Spatial and Temporal Regulation of Phytochrome A, Phytochrome B Levels and Distribution in Maize and Tomato Seedlings

A Thesis Submitted for The Degree of

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By

K. Ramachandra Reddy

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad- 500 046
INDIA

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Enrollment No. PL 5990

Department of Plant Sciences
School of Life Sciences
University Of Hyderabad

Certificate

This is to certify that the thesis entitled **"Spatial and Temporal Regulation of Phytochrome A, Phytochrome B Levels and Distribution in Maize and Tomato Seedlings"** is based on the results of the work done by **Mr K. Ramachandra Reddy** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University or Institution.

Prof A R Reddy
Head
Department of Plant Sciences

Prof RP Sharma
Supervisor

Prof.A R Reddy
Dean
School of Life Sciences

Department of Plant Sciences
School of Life Sciences
University of Hyderabad

Declaration

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of **Prof R P Sharma** and that this has not been submitted for a degree or diploma in any other University.

K. Ramachandra Reddi

Dated: 13 November 1995 **K. Ramachandra Reddi**
Place: Hyderabad

Prof RP Sharma
Supervisor

TO MY PARENTS

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ABBREVIATIONS

A = absorbance
au = *aurea*
BL = blue light
BSA = bovine serum albumin
cop = constitutive photomorphogenesis
d = day
det = de-etiolation
ein = elongated internode
EODFR = end-of-day far-red
EDTA = ethylene diamine tetraacetic acid
fhy = far-red hypocotyl
fre = far-red elongated
fri = far-red insensitive
FRL = far-red light
FW = fresh weight
h = height
h = hour
HA = hydroxyapatite
hy = elongated hypocotyl
kD = kilodalton
KP buffer = potassium phosphate buffer
l = length
lip = light independent photomorphogenesis
mAP = monoclonal antibodies of pea PHY A
mAT = monoclonal antibodies of tobacco PHYB
min = minutes
MW = molecular weight
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate buffer saline
PCB = phycocyanobilin
PEI = polyethylenimine
pew = partially etiolated in white light
Pr = red light absorbing form of phytochrome
Pfr = far-red light absorbing form of phytochrome
PHY = phytochrome apoprotein
PHY = phytochrome gene
Phy = phytochrome holoprotein
Phy = phytochrome gene mutant
PMSF = phenylmethylsulfonyl fluoride
POR = protochlorophyllide oxidoreductase
PVP = polyvinyl pyrrolidone
PVDF = polyvinylidenedifluoride
RL = red light
SAR = specific absorbance ratio
SDS = sodium dodecyl sulphate
SRID = single radial immunodiffusion
TBST = tris buffer saline tween 20
TCA = trichloroacetic acid
TEMED = N,N,N',N'-tetramethyl ethylene diamine
tri = temporarily red light insensitive
TRIS = (tris[hydroxymethyl]aminomethane)
UV = ultra-violet
w = width
WL = white light
WT = wild-type

CONTENTS

CHAPTER 1

INTRODUCTION

The sessile nature of plants has forced the evolution of several specific sensory and adaptive mechanisms, to adjust to the exigencies imposed on them by environmental fluctuations. The most important among the environmental factors are light, water and temperature, for which plants have evolved specific adaptive mechanisms. Light is a must for the survival of plants, to drive photosynthesis, where light is used as a source of energy. In addition, plants also use light as a source of information by detecting its direction, duration, and spectral quality.

Plants estimate the duration of light by responding to the length of the day, a phenomenon called photoperiodism, which also provides the plant with reliable information about the passage of the seasons (Shropshire and Mohr 1983). Several morphogenetic events in plants such as flowering, dormancy, formation of storage organs etc., are known to be initiated by photoperiodic stimulus.

The perception of the direction of light yields important information, enabling plants to optimize their position in the natural environment by appropriate orientation movements, called phototropism. Most of the photosynthetic mobile algae can move towards a favorable, or away from a harmful light, a phenomenon called phototaxis. Phototropism as well as phototaxis are effective mechanisms of movement to optimize the availability of light for plants.

Plants have also evolved mechanisms to sense the spectral quality of light impinging on them. Since plants largely use the blue and the red wavebands of light in photosynthesis, the transmitted or the reflected light in plant canopies is rich in the far-red region of the spectrum. The perception of enriched far-red light initiates adaptive mechanisms in plants to offset the disadvantage of growing in shade or close to other plant species.

To detect and coordinate all these light mediated physiological responses, plants have evolved specialized photoreceptors. These photoreceptors detect the light spectrum and adjust the growth and development of plants according to the changes in the environment. To date, three groups of photoreceptors have been detected which specifically sense UV-B (280-320 nm), UV-A/blue (320-500 nm) and red/far-red

(600-750 nm) regions of the spectrum. It is believed that these three groups of photoreceptors act both independently as well as cooperatively with each other.

Blue light plays a role in directional responses-phototropism, morphogenic responses-inhibition of hypocotyl elongation, movement of guard cells and biosynthesis of pigments. Though the biochemical nature of this receptor is not known, a gene responsible for some of the blue light mediated responses has recently been cloned from the *hy4* mutant *of Arabidopsis* (Ahmad and Cashmore 1993). The molecular nature of other blue absorbing photoreceptors is not known. The molecular identity of UV-B receptors has not yet been elucidated. Several physiological responses are known to be specifically mediated by UV-B photoreceptors like phototropism (Baskin and Iino 1987), hypocotyl growth (Ballaré et al., 1991) and hormone inactivation (Tevini 1991) etc.

In contrast to the above two groups of photoreceptors, the molecular identity of the photoreceptor perceiving the red/far-red region of the spectrum has been known since 1959, when it could be detected in the crude extracts of maize seedlings by Butler et al., (1959), who named it as phytochrome. Phytochrome has been studied extensively both at the biochemical and molecular levels. Phytochrome is a biliprotein, blue in color, with a monomeric molecular weight of about 116-127 kD, and has an open chain tetrapyrrole as a chromophore (Furuya 1993; Pratt 1995). Phytochrome in plants principally functions to monitor the duration of day length and change in the spectral quality of light. It is involved in several morphogenic responses throughout the life cycle of plants.

It is known that several light mediated physiological responses like hair formation on epidermis, anthocyanin induction in the sub-epidermal layer, stomatal opening in guard cells, chlorophyll formation in mesophyll and bundle sheath cells are restricted to a specific cell or cell layer. Apparently, these responses are likely to be triggered by the photoreceptor located within these cells or cell layers. However, the exact location of the photoreceptor and the relationship between the level of the photoreceptor and the physiological response is not established clearly. In the case of phytochrome, where studies have been carried out about its distribution, the amount of phytochrome was found to be more in the young meristematic cells. It is however not

clear, if such a localization has a direct bearing to its role in growth regulation, but preliminary evidence has favored the idea of site specific action of phytochrome.

The advent of recent techniques of molecular-genetic analysis has revealed that the red/far-red sensing photoreceptor consists of several sub-species (Pratt 1995). Even the diminutive *Arabidopsis* plants possess five genes of phytochrome, which perform discrete functions to regulate the photomorphogenetic phenomena. The discovery of multiple phytochrome species has necessitated the examination of the mode of the action and distribution of each of these species in plants.

Only a limited information is available regarding the comparative distribution of different phytochrome species in plants. Most of the earlier studies have examined only the distribution of the phytochrome species which is predominantly present in etiolated tissue. In the presence study, the distribution of two species of phytochromes was examined using immunochemical methods. The study was also directed towards deciphering the interrelationship between the spatial and temporal expression and the distribution pattern of two phytochrome species namely phytochrome A (light-labile) and phytochrome B (light-stable) in a monocot and a dicot species. In addition, attempts were made to rescue phytochrome to its spectrally active form in the *aurea* mutant of tomato using the exogenously supplied chromophore, phycocyanobilin.

CHAPTER 2

REVIEW

Plants have evolved specialized photosensory mechanisms to interpret the light environment, so that they can suitably regulate their metabolism, growth and development to optimize their survival in the Nature. Using these photosensory mechanisms, plants can detect the intensity, direction, duration and spectral quality of light. The light signal is involved in some way in the initiation or regulation of almost all facets of plant development such as, seed germination, tropic responses, stem growth, leaf expansion, Chloroplast biogenesis, pigmentation, flowering and senescence (Kendrick and Kronenberg 1986).

The photosensory mechanism of plant consists of different photoreceptors to sense the entire range of the visible spectrum. So far, at least three groups of photoreceptors, have been detected by action spectroscopic studies, absorbing specifically in three spectral regions of light - UV-B (280-320 nm), blue/UV-A (320-500 nm) and red and near red region (600-750 nm). It is believed that these photoreceptors independently as well as co-operatively control photomorphogenesis of plants. These pigments detect one or more properties of the light environment such as the quality, quantity, direction and duration of light and suitably modulate growth and development in response to these conditions. The present review deals mainly with phytochrome: its expression, regulation and distribution.

2.1. Blue/UV-A photoreceptors

Blue light plays a role in directional responses like phototropism, morphogenic responses like inhibition of hypocotyl elongation, movement responses like stomatal opening, and metabolic responses like biosynthesis of pigments and gene expression (Kaufman 1993). Although blue-light responses in plants were reported by Darwin over a century ago, so far no chemically defined blue/UV photoreceptor, often referred to as cryptochrome, absorbing specifically in blue spectral regions of the light has been identified or isolated. Among the several molecules which absorb in the blue/UV spectrum, flavins are considered as the most likely candidates for chromophore, although carotenoids and pterins are also believed to be suitable candidates. In fact, Quiñones and Zeiger (1994) have recently presented compelling

evidences that carotenoids like zeaxanthin act as chromophore for phototropism in maize coleoptiles.

The molecular identity of the apoproteins likely to act as blue/UV-A photoreceptor is also not known. Recently, a gene for a likely UV/BL photoreceptor has been cloned from the *hy4* mutant of *Arabidopsis.* The *hy4* mutants do not display blue-light dependent inhibition of hypocotyl elongation (Koornneef et al., 1980). Ahmad and Cashmore (1993) isolated the gene corresponding to the *hy4* locus of *Arabidopsis thaliana* using t-DNA tagging. The *hy4* gene encodes a protein with significant homology to a very rare class of flavoprotein that catalyzes BL dependent reactions. Moreover, the action spectrum of BL inhibition of hypocotyl elongation matches the absorption spectrum of the homologous long wavelength class of photolyases. On the basis of the hydropathy profile, HY4 appears to be a soluble protein like phytochrome. The *hy4* locus shows a close homology of amino acid sequence with microbial DNA photolyases. The photolyases are a class of flavoproteins that catalyze the light dependent repair of pyrimidine dimers in DNA damaged by UV light (Sancar and Sancar 1988). The homology of amino acid sequence of HY4 with microbial photolyases was high in the regions of chromophore binding site which is likely to be a flavin. However, a tryptophan residue which is conserved in microbial photolyases and essential for specific recognition of the pyrimidine-dimer substrate, is not conserved in HY4. The molecular nature of other blue absorbing photoreceptors is not known, but the study of other blue light mutants has pointed to the presence of additional blue photoreceptors for controlling photomorphogenesis and phototropism (Khurana and Poff, 1989).

Since phytochrome also absorbs blue and UV light, it has been difficult in the past to exclude the possibility that phytochrome may be involved in many blue-light mediated responses. The usage of photomorphogenic mutants defective in BL perception has now shown that, 1) low fluence blue responses are mediated by phytochrome, 2) high fluence responses are mediated by BL receptors, and 3) there is an independent photoreceptor for UV-A perception (Liscum and Hangarter 1994). These studies point to the presence of at least three distinct photoreceptors for blue responses, one for UV-A, another for hypocotyl elongation, and a third for phototropism.

2.2. UV-B receptors

Though physiological studies have clearly established the presence of UV-B photoreceptor in a number of species (Wellmann 1971; Drumm-Herrel and Mohr 1981), to date, the biochemical or molecular identity of these receptors has not been elucidated. Using photomorphogenic mutants and flavin quenching compounds, Ballaré et al., (1995) showed that it is likely that flavins act as chromophores for UV-B photoreceptors. There are many physiological responses known to be mediated by UV-B receptors such as phototropism (Baskin and Iino 1987), hypocotyl growth (Ballaré et al., 1991), flavonoid induction (Tevini et al., 1981, 1991; Beggs and Wellmann 1985), hormone inactivation (Tevini et al., 1991) etc. UV-B and blue/UV-A were the most effective wave bands in stimulating anthocyanin synthesis. A step has been taken in the direction of identifying the UV-B receptor by isolation of UV-B mutants such as *uvr1* and *uvh1*. These mutants were found to be hypersensitive to UV-B radiation (Britt et al., 1993; Harlow et al., 1994).

2.3. Phytochrome

The advent of molecular-genetic analysis and its application to photomorphogenesis revealed several additional facets governing plant development, particularly with respect to the phytochrome molecule, its function and signal transduction. Phytochrome is encoded by a small multi-gene family in *Arabidopsis* consisting of at least five genes (Quail 1991). However, information on the molecular properties of phytochrome is restricted only to phytochrome A, which is the only photoreceptor to be purified to homogeneity. It is a bluish chromoprotein of about 116-127 kD monomeric size, with an open chain tetrapyrrole as a chromophore (Kendrick and Kronenberg 1986). The tetrapyrrole chromophore is linked to the apoprotein moiety via a thioether linkage at a cystein residue 321 (Lagarias and Rapoport 1980; Hershey et al., 1985). This chromophore absorbs light and brings about conformational changes in the apoprotein.

Phytochrome exists in two photointerconvertible forms; Pr- the red-light absorbing form which converts to Pfr form after absorbing RL; and Pfr- the far-red absorbing form which converts to Pr form after absorbing FRL. Photoconversion of Pr to Pfr likely involves a Z to E isomerization about the C15 double bond between the C- and D-tetrapyrrole rings of the phytochrome chromophore (Terry et al., 1993).

Phytochrome is synthesized in the Pr form, and accumulates as a soluble cytoplasmic protein. When seedlings are exposed to light, the Pr form of phytochrome converts into the Pfr form, which initiates many photoresponses including the alteration of plant gene expression, involving either transcriptional activation or repression of specific genes (Quail 1991; Thompson and White 1991), bringing changes in several metabolic pathways (Pratt 1982).

2.4. Types of phytochrome

Physiological (Hillman 1967), spectrophotometric (Jabben and Holmes 1983), biochemical and immunochemical (Shimazaki et al., 1983; Tokuhisa and Quail 1983, 1987, 1989; Shimazaki and Pratt 1985 1986; Tokuhisa et al., 1985; Cordonnier et al., 1986; Pratt et al., 1991; Wang et al., 1991) studies demonstrated that there are at least two pools of phytochrome in plants. The most abundant species of phytochrome, which is present in the etiolated tissues in plants is referred to as type I phytochrome (Quail 1991), it is light-labile and present at substantially lower levels in green tissue when compared to etiolated tissue. Since type I phytochrome is encoded by *PHYA* genes it can be referred to as phytochrome A (Quail 1991). Phytochrome A has been purified and extensively characterized. Type II phytochrome is light-stable and present at approximately the same levels in light-grown and etiolated tissues. Molecular studies on this phytochrome has made it clear that type II phytochrome consists of several phytochrome species (Quail 1991).

2.4.1. Phytochrome types: biochemical evidences

Butler (Siegelman and Butler 1965) was the first to propose the existense of multiple phytochrome species in plants. A similar suggestion was made by Heim et al., (1981) on the basis of spectroscopic examination of the kinetics of phytochrome degradation in light-grown tissues of *Amaranthus caudatus.* Subsequently several groups reported immunological evidences for the presence of more than one phytochrome. Tokuhisa et al., (1985) reported that phytochrome in light-grown oat seedlings differed both spectrally and immunochemically from that purified from etiolated oat seedlings. Shimazaki and Pratt (1985) also found that chlorophyllous oat leaves contained a phytochrome which was immunologically distinct from the most abundant phytochrome present in etiolated oat and pea. Pratt et al., (1991) prepared seven different monoclonal antibodies using phytochrome purified from green oat

leaves. Out of these only one cross reacted with the abundant phytochrome species present in etiolated oat shoots. Using monoclonal antibodies raised against different domains of phytochrome isolated from light-grown oat seedlings, Wang et al., (1991) found three phytochrome apoproteins in oat seedlings with molecular weights of 123 kD, 124 kD and 125 kD. The 124 kD phytochrome protein accumulated in relatively high levels in etiolated seedlings. The other two phytochromes accumulated in relatively low levels and had almost the same level in both light- and dark-grown seedlings.

Abe et al., (1989) compared the partial amino acid sequences of the phytochrome purified from green pea leaves with phytochrome purified from etiolated tissues. They found that the N-terminal segment of the green tissue phytochrome is different from that of the etiolated tissue phytochrome, and is likely derived from a gene other than that encoding the more abundant phytochrome. The multiple species of phytochrome have been detected in several species by immunodetection. For example, in *Arabidopsis* three different apoproteins of phytochrome namely, phytochrome A, phytochrome B and phytochrome C were immunodetected by Parks and Quail (1993). In tomato, both phytochrome A and phytochrome B were detected using monoclonal antibodies raised against phytochrome A of pea (mAP) and monoclonal antibodies raised against phytochrome B of tobacco (mAT) (Sharma et al., 1993).

2.4.2 Phytochrome types: molecular evidences

The first unequivocal evidence for the existence of multiple phytochrome species was provided by molecular cloning. In *Arabidopsis,* phytochrome was found to be encoded by a small multi-gene family consisting of five distinct apophytochrome-encoding genes. The full-length cDNAs representing all five of these, *PHYA*, *PHYB* and *PHYC* (Sharrock and Quail 1989), *PHYD* and *PHYE* (Clack et al., 1994) have also been cloned and sequenced. In tomato, five genes have been cloned and sequenced *{PHYA, PHYB1, PHY2, PHYE, PHYF)* and the presence of at least three more genes have been predicted (Pratt 1995).

In sorghum, three *PHY* named as *PHYA, PHYB, PHYC* have been identified by sequence comparisons of PCR fragments to known *PHY* (Cordonnier-Pratt et al., 1994). In rice both *PHYA* and *PHYB* have been sequenced (Kay et al., 1989; Dehesh

et al., 1991) and *PHYC* has been reported in a preliminary fashion (Quail et al., 1991). In addition, complete cDNA sequences have been reported for *PHY. A* and *PHYB* from potato (Heyer and Gatz 1992a,b), and tobacco (Adam et al., 1993; Kern et al., 1993).

It is now known that type I phytochrome, which is light-labile, is encoded by the PHYA gene. Type II phytochrome consists of several phytochrome proteins-phytochrome B, phytochrome C etc., that are light-stable (Quail 1991). The amino acid sequences of these proteins diverged from each other during evolution (Schneider-Potesch et al., 1994). Among these phytochrome B is the most primitive and the other phytochromes have apparently evolved from it.

2.5. Purification of phytochromes

To unravel the molecular mechanism by which phytochrome regulates developmental responses in plants, a knowledge of its structure is necessary. To decipher the structure, structure-function relationship and other biochemical properties, it is necessary to purify phytochrome. Since distinct phytochrome species have been identified in higher plants, their individual purification becomes necessary to understand their role.

2.5.1. Assay

Purification of phytochrome started promptly after its discovery, but it took almost twenty-four years to develop a successful protocol for purifying the phytochrome molecule with properties apparently identical to those of the native phytochrome (Vierstra and Quail 1983). Several problems are encountered during phytochrome purification. Firstly, due to the interference of other pigments like chlorophyll, it is very difficult to quantitate and purify phytochrome from green tissues. Secondly, Phytochrome also undergoes rapid degradation in light, particularly phytochrome A. Moreover, phytochrome is highly susceptible to proteases liberated during homogenization.

During the process of purification, a good assay system should be available, to monitor the purified protein level at different stages of purification. Since the molecular properties of phytochrome are largely unknown and it is not an enzyme, its activity can not be monitored during purification using conventional methods. However, its chromoprotein nature and capacity to exist in two form has been highly useful to estimate it by spectrophotometry during purification.

Spectrophotometric assay can be accomplished by either direct measurement or by difference spectroscopy, making use of phytochrome's unique property to undergo photoreversible absorbance changes. The direct absorbance assay can be used for any clear solution which contains no appreciable absorption from other pigments in the red spectral region. While, the difference spectroscopic method is preferable while measuring samples containing other molecules absorbing in the visible region, this method has the advantage that it can be used to measure phytochrome in opaque samples such as intact tissues or turbid samples.

Phytochrome can also be quantitated using antibodies raised against it by different immunological techniques. Though these techniques have high sensitivity, are insensitive to other pigments and can detect spectrally aberrant molecules, but these techniques can not discriminate between spectrally active and inactive phytochrome molecules.

2.5.2. Purification of light-labile phytochrome

To date only one light-labile phytochrome i.e. phytochrome A has been found. Earlier attempts to purify phytochrome A were based on the procedure established by Siegelman and Firer (1964). They reported 60 fold purification from isolated oat seedlings using ammonium Sulfate fractionation, ion-exchange and gel filtration chromatography. Mumford and Jenner (1966) reported 750 fold purification of phytochrome from oats. This protein had a molecular weight of 55 kD to 62 kD. Hopkins and Butler (1970) obtained a similar preparation with the same molecular weight, with a little higher yield than that reported by Mumford and Jenner. This phytochrome molecule was subsequently shown to be derived artificially from a larger molecule with an apparent monomeric molecular weight of 120 kD by *in vitro* proteolysis during purification which could be prevented by inclusion of PMSF in buffers (Gardner et al., 1971). In order to decrease the time required for purification, Hunt and Pratt (1979) developed immunoaffinity purification procedure which yielded phytochrome with molecular weight of 120 kD. Smith and Daniels (1981) employed agarose-immobilized Cibacron Blue chromatography with a combination of brushite chromatography and purified phytochrome from rye with a molecular weight of 120 kD.

By cell-free synthesis of phytochrome apoprotein in both rabbit-reticulocyte lysate and wheat germ systems, using poly(A) RNA from etiolated oat shoots, Bolton and Quail (1982) reported that the monomeric molecular weight of the protein is 124 kD. Finally, Vierstra and Quail (1983) were able to develop a protocol with a few modifications to isolate the native form of phytochrome with a monomeric molecular weight of 124 kD. They included PMSF, a serine protease inhibitor, in the extraction buffer, and also maintained the protein in its Pfr form throughout the purification. With this protocol pure oat phytochrome A with a SAR value of 0.67 and yield of 13% was obtained (Table 2.1)

Thereafter, several groups improved upon the protocol based on Vierstra and Quail (1983) mainly to reduce the time required for purification and to increase the yield and purity. Litts et al., (1983) developed a different protocol which included pentylagarose chromatography step followed by two hydroxyapatite chromatography steps. They purified phytochrome with a monomeric molecular weight of 124 kD (70%) with a SAR value of 0.65 and about 20% yield. Datta and Roux (1985) concentrated phytochrome after hydroxyapatite chromatography with polyethylene glycol and subjected it to another hydroxyapatite chromatography step. They were able to complete the purification process within 14 h and obtained an average yield of 10%. This procedure is advantageous when compared to that of Litts et al., (1983) as it can be completed in shorter time due to the use of fast flow hydroxyapatite. This procedure also eliminates pentylagarose column used by Litts et al., (1983) and adds a Fractogel molecular sieve column step.

Grimm and Rüdiger (1986) developed a simple and rapid method for the isolation of 124 kD phytochrome from oat seedlings. These authors used the differential solubility of phytochrome and other contaminant proteins at different concentrations of potassium phosphate buffer to purify phytochrome. After hydroxyapatite chromatography, phytochrome was precipitated with ammonium Sulfate and the pellet was washed with 10 mM and 100 mM potassium phosphate buffer to eliminate contaminating proteins including the degraded products of phytochrome, i.e. 114/118 kD proteins. They also included a polyvinylpyrrolidone precipitation step for further purification. They reported an yield of 16% with a SAR value of 0.90-0.99. Chai et al., (1987) used ammonium Sulfate back extraction, hydroxyapatite

Table 2.1 Comparative evaluation of different methods of 124 kD phytochrome A purification in oat

Method	SAR	Yield (%)	No of columns	Time (h)
Vierstra and Quail (1983)	0.67	**13**	**3**	**48**
Lifts et al,, (1983)	0.65	**20**	**3**	**36**
Datta and Roux (1985)	0.8-0.92	**10**	**3**	**14**
Grimm and Rüdiger (1986)	0.92-0.99	**25**	**1**	**16**
Chai et al., (1987)	0.95-1.13	**22**	**2**	**>10**
Lapko and Song (1995)	>1.00	**40**	**1**	-

chromatography and Bio-Gel filtration chromatography and reported a SAR value of 0.99-1.13 and yield of 22% for the purified 124 kD oat phytochrome.

Nakazawa et al., (1990) purified native phytochrome from etiolated pea seedlings using several columns such as pentyl agarose, DEAE-Sepharose, phenyl Toyopearl, Red Toyopearl and Sephacryl S-300. They obtained pure phytochrome with a SAR value of 0.98 with a yield of 7% and molecular weight of 121 kD. Recently Lapko and Song (1995) reported a simple procedure for purification of phytochrome from oat seedlings, using a combination of ammonium Sulfate back extraction and phosphate washings. The phytochrome sample was further subjected to Toyopearl HW-65S gel filtration chromatography and pure phytochrome with a SAR value greater than 1 was obtained. For the first time, these authors could eliminate hydroxyapatite chromatography, which is the major time consuming step in the purification procedure by using the property of differential solubility of phytochrome.

2.5.3. Purification of light-stable phytochromes

Most information regarding the molecular properties of phytochrome has come from the studies on the phytochrome isolated from etiolated tissues. But physiological and *in vivo* spectrophotometric studies clearly implied presence of phytochrome(s) with different properties in green tissues. Recent molecular evidences confirmed the presence of a different pool of phytochrome- light-stable phytochromes. This light-stable pool of phytochrome consists of several distinct phytochrome species in higher plants. In *Arabidopsis*, four *PHY* genes have been cloned, encoding four light-stable phytochromes, namely phytochrome B, C, D and E. The discovery of multiple species of phytochromes initiated efforts to purify and characterize them individually. So far, purification of light-stable phytochrome has been attempted only in oat (Pratt et al., 1991) and pea (Abe et al., 1985) and in both instance only partial purification could be achieved. The attempts to isolate it from other species have not yet been reported.

2.5.3.1. Limitations in purification of light-stable phytochrome

The major limiting factor in achieving homogeneous purification of light-stable phytochrome is its extremely low amount that is 50-100 fold lower than that of light-labile phytochrome (Pratt 1982). In aqueous crude extract of green oat leaves, its level was estimated to be about 0.002-0.003% of the total proteins (Tokuhisa and

Quail 1989). Additionally the presence of pigments like chlorophyll in green tissues strongly interferes during spectrophotometric assay of phytochrome during purification (Pratt 1982).

Earlier approaches to this problem included the use of achlorophyllous tissue (Butler et al., 1963; Jabben and Deitzer 1978) or extracts of chlorophyllous tissue treated either with organic solvents (Taylor and Bonner 1967), with various precipitating agents (Hunt and Pratt 1979; Bolton and Quail 1981) or simply with respective fractionation using ammonium Sulfate and chromatography (Lane et al,. 1963).

2.5.3.2. Purification of light-stable phytochrome

Tokuhisa et al., (1985) developed a method to concentrate and estimate phytochrome from green oat tissues, reducing the chlorophyll content using polyethylenimine and ammonium Sulfate fractionation. The difference spectrum of the partially purified phytochrome showed two peaks; at 652 nm and 729 nm. Immunoblot probed with antibodies raised against 124 kD phytochrome, recognized two bands; a faint band with a molecular weight of 124 kD and another strong band with a molecular weight of 118 kD. Peptide mapping with *Staphylococcus aureus* V 8 protease has proved that 118 kD phytochrome from green oats was different from the 124 kD phytochrome from etiolated oats.

Tokuhisa and Quail (1989) extended the above findings by developing an improved protocol based on Vierstra and Quail (1983) method. After PEI and ammonium Sulfate fractionation, they included HA chromatography and PEI-agarose chromatography. They also found that PMSF, a serine protease inhibitor is not effective in controlling proteolysis. But iodoacetamide and leupeptin which block thiol specific proteases, are effective for the isolation of phytochrome from green plant tissues. With these modifications they reported about 55 µg yield of phytochrome per kilogram of plant tissue. This partially purified phytochrome showed a monomeric molecular weight of 118 kD on immunoblot. The difference spectrum maxima of this partially purified phytochrome were at 654 nm and 731 nm.

Immunoblot analysis of green-oat extracts prepared with non-denaturing buffers and incubated at 20°C for 2 h probed with antibodies raised against oat type I phytochrome showed a shift in phytochrome mobility from 118 kD to 112 kD. In the

immunoblot, the extracts from fresh or freeze-dried green oat tissue extracted directly into boiling sodium dodecyl sulfate-containing buffer also showed a predominant band with a molecular weight of 118 kD. In contrast, Cordonnier et al., (1986) reported a molecular weight of 121 kD to 125 kD for this phytochrome isolated from green oat shoots. Abe et al., (1985) partially purified phytochrome from green pea seedlings using immunoaffinity chromatography. They used agarose columns coupled to monoclonal antibodies raised against 121 kD pea phytochrome, the predominant species in dark-grown seedlings.

Pratt et al., (1991) developed a purification method for the large-scale isolation of phytochrome from light-grown oat seedlings. Using sequential polyethylenimine and ammonium Sulfate fractionation, followed by hydroxyapatite chromatography, they achieved 250 fold purification with about 0.6% yield of pure protein. The purified protein showed absorbance peaks at 659 nm and 720 nm. The monomeric molecular mass of the partially purified sample was 123 kD which is different from 118 kD value reported by Tokuhisa and Quail (1989). With this method the approximate yield was 100 μg of phytochrome per kilogram fresh weight of sample. This partially purified phytochrome sample was used to raise monoclonal antibodies (Pratt et al., 1991). Kidd and Lagarias (1990) purified phytochrome from *Mesotaenium caldariorum*, a unicellular green alga with a monomeric molecular weight of 120 kD and a SAR value of 0.78. The purified protein showed in the difference spectrum, two peaks, one at 646 nm and another at 720 nm.

In general, purification of phytochrome to homogeneity from green tissues was not successful in higher plants. Only one light-stable phytochrome, probably phytochrome B, has been partially purified. Further work is needed to purify other phytochrome species like phytochrome C, phytochrome D and phytochrome E etc. Overexpression of these proteins in transgenic mutants will offer a system for achieving homogeneous purification, though the overexpressed protein may not be exactly similar to the native phytochrome present in the plant system for structural studies. Cloning of phytochrome genes has enabled antibodies to be raised against particular conserved constructs to produce more universally applicable antibodies. López-Juez et al., (1992) raised monoclonal antibodies against tobacco phytochrome B by overexpressing PHYB C-terminal fragment in E. coli. Using this technique,

antibodies against phytochrome B and phytochrome C in *Arabidopsis* (Somers et al., 1991) and against phytochrome B in rice (Dehesh et al., 1991) have also been raised.

2.6. Chromophore biosynthesis and hoioprotein assembly

There are two convergent pathways in the biogenesis of the phytochrome hoioprotein. One involves the synthesis of the apoprotein and the other, the synthesis of the chromophore and its attachment to the apoprotein. The knowledge about the chemical path of phytochromobilin biosynthesis is lacking (Elich and Lagarias 1987). But it is known that the site of phytochromobilin synthesis is the plastid and it is exported to the cytoplasm (Terry and Lagarias 1991), where the formation of the hoioprotein occurs. Moreover, the synthesis of both the apoprotein and phytochromobilin are found to be independent.

The phytochrome chromophore bears a close structural resemblance to the chromophores of phycobiliprotein- phycocyanin and phycoerythrin; the light-harvesting pigments of rhodophyte and cryptophyte algae and cyanobacteria (Rüdiger et al., 1980; Lagarias et al., 1979). Heating these phycobiliproteins with methanol or strong acids, cleaves the thioether bilin-protein linkages and liberates free bilins that have been characterized by spectroscopy (Cole et al., 1967; Crespi et al., 1967). Cleaved bilin free acids have proven to be useful reagents for the study of the biosynthesis of the free phytochrome chromophore and its assembly with the phytochrome apoprotein. Because phytochromobilin has not been readily available, the intermediacy of this pigment in phytochrome biosynthesis has been inferred from the ability of phycocyanobilin to substitute for the natural chromophore precursor both *in vivo* (Elich et al., 1989) and *in vitro* (Elich and Lagarias 1989; Lagarias and Lagarias 1989; Wahleithner et al., 1991; Deforce et al., 1991; Kunkel et al., 1993).

The use of two inhibitors of plant tetrapyrrole synthesis, gabaculine (Gardner and Gorton 1985) and 4-amino-hexynoic acid (Elich and Lagarias (1987) have facilitated the analysis of the pathway of phytochrome chromophore biosynthesis. These inhibitor blocks phytochrome chromophore synthesis resulting in accumulation of phytochrome apoprotein *in vivo.* Etiolated oat seedlings grown in the presence of gabaculine, administered with 5-aminolevulinic acid, or with biliverdin, or with phycocyanobilin- putative precursors of chromophore, exhibited a rapid increase of spectrophotometrically detectable phytochrome (Elich and Lagarias 1987; Elich et al.,

1989). The phycocyanobilin derived photochromic adduct exhibited blue-shifted optical properties, thus providing a unique signature of this newly formed species. Similarly, ***hy1***, ***hy2*** mutants of *Arabidopsis* and ***pew1-1*** mutant of tobacco, characterized as chromophore deficient mutants, were rescued by feeding them with exogenously supplied tetrapyrrole chromophore analogs like biliverdin IXα and phycocyanobilin (Parks and Quail 1991; Kraepiel et al., 1994).

Elich and Lagarias (1989) showed that addition of phycocyanobilin to soluble extracts of 4-amino-5-hexynoic acid (an inhibitor of chromophore) treated seedlings results in a rapid increase in spectrally active phytochrome holoprotein *in vitro*. Further, it was proved that the formation of photochemically active holophytochrome is spontaneous and occurs in the absence of any enzyme or cofactors. Also, phytochrome apoprotein produced by *in vitro* transcription and translation of a full-length oat phytochrome cDNA clone combines with phycocyanobilin to produce a photoreversible holoprotein (Lagarias and Lagarias 1989; Wahleithner et al., 1991). It is believed that the phytochrome apoprotein itself may have the bilin C-S-lyase activity which is necessary for the attachment of the chromophore to the apoprotein.

In another study, it was shown that pea type I phytochrome apoprotein expressed in yeast assembled *in vitro* with phycocyanobilin to produce a photoreversible phytochrome like adduct (Deforce et al., 1991). Recently Li and Lagarias (1994) showed that yeast cells expressing recombinant oat apophytochrome A can take up exogenously supplied linear chromophore analogs to form photoactive holophytochrome *in situ*. In another study, the light-stable tobacco apophytochrome B expressed in yeast could be assembled with phycocyanobilin to give a photoreversible adduct (Kunkel et al., 1993). The ligation of the chromophore to the dimeric apoprotein resulted in a PHYB-phycocyanobilin adduct with the spectral properties of the Pr form of PHYB. Since the assembly of the chromophore to the apophytochrome is not only restricted to PHYA alone, but also takes place in the case of PHYB, it can be assumed that the same is also possible for other phytochromes like PHYC, PHYD etc.

2.7. Phytochrome expression and distribution

2.7.,1 Transcript level and distribution

In contrast to phytochrome A which has been extensively characterized both by spectrophotometry and immunocytochemistry, the expression of *PHY* encoded mRNA has been little investigated (Pratt 1995). Using *in situ* hybridization, in maize *PHYA* transcript was found to accumulate preferentially in the root cap (Johnson et al., 1991). This is consistent with PHYA distribution assessed in other grasses by immunocytochemistry (Pratt and Coleman 1974). Seelay and Colbert (1992) found that in 4-d-old etiolated oat seedlings, *PHYA* mRNA amount is maximal within 10 mm of the tip of the coleoptile and in the coleoptile node, with almost none in the enclosed primary leaf. These observations are again consistent with PHYA distribution assessed immunocytochemically (Pratt and Coleman 1974). These data support the assumption that PHYA mRNA accumulates in the same region of the etiolated seedlings as the PHYA protein. In potato, Heyer and Gatz (1992a) found *PHYA* transcripts to be most abundant in dark-grown sprouts and seedlings. However, in light-grown potato seedlings, *PHYA* mRNA was abundant in the root.

Other than the comparative analysis of *PHYA* and *PHYB* expression in rice (Dehesh et al., 1991), there has been no comparative analysis of *PHY* transcript level in a monocot. In rice, the level of *PHYB* transcripts are not much influenced by light, whereas PHYA mRNA is strongly photoregulated. The relative expression levels of different *PHY* in *Arabidopsis* has been well studied (Sharrock and Quail 1989; Clack et al., 1994). Clack et al., (1994) reported that all five *PHY* in *Arabidopsis* are generally expressed constitutively, the only significant exception being the down regulation of *PHYA* transcripts, either by light or as the plants age. On comparing the transcript levels of all five *PHY* in roots, rosette leaves, flowering bolt stems and whole flower, they found little organ specificity in distribution of the *PHY* mRNAs.

In contrast to the preceding observations made by the analysis of Northern blots, Somers and Quail (1995) followed the transcriptional activity of *PHYA* and *PHYB* promoter throughout the life cycle of *Arabidopsis*. This was done by preparing fusion between the appropriate *Arabidopsis PHY* promoter and the ß-glucuronidase (GUS) reporter gene and by expressing the construct in transgenic *Arabidopsis*.

Whenever a direct comparison can be made, the data are similar to those obtained by Northern blots. Somers and Quail (1995) found the *PHYA* promoter to have 10 fold lower activity in light-grown, when compared to etiolated seedlings, while, the *PHYB* promoter decreased only 2 fold in activity. Interestingly, the activity of *PHYA* promoter in light-grown seedlings was 2-5 fold greater in roots than in shoots which agrees with the observation made for *PHYA* mRNA in potato and oat.

With these same transgenic plants, Somers and Quail (1995) determined both the temporal and spatial distribution of *PHYA* and *PHYB* promoter. The major findings of their studies can be summarized as a) only slight differences were found between *PHYA* and *PHYB* with respect to the spatial distribution of their promoter activities b) both *PHYA* and *PHYB* are active throughout the life cycle in *Arabidopsis* and c) *PHYA* promoter activity is greater than that of *PHYB.* Both promoters are active in light and dark and in both shoots and roots. Moreover, activities of the two promoters are well correlated, with GUS accumulating in chlorophyllous cells, in both the mesophyll and the epidermis of light-grown cotyledons, in vascular tissue; essentially all the cells of 4 week-old leaves, in root tips, in chlorophyllous tissues, in sepals, carpels and in stamens. Discrete differences between the two activities are seen in mature pollen, where only the *PHYB* promoter is active, and in the roots, where only *PHYA* promoter is active.

More recently, Hauser et al., (1994) have assessed by Northern blotting the distribution of five *PHY* mRNA in tomato through its entire life cycle. As in *Arabidopsis,* the *PHYA* transcript is the most abundant throughout the life cycle of green house grown tomato plants. All the mRNAs increase in quantity during the first 5-6 d of development, irrespective of whether the seedlings are grown in darkness or light. All the transcripts begin to decline by 6-7 d except for *PHYE* which is abundant in older plants. The expression of the different *PHY* in tomato is constitutive, with *PHYA* mRNA almost always predominating just as in *Arabidopsis.* In contrast to all other transcripts whose abundance peaks at an early age, *PHYE* mRNA is several fold more abundant in mature plants than in seedlings. *PHYF* mRNA is present at a very low level in the seedling stage, but it reaches the level of other *PHY* transcripts in both leaves and reproductive organs of mature plants.

Adam. et **al., (1994)** demonstrated that in transgenic tobacco plants, all the transgenes, i.e., *PHY-A1-GUS*, *PHY-A2-GUS*, *PHY-A1-CAT* and *PHY-A2-CAT* were expressed identically as that of the *PHY-A* gene at all developmental stages. Their findings show that i) expression of both *PHYA* genes in tobacco is controlled by the same regulatory mechanism ii) *PHYA* mRNA abundance is mainly influenced by transcription rather than mRNA stability, iii) the transcription of the two *PHYA* genes is autoregulated by phytochrome and is not affected by the circadian clock. They also reported that the transcription of *PHYA* genes is down regulated very effectively by light in the early stage of development. Whereas at later stages of development, the down regulation by light becomes less effective and this transition is regulated developmentally. In addition, the expression of *PHYA* genes shows a characteristic organ specific pattern, with expression being maximum in the root tip, hook region and to lesser extent in cotyledons and vascular tissues.

2.7.2. Phytochrome expression: protein level

In order to understand the function of phytochrome, it is important to study its intra- and inter-cellular localization. The two different methods employed to study the localization of phytochrome are a) spectroscopic assay method b) immunological assay method.

2.7.2.1. Inter-cellular distribution

2.7.2.1.1. Spectrophotometry

After the advent of the spectroscopic assay for phytochrome, several investigators used it as a tool to establish its distribution in different tissues and organs. Using this technique, phytochrome has been detected in a wide variety of plant species. Briggs and Siegelman (1965) studied phytochrome distribution using the spectroscopic assay method. In monocots i.e. barley, oat and maize they reported that high amount of spectroscopically detectable phytochrome was present at the base and the apex of the coleoptile and leaf. The also found that phytochrome level was high at the mesocotyl node and very low in the remaining mesocotyl.

In light-grown plants, phytochrome measurements were made using tissues in which chlorophyll was eliminated by herbicide bleaching (Jabben and Deitzer 1978) or in tissues lacking chlorophyll such as roots, tubers and cauliflower curd. Phytochrome was detected in monocot and dicot leaves, roots, cotyledons, fruits,

inflorescence, hypocotyls, petioles, bulbs etc. (Kendrick and Kronenberg 1986). In Norflurazon bleached tissue which lacks chlorophyll, phytochrome was found throughout the secondary leaves of corn, primary leaves of oat seedlings and cotyledons of various dicot plants. By microbeam irradiation, Tepfer and Bonnett (1972) found that phytochrome was present in the root apex of *Convolvulus arvensis.* Gravitropism of roots of this plant was influenced by RL via phytochrome. In rhizomes of 12-14 week-old johnson grass plants, phytochrome was assayed *in vivo* and a basipetal decrease from the apex through the 8 nodes was found (Duke and William 1977). Though spectroscopic assay suffers from the disadvantage of non-specificity, it can be presumed that in etiolated seedlings it largely represents phytochrome A distribution, whereas in light-grown plants, that of light-stable phytochrome.

2.7.2.1.2. Immunocytochemistry

Pratt and Coleman (1971) were the first to apply immunocytochemical technique to study phytochrome distribution. This technique is superior to the spectroscopic assay, as it allows the examination of phytochrome in different tissues of the same organ. In coleoptiles of etiolated oat seedlings, immunochemically phytochrome was localized near the tip and at the coleoptilar node, at the tip of developing adventitious roots and in the root cap (Pratt and Coleman 1971). In etiolated pea seedlings, phytochrome was abundant in the subepidermal and the cortical cells (Saunders et al., 1983). In the epidermis, guard cells had high phytochrome content. Later Pratt and Coleman (1974) studied phytochrome distribution in etiolated grass seedlings by using an indirect antibody-labeling method. They confirmed that phytochrome amount was high near the tip of the coleoptile and the shoot apex in etiolated seedlings such as oat, rye, barley and rice.

Rice, barley and rye also had high concentrations of phytochrome in their leaf bases, but oat leaves were devoid of measurable phytochrome. In cross sections, its amount appeared to be more in the vascular tissues. In phytochrome A overexpressed transgenic tobacco also, phytochrome A was detected in the stem and the petiole vascular tissue, implicating the vascular tissue as a potential site of phytochrome A action (Jordan et al., 1995). In roots, high level of phytochrome was found only in the root caps, with lesser amount in other parts of the root.

In another study, Schwartz and Schneider (1987) studied phytochrome distribution using enzyme linked immunosorbent assay (ELISA) in maize seedlings. Phytochrome level was high in the coleoptile tip, root cap and shoot apex, whereas the mesocotyl and the leaf contained relatively low amounts of phytochrome. The maximal amount of phytochrome was present in 5 d-old dark-grown maize Seedlings.

Recently Wang et al., (1993a,b) studied the expression of three different phytochromes in etiolated oat seedlings using specific monoclonal antibodies by western blotting. They reported that in 3-d-old dark-grown seedlings, all the three phytochromes were expressed in three different organs i.e. the shoot, scutellum and the root. Phytochrome amount was high in the shoots followed by the scutellum and the root. But light-labile phytochrome was more abundant in the root than in the shoot or the scutellum of light-grown seedlings.

2.7.2.2. Intra-cellular localization

Microbeam irradiation experiments showed that phytochrome that controls Chloroplast orientation in the green algae *Mougeotia* (Haupt 1970) was associated with the periphery of the cytoplasm. Similar conclusions were obtained using the protonema of the fern *Adiantum* (Wada et al., 1983). When phytochrome was visualized by immunocytochemistry in non-irradiated etiolated plants, it was found to be distributed uniformly throughout the cytosol. Using immunofluorescence method Saunders et al., (1983) studied the subcellular localization of phytochrome in dark-grown pea seedlings in both cortical and stomatal guard cells of the epicotyl and found that phytochrome exhibits a homogeneous cytoplasmic distribution.

Cope and Pratt (1992) studied the intracellular distribution of phytochrome in hypocotyl hook of etiolated soybean by immunofluorescence technique using monoclonal antibodies. Cortical cells in the hook region were found to exhibit the strongest phytochrome associated fluorescence which is diffusely distributed throughout the cytosol in etiolated seedlings.

2.7.2.3. Sub-cellular localization

By spectroscopic assays of isolated organelles, phytochrome was found to be associated with amyloplasts, etioplasts, etioplast envelopes, Chloroplast envelopes, mitochondria, nuclei, plasma membrane and endoplasmic reticulum. However, most of these experiments represent the non-specific binding of phytochrome to these

fractions. The above reports of the presence of phytochrome in isolated organelles are in fact artifacts; as phytochrome being a charged molecule, binds to these organelles on extraction. Most interesting feature of phytochrome is its extractibility in the pellet after irradiation with RL in the presence of 10 mM $MgCl_2$. This pellet consists of ribonucleoprotein particles (Quail et al., 1973). However, this phenomenon was observed *in vitro* and may not have any biological meaning.

When plants are irradiated first with RL and then homogenized with Mg^{2+}, phytochrome was found in the pellet. One likely explanation of the observed sequestering of phytochrome *in vivo* in the **Pfr** form is its association with the ubiquitin linked degradation pathway. The hydropathy profile of amino acid sequences of all **phytochromes** strongly favors the view that phytochromes are soluble cytosolic proteins and have no amino acids sequence specifically directing them to the nucleus, endoplasmic reticulum, plasma membrane, Chloroplast or mitochondria.

By electron microscopy, phytochrome seemed to be associated with membranes including endoplasmic reticulum, plasma membrane and with mitochondria (Pratt and Coleman 1974). While the Pr form of phytochrome A is uniformly distributed, the Pfr form becomes sequestered immediately after its formation from the Pr form (Mackenzie et al., 1975) and this sequestering is photoreversible. Electron microscopic studies largely indicate that phytochrome is a soluble protein present in the cytosol of the cells and not associated with organelles or membranes.

2.8. Phytochrome degradation

The intracellular level of any protein is dependent on both, the rate of its synthesis and the rate of degradation. Thus, differential regulation of protein stability represents a potential mechanism for modulating gene expression. The level of phytochrome, particularly the light-labile species, is highly regulated by light. The synthesis and degradation of this protein and mRNA has extensively studied, whereas not much information is available about the other phytochrome species.

2.8.1. Phytochrome degradation-protein level

The intracellular concentration of phytochrome is known to be under both developmental and light control (Quail et al., 1973). Phytochrome molecule is

synthesized de novo in the Pr from, accumulating in the dark-grown tissue till a plateau is reached. Transfer to the light results in a rapid decline in phytochrome A level because the rate of degradation of Pfr form of phytochrome is much higher than that of Pr. Two alternative molecular mechanisms have been proposed to account for the rapid turnover of Pfr: the PEST mechanism (Rogers et al., (1986), and the ubiquitin pathway (Jabben et al., 1989; Shanklin et al., 1987, 1989). Oat phytochrome A contains a sequence motif (PEST) similar to several proteins which have short half-lives. Moreover, conversion of phytochrome to Pfr causes residues within the PEST sequence to be more accessible to proteases *in vitro* (Grimm et al., 1989). It is possible that the selective degradation of Pfr in vivo could be signaled by this sequence. However, the immunocytochemical localization of ubiquitin with sequestered phytochrome in RL exposed seedlings has been reported (Speth et al., 1987), which supports ubiquitin mediated proteolysis. But the pool size and the half-life of ubiquitin-phytochrome conjugates were unable to account for the rate of phytochrome degradation (Jabben et al., 1989). Definitive evidence supporting this mechanism of selective turnover is also yet to be obtained.

There is not much information about the synthesis and degradation of other phytochrome species. Since phytochrome B has been found immunochemically in more or less equal amounts in both light and dark-grown seedlings, probably its Pfr form exhibits equal stability as its Pr form. Moreover, all the available phytochrome A sequences contain a PEST motif, but the PHYB and PHYC sequences do not possess it, suggesting that the degradation pathway of different phytochrome species may differ.

2.8.2. Phytochrome degradation-mRNA level

The information about the accumulation and the level of transcripts is available mainly for phytochrome A. The translatable phytochrome A mRNA is only about 0.005% of the total poly(A)-RNA extractable from etiolated oat shoots. Production of Pfr by a brief pulse of RL results in dramatic decreases in both the transcription of phytochrome A genes and the abundance of phytochrome A mRNA (Colbert 1988). Phytochrome A mRNA level is highly light sensitive and even five seconds exposure to RL initiates a rapid decline to a lower level with a >90% decrease occurring within 2.5 h (Colbert et al., 1991). The Pfr form of phytochrome A induces

the decrease of phytochrome A mRNA and approximately 1 % Pfr was sufficient to decrease the mRNA level by 60% (Colbert et al., 1983). The effect of RL is revertible with immediate FRL exposure. Since phytochrome A mRNA levels increase again in light-grown oats that are returned to the dark (Gottman and Schäfer 1982), this negative feedback modulation appears to be reversible upon the depletion of Pfr in extended darkness.

Red-light regulation of phytochrome A mRNA abundance has also been investigated in etiolated seedlings of pea, cucumber, rice, zucchini, corn, tomato and *Arabidopsis thaliana.* Though the effect of RL on phytochrome A mRNA level varies from little change to massive decline, it induces a decrease similar to oat in rice seedlings (Kay et al., 1989) and pea seedlings (Tomizawa et al., 1989). Down regulation of PHYA mRNA in response to RL is less dramatic in etiolated zucchini (Lissemore et al., 1987) and in corn seedlings (Christensen and Quail 1989). In cucumber, 3 h of RL had reduced the level of phytochrome A mRNA to 20% of the initial abundance. Unexpectedly, this was followed by reaccumulation of phytochrome A mRNA up to 60% of dark zero control levels by 12 h after transfer to dark (Tirimanne and Colbert 1991).

Surprisingly, benzyladenine induced a similar decrease like RL in the abundance of phytochrome A mRNA in etiolated cucumber cotyledons. (Cotton et al., 1990). In *Arabidopsis,* PHYA mRNA abundance rapidly decreased after exposure of etiolated seedlings to continuous white-light, and by 5 h of transfer, the same level of mRNA that was present in continuous WL-grown seedlings was found (Shrarrock and Quail 1989). Transcription of phytochrome A genes was rapidly down regulated in response to RL in oat (Quail et al., 1986) and rice (Kay et al., 1989) seedlings. When oat seedlings were grown in continuous WL for 4 d followed by 3 d in darkness, both green and etiolated portions of the leaf accumulated *PHYA* transcripts. A saturating pulse of RL could down regulate these accumulated transcripts both in green and etiolated portions of the leaf (Edwards and Colbert 1990).

It is now believed that phytochrome mRNA itself is inherently unstable, having a half-life of about 1 h both before and after a RL irradiation of etiolated oat seedlings (Byrne et al., 1993). Further, it has been proved that the instability of oat phytochrome mRNA *in vivo* is independent of RL treatment (Seeley et al., 1992).

Recently Higgs and Colbert (1994) proposed two distinct pathways for oat phytochrome A mRNA degradation. The majority of the *PHY. A* mRNA molecules were degraded prior to the removal of the poly(A) tail by 5'→3' exoribonuclease. 25% of the *PHY. A* mRNA were poly(A) deficient which were degraded by a 3'→5' exoribonucleolytic removal of the poly(A) tail followed by both 5'→3' and 3'→5' exoribonuclease activity. These two distinct degradation pathways were poly some associated.

Regarding other phytochromes, not much information is available on mRNA synthesis and regulation. However, in *Arabidopsis* it has been shown that putative type II phytochrome encoding mRNAs *(PHYB* and *PHYC)* may be at least transiently induced by WL (Sharrock and Quail 1989).

2.9. Phytochrome mutants

Phytochrome mutants can be classified into two types. 1) Photoreceptor mutants: These mutants either lack one or the other phytochrome or have some kind of modified phytochromes. 2) Signal transduction mutants: These mutants have modification in some component of the signal transduction chain leading to the array of physiological responses under its control (Kendrick and Nagatani 1991).

2.9.1. Phytochrome deficient mutants

One of the distinct responses of phytochrome in etiolated seedlings is the suppression of hypocotyl elongation. Using this property as a screen, mutants defective in phytochrome functions were isolated. Koornneef et al., (1980) pioneered this screening in *Arabidopsis* and isolated a series of long hypocotyl mutants (*hy1*, *hy2*, *hy3*, *hy4*, *hy5* and *hy6).* Subsequently, *hy7* mutant was identified by Chory et al., (1989b). Similarly, long hypocotyl mutants also have been isolated in other species like tomato (Koornneef et al., 1981, 1985), cucumber (Robinson and Shail 1981) and Brassica (Devlin et al., 1992).

2.9.1.1. Phytochrome A deficient mutants

Mutants which display elongated hypocotyl in continuous far-red, but not in continuous red or WL are considered phytochrome A deficient mutants. Using this screen, phytochrome A deficient mutants have been isolated from *Arabidopsis,* tomato and tobacco. In *Arabidopsis fre1* (Nagatani et al., 1993), *fliy-2* (Whitelam et al.,

1993), *hy8* (Parks and Quail 1993) mutants are phytochrome A deficient. Molecular analysis of the above mutants revealed that, these mutants lack both spectrally and immunochemically detectable phytochrome A, possesses reduced or undetectable levels of *PHY.A* transcript and have structural alterations of the *PHY.A* gene (Whitelam et al., 1993). Interestingly, *hy8-S* mutant has spectrally active but biologically inactive mutated phytochrome A. Thus this mutant provides an opportunity to study the structure-functions relationship regarding light mediated signal transmission (Parks and Quail 1993).

2.9.1.1.1. *fri* mutants of tomato

fri (far-red insensitive) mutants were isolated by Van Tuinen et al., (1995a) by selecting two recessive mutants of tomato with slightly longer hypocotyls than the WT, one under low fluence rate RL and the other under low fluence rate BL. These two mutants are allelic and the loci is located on chromosome 10. In these mutants, immunochemically detectable phytochrome A is absent, but phytochrome B like polypeptide is present in normal amounts and a stable phytochrome pool can be readily detected by spectrophotometry. The *fri* mutants do not respond to FRL for cotyledon expansion and hypocotyl growth, but exhibit efficient chlorophyll biosynthesis and cotyledon expansion upon transfer to WL.

2.9.1.1.2. *pew* mutants of tobacco

In tobacco, *pew* (partly etiolated in white-light) mutants were isolated by Kraepiel et al., (1994). These mutants exhibit long hypocotyls and chlorotic cotyledons under WL. The *pew1-1* mutant is deficient in light-labile pool of phytochrome. However, this mutant has spectrally active light-stable pool of phytochrome. The failure of *in vivo* rescue experiments using biliverdin in *pew2-l* reveals that these mutants are defective in the *PHYA* gene.

2.9.1.2. Phytochrome B deficient mutants

Phytochrome B deficient mutants display an elongated hypocotyl in red and WL but not in FRL (Whitelam and Harberd 1994). Several phytochrome B deficient mutants have been isolated such as *hyS* mutants of *Arabidopsis*, (Nagatani et al., 1991; Somers et al., 1991; Reed et al.,. 1993), *lh* mutant of cucumber (López-Juez et al., 1992), *ein* mutant of *Brassica rapa* (Devlin et al., 1993), ma_3^R mutant of

sorghum (Childs et al., 1991, 1992) and *tri* mutant of tomato (Van Tuinen et al., 1995b). Among these, *lh*, *hy3* and *ein* mutants display pleiotropic phenotypes in field grown conditions that is similar to the shade avoidance response. These mutants are elongated and defective in end-of-day FRL response (EODFR) and they are also early flowering when compared to their isogenic wild-types (Kendrick and Nagatani 1991; Nagatani et al., 1991; Whitelam and Smith 1991). ma_3^R mutant of sorghum, a short day plant, is not elongated but shows photoperiod insensitivity (Childs et al., 1991).

Immunochemical analysis have shown that these mutants are deficient in phytochrome B (Devlin et al., 1993; López-Juez et al., 1992; Nagatani et al., 1991; Somers et al., 1991) except for ma_3^R which is reported to be missing in one of the less abundant phytochromes that predominates in light-grown tissues (Childs et al., 1992).

It has been shown that *hy3* mutation lies within the phytochrome B structural gene (Reed et al, 1993) and the typical phenotype of this mutant results from the deficiency of functional phytochrome B. Further studies have shown that, that mutation in *PHYB* structural gene, affects elongation of many plants (Reed et al., 1993) and in mutant seedlings the expansion of cotyledons is also inhibited (Parks and Quail 1993). Neff and Volkenburgh (1994) demonstrated that phytochrome B is necessary for light stimulated cell expansion in cotyledons *of Arabidopsis.*

2.9.1.3. Chromophore deficient mutants

hy1 and *hy2* mutants of *Arabidopsis* and *pew]-J* mutant of tobacco (Kraepiel et al., 1994) are shown to be real chromophore deficient mutants. *hy6* mutant of *Arabidopsis* and *aurea* mutant of tomato are probably defective in chromophore biosynthesis (Reed et al., 1992; Whitelam and Harberd 1994).

2.9.1.3.1. *hy1, hy2* and *hy6* mutants of *Arabidopsis*

hyl, hy2 and *hy6* mutants are deficient in phytochrome A mediated de-etiolation. These mutants have elongated hypocotyl in WL, RL and FRL. They also have slightly elongated hypocotyl in BL when compared to the isogenic WT (Koornneef et al., 1980). The adult mutant plants are yellow green in color, have increased apical dominance, reduced leaf area, and an overall spindly, elongated appearance (Koornneef et al., 1980; Chory et al., 1989b).

Further analysis of *hyl* and *hy2* properties revealed that they have reduced amount of chlorophyll and high chla/b ratio when compared to WT. The cells have reduced number of chloroplasts and reduced levels of photosynthetic proteins such as LHCP I and II. Thus they have deficiency in their photosynthetic apparatus. RL-induced cab gene expression in etiolated *hyl* mutants is reduced which is FR reversible. Also, the germination of *hyl* and *hy2* seeds is promoted by RL and these mutant seedlings display elongation growth response to a reduction in R/FR ratio.

It has been reported that dark-grown *hyl*, *hy2* and *hy6* mutants have greatly reduced levels of spectrally detectable phytochrome, but have WT levels of immunochemically detectable phytochrome A in both light- and dark-grown seedlings (Chory et al., 1989b; Parks et al., 1989). These observations suggest that the *hyl*, *hy2* and *hy6* mutants have normal levels of non-functional phytochrome A apoprotein. When *hyl* and *hy2* mutants were grown in light on a medium containing biliverdin IXα, a precursor of phytochrome chromophore, these mutant seedlings displayed a phenotype indistinguishable from that of the light-grown WT plants. Further, these seedlings showed similar levels of photochemically active phytochrome when compared to WT control seedlings. Most probably, these mutants are blocked at a stage of phytochromobilin biosynthesis prior to biliverdin IXa (Parks and Quail 1991).

Regarding the pleiotropic phenotype of these mutants, the deficiency in tetrapyrrole biosynthesis may have other effects on the physiology of these plants, in addition to its effect on phytochrome function. Therefore, some aspects of the *hyl* and *hy2* phenotypes such as the yellow green color may not be solely due to the defect in functional phytochrome.

2.9.1.3.2. *pew1-1* mutant of tobacco

Similar to *hyl* and *hy2* mutants of *Arabidopsis*, *pew1-1* mutants of tobacco exhibit long hypocotyls and chlorotic cotyledons under WL and contain spectrally inactive but immunochemically detectable phytochrome A in etiolated seedlings (Kraepiel et al., 1994). In these mutants, biliverdin restores light regulated responses and increases photoreversible phytochrome when grown in darkness indicating that *pewl-1* locus may be involved in chromophore biosynthesis (Kraepel et al., 1994).

2.9.1.3.3. *Aurea* mutant of tomato

The *aurea* mutant of tomato shares several characteristics similar to the chromophore deficient *hyl* and *hy2* mutants of *Arabidopsis*. When compared to WT, *aurea* seedlings show reduction in hypocotyl growth inhibition in WL, FRL, RL, BL and UV-A light (Koornneef et al., 1985; Adamse et al., 1988). They also show reduced amount of anthocyanin (Adamse et al., 1989), chlorophyll and retarded Chloroplast development (Koornneef et al., 1985; Ken-dror and Horwitz 1990; Neuhaus et al., 1993). Moreover, this mutant shows reduced photoregulation of the transcript levels of cab proteins of photosystem I and II (Sharrock et al., 1988; Oelmiiller and Kendrick 1991). Field-grown *aurea* plants have yellow-green leaves which are slightly juvenile in appearance. Though *aurea* has reduced amount of chlorophyll content, their growth and response to end-of-day-far-red (EODFR) treatments is normal (Adamse et al., 1988).

Like *hyl* and *hy2* mutants of *Arabidopsis*, etiolated *aurea* seedlings have reduced levels of spectrally detectable phytochrome A (Koornneef et al., 1985). But *aurea* differs from *hyl* and *hy2* in that it also has reduced immunochemically detectable phytochrome A (Parks et al., 1987; Oelmüller et al., 1989). Though *aurea* mutant contains normal levels of phytochrome mRNA which on *in vitro* translation yields phytochrome apoprotein, the protein fails to accumulate *in vivo* (Parks et al., 1987; Sharrock et al., 1988). Using PHYA specific antibodies Sharma et al., (1993) reported about 20% of the WT level of PHYA. Also, they showed that this PHYA protein is stable in light. Since light-grown *aurea* seedlings have WT levels of spectrophotometrically detectable phytochrome (López-Juez et al., 1990), and respond normally to EODFR irradiations, and low R/FR ratio by increasing elongation growth (Kendrick and Nagatani 1991, Whitelam and Smith 1991), the *aurea* mutant has been regarded being deficient in phytochrome A.

However, it has been shown that the *aurea* mutant has WT levels of translatable phytochrome mRNA (Sharrock et al., 1988) and the *aurea* locus and the phytochrome locus are located on different chromosomes (Reed et al., 1992). These observations suggest that the *aurea* mutant is probably chromophore deficient. It is assumed that phytochrome apoprotein accumulation does not occur because it is unstable in the absence of the chromophore. When *aurea* was transformed with oat

PHYA3 gene, no PHYA apoprotein was detected by western blotting in the transformant (McCormac et al., 1993). This may be due to some defect in the translation process in the *aurea* mutant, and the *aurea* phenotype may not arise due to the deficiency of chromophore. In another experiment, oat *PHY.A3* overexpressing line was crossed with *aurea* mutant as well as the phenotypically similar *yg2* mutant. *Au-PHYA3* and *yg2-PHYA3* double mutants accumulated PHYA apoprotein but they did not show any rescue of phenotype (Kendrick et al., 1994) indicating a probable deficiency in chromophore.

2.9.1.4. Signal transduction mutants

Mutants which contain a photoreceptor but lack photomorphogenic responses of that particular photoreceptor (Chory 1992; Whitelam et al., 1993) and also mutants which display light mediated phenotype in dark can be considered signal transduction mutants.

Mutants that express light induced genes at high levels in dark have been isolated. For example, several of the *det* (Chory et al., 1989a, 1991) and *cop* (Deng et al., 1991, Wei and Deng 1992) mutants of *Arabidopsis thaliana* and the *lip* (Frances et al., 1992) mutant of pea have been isolated on the basis of their abnormal deetiolated morphology in dark. Mutations identified in this way are pleiotropic and affect multiple down stream events or have no effect on light regulated gene expression. DET and COP are most likely to regulate early steps in the light regulated signal transduction pathway. The recessive nature of *det* and *cop* loci suggests that the WT gene products act to repress the photomorphogenic development in darkness, and light reverses this repressive action. Mutations in *cop J, cop8, cop9, cop10, cop 11* and *det1* results in the most pleiotropic phenotype. These six loci *may* be involved in the early steps of light signaling before the branched pathway that control individual developmental processes (Deng 1994). Recently it was found that severe mutations in all six loci lead to purple seed color, therefore they were named *FUSCA* loci (Castle and Meinke 1994).

cop1 locus encodes a novel protein that consists of a zinc binding motif at the N-terminus, a putative coiled-coil region, and several WD-40 repeats in the C-terminal half that is homologous to the ß-subunit of the trimeric G-proteins and is localized in the nuclei of dark-grown seedlings (von Arnim and Deng 1994). COP

may act as a negative transcriptional regulator. DET1 is a nuclear localized protein and negatively regulates transcription without binding directly to DNA (Pepper et al., 1994).

fhy1 and *fliy3* mutants of *Arabidopsis* are also considered as signal transduction mutants and probably these loci encode products which are involved in the signal transduction pathway of phytochrome A (Whitelam et al., 1993).

2.10. Phytochrome-role

Since there are some overlapping as well as separate functions for both phytochrome A and phytochrome B, probably some components in the signal transduction pathway are common for both phytochromes and some other components are specific to each phytochrome.

Under these circumstances, a lot of information is needed to ascertain the role of each phytochrome. Probably under natural field grown conditions, only light-stable phytochromes play an important role. Since double mutants for both *PHY.A* and *PHYB* are also able to survive in the natural field grown conditions, it can be assumed that other phytochromes, such as phytochrome C, D and E also have important roles. The exact physiological roles of these phytochrome remain to be determined. They may control specific aspects of photomorphogenesis, such as photoperiodism and flowering, that are possibly not assigned to phytochrome A or B (Vierstra 1993). The analysis of plants with specific mutations in these remaining phytochrome genes will be necessary to answer this question. In the absence of one phytochrome, the other phytochrome(s) may take up the role of the impaired phytochrome in order to help the plant survive. Probably this is an adaptation mechanism to overcome any spontaneous mutations that may occur in the long process of evolution.

The multiplicity of photoreceptors have raised questions about their relation and respective roles in adaptation of plant growth and development to changes in the light environment. Photomorphogenic mutants proved to be a valuable resource in assigning physiological roles to different phytochromes.

Phytochrome B deficient mutants have elongated hypocotyls when grown in WL and RL, but display near normal inhibition of hypocotyl elongation when etiolated seedlings are exposed to FRL (Koornneef et al., 1980; Devlin et al., 1992). Phytochrome B mutants also show reduced expansion of cotyledons and pigmentation

(Adamse et al., 1987; Chory 1992). These observations indicate that phytochrome B plays a role in some aspects of de-etiolation. Phytochrome B mutants do not show significant growth promotions by end-of-day FRL treatments (L6pez-Juez et al., 1990; Nagatani et al., 1991; Devlin et al., 1992) and respond only poorly to reductions in R:FR ratio (Whitelam and Smith 1991; Devlin et al., 1992). Phytochrome B is also responsible for shade-avoidance response to low R:FR ratio (Whitelam and Smith 1991; Robson et al., 1993). *PhyB* deficient *hy3* mutant flowers early compared to the wild-type plant (Goto et al., 1991; Whitelam and Smith 1991), indicating that phytochrome B helps to regulate the timing of events in response to the environment like growth rates and flowering time. However, phytochrome B appears not to be necessary for initiating these events, since these plants can go through the complete life-cycle in the absence of phytochrome B (Reed et al., 1993). Interestingly, Pr form of phytochrome B plays an active role in the regulation of gravitropism in dark-grown seedlings of *Arabidopsis* (Liscum and Hangarter 1993). Using *lh* mutant of cucumber, Saefkow et al., (1995) showed that Pr form of phytochrome B is an active positive regulator of development in elongated plants.

Phytochrome A deficient mutants have elongated hypocotyls when grown in 8 h light/16 h dark photoperiod compared to their WT (Johnson et al., 1994) and show normal height when grown in continuous WL (Whitelam et al., 1993). In contrast to phytochrome B deficient mutants, phytochrome A deficient mutants exhibit phenotypes indistinguishable from WT when grown in WL. This indicates that under field grown conditions, phytochrome A has a limited role. But it has been shown that phytochrome A plays an important role in photoperiod detection and mediates end-of-day FRL responses (Whitelam and Harberd 1994). Using transgenic BN1 seedlings of tobacco, which overexpress rice phytochrome A, Schäfer et al., (1994) showed that PHYA is also responsible for apical hook opening.

Based on phytochrome A and phytochrome B antagonistic signals in response to continuous red and far RL, Quail (1995) proposed a yin-yang type relationship between these two photoresponses. Continuous FRL induces deetiolation, but this response is suppressed by simultaneous irradiation with continuous RL absorbed by phytochrome A. Whereas, continuous RL induces deetiolation and this response is suppressed by simultaneous irradiation with continuous FRL. However, due to the

rapid light induced decline in the amount of phytochrome A in continuous far-red rich light, the balance in this mutual antagonism between phytochrome A and phytochrome B shifts rapidly during deetiolation from phytochrome A dominance initially, to phytochrome B dominance in fully deetiolated seedlings. Thus seedlings emerging from soil darkness into open sunlight appear to use primarily phytochrome B for deetiolation. Whereas, seedlings emerging into far-red enriched vegetational shade appear to use primarily phytochrome A for initial etiolation, and then later phytochrome B to signal the shade-avoidance response, presumed to enable shaded plants to strive to reach the top of the canopy rapidly (Quail 1995).

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant material

Oat *(Avena sativa* L.) seeds were obtained from Vikram Seeds, Neemuch, India. Maize *(Zea mays* L. cv. Ganga-5) and pearl millet *(Pennisetum americanum* L. cv. WCG-75) seeds were obtained from Andhra Pradesh State Seed Corporation, Hyderabad, India. *Aurea* mutant of tomato *(Lycopersicon esculentum* L.) and its isogenic wild type (cv. Alisa Craig) seeds were obtained from Dr. R.E. Kendrick, Netherlands. Rice *(Oryza sativa* L.), wheat *(Triticum aestivum* L.), tobacco *(Nicotiana tabacum* L.), and spinach *(Spinacea oleracea* L.) seeds were purchased from the local market.

3.2. Growth and harvesting of seedlings

3.2.1. Oat

Oat seeds were first washed with tap water and then twice with distilled water. The washed seeds were soaked overnight in distilled water at room temperature (20-25°C). Thereafter the oat seeds were rolled in germination papers. About five grams of seeds were placed in a single row at a distance of 2 cm inside the upper edge of the germination paper strips (10 cm *w* x 45 cm *l*). Thereafter, the papers were rolled tightly and tied with rubber bands. The paper rolls were placed in plastic trays (28 cm *w* x 39 cm / x 6 cm *h*) with their lower edges dipped in 4 cm of distilled water. Seedlings were grown either in total darkness or under continuous red-light at 25°C till the time of harvest. For purification of phytochrome A, 4 kg of oat seeds were sown for each experiment.

About 1 kg of oat shoots were excised at the mesocotyl node under dim green safe light (λ_{max} 530 nm). The excised shoots were kept on aluminum foil layered on ice. The shoots were irradiated with RL (λ_{max} 650 nm, fluence rate 6.0 Wm^{-2}) for 10 min prior to homogenization.

3.2.2. Spinach

Spinach leaves were either harvested fresh from the plants grown in the fields of the University or young and fresh leaves were purchased from the local market.

3.2.3. Maize

Maize seeds were first soaked for 12 h in distilled water and then sown on moist germination papers in plastic trays. The trays were covered with transparent glass plates for 4 days, then left open. The seedlings were watered everyday with distilled water. Seedlings were grown under continuous RL (λ_{max} 650 nm, 0.67 Wm^{-2}) or in complete darkness at 25°C.

To analyze distribution pattern of phytochrome, 5d-old seedlings were dissected into root, mesocotyl, coleoptile and primary leaf. In the coleoptile and the first leaf phytochrome distribution was analyzed by dissecting these organs into 1 cm long segments from the base to the tip. For both the leaf and the coleoptile, the segments were numbered from the base to the tip. The endosperm, scutellum (de-embryonated) and embryo were separated from the seeds imbibed for 24 h at 4°C in distilled water. After separation, the organs were thoroughly washed with distilled water to remove any adhering contaminants.

Rice, wheat, and pearl millet were also grown in a fashion similar to maize. Tobacco seedlings were grown in vermiculite in plastic boxes.

3.2.4. Tomato

Tomato seeds were first washed with distilled water and then surface sterilized by rinsing in 75% (v/v) ethanol for 5 min followed by several washes with distilled water. The seeds were soaked in distilled water overnight at 4°C before sowing on 1% (w/v) agar containing 0.6 mM KNO_3 in transparent plastic boxes (9.5 cm *w* x 9.5 cm / x 5 cm *h*). Thereafter, seedlings were grown either in complete darkness or under continuous WL.

For rescue experiments, of *aurea* mutant by exogenous chromophores, the seedlings were grown on agar medium supplemented with or without 66 µM phycocyanobilin (PCB). The required amount of PCB was mixed at 40°C before the solidification of agar. For the experiment of *in vitro* reconstitution, agar medium was prepared in 1 mM HEPES buffer, pH 7.4 containing 0.6 mM KNO_3.

3.3. Light sources

Red light (0.67 Wm^{-2}) was obtained by filtering the light from two cool white fluorescent tube lights (40 W) through two red Plexiglas sheets (λ_{max} 650 nm). Long

wavelength far-red light (6.0 Wm^{-2}) was obtained by using Schott interference filters (λ_{max} 756 nm). The green safe light (<0.01 Wm^{*2}) was obtained from a cool white fluorescent tube light wrapped in 6 layers of green cellophane paper (λ_{max} 530 nm). The intensity of the light sources was measured with a LI-COR radiometer.

3.4. Spectral assay of phytochrome

The amount of phytochrome present in different tissues or homogenates was estimated by recording the difference spectrum of phytochrome using Hitachi-557 spectrophotometer in either double beam or dual wavelength mode. The difference spectra were recorded at 4°C from 600 nm to 800 nm. The phytochrome sample was first irradiated with RL for 5 min to convert into Pfr form and the base line was recorded. Then, the sample was irradiated with FRL for 5 min to convert it to Pr form and the difference spectrum was recorded. The difference between the absorbance at 667 and 730 nm of the spectrum was expressed in terms of phytochrome units. A difference in the value of A (ΔA_{667}-A_{730}) = 0.001 was considered as one unit.

The amount of phytochrome in various tissues was quantified by difference spectroscopy of the tissue at 4°C after RL and FRL irradiation using Hitachi-557 spectrophotometer in dual wavelength mode. The tissues were excised into about 1 cm long segments and uniformly packed into a plastic cuvet.

3.5. Purification of phytochrome A from oat seedlings

3.5.1. Preparation of 10% (v/v) polyethylenimine

Twenty ml of 50% polyethylenimine (Sigma) was mixed with 60 ml of distilled water, cooled to 4°C and the pH was adjusted with 3 N HC1 to 7.8. The mixture was left overnight at 4°C and thereafter the pH was rechecked and adjusted to 7.8 if necessary. The volume was made upto 100 ml with distilled water and the solution was stored at 4°C.

3.5.2. Preparation of hydroxyapatite

Hydroxyapatite was prepared according to the method of Tiselius et al., (1956). One litre each of 0.5 M $CaCl_2$ and 0.5 M Na_2HPO_4 were slowly mixed in a beaker containing 100 ml of 1 M NaCl at a flow rate of 4 ml/min with continuous, slow stirring of only the surface of the solution. The supernatant was siphoned off and the precipitate (brushite) was washed twice with two litres of distilled water.

Thereafter, the brushite was suspended in two litres of distilled water, to which 50 ml of 40% (w/v) NaOH was added. The mixture was boiled for 1 h on a hot plate. The HA crystals were allowed to settle down for 10 min. The supernatant was siphoned off and the HA was washed twice with two litres of distilled water, followed by two washes with two litres of 10 mM Na-phosphate buffer, pH 6.8 followed by two more washes with two litres of 1 mM Na-phosphate buffer, pH 6.8. All the washings were carried out by heating for 15 min on a hot plate to enable the solution to reach the boiling point. Then, the crystals were allowed to settle down for 5 min and the supernatant was siphoned off. After the last wash, the supernatant was siphoned off and the HA was stored after dividing into 100 ml aliquots in 1 mM phosphate buffer, pH 6.8 in 1:1 (v/v) ratio at 4°C.

3.5.3. Extraction of phytochrome A

Phytochrome A was purified by using the procedure of Grimm and Rüdiger 1986). One kg of oat shoots was homogenized [1:1 (w/v) ratio] with pre-chilled (-20°C) extraction buffer containing 50% (v/v) ethylene glycol, 100 mM Tris-HCl, 140 mM ammonium Sulfate, 10 mM Na4-EDTA, 20 mM sodium bisulfite and 4 mM PMSF, final pH 8.3. at 4°C in a Waring blender at 4°C. The homogenate was filtered through two layers of cheese cloth and centrifuged at 16,000 x *g* for 20 min using a JA-10 rotor in J2-21 centrifuge (Beckman, USA).

3.5.4. Polyethylenimine precipitation

The supernatant was mixed with 10% (v/v) polyethylenimine (10 ml/litre) to a final concentration of 0.1% by continuous stirring for 10 min. It was left standing for 5 min before centrifuging at 16,000 x *g* for 20 min, and the supernatant was discarded.

3.5.5. Ammonium Sulfate precipitation

The above supernatant was first irradiated with RL for 5 min to keep the phytochrome in its Pfr form. To this sample, solid ammonium Sulfate (enzyme grade) was slowly added with continuous stirring for 20 min, to a final concentration of 42% (w/v) saturation. After the addition of ammonium Sulfate, the pH of the solution was adjusted to 7.8 by adding solid Tris base. The mixture was further stirred for 40 min and centrifuged at 30,000 x *g* for 30 min. The supernatant was discarded and the centrifuge tubes were drained by inverting them on absorbent paper. The pellet was

dissolved in resuspension buffer (4.5 ml per 1000 phytochrome units) containing 25% (v/v) ethylene glycol, 50 mM Tris-HCl, 5 mM tetra-sodium EDTA, 14 mM 2-mercaptoethanol and 2 mM PMSF with a final pH of 7.8 at 4°C. The resuspended sample was clarified by centrifugation at 30,000 x *g* for 20 min. The clear supernatant was used for HA chromatography.

3.5.6 Hydroxyapatite chromatography

Hydroxyapatite was packed into a column (4.5 cm *d* x 50 cm /). The void volume was determined by passing a few drops of methyl orange through the column. HA was equilibrated with equilibration buffer containing 25% (v/v) ethylene glycol, 50 mM Tris-HCl, pH. 7.8, 5 mM Na4-EDTA, 14 mM 2-mercaptoethanol, 70 mM ammonium Sulfate and 2 mM phenylmethylsulfonyl fluoride. Phytochrome sample was applied slowly on the surface of HA column. The column was washed with three column volumes of washing buffer containing 50 mM Tris-HCl, 5 mM Na4-EDTA, and 14 mM 2-mercaptoethanol with a final pH of 7.8.

Phytochrome was eluted using a linear gradient of 5-200 mM potassium phosphate buffer, pH 7.8 containing 5 mM EDTA and 14 mM 2-mercaptoethanol. Five ml fractions were collected using a fraction collector in drop mode. Fractions were checked for phytochrome levels and those containing at least 15 units per ml of phytochrome were pooled. Phytochrome sample was exposed to RL for 5 min and pelleted with 3.3 M ammonium Sulfate solution containing 50 mM Tris-Cl, pH 7.8 (0.6 ml/ one ml phytochrome sample) to a final concentration of 42% saturation. The mixture was centrifuged at 20,000 x *g* for 30 min and the supernatant was discarded. The tubes were drained by inverting them on tissue paper towels to remove traces of ammonium Sulfate solution.

3.5.7. Phosphate buffer washings

The phytochrome containing pellet was washed in 10 mM KP buffer, pH 7.8 (0.65 ml buffer per 1000 phytochrome units), by subjecting to slow shaking for 20 min and centrifuged at 20,000 x *g* for 20 min. Then the pellet was washed with 100 mM KP buffer, pH 7.8 (0.85 ml buffer per 1000 units of phytochrome) in a similar fashion. The pellet was dissolved in 10 mM KP buffer, pH 7.8 (0.9 ml buffer per 1000

units of phytochrome) and centrifuged at 20,000 x *g* for 25 min. The phytochrome containing supernatant was used for further purification.

3.5.8. Precipitation with polyvinylpyrrolidone

The final purification was achieved by mixing 1.35 g of soluble polyvinylpyrrolidone (PVP-40, Sigma) to 1000 units of phytochrome sample. PVP-40 was added slowly to the phytochrome sample and subjected to slow shaking for 20 min and precipitated by centrifuging at 30,000 x *g*. The pellet was rinsed with 2-3 drops of distilled water to remove the remaining traces of PVP-40. The purified phytochrome was stored at -70°C for further spectroscopic and immunochemical characterization.

3.6. Purification of phytochrome B from spinach seedlings

3.6.1. Extraction of phytochrome B

The initial steps of phytochrome B purification was essentially similar to that described by Pratt et al., (1991). The entire purification work was carried out under green safe light. Spinach leaves harvested from field grown plants were chopped into small pieces. The tissue was extracted in 1 kg lots in a Waring blender with 750 ml of extraction buffer to which 12 ml of 2.5 M Tris base was added. The extraction buffer consisting of 0.1 M Tris-Cl, 0.14 M ammonium Sulfate, 10 mM EDTA and 50% (v/v) ethylene glycol, pH 8.3 at 4°C, was pre-chilled to -20°C prior to use. Iodoacetamide was added freshly to a final concentration of 18 mM to the extraction buffer and 10 mM in all the subsequent buffers as a protease inhibitor. In addition, 2.0 g of sodium bisulfite and a few drops of Antifoam A were added at the time of homogenization of the tissue. The homogenate was filtered through 4 layers of cheese cloth and the filtrate was centrifuged at 16,000 x g for 20 min.

3.6.2. Polyethylenimine precipitation

The phytochrome containing supernatant was mixed with PEI to a final concentration of 0.1% (v/v) by adding 10 ml of 10% PEI solution per 1 litre of the extract, stirred for 5 min and centrifuged for 20 min at 16,000 x *g*. The supernatant was again mixed with PEI, at a lower concentration [0.05% (v/v)], stirred and centrifuged at 16,000 x *g* for 20 min. The phytochrome containing supernatant was exposed to RL for 5 min on ice.

3.6.3. Ammonium Sulfate precipitation

The above supernatant was mixed with saturated ammonium Sulfate solution, pH 7.8 containing 10 mM iodoacetamide to a final concentration of 36% (v/v) saturation. After stirring for 5 min, the solution was centrifuged at 25,000 x *g* for 20 min. The pellet was dissolved in 50 ml of resuspension buffer containing 50 mM Tris-Cl, 5 mM EDTA, 10 mM iodoacetamide, 25% (v/v) ethylene glycol, pH 7.8 at 4°C and centrifuged at 25,000 x *g* for 20 min. The phytochrome containing supernatant was stored at -70°C. Four such preparations were accumulated for further purification by HA chromatography.

3.6.4. Hydroxyapatite chromatography

200 ml bed volume of HA was equilibrated with resuspension buffer containing 70 mM ammonium Sulfate. The samples from four preparations were pooled and mixed with HA and incubated for 1 h on a shaker at 4°C. Then, the sample was washed with 400 ml of washing buffer-I containing 50 mM Tris-Cl, 5 mM EDTA, 10 mM iodoacetamide, pH 7.8, followed by 400 ml washing buffer-II containing 5 mM potassium phosphate buffer, 5 mM EDTA, 10 mM iodoacetamide at pH 7.8. Phytochrome was eluted by step gradient using 50 ml each of 20, 50, 80, 120 and 200 mM of K-phosphate buffer containing 5 mM EDTA and 10 mM iodoacetamide at pH 7.8. The fractions were checked by immunoblotting and phytochrome B containing fractions were pooled and precipitated by adding saturated ammonium Sulfate solution containing 10 mM iodoacetamide, pH 7.8 to a final concentration of 30% (v/v) saturation. The solution was centrifuged at 25,000 x *g* for 20 min and the pellet was dissolved in 25 ml of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.8, and used for DEAE-Sepharose chromatography.

3.6.5. DEAE-Sepharose chromatography

Phytochrome sample was loaded on DEAE-Sepharose column (2.5 cm *d* x 25 cm /) which was pre-equilibrated with 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.8. After loading the phytochrome sample, the column was washed with equilibration buffer. The proteins were eluted using a step gradient consisting of 12 ml each of potassium chloride (50, 100, 200, 300, 400 and 500 mM) in 10 mM potassium phosphate buffer containing 1 mM EDTA. Fractions were

checked for the presence of phytochrome B by western blotting and the phytochrome B containing fractions were pooled and concentrated by precipitating with solid ammonium Sulfate to a final concentration of 42% (w/v). The pellet was dissolved in 10 mM potassium phosphate buffer and used for spectroscopic assay.

3.7. Protein estimation

Protein was estimated according to the procedure of Lowry et al., (1951). Total proteins in the crude extracts was estimated after precipitation with an equal volume of 10% trichloroacetic acid. The mixture was left for 30 min at -20°C and then centrifuged at 10,000 x *g* for 10 min. The precipitate was dissolved in 1 N NaOH. A standard curve for the protein estimation was prepared by using bovine serum albumin fraction V (Fig. 3.1). The absorbance of the protein sample was measured at 500 nm after 30 min incubation with the reagent mixture.

3.8. SDS-PAGE

The electrophoresis of denatured protein in polyacrylamide gel in the presence of an ionic detergent sodium dodecyl Sulfate (SDS) was essentially carried out following the procedure of Laemmli (1970).

One mm thick separating gels of 8 or 10% (w/v) were polymerized in 0.375 M Tris-HCl buffer, pH 8.8 containing 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.01% (v/v) TEMED. 5% (w/v) stacking gel was made in 0.125 M Tris-HCl, pH 6.8, containing 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.012% (w/v) TEMED. Samples were prepared in a sample buffer containing 50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.1% (w/v) bromophenol blue and 5% (v/v) ß-mercaptoethanol. The samples were boiled for 4 min at 100°C for uniform coating of the detergent. After cooling, the samples were loaded in the wells of the gel and electrophoresis was carried out at room temperature at a current of 10 mA and 20 mA in stacking and separating gels respectively. The gel running buffer was made of 25 mM Tris, 250 mM glycine, pH 8.3 and 0.1% (w/v) SDS.

3.9 Silver staining

The staining was carried out at room temperature with gentle shaking on a reciprocal shaker. The gels were stained according to the method of Blum et al., (1987). After electrophoresis, the gels were incubated in fixing solution containing

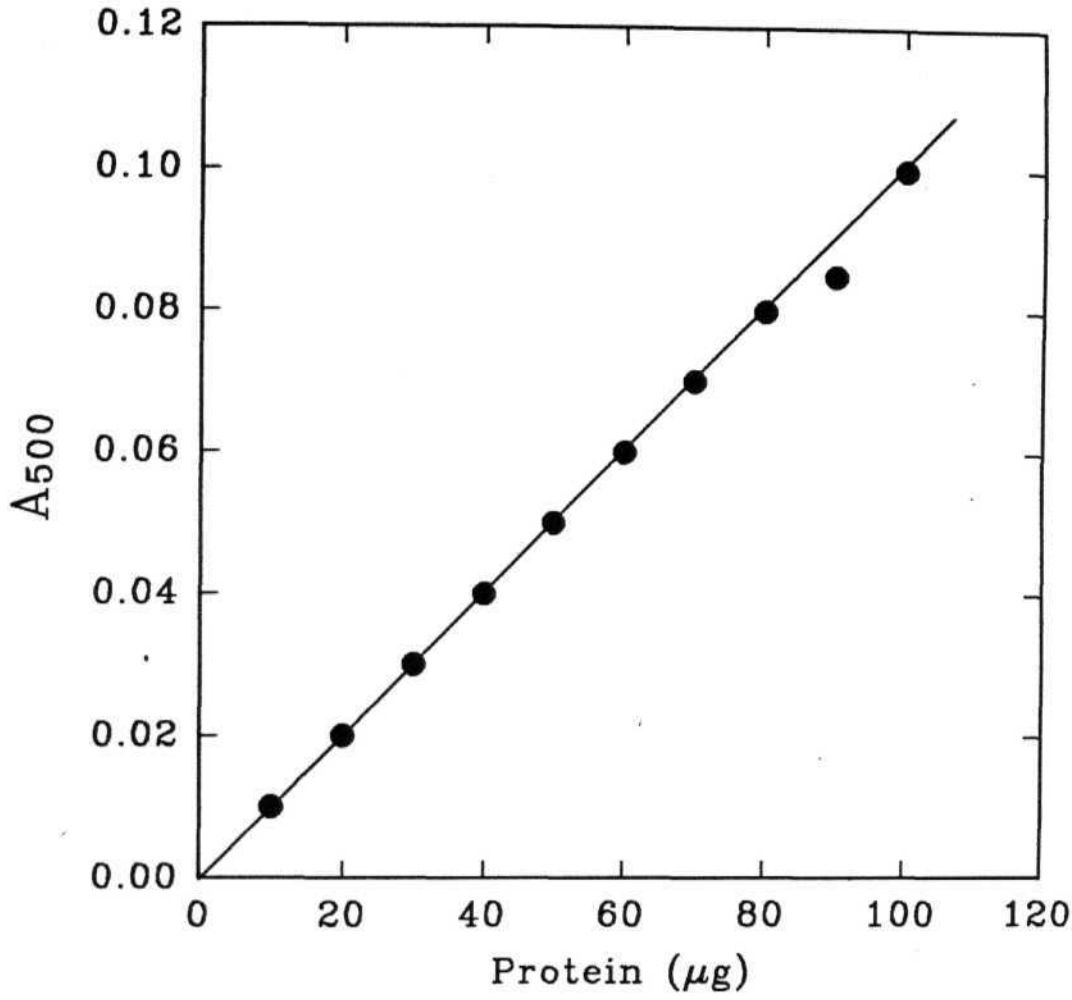

Figure 3.1. Standard curve for the estimation of protein (Lowry et al., 1951). Bovine serum albumin (fraction V) was used as the standard protein

50% (v/v) methanol, 12% (v/v) acetic acid and 0.0185% (v/v) formaldehyde for 1 h. Then they were washed three times with 50% (v/v) ethanol for 20 min each. The gels were pre-fixed with 0.02% (w/v) sodium thiosulfate for 1 min followed by three washes with distilled water for 20 sec each. The gels were impregnated with 0.2% (w/v) silver nitrate solution containing 0.028% (w/v) formaldehyde for 20 min, followed by washing twice with distilled water for 20 sec each. The protein bands were visualized by developing the gels with 6% (w/v) sodium carbonate solution containing 0.0185% (v/v) formaldehyde. After the protein bands became visible, the reaction was stopped by washing the gels with several changes of water and the gels were stored in fixer at 4°C.

3.10. Molecular weight determination

The molecular weight of phytochromes was determined by SDS-PAGE in 8 % polyacrylamide gels. The following molecular weight markers (SDS-6H, Sigma) were used as standards; myosin from rabbit muscle (205 kD), ß-galactosidase from E. coli (116 kD), muscle phosphorylase b from rabbit muscle (97.4 kD), bovine serum albumin (66 kD), ovalbumin (45 kD).

3.11. Gel documentation

The relative quantity of protein in the immunoblots were measured by scanning the bands using a gel documentation (Molecular Dynamics) system. Each band in the immunoblot was inserted in a separate window (box), such that the band fits exactly within the box. The total color intensity of the band was considered as its volume. This was done in order to minimize errors caused due to scanning at a single point within a band. The background value was deducted from the total value of the band. The resulting values were used to plot graphs.

3.12. Antibodies production

Four hundred micrograms of purified phytochrome A in 0.25 ml of 10 mM potassium phosphate buffer was mixed with 0.25 ml of phosphate buffer saline (PBS), containing 0.8% (w/v) NaCl and 0.02% (w/v) KCl in 10 mM phosphate buffer, pH 7.4. This phytochrome sample was mixed thoroughly with 0.5 ml of Freund's complete adjuvant. Using this mixture, multiple injections were given subcutaneously to a rabbit. Subsequently, booster injections were given at weekly intervals for next

three weeks using 200 µg of phytochrome each, mixed with an equal amount of Freund's incomplete adjuvant. The rabbit was bled during the 5th week and the antiserum was collected and stored in aliquots with 0.001% (w/v) sodium azide at -20°C. The pre-immune serum to be used as control was collected from the same rabbit prior to immunization.

3.13. Ouchterlony double immunodiffusion

Double immunodiffusion was performed on agar plates following the procedure of Ouchterlony (1949). 1% (w/v) agar solution was prepared in PBS buffer, pH 7.4, by heating. After cooling, the clear agar solution was mixed with sodium azide at a final concentration of 0.001% (w/v) and poured on glass plates to a thickness of about 2 mm and allowed to solidify on a horizontal surface at room temperature. Wells were punched in the gel with a steel template forming to a central well and 6 peripheral wells. The central well was loaded with oat PHYA antiserum and the antigens to be tested were loaded in the peripheral wells. After loading all the wells, the agar gels were incubated at 4°C in a humid chamber to avoid dehydration of the gel. The precipitin lines were visualized after 24 h against a dark field illumination and were photographed.

3.14. Single radial immunodiffusion

Single radial immunodiffusion was performed by following the procedure outlined by Mancini et al., (1965). Two fifty microlitres of oat anti-PHYA serum was mixed with 10 ml of 1% (w/v) agar solution prepared in PBS buffer, pH 7.4 at 40°C, and the mixture was poured on a glass plate (7.5 cm / cm x 2.5 cm *w*). After gelation of agar, circular wells were punched and filled with increasing amounts of phytochrome. The plates were incubated at 4°C for 48 h in a humid chamber. The precipitin lines were visualized against a dark field illumination and were photographed.

3.15. Preparation of Ponceau S

A stock solution of 100 ml Ponceau S was prepared by dissolving 2 g of ponceau S, 30 g of trichloroacetic acid and 30 g of sulfosalicylic acid in distilled water and the volume was made upto 100 ml. One part of the stock solution was diluted with 9 parts of distilled water to make the working solution. Electroblotted

membranes were incubated in the Ponceau S solution for 5 min to visualize the protein bands and then destained by washing several times with distilled water. After destaining, the membranes were subjected to western blotting.

3.16. Electroblotting

Proteins were electroblotted onto nitrocellulose or polyvinyldifluoride membranes (PVDF) using semi-dry blotting method. The membrane was cut into the size of the resolving gel and floated on the surface of distilled water for 5-10 min and dipped in water followed by transfer buffer containing 48 mM Tris base, 39 mM glycine, 20% (v/v) methanol and 0.037% (w/v) SDS. In the case of PVDF membrane, the membrane was first wetted with methanol for 5-10 min, washed with distilled water and soaked in transfer buffer. Whatman No. 3 chromatographic papers were cut to the size of the membrane and soaked in the transfer buffer.

After SDS-PAGE, the stacking gel portion was excised from the resolving gel and the bottom left corner of the gel was marked with a small cut. The gel was washed twice with distilled water followed by transfer buffer.

The semi-dry blotting was carried out in a custom made apparatus. Both anode and cathode graphite plates were washed thoroughly with distilled water. On the anode plate, three sheets of Whatman No 3 papers were layered carefully, avoiding trapping of any air bubbles between the sheets, the membrane was layered over it and a small cut was made in the bottom left comer of the membrane which coincides with the gel. The gel was carefully layered on top of the membrane on which three more sheets of Whatman papers were layered. Finally, the cathode plate was placed over the Whatman papers and the whole sandwich consisting of the plates, papers, membrane and gel was tightened by clamps. The blotting unit was connected to a power supply which was set to a constant current supply of 0.8 mA/sq. cm area for 1.5 h. After the run, the efficiency of the transfer of proteins onto the membrane was checked by staining the proteins with Ponceau S dye.

3.17. Western blotting

Immunoblotting was done following the procedure of Towbin et al., (1979) at room temperature. After electroblotting, the membranes were blocked for three min with blocking buffer containing 2% (v/v) polyoxyethylene sorbitan monolaurate

(Tween 20), 20 mM Tris-Cl, pH 7.5 and 500 mM NaCl. Immediately after blocking, the membranes were washed with a buffer containing 20 mM Tris-Cl, pH 7.5, 500 mM NaCl and 0.05% (v/v) Tween 20. Further, the membranes were washed three times with 20 ml of washing buffer (TBST) containing 20 mM Tris-Cl, 150 mM NaCl and 0.05% (v/v) Tween 20 for 10 min each. Next, the blots were incubated with primary antibody (1:500 dilution) for 1 h in incubation buffer (TBS) containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl and 1% (w/v) low fat skim milk powder. Thereafter, the blots were washed three times with TBST as above for 10 min each. Membranes were again incubated in TBS for 1 h containing appropriate secondary antibodies (1:30,000 dilution) conjugated with alkaline phosphatase. Thereafter, the blots were washed three times as above with TBST for 10 min each. The blots were stained by adding alkaline phosphatase substrate; nitro blue tetrazolium (50 mg/ml, 66 µl) and 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml, 33 µl) in 10 ml alkaline phosphatase buffer containing 100 mM Tris-Cl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. The reaction was stopped after the bands become clearly visible, by washing the blot with distilled water.

3.18. Immunoprecipitation

A known amount of phytochrome sample was split into two equal aliquots, one was incubated with an equal amount of pre-immune serum and another with anti-phytochrome serum for 1 h at 4°C with continuous shaking in darkness. Thereafter, both these samples were mixed with an equal volume of pre-swollen protein A sepharose 4B (same volume for both) and shaking was continued for one more hour. The samples were centrifuged for 5 min at 12,000 x *g* for 5 min and the phytochrome amount in the supernatants was checked spectrophotometrically. The immunoprecipitates (pellets) were washed twice with 1 ml buffer containing 50 mM Tris-HCl pH 7.5. 150 mM NaCl, 0.1% (v/v) nonidet P-40 (NP-40), 1 mM EDTA, 0.25% (w/v) gelatin and 0.02% (w/v) sodium azide. Then the pellets were washed with a buffer containing 10 mM Tris-HCl, pH 7.5 and 0.1% (v/v) NP-40. All the washings were done by dissolving the pellets with the buffers and stirring for 20 min at 4°C and centrifuging at 12,000 x *g* for 2 min. After the washes, the pellets were dissolved in 1x SDS gel-loading buffer. The proteins were denatured by heating at 100°C for 3 min and Protein A-Sepharose was removed by centrifugation at 12,000 *g*

for 2 min. The supernatants were analyzed by SDS-PAGE followed by western blotting.

3.19. Zn^{+2}-blotting

The location of phytochrome polypeptide on blots was also ascertained by using a chromophore fluorescence assay following the procedure of Berkelman and Lagarias (1986) as modified by Wahleithner et al., (1991). Purified oat phytochrome A was subjected to SDS-PAGE and transferred onto PVDF membrane (Immobilon P, Millipore). The membrane was incubated for 30 min in 1.3 M zinc acetate solution containing 20% (v/v) ethanol. Thereafter, the chromophore fluorescence was visualized in UV transilluminator through a red filter. The fluorescent band showing phytochrome was photographed using a red interference filter. After photographing, the membrane was washed several times with distilled water and subjected to western blotting with phytochrome A antibodies as described above. The position of the band in the Zn^{+2} blot was compared with that of the western blot.

3.20. In vitro proteolysis of phytochrome A

The protocol followed for *in vitro* proteolysis assay was essentially similar to that outlined by Parks et al., (1989). Phytochrome was extracted from shoots of 4½ d old dark-grown oat, maize, rice, wheat and pear millet seedlings with MOPS buffer, pH 7.8, consisting of 100 mM 3-(N-morpholino) propanesulfonic acid, 50% (v/v) ethylene glycol, 5 mM Na4-EDTA, 56 mM 2-mercaptoethanol without any protease inhibitors under green safe light as described (Sec. 3.5.3). The extract was split into two portions, one portion was irradiated with RL and another with FRL for 5 min on ice to convert phytochrome to its Pfr and Pr forms respectively. The samples were then incubated in dark at 25°C. Aliquots were drawn at different time points, and boiled with an equal volume of SDS-PAGE sample buffer containing 2 mM PMSF. Each lane was loaded with equal amount of protein, and after SDS-PAGE, the gel was electroblotted onto nitrocellulose membrane and immunostained using antibodies raised against oat phytochrome A.

3.21. Leaf blotting

Seven day-old maize leaves were used for leaf blotting. The epidermal layer was removed by rubbing evenly with sand paper, washed with distilled water and

dried by blotting on four layers of tissue paper. On an even surface of a glass plate two layers of Whatman No. 3 papers were placed followed by PVDF membrane which was pre-soaked in methanol followed by distilled water. The leaf was placed on it followed by two more layers of Whatman No. 3 papers and covered with another glass plate and pressed with hand for two minutes (Polston et al., 1991). Dark- and light-grown leaf prints were obtained and probed with either phytochrome A or phytochrome B specific antibodies.

3.22. In vivo and in vitro reconstitution experiments

3.22.1. Preparation of phycocyanobilin

Phycocyanobilin was prepared according to the method of Kunkel et al., (1993). Fifty grams of dry *Spirulina* cells were mixed with 300 ml of distilled methanol and incubated in a water bath at 90°C for a few minutes. The extract was filtered through a G-3 sintered glass funnel and the cells were washed with hot methanol. This step was repeated 4 times until the color of the cells became blue and the methanol contained only a small amount of chlorophyll. To these chlorophyll free cells, 300 ml of absolute methanol was added and it was stirred continuously for 3 h in a hot water bath at 90°C. The extract was filtered through a G-3 sintered funnel and the cells were discarded.

The extract was concentrated to a volume of 30 ml by using a rotatory evaporator in a water bath set at 50°C. This extract was mixed with 70 ml of diethyl ether and poured into a separating funnel. After shaking it with 100 ml of 1% (w/v) citric acid, the organic phase was separated. Most of the PCB was partitioned in the ether phase but a little amount of PCB was also present in the water phase. The PCB present in the water phase was reextracted with 50 ml of diethyl ether. The water phase was again extracted with the first solution (30 ml methanol and 70 ml diethyl ether). The PCB from the water phase was reextracted with another 50 ml of diethyl ether. This procedure was repeated as long as the extraction of the first solution (methanol-diethyl ether) yielded blue solutions of the water phase (citric acid). For separation of the two phases, several ml of saturated NaCl solution was added to the mixture.

The PCB/diethyl ether solution was mixed with 50 ml of $NaHCO_3$ (0.5% w/v). Since PCB is not stable at alkaline pH, ice cold solutions were used and these steps were completed as quickly as possible. The water phase was collected and the pH was adjusted to 5.5 by adding solid citric acid.

PCB was extracted from the water phase with 50 ml of chloroform. The water phase was discarded and sodium Sulfate crystals were added to the chloroform to remove traces of water. The chloroform solution was evaporated to 10 ml. This 10 ml of PCB solution was added slowly to centrifuge bottles consisting of 150 ml of hexane, while the hexane was continuously stirred. After a while, the PCB precipitated out as dark crystals which were collected by centrifuging at 5000 x *g* for 5 min.

The hexane was removed by aspiration and the PCB crystals were dissolved in chloroform, divided into small volumes and dried in a speed vac concentrator. The PCB crystals were stored at -20°C.

3.22.2. Phytochrome extraction for *in vitro* reconstitution experiment

All the procedures were performed at 4°C under green safe light. Shoots from 5 d-old dark-grown tomato seedlings were cut at the base and the tissue was frozen in liquid nitrogen. Phytochrome was extracted from the tissue using the extraction buffer in the ratio of 1:3 (w/v) which consisted of 50 mM Tris-Cl pH 8.3 at 4°C, 100 mM ammonium Sulfate, 25% (v/v) ethylene glycol, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM diethyl dithiocarbamate and 142 mM 2-mercaptoethanol. The extract was filtered through three layers of cheese cloth. The filtrate was mixed with PEI at a final concentration of 0.1% (v/v) for 10 min and centrifuged at 10,000 x *g* for 10 min. The supernatant was mixed with ammonium Sulfate to a final concentration of 42% (w/v) for 45 min and centrifuged at 25,000 x *g* for 25 min. The pellet was dissolved in resuspension buffer containing 50 mM Tris-Cl, pH 7.8, 1 mM PMSF, 0.7 µl/ml leupeptin and 1 mM dithiothreitol. The extract was clarified at 30,000 x *g* for 20 min and used for pigment incubations.

3.22.3. Pigment incubations

In vitro reconstitution experiments were performed by adding 5-10 µl of the phycocyanobilin stock solution in dimethyl sulfoxamide to give a final pigment

concentration of 33 μM to the phytochrome extracts. The mixture was incubated at different time points and clarified by centrifuging at 20,000 x g for 20 min and used for spectroscopic assay as described earlier (Sec. 3.4).

CHAPTER 4

RESULTS

4.1. Optimal age of seedlings for phytochrome purification

It is known that in germinating seedlings, the amount of phytochrome varies considerably during development in an age dependent fashion. In order to determine the optimal age of the oat seedlings for extraction of phytochrome, the amount of phytochrome in shoots was quantitated using both *in vivo* and *in vitro* spectroscopic methods. At different time points from sowing, oat shoots were harvested; and phytochrome amount *in vivo* as well as in crude extracts was estimated and quantitated on the basis of gram fresh weight (Fig. 4.1). The maximal level of phytochrome was found in 3.5 d-old dark-grown seedlings and thereafter its amount gradually declined upto 8-d. Although 3.5 d-old seedlings possessed more amount of phytochrome, they were tiny (1-2 cm in length) and yielded little fresh mass. Therefore, phytochrome was purified from 4.5 d-old dark-grown oat seedlings which yielded reasonably high amount of tissue with high amount of phytochrome.

4.2. Purification of phytochrome A

4.2.1. Extraction conditions

All the purification steps were carried out under green safe light at 0°C. The procedure was completed as quickly as possible to minimize degradation and protease inhibitors were also added at each step of purification. The steps adopted for purification of phytochrome are summarized in Fig 4.2. Table 4.1 shows the purity and the yield of phytochrome at different stages of purification. The amount of phytochrome at various stages of purification was quantitated by spectroscopy. The relative purity was ascertained by SDS-PAGE by comparing aliquots drawn from different stages of purification (Fig. 4.5).

4.2.2. Extraction

Phytochrome was purified to homogeneity following the procedure of Grimm and Riidiger (1986) using 4.5 d-old dark-grown oat seedlings. Since phytochrome is susceptible to rapid proteolysis in its Pr form in crude extracts (Vierstra and Quail 1982b; Vierstra et al., 1984), the harvested shoots were exposed to RL for 5-10 min to

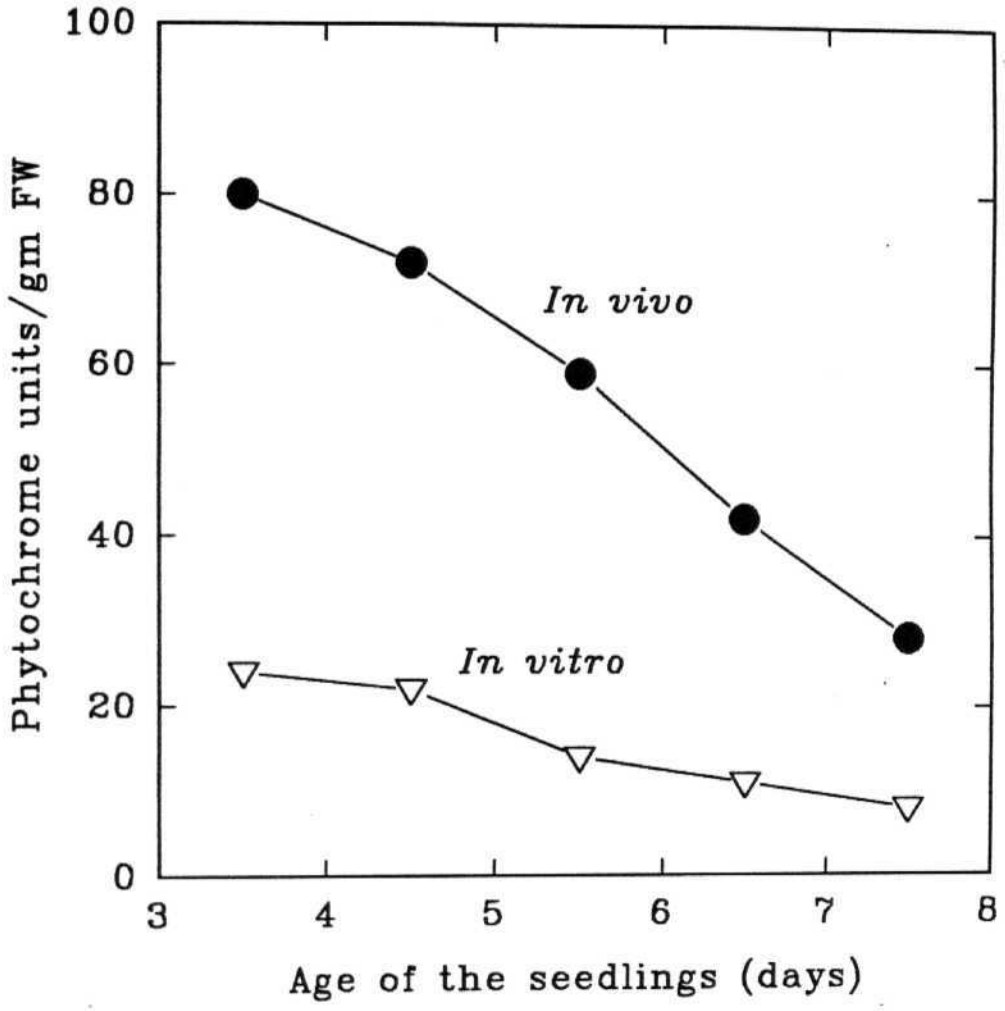

Figure 4.1. Time course of accumulation of phytochrome in oat seedlings. Oat seedlings were grown in continuous darkness from the time of sowing. At the time points (days) indicated, phytochrome level was estimated in oat shoots by spectroscopic assay, both in intact shoots *(in vivo)* and crude extracts *(in vitro).*

Purification of Oat Phytochrome A

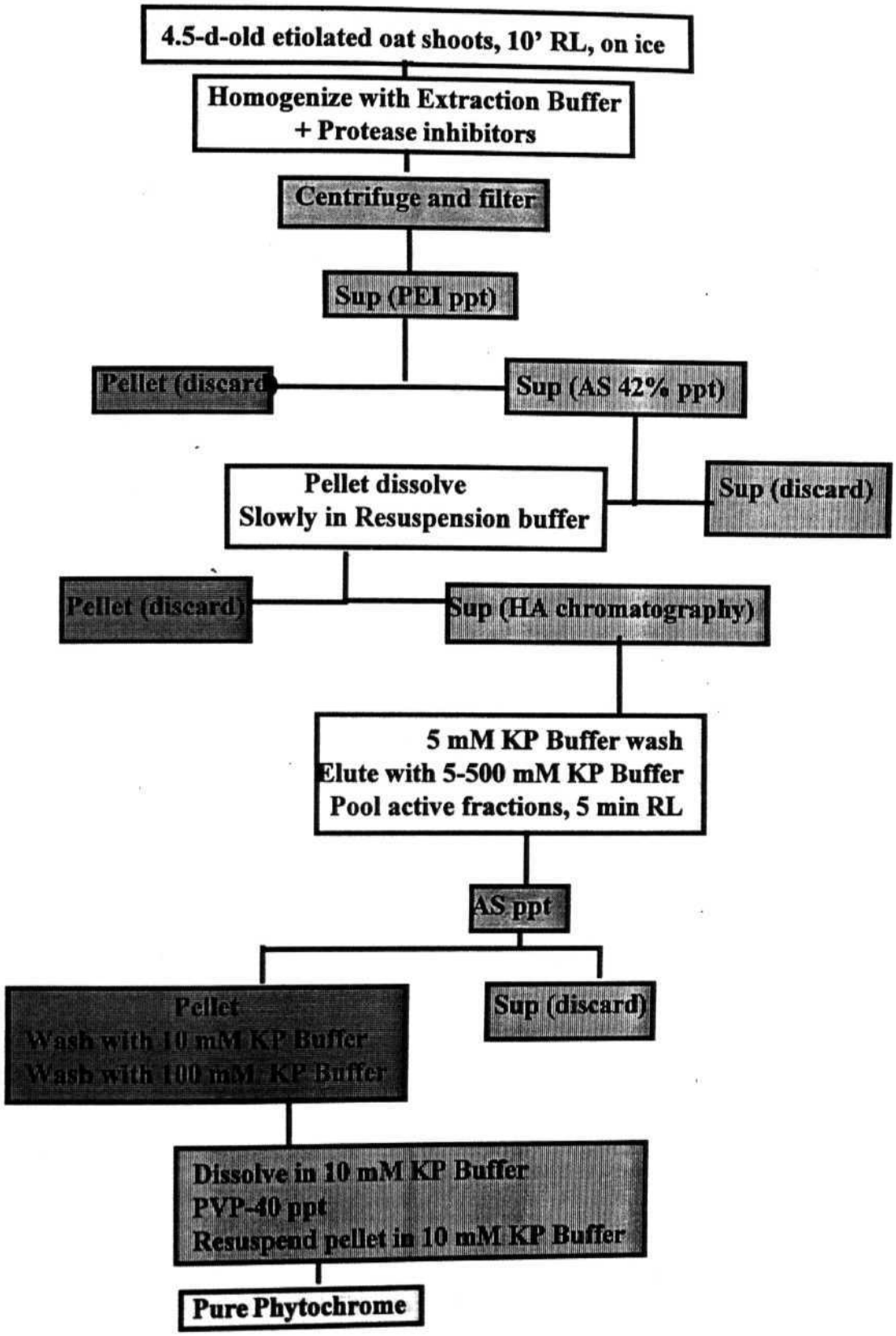

Figure 4.2 Protocol for the purification of oat phytochrome A [(Adopted from Grimm and **Rüdiger** (1986)]

convert phytochrome to its Pfr form, which is relatively stable. In addition, all the steps of purification were carried out at 4°C to minimize phytochrome degradation. The protease inhibitor, PMSF (4 mM) was added to the extraction buffer, just before homogenization to minimize proteolysis. It has been suggested that the presence of ethylene glycol, 140 mM ammonium Sulfate (Vierstra and Quail 1983), EDTA and sodium bisulfite in the homogenization buffer also minimize proteolysis (Pike and Briggs 1972; Vierstra and Quail 1982a). The extracts containing phytochrome were fortified with additional PMSF at different steps to further prevent proteolysis, since it is known that PMSF is unstable in aqueous buffers and addition of PMSF at each step safeguards phytochrome against the activated proteases.

4.2.3. Polyethylenimine precipitation

The phenolic substances and the nucleic acids present in the supernatant were eliminated by addition of PEI [0.1% (v/v)] and centrifugation. After centrifugation, the supernatant was again irradiated with RL to maintain the phytochrome in its Pfr form. At this stage the supernatant contained about 18 units/ml of phytochrome and almost 92% of the total phytochrome present in the crude homogenate could be recovered, which indicates that the loss of phytochrome if any was minimal.

4.2.4. Ammonium Sulfate precipitation

Phytochrome was precipitated by adding solid ammonium Sulfate to a final concentration of 42% (w/v) saturation, exercising a precaution to maintain the pH close to 7.8 by adding solid Tris base. The precipitate was collected by centrifugation. The pellet was dissolved in resuspension buffer by shaking on a reciprocal shaker and back extracted by centrifugation. At this stage about 60% of the phytochrome was recovered in the supernatant.

4.2.5. Hydroxyapatite chromatography

Hydroxyapatite chromatography has been employed as a major step in phytochrome purification to eliminate other proteins (Vierstra and Quail 1983). After this step it was possible to visualize the absorbance spectrum of phytochrome samples instead of difference absorbance spectrum. Fig. 4.3 shows the elution pattern of phytochrome subjected to HA chromatography. Since the flow rate in HA columns are known to decrease with continuous use, a peristaltic pump was employed to

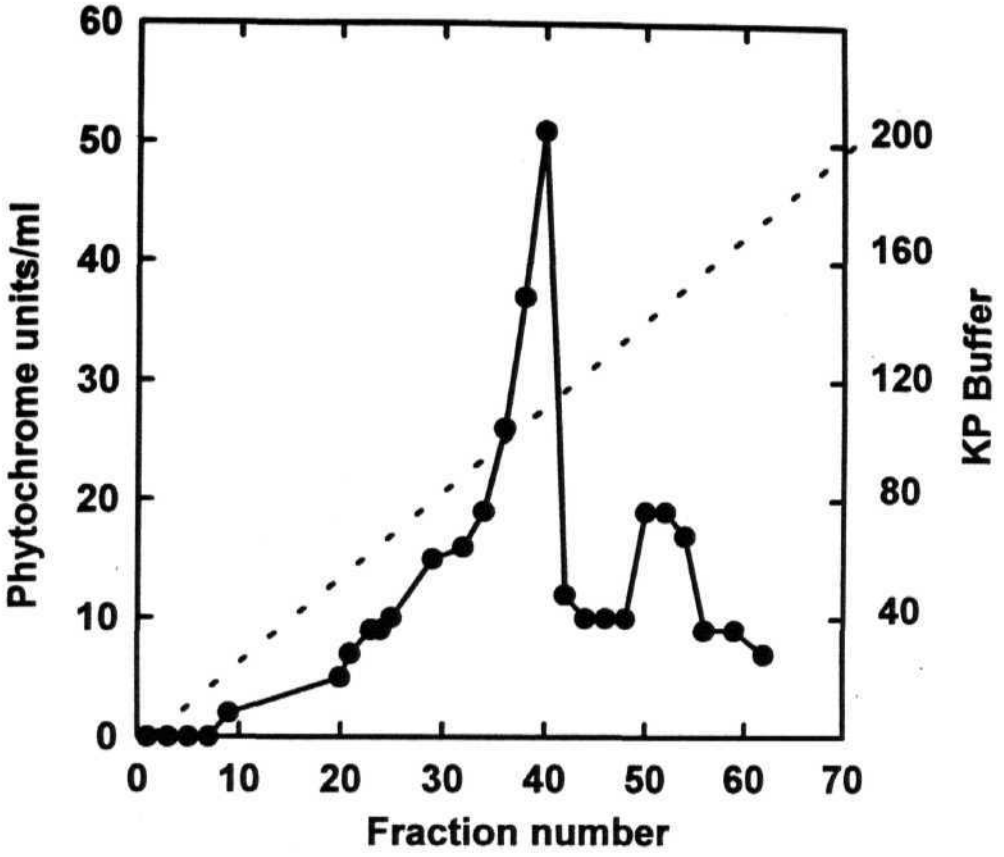

Figure 4.3. The elution pattern of oat phytochrome A during hydroxyapatite chromatography. The ammonium Sulfate precipitate after back extraction was applied onto HA column and the unbound protein was washed with washing buffer. Phytochrome was eluted using a linear gradient of 5-200 mM KP buffer and 5 ml fractions were collected. The fractions were checked spectroscopically for phytochrome amount. The values are expressed as phytochrome units/ml.

increase the flow rate of the column. All the fractions containing more than 15 units/ml of phytochrome were pooled. The pooled sample was first irradiated with RL for 5 min and then precipitated with a buffer (0.6 ml per ml of phytochrome sample) containing 3.3 M ammonium Sulfate in 50 mM Tris-Cl, pH 7.8.

4.2.6. Phosphate washings

Phosphate washings were included to eliminate contaminants including the degraded products of phytochrome i.e., 114/118 kD phytochrome. The pellet was first dissolved in 10 mM KP buffer (1 ml/4.5 units), stirred for 20 min and centrifuged, followed by a similar wash with 100 mM KP buffer. Finally the pellet was dissolved in 10 mM KP buffer and clarified by centrifugation.

4.2.7. Polyvinylpyrrolidone precipitation

The purification of phytochrome to near homogeneity was achieved by PVP-40 precipitation. The phytochrome containing supernatant was mixed with 150 mg PVP-40/ml of phytochrome solution and after stirring, phytochrome sample was collected in the pellet by centrifugation. The traces of PVP-40 was removed by rinsing the pellet with 2-3 drops of distilled water. The pellet was resuspended in 10 mM KP buffer (0.9 ml/mg phytochrome sample). This purified phytochrome sample was checked for its quantity and purity. A SAR value of 0.75 was obtained for the purified phytochrome (Fig. 4.4).

4.2.8. Storage of purified phytochrome

The purified phytochrome sample was stored as 0.5 ml aliquots in liquid nitrogen.

4.3. Characterization of purified phytochrome

Phytochrome purity was checked in two different ways, by spectrally monitoring specific absorbance ratio (Fig. 4.4) and by SDS-PAGE followed by silver staining (Fig. 4.5).

4.3.1. Spectral characterization

The ratio of the absorbance at 667 nm to that of the absorbance at 280 nm have been normally used as a parameter to estimate phytochrome purity. The specific absorbance ratio (SAR) was determined by scanning the phytochrome sample from 800 to 200 nm in the Pr form (Fig. 4.4). Even though, the purity of the phytochrome

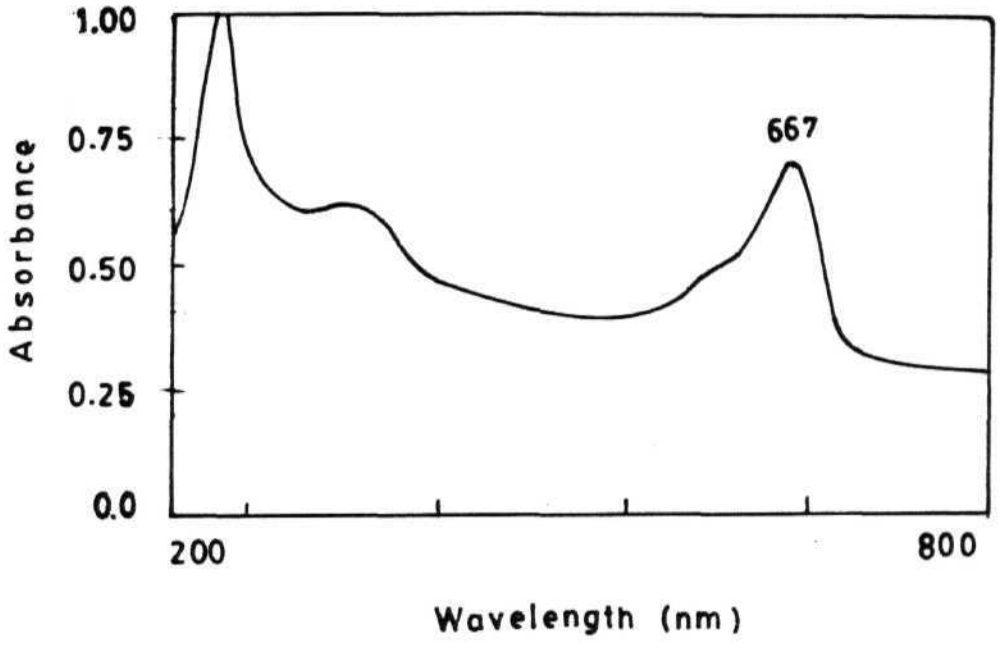

Figure 4.4. The absorbance spectrum of purified phytochrome A obtained after PVP-40 precipitation. Purified phytochrome A was first converted to Pr form and the spectrum was recorded by scanning from 200 nm to 800 nm.

Table 4.1. Purification and yield of phytochrome A from 2 kg of dark-grown oat shoots

Purification step	Volume (ml)	Pr A_{667}	Phytochrome (Units)	SAR $A_{667/280}$	Yield (%)
Crude extract	2750	0.03	46750	nd	100
PEI supernatant	2600	0.03	42900	nd	92
AS ppt	200	0.15	27885	nd	60
HA eluate	250	0.14	18020	0.12	39
PVP ppt	7.5	0.82	6692	0.75	15

nd- not determined

samples cannot be determined by merely checking the SAR value, in general this value is taken to represent the relative purity of phytochrome samples (Briggs and Rice 1972). A SAR value of 0.75 was obtained with the purified sample indicating that a reasonable degree of phytochrome purity was achieved.

4.3.2. SDS-PAGE

Aliquots of protein samples from different stages of purification were boiled with SDS-PAGE sample buffer, separated on 8% gels followed by silver staining. A single band was obtained with a molecular weight of 124 kD in the purified sample (Fig. 4.5). Since silver staining can detect proteins from 0.1 to 1.0 ng concentration, even when 0.5 μg of pure protein was loaded no contaminating bands were observed. The purified phytochrome A was injected subcutaneously into rabbits to raise polyclonal antibodies.

4.3.3. Ouchterlony double immunodiffusion

The specificity of antibodies against oat phytochrome A and maize phytochrome A was checked by double immunodiffusion. The oat anti-PHYA antibodies were found to cross react with phytochrome from dark-grown oat and maize samples. No precipitin lines could be observed with light-grown oat and maize samples (Fig. 4.6A). In order to check antigenic similarity between oat and maize phytochrome A, alternate wells were loaded with dark-grown oat and maize extracts. It was observed that the precipitin lines generated against oat and maize samples fused completely indicating a near identity in the antigenicity of phytochrome A of oat and maize. (Fig. 4.6B).

4.3.4. Single radial immunodiffusion

Single radial immunodiffusion (SRID) is often used to quantitate the amount of antigen in a particular sample, (Mancini et al., 1965) by loading it into a well punched in antibody containing gel. Antigen-antibody reaction generates a circular precipitin ring, the diameter of which is proportional to the amount of the antigen loaded. Fig. 4.6C shows that when oat phytochrome was subjected to SRID the diameter of the precipitin ring increased in proportion to the amount of protein loaded.

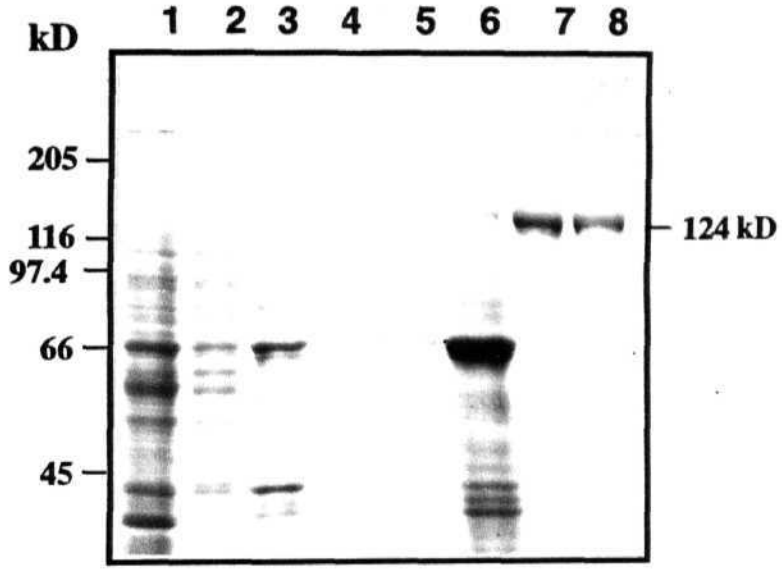

Figure 4.5. Monitoring of phytochrome A purity at different steps of purification by SDS-PAGE. The aliquots drawn at different stages of purification were analyzed by SDS-PAGE. The proteins bands were visualized by silver staining. Lane 1, crude extract; lane 2, PEI supernatant; lane 3, ammonium Sulfate precipitation; lane 4, hydroxyapatite column washing, lane 5, hydroxyapatite eluate; lane 6, ammonium Sulfate II precipitation; lane 7, phos-

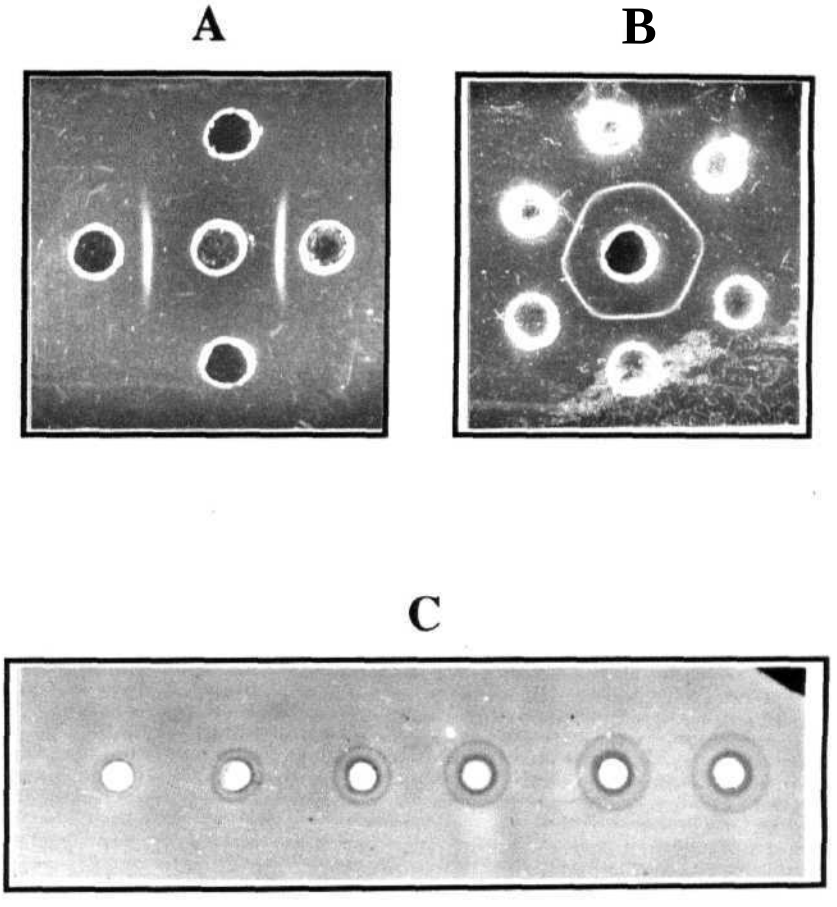

Figure 4.6 Ouchterlony double immunodiffusion (A, B) and single radial immunodiffusion (C) of phytochrome A from dark- and light-grown oat and maize extracts. For double immunodiffusion the central wells were loaded with oat anti-PHYA antibodies. The number in parentheses indicate the position of antigen in the peripheral wells.

A. The crude extracts of light-grown oat (1), dark-grown oat (2), light-grown maize (3), and dark-grown maize (4) were loaded in the peripheral wells.

B. The crude extracts of dark-grown oat (1, 3, 5) and maize (2, 4, 6) were loaded in the peripheral wells.

C. Single radial immunodiffusion of purified phytochrome A: Increasing concentrations of oat phytochrome A (1-6 μg) was loaded in the wells, punched in agarose gel containing 2.5% oat anti-PHYA antibodies.

4.3.5. Light induced down regulation

Phytochrome A is distinguishable from other phytochromes by an extreme reduction in its amount on exposure of dark-grown seedlings to light. This light-induced down regulation of phytochrome A protein level is a consequence and contribution of several mechanisms such as decreased transcription of PHYA mRNA (Colbert et al., 1983), decreased stability of PHYA mRNA (Seelay et al., 1992), and Pfr specific degradation of phytochrome by ubiquitin mediated proteolysis (Shanklin et al., 1987,1989) This light-induced down regulation of phytochrome A was taken as a diagnostic test to check the specificity of the phytochrome A antibodies. Fig. 4.7 shows that when equal amounts of proteins from both dark- and light-grown extracts from oat were separated on SDS-PAGE, phytochrome A specific signal on western blot was visible only for the dark-grown samples, the signal from the light-grown samples was below the level of detectability. Similarly, when seedlings grown in continuous darkness were transferred to light, PHYA level was found to be reduced.

4.3.6. Immunoprecipitation

Immunoprecipitation of phytochrome A from crude extracts was used to check the specificity of the antibodies. Phytochrome sample extracted from dark-grown oat seedlings was split into two equal aliquots and mixed with equal volumes of immune and pre-immune sera. Table 4.2 shows that in case of pre-immune serum, a considerable amount of phytochrome A, i.e., more than 83% was retained in the supernatant. Whereas in case of immune serum, only 16% of phytochrome A was retained in the supernatant. The reduction of phytochrome A level in the supernatant of immune serum is probably due to its precipitation.

The supernatants and pellets of the above samples were probed with phytochrome A antibodies in an immunoblot. While the supernatant of pre-immune serum gives phytochrome A band, no such band was detectable with the supernatant of immune serum. The reduction of the band intensity is taken as a proof for the immunoprecipitation of phytochrome (Fig. 4.8). This was further confirmed by the observation that only in the immunoprecipitate of immune serum treated extracts, phytochrome A band is visible.

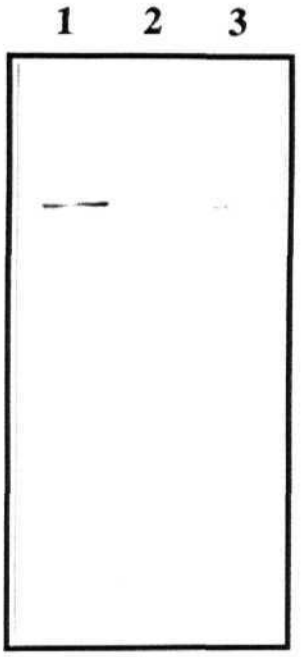

Figure 4.7. Immunoblot of crude extracts of dark- and light-grown oat seedlings. Total proteins (25 |ig) extracted from 5 d-old dark- (1), light-grown (2) seedlings, and seedlings transferred from dark to light (3) for 4 h, were separated on SDS-PAGE, transferred onto PVDF membrane and probed with oat anti-PHYA antibodies.

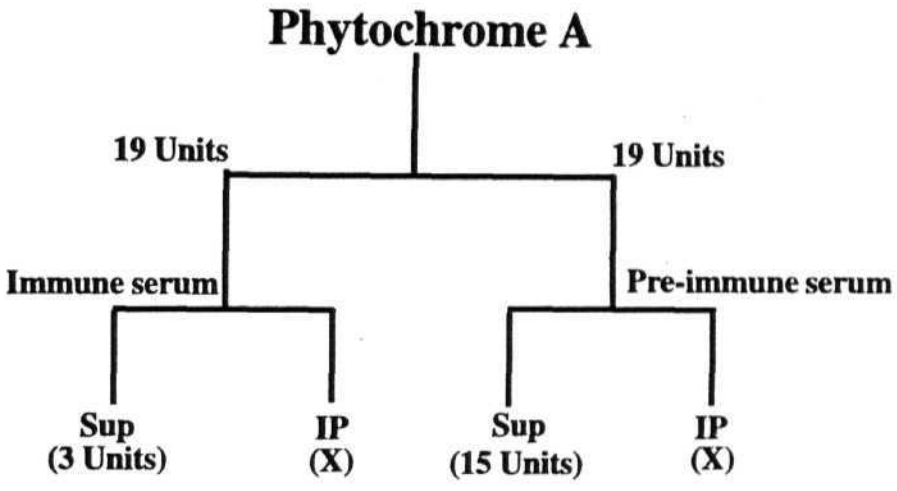

Table 4.2. Spectral values of phytochrome at different steps of immunoprecipitation. Crude exract from 4.5 d-old dark-grown oat seedlings was concentrated by ammonium Sulfate precipitation, and were incubated with either pre-immune serum or oat anti-PHYA serum. Phytochrome amount was measured sepctrally both in the supernatants (Sup) and immunopellets (IP).

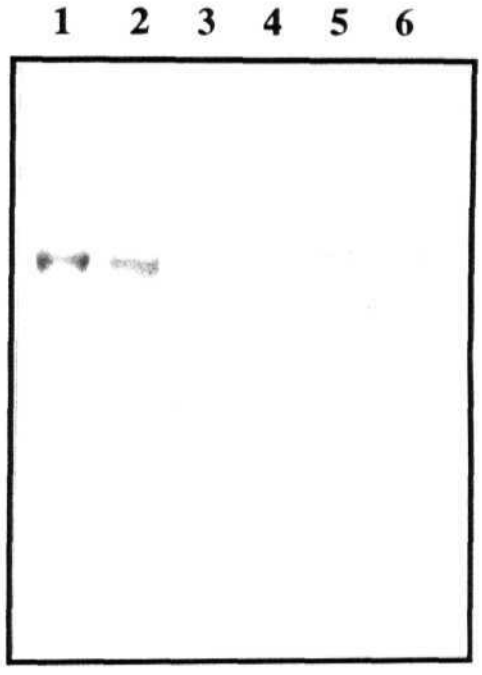

Figure 4.8. Immunoprecipitation of phytochrome A. Crude extract from 4.5 d-old dark-grown oat seedlings was concentrated by ammonium Sulfate precipitation, and were incubated with either pre-immune serum or oat anti-PHYA serum. Total proteins from supernatants and pellets were subjected to SDS-PAGE and immunoblotted. Lane 1, pure phytochrome A (control); lane 2, crude extract; lane 3, supernatant of pre-immune serum; lane 4, supernatant of immune serum; lane 5, pellet of immune serum; lane 6, pellet of pre-immune serum.

4.3.7. Zn^{+2} fluorescence

Berkelman and Lagarias (1986) demonstrated that phytochrome protein can be detected on gels by the interaction of Zn^{+2} ions with chromophore which induces orange fluorescence under UV light. Using this specific phytochrome property, purified phytochrome A after electroblotting was subjected to Zn^{+2} fluorescence (Fig. 4.9A). The electroblot under UV showed only a single orange band close to the molecular weight of the purified phytochrome. This was further confirmed by the fact that the immunoblot too showed a single band corresponding to the fluorescent band (Fig. 4.9B).

4.3.8. *In vitro* proteolysis

Vierstra and Quail (1982a,b) showed that out of the two photointerconvertable forms of phytochrome A, the Pr form was more susceptible than the Pfr form to proteases in the crude homogenate. This susceptibility of the Pr form has been used as a diagnostic tool to see if phytochrome exists in two specific forms. The specificity of phytochrome antibodies was determined using differential proteolysis of Pr and Pfr forms, after incubation of phytochrome samples at room temperature. Fig. 4.10 shows that the Pr form of oat phytochrome is more susceptible to proteases in comparison to the Pfr form. A second band resulting from the degradation of the Pr form was seen just below the main band. This confirmed that phytochrome A antisera is specific for phytochrome A. In oat, Pr specific degradation was initiated within 15 min of incubation. In contrast, in other monocots such as maize, pearl millet, rice and wheat, though the Pr form is susceptible to proteolysis, it degrades slowly when compared to oat. A. Whereas in oat, the Pr specific degradation starts after 15 min, in other species such as maize, rice and pearl millet the degradation is seen only after 8 h of incubation. In case of wheat, the Pr specific degradation is not very prominently visible (Fig. 4.12).

4.3.9. Cross reactivity with other species

In order to determine the cross reactivity of PHYA antibodies with phytochrome A from different species, the crude extracts from various monocot and dicot species were blotted and probed with oat anti-PHYA antibodies. Fig. 4.11 shows that a positive signal is seen with all monocots such as oat (124 kD), maize (127 kD),

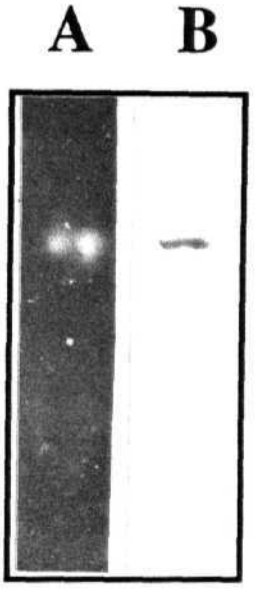

Figure 4.9. Zn^{+2} fluorescence and immunoblot of purified phytochrome. Pure phytochrome A protein was subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was incubated in 1.3 M zinc acetate solution for 30 min. The fluorescent band was visualized under UV and photographed (A). The same membrane was extensively washed and probed with anti-PHYA antibodies (B).

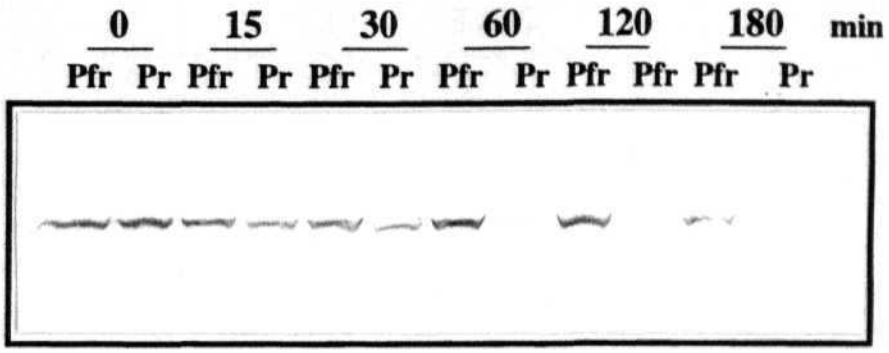

Figure 4.10. Susceptibility of Pr and Pfr forms of oat phytochrome A to endogenous proteases. Crude extracts from shoots of 4 d-old dark-grown seedlings after irradiation with either red light or far-red light were incubated in darkness at 25 °C. At the indicated time points, aliquots were withdrawn and subjected to immunoblotting, after boiling with SDS-PAGE buffer and electrophoresis.

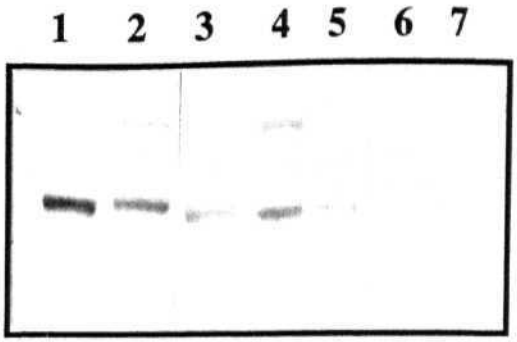

Figure 4.11. Cross reactivity of phytochrome A from different monocot and dicot species against oat anti-PHYA antibodies. Crude extracts from shoots of oat (lane 1), maize (lane 2), wheat (lane 3), rice (lane 4) pearl millet (lane 5), tomato (lane 6) and tobacco (lane 7) **were** subjected to immunoblotting.

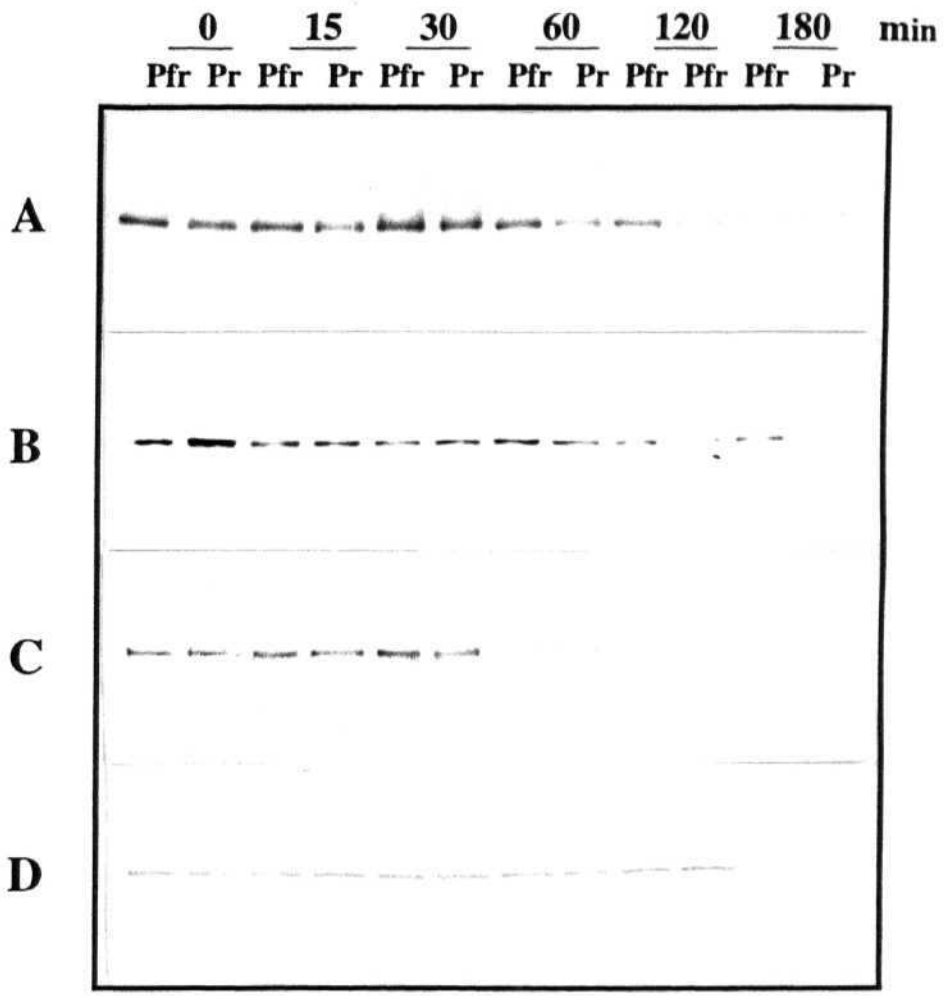

Figure 4.12. Susceptibility of Pr and Pfr forms of phytochrome A to endogenous proteases in different monocot species. Crude extracts from shoots of 4-d old dark-grown seedlings after irradiation with either red light or far-red light were incubated in darkness at 25°C. At the time points indicated, aliquots were withdrawn and subjected to immunoblotting after boiling with SDS-PAGE buffer and electrophoresis. A, maize; B, pearl millet; C, rice; and D, wheat.

wheat and pearl millet, but no signal is seen with dicots such as tomato and tobacco. The specificity of the antibodies against different monocot phytochrome A was also ascertained by the *in vitro* proteolysis of phytochrome A as a diagnostic tool (Fig. 4.12) confirming that these phytochrome A antibodies specifically detect phytochrome

4.3.10. Determination of titer value of phytochrome A antibodies

In order to determine the titer of the phytochrome A antibodies, different concentrations of pure phytochrome protein (0.1 ng to 50 ng) was separated by SDS-PAGE and subjected to immunoblotting. Phytochrome A specific antibodies could detect the protein upto a concentration of 5 ng (Fig 4.13).

4.4. Purification of Light-stable phytochrome

The amount of phytochrome in green leaves was found to be 100-fold less than phytochrome A in dark-grown seedlings and is about 0.002-0.003% of the total soluble proteins (Tokuhisa and Quail 1989). This low amount makes the purification of light-stable phytochrome to homogeneity far more difficult and other pigments such as chlorophyll strongly interfere with the spectroscopic assay of phytochrome. This makes the purification difficult. In spite of these problems, an attempt was made to purify phytochrome from green plant tissues.

4.4.1. Extraction conditions

Phytochrome B was purified partially by following the procedure of Pratt et al., (1991a) with some modifications. Iodoacetamide and leupeptin were included as protease inhibitors besides other inhibitors such as PMSF and 2-mercaptoethanol. Most of the initial steps in the purification were similar to the phytochrome A purification. In order to eliminate phenolic substances and nucleic acids, PE1 was included at a final concentration of 0.1% (v/v). During the PEI step, most of the chlorophyll which interferes with the spectral measurement was eliminated, therefore an extra PEI step, at a final concentration of 0.05% was included to further reduce the chlorophyll content.

4.4.2. Hydroxyapatite chromatography

During the HA chromatography, step gradient of increasing concentrations of KP buffer i.e., 20, 50, 80, 120, and 200 mM KP was used to elute phytochrome. All

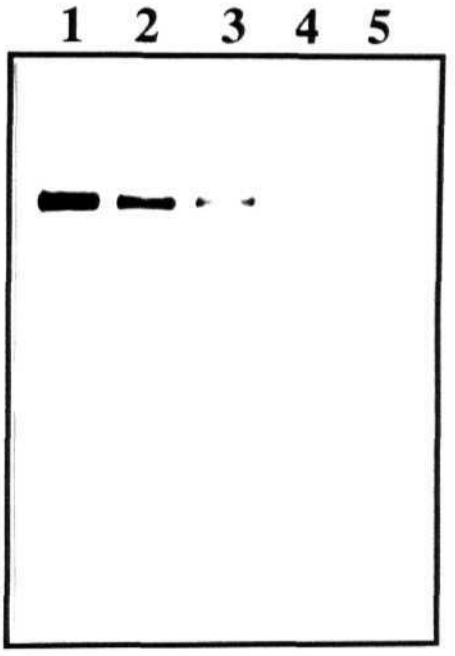

Figure 4.13. Detection limit of oat anti-PHYA antibodies against purified phytochrome A. The aliquots containing different concentrations of oat phytochrome A as indicated below were subjected to immunoblotting. Lane 1, 50 ng; lane 2,25 ng; lane 3, 5 ng; lane **4, 1** ng; **lane 5,0.1 ng.**

the eluates were checked immunochemically by using phytochrome B specific antibodies. Fig. 4.14A shows that a strong phytochrome B signal is present in 50, 80 and 120 mM KP buffer eluates. Weak signals were also found in other eluates. Eluates of 50, 80 and 120 mM KP buffer were pooled and concentrated by ammonium Sulfate precipitation.

4.4.3. DEAE-Sepharose chromatography

DEAE-Sepharose chromatography was used to further improve the purification of phytochrome obtained by HA chromatography. Phytochrome was eluted using an increasing concentration of KP buffer i.e., 50, 100, 200, 300, 400, and 500 mM. All the fractions were checked immunochemically for the presence of phytochrome B (Fig. 4.14B). Though phytochrome B signal was found in all the fractions, those fractions which showed intense bands for phytochrome B were pooled. The eluates of 100, 200 and 300 mM KP buffer were pooled and concentrated with ammonium Sulfate. At this stage of purification difference spectrum of phytochrome B could be recorded. (Fig. 4.15). The effectiveness of this protocol for purification of phytochrome B was checked by using western blotting. Using aliquots drawn from different stages of purification, SDS-PAGE was performed, proteins were electroblotted on to PVDF membrane and probed with PHYB specific antibodies (Fig. 4.16).

4.5. Distribution of PHYA and PHYB in maize seedlings

4.5.1. Specificity of PHY A antibodies

In seedlings of maize, proteins cross reacting with PHYA and PHYB specific antibodies were detected after immunoblotting. The specificity of oat anti-PHYA antibodies against maize PHYA was ascertained by Ouchterlony double diffusion (Fig. 4.6A,B) and the down regulation of phytochrome A after transfer of etiolated seedlings to light (Fig. 4.7). Additionally, maize phytochrome A as detected by oat antibody was less susceptible to degradation by proteases in crude extracts after photoconversion to the Pfr form (Fig. 4.12A). Further, the molecular weight of the protein recognized by oat antibody was equal to the molecular weight of maize PHYA i.e. 127 kD (Vierstra et al., 1984; Biermann et al., 1994) and close to the predicted value on the basis of its gene sequence i.e. 125 kD (Christensen and Quail 1989).

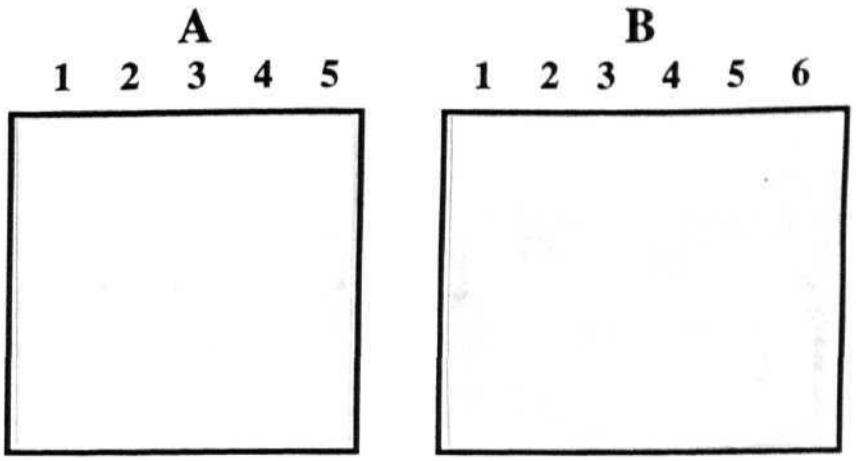

Figure 4.14. A. The elution pattern of PHYB during hydroxyapatite chromatography. Phytochrome B was eluted using step gradient of different concentrations of KP buffer. Lane 1, 20 mM; lane 2, 50 mM; lane 3, 80 mM; lane 4, 120 mM; lane 5, 200 mM.

B. The elution pattern of **PHYB** during DEAE-Sepharose chromatography. Phytochrome B was eluted using step gradient of different concentrations of KP buffer. Lane 1, 50 mM; lane 2, 100 mM; lane 3, 200 mM; lane 4, 300 mM; lane 5, 400 mM; lane 6, 500 mM.

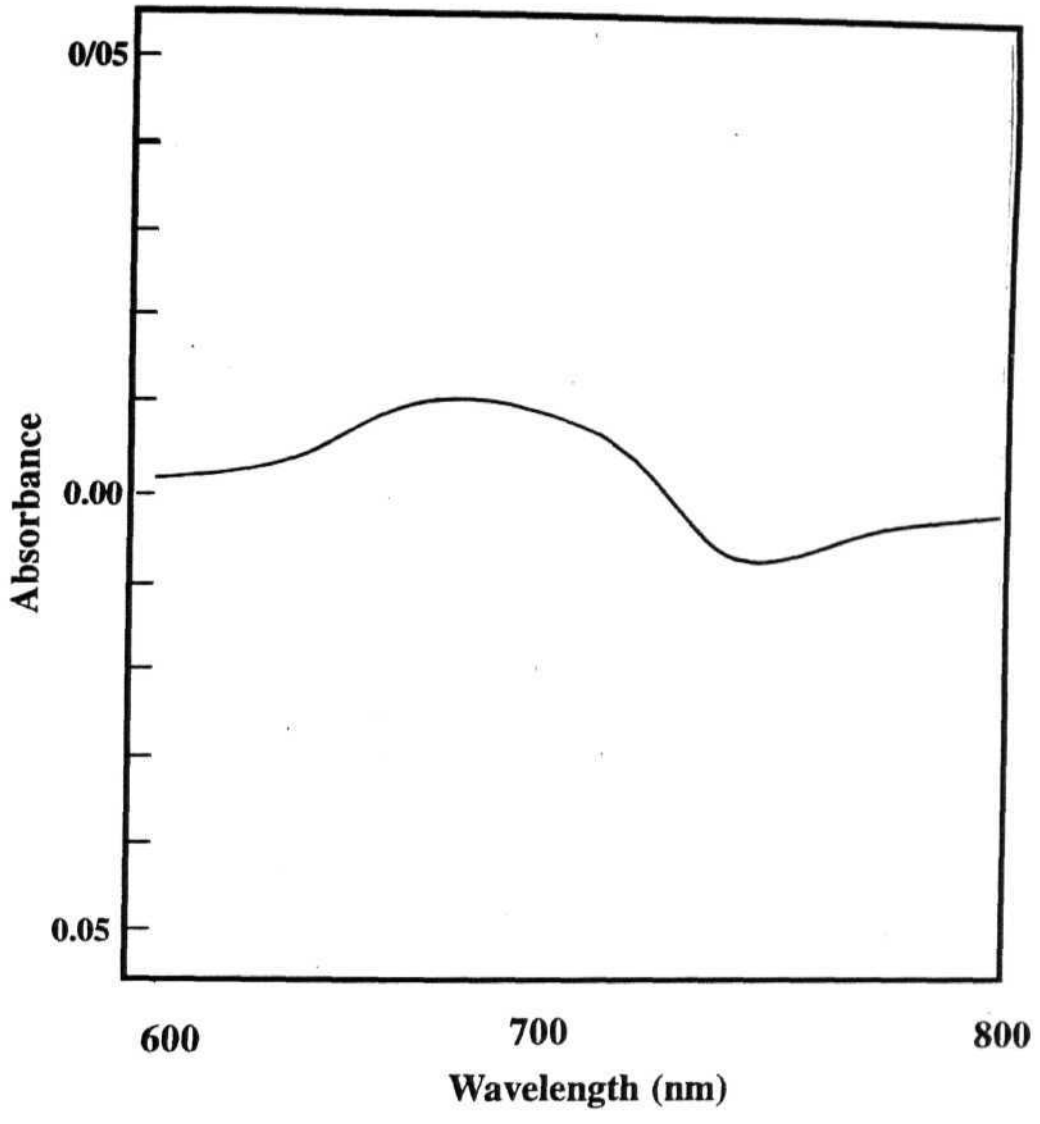

Figure 4.15. The difference spectrum of phytochrome purified from green spinach leaves after DEAE-Sepharose chromatography. The absorption value of Pfr form was stored as base line and after converting the sample to Pr form, the difference spectrum was recorded.

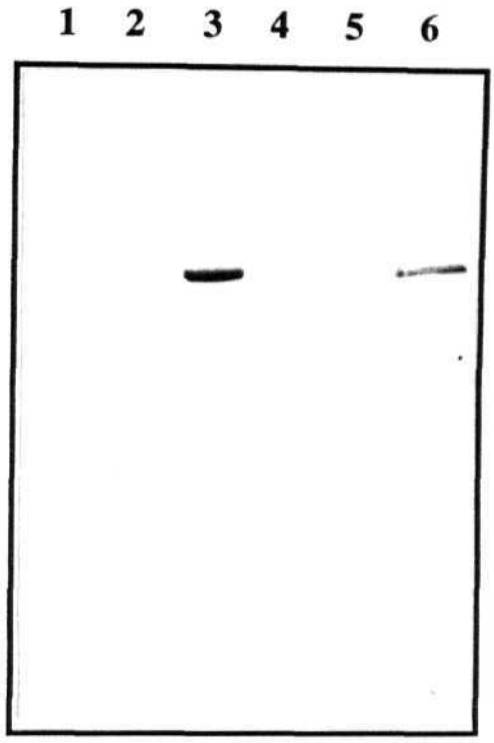

Figure 4.16. Monitoring of **PHYB** purity at different steps of purification by western blotting. Aliquots were drawn during different stages of purification and analyzed by western blotting. Lane 1, crude extract; lane 2, PEI supernatant; lane 3, ammonium Sulfate precipitation; lane 4, hydroxyapatite column wash; lane 5, DEAE-Sepharose column wash; lane, 6. DEAE-purified phytochrome.

4.5.2. Specificity of PHYB antibodies

The specific cross reactivity between monoclonal antibodies raised against tobacco PHYB and maize PHYB apoprotein was ascertained by comparative immunoblotting. The MW of immunodetected PHYB in rice according to this study is about 123 kD which is close to the MW of rice PHYB (122 kD) observed on immunoblots in another study (Wagner et al., 1991). Likewise, in maize these antibodies recognized a PHYB-like protein with a MW of 122 kD. The close correspondence between the MW of maize PHYB detected by tobacco monoclonals and the observed MW of rice PHYB was taken as evidence for the specific recognition of a PHYB-like protein in maize and rice. The above monoclonals also recognized PHYB-like proteins of similar MW in oat and wheat (Fig. 4.17).

4.5.3. Distribution and temporal expression of PHYA and PHYB

The question of whether PHYA and PHYB are inherited in maize seeds from the phase of desiccation of the seeds or resynthesized during the germination was examined by studying the distribution and temporal expression of phytochrome level in the seeds. On dissecting maize seeds incubated in darkness for 24 h after imbibition into scutellum without embryo, embryonic axes and kernels without scutellum and embryo, both PHYA and PHYB could be detected only in the embryonic axes and the scutellum, but not in the deembryonated kernels. In seeds imbibed under RL in the above organs, the levels of PHYA was strongly down-regulated and a similar decline was also noticed for PHYB (Fig. 4.18A,B). Neither of the two phytochrome species, could be detected in the embryonic axes isolated from dry seeds, and also in seeds imbibed for 24 h at 4°C. In germinating seeds, the embryonic axes accumulated PHYA and PHYB, which could be detected respectively 24 and 12 h after sowing. Levels of both phytochrome species peaked at 24 h and declined thereafter and by 72 h very little phytochrome could be detected (Fig. 4.18C,D).

4.5.4. Distribution of PHYA and PHYB in different organs

After the appearance of organs the relative levels of phytochromes were analyzed in different organs of maize seedlings. In 5 d-old dark-grown seedlings, PHYA was present in all the organs of the seedlings such as the root, mesocotyl, coleoptile and first leaf. In etiolated seedlings, the amount of PHYA was maximal in

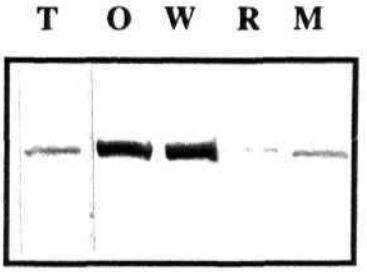

Figure 4.17. Cross reactivity of PHYB-like proteins in different monocot and dicot species against tobacco PHYB monoclonal antibodies. The PHYB like protein were detected using monoclonal antibodies raised against tobacco PHYB. Crude extracts (25 μg protein) from shoots of tobacco (T), rice (R), wheat (W), maize (M) and oat (O) after boiling with SDS-PAGE buffer were subjected to electrophoresis and immunoblotting. The value below each lane indicates the MW of the immunostained band.

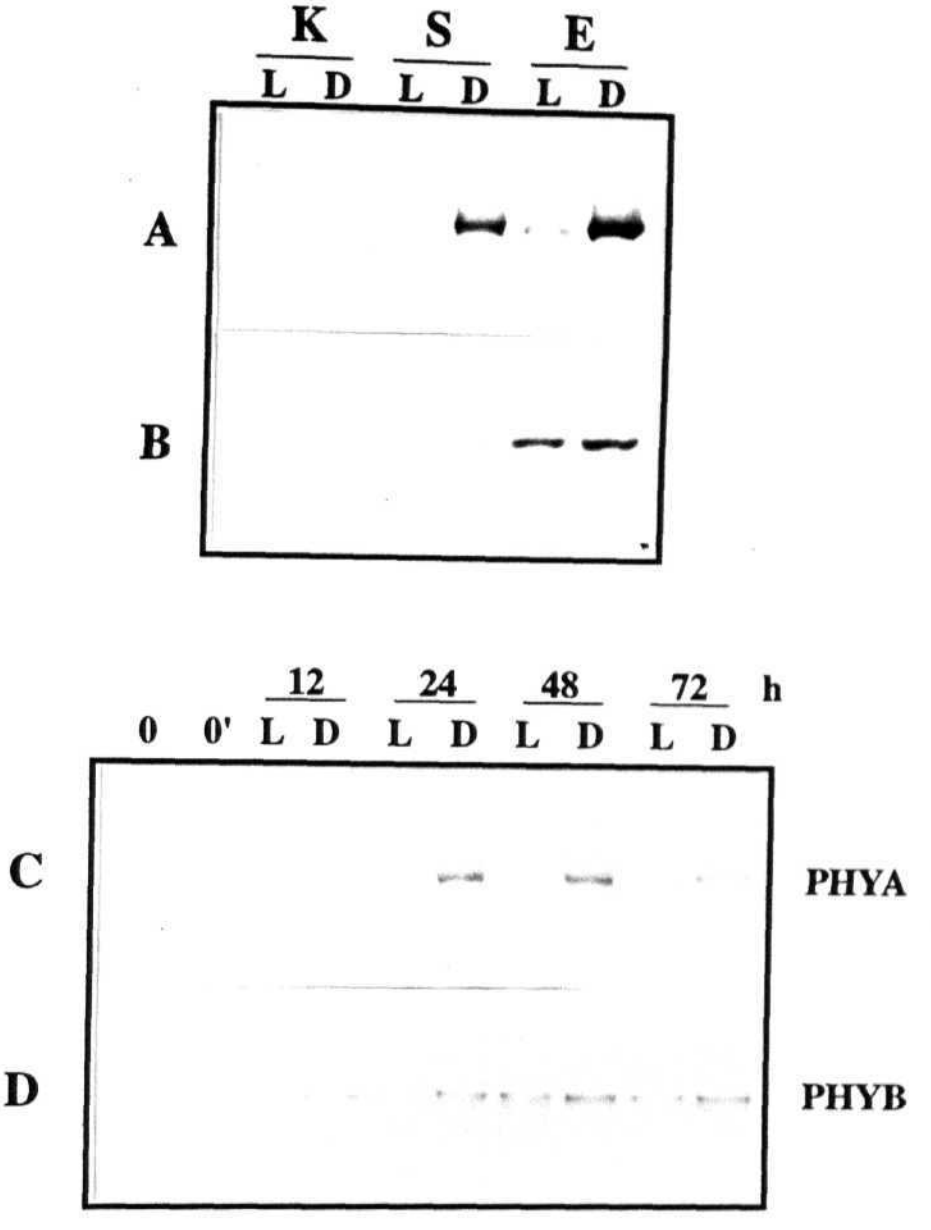

Figure 4.18. Spatial distribution and temporal regulation of PHYA and PHYB levels in germinating seeds. A-B. Distribution of PHYA and PHYB in different parts of maize seeds. Scutellum (S), embryo (E) and deembryonated kernel (K) were separated from maize seeds 24-h after sowing in dark (D) or red light (L). After homogenization and centrifugation, aliquots containing an equal amount of protein (25 μg) were subjected to SDS-PAGE and immunoblotting for PHYA (A) and PHYB (B). C-D. Temporal regulation of PHYA (C) and PHYB (D) levels in embryonic axes of germinating maize seeds in dark (D) and red light (L). For time course study, embryonic axes were either isolated from dry seeds (0) or at the indicated time points (h) after sowing and extracts were subjected to immunoblotting. Embryonic axes isolated from seeds imbibed at 4°C for 24 h has also been included for comparison (0').

the leaf, followed by coleoptile and only a low amount was detected in the root and mesocotyl. In all of the above organs, the amount of PHYA was below the detectability limit in light-grown seedlings. The distribution pattern of PHYB in maize seedlings was somewhat different when compared to that of PHYA. First, there was no excessive accumulation of PHYB in organs such as leaf or coleoptile, as observed for PHYA. However, PHYB level in these organs was much higher than in the other organs. Second, unlike PHYA, PHYB could be detected in both dark- and light-grown seedlings, but its level in the light-grown seedlings was a little lower than dark-controls indicating a mild down regulation of PHYB (Fig. 4.19A,B). In the mesocotyl and the root, the level of PHYA and PHYB in the mesocotyl node and the root cap was higher when compared to the entire organ, indicating that phytochromes preferentially accumulate in the meristematic regions (Fig. 4.19C,D).

4.5.5. Temporal expression of PHYA and PHYB in maize leaf

The comparison of the levels of PHYA and PHYB in various organs of maize seedlings indicated that both phytochrome A and phytochrome B species preferentially accumulate in the leaf and coleoptile. Therefore, the temporal and spatial distribution of phytochromes in these organs was studied in detail. In 4-d-old etiolated seedlings, in which the leaf was about 2 cm long, PHYA could be detected.

PHYA level was maximal in the first leaf of 4 d-old dark-grown seedlings. During the next 4 days while the leaf elongated from 2 to 8 cm long, the PHYA level declined severely with the age of the seedlings, its level being very low in the 8 d-old leaf. In the light-grown leaves, very little PHYA could be seen in young leaves, and thereafter its level was below the detectability limit (Fig. 4.20C). In comparison, the level of PHYB in dark-grown leaves remained almost constant till 6 d and declined thereafter. Though PHYB did not show as massive a decline as PHYA, its level also declined with age of the seedlings, and by 8 d, its level was below the detectability limit. Light-triggered down regulation of PHYB level was dependent on the age of the leaf, while it was mild in the younger leaves, it was more severe in the older leaves, and only a little PHYB was present in 8 d-old leaves (Fig. 4.20D).

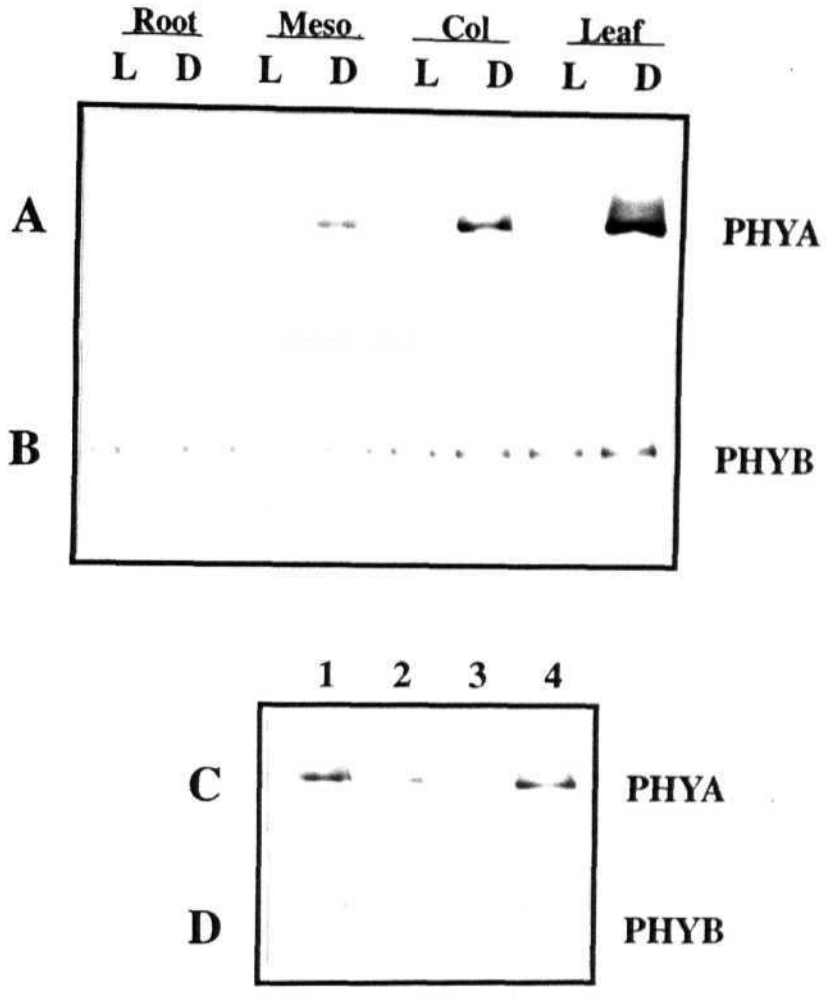

Figure 4.19. Distribution of PHYA and PHYB in different organs of maize seedlings. A-B. Red light-(L) and dark-grown (D) grown 5 d-old maize seedlings were excised into root, mesocotyl **(Meso),** coleoptile **(Col)** and first leaf. Crude extracts were subjected to immunoblotting as described in Fig. 1. and probed with PHYA (A) and PHYB (B) antibodies. C-D. Level of PHYA (C) and PHYB (D) was also compared between the root tip (1) and root (2), mesocotyl (3), and mesocotyl node (4). Extracts were subjected to immunoblotting as described in Fig. 4.17.

4.5.6. Temporal expression of PHY A and PHYB in coleoptile

It was observed that 4 d-old coleoptile possessed a maximal level of PHYA. During further growth, while the length increased from 2 cm to 5 cm, the level of phytochrome declined. No significant amount of PHYA could be detected in the light-grown coleoptiles (Fig. 4.20A, Fig. 4.21 A). PHYB level was higher in the dark-grown coleoptiles when compared to that of the light-grown coleoptiles, and declined slowly with age, and by 8 d the level of PHYB was below the detectability limit in the light-grown coleoptile, whereas in the dark-grown coleoptile, the level of PHYB can be observed till 8 d (Fig. 4.20B, 4.21B).

4.5.7. Spatial distribution of PHYA and PHYB in leaf

Since the dark-grown maize leaf showed a distinct pattern of PHYA accumulation, its distribution profile in the leaves was analyzed during the course of the leaf development. Leaves were cut into 1 cm long segments from the base to the tip and the phytochrome level was studied by western blotting. In very young leaves (2 cm) the leaf base segment contained high level of PHYA than tip segment, but during the subsequent growth of the leaf, PHYA preferentially accumulated to two different regions viz. the leaf base and the upper half of the leaf (Fig. 4.26A).

Spectral estimation of phytochrome level along the leaf length revealed that in the 7 d-old leaf of dark-grown seedlings, phytochrome level is higher at the leaf base, and it then declines in the middle region of the lower half of the leaf, and is higher again in the upper half of the leaf (Fig. 4.23). Immunoblotting of phytochromes confirmed the above pattern of phytochrome distribution in dark-grown leaves. Both PHYA and PHYB followed a pattern similar to the distribution of spectrally active phytochrome in maize leaf with a high level at the leaf base and at the upper half of the leaf (Fig. 4.22A,C,D). The densitometric scanning data show that both phytochromes follow a similar pattern of distribution along the length of the leaf. PHYA concentration is high at the basal 1 cm regions of the primary leaf, but declines thereafter in the middle portion of the leaf by almost 6-fold. In the top half of the leaf however, the PHYA concentration increases by 8-fold when compared to the middle portion of the leaf (Fig. 4.24A). PHYB is less expressed when compared to PHYA in dark-grown leaves. However, the distribution pattern is similar to that of PHYA. The

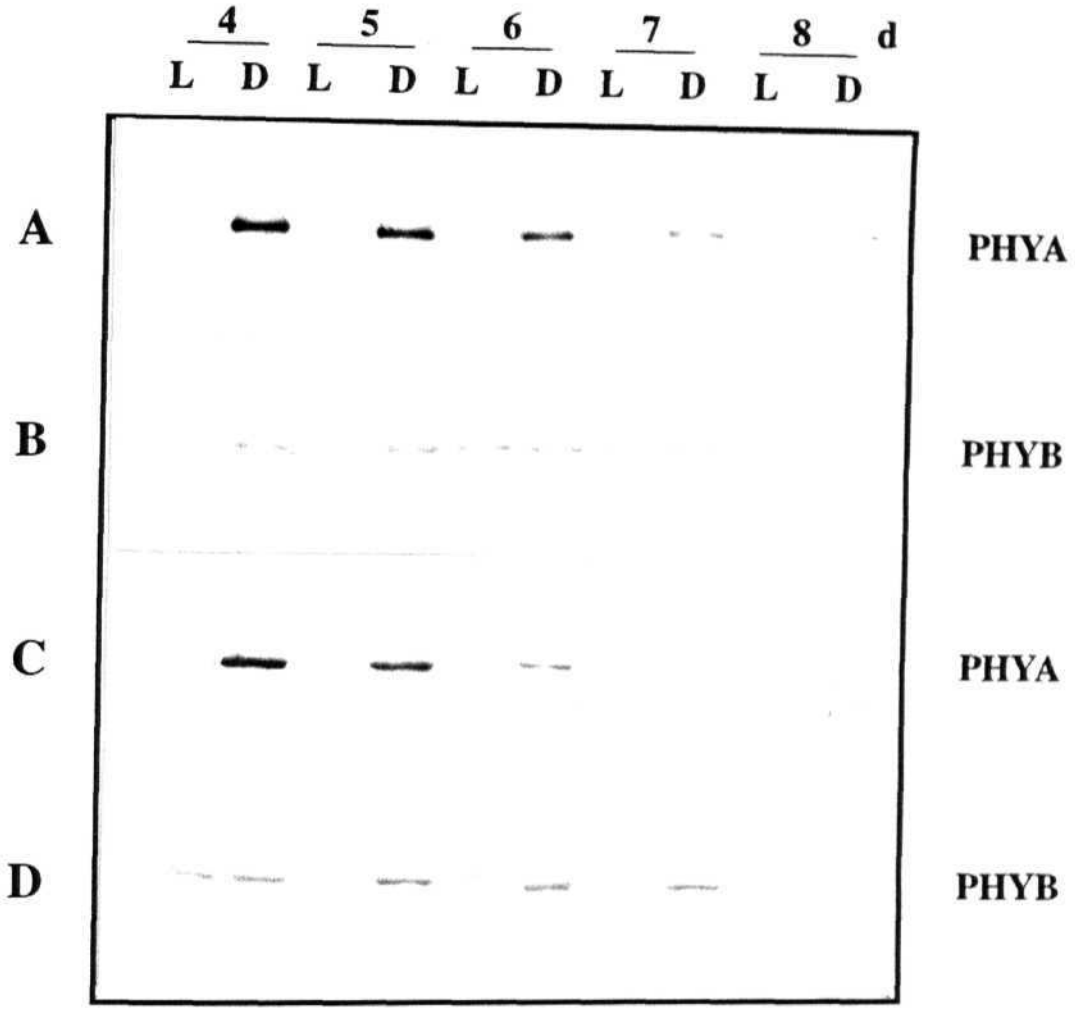

Figure 4.20. Temporal regulation of PHYA (A, C) and PHYB (B, D) levels in the leaf (C, D) and coleoptile (A, B) of maize seedlings grown in dark (D) and red light (L). For time course study, coleoptiles or leaves were excised from the seedlings at the indicated time points (d) after sowing. Extracts were subjected to immunoblotting as described in Fig. 4.17.

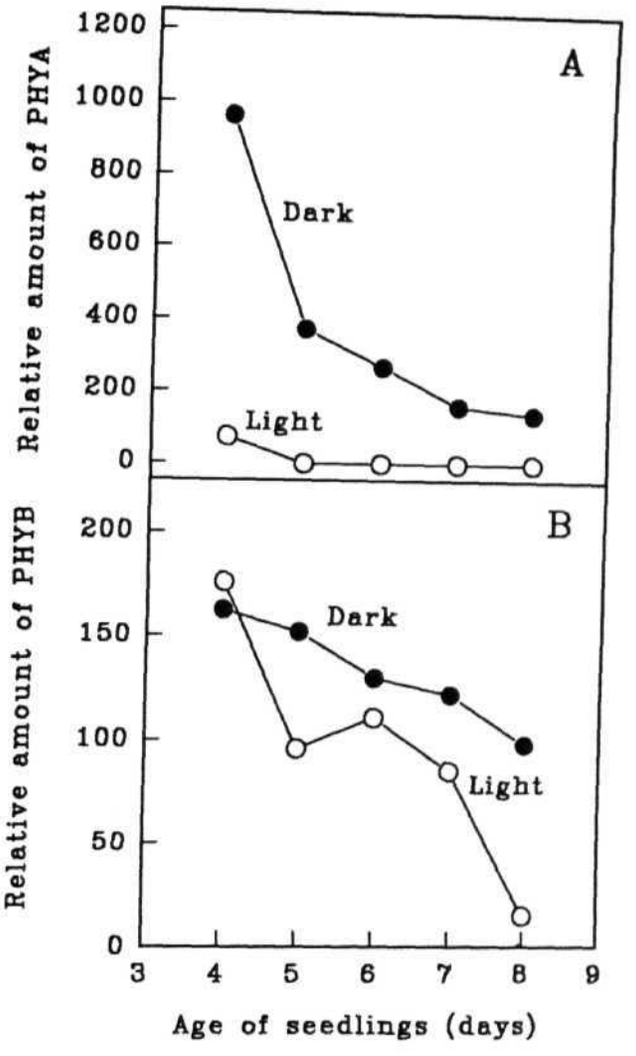

Figure 4.21. Time course of accumulation of PHY A and PHYB in the coleoptiles of maize seedlings grown in the darkness and light. The level of PHYA (A) and PHYB **(B)** was quantitated by densitometric scanning of the blots displayed in Fig. 4.20.

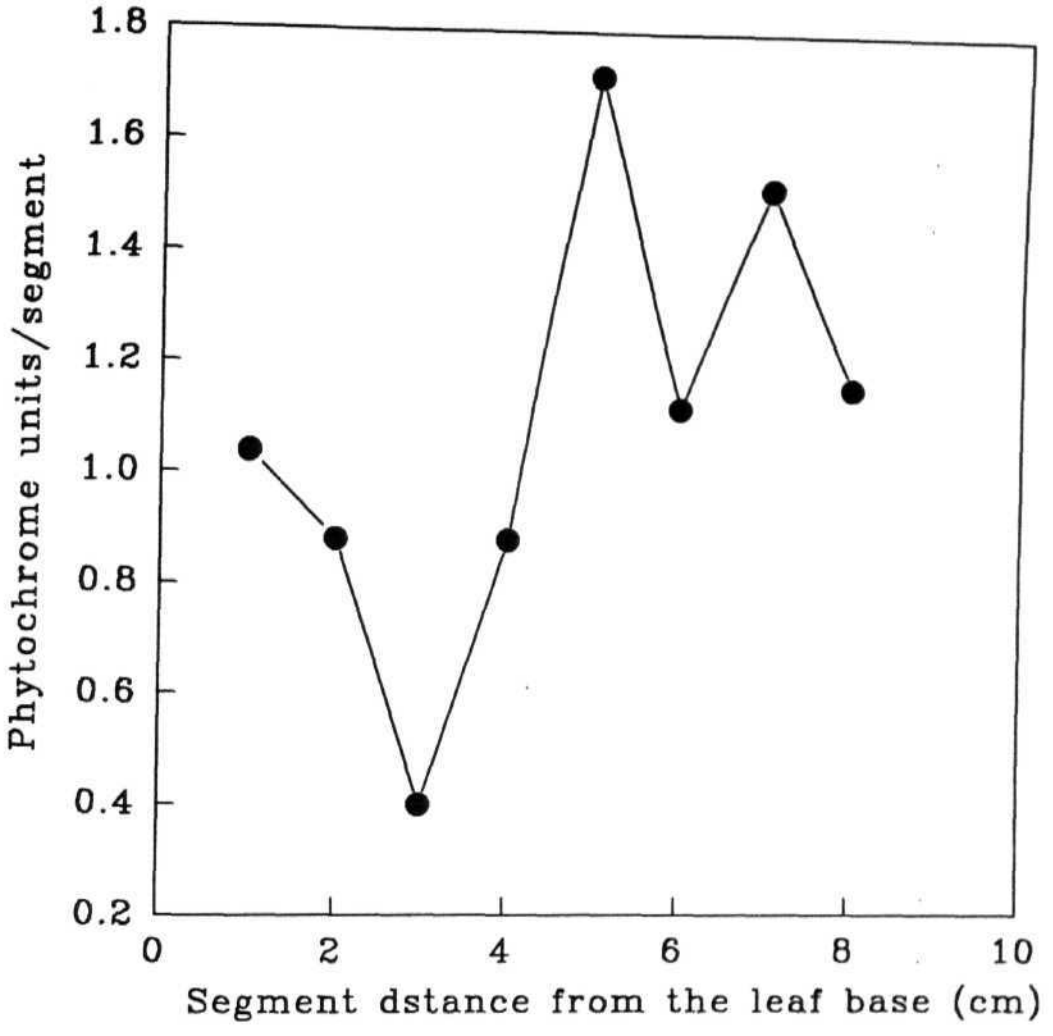

Figure 4.23. The level of spectrally active phytochrome (units/segment) in the segments of etiolated maize leaf. The first leaf from 7 d-old dark-grown maize seedlings was excised into 1 cm long segments from the base to the tip. The amount of phytochrome in segments was estimated spectroscopically and was expressed as PHYA units/segment.

level of PHYB was high at the base, declined 5-fold in the middle portion of the leaf and increased 9-fold at the tip (Fig. 4.24B). In the light-grown leaf, while the PHYA was below the level of detection due to its light-induced down-regulation of PHYA (Fig. 4.22 B), the PHYB distribution was essentially similar to the dark-grown leaves (Fig. 4.22D).

4.5.8. Leaf blotting

Tissue printing of the leaf confirms the distribution pattern of both phytochromes obtained by spectroscopic data as well as immunoblots. In these blots the PHYA and PHYB signal was found more in the base and upper half of the leaf. Positive signal was also found in the mid rib region i.e. vascular tissues (Fig. 4.25).

4.5.9. Distribution of protochlorophyllide oxidoreductase

The distribution pattern of protochlorophyllide oxidoreductase level in the maize leaf was also studied. It is a protein which is similar to phytochrome, undergoes down-regulation in light-grown seedlings (Forreiter et al., 1990). In maize leaves, contrary to PHYA and PHYB distribution pattern, the protochlorophyllide oxidoreductase level increased progressively from the base towards the leaf tip (Fig. 4.22 E,F). This pattern was observed in both light and dark-grown maize leaves, although the level of POR was much lower in the light-grown leaves when compared to the dark-grown leaves. It is evident from these results that though light induces a decline in the level of PHYB and protochlorophyllide oxidoreductase, it does not alter the distribution profile of these proteins along the length of the leaf.

On 4 d, 5 d, and 6 d, in contrast to PHYA, protochlorophyllide oxidoreductase, which was also present in nearly equal amount in two segments of 4 d-old dark-grown leaves accumulated preferentially in the tip region of the leaf during the course of leaf development (Fig. 4.26C).

4.5.10. Distribution of PHYA in the coleoptile

Similarly, during the growth of the coleoptile from 4 d to 8 d, a gradient towards the tip was found (Fig.4.26B). PHYA showed a preferential accumulation at the tip. On 4 d the length of the coleoptile is about 2 cm and phytochrome A was found more in the basal segment and relatively less at the tip. During the subsequent

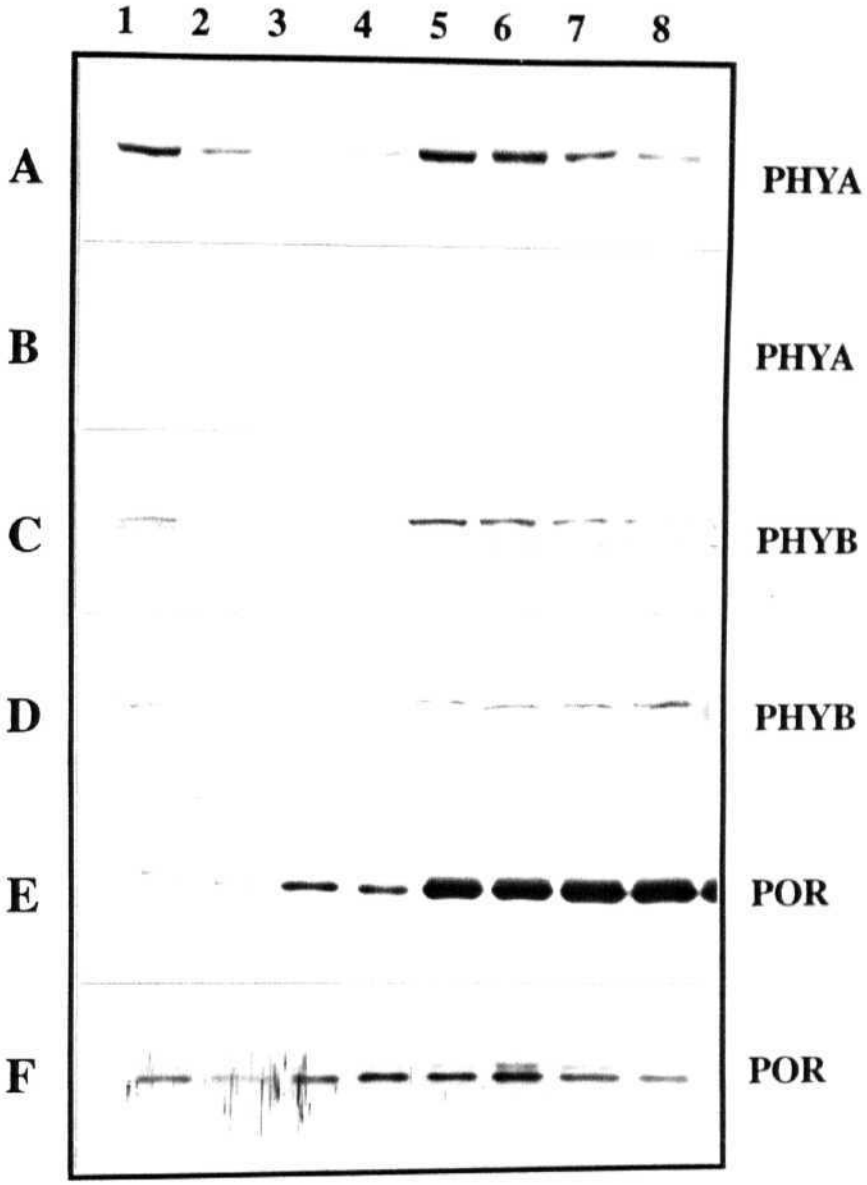

Figure 4.22. Distribution of PHYA, PHYB and protochlorophyllide oxidoreductase **(POR)** along the length of the maize leaf. The first leaf from 7-d-old dark- (A, C, E) and red-light (B, D, F) grown seedlings was excised into 8 segments of 1 cm length from the base to the tip. Extracts (50 ug protein) were subjected to immunoblotting as described in Fig. 4.17 and were probed with PHYA (A, B) and PHYB (C, D) antibodies. For protochlorophyllide oxidoreductase (E, F), crude extracts (2.5 µg protein) were subjected to immunoblotting using polyclonal antibodies raised against barley protochlorophyllide oxidoreductase. The lane numbers on the top of the gel correspond to the segment number with reference to the leaf base.

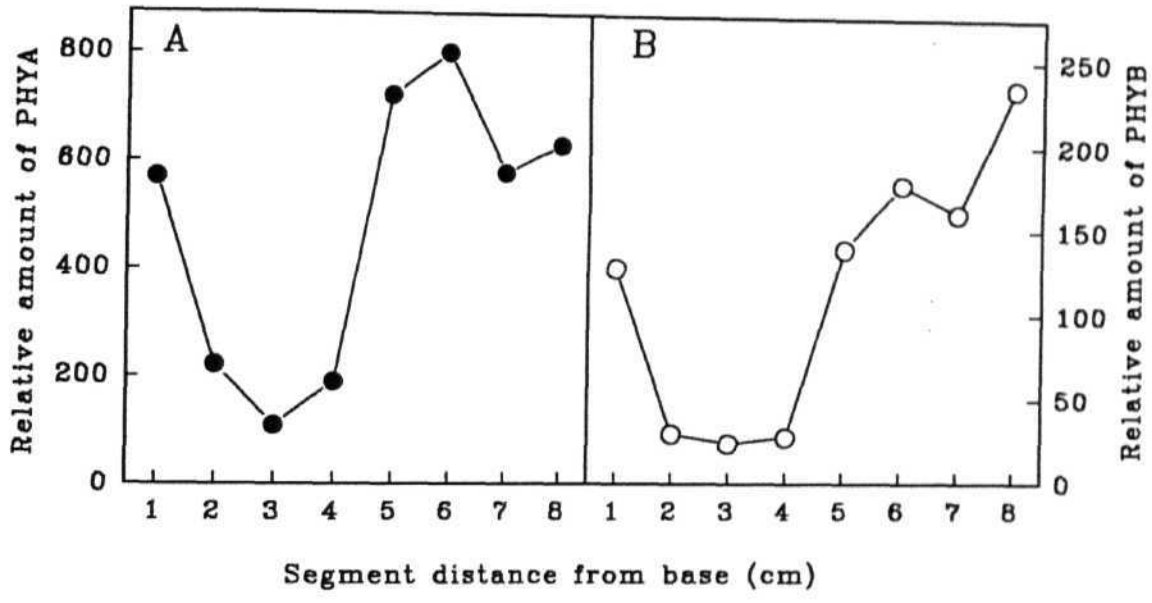

Figure 4.24. Distribution of PHYA and PHYB along the length of the maize leaf. The first leaf from 7 d-old dark-grown maize seedlings was excised into 1 cm long segments from the base to the tip. The segments were numbered from the base to the tip. The respective segments after homogenization were subjected to immunoblotting. The levels of PHYA (A) and PHYB (B) were quantitated by densitometnc scanning of immunoblots displayed in the Fig. 4.22A (A) and C (B).

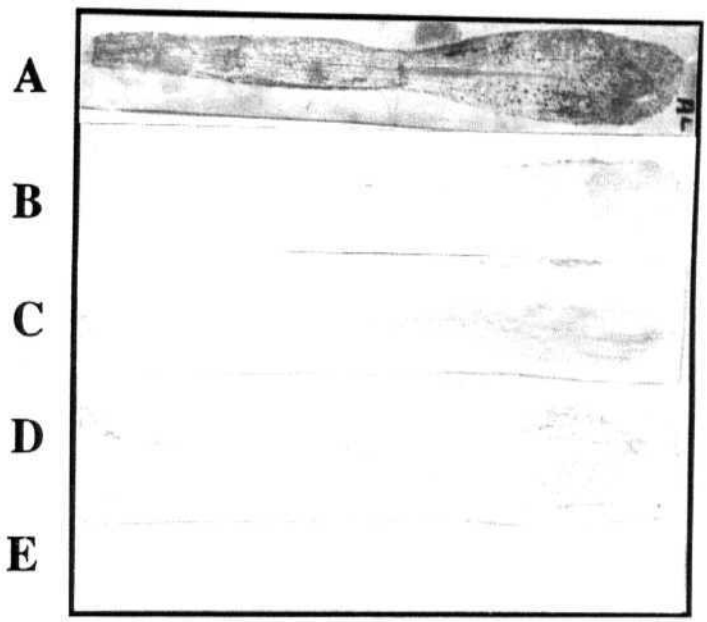

Figure 4.25. Tissue printing of 7 d-old primary leaf of maize. The epidermal layer was removed by rubbing with sand paper, and the leaves were pressed on PVDF membrane. The membranes were probed against PHYA and PHYB antibodies. A. PHYA (dark) B. PHYA (light) C. PHYB (dark) D. PHYB (light) and E. control (dark) probed against pre-immune serum.

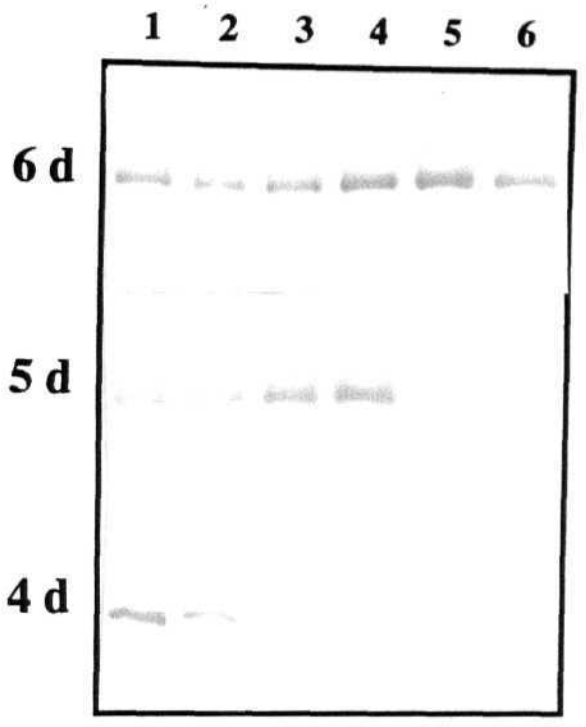

Figure 4.26 A Spatial distribution of PHYA in the leaf excised from 4 d to 6 d-old dark-grown seedlings. Extracts (25 fig protein) were subjected to immunoblotting using its antibodies as described in Fig. 4.22. The lane numbers on the top of the gel correspond to the segment number with reference to the leaf base.

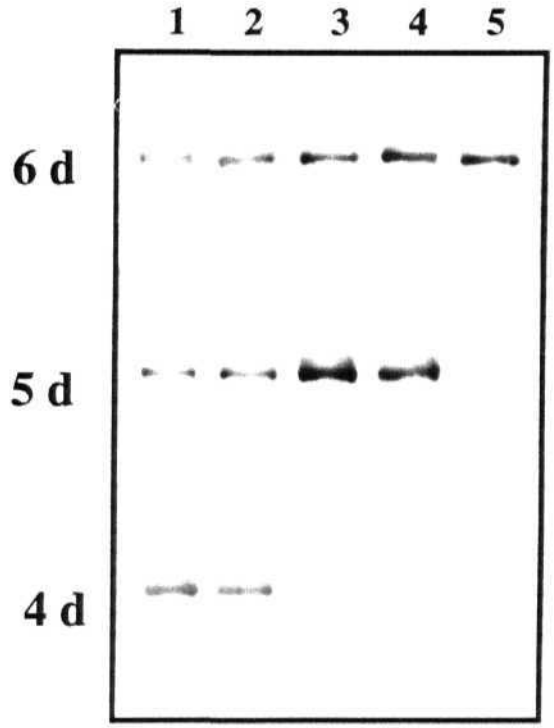

Figure 4.26 B Spatial distribution of PHYA in the coleoptile excised from 4 d to 6 d-old dark-grown seedlings. Extracts (25 μg protein) were subjected to immunoblotting as described in Fig. 4.17. The lane numbers on the top of the gel correspond to the segment number with reference to the coleoptile base.

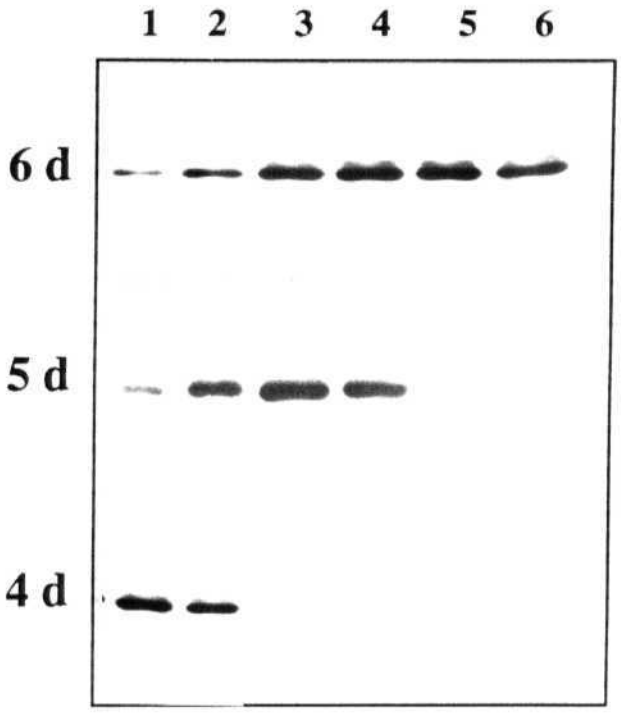

Figure 4.26 C Spatial distribution of protochlorophyllide oxidoreductase in the leaf excised from 4d to 6d old dark-grwon seedlings. Extracts of protochlorophyllide oxidoreductase were subjected to immunoblotting using its antibodies as described in Fig. 4.22. The lane numbers on the top of the gel correspond to the segment number with reference to the leaf base.

growth up to 6 d the PHYA level was found to increase gradually from the base to the tip.

4.5.11. PHYA and PHYB levels in the leaf during dark to light transition

In the leaves of 5 d-old dark-grown maize seedlings, RL induced a rapid decline in the level of PHYA within 2 h, and during the next 24 h it declined gradually to a very low level. In order to check the effect of light on phytochromes, and to observe the kinetics of degradation, 5 d-old dark-grown seedlings were transferred to RL and at different time points leaves were separated and checked for phytochrome A and phytochrome B levels by immunoblotting. Exposure to RL induced a rapid decline in the level of PHYA within 2 h and it gradually declined to a very low level during the next 24 h (Fig. 4.27A-A). Likewise, exposure to RL also initiated a decline in the PHYB level in the leaf (Fig. 4.27A-B). Densitometric scanning data shows that during the transition from dark to RL, PHYA level in the leaf declined gradually. Within 4 h of transfer there was a 10-fold decline in the PHYA level and within the next 8 h there was a decline of 38-fold (Fig. 4.28A).

4.5.12. PHYA level in coleoptile during dark to light transition

The light effect and the kinetics of light-induced down regulation in a different organ i.e., coleoptile, was studied. When 5 d-old dark-grown seedlings were exposed to RL and coleoptiles were separated and checked for PHYA level. The PHYA level decreased drastically by 4 h and it decreased gradually to the level of below the detectability limit (Fig.4.27A-C).

4.5.13. PHYA and PHYB levels in the leaf during light to dark transition

In order to check the kinetics of resynthesis of PHYA, 5 d-old light-grown seedlings were transferred to darkness and at different time points, the primary leaf was separated and the level of both PHYA and PHYB were checked by immunoblotting. Figure 4.27B shows that while in the 5 d-old light-grown leaf PHYA level is below the detectability limit, on transfer to darkness, PHYA reaccumulated within 8 h and thereafter its level increased steadily in the leaf (Fig. 4.27B-A). Similarly, the transfer of the seedlings to darkness also stimulated the synthesis of

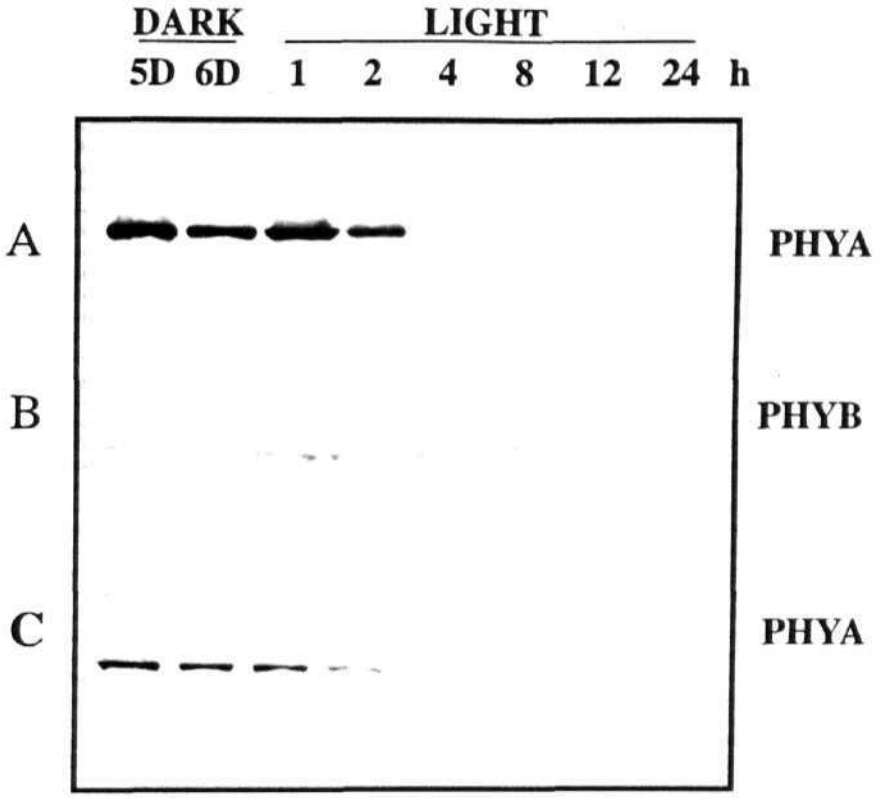

Figure 4.27 A. Time course of PHYA and PHYB levels after dark-to-light transition. 5 d-old dark-grown (5D) seedlings were transferred to red light for 24 h or kept in darkness (6D) as control. At the time points (h) indicated, PHYA (A) and PHYB (B) levels were analyzed in the leaves. Similarly, in coleoptile the level of PHYA (C) was analyzed at the indicated time points. Extracts were subjected to immunoblotting as described in Fig. 4.17.

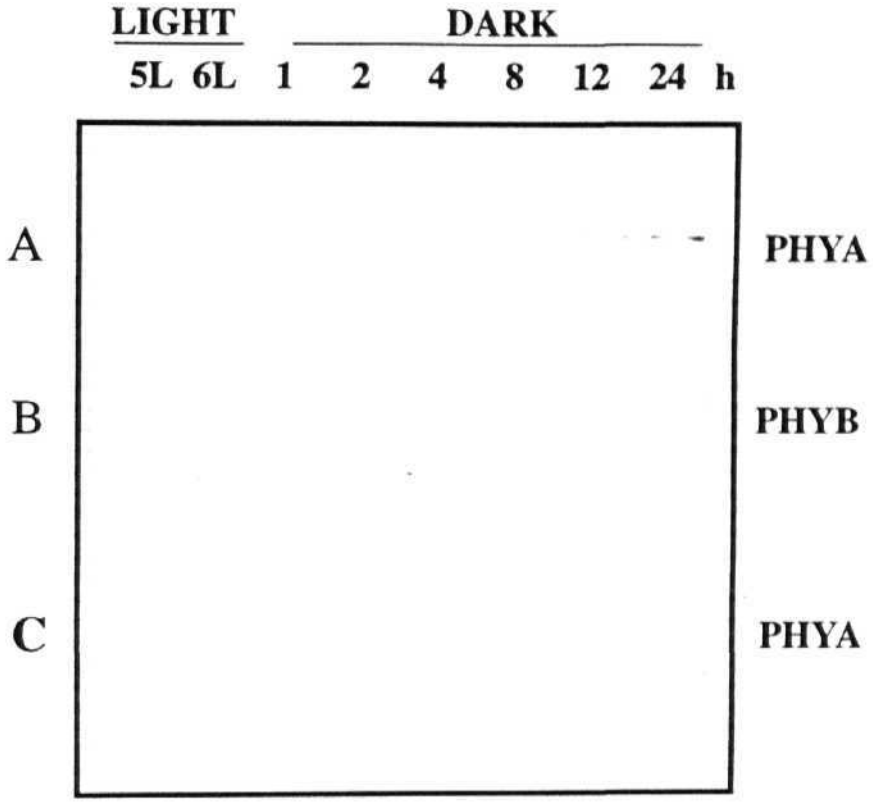

Figure 4.27 B. Time course of PHYA and PHYB levels after light-to-dark transition. 5 d-old light-grown (5L) seedlings were transferred to red light for 24 h or kept in light (6L) as control. At the time points (h) indicated, PHYA (A) and PHYB (B) levels were analyzed in the leaves. Similarly, in coleoptile the level of PHYA (C) was analyzed at the indicated time points. Extracts were subjected to immunoblotting as described in Fig. 4.17.

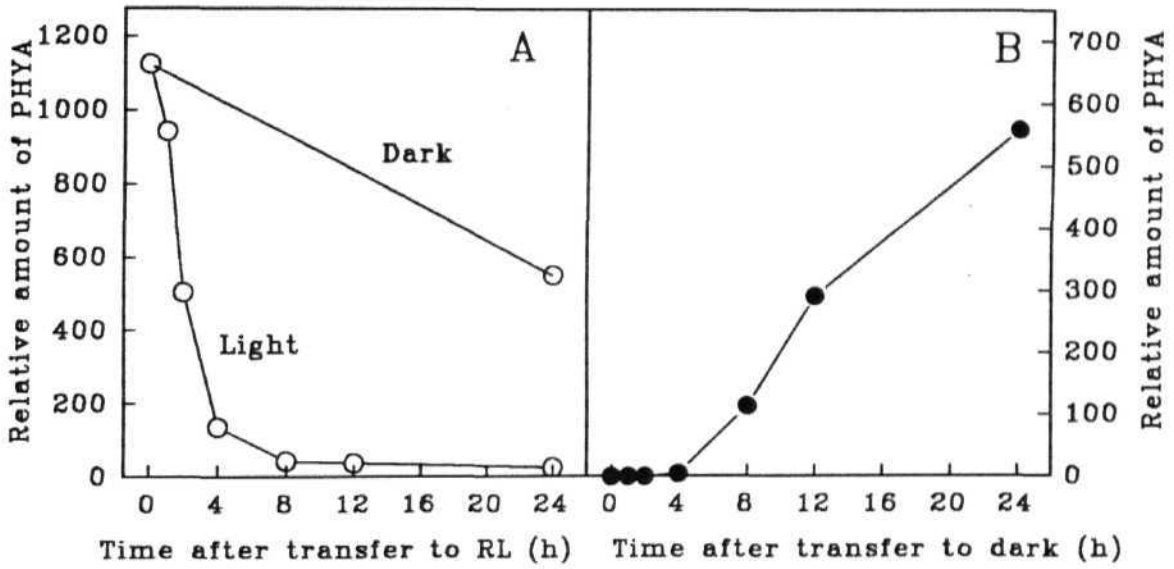

Figure 4.28. Time course of PHYA level after dark to light (A) or light to dark (B) transitions. A. Five day old dark-grown maize seedlings were transferred to red light for 24 h or kept in darkness as control (Fig. 4.27 A). B. Five day old red light-grown maize seedlings were transferred to darkness for 24 h or kept in light as control (Fig. 4.27 B). At the time points (h) indicated, PHYA level was quantitated. The level of PHYA was quantitated by densitometric scanning of immunoblots displayed in the Fig. 4.27 A and 4.27 B.

PHYB in the leaf (Fig. **4.27B-B).** The reaccumulation of PHYA started with a lag period of 4 h and by 24 h there was an increase of 500-fold in the PHYA protein level (Fig.4.28B).

PHYA and PHYB distribution in maize leaf showed a typical pattern with more amount of PHYA in the leaf base and in the upper half of the leaf, indicating a likely effect of cell maturity gradient on the phytochrome level. Therefore, the effect of cellular position on resynthesis of PHYA in maize leaves was examined after transferring 5 d-old light-grown seedlings to darkness. In maize leaves transferred to darkness, PHYA preferentially accumulated in the region of the leaf tip and to some extent in the leaf base by 12 h. On further growth in darkness, its level also increased in other segments of the upper half of the leaf too, and by 48 h, the distribution pattern was identical to that of the dark-control seedlings of the same age (Fig. 4.29).

4.5.14. PHYA level in the coleoptile during light to dark transition

Similarly the resynthesis of PHYA in the coleoptile was studied during the light-to-dark transition of the seedlings. 5 d-old light-grown seedlings were transferred to darkness and coleoptile was separated and checked for PHYA level by immunoblotting. As PHYA is light sensitive, its level was below the detectability limit in light and resynthesis took place during the dark transition. PHYA signal was detected about 8 h after transfer by irnmunoblotting (Fig. 4.27B-C).

4.6. Distribution of PHYA and PHYB tomato seedlings

Studies on PHYA and PHYB distribution in maize seedlings particularly in different segments of the leaf shows that both the proteins have a similar distribution profile along the length of the dark-grown leaf. In order to see if distribution of both PHYA and PHYB is correlated, we used the *aurea* mutant of tomato as a material. Tomato *aurea* mutant possesses phytochrome A apoprotein which can be detected immunochemically but it is spectrally inactive. However, *aurea* does possess spectrally active PHYB. Therefore, in this mutant it is possible to study the distribution pattern of PHYB in the absence of active phytochrome A. It also provides the distribution of phytochrome apoprotein in the absence of chromophore and/or uncoupled with chromophore. Monoclonal antibodies raised against PHYA of pea (mAP), and PHYB of tobacco (mAT) were used to detect PHYA and PHYB

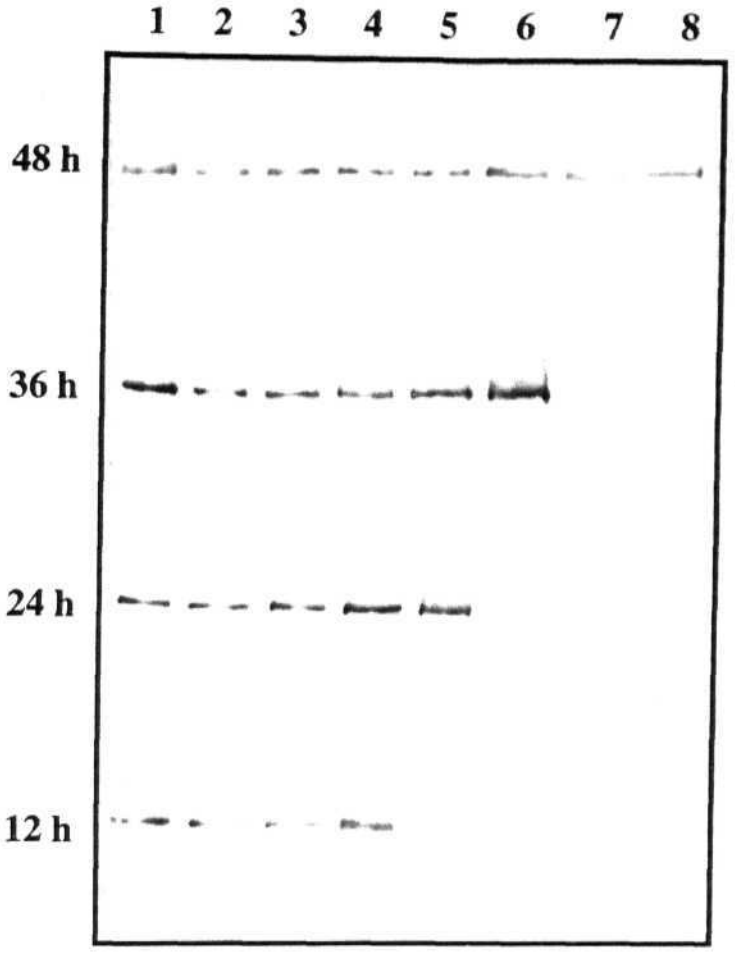

Figure 4.29. Profile of reaccumulation of PHYA level along the length of maize leaf. 5-d-old red light-grown seedlings were transferred to darkness for 48 h. At the time points (h) indicated, PHYA level was analyzed in 1 cm long segments excised from the first leaf. Extracts (25 μg protein) were subjected to immunoblotting as described in Fig. 4.17 and were probed with PHYA antibodies. The lane numbers on the top of the gel correspond to the segment number with reference to the leaf base.

respectively in tomato seedlings. The specificity **of** these antibodies against **PHYA and PHYB in** tomato was established by Sharma et al., (1993).

4.6.1. Time course of PHYA and PHYB

The levels of PHYA and **PHYB** in developing tomato seedlings was monitored in the shoots. In dark-grown WT seedlings, PHYA amount was maximal on the 5 d and thereafter declined with age. In the light-grown WT seedlings, PHYA **level** was below the detectability limit due to the light-induced down regulation. **PHYB** level was maximal in the 4-5 d old seedlings and declined with age. It was also found that PHYB level is slightly reduced in the light-grown seedlings when compared to the dark-grown seedlings (Fig. 4.30).

Densitometric quantitation of the blots show that in WT tomato, the amount of PHYA was maximal on the 5 d in dark-grown seedlings. Thereafter, it declined steadily up to the 8 d, and by this time it was about 1.8-fold less than 5 d-old seedlings (Fig.4.31A). In light-grown seedlings, its level was below the detectability limit. In case of PHYB, the maximal amount of PHYA was found on the 4 d, which gradually declined by 1.4-fold by 8 d. Both light and dark-grown seedlings had almost similar levels of PHYB, but in general the level of PHYB in light-grown seedlings was slightly lower than that of PHYB in dark-grown seedlings (Fig.4.31B).

In the *aurea* mutant also both PHYA and PHYB amounts were maximal at 5 d and declined with age. The decline was more pronounced for PHYA whereas PHYB level was nearly the same (Fig. 4.30). Though *aurea* PHYA lacks chromophore the accumulation of its apoprotein was similar to that observed in WT which possess spectrally active phytochrome. Similar to WT, almost equal amounts of PHYB was found in both light- and dark-grown seedlings of *aurea* mutant and followed the same pattern (Fig.4.31C,D).

4.6.2. Distribution of PHYA and PHYB in different organs

In order to study the localization of both PHYA and PHYB in the developing tomato seedlings, 5 d-old light- and dark-grown tomato seedlings were dissected into three major organs i.e., root, hypocotyl and cotyledon. PHYA and PHYB levels were checked in each of these organs by immunoblotting.

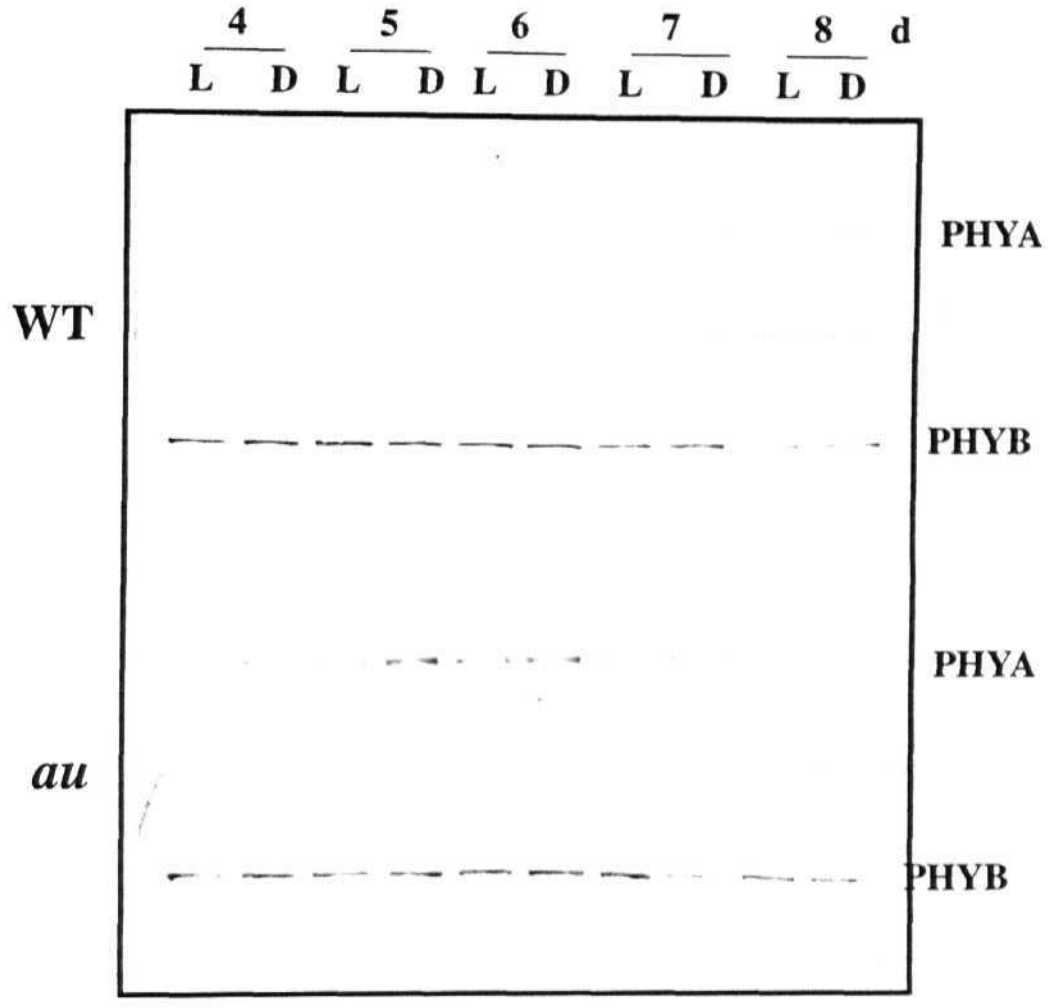

Figure 4.30. Temporal regulation of PHYA and PHYB levels in the WT and *au* seedlings grown in dark (D) and light (L). For time course study, shoots were excised at the time indicated (d) after sowing. Extracts were subjected to immunoblottings to detect PHYA and PHYB protein levels.

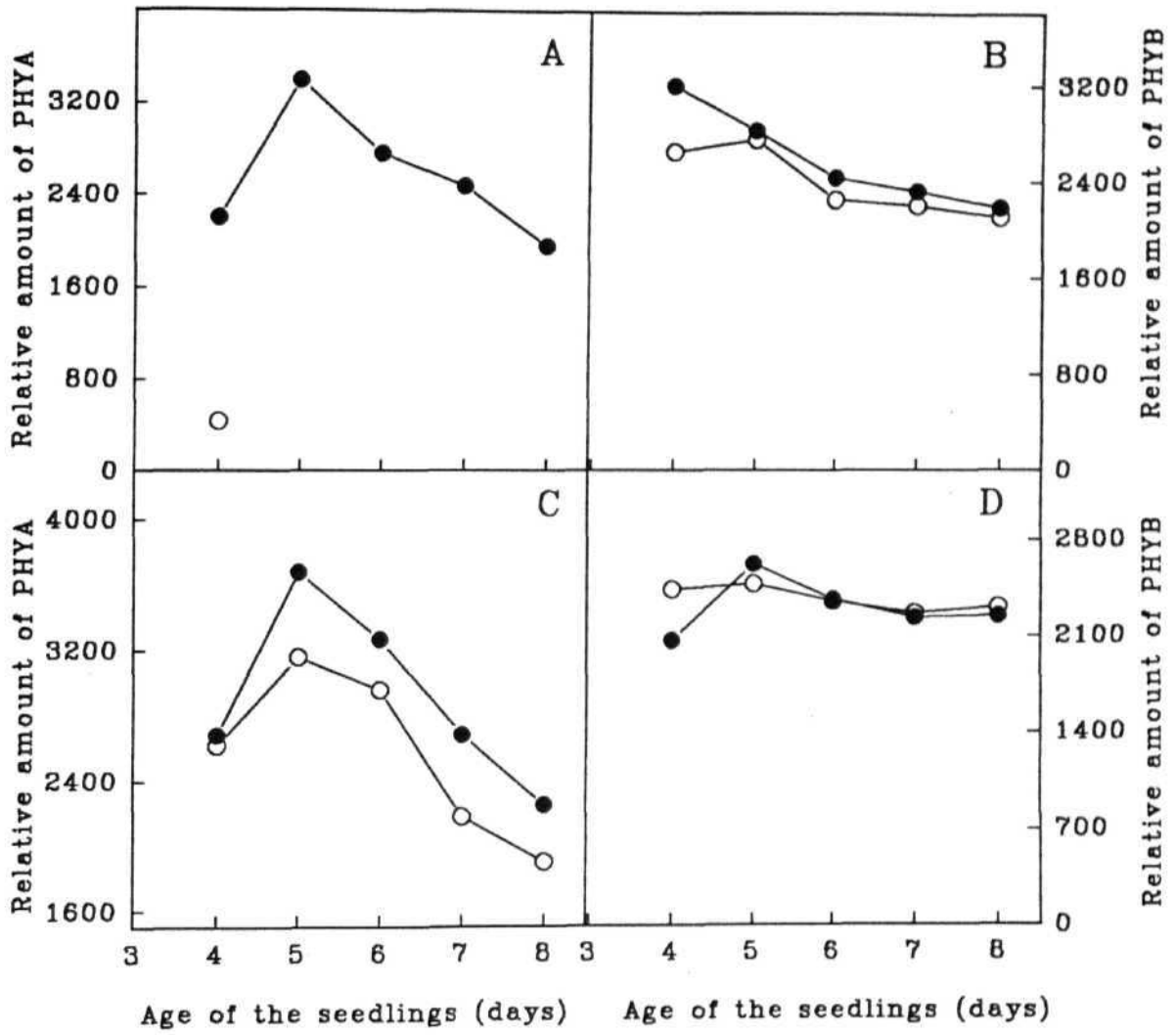

Figure 4.31. Temporal regulation of PHYA (A, C) and PHYB (B, D) levels in the WT (A, B) and *aurea* (C, D) seedlings of tomato grown in the dark (•) and light (○). The level of PHYA and PHYB was quantitated by densitometric scanning of immunoblots displayed in the Fig. 4.30.

In the light-grown WT seedlings, PHYA level was very low or below the detectability limit when compared to the dark-grown seedlings. Only in hypocotyl a little PHYA could be detected in the light-grown seedlings. In cotyledons and roots its amount was below the detectability limit probably due to the light-induced down regulation.

In the dark-grown WT seedlings, PHYA level was highest in the hypocotyl followed by the cotyledon, and in the root its level was found to be below the detectability limit. PHYB was detectable in both light- and dark-grown seedlings and its amount in dark-grown seedlings was high compared to the light-grown seedlings. But the difference in its amount in both light- and dark-grown seedlings was less. A slight reduction induced by light could be observed particularly at least in the hypocotyl (Fig. 4.32).

In the *aurea* mutant, due to the lack of light-induced down regulation, PHYA could be detected in both light- and dark-grown seedlings. The organ specific distribution pattern was similar to WT i.e., its amount was high in the hypocotyl followed by the cotyledon. In the root its amount was below the detectability limit, both in the light- and dark-grown seedlings (Fig. 4.32).

4.6.3. PHYA and PHYB levels in dark to light transitions

The effect of light on PHYA and PHYB levels was studied by transferring dark-grown seedlings to light. In WT, PHYA level rapidly declined and by 4 h its amount was below the detectability limit. In the case of PHYB, there was a slight initial decline for about 4 h after which the level remained the same for the next 24 h. (Fig. 4.33, Fig. 4.34A,B). In *aurea* no significant difference could be detected in both PHYA and PHYB levels after transfer to light. In *aurea,* PHYA does not show light-induced down regulation (Fig. 4.33, 4.34C,D)<

4.6.4. PHYA and PHYB levels in light to dark transitions

When 5 d-old light-grown WT seedlings were transferred to dark, PHYA reaccumulated steadily for a period of 24 h. The accumulation started within a lag phase of 2 h. In case of PHYB, the level remained steady through out the period of transfer (Fig. 4.35, Fig. 4.36A,B). In the *aurea* mutant, both PHYA and PHYB

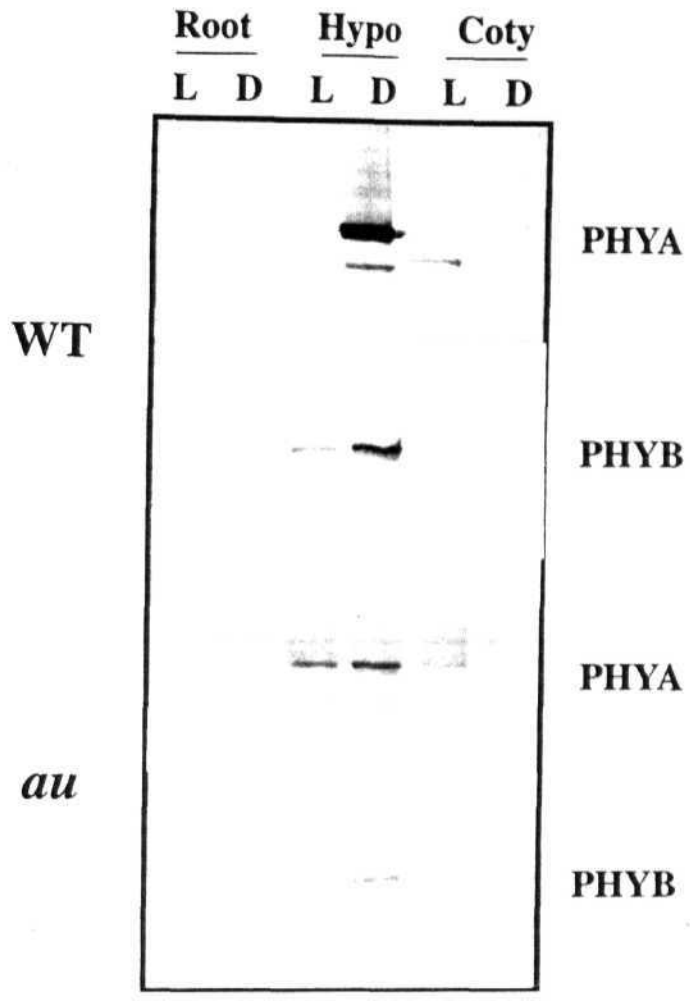

Figure 4.32. Distribution of PHYA and PHYB in different organs of tomato seedlings. 5 d-old light- (L) and dark-grown (D) WT and *aurea {au)* seedlings were excised into root, hypocotyl **(Hypo)** and cotyledon **(Coty).** Crude extracts were subjected to immunoblotting and probed with PHYA (mAP) and PHYB (mAT) antibodies.

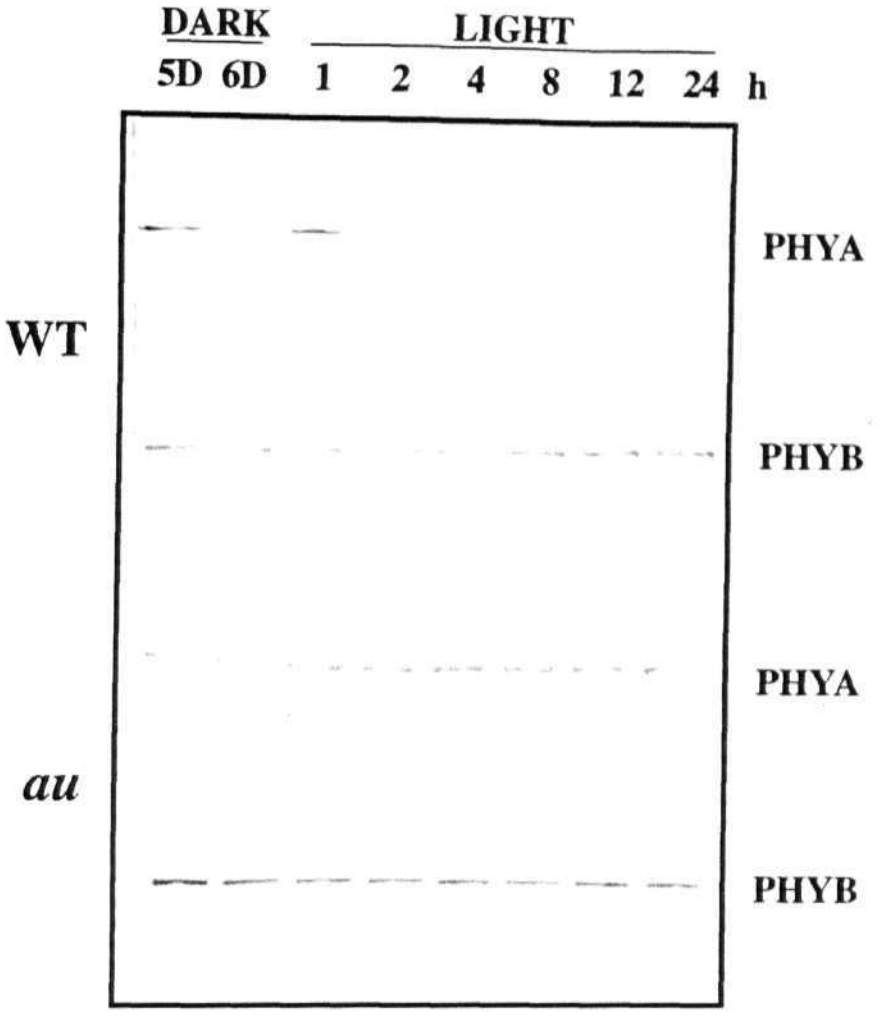

Figure 4.33. Time course of PHYA and PHYB levels after dark-to-light transition. 5 d-old dark-grown (5D) seedlings were transferred to light for 24 h or kept in darkness (6D) as control. At the time points (h) indicated, PHYA and PHYB levels were analyzed in the WT and *au* seedlings. Extracts were subjected to immunoblotting.

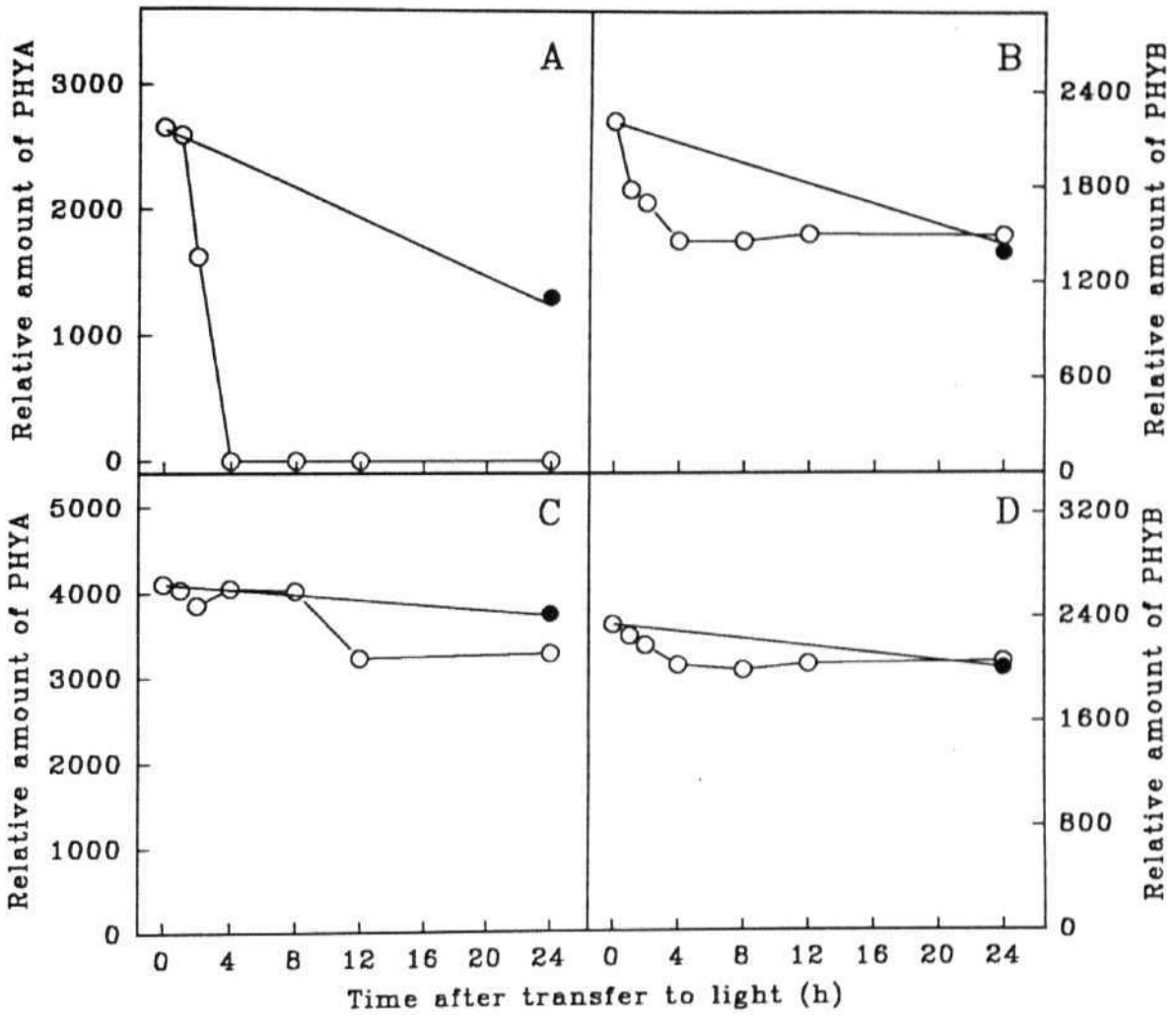

Figure 4.34. Time course of PHYA (**A, C**) and PHYB (**B, D**) levels in the WT (**A, B**) and *aurea* (**C, D**) seedlings of tomato after dark to light transition. Five d-old dark-grown WT seedlings were transferred to light for 24 h (○) or kept in darkness (●) as control. Similarly, 5 d-old dark-grown *au* seedlings were transferred to light for 24 h (○) or kept in darkness (●) as control. At the time points (h) indicated, PHYA and PHYB levels were quantitated. The level of PHYA and PHYB was quantitated by densitometric scanning of immunoblots displayed in the Fig. 4.33.

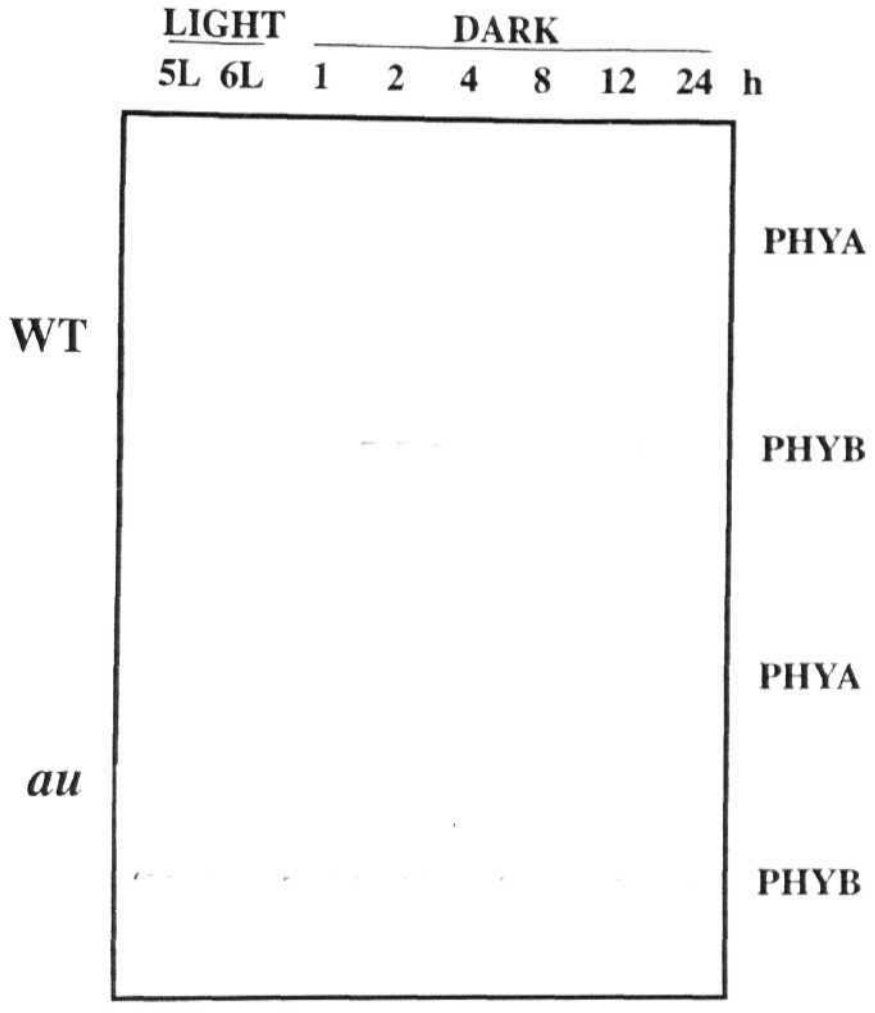

Figure 4.35. Time course of PHYA and PHYB levels after light to dark transition. 5 d-old light-grown (5L) seedlings were transferred to darkness for 24 h or kept in light (6L) as control. At the time points (h) indicated, PHYA and PHYB levels were analyzed in the WT and *au* seedlings. Extracts were subjected to immunoblotting.

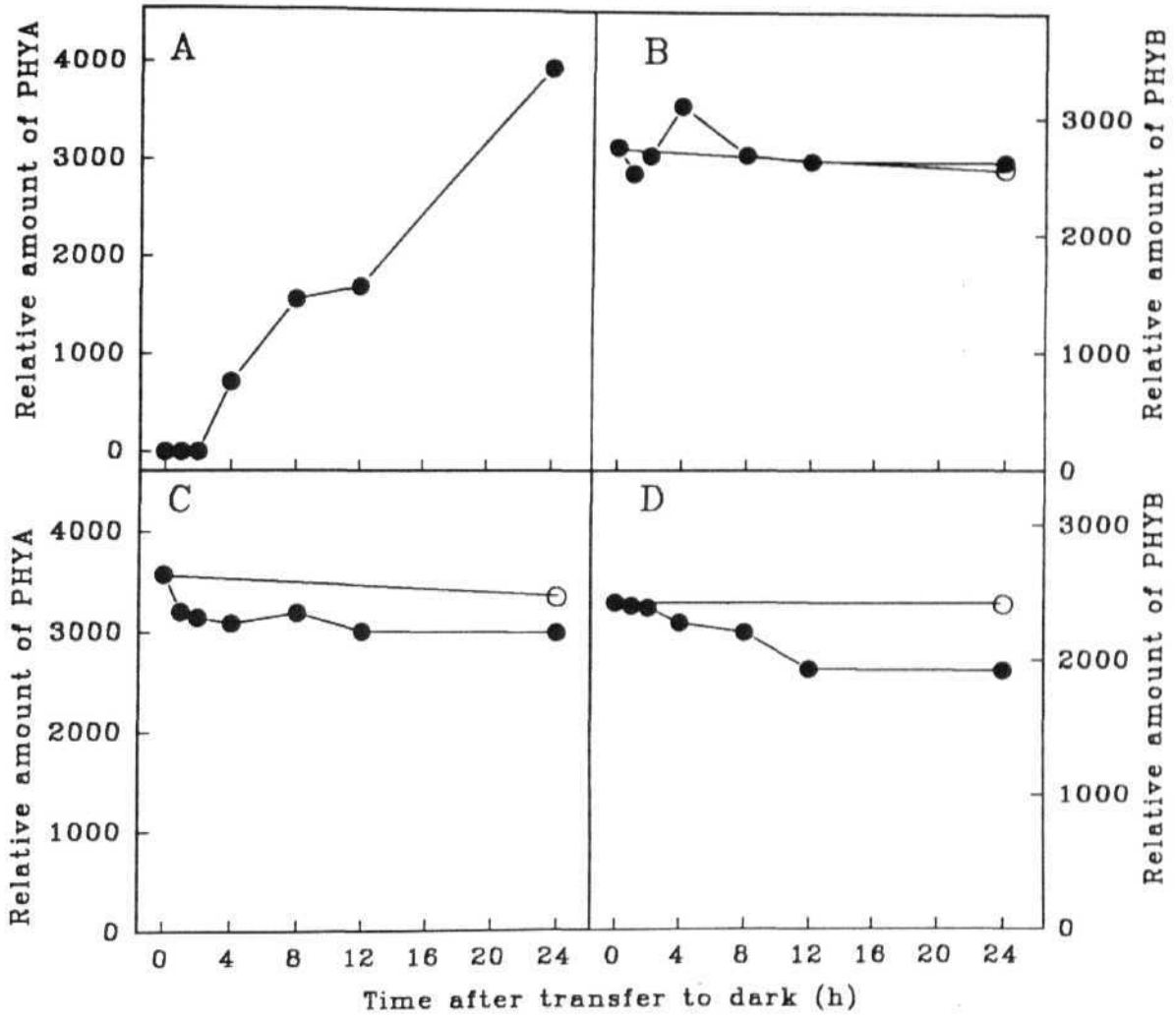

Figure 4.36. Time course of PHYA (**A, C**) and PHYB (**B, D**) levels in the WT (**A, B**) and *aurea* (**C, D**) seedlings of tomato after light to dark transition. Five d-old light-grown WT seedlings were transferred to dark for 24 h (●) or kept in light (○) as control. Similarly, 5 d-old light-grown *au* seedlings were transferred to dark for 24 h (●) or kept in light (○) as control. At the time points (h) indicated, PHYA and PHYB levels were quantitated. The level of PHYA and PHYB was quantitated by densitometric scanning of immunoblots displayed in the Fig. 4.35.

remained at a steady level and on transfer to dark not much effect was observed (Fig. 4.35, Fig. 4.36C,D).

4.7. In vivo rescue and in vitro reconstitution of aurea

There have been several speculations as to why the *aurea* mutant does not possess active phytochrome. Among the possibilities considered are; *aurea* lacks chromophore biosynthesis and *aurea* lacks a chaperone which may fold the protein to the needed configuration. The possibility of *aurea* being a chromophore deficient mutant was examined by attempting to rescue it by feeding it with chromophore *in vivo* and rescue the phenotype. Attempt was also made to reconstitute PHYA of the *aurea* mutant by incubating it with purified phycocyanobilin.

4.7.1. Preparation of phycocyanobilin

PGB was isolated from *Spirulina* using the procedure of Kunkel et al., (1993). Most of the chlorophyll was extracted and removed by hot methanol. Then phycocyanobilin was isolated by methanolysis by adding 300 ml of absolute methanol to 50 g of *Spirulina* cells and boiled for 3 h. The PGB was purified by changing different kinds of ether-water phases. Isolated PGB was checked spectrally, two peaks one at 377 nm and another at 385 nm were reported (Fig. 4.37). This purified PGB was used for *in vivo* rescue and *in vitro* reconstitution experiments using *aurea* mutant of tomato.

4.7.2. *In vivo* rescue of tomato *aurea* mutant

There are four major morphological differences between light-grown *aurea* and WT seedlings. In *aurea*, when compared to WT 1) elongated hypocotyl, 2) unopened hook, 3) unexpanded cotyledons and 4) reduced amounts of chlorophyll was observed. All these differences were taken collectively as parameters to observe whether *aurea* was rescued. If the mutant was rescued, changes in the above mentioned morphological features can be expected, leading to the appearance of WT phenotype. *Aurea* seedlings were grown right from the time of sowing in the presence or absence of PGB, both in light and dark, along with its isogenic WT for comparison. No morphological changes were observed in the *aurea* mutant in the presence of PGB in any of the above four listed parameters indicating the lack of rescue of the phenotypes (Fig. 4.38).

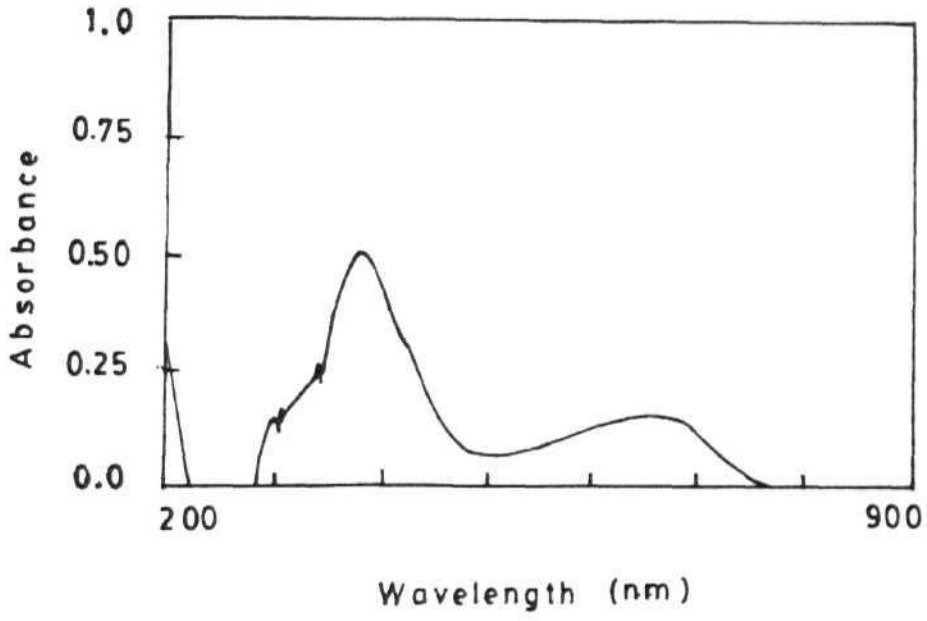

Figure 4.37. The absorption spectrum of purified phycocyanobilin. Purified phycocyanobilin was scanned from 200 nm to 800 nm.

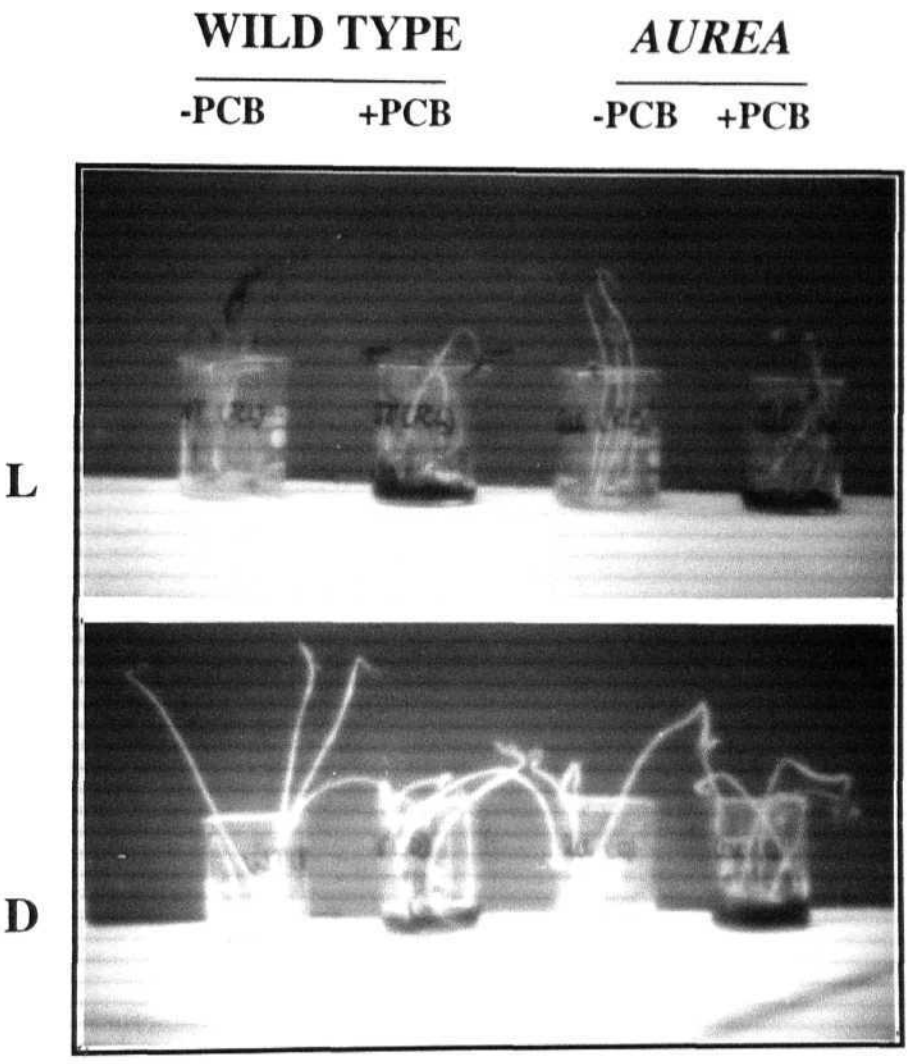

Figure 4.38. *In vivo* rescue of *aurea* mutant of tomato. Wild type and *aurea* seedlings were grown in the presence **(+PCB)** or absence **(-PCB)** of phycocyanobilin either in light (L) or in darkness (D).

4.7.3. *In vitro* reconstitution *of aurea* PHYA apoprotein

It is likely that phytochrome in *aurea* could not be rescued *in vivo* due to the lack of uptake of PCB into the cells. Therefore, an attempt was made to reconstitute *in vitro* by incubation of phytochrome A apoprotein with PCB. Since the level of PHYA is about 20% of WT (Sharma et al., 1993), five times more tissue from *aurea* seedlings was used in order to equalize the amount of PHYA level with WT. In order to test the ability of PHYA apoprotein from *aurea* to couple with PCB under *in vitro* conditions, the apoprotein was extracted using 25% ethylenc glycol buffer. Most of the nucleic acids and phenolic substances were removed by precipitating with PEI and phytochrome sample was concentrated with ammonium Sulfate at a final concentration of 42% saturation. The pellet was dissolved in 1 ml of resuspension buffer and used for pigment incubations with PCB for different time periods. After different time periods of incubations, the extracts were checked spectroscopically for the active PHYA. However, no active spectrum of PHYA could be recorded in the *aurea* mutant even after incubating with PCB for 12 h (Fig. 4.39).

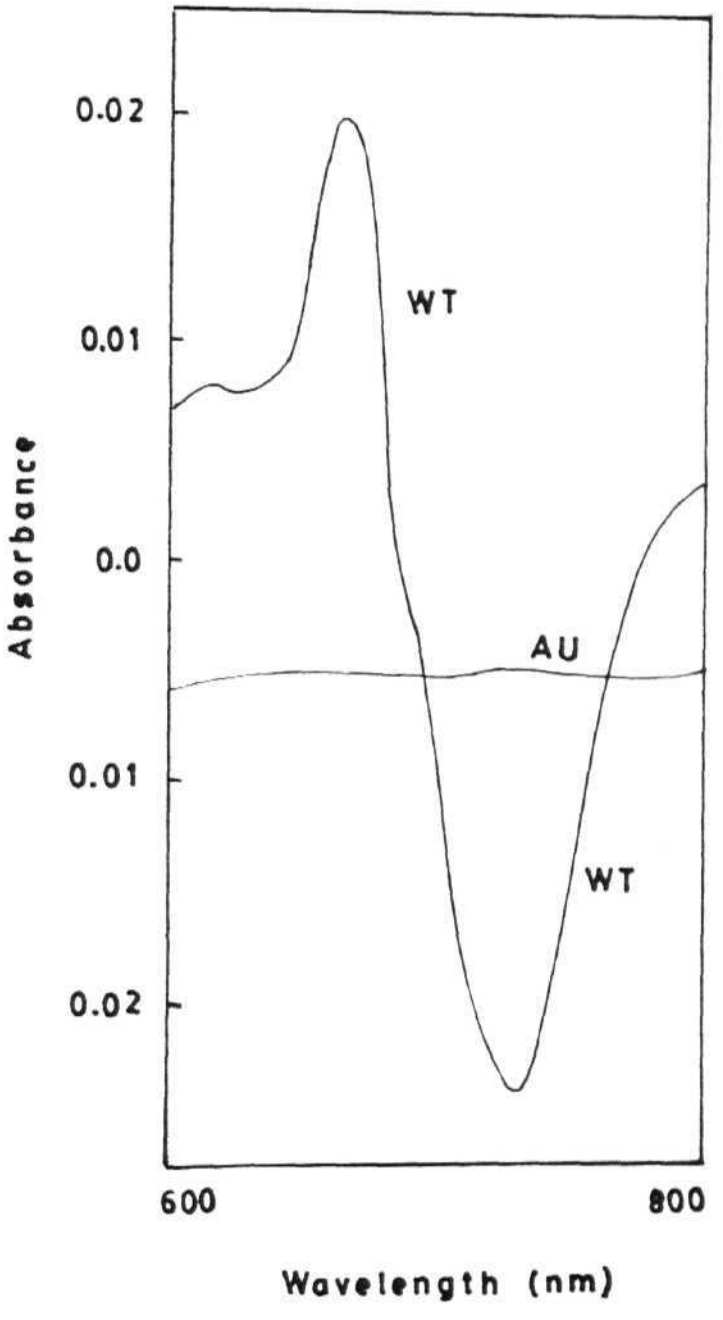

Figure 4.39A. ***In*** *vitro* reconstitution of phytochrome A in *aurea* mutant of tomato. The difference spectra were recorded using ammonium Sulfate precipitate of crude extracts of 5 d-old dark-grown wild type (WT) and *aurea (au)* seedlings before incubation with PCB.

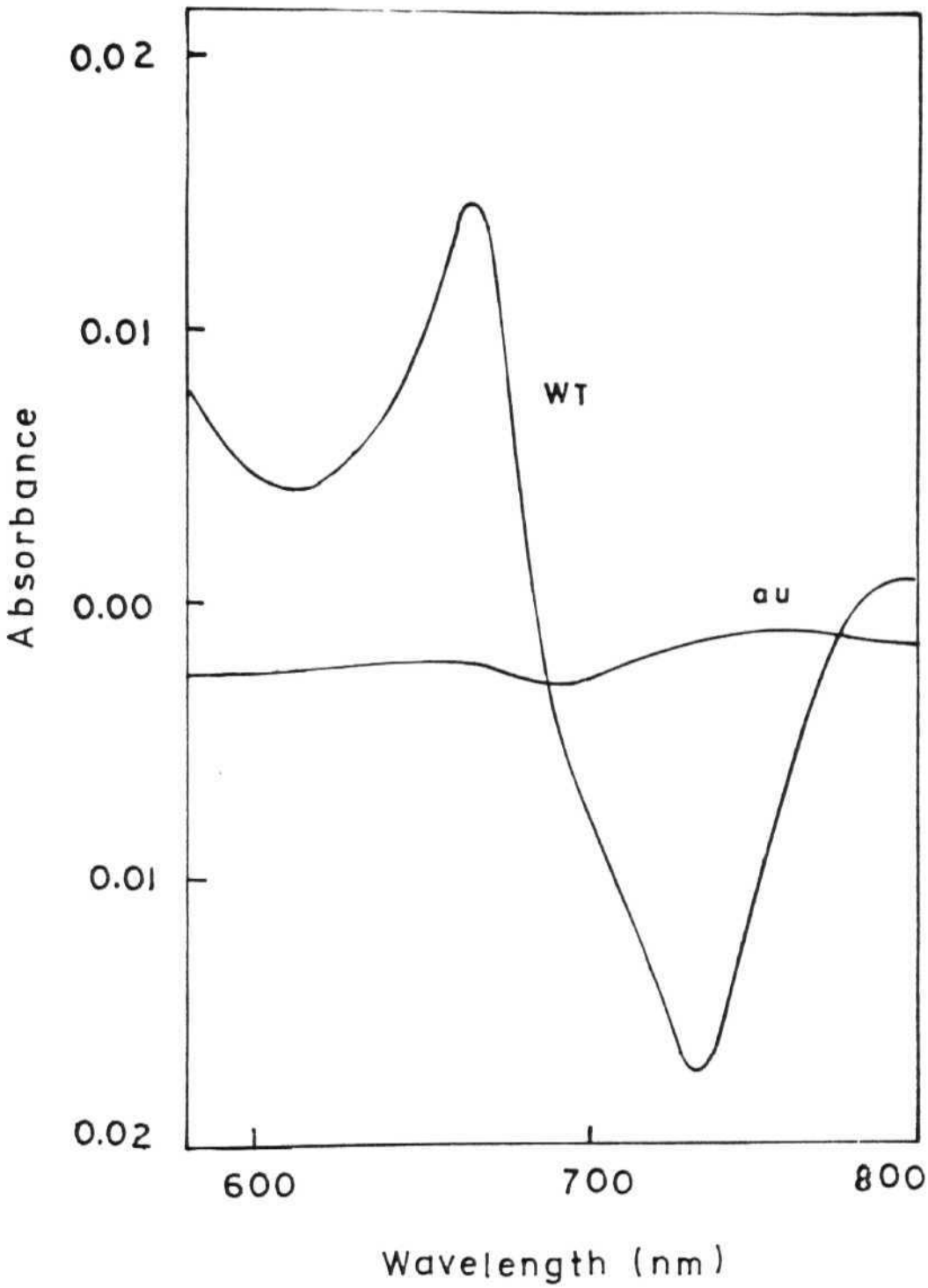

Figure 4.39B. *In vitro* reconstitution of phytochrome A in *aurea* mutant of tomato. The difference spectra were recorded using ammonium Sulfate precipitate of crude extracts of 5 d-old dark-grown wild type (WT) and *uurea (au)* seedlings after incubation with PCB.

CHAPTER 5

DISCUSSION

Photomorphogenesis, a process encompassing light-mediated developmental changes is mediated by at least three groups of photoreceptors consisting of blue/UV-A, UV-B and phytochrome. These photoreceptors independently as well as collectively control several facets of morphogenesis throughout the life cycle of the plants. Most information about photomorphogenesis, the mode of light perception and signal transmission, is known about phytochrome. However, the mode of action of phytochrome in controlling photomorphogenesis is yet to be fully deciphered. Since phytochrome is present in all the organs of plants, it may be assumed that it is also present in all living cells of plants and perhaps it also acts within a cell.

The recent evidences indicating the multiplicity of phytochrome species have however, engendered questions about the relative functions and distribution of the different phytochrome species. Current evidences indicate that these phytochrome species basically consist of two groups; light-labile and light-stable phytochrome. Since the relative levels of these two groups of phytochrome are likely to be different in plants during the day and night period, the question about the relative functions of the different phytochrome species is further complicated. One of the current approaches to resolve the question of the functions of phytochrome is to understand the distribution of the different phytochrome species at the sub-cellular and organ level during the different developmental stages of plants. However, all earlier studies on phytochrome distribution by spectroscopy, immunocytochemistry or ELISA represented only the distribution of light-labile phytochrome, i.e., phytochrome A which accumulates only in dark-grown seedlings.

At the moment it is not known if all the phytochrome species co-exist in all cells or follow an independent profile of regulation and distribution in different tissues and organs. In the present study, the distribution and the comparative levels of two phytochromes. PHYA and PHYB in maize- a monocot, and in tomato- a dicot was analyzed, using immunoblotting. Also, an attempt was made to understand the nature of the molecular defect of phytochrome A in the *aurea* mutant of tomato.

5.1. Assay of phytochrome

Butler et al., (1959) were the first to detect phytochrome *in vivo* by red/far-red reversible spectral alteration of phytochrome in etiolated maize seedlings using a double wavelength spectrophotometer. Thereafter, photointerconvertibility of phytochrome between its Pr and Pfr forms became the basis for the spectrophotometric assay to detect and quantify phytochrome molecule *in vivo* and *in vitro.* Though purified phytochrome can be measured directly by a normal spectrophotometer, such machines fail to measure the absorption spectrum of phytochrome precisely in tissues or in crude extracts because of the existence of interfering pigments such as chlorophyll and protochlorophyll absorbing in the red region. Since no other plant pigment shows photoreversible absorption changes, a difference spectra of phytochrome after actinic red and far-red light clearly distinguishes phytochrome from other pigments in tissues or in crude extracts (Butler et al., 1959). Spectrophotometric assay is however, besieged with several draw backs such as;

a) It cannot detect non-chromophore containing or non-photoreversible phytochrome.

b) The presence of chlorophyll in green tissues strongly interferes with phytochrome assay by screening and fluorescence artifacts.

c) It is difficult to detect and distinguish the different forms of phytochromes due to the overlapping of absorbance and low amount of phytochrome in green and mature tissue.

In this study, in order to monitor phytochrome distribution, an immunological assay was employed, which has several advantages;

a) high sensitivity,

b) insensitivity to other pigments such as chlorophyll,

C) ability to detect spectrally aberrant molecules and

d) possibility of distinguishing different forms of phytochromes by using specific antibodies.

Immunological assay, however, suffers from the defect that it cannot distinguish between the spectrally active and inactive proteins. Therefore, detection of phytochrome by immunoassay does not provide a correlation between its level and the biological activity of phytochrome.

5.2. Purification of phytochrome A

Purification of phytochrome is of great interest for several reasons. It is necessary to find out the structure-function relationship of the molecule including its crystal structure which has not yet been done mainly due to the difficulty in purifying phytochrome in large amounts. It is also necessary to purify it in order to raise specific antibodies in order to use them in distribution and expression studies.

The main objective in purifying phytochrome A in the present study was to raise specific polyclonal antibodies against it and use these antibodies to study the spatial and temporal expression and distribution of PHYA at different developmental stages in the various organs and tissues of maize seedlings. Due to the availability of several well standardized protocols to isolate phytochrome A from oat, it was selected as the plant material. Moreover, antibodies raised against oat phytochrome A also recognizes PHY A in maize due to the high homology between PHY A of oat and maize (Vierstra et al., 1984; Biermann et al., 1994). Phytochrome A was purified by using the method of Grimm and Riidiger (1986). A protocol suitable for purifying phytochrome from one plant species may not necessarily work for the purification of phytochrome from another species. For example, binding to and elution from affi-gel blue of **full** length oat, *Secale*, and cucurbita phytochrome require entirely different sets of conditions (Smith and Daniels 1981; Vierstra and Quail 1983, 85).

There are three land mark steps in phytochrome purification which have greatly aided phytochrome purification; the first among them was the demonstration that HA/brushite columns can greatly increase the purity of phytochrome A (Siegelman and Frier 1964). The second step was the demonstration that phytochrome is highly susceptible to proteolysis and the inclusion of proteolytic inliibitors such as PMSF help in preserving its integrity (Gardner et al., 1971). Finally, the demonstration that the Pfr form of phytochrome is more resistant to *in vitro* proteolysis; so that it became easier to purify phytochrome in the Pfr form with nearly native molecular mass (Vierstra and Quail 1983).

Subsequent to these land marks, attempts were made to reduce the time required to purify phytochrome in its native form with high SAR value. Among the several methods available. Grimm and Riidiger (1986) method is easier to follow as it requires only one chromatography step and yields high SAR value phytochrome. In

addition, the total time required for the completion of the entire purification protocol is also less. Considering the above advantages, this method was followed for the purification of phytochrome A.

5.2.1. Extraction of phytochrome

From the biochemical point of view, the purification of a given protein should be attempted at a stage where its specific activity is at the peak level. Since the phytochrome content of germinating seedlings depends on the age of the seedlings, the temporal course of the accumulation of phytochrome was studied, in order to select the optimal age of seedlings for phytochrome extraction. Using *in vivo* spectrophotometry it was found that the shoots of 4.5 d-old dark-grown oat seedlings were rich in phytochrome and yielded enough tissue for large scale purification.

Several precautions against denaturation and proteolysis were exercised in order to minimize phytochrome degradation. First among these was the irradiation of the harvested tissue on ice with red-light to convert phytochrome to Pfr form before homogenization. Since ethylenc glycol protects phytochrome from proteases, 50% (v/v) ethylene glycol and 140 mM ammonium Sulfate were used in the extraction buffer. PMSF was added freshly to prevent phytochrome degradation due to the activity of serine proteases. In the extraction buffer, 2-mercaptoethanol was added to ensure that no phenolic impurities were associated with the phytochromc preparations during the extraction procedure. Tetra-sodium EDTA and sodium bisulfite were also included, as they contribute in the inhibition of proteases activity (Pike and Briggs 1972; Vierstra and Quail 1982a).

5.2.2. Hydroxyapatite chromatography

Initially, the isolation of phytochrome was done using brushite chromatography (Siegelman and Frier 1964). Later it was found that HA gives higher yield of phytochrome. HA is a form of calcium phosphate prepared by the treatment of brushite with a strong base. The mechanism of binding and purification by HA is apparently similar to that of brushite (Roux et al., 1975). But in comparison to brushite the binding capacity of HA to proteins is high. Phytochrome was bound to HA using a low molarity phosphate buffer followed by elution with a linear gradient

of KP buffer (5-200 mM). This step resulted in about ten-fold purification of phytochrome and also eliminated most of the phenolic substances.

5.2.3. Phosphate washings

The main difference between Grimm and Rüdiger (1986) protocol with other protocols is the inclusion of the phosphate buffer washes. By using the specific solubility of phytochrome and other contaminating proteins at different concentrations of phosphate buffer, it was possible to eliminate other proteins as well as contaminating degraded phytochrome samples of 118 kD. Phytochrome pellet obtained after HA chromatography was first washed with 10 mM phosphate buffer, which does not solubilize phytochrome, as the concentration of ammonium Sulfate is very high i.e. about 2% saturation (Grimm and Rüdiger 1986). During the subsequent wash with 100 mM phosphate, native phytochrome was only slightly soluble, whereas this wash removed most of the 60 kD and 114/118 kD degraded phytochrome. Lastly, phytochrome was purified to a high degree by precipitating it with soluble PVP-40. Though phytochrome obtained showed a SAR value of 0.75, on SDS-PAGE it showed only a single band of 124 kD and was judged pure.

Recently, Lapko and Song (1995) have isolated native phytochrome without using HA chromatography by employing the specific solubility of phytochrome and other proteins at different concentrations of phosphate buffer. Till very recently, HA chromatography was unavoidable for the isolation of phytochrome, but Lapko and Song (1995) purified phytochrome to a very high SAR value by phosphate washings and gel filtration using Toyopearl HW-65S column.

5.3. *Characterization of purified phytochrome A*

5.3.1. Spectral characterization

In vivo spectroscopy of phytochrome shows that Pr and Pfr forms have absorption maxima at 667 nm and 730 nm respectively. The absorbance spectra of 124 kD phytochrome purified in the present study showed maxima in Pr form at 667 and 280 nm with a specific absorbance ratio of 0.75 (Fig. 4.4). In the Pfr form, the absorbance maxima was at 730 nm. It is known that in case of truncated phytochrome species (118/114 kD). the absorbance maxima for Pfr shifts to shorter wavelength in both far-red and blue regions of the spectrum. i.e. from 730 to 724 nm and from 400 to 390 nm (Hunt and Pratt 1979; Vierstra and Quail 1983). Additionally, the

absorbance of truncated phytochrome species at 730 nm is also known to be substantially lower than native phytochrome species (Vierstra and Quail 1982b).

Since the observed absorption maxima values of purified phytochrome are similar to that of native phytochrome, it clearly supports the notion that the purified phytochrome A consists mainly of native phytochrome molecule with a molecular weight of 124 kD. This is further substantiated by the observation that purified phytochrome shows a single band with a molecular weight of 124 kD on SDS-PAGE (Fig. 4.5). This purified phytochrome was blue in color and could be exposed to white light and left at room temperature without any loss by degradation as it was essentially free of proteases.

5.3.2. Immunological characterization

Polyclonal antibodies were raised against oat phytochrome A by injecting purified protein into rabbits. The specificity of these antibodies against maize and oat phytochrome A was confirmed by using several immunological techniques.

5.3.2.1. Ouchterlony double immunodiffusion

On Ouchterlony double immunodiffusion, a precipitin line formed only with crude extracts of dark-grown oat and maize seedlings but not with light-grown seedlings (Fig. 4.6A). Evidently the lack of precipitin line against light-grown seedlings signifies the drastically low level of phytochrome A in these plants. The complete fusion of precipitin line against maize and oat phytochromes (Fig. 4.6B) clearly supports the occurrence of a high degree of antigenic similarity between phytochrome A of these two species (Vierstra et al., 1984).

5.3.2.2. Immunoprecipitation

The specificity of oat phytochrome A antibodies against oat phytochrome was also characterized by immunoprecipitation (Fig. 4.8). Immunoprecipitation of phytochrome A was accompanied by reduction in spectrally active phytochrome in the supernatant indicating that the antibodies precipitated phytochrome forming an antigen-antibody complex. In contrast, no significant reduction of spectral value was observed with the pre-immune serum (Table. 4.2). Since crude extracts were used for immunoprecipitation. any cross reaction with other proteins would have been revealed in western blotting. The immunoprecipitated phytochrome on blotting migrated to the

same position as that of purified phytochrome A, indicating that these antibodies precipitated only phytochrome A molecules specifically.

5.3.2.3. Zn blot assay

The specificity of antibodies against phytochrome was further ascertained by performing comparative Zn^{+2} blot (Berkelman and Lagari as 1986) and immunoblot. It is known that the structure of the chromophore of phytochrome changes in the presence of Zn^{+2} ions. The linear tetrapyrrole chromophore of phytochrome in the presence of Zn^{+2} ions partially cyclyzes, leading to some conformational changes in the apoprotein (Sommer and Song 1990). In the presence of Zn^{+2}, phytochrome emits orange fluorescence under UV light which can be used as a very specific tool to identify phytochrome (Berkelman and Lagarias 1986). Visualizing purified oat phytochrome in the presence of Zn after blotting onto PVDF membrane under UV light revealed a single orange fluorescent band (Fig. 4.9). The position of the fluorescent band corresponded with the band obtained after western blotting, when the same membrane was also probed with phytochrome A antibodies. Since in plants, no other protein is known to yield Zn^{+2} induced fluorescence except phytochrome, it is evident that purified oat phytochrome is a holoprotein with a tetrapyrrole chromophore and is purified to a high degree.

5.3.2.4. Cross-reactivity of PHYA antibodies with other species

Ouchterlony double diffusion revealed that oat anti-PHYA antibodies recognize maize phytochrome A. The antigenic identity of maize phytochrome A was further checked by western blotting. Oat antibodies recognized a single band in oat with a molecular weight of 124 kD and a 127 kD band in maize. The observed values were similar to the molecular weights reported by Vierstra et al., (1984) for oat and Biermann et al., (1994) for maize.

The cross reactivity of the oat antibodies against phytochrome A of different species was checked by immunoblotting. While all the monocot species checked, i.e., maize, pearl millet, wheat and rice, cross reacted with oat antibodies, these antibodies did not react with phytochrome A from dicot species such as tomato and tobacco (Fig. 4.11. In general, monocot phytochrome antibodies were specific to monocot species

and did not cross react with phytochrome A of dicot species, except in a few cases like in pea (Cundiff and Pratt 1975) and in zucchini (Cordonnier and Pratt 1982) where a weak cross reaction was observed against oat phytochrome A antibodies,

5.3.2.5. In vitro proteolysis assay

A specific diagnostic test to ascertain the specificity of the antisera against phytochrome A is the *in vitro* proteolysis assay (Parks et al., 1989), Among the two forms of phytochrome, the Pfr form is relatively stable when compared to the Pr form under *in vitro* conditions. Taking advantage of this phenomenon, the specificity of phytochrome A antibodies was ascertained. The western blots of phytochrome extracts incubated at room temperature showed a gradual degradation of the Pr form, whereas its counterpart, the Pfr form was more stable. In the beginning, both Pr and Pfr forms were present in equal amounts, but with increasing time, the degradation of Pr form was more rapid, and the phytochrome band was almost undetectable by 180 min of incubation on the immunoblot.

The profiles of Pr and Pfr forms *of* phytochrome on *in vitro* proteolysis of maize was found to be different from oat with respect to the generation of truncated phytochrome species. It was reported that oat phytochromc A in Pr form is degraded to 118 kD species *in vitro* and the same was observed in the blot (Fig. 4.10). However, for maize, the formation of a band of molecular weight of 114/118 kD was not noticed (Fig. 4.12A). Probably, the relative amount of degraded phytochrome A was below the level of detectability in maize. Such a difference may also arise as the N-terminus of oat and maize phytochrome A are different from each other and the N-terminus of maize phytochrome A is less susceptible to proteolysis. In other species also, such as pearl millet, rice and wheat (Fig. 4.12B,C,D) the truncated phytochrome band was not observed. However, in contrast to oat, where the Pr form specific degradation was evident within 15 min, in other species such as maize, pearl millet and rice, the degradation was seen after 8 h. In case of wheat, the degradation was not clear even after 12 h.

5.4. Purification of phytochrome B

The existence of more than one phytochrome in higher plants necessitates the purification of each species to homogeneity to understand their respective structure

and mode of action at the molecular level. So far, because of its relative abundance, only phytochrome A has been purified to homogeneity from plants, and has been characterized at the physico-chemical level. The phytochrome predominating in green tissues was observed to be both immunochemically and spectrally distinct from the abundant phytochrome A (Tokuhisa et al., 1985, Shimazaki and Pratt 1985). Spectroscopic studies with partially purified phytochrome from green oat showed a blue shift of about 14 nm in the Pr minus Pfr difference spectrum maxima, and a blue shift of 8 nm in the isosbestic point in comparison to phytochrome A (Tokuhisa et al., 1985). So far purification of green tissue phytochrome has been attempted with some reasonable degree of success only from oat (Pratt et al., 1991a) and pea (Abe et al., 1985). We attempted to purify it from spinach as the leaves are relatively low in proteases and available throughout the year.

Initial steps of purification were essentially the same as described by Pratt et al., (1991a). The elimination of chlorophyll was achieved by PE1 precipitation and this step was repeated once again with a lower concentration of PE I to further reduce the chlorophyll content in the supernatant. The low amount of spectrally active phytochrome however, precluded the spectral assay of phytochrome during purification to monitor the fractions. Therefore, the phytochrome amount was monitored during purification by western blotting using PHYB specific antibodies. An obvious disadvantage of the protocol used was that the purification was more selectively directed for PHYB as other phytochrome species may have different elution profile on chromatography. Nevertheless, since PHYB appears to be the more predominant species of light-stable phytochrome it was easy to monitor it by western blotting. The lack of antibodies against phytochrome species other than PHYB also made it impossible to monitor the levels of other phytochrome species during purification.

Though Pratt et al., (1991a) and a few other authors were able to obtain a difference spectrum after concentrating the active fractions after HA chromatography, in spinach difference spectrum could not be obtained at this step. However, after DEAE-Sepharose chromatography, followed by concentrating the active fractions, the presence of phytochrome was revealed by differential spectroscopy.

The presence of extremely low amount of phytochrome in green tissues of spinach precluded further purification of PHYB by conventional means. However, other investigators have obtained PHYB apoprotein or a part of it from tobacco (L6pez-Juez et al., 1992), rice (Wagner et al., 1991), and PHYB and PHYC from *Arabidopsis* (Somers et al., 1991) by cloning the respective genes and overexpressing either in yeast or E. coli. Such proteins have been used to generate specific monoclonal antibodies against the apoproteins and one such monoclonal was used in this study to understand PHYB distribution in maize. Not only these apoproteins can be produced in bulk, PHYB could also be reconstituted to its spectrally active form fry assembling with PCB *in vitro* (Kunkel et al., 1993). Perhaps assembly with phytochromobilin could yield much higher amounts of phytochrome protein in the native form and might obviate the need to purify phytochrome from green plant tissue. Nevertheless, purification from green plants, though cumbersome and tedious, is desirable to find out if phytochrome protein *in vivo* is modified by glycosylation or any other mechanism to name a few. An ideal approach is perhaps to link *PHYB* gene to strong promoters such as CaMV promoter and purify it from green seedlings which would have more PHYB but little PHYA to interfere with the purification. Such an approach would be ideal to purify phytochrome from green tissues with its natural chromophore.

5.4.1. Distribution of PHYA and PHYB in maize seedlings

The fact that plants possess multiple phytochromes led us to study the distribution of different phytochromes in maize and tomato seedlings. Based on several evidences it is now believed that different species of phytochromes are likely to play different physiological roles (Smith and Whitelam 1990). The information on the distribution of individual phytochrome species is extremely limiting. Essentially all the earlier information on phytochrome distribution represents only phytochrome A distribution. For instance, the accumulation of phytochrome during germination and seedling development of oats had been followed spectrophotometrically (Briggs and Siegelman 1965; Jabben 1980), immunochemically (Hunt and Pratt 1979) and a combination of both the approaches (Shimazaki et al., 1983; Tokuhisa and Quail 1987). But though the spectrophotometric data on etiolated seedlings theoretically represented the collective abundance of different phytochromes, the fact remained that

the high abundance of phytochrome A masked the distribution of other phytochromes. The availability of monoclonal antibodies specific for individual phytochromes or polyclonal antibodies that discriminate at least partially among different phytochromes have now made possible the examination of the expression of different phytochromes at different stages of the life cycle of plants.

5.4.2. Specificity of antibodies against maize PHYA

Several experiments such as double immunodiffusion, western blotting, *in vitro* proteolysis described in the earlier sections clearly supports the notion that oat phytochrome A antibodies recognize maize PHYA. Since maize and oat PHYA possess 88% homology in their amino acid sequence (Okamoto et al., 1993), oat PHYA antibody against maize PHYA was used. The specificity *of* an oat PHYA antibody against maize PHYA was established by light-mediated down-regulation of PHYA and also by the susceptibility of the Pr form of PHYA to endogenous proteases (Parks et al., 1989). Furthermore, the MW of the immunoblotted band for PHYA matched with the reported MW of maize PHYA (Biermann et al., 1994) and was found to be close to the reported MW of maize (Christensen and Quail 1989).

5.4.3. Specificity of antibodies against maize PHYB

The question whether tobacco PHYB antibodies can recognize maize PHYB needs an intensive examination before using them as a tool to study PHYB distribution in maize. The comparison of the gene sequences of different phytochromes have shown that while the sequences of PHYA diverge about 50% between monocots and dicots, the gene sequences of PHYB are more conserved between angiosperms (Dehesh et al., 1991, Okamoto et al., 1993). PHYB of *Arabidopsis* and rice (Dehesh et al, 1991), and also of rice and tobacco (Kern et al., 1993), share amino acids identity close to 77.6% and 76.4% respectively. In addition, monocot and dicot PHYB possess very similar hydropathy profiles over the entire length of their amino acid sequence (Dehesh et al., 1991) and therefore may also possess antigenically similar epitopes.

In view of the above observations, the possibility of detection of maize PHYB fry tobacco anti-PHYB monoclonal antibodies was ascertained. The fact that the percentage of homology *of Arabidopsis* PHYB to tobacco (77.6%) is nearly similar to

the homology between tobacco and rice (76.4%) (Kern et al., 1993), and that the tobacco monoclonals against PHYB specifically detect *Arabidopsis* PHYB in wild type but not in PHYB deficient mutant (Nagatani et al., 1991, 1993), strengthens the possibility of detection of monocot PHYB. Such a possibility is further supported by the fact that the PHYB sequence between *Arabidopsis* and rice shares a homology of 73%. Moreover, monoclonals against monocot-rice PHYB can specifically recognize dicot- *Arabidopsis* PHYB protein and do not recognize *Arabidopsis* PHYA and PHYC apoproteins (Wagner et al., 1991). Wang et al., (1991) have also observed that a monoclonal antibody against light-stable pea phytochrome can recognize a phytochrome species abundant in light-grown oat leaves.

So far, tobacco anti-PHYB monoclonals have been used to detect PHYB in *Arabidopsis* (Nagatani et al., 1991), cucumber (López-Juez et al., 1992) and tomato (Sharma et al., 1993). The results obtained in this study indicate that the above antibodies also recognize PHYB like apoproteins in monocot species such as oat, rice, maize and wheat. Immunoblots of PHYB from these species show immunostained bands in the region of the observed molecular weight of PHYB. For example, the MW of immunodetected PHYB of rice in this study is about 123 kD, which is close to the MW of rice PHYB (122 kD) observed on immunoblots in another study (Wagner et al., 1991). Since the PHYB sequence diverges by 5()% from other phytochrome species, the chances of detection of a species other than PHYB is remote. In fact, most oat monoclonals against phytochrome purified from green oat shoots fail to cross-react with the 124 kD phytochrome species which arc abundant in etiolated oat seedlings (Pratt et al., 1991b). Taken together, the above evidences largely favor the view that the tobacco monoclonal antibody mixture is most likely to detect PHYB present in maize seedlings.

Although the arguments presented above favor the view that the antibodies used in this study recognizes PHYA and PHYB like proteins in maize, the likelihood of these antibodies recognizing an additional phytochrome species other than PHYA and PHYB, also merits consideration. For example, Tokuhisa et al., (1985) observed that rabbit polyclonal antibodies against PHYA recognized two phytochrornes and similarly certain monoclonals against oat PHYA can detect a second phytochrome (Shimazaki and Pratt 1985; Tokuhisa et al., 1985). Since the molecular weights of

PHYA and PHYB on immunoblots are widely different, it is highly unlikely that the PHYA antibodies used in this study recognized PHYB or vice versa. Nevertheless the possibility of recognition of an additional phytochrome species other than PHYA or PHYB by the antibodies is plausible and can not be unequivocally excluded. Nevertheless, presently the lack of knowledge about all the phytochrome species in maize and of specific antibodies against each of them precludes examination of the above possibility.

5.5. Distribution of PHYA and PHYB in maize seedlings

5.5.1. Distribution of PHYA and PHYB in different organs

In etiolated maize seedlings, both PHYA and PHYB are present in all the organs of the seedlings. The greatest amount of phytochrome was reported to accumulate in the meristematic regions of the plants like the coleoptile and the leaf tip in barley, oat and maize (Briggs and Siegelman 1965). Pratt and Coleman (1974) also reported high level of phytochrome near the tip of the coleoptile and shoot apex in oats but little in leaves. Whereas in barley and rice the primary' leaf bases were found to contain high level of phytochrome, in corn it was found to be more uniformly distributed. Schwartz and Schneider (1987) reported the highest amount of phytochrome in the coleoptile tip, leaf tip and root cap of corn. Wang et al., (1993b) found that all the three phytochromes, i.e., 124 kD, 123 kD, and 125 kD are expressed in a similar manner in light- and dark-grown oat seedlings. In all the studies, each of the three phytochromes were found to be abundant more in the shoots and least in the root.

The observed distribution of PHYA in etiolated maize seedlings was broadly similar to that observed in earlier spectroscopic (Briggs and Siegelman 1965) and immunochemical (Schwartz and Schneider 1987; Wang et al., 1993a,b) studies on maize seedlings. In this study PHYA seemed to preferentially accumulate in the leaf and also in the encircling coleoptile. In comparison, in the other organs such as roots and mesocotyl, the amount of PHYA was significantly lower than in the leaf or coleoptile, barring the mesocotyl node and the root tip which accumulate PHYA (Fig. 4.19).

The relative abundance of PHYA protein in maize seedlings was similar to the abundance of PHYA mRNA in oat seedlings where a maximal amount of mRNA was detected in the coleoptile and the mesocotyl node (Seeley and Colbert 1992). While Seeley and Colbert (1992) could not detect a significant level of PHYA mRNA in oat leaf, the maize leaf possessed a significant amount of PHYA protein.

5.5.2. PHYA and PHYB level in maize seed

The presence of phytochrome in unimbibed seeds is of interest because it is likely that this phytochrome may be responsible for controlling light-mediated germination. In unimbibed pea seeds, Konomi et al., (1985) reported the presence of about 7 ng of phytochrome per embryonic axis and its level rapidly increased to 200 ng per axis on imbibition in darkness. Tokuhisa and Quail (1987) reported that 124 kD phytochrome in oat seeds increased by 200-fold on imbibition for 72 h in darkness but not in light. Wang et al., (1992) found 125 kD, 124 kD and 123 kD phytochromes in dry oat seeds. Similar to 124 kD phytochrome, which is light-labile, 125 kD and 123 kD phytochromes which are light-stable also accumulate to higher levels in darkness than in light. Unlike Wang et al., (1992), neither PHYA nor PHYB could be detected in the embryonic axes isolated from dry maize seeds in this study. However after imbibition, both PHYA and PHYB species could be detected in the embryonic axes and the scutellum but not in the de-embryonated kernels (Fig. 4.18A,B).

5.5.3. Temporal regulation of PHYA and PHYB level

Accumulation of PHYA and PHYB in different organs distinctly shows an organ-specific accumulation and a temporal regulation of their levels. In the embryonic axes isolated from dark-grown seedlings, PHYB appeared earlier than PHYA and their levels peaked at 24 h from sowing (Fig. 4.18C,D). Since in the etiolated seedlings the PHYA level differs considerably in the different organs, it can be presumed that the accumulation of PHYA is determined in an organ-specific manner. As expected, light-grown seedlings possess only a little amount of PHYA, and PHYB seems to be the more dominant species in these seedlings.

Higher levels of PHYA and PHYB appear to be associated with younger organs, and the PHYA level, which was maximal in the 4-5-d-old leaf and coleoptile declined significantly during further growth. A similar age-dependent decline was

also noticeable in the level of PHYB in the leaf. It is obvious from the profiles of PHYA and PHYB levels that the massive accumulation of PHYA is distinctively restricted to the shoot of younger etiolated seedlings. 4 d-old dark-grown seedlings have very high amount of phytochrome A which declines rapidly, and by 8 d it is almost below the limit of detection. Whereas the amount of phytochrome A is high in dark-grown seedlings, in light-grown seedlings, it is almost two-fold lower. Phytochrome B levels in dark- and red light-grown seedlings arc similar on 4 d, and subsequently, both decline with age. Phytochrome B is also significantly down regulated and the amount of phytochrome B in light is lower than that of dark (Fig. 5.20). However, the aging of seedlings does not preclude the reaccumulation of phytochromes, and on dark-adaptation of the seedlings, both PHYA and PHYB levels augment significantly.

5.5.4. Distribution of PHYA and PHYB in maize leaves

The profile of PHYA distribution in the coleoptile supports the notion that in cereals it specifically accumulates in the coleoptile tip (Prati and Coleman 1974). The observed uniform distribution of PHYA in maize shoots by Pratt and Coleman (1974) appears to be true only for very young seedlings. In older maize seedlings, the level of PHYA in the leaf is higher in the upper half of the leaf and in the region close to the meristematic base. This is almost similar to the accumulation profile of PHYB. The above profile of phytochrome accumulation is at variance with protochlorophyllide oxidoreductase which also preferentially accumulates in dark-grown leaves but follows an acropetal gradient with maximal concentration towards the tip (Fig. 4.22).

In monocots like maize, leaves arc derived from the outer two layers of the shoot apical meristem (Poethig 1987). The leaf primordium divides anticlinally giving rise to the outer most layer of the shoot apex which forms the epidermis. The apical subsurface layers divide anticlinally as well as periclinally to form the mesophyll (Steeves and Sussex 1989). Once the leaf primordium is formed, further growth is restricted to the basal meristem. As the leaf elongates by continual cell division at the basal meristem, a gradient of cell maturity is formed, with the most mature cells being localized at the tip of the leaf and the younger cells localized at the base (Nelson and Langdale 1992).

The profile of phytochrome distribution in maize leaf appears to be determined by the above innate gradient of cell maturity. PHYA and PHYB follow similar pattern of distribution in the maize leaves. In the dark-grown leaves the basal portion of the primary leaf has high amount of PHYA which declines almost 6-fold in the middle of the leaf and increases at the tip of the leaf by about 6-8-fold. In light-grown 7 d-old primary leaf, the PHYA level is below the detectability limit. In case of PHYB, the leaf base has high amount of phytochrome which declines 5-fold in the middle of the leaf and picks up by almost 9-fold at the tip (Fig. 4.23). Similar pattern is found in dark-grown primary leaf for PHYB. On transferring the light-grown seedlings to darkness, PHYA reappears with a profile similar to that of the seedlings grown in continuous darkness (Fig. 4.29).

The fact that light does not seem to alter this pattern, is evident from the observation that though light reduces the level of both PHYB and protochlorophyllide oxidoreductase (Forreiter et al., 1990), their distribution pattern remains the same in the light-grown leaf (Fig. 4.22). Moreover, on transferring light-grown seedlings to darkness, PHYA reappears in the leaf with a distribution profile similar to that of dark-grown leaves. In view of the close similarity in the distribution of PHYA and PHYB levels along the length of maize leaves, it can be speculated that the expression of these two phytochromes may be regulated to some extent by the same gradient of cell maturity, at least in leaves. However, the above speculation is conjectural in nature as the cell and tissue level distribution of these phytochromes is still to be elucidated.

A comparison of the profiles *of* phytochrome distributions in this study and phytochrome-mediated induction of several plastidic and cytosolic enzymes along the length of maize leaves (Nelson and Langdale 1992) indicated a clear lack of correlation between the distribution of these photoreceptors and photoinduced responses in monocot leaves. In the past, such absence of correlation between phytochrome level and photoresponse was attributed to the existence of multiple pools of phytochrome and was termed as "paradox" (Hillman 1967). Results obtained in this study do not attempt to correlate the photoreceptor distribution with known photoresponses in maize leaves. In fact, recent studies indicate that though a photoreceptor such as phytochrome functions in a cell autonomous fashion (Neuhaus

et al., 1993), a **photoresponse would** only **be remotely connected to the photoreceptor level** and would rather **result from** a change in **the level,** or **the** presence/absence **of** trans-acting transcriptional factors controlling gene expression **(Vierstra** 1993; **Deng 1994).**

5.5.5. Both PHYA and PHYB are down regulated in maize seedlings

Several lines of physiological and biochemical evidences have indicated that phytochrome consists of a light-labile and a light-stable pool (Furuya 1993), which have been shown to belong to PHYA and other green-tissue specific phytochromes respectively. Immunoblotting of PHYB and PHYC from *Arabidopsis* (Somers et al., 1991), and PHYB in tomato (Sharma et al., 1993) and cucumber (López-Juez et al., 1992) showed nearly identical levels in etiolated and green tissues respectively. In contrast, in all the organs of maize, the PHYB level was consistently lower in the light-grown seedlings than in the etiolated seedlings. However, in comparison to PHYA, which drastically declined on exposure to light, PHYB was rather stable and declined partially (Fig. 4.27A). These results are essentially similar to oat, where the level of green-tissue specific phytochrome declined on exposure to light (Wang et al., 1993a).

Interestingly, light suppresses the accumulation of PHYA even in germinating seeds, and seeds germinated in light fail to accumulate an immunodetectable level of PHYA (Fig. 4.18). Similarly in oat embryo, Hilton and Thomas (1987) reported that while red light suppressed the accumulation of immunochemically active phytochrome likely to be phytochrome A, it did not prevent the accumulation of light-stable pool of phytochrome. In contrast, Wang et al., (1993a) did not find any effect of light on the phytochromes level during the first 48 h of germination in oat seeds. The effect of light on down-regulation of PHYA and PHYB level was impermanent in nature. Dark adaptation of continual light-grown seedlings elevated the PHYB level in the maize leaf after 12 h. Likewise, though continual red light grown maize leaves and coleoptiles were virtually devoid of PHYA, on dark adaptation, PHYA reaccumulated in the leaf and the coleoptile (Fig. 4.27B).

On dark to light transition, there was almost 500-fold decrease in the level of PHYA in leaf within 8 h. On light to dark transition, from levels below the detection limit. PHYA level increased by 100-fold within a period of 8 h and 600-fold within 24

h and attained a pattern similar to that of dark-grown leaves. In wheat also, after 8 h light period only low levels of PHYA remained as judged by EL1SA (Carr-Smith et al., 1994). When these plants were transferred to darkness, PHYA reaccumulated rapidly, and within 24 h, phytochrome level increased by 12-fold. Schwartz and Schneider (1987) also reported a rapid light-mediated degradation of PHYA in 5 d-old dark-grown maize seedlings. They reported that the degradation starts with a lag phase in the coleoptile, shoot apex, and mesocotyl but with no lag phase in the leaf.

It is now accepted that the PHYA down-regulation in oat is brought about by a combination of several phenomena such as the switch-off of PHYA gene transcription, selective degradation of its mRNA and ubiquitin mediated proteolysis of PHYA protein (Vierstra 1993; Higgs and Colbert 1994). At present the involvement of the above mechanism(s) in the down regulation of PHYB is not known. It is equally plausible that it may result from the operation of a mechanism other than those existing for PHYA.

Edwards and Colbert (1990) reported rapid increase in phytochrome A mRNA during dark adaptation in oat leaves, whereas red light-induced the down regulation of PHYA mRNA both in etiolated and green portions of oat leaves. Both green and etiolated portions were also capable of accumulating PHYA mRNA during dark adaptation, but there was at least a 24 h lag period before the mRNA accumulation began. Seelay et al., (1992) reported that the half life of PHYA mRNA in total RNA is 90 min in oat seedlings treated with red light. This is much shorter than the average half life of 30 h reported for most poly(A)$^+$ RNA in soybean culture cells (Silflow and Key 1979). They suggested that the PHYA mRNA was inherently unstable and the instability was Pfr independent. The distribution of PHYA mRNA was found to correspond to that of the protein (Seeley and Colbert 1992). Thus PHYA mRNA is mostly found in the same areas as PHYA protein in corn. This correlation was also found in pea seedlings (Tomizawa et al., 1991).

The results obtained in this study regarding the distribution of PHYA and PHYB in maize seedlings indicate that the profile of distribution and temporal regulation of the PHYA and PHYB levels appears to be controlled by a common mechanism in maize seedlings. The observed semblance between the accumulation and the distribution of the phytochrome species possibly results from their co-

regulation of synthesis during development. A detailed **molecular-genetic analysis of the** distribution and function of all the phytochrome species is nevertheless needed to understand their respective roles and **interaction between different phytochrome** species in controlling plant development.

5.6. Distribution of PHYA and PHYB in tomato seedlings

In maize both phytochrome A and phytochrome B are present in all the organs and follow a similar distribution pattern. It is not known if the expression of different members of the phytochrome family is autonomous of each other or **there** is some co-ordination in their expression and distribution. In the latter case the level or **the** activity of one phytochrome species may alter the distribution of other species. This aspect of the PHYA and PHYB expression and distribution was analyzed using the *aurea* mutant of tomato along with its isogenic WT as control. *Aurea* mutant is believed to be a chromophore deficient mutant, and has drastically reduced level of spectrally active phytochrome A. However, phytochrome A apoprotein can be detected immunologically at 1/5 level of WT (Sharma et al., 1993). At the same time *aurea* mutant possess nearly normal level of phytochrome B which is spectrally active. Thus, the *aurea* mutant can be used as a system to analyze the distribution profile of phytochrome B in the absence of active phytochrome A. It also provides an opportunity to study the distribution profile of the apoprotein without the chromophore attachment i.e., PHYA.

5.6.1. Specificity of antibodies against PHYA and PHYB

Tomato PHYA was detected using specific antibodies raised against PHYA of pea and PHYB of tobacco detecting PHYA (117 kD) and PHYB (114 kD) respectively in tomato (Sharma et al., 1993).

5.6.2. Distribution of PHYA and PHYB in different organs

Studies on the distribution and the temporal accumulation of phytochrome may provide an indication of the possible functions of the different phytochrome species. While it is known that phytochrome consists of several species, it is not clear how different phytochrome species arc distributed within different organs and cells. Many of the phytochrome responses specifically associated with green tissues are localized at specific sites in different organs. For example, the internode is the site for

proximity perception (Ballaré et al., 1990) whereas the leaf is the site for detection of photoperiodic signals (Kendrick and Kronenberg 1986).

Spatial accumulation of phytochrome in dicot seedlings have been examined in detail only in a few cases. One of the regions which shows maximal sensitivity to light is the hypocotyl hook, which is believed to possess higher level of phytochrome than any other organ (Kendrick and Kronenberg 1986). Since in germinating seedlings, the hook is supposed to be the first tissue which would be exposed to light on emerging from the soil, it is logical to assume an enrichment of phytochrome in this organ.

Both PHYA and PHYB were detected in the hypocotyl and the cotyledons of tomato seedlings. The observed distribution pattern of PHYA and PHYB in the etiolated tomato seedlings of WT reveals that the amount of both phytochromcs were high in the hypocotyl which was followed by the cotyledon. In the root, both PHYA and PHYB levels were below the detectable limit. In the light-grown WT seedlings, PHYA level was below the delectable limit due to its light-mediated down regulation, whereas PHYB level remained same as that of the control i.e., dark-grown seedlings. (Fig. 4.32). There was not much light-induced down regulation of PHYB in tomato as observed in maize. When these results were compared to the *aurea* mutant of tomato, the distribution profile of both PHYA and PHYB was found to be the same as WT. Since PHYA level in *aurea* does not decline in light-grown seedlings, a similar pattern of PHYA distribution was obtained as that of dark-grown WT or *aurea* seedlings.

The discovery of two closely related homologues of *PHYB* genes in tomato however pose questions like whether the mAT antibodies used in the present study recognizes both PHYB apoproteins or only one of them. Since at the moment information about the homology between the gene sequences of both PHYB of tomato and also with other species such as *Arabidopsis,* tobacco, rice is not available, this question remains open. It would be however, of interest to examine in future if both sub-species of PHYB carry out the same physiological function, or trigger their own independent physiological action.

In 5 d-old etiolated pea seedlings, Briggs and Siegelman (1965) found that the apical node and the bud had the highest level of phytochrome and the root had the

least. In bean seedlings, **the** distribution pattern was similar to that of **peas** (Briggs **and** Siegelman 1965). In sunflower also the apical bud was found to contain the highest amount of phytochrome which gradually declined up to the base of **the** hypocotyl. Thus in dicots also, the highest level of phytochrome is concentrated in the meristematic tissues.

5.6.3. Temporal regulation of PHYA and PHYS level

In dark-grown WT tomato PHYA level increased upto 5 d. thereafter **the** PHYA level declined gradually upto 8 d after germination. PHYA was undetectable in light-grown WT seedlings. In the case of PHYB, the decline is not very drastic and more or less uniform level of PHYB was maintained up to the 8 d of germination both in light- and dark-grown seedlings. In the *aurea* mutant, PHYA could be detected both in light- and dark-grown seedlings. The pattern however, remained the same as that of WT with the highest amount of phytochrome being present on the 5 d after germination (Fig. 4.30). In light-grown seedlings, the PHYA level was only slightly lower than in dark-grown seedlings by about 1.2-fold. PHYB level in the *aurea* mutant remained almost steady from 4 d to 8 d in both light- and dark-grown seedlings though a slight decline was observed with age (Fig. 4.31). The presence of similar amounts of PHYB in both light- and dark-grown seedlings points to its important role under natural conditions.

Overall, the expression of PHYA protein in etiolated seedlings is higher than that of PHYB which confirms the previous finding on promoter activities of the two phytochromes (Somers and Quail 1995). In *Arabidopsis*, Somers and Quail (1995) found strong differences in the transcriptional activities of PHYA and PHYB promoters. In dark-grown seedlings PHYA promoter was 20-fold more active than PHYB promoter in both shoots and roots. Also, each promoter was found to be 4-fold more active in shoots than in roots. In light-grown seedlings, PHYA promoter activity was 20-fold lower than in dark, whereas PHYB promoter decreased only by 2-fold.

These results are consistent with the data on PHYA and PHYB protein accumulation in dark-grown tomato. Hauser et al., (1994) assessed the distribution of five *PHY* mRNAs in tomato throughout its life cycle. The spatial expression patterns of each of the five *PHY* transcripts were quantitated in roots, hypocotyls, cotyledons and plumules of 21 d-old green house grown plants. In 56 d-old plants the distribution

was studied in floral buds, flowers, fruits and leaf buds. Except for *PHYA* mRNA each of the other transcript level was generally about 10-fold less abundant in roots than in shoots. *PHYA* mRNA was 2-3-fold more abundant than *PHYB1*, *PHYB2* **and** *PHYE* transcripts and about 50-fold more abundant than *PHYF* mRNA. In the older plants *PHYA* transcripts were again the most abundant. *PHYF* mRNA was relatively abundant in floral buds, flowers, and fruits. These results also agreed with the relative mRNA abundance of PHYA and PHYB in dark- and light-grown *Arabidopsis* seedlings (Sharrock and Quail 1989; Clack et al., 1994). Adam et al., (1994) demonstrated in transgenic tobacco plants that the *PHYA* mRNA abundance was mainly influenced by transcription rather than mRNA stability. Transcription of *PHYA* gene was down regulated very effectively by light in the early stage of development, whereas at later stages the down regulation became less effective. The expression of *PHYA* gene was maximum in the root tip, hook region, cotyledons and vascular tissue.

The results on transcript accumulation in light- and dark-grown tomato seedling, however, do not present a clear picture about the relative levels of the respective phytochromes in the seedlings. For example, though light-grown tomato seedlings show only 20-fold reduction of transcript level of PHYA, whereas the PHYA protein level drop below the level of detectability. This happens because light induces selective decline of PHYA protein among all phytochromes. In comparison, while the level of PHYB transcript declines by 2-fold in light and dark, there is little perceptible difference in PHYB protein level in light and dark. A dark-grown seedling may have PHYA protein in great excess in comparison to PHYB, whereas in light-grown seedlings the level of these proteins may be nearly equal.

This difference in relative level of PHYA and PHYB protein level may also affect the expression of phytochrome A response, as perception of RL by phytochrome B continues to be at the same level in dark- and light-grown seedlings, the perception of FRL by phytochrome A in terms of its absolute level, would be 100-500 fold less in light-grown seedlings than dark-grown seedlings.

Moreover, the mere presence of transcripts of PHYA or PHYB really does not ensure the availability of these proteins in the cells. In fact, in mature tomato leaves, the level of PHYA is extremely low, and it can be detected in crude extracts only after

ammonium Sulfate precipitation (Sharma et al., 1993). Although a clear cut relationship between the levels of the respective phytochrome and the photoresponse is not known and can only be speculated, it is assumed that phytochrome B plays an important role in the physiology of green plants.

Similar to maize, the accumulation of PHYA and PHYB in different organs distinctly showed an organ-specific accumulation and a temporal regulation of their levels. High amount of PHYA and PHYB were associated with younger seedlings. On day five the level of both phytochromes peaked, and declined significantly during further growth. A similar age dependent decline was also noticeable in the level of both PHYA and PHYB in the *aurea* mutant. Again in *aurea*, the level of PHYA was the same in both light- and dark-grown seedlings, showing a lack of light-induced down regulation as observed in WT.

5.6.4. Dark to light transition

When seedlings grown in continuous darkness were exposed to light, a gradual decline in the phytochrome A level with increasing exposure time was observed in WT. Whereas in the *aurea* mutant, due to the lack of light-induced down regulation, not much difference in the PHYA level was observed. PHYB level in *aurca* followed the same pattern as PHYB in the WT seedlings. On comparing the relative levels of PHYA and PHYB, a drastic decline of PHYA level was observed in WT on exposure to light, whereas PHYB level decreased to a much lesser extent (Fig. 4.33).

It was found that when 5 d-old dark-grown WT seedlings were transferred to light, PHYA underwent rapid down regulation and by 24 h the level was below detectability. In the case of PHYB however, there was no down regulation of the protein level which was maintained al a steady state after a slight initial decline. In the case of the *aurea* mutant, PHYA did not show any down regulation on transfer to light for 24 h (Fig. 4.33). This result is in agreement with an earlier study on tomato (Sharma et al., 1993). In the *aurca* mutant PHYA is believed to lack the chromophore due to which it does not undergo down regulation. PHYB level in the *aurca* mutant is similar to that of PHYB level in WT, where a steady state of the protein level was maintained for 24 h after transfer to light.

The regulation of PHYA level in oat is exercised at several levels such as transcription, mRNA stability and down regulation of the protein by proteolysis.

While the last mechanism is ruled out for PHYA protein in *aurea*, still the correlation in the level of dark- and light-grown seedlings indicate that other two mechanisms are also not effective in the *aurea* mutant. Evidently in light- and dark-grown seedlings the transcription of *PHY* genes and the mRNA stability are nearly the same leading to the lack of decline in PHYA level in *aurea*. In fact, Boy lan and Quail (1989) found that few seconds of red light is enough to decrease the mRNA level in many species like oat, corn etc. But it has been shown that even 3 h of red light exposure has less effect on type I mRNA level in tomato, while the phytochrome protein level is drastically decreased. It is not known whether the production of Pfr has no effect on the transcription of phytochrome genes, or whether the phytochrome mRNAs produced in these species are relatively stable, such that, even if transcription is dramatically decreased, the mRNA persists at a high level.

5.6.5. Light to dark transition

When light-grown seedlings were transferred to darkness, PHYA reappeared in the WT with similar kinetics as maize. A mild increase in PHYB level was also observed. In the *aurea* mutant, a small amount of both PHYA and PHYB accumulation was observed (Fig. 4.35). Analysis of both PHYA and PHYB in the *aurea* mutant of tomato, showed that the distribution and accumulation profile of both PHYA and PHYB in this mutant was similar to WT. This indicates that the lack of spectrally active phytochrome A does not change the distribution profile of phytochrome B in this mutant.

When 5 d-old light-grown WT seedlings were transferred to dark, PHYA accumulation started with a lag period of about 4 h, after which the level of PHYA increased steadily over the next 24 h. This result is similar to that obtained for maize where a lag period of about 4 h was observed. In *aurea* mutant both PHYA and PHYB levels were maintained at a steady level throughout the duration of 24 h transfer period (Fig. 4. 36).

Light-to-dark and dark to light transition experiments clearly support the notion that PHYA present in the *aurea* mutant is spectrally inactive and its accumulation in the tissues is independent of the light environment. No decline in PHYA level was observed when *aurea* seedlings were exposed to light. Similarly no

increase in PHYA level was noticed when *aurea* seedlings were transferred to darkness.

In both maize and tomato, dark to light and light to dark transition experiments clearly show a decline and reaccumulation of PHYA respectively. While in this study, the circadian level of PHYA in seedlings grown under normal light/dark cycles was not checked, it is tempting to speculate that such a decline/increase should result in a relatively higher PHYA level at dawn and very level at dusk. Whether such a diurnal fluctuations of PHYA level has any significance in the perception of dawn and dusk and other photobiological phenomenon associated with it is unknown. However, Adam et al., (1994) demonstrated in transgenic tobacco plants that the transgenes *PHY-A1-GUS*, *PHY-A2-GUS*, *PHY-A1-CAT* and *PHY-A2-CAT* were expressed identically as that of the *PHYA* gene at all developmental stages and the transcription of these genes is autoregulated by phytochrome and is not affected by the circadian clock.

In *Arabidopsis* out of the five *PHY* genes studied only PHYA gene exhibited a significant light regulation (Clack et al., 1994). In mature *Arabidopsis* all the five mRNAs were found to be present at fairly uniform levels in roots, leaves, stem and flowers. Somers and Quail (1995) reported in *Arabidopsis* by PHY promoter/GUS constructs that PHYA promoter has 10-fold lower activity in light-grown seedlings when compared to dark-grown seedlings, whereas PHYB promoter activity in light decreased by only 2-fold. Somers and Quail (1995) also found very slight differences in the PHYA and PHYB spatial distribution in *Arabidopsis.* Clack et al., (1994) also found that all the five genes were expressed in a similar manner in all the organs of *Arabidopsis.* Both PHYA and PHYB promoters were active in shoots and roots and their activities were well correlated in light and dark. Thus in *Arabidopsis* few differences were found in the *PHY* expression pattern apart from the stronger photoregulation *of PHYA* transcription. This is consistent with the results obtained in tomato. In green house grown tomato plants Hauser et al., (1994) found that PHYA mRNA was 10-fold abundant in shoot than in root. But overall, PHYA mRNA was 2-3-fold higher than PHYB and PHYE and 50-fold more than PHYF mRNA. In this study it was observed that the defect in phytochrome holoprotein does not affect the

distribution pattern of phytochrome A apoprotein. Thus it was found that the expression of phytochromes are regulated independently of one another.

5.7. In vivo and in vitro reconstitution experiments

Phytochrome, like algal proteins phycocyanin and phycoerythrin, is a biliprotein and therefore, possesses a linear tetrapyrrole as a chromophore. The chromophore of algal biliprotein, phycocyanobilin is closely similar to phytochromobilin. Algal biliproteins being photosynthetic accessory' pigments, are present in greater abundance, and phycocyanobilin has been purified and characterized for its properties. Relatively, phytochromobilin cannot be purified in large quantity as phytochrome is a low abundance regulatory protein. Nevertheless, similarity of these two chromophores in structure at least indicates that these two may share at least some steps in their biosynthetic pathway. It has been shown that the initial steps in both phycocyanobilin and chlorophyll biosynthesis show a common pathway and the pathway branches out in two different directions after formation of porphobilinogen.

Since phytochrome is a chromoprotein, it is considered possible that in the absence of the chromophore. the protein is unstable and is rapidly turned out. However, such a possibility was ruled out by demonstrating that gabaculine (an inhibitor of chromophore biosynthesis) treated seedlings though show a reduced accumulation of spectrally active phytochrome, nevertheless the amount of immunochemically detectable phytochrome is higher than spectrally active phytochrome. Therefore, phytochrome apoprotein is stable in the absence of chromophore and this is also evident by the fact that *hy1* and *hy>2* mutants of *Arabidopsis* arc strongly reduced in chromophore level but show PHYA level close to WT.

Taking advantage of uncoupling of different phytochrome apoprotcins synthesis and chromophore synthesis, Elich and Lagarias (1987) showed that on feeding chromophore depleted oat shoots which have drastically reduced PHYA level with biliverdin IXα a putative precursor of phytochromobilin, the amount of spectrally active phytochrome increased within a few hours. Moreover, such a restoration of spectral activity has also been shown in chromophore lacking phytochrome apoprotein by feeding phycocyanobilin to shoots. This experiment

clearly showed that phytochrome can use PCB as a chromophore and restore spectral activity and perhaps also biological activity. Elich and Lagarias (1989) further demonstrated that even in crude extracts, it is possible to restore spectral activity of phytochrome using phycocyanobilin.

Taking a cue from these studies, Parks and Quail (1991) demonstrated that on growing *hyl* and *hy2* seedlings of *Arabidopsis* on a medium containing biliverdin IXα, phycocyanobilin and other precursors of pathway, the mutant phenotype can be rescued to WT. Apparently, the chromophore analog could be converted to phytochromobilin as spectrally active phytochrome could be detected subsequent to chromophore feeding. A similar rescue of phenotype was obtained *for pew1-1* mutants of tobacco, where biliverdin restored the phenotype of the mutant and also spectral activity of phytochrome *in vivo* (Kraepiel et al., 1994).

However, similar feeding experiments of *aurea* with biliverdin (Sharma, unpublished results) and phycocyanobilin in this study showed no rescue of phenotype. Moreover, no spectrally active phytochrome could be detected *in vivo* on spectroscopic estimation. The above failure perhaps could have stemmed from two possibilities. First, PCB failed to penetrate into the *aurea* cells. While such a possibility is not likely, as in *hyl* and *hy2* seedlings grown on PCB, the phytochrome could be fully reconstituted and the phenotype rescued. The second possibility is that PCB entered the cells but some additional factors such as chaperonin may be lacking in the *aurea* mutant, which led to the failure to generate spectrally active phytochrome.

This possibility was examined by directly incubating phytochrome polypeptide from *aurea* mutant with PCB *in vitro.* Besides, the successful reconstitution of phytochrome from chromophore depleted phytochrome on incubation with PCB *in vitro* (Elich and Lagarias 1989), phytochrome polypeptide synthesized in yeast can also be reconstituted using PCB *in vitro* (Lagarias and Lagarias 1989; Deforce et al., 1991) and also *in vivo* (Li and Lagarias 1994) indicating that phytochrome apoprotein itself may possess bilin C-S-lyase activity. However, results obtained in this study did not show such a spectral reconstitution of phytochrome A under experimental conditions derived from Lagarias protocols. While this experiment does suggest that phytochrome of *aurea* is defective in

assembly of spectrally active phytochrome, **alternate interpretations are also possible.** One possibility is that the extraction conditions and extraction medium used for oat may not be ideal for tomato phytochrome, therefore it fails to reconstitute. The improvement of extraction conditions may perhaps help in the coupling of the chromophore to apoprotein.

However, WT tomato seedlings grown in the presence of gabaculine, and the incubation of the crude extract with PCB did not lead to the rescue of the phenotype (Sharma unpublished results). The WT seedlings grown in the presence of gabaculine and PCB also failed to show *in vitro* reconstitution of phytochrome. Therefore, the possibility of lack of coupling between apoprotein and chromophore by the absence of some factors is not very favorable.

Alternative approach of using a transgenic *aurca* plant consisting of phytochrome A from oat yielded similar results albeit with different interpretation. It was found that transgenic tomato *aurca* had the oat gene but no PHY A protein. The failure of phytochrome A protein accumulation may be due to the absence of chaperonin (McCormac et al., 1993). Further it was found that though the oat PHYA protein was expressed in transgenic *aurca* mutant of tomato, it was spectrally inactive (McCormac et al., 1993). This experiment provides a proof that *aurca* is a mutant of chromophore biosynthesis, but a demonstration is still needed that the expressed oat phytochrome can be reconstituted *in vitro* and *in vivo* by PCB.

Therefore it is still an open question, in the absence of the above details, as to whether phytochrome A is spectrally inactive in *aurca* due to the lack of chromophore or is a compound like chaperonin needed for the assembly. In this context, comparison *of* the *pew* mutant of tobacco becomes necessary (Kraepiel et al., 1994). Among the two *pew* mutants, *pew1-1* was found to possess spectrally inactive phytochrome A apoprotein level close to WT. This mutant could be rescued by exogenous bilivcrdin with restoration of spectral activity. In comparison, *pew 1-2* mutant possesses only reduced level of phytochrome A protein and could not be rescued by biliverdin. This mutant is also like *aurca* since it consists of nearly normal level of light-stable phytochrome with reduced phytochrome A amount. Taking together these data, and in the absence of alternative proof that *aurca* is a true chro-

mophore mutant, the possibility that phytochrome assembly with chromophore in *aurea* needs additional factors still remains open and needs further experimentation.

CHAPTER 6

SUMMARY

The present study was undertaken with an aim to study **the** spatial and temporal distribution of PHYA and PHYB in various organs at different developmental stages of light- and dark-grown maize, and tomato seedlings. The distribution was studied by purifying PHYA from dark-grown oat seedlings followed by raising of monospecific antisera against the purified phytochrome A. An attempt was also made to purify phytochrome B from field grown spinach leaves. The mAP and mAT used in this study were raised against phytochrome A of pea and phytochrome of B tobacco respectively. The distribution of phytochrome A and phytochrome B was studied using the above antibodies. An attempt was made to rescue the *aurea* mutant of tomato by feeding PCB. *In vitro* reconstitution of PHYA by co-incubation with PCB was also attempted.

Phytochrome A was purified to homogeneity by the procedure of Grimm and Rüdiger (1986) using 4.5 d-old dark-grown oat seedlings. The spectral analysis revealed that the purified phytochrome had absorption maxima at 667 nm and 280 nm in the Pr form and a SAR value of 0.75. The analysis of the physico-chemical properties of purified phytochrome revealed a single band with a monomeric molecular weight of 124 kD.

The purified phytochrome was injected into rabbits to obtain polyclonal antisera. The antisera were found to be specific against oat PHYA and showed a similar cross reactivity with PHYA of maize. The specificity of the antisera was also ascertained by western blotting, immunoprecipitation, Zn fluorescence, and *in vitro* proteolysis assay. The specificity of phytochrome B antibodies (mAT) against PHYB of maize was ascertained by comparative western blotting. The specificity of mAP and mAT against PHYA and PHYB respectively in tomato were ascertained by Sharma et al., (1993).

In the present study, the distribution of both PHYA and PHYB was ascertained by immunoblotting using specific antibodies against these two phytochrome species. In maize seeds germinating in darkness, both PHYA and PHYB could be detected only in the embryonic axes and the scutellum but not in the de-

embryonated kernels. However, in seeds germinating **under continuous red-light, the** level of PHYA was strongly down-regulated, and some amount of decline was also noticed for PHYB in both the scutellum and the embryonic axis.

Phytochrome A was detected in all the organs such as the root, mesocotyl, coleoptile and first leaf of 5 d-old dark-grown maize seedlings. PHYB was detected both in light and dark-grown seedlings in a similar fashion. In both cases, the primary leaf and the coleoptile had the maximal level of phytochrome. During the growth of the seedlings, the levels of both PHYA and PHYB were maximal on 4-5 d and thereafter declined up to 8 d. In comparison to PHYA, PHYB declined slowly with age both in the leaf and the coleoptile.

The distribution of phytochrome was checked along the length of the leaf in 1 cm long segments from the base to the tip by *in vivo* spectrophotometry. Immunoblots showed a similar pattern of PHYA and PHYB distribution in the primary leaf. The level of PHYA and PHYB were higher at the leaf base, decreased in the middle region and again increased in the upper half of the leaf. In the light grown leaf, while PHYA was below the level of detection, PHYB distribution was essentially similar to that of the dark-grown leaves. The distribution pattern of protochlorophyllide oxidoreductase, another light induced down-regulated protein, was different from that of the phytochromes, with a gradual increase in its level from the base to the tip both in the light and dark-grown leaves.

Exposure to red light induced a rapid decline in PHYA level in the leaf and coleoptile within 2 h, and a gradual decline to a very low level was observed during the next 24 h was observed. Likewise, exposure to red light also initiated a small decline in the PHYB level in the leaf. On dark adaptation of light grown seedlings, PHYA reappeared within 8 h, and thereafter its level increased both in the leaf and the coleoptile up to 24 h. Interestingly, the distribution pattern of resynthesized PHYA along the length of the leaf was similar to that of the continuous dark-grown seedlings.

Similar to maize, PHYA and PHYB distribution was also ascertained in WT tomato seedlings, and the profiles were compared with that of a photomorphogenic mutant- *aurea*, which is believed to be a chromophore deficient mutant. In the dark-grown WT seedlings, PHYA level was more in the hypocotyl followed by the

cotyledon, whereas PHYB level was similar in both light and dark-grown seedlings. In light-grown WT seedlings, PHY A was drastically down regulated. In contrast, in the *aurea* mutant, due to the lack of down-regulation of PHYA, both PHYA and PHYB could be detected as observed in light- and dark-grown WT seedlings. In the roots of both WT and *aurea,* phytochrome signal was below the detectability limit.

The time course profile of phytochrome from dark-grown seedlings revealed that PHYA and PHYB were maximal on the 5 d and then declined gradually. In contrast, in the light-grown seedlings, very low amount of PHYA could be detected even in 4 d-old seedlings and thereafter, its level was below detectability. Similar to maize, red light induced PHYA down regulation in etiolated WT tomato but not in *aurea.* In tomato too, on transfer to darkness PHYA reappeared after 8 h.

Since it is assumed that *aurea* is a chromophore deficient mutant, both *in vivo* rescue (Parks and Quail 1991) and *in vitro* reconstitution (Terry and Lagarias, 1991) of phytochrome A using purified phycocyanobilin (Kunkel *et al.,* 1993), a phytochrome chromophore analog were attempted. However, in both cases no rescue of the phenotype or spectrally active phytochrome could be observed.

In summary, this study highlights that the spatial and temporal expression of both phytochrome A and phytochrome B are not co-regulated and the distribution pattern of phytochrome B does not alter in the absence of active phytochrome A. Also, in the absence of attached chromophore, phytochrome apoprotein expression and distribution in *aurea* remains the same as in WT. The defect in phytochrome A in *aurea* may not be due to the lack of chromophore, but due to some other defect.

CHAPTER 7

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