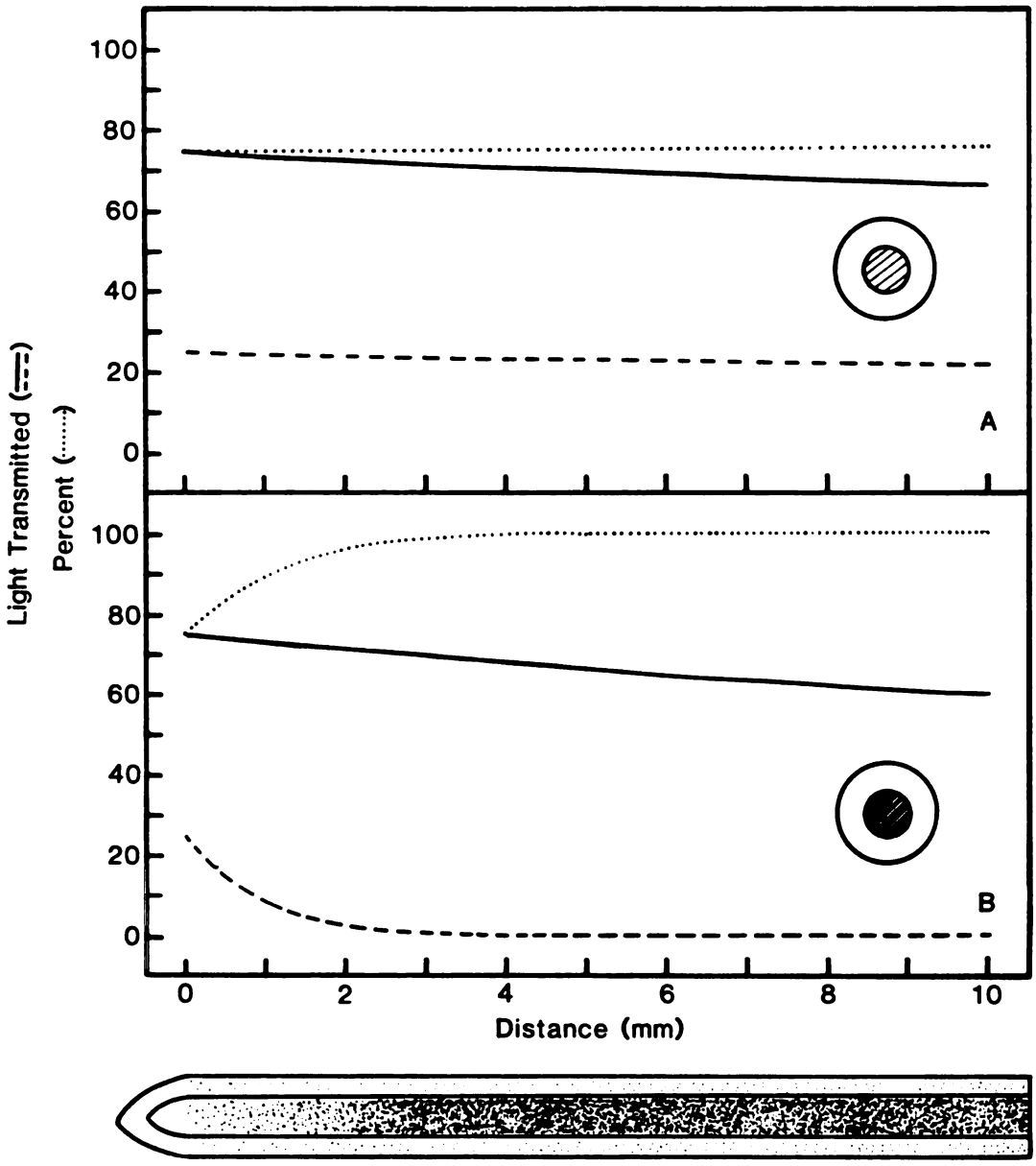
Light Transmitted (=)
Percent (.....)
100
80
60
40
20
0
A
B
0
2
4
6
8
10
Distance (mm)

Figure 26

Summary

Two models have been proposed to explain why light is transmitted mainly along the coleoptile rather than the inner leaf. Experimental evidence has supported a preferential path theory (i.e. optical coherence) (Mandoli and Briggs, 1982a). However, the necessity to invoke the property of total internal reflection tends to complicate this proposal. The model presented here argues that a primary path of light along the coleoptile can be described simply on the basis of differential light attenuation between the coleoptile and the inner leaf. This analysis has not attempted to show that optical coherence does not exist along the shoot tissue. It is quite possible that both are operative to some unknown degree, although this has not been addressed at present.

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